Studies on the expression and secretion of adipokines in canine white adipose tissue

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Vivien Hartley Ryan

May 2008

“ Copyright © and Moral Rights for this thesis and any accompanying data (where applicable) are retained by the author and/or other copyright owners. A copy can be downloaded for personal non-commercial research or study, without prior permission or charge. This thesis and the accompanying data cannot be reproduced or quoted extensively from without first obtaining permission in writing from the copyright holder/s. The content of the thesis and accompanying research data (where applicable) must not be changed in any way or sold commercially in any format or medium without the formal permission of the copyright holder/s. When referring to this thesis and any accompanying data, full bibliographic details must be given, e.g. Thesis: Author (Year of Submission) "Full thesis title", University of Liverpool, name of the University Faculty or School or Department, PhD Thesis, pagination.”

Declaration

I declare that the research reported in this thesis has been carried out by me at the Obesity Biology Unit, School of Clinical Sciences, University of Liverpool, and help which was received for any part of the work has been duly acknowledged. I believe that the contents of this thesis are wholly original, except where references are made. The work reported in this thesis has not been presented for any other degree.

Vivien Hartley Ryan BSc (Hons) BVSc (Hons) MRCVS

University of Liverpool

May 2008

"The devil has put a penalty on all things we enjoy in life. Either we suffer in health or we suffer in the soul or we get fat"

Albert Einstein, 1879-1955

Acknowledgments

I would hereby like to thank the following people for their assistance in completing this work:

Prof P. Trayhurn[1] for providing the required resources and unlimited supervision, and for his patience and belief in my work and abilities.

Dr A. J. German[2] for his constant enthusiasm and support, as well as for his tireless optimism and supervision throughout my studies.

Dr S. Wood[1] for his expert technical advice and kind assistance throughout the work.

Mr L. Hunter[1] for his invaluable practical assistance, patience and advice.

Ms R. Steen, Mrs S. Batchelor, Miss L. Hutchings, Miss N. Billing[3] & final year veterinary students at the University of Liverpool for performing the neutering of the animals which allowed the samples of tissue used in the study to be collected.

Dr M. R. Peeraully[1] for his technical advice and expertise throughout the work.

Dr P. G. Muniesa[1] for his kind help and support.

Dr S. Al-Naimi[1] for her assistance and support.

Dr A. C. German[2] for her guidance and tireless support.

Mr G. Gilligan[4] for all his logistical help in sample collection.

Dr J. Palomero[5] for his help in obtaining the images used in this study.

Mr N. Usher[1] for his assistance.

The Biotechnology and Biological Sciences Research Council for the award of a BBSRC Research Studentship for this project.

Waltham Pet Care and Nutrition for the generous award of funds

My husband Mr S. P. Ryan, Filomena, George and Magda for their support.

[1]Obesity Biology Unit, School of Clinical Sciences, University of Liverpool, Liverpool, UK; [2]Department of Veterinary Clinical Sciences, University of Liverpool, Small Animal Teaching Hospital, Leahurst, Wirral, UK; [3]Fern Grove Veterinary Surgery, University of Liverpool, Crown St, UK; [4]Department of Veterinary Preclinical Sciences, University of Liverpool, UK; [5]Cell Pathophysiology Group, Division of Metabolic & Cellular Medicine, School of Clinical Sciences, University of Liverpool, UK.

Abbreviations

Acrp30	adipocyte complement-related protein of 30 kDa
AgRP	agouti-related peptide
AMPK	5'-AMP-activated protein kinase
ARC	arcuate nucleus
ASP	acylation-stimulating protein
ATP	adenosine triphosphate
BAT	brown adipose tissue
BCS	body condition score
BDNF	brain-derived neurotrophic factor
11β-HSD1	11β-hydroxysteroid dehydrogenase
BLAST	basic local alignment sequence search tool
BMI	body mass index
BMR	basal metabolic rate
BSA	bovine serum albumin
BPB	bromophenol blue
BSB	block and sample buffer
cAMP	cyclic adenosine 3',5'-monophosphate
CART	cocaine and amphetamine-related transcript
CB	cannabinoid receptor
CCK	cholecystokinin
cDNA	complementary DNA
C/EBP	CAAT/enhancer-binding protein
CNS	central nervous system
CoA	acetyl coenzyme A
CRF	corticotrophin releasing factor
CRP	C-reactive protein
CSF	cerebrospinal fluid
C_T	threshold cycle
DEXA	dual energy x-ray absorptiometry
DF	dilution factor
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dsDNA	double-stranded DNA
DVC	dorsal vagal complex
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FAM	6-carboxyfluorescein
FCS	foetal calf serum
FIAF	fasting-induced adipose factor
FRET	fluorescence resonance energy transfer
GHS-R	growth hormone secretagogue receptor
GLP-1	glucagon-like peptide 1
GLUT 4	insulin-dependent glucose transporter 4
GPDH	glycerol-3-phosphate dehydrogenase
GRα	glucocorticoid receptor α
HCl	hydrochloric acid
HDL	high-density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIF-1α	hypoxia inducible factor-1 alpha
hMADS	human multipotent adipose-derived stem cells
HMW	high molecular weight
HRP	horseradish peroxidise
HSL	hormone sensitive lipase
H_2SO_4	sulphuric acid
IBMX	3-isobutyl-1-methylxanthine
Ig	immunoglobulin
IGF1	insulin growth factor-1
IL	interleukin
IRS-1	insulin receptor substrate 1
JAK	janus-activated kinase
LHA	lateral hypothalamic area
LMW	low molecular weight
LPL	lipoprotein lipase
LPS	lipopolysaccharide

mAb	monoclonal antibody
MCP-1	monocyte chaemoattractant protein-1
MC3R	melanocortin 3 receptor
MC4R	melanocortin 4 receptor
MCH	melanocortin concentrating hormone
MIF	macrophage migration inhibitory factor
mRNA	messenger RNA
α-MSH	α-melanocyte stimulating hormone
MT	metallothionein
NAD	nicotinamide adenine dinucleotide
NEFA	non-esterified fatty acids
NFκB	nuclear factor κB
NGF	nerve growth factor
NPY	neuropeptide Y
NSAID	non-steroidal anti-inflammatory drug
NT	neurotrophin
NTC	non-template control
Ob-R	leptin receptor
OXM	oxynotmodulin
pAb	polyclonal antibody
PAI-1	plasminogen activator inhibitor -1
PBS	phosphate buffered saline
PBE3B	phosphodiesterase 3B
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3 kinase
POMC	pro-opiomelanocortin
PP	pancreatic polypeptide
PPARs	peroxisome proliferator-activated receptors
PBN	parabrachial nucleus
PVN	paraventricular nucleus
PYY	peptide YY
qPCR	real-time PCR
R	raw fluorescence reading in arbitrary units
RAAS	rennin-angiotensin aldosterone system

RBP	retinol binding protein
RD	reagent diluent
RNA	ribonucelic acid
RNAPolIIa	RNA polymerase IIa
ROS	reactive oxygen species
ROX	carboxy-X-rhodamine
rRNA	ribosomal RNA
Rsq	fit of data to standard curve plot for qPCR.
RT	reverse transcription
RXR	retinoic X receptor
SAA	serum amyloid A
SBT	Staffordshire bull terrier
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error of the mean
SGBS	Simpson-Golabi-Behmel syndrome
SNS	sympathetic nervous system
SOCS-3	suppressor of cytokine signalling-3
ssDNA	single-stranded DNA
STAT	signal transducer and activator of transcription
SV	stromal vascular fraction
T_a	annealing temperature
TAMRA	6-carboxytertamethylrhodamine
Taq polymerase	*Thermus aquaticus* polymerase
TBE	tris-borate-EDTA
TG	triacylglycerol
TGs	triglycerides
TGFβ	transforming growth factor-β
TLR	toll-like receptor
T_m	melting temperature
TMB	tetramethyl benzidine
TNFα	tumour necrosis factor-alpha
Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloride
TZD	thiazolidinediones
UCP-1	uncoupling protein 1

UK	United Kingdom
USA	United States of America
UV	ultra-violet light
VEGF	vascular endothelial growth factor
VLDL	very-low-density lipoprotein
VMN	ventromedial nucleus
WAT	white adipose tissue
WHO	world health oraganisation
ZAG	zinc-α2-glycoprotein

Table of contents

Section	*Title*	*Page*
	Title page	i
	Declaration	ii
	Acknowledgments	iii
	Table of contents	x
	List of figures	xiii
	List of tables	xvii
	Abstract	xviii
Chapter 1	**Introduction**	**1**
1.1	Obesity	2
1.2	Energy balance and body weight regulation	17
1.3	White adipose tissue	26
1.4	Endocrine function of white adipose tissue	34
1.5	11β-hydroxysteroid dehydrogenase-1	60
1.6	Aims of the thesis	63
Chapter 2	**Materials and Methods**	**66**
2.1	Animals	67
2.2	Preadipocyte primary culture	69
2.3	Total RNA isolation from tissues and cells	74
2.4	Reverse-transcription polymerase chain reaction	80
2.5	Real-time polymerase chain reaction	89
2.6	Enzyme-linked immunosorbent assay	98
2.7	Statistical analysis	104
Chapter 3	**Adipokine gene expression in canine white adipose tissues**	**105**
3.1	Introduction	106
3.2	Methods	108
3.3	Results	109
3.4	Discussion	119

Chapter 4	**Establishment of a canine primary cell culture protocol: Adipokine gene expression and protein secretion during canine adipocyte differentiation and development**	**124**
4.1	Introduction	125
4.2	Methods	127
4.3	Results – Part A	131
4.4	Results – Part B	138
4.5	Discussion	161
Chapter 5	**Adipokine expression and secretion by canine adipocytes in primary culture: Regulation by the inflammatory mediators TNFα, IL-6 and LPS**	**171**
5.1	Introduction	172
5.2	Methods	174
5.3	Results	175
5.4	Discussion	192
Chapter 6	**Adipokine expression and secretion by canine adipocytes in primary culture: Regulation by PPARγ agonists and Dexamethasone**	**198**
6.1	Introduction	199
6.2	Methods	200
6.3	Results	201
6.4	Discussion	216
Chapter 7	**11β-HSD-1 expression by canine adipocytes in primary culture: Regulation of inflammatory mediators, PPARγ agonists and dexamethasone**	**220**
7.1	Introduction	221

7.2	Methods	222
7.3	Results	224
7.4	Discussion	231
Chapter 8	**Discussion**	**235**
8.1	Introduction	236
8.2	Adipokine gene expression in canine white adipose tissues	237
8.3	Adipokine gene expression and secretion in canine visceral and subcutaneous adipocytes during differentiation *in vitro*	238
8.4	Effect of inflammation-related agents and pro-inflammatory cytokines on adipokine expression and secretion	238
8.5	Effect of PPARγ agonists and dexamethasone on adipokine expression and secretion	240
8.6	Regulation of adipokine expression in canine lipomatous tissue by treatment with dexamethasone and TNFα	242
8.7	11β-HSD-1 expression in canine adipose tissue and adipocytes in primary culture	243
8.8	Concluding comments	245
Appendix		**246**
I	Publications and presentations associated with the thesis	247
II	Reagents and equipment	248
References		**254**

List of figures

Figure	*Title*	*Page*
1.1	Waltham S. H. A. P. E ™ guide for dogs	6
1.2	Neuroanatomical model representing regulation of food intake and body weight	21
1.3	Evolving views of the biological function of white adipose tissue	35
1.4	Molecular pathways integrating inflammatory responses with insulin action	46
1.5	Differential function and tissue expression of 11β-HSD isozymes	62
2.1	DEXA scan image	68
2.2	Checking mRNA integrity from a sample of total RNA	79
2.3	Detection chemistries used in qPCR	91
2.4	Validation of the $2^{-\Delta\Delta C_T}$ method	97
2.5	ELISA standard curve	101
3.1	Canine PPARγ2 sequence alignment	110
3.2	Expression of adipocyte related genes by canine white adipose tissue	111
3.3	Expression of leptin by canine white adipose tissue	113
3.4	Expression of adiponectin by canine white adipose tissue	114
3.5	Expression of pro-inflammatory adipokine genes by canine white adipose tissue	115
3.6	Quantification of the level of expression of pro-inflammatory adipokine genes by canine white adipose tissue	116
4.1	Comparison between Wu and Eisele protocols	135
4.2	Depot comparison using the protocol by Wu *et al.*, 2001	136
4.3	Comparison of well size as a potential stress for	

cells in culture 137

4.4 Time course of differentiation and development of canine adipocytes in primary culture 141
4.5 Time course of gene expression of adipose tissue-related markers in canine adipocytes in primary culture 142
4.6 Time course of leptin gene expression in adipocytes of the subcutaneous inguinal depot differentiated in primary culture 143
4.7 Time course of leptin gene expression in adipocytes of the gonadal depot differentiated in primary culture 144
4.8 Time course of adiponectin gene expression and protein secretion in adipocytes of the subcutaneous inguinal depot differentiated in primary culture 145
4.9 Time course of adiponectin gene expression and protein secretion in adipocytes of the gonadal depot differentiated in primary culture 146
4.10 Time course of pro-inflammatory adipokine gene expression in adipocytes of the subcutaneous inguinal depot differentiated in primary culture 150
4.11 Time course of TNFα gene expression and protein secretion in canine subcutaneous inguinal adipocytes in primary culture 151
4.12 Time course of TNFα gene expression and protein secretion in canine gonadal adipocytes in primary culture 152
4.13 Time course of IL-6 gene expression and protein secretion in canine subcutaneous inguinal adipocytes in primary culture 153
4.14 Time course of IL-6 gene expression and protein secretion in canine gonadal adipocytes in primary culture 154

4.15 Time course of MCP-1 gene expression and protein secretion in canine subcutaneous inguinal adipocytes in primary culture 157

4.16 Time course of MCP-1 gene expression and protein secretion in canine gonadal adipocytes in primary culture 158

4.17 Time course of NGF gene expression and protein secretion in canine subcutaneous inguinal adipocytes in primary culture 159

4.18 Time course of NGF gene expression in canine gonadal adipocytes in primary culture 160

5.1 Human and canine TNFα coding sequence (mRNA) and amino acid sequence alignment 177

5.2 Regulation of adipokine gene expression in canine adipocytes by human recombinant TNFα 178

5.3 Regulation of adipokine gene expression in canine subcutaneous inguinal adipocytes by canine recombinant TNFα 180

5.4 Regulation of adipokine gene expression in canine gonadal adipocytes by canine recombinant TNFα 181

5.5 Regulation of adipokine protein secretion in canine adipocytes by canine recombinant TNFα 184

5.6 Regulation of adipokine gene expression in canine adipocytes by canine recombinant IL-6 186

5.7 Regulation of adipokine protein secretion in canine adipocytes by canine recombinant IL-6 187

5.8 Regulation of adipokine gene expression in canine adipocytes by LPS 189

5.9 Regulation of adipokine protein secretion in canine adipocytes by LPS 191

6.1 Chemical structures of four anti-diabetic thiazolinediones 203

6.2 Regulation of adipokine gene expression in canine

	subcutaneous inguinal adipocytes by rosiglitazone	204
6.3	Regulation of adipokine gene expression in canine gonadal adipocytes by rosiglitazone	205
6.4	Regulation of adipokine protein secretion in canine subcutaneous inguinal adipocytes by rosiglitazone	206
6.5	Regulation of adipokine gene expression in canine subcutaneous inguinal adipocytes by ciglitazone	209
6.6	Regulation of adipokine protein secretion in canine subcutaneous inguinal adipocytes by ciglitazone	210
6.7	Regulation of adipokine gene expression in canine subcutaneous inguinal adipocytes by dexamethasone	213
6.8	Regulation of adipokine protein secretion in canine subcutaneous inguinal adipocytes by dexamethasone	214
7.1	Expression of 11β-HSD-1 by canine white adipose tissue	225
7.2	Time course of gene expression of 11β-HSD-1 in canine adipocytes in primary culture	227
7.3	Regulation of 11β-HSD-1 gene expression in canine adipocytes by inflammatory mediators	228
7.4	Regulation of 11β-HSD-1 gene expression in canine adipocytes by PPARγ agonists and dexamethasone	230

List of tables

Table	*Title*	*Page*
1.1	Medical criteria used to define the Metabolic Syndrome	9
2.1	Sequences of RT-PCR primers	85
2.2	Sequences of primers and TaqMan probes used for qPCR	93

Abstract

Obesity is now one of the most common medical disorders of companion animals (dogs, cats and horses) and is defined by the expansion of white adipose tissue mass. This tissue is now known to be major endocrine and secretory organ, releasing different protein hormones and signals termed adipokines. A number of these are linked to inflammation and immunity, and a role for inflammation-related adipokines in the development of obesity-associated diseases in humans is increasingly recognised. The specific aim of this project was to examine the extent to which canine adipose tissue produces various adipokines and to determine some of the factors which regulate their production. The Staffordshire bull terrier was the main breed employed.

Studies by RT-PCR indicated that the major adipose tissue depots in the dog (subcutaneous inguinal, gonadal, omental, per-renal and falciform ligament) expressed major adipokine genes including leptin, adiponectin, TNFα, IL-6 and MCP-1. Using real-time PCR, no significant difference was found in inflammatory adipokine mRNA levels between the depots examined. However, mRNA levels of the adipocyte hormone leptin were found to be higher in four out of the five white adipose tissue depots examined, in female compared to male dogs. A primary cell culture system was established in which fibroblastic preadipocytes from both visceral and subcutaneous depots were successfully isolated and induced to differentiate into mature adipocytes as a tool for examining adipokine expression and secretion *in vitro*.

The expression profile of the adipocyte hormones leptin and adiponectin, as well as that of a series of inflammation-related adipokine genes, was examined during the differentiation of canine preadipocytes to adipocytes from both depots. Expression for some genes, notably leptin and adiponectin, was highly differentiation-dependent, being evident only after the induction of differentiation. In contrast, that of other adipokines such as NGF and TNFα was similarly expressed both before and after differentiation. Canine white adipocytes of both visceral and subcutaneous depots were found to synthesise and release several protein-factors including adiponectin,

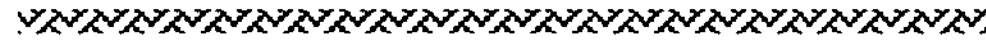
TNFα, IL-6, MCP-1 and NGF and as such these can be referred to as adipokines in the dog.

The effects of several agents, including the pro-inflammatory cytokines TNF-α and IL-6, and LPS, on the expression and secretion of adipokines by canine adipocytes were investigated. Treatment of differentiated adipocytes with these agents led to substantial increases in the mRNAs encoding several adipokines, including IL-6 and MCP-1. In contrast, dexamethasone and the PPARγ agonist rosiglitazone, both of which have anti-inflammatory effects, led to a marked reduction in the level of expression and secretion of several inflammation-related adipokines.

Studies reported herein also show that the intracellular enzyme 11β-HSD-1, responsible for the conversion of inactive cortisone to cortisol, is expressed in all five main white adipose tissue depots in the dog. Although there was little difference in level of expression between depots, 11β-HSD-1 mRNA level was found to be higher in the omental depot of female than in male dogs. Both pro-inflammatory agents, and agents with an anti-inflammatory effect, such as the PPARγ agonist rosiglitazone, increased the level of expression of 11β-HSD-1 in cells of both visceral and subcutaneous depots. Thus, the local regulation of cortisol metabolism in canine adipocytes may be in a delicate balance, where regulation by inflammatory and anti-inflammatory agents may play a significant role.

In conclusion, these studies indicate that canine white adipocytes, from both visceral and subcutaneous depots, are able to synthesise and actively secrete several key adipokines. Strong evidence has been provided that canine adipocytes are able to synthesise and actively secrete these adipokines in response to specific stimuli and this may have a significant role in the metabolic profile of the dog in obesity.

Chapter 1



Introduction

1.1 Obesity

Obesity stems from an imbalance between levels of energy intake and expenditure. Prolonged excessive energy (food) intake coupled with low levels of energy expenditure lead to a state of positive energy balance where excessive deposition of energy in the form of triglycerides in white adipose tissue (WAT) occurs. Obesity is the main nutritional disorder affecting both humans and companion animals (dogs, cats & horses) in westernised society (Shibata *et al.*, 2003; Kearns *et al.*, 2006; Bach *et al.*, 2007; Wells *et al.*, 2007). In humans, it has reached epidemic proportions in countries such as the USA and it is also a growing concern worldwide, including in developing nations. Factors in an individual's lifestyle such as decreased level of physical activity, coupled with greater availability of highly palatable, cheap, energy-rich foods are thought to be the main contributor for the global rise in levels of human obesity.

However, susceptibility to obesity is also dependent upon genetic background. The heritability of obesity, is considered to be relatively high (up to 60-70%), and there is evidence that several major genes contribute to the variation in obesity related phenotypes in humans (O'Rahilly & Farooqi, 2006). However, the current levels of obesity cannot be attributed to genetic changes, for which the timescale would be too short, but rather to environmental and socio-economic factors coupled with both genetic and epigenetic factors (Rolls, 2007; Speakman *et al.*, 2007). In dogs, it has been shown that factors related to lifestyle such as the socio-economic and dietary habits of owners as well as breed susceptibility influence the development of obesity (Colliard *et al.*, 2006).

1.1.1 Clinical definition and prevalence of obesity

Obesity can be defined as a condition resulting from a positive energy balance, where energy intake exceeds expenditure leading to weight gain and excess body fat. A working definition of overweight for dogs has been suggested as an excess of 10%, and obese when weight exceeds 20%, above the ideal level recommended for a certain breed (Lund *et al.*, 2006). It is critical to have a method for the diagnosis of obesity. Imprecise methods would either over or under diagnose the condition resulting in shifts in estimates of its incidence and prevalence (Misra, 2003).

1.1.1.1 BMI in humans

Obesity can be measured using various advanced imaging techniques such as dual-energy X-ray absorptiometry (DEXA) and magnetic resonance imaging (MRI). However, these methods require expensive equipment and are therefore limited to a clinical research setting. Alternatively, body mass index (BMI) can be used indirectly to quantify overweight and obesity, and is defined as the weight in kilograms divided by the square of the height in metres (kg/m^2). This is a valid and reproducible method that is cheap and convenient for the assessment of the weight status of an individual in the clinical setting (where weight is a surrogate for body fatness) (O'Neill *et al.*, 2007). The World Health Organisation (WHO) has proposed a classification of overweight and obesity that applies to adult men and women of a BMI of ≥ 25 kg/m^2 as overweight, ≥ 30 kg/m^2 as obese and ≥ 40 kg/m^2 as morbidly obese (WHO, 2007).

These cut off points were derived primarily in European and North American populations. There is strong ongoing debate as to whether these criteria are appropriate for non-Europeans where, for example, people from China or South Asia have higher levels of blood glucose and blood pressure for a given BMI compared to Caucasians (Razak *et al.*, 2007). Thus, for most such populations, the reported BMI cut-offs would be lower than those currently accepted (Misra, 2003). This has been taken into account in the current WHO recommendations where the cut-off points of 23, 27.5, 32.5 and 37.5 kg/m^2 are added to the current classification as points for public health action. For the medical management and treatment of overweight and obesity in humans BMI alone should not be considered diagnostic.

Measurement of the presence of abdominal obesity based on a measure of waist-to-hip ratio (WHR) is also important (Kuczmarski & Flegal, 2000), and several studies indicate a link between increased adiposity in the upper part of the body male-type fat distribution (i.e., visceral or abdominal) and increased risk of obesity-associated diseases (Vgontzas *et al.*, 2003; Wajchenberg *et al.*, 2003; Bray, 2004). In contrast, those with fat stores in gluteal-femoral or peripheral depots, the so called lower body obesity or female-type fat distribution, have been found to have a lower risk of morbidity/mortality from these diseases (Wajchenberg *et al.*, 2003). Visceral adipose tissue has been found to be functionally and morphologically different from

subcutaneous adipose tissue in that visceral adipocytes have a higher fatty acid turnover and are less responsive to the anti-lipolytic effects of insulin compared to adipocytes from subcutaneous depots (Wajchenberg *et al.*, 2003).

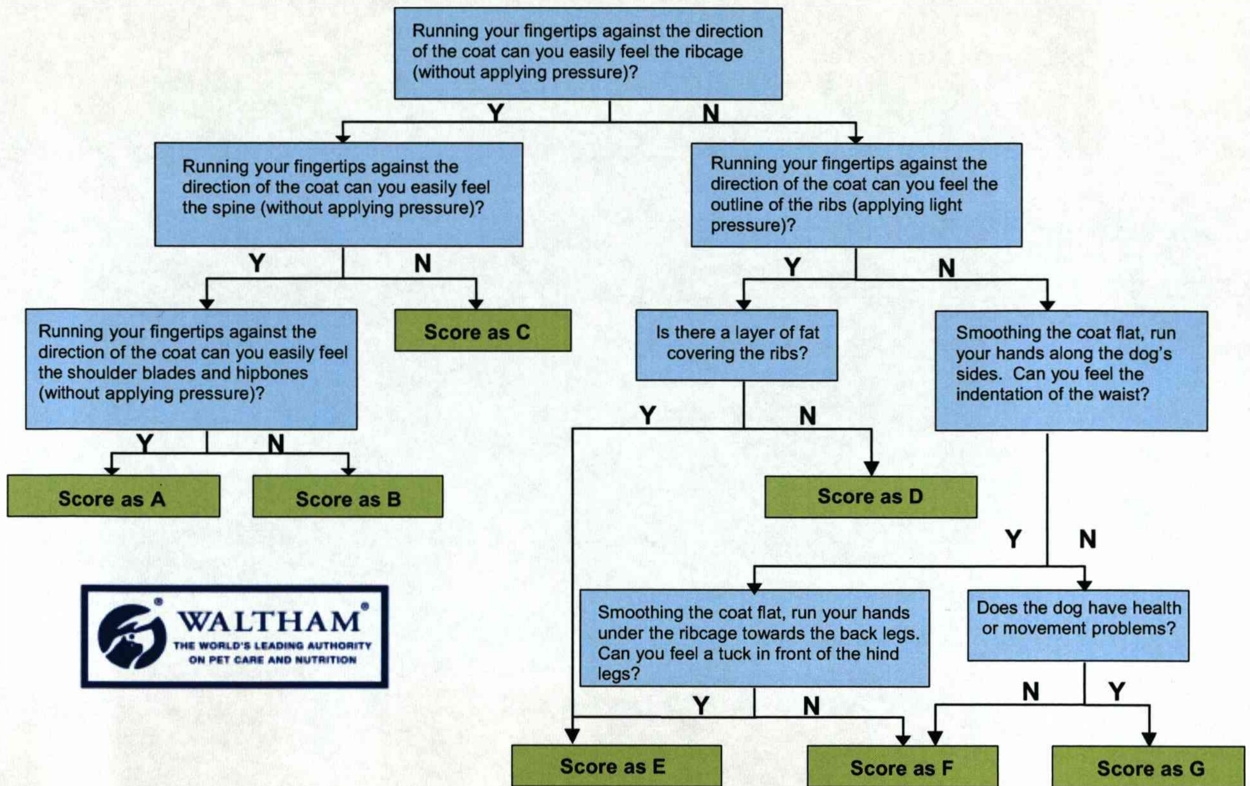
Recent figures indicate that 23% of men and 25% of women in the United Kingdom (UK) are obese. This represents an approximate trebling in the prevalence of obesity since 1980 (Rennie & Jebb, 2005). There has also been an alarming rise in the levels of childhood obesity, with 22% of boys and 28% of girls aged 2-15 being overweight or obese (Rennie & Jebb, 2005). Data for other European countries (excluding the UK) estimate that 43.5% of Europeans aged 50 to 79 years are overweight and 18.2% are obese (Peytremann-Bridevaux *et al.*, 2007). Figures for the United States of America (USA) estimate that approximately one third of adults are obese (Marcason, 2007), and 17.1% of children and adolescents are overweight (Ogden *et al.*, 2006). Levels of obesity in some lower-income and transitional countries is as high, or even higher than, the prevalence reported in developed nations and rates seem to be rising increasingly rapidly (Filozof *et al.*, 2001; Wijesinghe, 2006; Hossain *et al.*, 2007).

1.1.1.2 Companion animals and Body Condition Score

The measurement of body composition in any species is vital not only for assessment and control of obesity, but also for the re-alimentation of sick animals and the evaluation of energy requirements (Munday, 1994). All measures of adiposity in dogs involve defining body composition, or the relative amounts of the various biological components of the body, namely fat and lean body mass (German, 2006). Various techniques are available to measure body composition and can either be direct (chemical analysis of a cadaver) or indirect (based upon the model of the body as distinct compartments) (Munday, 1994). Anthropomorphic measurements such as skin-fold thickness in humans, DEXA analysis, MRI, ultrasonography (subcutaneous and abdominal), isotope dilution (deuterium and tritium oxide) are just a few of the techniques used, mainly in a research setting (Wilkinson & McEwan, 1991; Munday *et al.*, 1994; Toll *et al.*, 1994; Morooka *et al.*, 2001; Speakman *et al.*, 2001; Mawby *et al.*, 2004).

Clinically, morphometric methods (defined as the measurement of 'form') are the most common way to assess body condition in companion animals. The three main morphometric approaches are measurement of skin fold thickness (not suitable in cats or dogs due to the large amount of subcutaneous fascia), dimensional evaluations (in which various measurements of stature and weight are combined) and body condition score (BCS) (German, 2006). Body condition scoring is a subjective semi-quantitative assessment of body condition with a range of categories (each given a number) from cachectic to severely obese. A number of different schemes are in existence, of which the most widely-adopted in general practice is a 9-point scheme (Laflamme & Kuhlman, 1995), whereas a newly devised 7-point scheme has been shown to be suitable for scoring by owners (German *et al.*, 2006). Both systems have been validated by DEXA and assess both palpable and visual characteristics to correlate abdominal and subcutaneous fat as well as superficial musculature with degree of adiposity (Fig. 1.1).

Figure 1.1 Waltham S.H.A.P.E ™ guide for dogs



S.H.A.P.E.™ Score	Description
A	**Extremely Thin** Your dog has a very small amount or no total body fat. Recommendation: Seek veterinary advice promptly.
B	**Thin** Your dog has only a small amount of total body fat. Recommendation: Seek veterinary advice to ensure your dog is offered the appropriate amount of food. Reassess using the S.H.A.P.E.™ chart every 2 weeks.
C	**Lean** Your dog is at the low end of the ideal range with less than normal body fat. Recommendation: Increase food offered by a small amount. Monitor monthly using the S.H.A.P.E.™ chart and seek veterinary advice if no change.
D	**Ideal** Your dog has an ideal amount of total body fat. Recommendation: Monitor monthly to ensure your dog remains in this category and have him/her checked by the veterinarian at your next visit.
E	**Mildly Overweight** Your dog is at the upper end of the ideal range with a small amount of excess body fat. Recommendation: Seek veterinary advice to ensure your dog is offered the appropriate amount of food and consider increasing activity levels. Avoid excessive treats and monitor monthly using the S.H.A.P.E.™ chart.
F	**Moderately Overweight** Your dog has an excess of total body fat. Recommendation: Seek veterinary advice to implement safely an appropriate weight loss plan including increasing activity levels. Reassess using the S.H.A.P.E.™ chart every 2 weeks.
G	**Severely Overweight** Your pet has a large amount of excess total body fat that is affecting its health and well being. Recommendation: Seek veterinary advice promptly to introduce a weight loss plan to reduce your dog's weight, increase activity levels and improve health.
NB: Some breeds and different life-stages may have different ideal S.H.A.P.E scores. Consult your veterinarian if you are unsure.	

TM Registered Trademark © 2005 Mars, Incorporated

Obesity is one of the most common medical disorders of dogs and, the most common form of malnutrition in dogs in western society (Edney & Smith, 1986; Mawby *et al.*, 2004; McGreevy *et al.*, 2005). Unfortunately, the study of the pathogenesis and treatment of naturally-occurring obesity in animals has not kept pace with the perceived increase in the frequency of the disease (Buffington, 1994). Previous estimates of the prevalence of obesity in clinical practice have suggested that 25 to 33% of dogs are overweight (Markwell *et al.*, 1990). However, recent figures from Australia and France estimate 33.5% and 38.8% of dogs as being overweight, while 7.6% and 5% as obese (McGreevy *et al.*, 2005; Colliard *et al.*, 2006; Lund *et al.*, 2006; Holmes *et al.*, 2007).

Factors which are thought to contribute to this include living indoors, inactivity, age (with older dogs being increasingly predisposed) (Donoghue *et al.*, 1991; McGreevy *et al.*, 2005; Colliard *et al.*, 2006) and being neutered (Edney & Smith, 1986; Markwell *et al.*, 1990; Colliard *et al.*, 2006). Neutering has been shown to be one of the predisposing factors for obesity in the dog, with effects on energy balance through increased appetite coupled with reduced levels of activity. In addition, neutering may predispose animals to obesity by reducing the concentration of oestrogens and androgens that induce behaviours associated with breeding, including searching for a mate. Furthermore, oestrogen contributes to leptin sensitivity in the central nervous system (CNS), its removal may therefore contribute to leptin resistance in companion animals (Clegg *et al.*, 2006) (Mayes & Watson, 2004).

Over the past few hundred years considerable inbreeding has occurred in the dog population to generate specific genetic isolates referred to as breeds. These have both physical and in some cases physiological characteristics which are specific to a certain breed and which differ among the dog population (Sutter & Ostrander, 2004). One such example is hip osteoarthritis due to degenerate joint disease, which is seen in German shepherd dogs, rottweilers, Labrador retrievers and golden retrievers (Smith *et al.*, 2001).

Overweight and obesity has been linked to breeds such as the Labrador retriever (Colliard *et al.*, 2006), cocker spaniel, long-haired dachshunds, Shetland sheepdogs, cairn terriers, Cavalier King Charles spaniels, beagles and basset hounds (Edney &

Smith, 1986). Moreover, it has been suggested that obese owners may be more likely to own obese dogs (Mason, 1970; Edney & Smith, 1986; Holmes *et al.*, 2007). However, it is not clear whether this is related to a failure to exercise their dogs, a relationship between overeating and overfeeding or a failure to recognise obesity in their pets. It is likely to be a complex mixture of these, with many owners failing to estimate accurately the weight of their dogs or to consider their pet as overweight (Mason, 1970; Colliard *et al.*, 2006).

1.1.2 Metabolic syndrome

The metabolic syndrome as defined in humans, represents a group of metabolic and vascular disorders which increase the risk of an individual developing type II diabetes mellitus and cardiovascular disease (Reisin & Alpert, 2005). Over the past two decades, the definition has been modified to include measures of blood pressure and albumin-to-creatinine ratio due to the rising levels of diabetic nephropathy (Hossain *et al.*, 2007). The most recently described definition was proposed by the International Diabetes Federation, and it requires the presence of central (visceral) obesity with specific ethnic cut-off points plus two additional factors described in Table 1.1. Adopting ethnic-specific criteria, improves the usefulness of the term 'metabolic syndrome' in identifying individuals at risk from obesity related co-morbidities (Banerjee & Misra, 2007).

Type II diabetes mellitus, also known as non-insulin-dependent diabetes, results from an inability of the tissues in the body to respond appropriately to insulin produced by pancreatic β-cells (Yach *et al.*, 2006). Its prevalence has increased dramatically in the last decade, primarily because of the link with obesity. In the 1950s 200,000 people were diagnosed with type II diabetes in the UK, whereas in 2004 the figure stood at 1.8 million (Diabetes, 2005). In other countries such as the USA, it is estimated that the number of individuals diagnosed with diabetes will increase by 165% between the years 2000 and 2050, with the most rapid increases occurring in older and minority populations (Narayan *et al.*, 2003). The risk of developing diabetes increasing with increasing BMI; individuals with a BMI >30 kg/m^{-2} are 10 times more likely to develop type II diabetes mellitus than those with a BMI < 25 kg/m^{-2} (Ayata *et al.*, 2000).

Cardiovascular disease is also strongly associated with obesity, especially central obesity, independent of gender (Carr & Brunzell, 2004). The obese have higher incidences of coronary heart disease, arthrosclerosis, hypertension and dyslipidaemias (Shaw *et al.*, 2005). A meta-analysis, that collected data from 26 separate studies, found coronary heart disease to be the most common cause of premature death in obese individuals (McGee, 2005). Furthermore, a 16-year prospective study has linked obesity with an increased risk of death from various forms of cancer, such as those of the breast, prostate and ovary in individuals in the United States (Calle & Thun, 2004). Other co-morbidities associated with obesity in humans include renal disease (mainly characterised by diabetic nephropathy) (Rutkowski *et al.*, 2006), osteoarthritis (Pottie *et al.*, 2006) and respiratory impairment (including obstructive sleep apnoea) (Vgontzas *et al.*, 2003).

Table 1.1 Medical criteria used to define the Metabolic Syndrome

Criteria	Defining levels
Serum triglyceride level, mg/dl	>150 or specific treatment
Serum high density lipoprotein cholesterol, mg/dl	< 40 (male), <50 (female) or specific treatment
Blood pressure, mmHg	≥130/85
Fasting plasma glucose, mg/dl	≥100
Albuminuria, mg/g albumin-to-creatinine ratio	≥30

Table adapted from (Diabetes, 2005).

In a manner similar to humans, obesity has a detrimental effect on the health and longevity of dogs (German, 2006). Co-morbidities associated with obesity in dogs include orthopaedic disease where obesity is a major risk factor for the development of osteoarthritis (Kealy *et al.*, 2000; Smith *et al.*, 2001). A prospective clinical trial carried out in the United States found that weight reduction alone leads to a substantial improvement in clinical lameness in dogs suffering from osteoarthritis (Impellizeri *et al.*, 2000). Cats, unlike dogs, most often suffer from a condition which resembles type II diabetes in humans and obesity therefore is a major risk factor in this species (Nelson *et al.*, 1990; Feldhahn *et al.*, 1999).

In contrast, dogs more commonly suffer from a form of diabetes resembling type I diabetes mellitus in humans caused by an autoimmune-mediated destruction of pancreatic β-cells. Dogs with this form of diabetes are prone to developing ketoacidosis and need exogenous insulin for survival (Hoenig, 2002). In dogs, obesity causes marked insulin resistance leading to hyperinsulinaemia and impaired glucose tolerance (Bailhache *et al.*, 2003; Larson *et al.*, 2003; Gayet *et al.*, 2004), and this has been linked to an increased risk of type II diabetes (Klinkenberg *et al.*, 2006; Lund *et al.*, 2006).

In a similar manner to obstructive sleep apnoea in humans, dogs experience an increased expiratory airway resistance with obesity (Bach *et al.*, 2007). In addition, obesity is an important risk factor for the development of tracheal collapse in small breeds of dogs (White & Williams, 1994). Other respiratory diseases which can be exacerbated by obesity include laryngeal paralysis and brachycephalic airway syndrome. Obesity also leads to abnormal lipid profiles in dogs in a similar manner to obese humans, characterised by increases in plasma triacylglycerol and very-low-density lipoprotein (VLDL) concentrations and lower plasma high-density lipoprotein (HDL) cholesterol concentrations (Bailhache *et al.*, 2003).

Furthermore, obesity is thought to be a risk factor for fatal acute pancreatitis in dogs (Hess *et al.*, 1999). Dietary restriction has been shown to increase longevity in both humans and rodents (Fontana & Klein, 2007), as well as dogs where it is also associated with a longer median time to first treatment for chronic diseases such as osteoarthritis (Kealy *et al.*, 2002; Larson *et al.*, 2003; Lawler *et al.*, 2005). Although animals also suffer from diseases associated with obesity, many of their specific disease-associations are different from those of obese humans (Buffington, 1994). For example, of the tumours commonly associated with obesity in humans, only adenocarcinoma of mammary tissue has been reported as significantly correlated with obesity in dogs (Sonnenschein *et al.*, 1991).

1.1.3 Treatment of obesity

Given the scale of the obesity epidemic and the profound implications for public health, treatment is directed towards those at greatest risk of developing co-morbidities associated with obesity. Although weight loss is one aim of therapy, the main goal is an improvement in metabolic parameters coupled with long-term weight loss (Wilding, 2007). The two most conventional approaches used are those of calorie restriction, usually involving both a decrease in the overall amount consumed as well as a switch to a low-calorie diet, combined with increased physical activity. Cognitive behavioural therapy is also vital in achieving long-term weight loss (Wilding, 2007).

Diet and exercise has been the cornerstone of weight management therapy in humans, but this has been found to have limitations, especially in terms of weight maintenance. Thus, pharmacotherapy has also become an important aspect of obesity management (Hanif & Kumar, 2002). Three drugs are currently licensed for the treatment of obesity in humans in the UK; these are orlistat, sibutramine and rimonabant (Wilding, 2007). Orlistat is a gastrointestinal lipase inhibitor which acts to reduce intestinal fat absorption. It has been shown to be both effective and cost-effective in the treatment of obese patients (Lacey *et al.*, 2005; Padwal & Majumdar, 2007). Sibutramine acts on the CNS to inhibit the reuptake of norephinephrine and serotonin thereby amplifying satiety signals inducing a sensation of fullness. In addition, it is thought to also have important thermogenic effects (Aronne, 2007). Rimonabant is a cannabinoid receptor Type 1 blocker preventing hyperphagia (Gelfand & Cannon, 2006). In clinical trials, both have been shown to produce greater weight loss than lifestyle alone and importantly, to help maintain weight loss (Wilding, 2007).

Thiazolidinediones (TZDs) are peroxisome proliferator-activated receptor (PPARγ) ligands which are used clinically to improve insulin sensitivity in type II diabetes patients. PPARs are ligand-dependent transcription factors which after ligand binding and activation are able to either enhance or repress expression of target genes. Three PPARs have been identified, each with differential expression patterns and physiological functions. PPARδ (also known as PPARβ) is ubiquitously expressed, with highest level of expression in skin, brain and adipose tissue. Deletion

of PPARδ in animal models causes alterations in these target tissues, such as impaired wound healing through effects on keratinocyte proliferation (Hammarstedt *et al.*, 2005). PPARα is predominantly expressed in the liver, heart, skeletal muscle and also in the vascular wall. It is involved in the regulation of genes related to fatty acid metabolism in the liver (Kersten *et al.*, 2000a).

PPARγ exists as two isoforms, PPARγ1 and γ2, that differ in their N-terminal end as a result of alternative promoter usage and differential splicing (Zhu *et al.*, 1995). PPARγ1 has a similar expression pattern to PPARα while PPARγ2 is predominantly expressed in adipose tissue where it regulates adipocyte differentiation and fatty acid storage (Ren *et al.*, 2002; Hammarstedt *et al.*, 2005). By activating PPARγ receptors in adipocytes and promoting adipocyte differentiation, these agents are thought to promote a redistribution of fat from liver and muscle into adipocytes, thereby improving insulin sensitivity (Shulman, 2000). PPARγ2 knock-out mice have a reduced fat mass, lower lipid accumulation and impaired insulin sensitivity (Zhang *et al.*, 2004). PPARγ agonists such as rosiglitazone (Avandia) and pioglitazone (Actos) have been used clinically to improve insulin sensitivity in type II diabetes. Rosiglitazone has been shown to improve insulin sensitivity directly by reducing inhibitory serine phosphorylation of insulin receptor substrate-1 (IRS-1) whereas pioglitazone improves insulin sensitivity by increasing IRS-2 gene expression (Hammarstedt *et al.*, 2005).

Another important class of PPAR agonists are fibrates. They lower plasma triglyceride levels by stimulating fatty acid oxidation and reducing apoCIII expression and increasing HDL levels by induction of apolipoprotein AI and apolipoprotein AII, both effects mediated by binding to PPARα. Fibrates may also have hypoglycaemic and thus anti-diabetic effects as a consequence of their hypolipidaemic action (Kersten *et al.*, 2000a).

Surgical procedures are currently only recommended for those suffering from severe obesity (BMI ≥40 kg/m^{-2} or ≥35 kg/m^{-2} with co-morbidities), who have not been able to lose weight through other treatment strategies. The three approaches most commonly used include laparoscopic gastric banding, gastric bypass or a duodenal switch (Wilding, 2007).

1.1.3.1 Treatment of obesity in dogs

In dogs, dietary therapy and increased level of exercise is paramount in developing an effective weight loss management programme. The reduction in the amount fed should be tailored to the individual patient and this is best achieved through the use of a purpose-formulated diet (German, 2006). An important development for the pharmaceutical management of body weight in dogs is the recent introduction of dirlotapide and mitratapide (EMEA, 2007; Wren *et al.*, 2007). These are microsomal triglyceride transfer protein inhibitors, which act primarily within the endoplasmic reticulum of enterocytes preventing the formation and release of chylomicrons into the lymphatics. This leads to fat malabsorption resulting in a 10% reduction in energy intake (Gossellin *et al.*, 2007).

Following administration, there also appears to be a marked reduction in food intake, which accounts for 90% of the total reduction in energy intake, and limited evidence suggests that this may be related to the release of peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) into the circulation (Wren *et al.*, 2007). PYY is a 36 amino acid peptide related to neuropeptide Y (NPY) and is co-secreted with GLP-1. Administration has been found to decrease food intake in both rodents and humans through inhibition of NPY production in the brain (le Roux & Bloom, 2005). Lifestyle management including increasing exercise and behavioural management (decrease in begging behaviour) is also essential (Yaissle *et al.*, 2004). Owner education is key to achieving long-term weight loss, with one study reporting that 31% of owners of obese pets considered their dog's weight to be 'just right' (Mason, 1970).

In human and companion animals, weight loss is accompanied by an improvement in glycaemic profile (Larson *et al.*, 2003; Phelan *et al.*, 2007), with a reduction in the odds of developing the metabolic syndrome (Phelan *et al.*, 2007). In dogs, as in humans, failure to maintain weight loss is a common outcome of strategies for weight reduction (Laflamme & Kuhlman, 1995). Thus, long-term monitoring and regular weighing are essential to ensure long-term weight loss (Yaissle *et al.*, 2004; German, 2006).

1.1.4 Obesity as an inflammatory state

Obesity is characterised by a state of chronic low-grade systemic inflammation (Trayhurn & Wood, 2004; Fantuzzi, 2005). Levels of inflammatory markers such as C-reactive protein (CRP) as well as several proinflammatory cytokines including interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα), are raised systemically in obese individuals compared with lean subjects (Das, 2001). Weight-loss in these subjects has been shown to reverse this low-grade inflammatory state and to improve insulin sensitivity (Manco *et al.*, 2007). As will be discussed in section 1.4, adipose tissue is an important source of these pro-inflammatory cytokines and acute phase proteins (Trayhurn & Wood, 2004; Ahima, 2006). Thus adipose tissue itself is thought to be an important source of raised circulating levels of these proinflammatory cytokines in obesity (Yudkin *et al.*, 1999), providing a link between obesity, insulin resistance and the metabolic syndrome (Trayhurn, 2005a; Phelan *et al.*, 2007).

One proposed explanation for the increased production of these pro-inflammatory cytokines is that the expansion of WAT in obesity leads to a general state of hypoxia with a decreased oxygen perfusion of the tissue (Lolmede *et al.*, 2003; Trayhurn & Wood, 2004; Trayhurn *et al.*, 2008). This leads to increased levels of pro-inflammatory cytokines in response to the transcription factor hypoxia inducible factor-1α (HIF-1α) (Wood *et al.*, 2007). HIF-1α is a heterodimeric protein composed of α and β subunits. The β subunit is constitutively expressed, whilst expression of the α-subunit is induced under hypoxic conditions leading to a functional protein (Wenger, 2002). It is a key transcription factor involved in cellular response to hypoxia, and studies have shown both a markedly decreased production of the insulin-sensitising hormone adiponectin and increased expression of PAI-1 through induction of HIF-1α (Chen *et al.*, 2006).

Another proposed mechanism for the observed activation of inflammatory pathways in obesity is a link to the induction of stress in the endoplasmic reticulum, which is involved in protein synthesis and post-translational processing, leading to impaired insulin signalling (Ozcan *et al.*, 2004). Furthermore, studies in rodents and humans have also linked obesity to an increase in the production of reactive oxygen species (ROS) within WAT itself, leading to oxidative stress and elevated production of pro-

inflammatory markers (Furukawa *et al.*, 2004). It is important to note that the different proposals for the basis for the inflammatory response in adipose tissue in obesity are not mutually exclusive; where, for example, hypoxia has been reported to induce endoplasmic reticulum stress and the generation of ROS (Koumenis *et al.*, 2002; Carriere *et al.*, 2004).

Macrophages are components of adipose tissue and actively participate in its activities (Fantuzzi, 2005). The number of macrophages present in WAT is directly correlated with adiposity and with adipocyte size in both humans and mice, with no significant differences between depots (Weisberg *et al.*, 2003; Xu *et al.*, 2003; Curat *et al.*, 2004). Despite clear similarities between macrophages and adipocytes having been reported, and the fact that preadipocytes can differentiate into macrophages, these two cell types are distinct (Cousin *et al.*, 1999).

Experiments have shown that WAT macrophages are derived from the bone marrow, indicating that macrophages present in WAT do not derive *in situ* from differentiation of preadipocytes, but rather from circulating monocytes infiltrating WAT (Weisberg *et al.*, 2003). This process is augmented in the obese with the increased expression of monocyte/macrophage chemoattractant protein-1 (MCP-1) (a chemoattractant for monocytes and macrophages) and macrophage migration inhibitory factor (MIF) (responsible for increasing the life span of macrophages thus prolonging inflammatory responses) by WAT in the obese (Weisberg *et al.*, 2003; Dahlman *et al.*, 2005; Skurk *et al.*, 2005a).

Obesity is thought to induce a phenotypic switch in adipose tissue macrophage phenotype from an M2 or 'alternatively activated' state in lean individuals to an M1 or 'classically activated' state in the obese, capable of increasing gene expression of pro-inflammatory cytokines such as TNFα, which (as will be discussed in section 1.4.3), is thought to have an important role in the development of insulin resistance (Permana *et al.*, 2006; Lumeng *et al.*, 2007; Zeyda *et al.*, 2007). Thus the arrival of macrophages is likely to result in a significant amplification of the inflammatory state within adipose tissue, involving extensive cross-talk between macrophages and adipocytes themselves (Trayhurn, 2005a).

1.1.5 Study of obesity

The study of obesity and energy balance has been greatly aided by the use of several rodent models. The obese nature of these rodents may result from spontaneous mutations, or deliberately induced via genetic manipulation. The most well characterised model used for the study of obesity has been the *ob/ob* (obese) mouse, which displays clinical signs such as type II diabetes mellitus, hyperphagia, and profound obesity (Zhang *et al.*, 1994). A recessive mutation of the *Ob* gene (leptin) on chromosome 6 was found to result in the non-functional form of the leptin hormone seen in affected mice. The Zucker *fa/fa* (fatty) rat and its mouse homolog the *db/db* (diabetic) mouse, also display the obese phenotype as well as type II diabetes mellitus. However, in both these models, the defect is due to a point mutation in the gene coding for the leptin receptor (Chua *et al.*, 1996).

The yellow A^y (Agouti) mouse develops the obese phenotype, moderate hyperphagia and reduced thermogenesis due to over-expression of agouti peptide in the hypothalamus leading to antagonism of the melanocortin 4 receptor (MC4R) (Beck, 2000). A splicing defect in the *tub* gene leads to mature-onset obesity and neurosensory deficits in the *tubby* mouse. Although *tubby*-like proteins are thought to function in intracellular signalling, possibly in relation to insulin metabolism, their function is as-yet not fully understood (Carroll *et al.*, 2004). A mutation in the gene coding for carboxypeptidase E in the *fat* mouse results in very low levels of this protein (which is involved in proinsulin and pro-opiomelanocortin (POMC) production) and dysregulation of several other neuropeptides, leading to obesity despite the absence of overt hyperphagia (Beck, 2000). Further insights into the molecular mechanisms underlying the development of obesity have arisen through the use of transgenic and knockout mice, in which the aim has been to identify the genetic factors that underlie the complex, polygenic inheritance of obesity (Brockmann & Bevova, 2002).

At the time of writing, there are no recognised molecular models of obesity in the dog. However, the dog offers distinct advantages over rodents in the study of obesity: firstly, obesity can be induced in the dog through modest changes in dietary composition (Kim *et al.*, 2003); second, the dog has a long history of accurate methods for metabolic assessment; third, longitudinal studies may be performed

allowing multiple physiologic measurements and full control over dietary intake and assessment of energy expenditure (Bergman *et al.*, 2007). In addition, dog breeds offer all the advantages of geographically isolated human populations but with a higher degree of isolation, narrower bottlenecks and much better genealogical records (Ostrander & Kruglyak, 2000).

Many of the ~ 400 modern dog breeds also show a high prevalence of specific diseases including cancers, heart disease, epilepsy, cataracts and hip dysplasia (Patterson, 2000). Most of these diseases are also commonly seen in the human population and the clinical manifestations in the two species are often similar (Sargan, 2004). The high prevalence of specific diseases in certain breeds suggests that a limited number of loci are involved, making their identification easier in the dog than humans (Chase *et al.*, 2002).

In 2005, the dog became the fifth mammal to have its genome fully sequenced (Lindblad-Toh *et al.*, 2005). More than 650 million base pairs (>25%) of the dog sequence align uniquely to the human genome (Kirkness *et al.*, 2003). The canine genome is a powerful tool for identifying genetic factors which contribute to human health and disease (Ostrander & Comstock, 2004) (Mellersh, 2007). It is hoped that over the next few years advances in several areas of disease research, including that of the inheritance of common skin diseases such as atopic dermatitis in both dogs and humans, will help to improve the understanding of the genetic basis of disease (Pennisi, 2007).

1.2 Energy balance and body weight regulation

On a short term basis (hour-to-hour), energy intake and expenditure may not match. This is why it is necessary to have short-term storage compounds such as glycogen and triacylglycerol (TG). In the long term, if energy intake consistently exceeds energy expenditure, triglycerides (TGs) accumulate in WAT leading to obesity.

1.2.1 Energy intake

In order to maintain a relatively constant body weight, intake should be balanced over the long term by an equal amount of energy expenditure. Failure to do so, will

result in an imbalance, leading on one hand to weight gain (in the event of excessive intake, as outlined above) and on the other hand to wasting and cachexia (if there is a net energy deficit). Energy is used for basic metabolic processes (basal metabolic rate, BMR) and also for the performance of physical activity. This mechanism of long-term storage is vital for the survival of several species through hibernation and migration (Speakman *et al.*, 2007).

For the majority of adult animals, energy intake is normally closely matched to energy expenditure leading to the maintenance of a stable body weight (Frayn, 2003). Therefore, a period of weight gain due to overfeeding would be compensated for by a subsequent reduction in meal size, inducing weight loss until the original body weight is regained. Conversely, depletion of fat stores due to decreased intake would be reversed by increased intake once food is again freely available. This led to the concept of a 'set-point' for body weight originally suggested by the British physiologist G.C. Kennedy, in the 1950s, linking central control of energy intake by the brain with the body's fat stores (Kennedy, 1953).

1.2.1.1 Central control of appetite

Over half a century ago, the hypothalamus emerged as an important site for appetite regulation. This was supported by studies showing that lesions of specific hypothalamic areas generate marked and consistent effects on the regulation of body weight. For instance, bilateral lesions of several of the mediobasal hypothalamic nuclei, including the arcuate nucleus (ARC), the ventromedial nucleus (VMN) or paraventricular nucleus (PVN) leads to marked hyperphagia, weight gain, decreased physical activity and neuroendocrine abnormalities such as hypogonadism (Stanley *et al.*, 2005). Conversely, lesions of the lateral hypothalamic area (LHA) induce hypophagia, increased energy expenditure and weight loss (McMinn *et al.*, 2000).

More recent studies suggest that these areas contain peptide systems which are important in body weight regulation. Thus, neuronal circuits signalling through specific neuropeptides are vital in the regulation of energy homeostasis. Identification of these central effectors is an ongoing process that is accomplished, in part, by quantifying changes in expression and release of neuropeptides in candidate

hypothalamic areas in response to interventions that alter energy balance, for example food restriction and re-feeding (McMinn *et al.*, 2000).

Within the ARC, there are two distinct subpopulations of neurones associated with food intake. One has a medial location and expresses orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), whilst the other has a more lateral location and express anorexigenic peptides cocaine and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC). Peripheral signals, particularly leptin, act on these neurones, which project to secondary hypothalamic nuclei and the CNS, where the release of further peptides is modulated (Stanley *et al.*, 2005).

The most potent orexigenic neuropeptide currently known is NPY, with central administration resulting in marked hyperphagia and chronic obesity. There are several known NPY receptors all of which are expressed in the hypothalamus. Whilst NPY may mediate its effects via a combination of these receptors, the Y_1 and Y_5 subtypes appear to be the most important with regard to the regulation of energy homeostasis. NPY has recently been classified as an adipokine being expressed and secreted by human adipocytes (Kos *et al.*, 2007). Adipocyte derived NPY is thought to mediate a reduction in leptin secretion and thus may have implications for the central control of appetite (Kos *et al.*, 2007). AgRP and NYP neurones are co-localised within the ARC with AgRP acting as a potent agonist to both melanocortin 3 and 4 receptor (MC3R and MC4R), thereby antagonising the effects of α-MSH (α-melanocyte stimulating hormone) a potent anorexigenic peptide (Stanley *et al.*, 2005).

There is increasing evidence that the endocannabinoid system plays a central role in regulating metabolism and body composition by enhancing the central orexigenic drive and increasing peripheral lipogenesis. The cannabinoids exert their pharmacological action through the interaction with specific receptors CB_1 and CB_2 (cannabinoid receptors 1 & 2). The CB_1 receptors are primarily located in the brain and adipose tissue, but are also found in the myocardium, vascular endothelium and sympathetic nerve terminals, while CB_2 receptors are primarily located in lymphoid tissue and peripheral macrophages (Gelfand & Cannon, 2006). Both

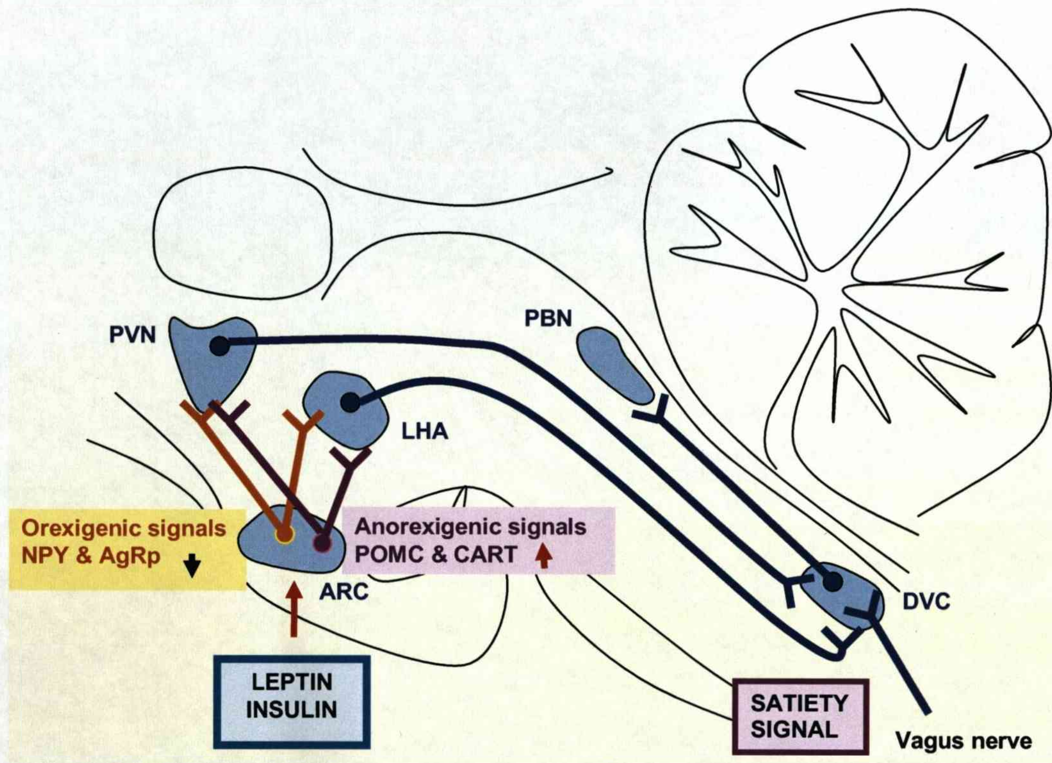
endocannabinoid receptors and their ligands are present in all tissues which play an important role in the regulation of food intake. However, it is the CB_1 receptor which is mainly responsible for the orexigenic effect of endogenous cannabinoids (Gelfand & Cannon, 2006). Antagonism of this receptor by specific drugs such as rimonabant leads to hypophagia and weight loss, and as mentioned earlier, it is currently marketed for the treatment of human obesity. Other orexigens include MCH (melanocortin concentrating hormone), orexin-A, galanin and nitric oxide (Stanley *et al.*, 2005).

In contrast to orexigenic peptides, anorexigenic peptides suppress appetite, and these include α-MSH, which is derived from POMC expressed by the ARC neurones, and is the main endogenous ligand for MC3R and MC4R (McMinn *et al.*, 2000) (Fig. 1.2). CART has been found to be co-expressed with α-MSH in the lateral ARC. Food deprivation induces a marked decrease in CART mRNA within the ARC, while peripheral administration of leptin has the opposite effect. Other anorexigenic neurotransmitters include serotonin and glucagon-like peptide 1 (GLP-1), neurotensin and corticotrophin releasing factor (CRF) (Wilding, 2002).

The criteria defining candidates for the long-term hormonal control of appetite regulation (Schwartz & Porte, 2005) include:

- Circulating plasma levels should be proportional to body fat content
- The factor should be able to enter the brain in proportion to its circulating level
- Administration of the signal would have anorexigenic effects, whilst a deficit of the signal should have the opposite effect
- A signal transduction pathway mediating the effects of the signal should be present in areas of the brain known to control appetite.

Figure 1.2 Neuroanatomical model representing regulation of food intake and body weight



Leptin secrected from WAT and insulin from pancreatic β-cells both circulate in proporition to adipose stores and both hormones entre the CNS through a saturable uptake mechanism, the blood brain barrier. Once in the CNS, they bind their receptors located on neurones of the ARC and alter the expression of neuropeptides responsible for the regulation of energy balance; anabolic neurones (NPY/AgRP) are inhibited, and catabolic neurones (α-MSH/CART) are upregulated. PVN, praventricular nucleus; DVC, dorsal vagal complex; LHA, lateral hypothalamus; ARC, arcuate nucleus; PBN, parabrachial nucleus.

Insulin can be considered as one such signal since plasma levels rise in adiposity. Binding sites for insulin have been localised to nuclei of the brain associated with energy homeostasis and a saturable transport system that carries insulin from the circulation into the brain has been identified. The binding of insulin to its recepots centrally leads to appetite suppression and, therefore, to a decrease in energy intake. Although insulin remains a candidate humoral adiposity signal, several observations suggest that additional peripheral signals such as leptin are also vital to body weight regulation (McMinn *et al.*, 2000). Leptin has pervasive effects on the hypothalamic control of food intake by upregulating the expression of anorexigenic neuropeptides and downregulating that of orexigenic neuropeptides (Trayhurn & Bing, 2006), these will be discussed in greater detail in section 1.4.1

1.2.1.2 Peripheral signals

The long-term regulation of food intake, involves the hypothalamic effects of insulin and leptin. In contrast, short-term control of food intake on a meal-to-meal basis involves neural and humoral signals from the gastrointestinal tract and liver generated in response to nutrient intake (McMinn *et al.*, 2000). Generally, these signals serve to produce satiety, bringing about the end of a meal. One of these hormones is cholecystokinin (CCK). This is released from the duodenum after food ingestion and acts to enhance satiety both at a local level by acting on CCK-A receptors, and centrally within the hypothalamus through CCK-B receptors (Wilding, 2002). Peptide YY (PYY) is secreted from L cells in the intestine, its level of release being closely correlated with the level of calorie intake. Administration of PYY has been shown to reduce food intake in both rodents and humans, and it may also act on NPY neurones in the ARC inducing anorexia (Stanley *et al.*, 2005).

Ghrelin is a potent orexigenic factor produced by gastric oxyntic cells and is the natural ligand of the growth hormone secretagogue receptor (GHS-R). Ghrelin secretion rises during fasting and is suppressed following feeding. Administration of ghrelin strongly stimulates endogenous growth hormone release centrally through binding to hypothalamic and pituitary GHS-R (Espelund *et al.*, 2005). Ghrelin is derived from a prohormone by posttranslational processing. It has been established that another peptide exists, that is also derived from proghrelin, and whose effects on appetite are opposite to those of ghrelin; this peptide has been named obestatin

(Zhang *et al.*, 2005). Obestatin appears to act as an anorexic hormone decreasing food intake, gastric emptying and jejunal motility (Zhang *et al.*, 2005). Other satiety hormones produced by the gut include pancreatic polypeptide (PP), bombesin and glucagon (Stanley *et al.*, 2005).

1.2.2 Energy expenditure

Energy is continuously being expended over a 24 h period. Some of this energy represents requirements for BMR, physical activity and diet induced thermogenesis. The BMR is closely related to the amount of non-fat tissue or lean body mass. The larger the fat free mass, the higher the BMR. BMR is also regulated by hormones, primarily thyroid hormone. During conditions of starvation or food deprivation thyroid hormone levels fall and BMR decreases (Frayn, 2003). As mentioned earlier (see section 1.1.4) increasing energy expenditure, by increasing levels of exercise, goes hand-in-hand with methods of decreasing food intake used to treat obesity.

1.2.2.1 Thermogenesis

Thermogenesis (heat production) plays a role in all metabolic processes and consists of two major categories, obligatory and facultative (Himms-Hagen, 2001). Obligatory thermogenesis is the equivalent of BMR, whereas facultative thermogenesis represents heat production which can be switched on and off according to different circumstances. Facultative thermogenesis can be further subdivided into heat generated by shivering of skeletal muscles in response to cold (cold-induced shivering), non-shivering thermogenesis, voluntary activity (exercise), non-exercise activity (fidgeting) and diet-induced thermogenesis. Adaptive thermogenesis occurs in response to changes in environmental temperature or diet, with the aim of maintaining body temperature or energy homeostasis. Adaptive thermogenesis has a genetic component, and can compensate for the prescribed energy deficit in weight loss programs (Major *et al.*, 2007).

Non-exercise activity thermogenesis is argued to be a valuable component of energy expenditure in humans, especially for those living a sedentary lifestyle. It has been proposed to be a key factor in the inter-individual variation in human susceptibility to gain weight, as activation of non-exercise induced thermogenesis in response to higher energy intake may help to dissipate excess energy and prevent weight gain

(Levine, 2004). Diet-induced thermogenesis is thought to have evolved as a mechanism for regulating energy balance and has been shown to take place in brown adipose tissue (BAT) (Rothwell & Stock, 1997). As with non-exercise induced thermogenesis, individual variation in the capacity to activate diet-induced thermogenesis (although not necessarily through BAT), may play a role in the propensity to develop obesity. Other mechanisms of thermogenesis have, of course been proposed; including substrate cycles and sodium pump (Himms-Hagen, 1981; Braun *et al.*, 1996).

1.2.2.2 Brown adipose tissue

Mammals contain two different types of adipose tissue: white and brown, which form part of 'the adipose organ' (Cinti, 2005). These have distinct anatomy and physiology and are organised in distinct depots, which can be broadly categorised as visceral or subcutaneous. BAT is specialised for the generation of heat through non-shivering thermogenesis and, in this tissue, the stored lipid droplets serve primarily as a fuel for the production of heat (Trayhurn, 2007). Compared with WAT, BAT has a rich vascular supply, dense with multiple capillaries. This, in combination with densely packed mitochondria at the ultrastructural level account for its 'brown' colour.

Unlike white adipocytes, brown adipocytes have a centrally located nucleus and lipids within brown adipocytes are organised into multiple smaller 'multilocular' droplets (Avram *et al.*, 2005). Noradrenergic fibres in WAT are mostly confined to the capillary wall, whereas in BAT they directly interface with the plasma membrane of individual adipocytes (Cinti, 2005). Increased sympathetic activity in BAT results in both hypertrophic and hyperplastic expansion of the tissue, combined with an increase in blood flow and utilisation of lipid and carbohydrate substrates for oxidative metabolism and increased heat production *per se* (Avram *et al.*, 2005).

The thermogenic ability of BAT has several physiological functions. The tissue was originally observed in hibernating mammals, being referred to as the 'hibernation gland' (Cannon & Nedergaard, 2004). In rodents, energy balance and body weight are subject to regulation by BAT thermogenesis in relation to diet-induced thermogenesis. Rats fed on a palatable 'cafeteria' diet increase their BAT activity

and do not become obese despite having a higher energy intake (Rothwell & Stock, 1983). Furthermore, experiments on transgenic mice have shown that over-activity of BAT results in resistance to obesity. However, hypoactivity of the tissue does not necessary lead to the obesogenic phenotype (Klaus, 2001a).

BAT is also present in humans where its development begins at around the 20th week of pregnancy and continues until shortly after birth. In the neonate and newborn, BAT can be found in several areas including: the interscapular region (shoulder), perivascularly, in the strap muscles of the neck, surrounding the abdominal aorta, the pancreas, adrenal glands and kidneys (Ricquier & Bouillaud, 2000). In small mammals, such as rodents, BAT persists throughout life. However, in large mammals and humans, until recently, it was thought that all brown fat underwent morphologic transformation to white adipocyte morphology and consequently that there were no discrete collections of BAT in human adults (Avram *et al.*, 2005). Recent evidence from studies using fluorodeoxyglucose positron emission tomography to trace tumour metastasis has identified BAT, with prevalence in the range of some tens of percent in the adult human population. Furthermore, as expected, BAT activity in man was found to be stimulated by cold and sympathetic nervous system (SNS) activity (Nedergaard *et al.*, 2007).

1.2.2.3 Mitochondrial uncoupling proteins

The thermogenic activity of BAT is mediated by the uncoupling of mitochondrial respiration. In BAT, the proton electrochemical gradient generated during the production of ATP by mitochondrial F_0-F_1 ATPase during β-oxidation of fatty acids is dissipated by a protein uniquely expressed by BAT; uncoupling protein 1 (UCP-1) (Dulloo & Samec, 2000). The net effect of activation of UCP-1 by the SNS, cold stimulus or diet is that substrate oxidation is effectively uncoupled from phosphorylation, with a resultant increase in heat production. Thermogenesis by BAT is also subject to hormonal control by insulin, thyroid hormone and possibly leptin via the SNS (Ricquier & Bouillaud, 2000; Klaus, 2001a). Brown adipocytes, including those from neonatal dogs, express a combination of adrenoceptor isoforms α_1, β_1, β_2 and β_3, with β_3-adrenoceptors being the most abundant and α_2 being completely absent in the case of the dog (Champigny *et al.*, 1991; Lin & Li, 2004).

The SNS acts on the tissue mainly via these receptors to induce UCP-1 gene expression and thereby modulate thermogenesis (Bray, 2004).

Over the past decade there has been a rise in interest in the roles of UCP homologues. These are members of the same UCP family based on their high sequence homologies and include UCP-2, UCP-3, UCP-4 and BMCP-1. Unlike UCP-1, which is expressed only in BAT, UCP-2 is expressed ubiquitously and UCP-3 is highly expressed in skeletal muscles and BAT, whereas UCP-4 and BMCP-1 are expressed solely in the brain (Dulloo & Samec, 2000). Despite the initial enthusiasm for the hypothesis that by analogy to UCP-1 in BAT, these UCPs may mediate thermogenesis in other tissues and thus control adaptive thermogenesis in sites other than in BAT, this is unlikely and their role in metabolic regulation is not yet fully understood (Ricquier & Bouillaud, 2000).

1.3 White adipose tissue

Obesity is characterised and defined by the expansion in adipose tissue mass. Adipose tissue can also be found diffusely associated around or within organs and also in close association with blood vessels (Cinti, 2001). Whereas BAT utilises TGs for the generation of heat through non-shivering thermogenesis, WAT stores TGs providing a long-term fuel reserve (Trayhurn, 2007). Discrete depots are predominantly composed of either white or brown fat, but both white and brown adipocytes may be found in any particular depot, which may reflect the ability of mature adipocytes to trans-differentiate between the two types (Cinti, 2005).

1.3.1 Morphology of white adipose tissue

Mature adipocytes comprise only one third of adipose tissue mass. The remaining two thirds is composed of a combination of small blood vessels, nerve tissue and fibroblasts collectively referred to as the stromal vascular (SV) fraction (Cinti, 2001). In addition, as alluded to earlier, adipose tissue may be infiltrated by macrophages, particularly in obesity (Weisberg *et al.*, 2003). Histologically, white adipocytes are spherical and much larger than their brown counterparts ranging in diameter from 15-150 μm (Cinti, 2001). Lipids within white adipocytes are normally organised within one large 'unilocular' droplet which occupies the majority of the intracellular

space, compressing the cytoplasm and the nucleus into a thin visible rim (Avram *et al.*, 2005). They also have fewer, less well-developed mitochondria when compared to brown adipocytes (Cinti, 2005). WAT is well-vascularised and receives both sympathetic and sensory innervation (Cinti, 2005). Recently, evidence has been presented for a parasympathetic supply to WAT (Kreier *et al.*, 2002; Romijn & Fliers, 2005). However, this has been seriously questioned with strong counter-evidence being presented (Giordano *et al.*, 2006).

1.3.2 Innervation of white adipose tissue

The SNS has regulatory influences on a wide range of WAT functions. In adipose tissue, catecholaminergic effects appear to be modulated by α_2 and β-adrenergic receptors, with β_3 being the key subtype present in rodents (Rayner & Trayhurn, 2001). Activation of β-receptor subtypes stimulates lipolysis, but the degree to which each subtype is involved in the lipolytic process varies according to fat pad, species, gender and degree of obesity (Lafontan *et al.*, 1995). The α_2-acrenoceptor, like β_3-adrenoceptors, is G-protein coupled but decreases rather than increases adenyl cylase levels (Rayner, 2001). Therefore, α_2-adrenoceptor stimulation may be expected to have the opposite effects to β_3-receptor stimulation.

Thus the effectiveness of catecholamine-stimulated lipolysis depends on the balance between β- and α_2-adrenoceptor activation. That is, when β-adrenoceptor activation predominates, lipolysis is stimulated via activation of hormone sensitive lipase (HSL). Conversely, when α_2-adrenoceptor activation predominates lipolysis is inhibited (Bartness & Bamshad, 1998). The β_3-adrenoceptor is the predominant β-subtype in adipose tissue of rodents (although not in humans were they have been estimated to be only 20% of the total population of β-adrenoceptors) (Rayner, 2001). In dogs, all 3 adrenoceptor subtypes have been detected and in a manner similar to humans, lipolysis appears to be mainly mediated thorough β_1 and β_2 subtypes with activation of the β_3-subtype only occurring at higher levels of catecholamine stimulation (Galitzky *et al.*, 1993).

Studies in rodents have shown that sympathetic denervation results in increased fat pad weight, while stimulation of sympathetic nerves to WAT leads to fatty acid secretion (Bartness & Bamshad, 1998). The SNS has also been strongly implicated in

the modulation of fat cell number. *In vivo* studies using Siberian hamsters have demonstrated that denervation of inguinal subcutaneous WAT depots promotes increases in fat cell number without affecting fat cell size. These differences in adipocyte proliferation between depots have been linked to neuroanatomical origins and function of the SNS innervation to each individual depot (Youngstrom & Bartness, 1998). The SNS has been shown to modulate the secretion of key proteins by adipocytes including that of the adipocyte hormone leptin (Trayhurn *et al.*, 1995). This will be discussed in greater detail in section 1.4.1.

1.3.3 White adipose tissue function

As demonstrated by various models of lipodystrophy, white adipose tissue is essential to the maintenance of energy and metabolic homeostasis, whereby the absence of WAT leads to marked insulin resistance, and other clinical signs including hypertrophy of skeletal and cardiac muscle as well as hepatic cirrhosis (Ganda, 2000). Over the past decade, the idea of WAT acting merely as a storage depot for TGs has become obsolete. With the discovery of the hormone leptin in 1994 (Zhang *et al.*, 1994), there has been an appreciation of WAT as an endocrine organ whose function in this respect will be discussed in detail in section 1.4.

1.3.3.1 Lipid metabolism

After a meal, adipose tissue buffers digested nutrients in the form of fatty acids stored as TG. This provides a reservoir of energy stores which can be utilised in times of fasting. The energy density of lipid is twice that of carbohydrate or protein (39.1 kJ/g compared to 15.4-17.5 kJ/g for protein and carbohydrate) and because, in contrast to carbohydrates, TGs can be stored with little associated water, lipid storage is 8- 12-fold more efficient in energy terms compared to either carbohydrate or protein (Klaus, 2001b). In states of positive energy balance, lipoprotein lipase (LPL) produced by adipocytes is exported to the capillary endothelium, where hydrolysis of TGs in lipoprotein particles occurs releasing fatty acids which can diffuse into the interstitial space reaching the adipocyte. The activity of LPL in adipose tissue is stimulated by insulin. Chronic treatment of human adipocytes with insulin has been shown to stimulate both lipolysis and LPL protein expression (McTernan *et al.*, 2002b). *In vivo,* adipose tissue LPL activity is mediated in response to a post-prandial elevation in insulin and blood glucose concentration.

In states of negative energy balance, energy is released from fat stores by lipolysis by HSL. The activity of HSL is very closely regulated by phosphorylation brought about by raised levels of cyclic adenosine 3', 5'-monophosphate (cAMP), in response to adrenaline (systemically) or noradrenaline (released by sympathetic nerves) (Frayn, 2003). Although the SNS is the major regulator of lipolysis in WAT, there is also regulation via nutrient and hormonal signals, including glucose and insulin (which both decrease the activity of HSL) (Ahima & Flier, 2000) (Bartness & Bamshad, 1998). The increase in insulin-induced lipolysis in human adipocytes is not explained by a concomitant rise in HSL expression. This may explain, in part, why hyperinsulinaemia coexists with increased circulating non-esterified fatty acids and increased adiposity in humans suffering from obesity and type II diabetes mellitus (Trayhurn *et al.*, 2008) (McTernan *et al.*, 2002b).

1.3.3.2 Glucose metabolism

Adipocytes contribute to glucose metabolism in several ways. First, adipose tissue, in conjunction with heart and skeletal muscle, are the main tissues to express the insulin-dependent glucose transporter (GLUT 4). Once within the adipocyte, glucose provides the substrate for *de novo* fatty acid and glycerol synthesis (lipogenesis). Second, increased lipolysis of TGs in adipocytes generates increased levels of circulating free fatty acids. In the liver, these have a negative regulatory role on several important functions including hepatic clearance of insulin and insulin-mediated suppression of hepatic glucose production, while at the same time exerting a positive effect on the rate of gluconeogenesis. The net effect of free fatty acids in the liver, therefore, results in the promotion of increased plasma glucose concentrations (Avram *et al.*, 2005).

1.3.3.3 Other functions of white adipose tissue

Additional functions of adipose tissue include thermal insulation which is particularly important in minimising heat loss in aquatic mammals. Adipose tissue also affords a degree of mechanical protection when interposed between body structures and soft tissues; an example is the eye socket area. Fat stores can also act as a source of 'metabolic water' (which is a bi-product of β-oxidation) (Klaus, 2001b).

1.3.4 Adipocyte differentiation and development

Adipose tissue mass can be increased in one of two ways: hypertrophy of existing adipocytes by increased intracellular lipid accumulation or hyperplasia of adipose depots by an increase in the number of mature adipocytes (Wabitsch *et al.*, 2003). The latter is achieved by inducing differentiation of pre-existing preadipocytes into mature adipocytes (Gregoire, 2001). These preadipocytes (fibroblast-like cells committed to becoming adipocytes) along with pleuripotent precursor cells found in adipose tissue can be induced to differentiate into mature adipocytes. Unlike many other cell types, this ability to increase the quantity of mature fat cells is retained into adulthood (Gregoire, 2001).

The process of adipocyte differentiation (adipogenesis) is complex and highly controlled. At the cellular level, committed preadipocytes have to withdraw from the cell cycle, undergoing a period of growth arrest, before conversion to mature adipocytes. This is normally achieved through contact inhibition. However, contact inhibition *per se* is not a pre-requisite for adipogenesis since cells plated at low densities in serum-free medium still undergo differentiation (Gregoire, 2001). Differentiation is also dependent on hormonal and nutritional signalling, where insulin (in supra-physiological concentrations) serves to accelerate lipid accumulation (Gregoire *et al.*, 1998). The onset of differentiation is associated with the promotion of gene expression and the initiation of a complex transcription cascade. Two families of transcription factors, CAAT/enhancer binding proteins α, β and δ (C/EBP-α and C/EBP-β and C/EBPδ) and the PPAR family of transcription factors (mainly PPARγ) have been shown to activate transcription of adipocyte specific genes and are involved in the growth arrest necessary for adipocyte differentiation (Gregoire *et al.*, 1998).

During adipogenesis, stimulation of C/EBP-β and δ occur early in clonal expansion in response to exposure of preadipocytes to adipogenic hormones (Lane *et al.*, 1999). These then induce the expression of PPARγ, leading to the production of PPARγ ligands. Once PPARγ has been activated, it then controls terminal adipogenesis by inducing expression of C/EBPα, which is required for the production of adipogenic genes and terminal differentiation (Farmer, 2005).

1.3.5 Adipose tissue and adipocyte studies

In vivo studies enable the full integrated physiological response to a stimulus to be characterised. Certain investigations, such as response to fasting/feeding or cold exposure, can only be conducted using living animals. Studies investigating the effect of compounds *in vivo* require the use of a range of techniques for the administration of different agents including oral administration, internal sampling via cannulae, and direct injection.

One key disadvantage of *in vivo* studies is that it can be problematic to identify the specific causal agent that leads to an effect, as certain stimuli may be acting directly or indirectly via downstream mediators. *In vitro* studies have the advantage in this regard, where if an effect is observed it is likely to be due to a specific and direct response to the applied stimulus. Disadvantages of *in vitro* work include the lack of interaction between cultured cells and endocrine and neural signals which would occur in the whole animal, and the absence of paracrine interactions with other cell types. In addition, substrate availability and clearance of metabolites is likely to differ in culture from that provided by the vascular system. As far as possible, treatment agents used *in vitro* are used at concentrations likely to be found at the cellular level *in vivo*.

Currently, three main *in vitro* systems are in use for investigating adipocyte physiology: isolated mature adipocytes, primary culture of preadipocytes and clonal cell lines. Mature adipocytes can be harvested from freshly dissected adipose tissue by collagenase digestion and once isolated, these cells are fragile and can only be maintained in culture for a short period (up to 48 h) (Fain *et al.*, 2003). It can be argued that this is the closest to the *in vivo* situation, as it involves only limited intervention in transferring the cells from the animal to the culture system. However, the *in vitro* culture of primary adipose cells, at least in rodents, may be associated with down-regulation of GLUT4 mRNA and the induction of many genes encoding inflammatory mediators such as TNFα and IL-6 (Ruan *et al.*, 2003). The mechanisms underlying these alterations are unknown, but there appears to be a strong link between the induction of these proinflammatory mediators and collagenase digestion (Ruan *et al.*, 2003).

Adipose cells differentiate well in culture, and *in vitro* differentiation leads to activation of most of the same set of genes characteristic of adipose tissue *in vivo* (Spiegelman *et al.*, 1993). This makes preadipocyte conversion an attractive model for the study of mammalian cell differentiation. Both primary culture and cell lines involve the differentiation of preadipocytes into mature adipocytes. There are three main agents (which form part of the 'adipogenic cocktail') used to induce differentiation *in vitro*; insulin, dexamethasone (representative of natural glucocorticoids) and 3-isobutyl-1-methylxanthine (IBMX; acts to raise intracellular concentrations of cAMP) (Gregoire, 2001). In addition, the use of PPARγ agonists, has also been adopted in several protocols as part of the adipogenic cocktail for *in vitro* primary adipocyte cell culture (Hauner *et al.*, 2001; Eisele *et al.*, 2005).

Primary culture involves the isolation of fibroblastic preadipocytes from adipose tissue, which are induced to differentiate into mature adipocytes (Hauner *et al.*, 2001). These cells can then be maintained in culture for a period of 2-4 weeks. A drawback to this technique is that a significant quantity of starting material is required, as preadipocytes make up only a small portion of WAT. In addition, careful isolation is needed in order to separate preadipocytes from fibroblasts and other components of the SV fraction. Cells in primary culture tend to have a low proliferative capacity and repeated subculture results in a rapid decline in their ability to differentiate into mature adipocytes. However, one significant advantage of the primary culture system is that it is only one step removed from the culture of mature adipocytes, and as much may be just as reliable (Hauner *et al.*, 2001).

Clonal cells lines such as murine 3T3-L1 and 3T3-F442A cells spontaneously arrest growth at confluence and can be induced to differentiate after treatment with adipogenic compounds (Spiegelman *et al.*, 1993). These have several advantages over cells isolated from tissue in that being immortal allows an endless supply of cells, as long as they are passaged and stored correctly. Clonal lines also provide a homogeneous cell population and therefore are more likely to yield consistent and reproducible results. One disadvantage is that due to genetic modifications which had to take place in order for them to gain their immortality (many such cell lines are

derived from tumour cells); their properties may differ from those isolated from tissues and cultured using primary culture.

Although the use of murine clonal cell lines such as the 3T3-L1 and 3T3-442A is well established, until very recently, few studies have described the use of such cell lines in human preadipocytes. One such study involves the generation of a human preadipocyte clonal cell line, 'human multipotent adipose-derived stem cells' (hMADS). These have been shown to display the key features of human adipocytes once differentiated *in vitro* (Rodriguez *et al.*, 2004; Dani, 2006). A human preadipocyte cell strain has also been shown to be a useful *in vitro* tool in the study of adipocyte metabolism. These cells are derived from the subcutaneous white adipose tissue of an infant with Simpson-Golabi-Behmel syndrome (SGBS). This is characterised by generalised tissue overgrowth and clinically affected individuals suffer from expanded subcutaneous WAT depots and other changes including a disproportionately large head with prominent features (Wabitsch *et al.*, 2001). Due to their lack of immortality these cells cannot be referred to as a cell line. However, they show a high capacity for adipose differentiation over many generations and the differentiated cells are functionally indistinguishable from human adipocytes differentiated in primary culture (Wabitsch *et al.*, 2001).

At present no canine adipose tissue cell lines have been characterised. Thus to date, work on canine adipocytes *in vitro* has involved the use of a primary cell culture system. Two methods currently in the literature describe the culture of primary preadipocytes from various depots, including visceral (omental, perirenal and gonadal) and subcutaneous (abdominal and inguinal-subcutaneous) (Wu *et al.*, 2001; Eisele *et al.*, 2005). Canine preadipocytes in primary culture can be induced to differentiate in a preconfluent state (Eisele *et al.*, 2005), in a similar manner to human primary cell culture (Hauner *et al.*, 2001). Therefore, cellular interaction does not appear to be a pre-requisite for differentiation. Both systems also make use of different 'adipogenic cocktails' with cortisol and the PPARγ agonist rosiglitazone used in one system in a similar manner to that used for human primary cell culture (Hauner *et al.*, 2001; Eisele *et al.*, 2005). While the use of a non-steroidal anti-inflammatory drug (NSAIDs) indomethacin is present in the adipogenic cocktail of the other published protocol (Wu *et al.*, 2001). Both systems appear to be equally

successful in terms of inducing preadipocyte differentiation, with cells from the subcutaneous inguinal depot being the most susceptible to differentiation *in vitro* (Wu *et al.*, 2001).

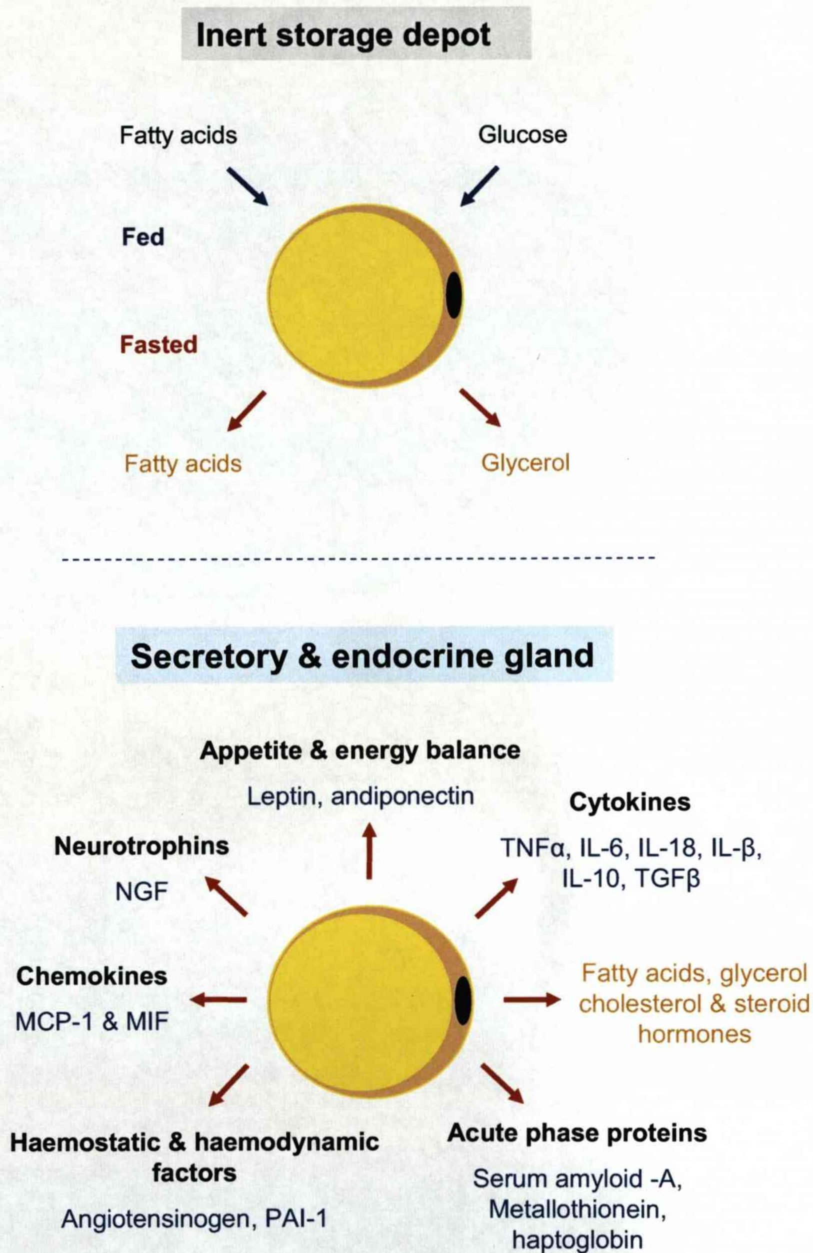
1.4 Endocrine function of white adipose tissue

Although once considered a passive fuel depot, adipose tissue is now recognised as an active endocrine organ that communicates with the brain and peripheral tissues by secreting a wide range of hormones and protein factors, collectively termed adipokines (Trayhurn & Beattie, 2001; Trayhurn, 2005c; Ronti *et al.*, 2006). Previously, these secreted proteins were referred to as adipocytokines (Fasshauer & Paschke, 2003; Pittas *et al.*, 2004). This is potentially misleading as it might be taken to infer that all adipocyte-secreted proteins are in fact cytokines, which is not the case. More than 50 of these different proteins have now been characterised; the 'adipokinome', together with lipid moieties released by the adipocyte, constitute the 'secretome' of the fat cell.

This description is restricted to proteins secreted by adipocytes themselves and not by WAT as a whole, as this would include proteins secreted by other cells types found in WAT such as macrophages (Trayhurn & Wood, 2004).

It is important to note that, quantitatively, the main components of the adipocyte secretome are fatty acids from TG lipolysis. Cholesterol and retinol are two lipid moieties secreted into the circulation by white adipocytes and, although the tissue is unable to synthesise steroids *de novo*, WAT expresses the enzymes necessary for the conversion of androstenedione to testosterone, androgens to oestrogens and oestrone to oestradiol. These are, therefore, also secreted by white adipocytes (Trayhurn & Beattie, 2001). The current view of the key biological function of WAT is summarised in Fig. 1.3. This includes key examples of its secretory products which will be discussed in further detail in later sections.

Figure 1.3 Evolving views of the biological functions of white adipose tissue



Adapted from (Trayhurn & Wood, 2004; Romijn & Fliers, 2005)

Adipocytes were previously considered to be inert storage depots releasing fuel as fatty acids and glycerol in time of fasting or starvation. It has become increasingly clear that adipocytes are endocrine glands which secrete important hormones, cytokines, vasoactive substances and other peptides. This figure outlines the range of such proteins secreted from WAT, although it does not aim to be a comprehensive list. Many of the proteins mentioned will be discussed in detail in the following sections. TNFα, tumour necrosis factor-α; IL, interleukin; PAI-1, plasminogen activator inhibitor-1; TGFβ, transforming growth factor-β; MIF, macrophage migration inhibitory factor; MCP-1, monocyte chemoattractant protein-1.

1.4.1 Leptin

The identification of the *Ob* (obese) gene and its protein product, leptin (also known as the OB protein) in 1994 (Zhang *et al.*, 1994), has been pivotal in the development of the current understanding of WAT as an endocrine organ (Trayhurn & Beattie, 2001). Leptin (Greek *leptos*, meaning thin or small), is synthesised as an 18 kDa pro-hormone, which is cleaved to yield a 16 kDa mature 'cytokine-like' protein. Although quantitatively, WAT is the main site of *Ob* gene expression and leptin secretion (both exclusively by differentiated adipocytes), leptin is also produced by several cell types in other organs. For example, in follicular papilla cells of hair follicles (Iguchi *et al.*, 2001), in the gastric cells (Cinti *et al.*, 2000), BAT (Moinat *et al.*, 1995), the placenta (Hoggard *et al.*, 1997a) and skeletal muscle (Wang *et al.*, 1998), and in oestoblasts (Reseland *et al.*, 2001). In each of these cases, it is probable that the effect of leptin is of a local (autocrine/paracrine) nature rather than endocrine (Trayhurn & Bing, 2006).

Despite the range of tissue specific sites of leptin synthesis, WAT is quantitatively the main site of production of the hormone and is thus the primary determinant of circulating levels. This is reflected in the close link between the degree of adiposity and circulating leptin levels (Maffei *et al.*, 1995). As mentioned earlier, the underlying mutation responsible for the obese phenotype of the *ob/ob* mouse is in the *Ob* gene itself, which thus encodes a non-functional leptin protein. The mutations responsible for the obese phenotype of *db/db* mice and *fa/fa* rat on the other hand, are in the leptin receptor gene (Chua *et al.*, 1996). Thus, obese mutants can occur involving either the hormone or its receptor. Although examples of human obesity due to mutations in the *Ob* gene and *Ob* receptors have been documented (Clement *et al.*, 1996; Montague *et al.*, 1997a), these are rare.

1.4.1.1 Functions of leptin

Leptin is a satiety factor, and there is no doubt that it has a substantial effect on food intake (Campfield *et al.*, 1995; Mercer *et al.*, 1997) Leptin also has a stimulatory effect on energy expenditure, thus interacting with both components of the energy balance relationship (Campfield *et al.*, 1995). Other functions of leptin include a key role in the reproductive system. *Ob/ob* mice are infertile, and exogenous leptin restores their fertility (Chehab *et al.*, 1996). Leptin has been increasingly described

as a general 'metabolic' hormone, in that there is an ever expanding range of processes with which it is thought to interact. These include stimulation of glucose utilisation (particularly in skeletal muscle) (Kamohara *et al.*, 1997), inhibition of insulin secretion from pancreatic β-cells (Emilsson *et al.*, 1997) and the stimulation of lipolysis in adipocytes (Fruhbeck *et al.*, 1997).

Leptin also has an important role in the regulation of insulin sensitivity, as demonstrated by the improved insulin sensitivity in a mouse model of severe lipoatrophic diabetes when leptin was over-expressed (Ebihara *et al.*, 2001). Leptin has also been shown to improve insulin action on both the inhibition of hepatic glucose production and in the stimulation of glucose uptake in rats (Barzilai *et al.*, 1997). Furthermore, leptin has been found to reduce significantly insulin-mediated glucose uptake in isolated mature human omental and subcutaneous adipocytes (Zhang *et al.*, 1999). Leptin appears to be a significant modulator of both immune and inflammatory responses, including in the activation of neutrophils, macrophages and natural killer cells and lymphocyte proliferation (Otero *et al.*, 2005).

1.4.1.2 Leptin receptors

The leptin receptor (Ob-R) is a product of the *Lepr* gene (Tartaglia *et al.*, 1995). Due to alternative splicing of a single gene product, the leptin receptor has a series of different splice variants (Ob-Ra to Ob-Rf) (Lee *et al.*, 1996). This level of variation is unsurprising given the range of functions of leptin. All splice variants share the same extracellular and transmembrane domains, but differ in the nature of their intracellular domains (Ahima & Osei, 2004). Of the short forms of the receptor, Ob-Ra has been found to be highly expressed in the choroid plexus and is thought to facilitate leptin transport across the blood-brain-barrier (McClain, 1998). Of these, the variant which has received most attention is Ob-Rb. This is the 'long form' of the receptor containing a full-length intracellular domain (Lee *et al.*, 1996).

The Ob-Rb receptor has been identified in a number of tissues, including the kidney, adrenal medulla, pancreatic β-cells and adipose tissue (Emilsson *et al.*, 1997; Guan *et al.*, 1997; Hoggard *et al.*, 1997b). *In situ* hybridisation studies have demonstrated the presence of the leptin receptor in several regions of the rat CNS, including the choroid plexus, the leptomeninges and importantly the hypothalamus, with Ob-Rb

having a particularly high level of expression in regions of the hypothalamus such as the ARC (Mercer *et al.*, 1996; Guan *et al.*, 1997). Further studies, looking at the presence of the Ob-Rb protein by immunohistochemistry, have further supported the hypothalamus as being a clear target for leptin (Hakansson *et al.*, 1998), which is in accordance with the role of this region in the brain in terms of the regulation of energy balance (Trayhurn *et al.*, 1999).

Ob-Rb regulates gene transcription via janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins (Vaisse *et al.*, 1996). Evidence also suggests that in addition to the JAK-STAT signalling pathway, leptin activates phosphatidylinositol-3 kinase (PI3K) and phosphodiesterase 3B (PDE3B) reducing cAMP levels in the hypothalamus, where intra-hypothalamic cAMP administration has been found to increase food intake in mice (Gillard *et al.*, 1997). The state of hyperleptinaemia in obesity will be discussed in detail in section 1.4.1.5.

1.4.1.3 Regulation of leptin expression and secretion

Plasma leptin concentrations are subject to complex regulation (Sahu, 2004; Howard & Flier, 2006). Systemically, leptin concentrations and are subject to diurnal variation with a nocturnal peak, although a pulsatile release pattern is also observed on top of this circadian rhythm (Fruhbeck *et al.*, 1998). The expression of the *Ob* gene and circulating leptin concentrations are acutely regulated by nutritional status, with a rapid fall in both occurring in rodents when subject to fasting, or acute cold exposure, and a substantial rise on overfeeding and re-warming (Trayhurn *et al.*, 1995). This is suggestive of an important role of the SNS in leptin production. This is supported by leptin gene expression being inhibited by the direct administration of noradrenaline and the sympathomimetic drug isoprenaline (Trayhurn *et al.*, 1995). These agents have subsequently been shown to exert the same effects on leptin production by adipocytes in culture (Gettys *et al.*, 1996; Mitchell *et al.*, 1997).

The production of leptin is therefore regulated by the SNS through a negative feedback mechanism (Mark *et al.*, 2003). Further evidence of this is provided by rodent studies where increases in leptin mRNA and circulating levels are seen after a blockade of noradrenaline synthesis (Rayner *et al.*, 1998). This negative feedback loop has also been found to exist in humans, as well as rodents, where the

administration of adrenaline (Carulli *et al.*, 1999) or isoprenaline (Pinkney *et al.*, 1998) to human subjects led to raised circulating levels of leptin. While in rodents, the SNS appears to mediate its effects on leptin through β_3-adrenoceptors (Gettys *et al.*, 1996; Trayhurn *et al.*, 1996); in humans it is likely to occur through β_1 and β_2 receptors (Rayner & Trayhurn, 2001).

Leptin production is also subject to control by other factors, particularly insulin, whose effect is likely to be mediated via the stimulation of glucose metabolism in adipose tissue (Havel, 2001). Administration of TZDs has been shown to inhibit leptin gene expression, indicating that the *Ob* gene is under the transcriptional control of PPARγ (De Vos *et al.*, 1996). Endogenous glucocorticoids such as cortisol and growth hormone may also regulate leptin secretion, although they are unlikely to play a major role (Havel, 2001). Oestrogen treatment has been shown to raise leptin concentrations (Castracane *et al.*, 1998) and increase leptin sensitivity (Clegg *et al.*, 2006), with exogenous testosterone having the opposite effect (Blum *et al.*, 1997). In addition, leptin mRNA levels have been found to differ between WAT depots. Higher levels are found in subcutaneous compared to visceral depots, and therefore, unsurprisingly higher levels are seen in women rather than men (Montague *et al.*, 1997b). Leptin levels have been shown to rise in response to acute infection, sepsis and increased levels of inflammatory cytokines such as TNFα and IL-1β (Sarraf *et al.*, 1997; Zumbach *et al.*, 1997; Faggioni *et al.*, 1998; Gualillo *et al.*, 2000).

1.4.1.4 The role of leptin in appetite control

Circulating leptin is transported into the brain across the blood-brain-barrier by a saturable process, which is independent of insulin (Banks *et al.*, 1996), where the short form of the leptin receptor (Ob-Ra) is thought to be particularly important. Once in the CNS, leptin interacts via the long-form of the receptor (Ob-Rb), with both orexigenic and anorexigenic pathways. The expression of orexigens such as NPY, MCH, AgRP and orexin-A are inhibited by leptin, while those of anorexigenic peptides such as POMC (the precursor to α-MSH), CART and CRF are upregulated by leptin (McMinn *et al.*, 2000). Furthermore, the endocannabinoid system is also considered to be inhibited by leptin, where acute leptin administration decreases the hypothalamic level of endogenous cannabinoids in both rats and the *ob/ob* mouse (Di Marzo *et al.*, 2001).

1.4.1.5 Leptin in the obese state; the concept of leptin resistance

Since the first reports of elevated serum leptin levels in the obese (Considine *et al.*, 1996), and the clear failure of these high levels to produce changes in energy intake, it has been proposed that the obese are 'leptin resistant' in a manner analogous to type II diabetics being resistant to the effects of insulin. However, there is little direct evidence for leptin resistance in humans (Arch *et al.*, 1998). In terms of the analogy with type II diabetes, insulin levels rise after a meal, not due to insulin resistance, but in response to a raised influx of glucose. Similarly, leptin might be raised in obesity not due to leptin resistance, but because of elevated fat stores (Arch *et al.*, 1998). Proposed mechanisms underlying leptin resistance in the obese include; decreases in the hypothalamic leptin levels (Banks *et al.*, 1999) and receptor expression; and impairment in leptin signalling, all offering increased susceptibility to diet-induced obesity in affected individuals (Zhang & Scarpace, 2006)

1.4.1.6 Leptin and companion animals

Rising levels of obesity in companion animals have led to increased research into molecular mechanisms underlying obesogenic pathways in these species (Iwase *et al.*, 2000b; Sasaki *et al.*, 2001). Leptin gene expression has been documented in all five main WAT depots in the dog, with expression being confined to mature adipocytes (Eisele *et al.*, 2005). In addition, canine adipocytes *in vitro* have been shown to express leptin. However, unlike human adipocytes (Wang *et al.*, 2004), expression has been documented in both pre- and post-differentiation (Eisele *et al.*, 2005), with higher levels of expression post-differentiation, as is also the case in both rodents and humans (Wang *et al.*, 2004; Eisele *et al.*, 2005).

In a similar manner to humans and rodents, plasma leptin concentrations in companion animals increase with rising levels of adiposity (Appleton *et al.*, 2000; Sagawa *et al.*, 2002; Kearns *et al.*, 2006). However, in dogs, unlike humans and rodents where leptin levels are higher in females, there appears to be no association between sex and systemic levels of leptin (Ishioka *et al.*, 2002; Ishioka *et al.*, 2007). In addition, it has been proposed that leptin has less of an effect in terms of decreasing food intake and body weight in obese animals than other factors such as neutering status, suggesting the presence of leptin resistance (Jeusette *et al.*, 2006).

However, a study investigating leptin concentrations in the cerebrospinal fluid (CSF) of lean versus obese dogs has shown no significant difference in levels before or after weight gain suggesting no decrease in leptin transport across the blood-brain-barrier (Nishii *et al.*, 2006). Thus, to date there is no evidence of impaired transport of leptin into the CNS or of a 'leptin resistant' state in the dog.

1.4.2 Adiponectin

Adiponectin, also called GB-28, apM1, AdipoQ and Acrp30, is an adipose tissue-specific protein, which circulates at high levels in human plasma (Diez & Iglesias, 2003). Adiponectin circulates as three discrete protein complexes in mice and humans: a 90-kDa trimer and a 180 kDa low molecular weight (LMW) hexamer, and a higher order complex (> 360 kDa) comprised of 4 to 6 trimers termed the high molecular weight (HMW) form. The LMW hexameric from of the protein is thought to be required for its basal activity. In contrast, the HMW form selectively disappears (with no increase in the LMW form) in mice treated with insulin or glucose, suggesting that HMW adiponectin complexes in serum represent a precursor pool that can be activated by metabolic stimuli and subsequently dissociate into the bioactive LMW form (Pajvani *et al.*, 2003).

Studies have shown that the ratio of HMW to HMW plus LMW forms, and not necessarily absolute levels of the different forms, can be used as a quantitative indicator of insulin sensitivity in humans (Pajvani *et al.*, 2004). Furthermore, there appears to be a sexual dimorphism in adiponectin levels in humans, with females having significantly higher circulating concentrations than males (Arita *et al.*, 1999). A similar dimorphism also exists in terms of adiponectin complexes; the vast majority of these in the male being the LMW form, whereas in females, the two complexes are more equally distributed (Pajvani *et al.*, 2003). In the dog, a protein band at 180 kDa, which is analogous to the LMW form in humans, has been identified. However, the HMW form has not yet been isolated via either sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or Western immunoblotting techniques. Despite this, data from velocity sedimentation studies has suggested that the HMW form is also present in canine serum (Brunson *et al.*, 2007).

1.4.2.1 Functions of adiponectin

Unlike most adipokines, adiponectin levels are inversely related to body weight, with reduced expression occurring in obesity and type II diabetes mellitus (Abbasi *et al.*, 2004). There also appears to be inverse correlations between plasma adiponectin concentrations and risk factors associated with cardiovascular disease, such as impaired vasoreactivity and levels of CRP (whose levels are closely related to parameters associated with cardiovascular disease and the metabolic syndrome) (Ouchi *et al.*, 2003; Putz *et al.*, 2004). Adiponectin has also been documented to have anti-artherogenic properties including as an endogenous angiogenesis inhibitor and thus may have therapeutic benefits in the treatment of angiogenesis-dependent diseases (Brakenhielm *et al.*, 2004).

Impaired glucose and lipid metabolism, a hallmark of obesity and type II diabetes mellitus, leads to an increase in lipid stores in insulin target tissues such as muscle and liver, thereby leading to insulin resistance (Shulman, 2000). Adiponectin acts as an insulin sensitiser by increasing fatty-acid oxidation, which reduces the TG content of these tissues in obese and type II diabetic mice (Yamauchi *et al.*, 2001). Complementary DNA-encoding adiponectin receptors (AdipoR1 and AdipoR2) have been cloned (Yamauchi *et al.*, 2003). AdipoR1 is found abundantly in skeletal muscle, whereas AdipoR2 is expressed predominantly in the liver. These receptors are suggested to mediate the insulin sensitising effect of adiponectin via an increase in activity of 5'-AMP-activated protein kinase (AMPK) (an enzyme concerned with fatty-acid oxidation and glucose metabolism in liver and skeletal muscle) (Yamauchi *et al.*, 2002).

Both receptors are expressed in human adipose tissue and isolated adipocytes, with levels of AdipoR1 expression being higher than those for AdipoR2. Thus it is possible that adiponectin may have similar effects on fatty acid oxidation and glucose metabolism in white adipose tissue (Rasmussen *et al.*, 2006). Evidence from rodent experiments also suggests that adiponectin may act centrally through the melanocortin pathway in mice to decrease body weight (Qi *et al.*, 2004), although the exact mechanism through which this is thought to occur remains to be elucidated.

Besides the well-established anti-artherogenic and insulin sensitising properties of adiponectin, evidence also supports a strong anti-inflammatory function (Tilg & Wolf, 2005). Studies have demonstrated that adiponectin suppresses production of the potent pro-inflammatory cytokine TNFα by monocytes, as well as attenuating its biological effects systemically in humans (Ouchi *et al.*, 2001). In addition, hypoadiponectinaemia has been associated with raised levels of several pro-inflammatory cytokines such as IL-6 and IL-8 and TNFα itself in obese individuals (Engeli *et al.*, 2003a), where it is suggested that raised levels of these endogenous cytokines may be directly responsible for the inhibition of adiponectin secretion (Bruun *et al.*, 2003a).

In humans, omental adipocytes *in vitro* have been found to express little adiponectin when compared to other depots. Furthermore, studies on samples obtained from diabetic patients have shown that once diabetic status is reached, non-visceral depots have a reduced capacity to express adiponectin. Thus, depot specific expression of adiponectin may influence the pattern of serum adiponectin concentrations and associated disease risk (Fisher *et al.*, 2002).

Administration of TDZs such as the PPARγ agonist rosiglitazone, which has potent anti-inflammatory effects, has been shown to increase serum adiponectin levels and is used clinically to treat type II diabetes mellitus in humans (Ghanim *et al.*, 2006). In experiments carried out in rodents, the use of a PPARα agonist has been shown to enhance the effects of rosiglitazone on serum adiponectin concentrations and the expression of AdipoRs in WAT of mice (Tsuchida *et al.*, 2005). Further evidence, of the role of adiponectin in obesity, comes from rodent studies where adiponectin enhances energy expenditure as well as impairing adipocyte differentiation in obese mice (Bauche *et al.*, 2007).

1.4.2.2 Adiponectin and companion animals

Plasma adiponectin concentrations have been found to decrease in dogs, in a manner similar to humans, with increasing body weight and similarly subsequent weight loss results in a rise in serum adiponectin levels (Ishioka *et al.*, 2005b). Like leptin, adiponectin gene expression has also been documented in all five main WAT depots in the dog, where expression is again confined to mature adipocytes and not cells of the SV fraction (Eisele *et al.*, 2005). Canine adipocytes *in vitro* also express mRNA for adiponectin and, in a manner similar to humans; expression is confined to mature adipocytes (Wang *et al.*, 2004; Eisele *et al.*, 2005). Serum concentrations of adiponectin in dogs have been found to range from 2.6 to 73.5 μg/ml in some reports (Ishioka *et al.*, 2005b), with others reporting much lower serum concentrations of the order of 0.85 to1.5 μg/ml (Brunson *et al.*, 2007). Those in the latter range are comparable to levels found in humans and rodents (Chandran *et al.*, 2003). It is widely recognised that atherosclerosis and type II diabetes are rare in dogs compared to other species (Ettinger & Feldman, 2000). Therefore, the role of adiponectin as an insulin sensitiser and whether high levels of the adipokine are associated with improvement in metabolic parameters in the obese dog remains to be proven.

1.4.3 Tumour necrosis factor-α

TNFα is synthesised as a 26-kDa transmembrane pro-hormone, which subsequently undergoes proteolytic cleavage to yield a 17-kDa soluble TNFα molecule. Despite differences in both size and location, both forms of TNFα are capable of mediating biological responses, and together may be responsible for both local and systemic actions of this cytokine. Plasma levels of TNFα are raised in obesity in both humans and dogs (Hotamisligil *et al.*, 1997; Gayet *et al.*, 2004). TNFα acts on two distinct cell surface receptors, type 1 and 2 (TNFR1 and TNFR2). To date, most known cellular functions of TNFα have been ascribed to signals transduced by TNFR1, with TNFR2 being thought to play a modulatory role in ligand trafficking. Both TNFα receptors can also be released from the cell surface through proteolytic cleavage and exist in soluble form. Circulating levels of these receptors have been found to be elevated in many pathological states including obesity (Hotamisligil *et al.*, 1997), and may serve to modulate the bioactivity of TNFα both spatially and temporally (Sethi & Hotamisligil, 1999). Binding of TNFα to either of its receptors, activates multiple kinases and phosphatases thereby utilising all major transduction pathways

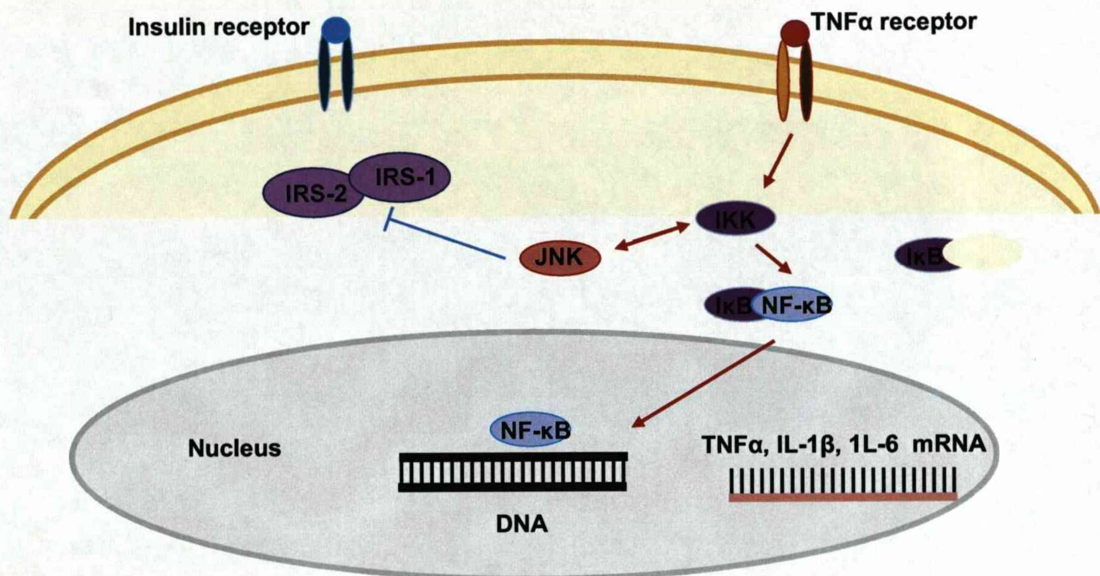
(Vilcek & Lee, 1991). One such pathway is that resulting in the activation of nuclear factor κB (NFκB), see Fig. 1.3.

1.4.3.1 Role of TNFα in insulin resistance

TNFα production by WAT is well documented, with neutralisation of the protein in obese rodent models leading to improvements in insulin sensitivity (Hotamisligil *et al.*, 1993). However, whilst TNFα is recognised as an adipokine being synthesised and released from adipocytes, it may be that, at least in humans, the majority of the production occurs in cells of the SV fraction of WAT (Fain *et al.*, 2004). With the increased influx of macrophages into WAT depots in the obese (Weisberg *et al.*, 2003), this could contribute significantly to the chronic inflammatory state seen in obesity.

The role of TNFα in insulin resistance is thought to occur at various levels, both centrally in the hypothalamus and in adipocytes themselves, with TNFα being firmly established as an important mediator of obesity-related insulin resistance (Hotamisligil *et al.*, 1993; Hotamisligil *et al.*, 1994; Hotamisligil *et al.*, 1995). Both diet and drug treatment of obesity has been shown to improve insulin sensitivity, and correlates with a decreased production of TNFα (Hotamisligil *et al.*, 1995). The inhibitory actions of TNFα can also be reversed by insulin-sensitising drugs such as TZDs, which *in vivo* coincide with a decrease in the levels of TNFα expression (Miles *et al.*, 1997). In terms of glucose transport, TNFα has been shown to down-regulate GLUT 4, thereby decreasing insulin-stimulated intracellular glucose transport in adipocytes (Stephens *et al.*, 1997). TNFα has also been shown to act on the proximal steps of insulin signalling by an increase in phosphorylation of IRS-1 at serine residues, inhibiting down-stream events of the insulin signalling pathway (Hotamisligil *et al.*, 1994), see Fig 1.4. Such modification of IRS-1 has been observed in obesity and insulin resistance (Hotamisligil *et al.*, 1993). Conversely, in 3T3-L1 adipocytes, treatment with TNFα decreases IRS-1 mRNA levels, but does not appear to have an effect on down-stream events in insulin signalling (Stephens *et al.*, 1997).

Figure 1.4 Molecular pathways integrating inflammatory responses with insulin action



Adapted from (Shoelson *et al.*, 2003; Hotamisligil, 2006)

NF-κB is a transcription factor which is activated by inflammatory cytokines such as TNFα. It drives the production of numerous proinflammatory cytokines including IL-β, IL-6 and TNFα itself. Under basal conditions, NF-κB is inhibited by IκB and remains in the cytoplasm and upon activation, the kinase complex referred to as IKK is activated and catalyses the phosphorylation of IκB. This leads to IκB degradation, liberating NF-κB which translocates into the nucleus and stimulates the transcription of several proinflammatory mediators. Expression of IKKβ (one of the kinases of the IKK complex), inhibits IRS-1/2 leading to TNFα-induced insulin resistance.

1.4.3.2 Role of TNFα in adipose tissue

There is also an increasing body of evidence to support a role for TNFα in modulating lipid metabolism in WAT. In obesity, the elevated levels of TNFα may contribute to the increase in basal lipolysis characteristic of adipocytes from obese subjects (Ramsay, 1996). Moreover, TNFα-deficient mice exhibit lower circulating free fatty acids and TGs than their wild-type littermates (Uysal *et al.*, 1997). Since elevated concentrations of free fatty acids have been associated with insulin resistance, the role of TNFα in mediating insulin resistance may be potentiated by increased lipolysis. TNFα has also been shown to down-regulate LPL activity and protein expression (Semb *et al.*, 1987), and in addition to inhibiting free fatty acid uptake, TNFα also acts to decrease the expression of key enzymes involved in lipogenesis (Doerrler *et al.*, 1994). Furthermore, TNFα has also been found to play an important role in the induction of apoptosis of both human preadipocytes and adipocytes *in vitro* (Prins *et al.*, 1997). Treatment of cells with dexamethasone, which has both anti-inflammatory and adipogenic properties, has been found to inhibit TNFα-induced adipocyte apoptosis (Zhang *et al.*, 2001).

Within adipose tissue itself, TNFα has been shown to be an important regulator of the expression of other pro-inflammatory adipokines. It has been shown to upregulate gene expression of IL-6, MCP-1, nerve growth factor (NGF), vascular endothelial growth factor (VEGF) (Wang *et al.*, 2005; Wang & Trayhurn, 2006), plasminogen activator inhibitor-1 (PAI-1) (Cigolini *et al.*, 1999; Wang & Trayhurn, 2006) and haptoglobin (Chiellini *et al.*, 2002; do Nascimento *et al.*, 2004) in adipocytes *in vitro*. TNFα also appears to have a substantial effect on its own level of gene expression, with a greater than 2,000-fold upregulation with acute treatment of human adipocytes *in vitro* (Wang & Trayhurn, 2006).

TNFα has been shown not only to inhibit adipocyte differentiation but also to be capable of suppressing the expression of adipocyte-specific genes in fully differentiated adipocytes (Ruan *et al.*, 2002; Wang & Trayhurn, 2006). Interestingly, the anti-adipogenic action of TNFα also appears to be reversed by subsequent treatment with adipogenic agents such as dexamethasone and indomethacin raising the possibility that adipocyte differentiation may be a reversible process under control of both positive and negative regulators (Sethi & Hotamisligil, 1999; Zhang

et al., 2001). The exact mechanism whereby TNFα alters the adipocyte differentiation programme is not clear. However, TNFα has been shown to down-regulate the expression of key transcription factors involved in adipogenesis, namely PPARγ and C/EBPα (Loftus & Lane, 1997). This may explain the parallel downregulation of adipose-specific genes (such as GLUT 4 and aP2) which contain binding sites for PPARγ and or C/EBPα in their promoters, upon treatment with TNFα (Williams *et al.*, 1992).

TNFα gene expression has been reported in canine adipocytes after collagenase digestion to separate mature adipocytes and cells of the SV fraction. Expression in canine WAT was not found prior to digestion thus indicating that mature adipocytes and cells of the SV fraction of dog WAT can express TNFα, but do not appear to do so in the tissue as a whole entity (Eisele *et al.*, 2005).

1.4.4 Interleukins

The interleukins are a group of cytokines that were first seen to be expressed by white blood cells (leukocytes, hence the *–leukin*) as a means of communication (*inter-*). Their role is to mediate and control immune and inflammatory responses. In addition to white blood cells, interleukins have been found to be expressed and secreted by other cell types including white adipocytes (Trayhurn & Wood, 2004).

IL-6 is a multifunctional cytokine and has a ubiquitous tissue distribution. Human WAT produces substantial amounts of IL-6 and this may represent 10-30% of circulating concentrations (Mohamed-Ali *et al.*, 1998). Circulating IL-6 concentrations are elevated in obesity and insulin resistance, and sympathetic stimulation has been shown to increase plasma IL-6 concentration in both humans and mice (Mohamed-Ali *et al.*, 2001). IL-6 is thought to play a direct role in insulin resistance by altering insulin signalling in hepatocytes (Senn *et al.*, 2002). This effect is mediated by the induction of SOCS3, which inhibits insulin-dependent insulin receptor autophosphorylation (Lagathu *et al.*, 2003; Senn *et al.*, 2003). Adipose tissue expression of IL-6 is stimulated by TNFα (Wang *et al.*, 2005; Wang & Trayhurn, 2006). Like TNFα, IL-6 decreases adipose tissue LPL activity and has been implicated in the WAT wasting that occurs in cancer cachexia (Greenberg *et al.*, 1992). Studies have also demonstrated a high rate of production of IL-6 in

adipose tissue of obese subjects, particularly in visceral depots. This suggests that as well as a central role, IL-6 also has an important autocrine and paracrine role as a regulator of regional adipose tissue metabolism (Fried *et al.*, 1998).

Other interleukins synthesised by WAT include IL-1β, IL-10 and IL-18 (Fain *et al.*, 2004; Juge-Aubry *et al.*, 2005; Wood *et al.*, 2005). IL1-β exerts its biological function by binding to the IL-1 receptor and activating the NFκB signalling pathway. IL-1β has been shown to mediate leptin induction in mice after an inflammatory stimulus (Faggioni *et al.*, 1998), and to stimulate IL-6 release from human adipocytes *in vivo* (Flower *et al.*, 2003). In a similar manner to IL-6, IL-1β levels are increased in the obese (Um *et al.*, 2004). Moreover, individuals with a combined raised level of IL-1β and IL-6 have an increased risk of type II diabetes and the metabolic syndrome (Spranger *et al.*, 2003). In addition, treatment of human adipose tissue explants with IL-β has been shown to decrease levels of adiponectin (Lihn *et al.*, 2004). Long-term treatment with IL-1β has been shown to induce insulin resistance in both human and rodent adipocytes (Lagathu *et al.*, 2006). This is thought to be mediated through decreased IRS-1 expression (Jager *et al.*, 2007).

In contrast to most cytokines secreted by white adipocytes, IL-10 is thought to play an anti-inflammatory role, where its level of expression is upregulated by pro-inflammatory agents such as lipopolysaccharide (LPS) and TNFα. Its level of expression is raised in both human and rodent obesity due to increased WAT mass (Juge-Aubry *et al.*, 2005). The plasma level of IL-18, a pro-inflammatory cytokine, is also elevated in obesity and its levels fall in response to weight loss (Esposito *et al.*, 2002). Increased serum IL-18 concentration has been associated with hypoadiponectinaemia in obesity independently of insulin resistance (Straczkowski *et al.*, 2007). Recently, it has been established that WAT is an important site of IL-18 gene expression, with a marked upregulation of expression by human adipocytes *in vitro* with TNFα treatment. However, there is mixed evidence as to whether it is secreted by adipocytes (Skurk *et al.*, 2005b; Wood *et al.*, 2005).

IL-6 gene expression has also been documented in canine adipocytes *in vitro*, with levels of expression being greater pre- compared to post-differentiation (Eisele *et al.*, 2005). This is unlike what is seen in human adipocytes, where levels of expression

appear to fall markedly post differentiation, followed by a gradual rise, until they reach pre-differentiation levels (Wang *et al.*, 2004).

1.4.5 Chemokines

Chemokines are low molecular weight peptides that play a central role in inflammatory processes by regulating leukocyte migration into sites of tissue damage. In excess of 50 chemokines have been described, and they exert their effects through G-protein-coupled receptors (Zlotnik *et al.*, 1999). As discussed earlier, obesity is associated with an increased infiltration of macrophages into WAT leading to an increase in the level of expression of several inflammation and macrophage-specific genes (Weisberg *et al.*, 2003; Xu *et al.*, 2003). This process of macrophage infiltration is thought to be regulated by chemokines secreted from adipocytes themselves; these include MCP-1, MIF and IL-8 (Gerhardt *et al.*, 2001). All three have been shown to be expressed in human preadipocytes *in vitro* and their level of expression is markedly upregulated by treatment with proinflammatory cytokines such as TNFα (Gerhardt *et al.*, 2001).

MCP-1, as the name suggests, is responsible for the attraction of macrophages and T-lymphocytes to sites of inflammation. Circulating levels of MCP-1 have been shown to be significantly raised in obesity (Kim *et al.*, 2006). TZDs have been found to reduce MCP-1 mRNA levels in WAT. The decreased adipocyte expression of MCP-1 by TZDs, results in decreased macrophage recruitment into the tissue, along with decreased expression of other inflammatory cytokines such as TNFα (Di Gregorio *et al.*, 2005). Studies have shown a potent stimulation of MCP-1 gene expression and protein secretion in human adipocytes *in vitro* with TNFα treatment (Wang *et al.*, 2004; Wang *et al.*, 2005). Since TNFα produced by adipocytes is known to induce insulin resistance, MCP-1 may play an important role in the mechanism of insulin resistance observed in obesity and type II diabetes mellitus. Studies, using human preadipocytes *in vitro*, have shown that levels of both MCP-1 gene expression and protein secretion increase during adipocyte maturation (Wang *et al.*, 2004).

Given that MCP-1 is expressed by adipocytes and plays a key role in monocyte recruitment into WAT, it is thought that obesity-related adipocyte expression of MCP-1 results in the recruitment of resident monocytes, which then themselves

secrete proinflammatory cytokines, contributing to the state of chronic-low grade inflammation seen in the obese state (Chen *et al.*, 2005; Di Gregorio *et al.*, 2005). MCP-1 has also been shown to have a direct angiogenic effect on endothelial cells (Salcedo *et al.*, 2000), and may thus be involved in the expansion and remodelling of adipose tissue in obesity. This indicates that MCP-1 may have other roles in adipocyte physiology in addition to inflammatory cell recruitment (Sartipy & Loskutoff, 2003).

MIF has been identified as a critical mediator of inflammatory responses and, like MCP-1, is also thought to play an active role in angiogenesis (Lue *et al.*, 2002). It is a potent inhibitor of monocyte/macrophage migration, and thus regulates macrophage accumulation in tissues. Human preadipocytes have been found to secrete MIF in a differentiation-dependent fashion, with maximal concentrations in mature adipocytes, although secretion is also evidenced in preadipocytes. Furthermore, MIF production by human adipocytes in culture has been shown to be positively correlated with donor BMI (Skurk *et al.*, 2005a), with raised circulating levels in the obese (Dandona *et al.*, 2004; Ghanim *et al.*, 2004) and falling levels after weight loss (Church *et al.*, 2005). Hence, MIF is thought to be an obesity-dependent mediator of macrophage infiltration of adipose tissue in obesity (Skurk *et al.*, 2005a).

Studies documenting the response of MIF to TNFα treatment have yielded contradictory results, where *in vitro* studies using human adipocytes, appear to show little or no change in the level of MIF gene expression with TNFα treatment (Wang *et al.*, 2005). This is in contrast to rodent adipocytes, where *in vitro* treatment of cells with TNFα led to a marked upregulation of both gene expression and protein secretion (Hirokawa *et al.*, 1997). MIF is also expressed by pancreatic β-cells and its production is regulated by glucose in a time- and concentration-dependent manner, with a progressive decrease in MIF action within the islets of Langerhans, contributing to β-cell dysfunction and diminished insulin release in type II diabetes mellitus (Waeber *et al.*, 1997). Therefore, in the presence of raised plasma concentrations of glucose, elevated MIF may be used as a marker for β-cell dysfunction (Church *et al.*, 2005). Thus MIF may affect the pathophysiology of both obesity and type II diabetes mellitus (Sakaue *et al.*, 1999).

IL-8 is a chemokine shown to be produced and released from human adipocytes both *in vitro* and *in vivo* and its level of production is stimulated by both IL-1β and TNFα (Bruun *et al.*, 2001). Circulating concentrations of IL-8 have been found to correlate with adiposity and insulin sensitivity, suggesting an involvement in obesity-related diseases (Bruun *et al.*, 2003b).

1.4.6 Nerve growth factor

Certain organs and cell types produce neurotrophic factors responsible for the control of the level of tissue innervation, which is essential for the maintenance and survival of sympathetic and certain sensory neurones (de Romo, 2007). NGF was the first target-derived neurotrophin to be identified, and is now recognised to be a member of a family of such proteins which include brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5, NT-6 and NT-7 and are all known to be expressed in the vertebrate brain. These proteins play key roles in the development of the nervous system (Hennigan *et al.*, 2007).

All members of the neurotrophin family bind, with different affinities, to the TrK family of receptor tyrosine kinases (TrkA, TrkB and TrkC) and with similar affinities to the p75 neurotrophin receptor (Salton, 2003). NGF exists as a pentameric protein complex consisting of three subunits (α, β and γ), and studies have determined that the β-subunit is that responsible for the biological activity of NGF (Shooter, 2001). The trophic functions of NGF have been ascribed to its interaction with TrkA, and are responsible for neuronal cell survival, proliferation and axonal growth (Chao & Hempstead, 1995; Scully & Otten, 1995). Activation of TrkA by NGF has also been reported to stimulate PPARγ activity in neurones, raising the possibility of a therapeutic role for NGF as an inducer of anti-inflammatory effects in neurological disorders (Fuenzalida *et al.*, 2005). In contrast, binding of NGF to p75 alone (or in combination with receptors other than TrkA), leads to the induction of programmed cell death during neuronal development and after neural injury (Frade & Barde, 1998). The interaction between p75 and the neurotensin receptor sortilin is a key aspect of this apoptotic effect, where sortilin binds to the unprocessed form of NGF (proNGF) and forms a complex with p75 which can then induce cell death (Nykjaer *et al.*, 2004).

1.4.6.1 Functions of nerve growth factor

As mentioned earlier, target tissues secrete specific neurotrophins, and these initiate programs of cellular differentiation, and support the survival of neuronal populations which express the appropriate receptors. NGF acts primarily, as a trophic factor for sympathetic and some neural crest-derived sensory neurones (Salton, 2003). However, NGF is also recognised to have a range of biological functions which include endocrine, immune and inflammatory roles (Scully & Otten, 1995; Levi-Montalcini *et al.*, 1996).

Several lines of evidence have been shown to implicate NGF in inflammation (Scully & Otten, 1995; Levi-Montalcini *et al.*, 1996). Monocytes and mast cells release NGF, and are a possible source of the elevated plasma concentrations of the neurotrophin in allergic states (Rost *et al.*, 2005). Inflammatory cytokines, such as IL-1β and TNFα, are found at sites of injury and induce NGF synthesis in both the peripheral and central nervous systems (Gadient *et al.*, 1990; Steiner *et al.*, 1991). NGF can also be released by non-neuronal cells under the influence of inflammatory mediators at sites of inflammation, thereby potentiating the immune response and enhancing regeneration of damaged nerves (Scully & Otten, 1995). Such an example is NGF secretion by human airway epithelial cells *in vitro* with treatment with IL-1β and TNFα. This effect can subsequently be reversed by treatment with dexamethasone (Pons *et al.*, 2001).

1.4.6.2 Nerve growth factor and adipose tissue

Due to its high degree of sympathetic innervation, much of the early work investigating the role of NGF and adipose tissue has focused on brown fat, where studies have demonstrated that NGF is produced by brown adipocytes (Nechad *et al.*, 1994; Nisoli *et al.*, 1996). It has now been established that NGF is expressed by all major WAT depots of mice was well as human white fat (Peeraully *et al.*, 2004), and that NGF is secreted from both rodent and human adipocytes in culture (Peeraully *et al.*, 2004; Wang *et al.*, 2004; Bullo *et al.*, 2005). In addition, the genes encoding the low-affinity p75 and the high-affinity TrkA NGF receptors are also expressed in WAT (Peeraully *et al.*, 2004). The proinflammatory cytokine TNFα, is a major factor in the stimulation of NGF gene expression and secretion, in both rodent (Peeraully *et al.*, 2004) and human adipocytes (Wang *et al.*, 2004; Wang & Trayhurn, 2006). This

is consistent with the role of the neurotrophin in inflammatory responses. NGF expression has been demonstrated in all main WAT depots in the dog, with expression being evident in both mature adipocytes and cells of the SV fraction (Eisele *et al.*, 2005). However, it is not established whether this important neurotrophin is in fact also an adipokine in the dog.

1.4.7 Haemostatic and haemodynamic factors

The renin-angiotensin aldosterone system (RAAS) is an important regulator of systemic blood pressure and renal electrolyte balance. Identification of the RAAS locally within adipose tissue is suggestive of a potential causal role for it in obesity-associated hypertension (Harte *et al.*, 2005). Circulating angiotensinogen is converted by renin to angiotensin II, which is a potent vasoconstrictor and key regulator of vascular tone. Although the liver is the main source of angiotensinogen, it is also produced and secreted by WAT (Engeli *et al.*, 2003b). In addition, human subcutaneous adipose tissue is a significant source of angiotensin II (Harte *et al.*, 2005). Adipocyte expression and secretion of angiotensinogen is stimulated by glucocorticoids and inhibited by insulin and TNFα (Aubert *et al.*, 1997; Aubert *et al.*, 1998; Wang *et al.*, 2005).

Angiotensinogen levels have been shown to fall in response to weight loss in humans with an associated reduction in expression in WAT. This is thought to contribute to the fall in blood pressure seen in obese hypertensive patients with subsequent weight loss (Engeli *et al.*, 2005). The PPARγ agonist rosiglitazone has been shown to downregulate the RAAS in subcutaneous adipose tissue, further contributing to the long-term reduction in blood pressure levels in humans suffering from obesity-related hypertension (Harte *et al.*, 2005). Angiotensinogen gene expression has been documented in the main WAT depots of the dog. Expression is also seen in canine adipocytes *in vitro*, with similar levels of expression pre- and post-differentiation (Eisele *et al.*, 2005).

The prothromboembolic factor PAI-1 is a key regulator of vascular haemostasis. By inhibiting the activation of plasminogen (the precursor to plasmin), it suppresses fibrinolysis (Sprengers & Kluft, 1987). Elevated levels of PAI-1 have been linked to insulin resistance (Juhan-Vague *et al.*, 1991). Adipose tissue expression and

circulating concentrations of PAI-1 are elevated in obese individuals (Koistinen *et al.*, 2000) and may represent the main source of elevated circulating plasma PAI-1 in obesity. The increased production of PAI-1 in the obese has been linked to several proinflammatory cytokines, including TNFα (Wang *et al.*, 2005) and IL-6, and is also known to be implicated in the increased risk of cardiovascular and atherosclerotic complications associated with obesity (Ziccardi *et al.*, 2002; Cao *et al.*, 2007). Like angiotensinogen, PAI-1 gene expression has been documented in canine adipocytes in primary culture, and in a similar manner, levels of expression appear not to differ pre- and post-differentiation (Eisele *et al.*, 2005).

1.4.8 Acute phase proteins

Acute phase proteins are a group of protein factors secreted mainly by the liver, with raised levels during the early part of the inflammatory response (Gabay & Kushner, 1999). However, several are also both synthesised and secreted by WAT and are thus recognised as adipokines. Acute phase proteins secreted by white adipocytes are a potential contributor to raised circulating levels seen in obesity (Trayhurn & Wood, 2004). One such protein is PAI-1; another example is serum amyloid A (SAA), a precursor of amyloid A, which is a constituent of the amyloid fibrils involved in amyloidosis. SAA is a well-characterised independent risk factor for coronary artery disease and AA amyloidosis. Serum SAA concentrations are correlated with BMI, adiposity and adipose tissue SAA mRNA levels (Poitou *et al.*, 2005), with WAT being a major expression site of SAA (Sjoholm *et al.*, 2005). Both WAT gene expression, and protein circulating concentrations, is under nutritional control and both decrease in response to weight loss. This indicates that, if amyloidosis is seen in the context of obesity, it is possible that production by adipocytes could be a contributing factor to amyloidosis seen in cardiovascular disease (Poitou *et al.*, 2005).

Haptoglobin binds free haemoglobin released by red blood cells in the event of haemolysis. During such events it sequesters iron found within haemoglobin, preventing iron-utilising bacteria from benefiting from events associated with haemolysis. As a result, haptoglobin is classified as an acute phase protein, and is a classical marker of inflammation, the expression of which has been documented in human, murine and canine white adipocytes *in vitro*. In all three models, expression

levels increase with differentiation, gene expression being virtually undetectable in preadipocytes (do Nascimento *et al.*, 2004; Wang *et al.*, 2004; Eisele *et al.*, 2005). A significant upregulation of haptoglobin expression has been documented in WAT of rodent models such as the yellow agouti and *ob/ob* mice (Chiellini *et al.*, 2002). Haptoglobin expression is stimulated by pro-inflammatory cytokines TNFα (Chiellini *et al.*, 2002) and IL-6, and inhibited by the TZD rosiglitazone in 3T3-L1 adipocytes (do Nascimento *et al.*, 2004). This is in contrast to human adipocytes, where TNFα treatment has been shown to reduce levels of gene expression after 24 h *in vitro*. Thus in contrast to rodents, it is unlikely that haptoglobin synthesis is stimulated in WAT as part of the inflammatory response (Wang *et al.*, 2004).

Metallothionein (MT) is a low-molecular-weight-metal-binding protein, the production of which is strongly induced in both the liver and kidney by the administration of heavy metals such as cadmium or zinc (Bremner & Beattie, 1990). The MT gene is strongly expressed in BAT, where it is suggested that it may play an important role as an antioxidant (Beattie *et al.*, 2000). It has also been demonstrated that both MT-1 and MT-2 genes are expressed in mature adipocytes of rodents, the mRNA being present in each of the main depots, with no substantial differences in the level of expression (Trayhurn *et al.*, 2000). It has been suggested that MT has an anti-oxidant role in WAT protecting fatty acids from oxidative damage (Trayhurn *et al.*, 2000). The differentiation of fibroblastic preadipocytes to adipocytes in primary culture has been shown to result in raised level of MT mRNA and increased secretion of MT protein (Trayhurn *et al.*, 2000).

MT gene expression in human adipocytes *in vitro* is upregulated by treatment with TNFα (Wang *et al.*, 2005). Gene expression for both MT-1 and MT-2 has also been documented in canine WAT; with expression being evident in all main canine WAT depots. As documented in humans (Do *et al.*, 2002), gene expression was evident for both genes in mature adipocytes and cells of the SV fraction (Eisele *et al.*, 2005). Gene expression was also evident in canine adipocytes *in vitro*, where unlike human adipocytes (Wang *et al.*, 2004), gene expression levels appear to be higher in mature cells compared to that seen in preadipocytes (Eisele *et al.*, 2005).

1.4.9 Resistin and visfatin

Resistin (also described as FIZZ3 and ADSF) is a 12.5 kDa cystine-rich protein that is secreted from adipocytes, is present in the circulation, and was initially thought to be the link between increased adiposity and type II diabetes mellitus by inducing insulin resistance (Steppan *et al.*, 2001). This was based on observations that diet-induced obese mice and genetically obese mice had raised levels of resistin, and subsequent immuno-neutralisation of resistin in these animals improved insulin sensitivity and hyperglycaemia. Resistin administration to normal mice, led to an impairment of glucose tolerance and insulin sensitivity. *In vitro* treatment of 3T3-L1 adipocytes with TZDs decreased levels of resistin gene expression (Steppan *et al.*, 2001). In humans, resistin mRNA concentrations have been found to be similar between abdominal subcutaneous and omental depots. However, quantitative comparisons have shown there to be a marked difference in the level of resistin mRNA expression between abdominal depots and depots located elsewhere in the body such as the thigh. (McTernan *et al.*, 2002a).

Resistin has been found to be both expressed and secreted by human adipocytes, with levels being higher in abdominal depots (subcutaneous abdominal and omental), compared to non-abdominal depots (thigh and breast) (McTernan *et al.*, 2002c). However, there is little evidence for a role for resistin as a link between obesity, insulin resistance and type II diabetes mellitus in humans, where administration of resistin has lead to the development of hepatic, but not peripheral, insulin resistance (Rajala & Scherer, 2003). Furthermore, in humans, unlike rodents, resistin is mainly secreted by macrophages in the SV fraction not by adipocytes themselves (Lehrke *et al.*, 2004). Studies carried out using human adipocytes reported barely detectable levels of resistin, and no differences were found in resistin gene expression when comparing cells from normal, insulin-resistant or type II diabetic individuals (Nagaev & Smith, 2001). In addition, reports have failed to establish either a clear mechanism by which resistin expression is regulated, or a definite physiological role for the protein (Rea & Donnelly, 2004). Thus, it appears that human and mouse resistin have different physiological roles and the postulated relationship of murine resistin and insulin resistance may not readily translate to humans (Savage *et al.*, 2001).

Visfatin, is a 52 kDa cytokine whose expression was primarily characterised in lymphocytes, but which has also been shown to be secreted by visceral WAT of both humans and mice, and is thus also an adipokine. Circulating concentrations have been shown to correlate with adiposity. Like resistin, it is also thought to be involved in glucose homeostasis and is a ligand for the insulin receptor. It appears to have insulin-like effects enhancing glucose uptake in 3T3-L1 cells and reducing glucose levels in mice. However, unlike insulin, visfatin levels have been shown to be unaffected by feeding or fasting (Fukuhara *et al.*, 2005). In addition, plasma concentrations of visfatin and visfatin mRNA expression have been correlated with measures of obesity, but not with visceral fat mass or waist-to-hip ratio in humans (Berndt *et al.*, 2005). Further studies into the physiological role of this novel adipokine may lead to new insights into glucose homeostasis including type II diabetes mellitus and the metabolic syndrome (Fukuhara *et al.*, 2005).

1.4.10 Proteins of lipid and lipoprotein metabolism

Adipsin was first identified on the basis of the differentiation-dependent expression of its mRNA in murine 3T3-F442A adipocytes (Spiegelman *et al.*, 1983). Adipsin cDNA was found to encode a new serine protease with a high homology to human complement factor D. Adipsin is required for the synthesis of acylation-stimulating protein (ASP), which is derived from the end product of the alternative complement pathway, complement C3a (Trayhurn & Beattie, 2001). ASP is involved in the uptake and esterification of fatty acids to TGs for storage in adipocytes and has an additive effect with that of insulin by allowing adipose tissue to self-regulate its lipid-storage capacity (Cianflone *et al.*, 1999). Furthermore, plasma levels of both adipsin and ASP are increased in human obesity (Cianflone *et al.*, 1999; Ahima & Flier, 2000). Although there is a close similarity between human and murine complement factor D, mouse adipsin expression appears to be specific to adipose tissue, whereas human complement factor D is reported to be synthesised mainly by cells of the macrophage/monocyte lineage (Barnum & Volanakis, 1985). With increased infiltration of WAT by macrophages in obesity, ASP may play a key role in adipose tissue metabolism and plasma TG clearance in the obese state.

Zinc-α2-glycoprotein (ZAG) is a lipid mobilizing factor which is over-expressed in several types of malignant tumour, and has been used as a cancer marker. Expression

has been detected in both mature rodent adipocytes and in cells of the SV fraction (Bing *et al.*, 2004). ZAG mRNA has been detected in 3T3-L1 cells both before and after the induction of differentiation, with levels rising as cells mature (Bing *et al.*, 2004). ZAG has been shown to stimulate lipolysis in a dose-dependent manner in rodent adipocytes *in vitro* (Hirai *et al.*, 1998). *In vivo* administration of ZAG to mice induces a rapid reduction in body fat stores and increases in serum free fatty acids via the activation of HSL. Both ZAG mRNA and protein have been detected in murine and human WAT. Thus, ZAG appears to play a key role in lipid metabolism and in the modulation of lipolysis in white adipocytes (Bing *et al.*, 2004).

Retinol binding protein-4 (RBP4) is a protein required for the transport of retinol (vitamin A) from the liver and adipose tissue to target tissues. It is highly expressed by WAT, although liver and kidney are also a major source of circulating levels (Trayhurn & Beattie, 2001). Raised serum concentrations have been reported in obese humans and mice, and it is thought that this may contribute to the development of insulin resistance in obesity and type II diabetes mellitus (Yang *et al.*, 2005). Fasting-induced adipose factor (FIAF) is an angiopoeitin-like protein which was originally identified as a result of it being a target gene for PPARα. Circulating levels have been found to rise on fasting (Kersten *et al.*, 2000b). It has been shown to inhibit LPL regulating TG accumulation by white adipocytes (Backhed *et al.*, 2004). Furthermore, fasting induces increased expression of FIAF in the pituitary. This is suggestive of a possible role for FIAF in both the peripheral and central regulation of energy balance (Wiesner *et al.*, 2004).

1.4.11 Growth factors

Adipokines include key growth factors and one such example is insulin-like growth factor-1 (IGF-1). This is secreted from WAT and stimulates preadipocyte differentiation and proliferation and further mediates the functions of growth hormone in the tissue (Hausman *et al.*, 2001). Transforming growth factor-β (TGFβ) is an adipokine whose production by WAT is increased in murine genetic models of obesity (*db/db* and *ob/ob*) (Samad *et al.*, 1998) and also in human obesity (Fain *et al.*, 2005). TGFβ is a negative regulator of adipocyte differentiation (Rahimi *et al.*, 1998), and stimulates production of PAI-1 by adipocytes (Samad *et al.*, 1998). It is likely to play a key role in the expansion of WAT in obesity, as well as being part of

the increased cardiovascular risk associated with obesity, due to its link with raised levels of PAI-1 production. One final adipokine which will be mentioned is vascular endothelial growth factor (VEGF). This is a potent angiogenic factor, whose plasma concentration is correlated with adiposity in *db/db* mice, and whose secretion has been found to be higher in visceral than subcutaneous WAT (Miyazawa-Hoshimoto *et al.*, 2005). Adipocyte VEGF production is stimulated by insulin, but suppressed by dexamethasone (Fain & Madan, 2005). VEGF is likely to be a key player in the process of neovascularisation associated with expanded WAT depots in obesity.

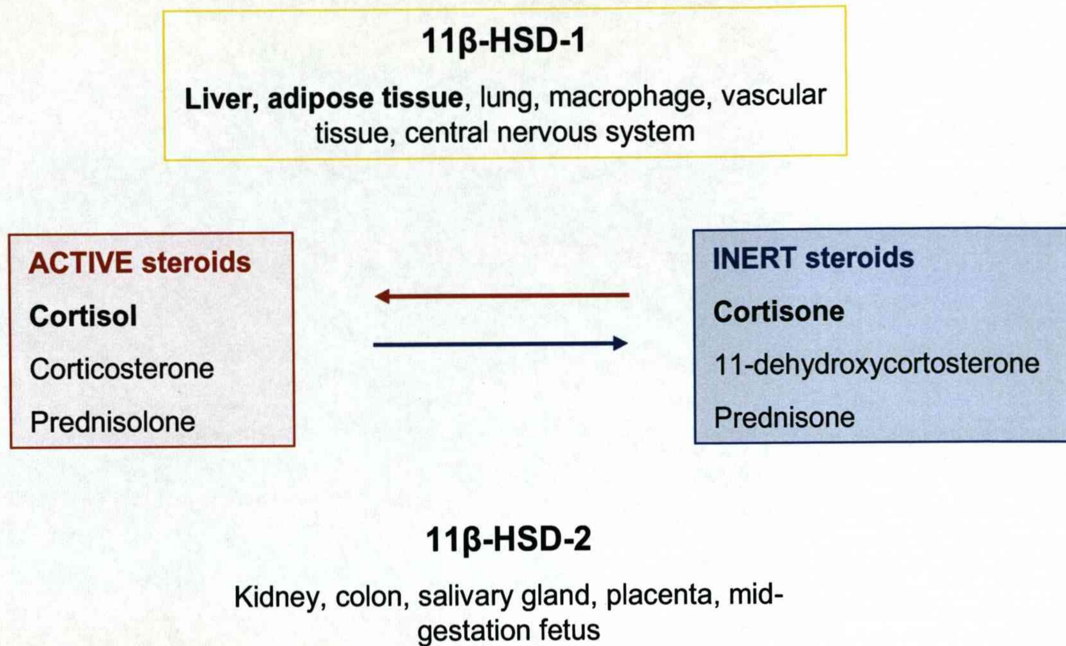
1.5 11β-hydroxysteroid dehydrogenase-1

Exposure to high circulating glucocorticoid concentrations, as found in Cushing's syndrome, causes a metabolic disease which resembles features of idiopathic metabolic syndrome including pronounced visceral obesity (Seckl *et al.*, 2004). However, idiopathic obesity is not associated with elevated circulating concentrations of glucocorticoids (Seckl *et al.*, 2004). Rather, it seems that intracellular generation of active cortisol from inactive cortisone locally in WAT, could amplify glucocorticoid signalling and may be responsible for co-morbidities associated with the metabolic syndrome in obesity (Stewart *et al.*, 1999; Seckl *et al.*, 2004; Sandeep *et al.*, 2005). A key factor in this process is the nicotinamide adenine dinucleotide (NAD)-dependent enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). Two 11β-HSD isozymes have been characterised and their function and tissue specific expression is summarised in Fig. 1.5.

It has been demonstrated that both 11β-HSD-1 mRNA levels and activity are elevated in visceral and abdominal subcutaneous WAT from obese individuals (Paulmyer-Lacroix *et al.*, 2002; Rask *et al.*, 2002; Alberti *et al.*, 2007) and in omental WAT of Zucker fatty rats (Livingstone *et al.*, 2000). Given the key association of visceral adipocity with metabolic and cardiovascular risk, it has been hypothesised that increased 11β-HSD-1 activity in visceral, rather than subcutaneous, adipose depots are responsible for the adverse metabolic consequences of obesity and the metabolic syndrome (Bujalska *et al.*, 1997; Stewart *et al.*, 1999). This has been supported by the observation that transgenic mice overexpressing 11β-HSD-1 in WAT develop visceral obesity and the metabolic syndrome phenotype (Masuzaki *et*

al., 2001). Conversely global knockout mice are protected from the metabolic consequences of dietary obesity (Morton *et al.*, 2004). In addition, increased local conversion of cortisone to active cortisol through the expression of 11β-HSD-1 has been primarily documented in omental compared to subcutaneous human adipocytes *in vitro* (Bujalska *et al.*, 1999).

Figure 1.5 Differential function and tissue expression of 11β-HSD isozymes



Adapted from (Seckl *et al.*, 2004)

11β-HSD-1 is predominantly a11-ketoreductase, and is thus responsible for the reactivation of inert cortisone into cortisol. In terms of isozymes, 11β-HSD-1, but not 11β-HSD-2, mRNA is expressed in white adipose tissue of both rodents and humans. 11β-HSD-2 is a high-affinity (low nM for cortisol) NAD(H)-dependent dehydrogenase which rapidly converts cortisol to inert cortisone. In adults, 11β-HSD-2 is primarily expressed in tissues where aldosterone induces its classical effects on sodium excretion, including the distal nephron, salivary glands and colonic mucosa.

Increased expression of 11β-HSD-1 in visceral WAT has been associated with hypoadiponectinaemia. It has thus been proposed that this could play a role in decreased levels of insulin sensitivity associated with obesity (Alberti *et al.*, 2007). Furthermore, 11β-HSD-1-driven cortisone reactivation regulates the synthesis and secretion of PAI-1 by WAT, suggesting that local cortisol metabolism in adipose tissue may be involved in increasing risk of cardiometabolic diseases in obesity (Ayachi *et al.*, 2006).

Mechanistically, glucocorticoids induce adipose tissue expansion by stimulating differentiation of preadipocytes (Xu & Bjorntorp, 1990; Liu *et al.*, 2006) and LPL-medated TG accumulation (Fried *et al.*, 1993). A key factor in this process is tissue levels of the intracellular glucocorticoid receptor α (GRα) and higher levels of GR have been found in omental compared to subcutaneous WAT (Rebuffe-Scrive *et al.*, 1990). The reasons for altered activity of 11β-HSD-1 in obesity are not fully understood. There is mounting evidence that it may be dysregulated secondarily to factors that are altered in obesity, including substrates for metabolism, hormones and inflammatory mediators (Wake & Walker, 2006). 11β-HSD-1 is a potent therapeutic target for the treatment of the metabolic syndrome, and although many specific inhibitors have now been developed (Tomlinson & Stewart, 2005), their efficacy in enhancing insulin sensitivity in WAT remains to be proven (Sandeep *et al.*, 2005). To date, no research has been carried out linking 11β-HSD-1 with WAT in the dog, and with rising levels of companion obesity, this remains, as yet, an unexplored area of canine obesity biology.

1.6 Aims of the thesis

Over the past two decades, there has been a radical change in the perception of the role of WAT in the regulation of energy balance and metabolism. It has been established that far from being a passive organ concerned with lipid storage, WAT is an important endocrine and secretory organ. In excess of 50 protein factors and signals secreted by white adipocytes, termed adipokines, have been characterised. Thus, it is now apparent that WAT is a complex secretory and endocrine organ with extensive roles involving not only whole body energy metabolism but also the central control of appetite and energy intake (Trayhurn, 2005c).

A key hypothesis is that adipose tissue-derived proinflammatory adipokines may play a direct role in the development of insulin-resistance and the associated pathologies of obesity (Hotamisligil, 2003; Hotamisligil, 2006). There is now considerable evidence for support of this concept, with both circulating concentrations and adipocyte expression of several proinflammatory adipokines being raised in obesity, including leptin, IL-6 and TNFα (Considine *et al.*, 1996; Mohamed-Ali *et al.*, 1997; Bastard *et al.*, 2000). In contrast, plasma concentrations of adiponectin (an adipokine which has a role as an insulin sensitiser) (Yamauchi *et al.*, 2001), are reduced in obesity (Abbasi *et al.*, 2004).

Unlike humans and rodents, the study of adipose tissue biology in the context of obesity and energy balance in companion animals is in its infancy. Despite several reports of the metabolic consequences of obesity (Impellizeri *et al.*, 2000; Gayet *et al.*, 2004), further insights into the metabolic pathways involved remain to be elucidated. With the rapid rise in levels of obesity seen clinically in companion animals, work in this area has recently begun in earnest.

Therefore, the work presented in this thesis centres on characterising adipokine production from canine WAT and adipocytes in primary culture. It further addresses the hypothesis that white adipocytes are a source of several protein factors actively secreted by canine adipocytes, and that synthesis is regulated by both pro-inflammatory cytokines as well as anti-inflammatory agents. Furthermore, levels of gene expression of the key enzyme 11β-HSD-1 responsible for the regulation of active glucocorticoid levels locally in WAT are also examined.

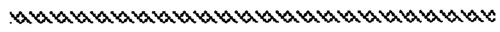
The first specific aim of the thesis was to investigate whether the expression of several proinflammatory adipokine genes could be detected in canine WAT. Subsequently, a robust system of primary culture of canine adipocytes was established with the aim of investigating whether these proteins where actively secreted by canine adipocytes, and therefore can be classified as adipokines in the dog.

Conventional RT-PCR was initially employed, with the more sensitive technique of qPCR used in subsequent parts of the study for quantitative comparisons. ELISA was used to detect levels of secreted protein in cell culture supernatant. Use of these techniques allowed the investigation of the following:

1. A comparison of adipokine gene expression in the main WAT depots of the dog.
2. The study of adipokine gene expression and protein secretion by canine adipocytes, from both subcutaneous and visceral depots, during differentiation and development in primary culture.
3. Examination of whether proinflammatory cytokines such as TNFα and IL-6 regulate adipokine gene expression and secretion by canine adipocytes *in vitro*.
4. Exploration of the effect of both pro-inflammatory agents and compounds with an anti-inflammatory role in adipocyte metabolism in canine adipocytes *in vitro*.
5. Examination of the effect of treatment with both pro- and anti-inflammatory mediators on the level of gene expression of the key enzyme 11β-HSD-1.

The project will provide important insights into obesity biology in the dog. By investigating whether synthesis and secretion of key adipokines occurs in canine WAT, it is expected that the understanding of the role of adipose tissue as an important endocrine organ in the dog will be significantly enhanced.

Chapter 2



Materials and Methods

2.1 Animals

Tissue samples were obtained from dogs euthanased at an animal shelter for reasons unrelated to the study, and from dogs undergoing surgery for routine neutering at the Fern Grove veterinary surgery, first opinion practice at the University of Liverpool. The study adhered to the University's Guidelines on Animal Ethics. The breeds included were Staffordshire bull terrier (SBT) and Labrador, and all animals were entire at the time of sample collection (not neutered). Animals used for tissue expression studies were 6 male and 7 female SBT dogs between the ages of 2-6 years (estimated on dentition). Their nutritional status prior to the study for logistical reasons was unknown.

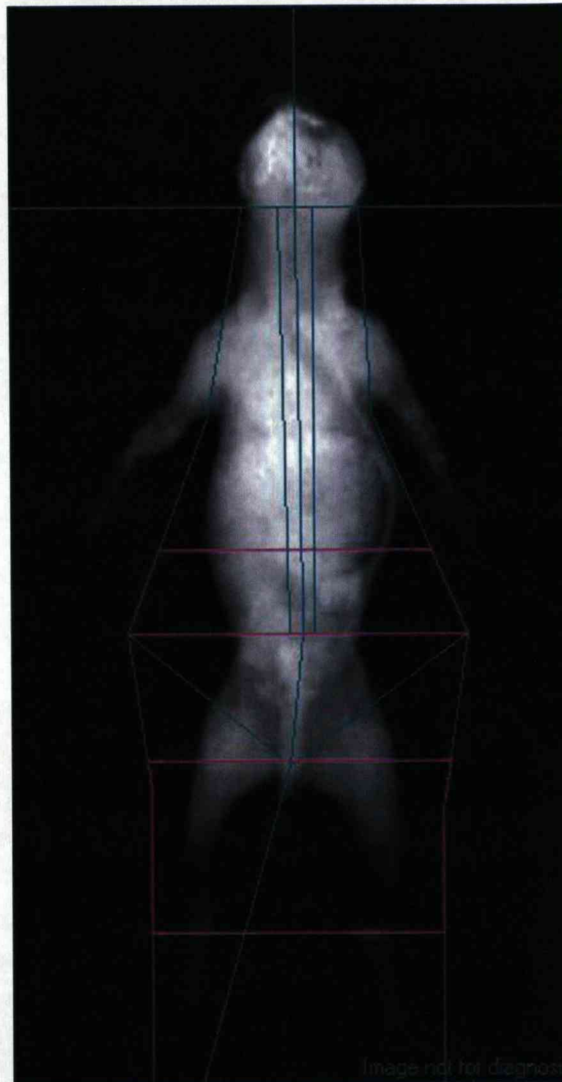
2.1.1 Tissue samples

Samples from liver, spleen, kidney, and from the five main WAT depots in the dog (subcutaneous inguinal, omental, peri-renal, gonadal and falciform ligament) were obtained within thirty minutes of euthanasia and immediately frozen in liquid nitrogen for future total RNA extraction. Further samples of subcutaneous inguinal WAT were taken for primary culture of canine preadipocytes. Once tissues were collected animals were subjected to a DEXA scan (GE Healthcare) to determine body composition (Fig. 2.1). Data was analysed using enCORE 2004 software (GE Healthcare).

2.1.2 Surgical samples

Samples from the broad ligament of entire bitches undergoing routine surgery for neutering at Fern Grove veterinary surgery, University of Liverpool were used for primary culture of canine preadipocytes. Samples of peri-lipomatous and lipomatous tissue from an entire male Labrador undergoing surgery for an excision biopsy of a lipomatous ventral abdominal mass were also used. All owners gave written consent for collection of samples.

Figure 2.1 DEXA scan image



This figure is a representative DEXA scan from an entire male SBT dog illustrating the typical image obtained from such a scan. DEXA is a non-invasive method of quantifying body composition (fat and lean content), which can be used in companion animals. It utilises x-ray of two different energy levels to distinguish the type and amount of tissue scanned. The x-ray source is positioned underneath the table supporting the subject, with the detector housed in an arm above. The detector measures the amount of x-rays which pass through the subject at any given time. X-rays of the two different energy levels are impeded differently by different tissues. The body can be compartmentalised and algorithms used to calculate both the quantity and type of tissue in each compartment (Toll *et al.*, 1994).

2.2 Preadipocyte primary cell culture

2.2.1 Reagents

All media and reagents for cell culture were prepared immediately using Corning filter systems (containing a 0.22 μm sterilising filter membrane) in a category II biological safety cabinet. Prepared media and buffers were pre-warmed to 37°C in a water bath and any excess discarded after use, unless stated otherwise.

(i) 10x HEPES (pH 7.4)

100 mM HEPES
12.5 mM sodium dihydrogen orthophosphate
12.5 mM disodium hydrogen phosphate
Autoclaved distilled water

Aliquots were stored at -20°C.

(ii) 10% BSA (pH 7.4)

A 10% BSA solution was made up in autoclaved distilled water and left overnight at 4°C to allow full dissolution prior to use. The pH was adjusted to 7.4 and aliquots were stored at -20°C.

(iii) 1x Phosphate Buffered Saline (PBS)

137 mM sodium chloride
2.7 mM potassium chloride
8.1 mM disodium hydrogen phosphate
1.5 mM potassium dihydrogen orthophosphate
Autoclaved distilled water

The pH adjusted to 7.2-7.4, and aliquots were stored at -20°C.

(iv) Transport Medium

15 mM HEPES
15 mM sodium hydrogen carbonate
33 μM D-biotin
17 μM D-panthothenate
1x DMEM

100 U/ml penicillin G sodium

100 μg/ml streptomycin sulphate

2.5 μg/ml amphotericin B

(v) Digestion Medium

1 mg/ml Collagenase

4 mg/ml BSA

20 mM HEPES

1x DMEM

100 U/ml penicillin G sodium

100 μg/ml streptomycin sulphate

2.5 μg/ml amphotericin B

(vi) Washing Medium

10% FCS

1x DMEM

100 U/ml penicillin G sodium

100 μg/ml streptomycin sulphate

2.5 μg/ml amphotericin B

(vii) Erythrocyte lysis buffer (pH 7.4)

155 mM ammonium chloride

10 mM potassium hydrogen carbonate

0.1 mM EDTA

(viii) Inoculation Medium

5% FCS

4 μM D-biotin

1x DMEM

100 U/ml penicillin G sodium

100 μg/ml streptomycin sulphate

2.5 μg/ml amphotericin B

(ix) Induction Medium

5% FCS

100 μM indomethacin

10 μg/ml insulin

0.5 mM IBMX

1x DMEM

100 U/ml penicillin G sodium

100 μg/ml streptomycin sulphate

2.5 μg/ml amphotericin B

(x) Feeding Medium

5% FCS

100 μM indomethacin

10 μg/ml insulin

1x DMEM

100 U/ml penicillin G sodium

100 μg/ml streptomycin sulphate

2.5 μg/ml amphotericin B

2.2.2 Collection of samples

On the day of collection, the required number of aliquots of 10x HEPES, 10% BSA and 1x PBS were thawed. The transport and digestion media were freshly prepared. After dissection, WAT samples from each depot were rinsed immediately in 1x PBS. A portion of each sample (2-5 g) was placed in transport medium for canine preadipocyte cell culture, and the remainder (1-2 g) as well as samples from liver, spleen and kidney, were snap frozen in liquid nitrogen for future total RNA extraction.

2.2.3 Fractionation method

The next steps were carried out in a category II biological safety cabinet. All items introduced into the cabinet were sprayed beforehand with 70% ethanol. Samples were transported from the dissection room to the laboratory within 30 min of collection. The sample in transport medium was placed in a Petri dish and carefully

dissected, removing any blood vessels and other non-adipose tissue. The cleaned WAT was then finely minced using disposable scalpels before being transferred into freshly prepared digestion medium in universals. The universals were sealed with Nescofilm, placed in an HB-1000 hybridisation oven, and rotated at 100 cycles/min at 37°C for 50-60 min (or until the tissue was digested). The universals were removed from the oven every 15 min during the digestion process and shaken by hand to encourage thorough digestion and homogenisation of the tissue.

2.2.4 Culture method

After digestion was complete, the disrupted tissue was centrifuged at 200x*g* for 10 min at room temperature. The mature adipocytes at the top of the supernatant were aspirated along with the supernatant into a sealed flask containing a solution of 1% Virkon, leaving the pellet of stromal vascular cells at the bottom. The pellet was then re-suspended in washing medium.

Once in washing medium the pellet was thoroughly homogenised and the suspension filtered through a 10 cm^2 100 μm pore size mesh, to remove any remaining mature adipocytes and blood vessels. The filtrate was centrifuged at 200x*g* for 10 min at room temperature. The supernatant was aspirated, washing medium added, and the filtration step repeated. After the second filtration step, the supernatant was aspirated and washing medium added. The cells were then re-suspended and homogenised thoroughly. The suspension was again centrifuged at 200xg for 10 min and the supernatant aspirated. This washing step was repeated, the supernatant aspirated and the stromal vascular pellet re-suspended in pre-warmed erythrocyte lysis buffer. Cells were kept in the buffer for 10 min to allow adequate lysis of any remaining erythrocytes. This was followed by centrifugation at 200xg for 10 min and aspiration of the supernatant. The remaining pellet was re-suspended in pre-warmed inoculation medium. An aliquot of this cell suspension was then stained with trypan blue and intact cells counted using a haemocytometer. Further inoculation medium was added to achieve the required plating density (40,000-60,000 cells/cm^2). Cells were seeded in 6-, 12 or 24-well plates as required and cultured at 37°C in an Mk II incubator (LEEC) with a humidified atmosphere of 5% CO_2/95% air.

After 24 h, gonadal cells were thoroughly washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells from the subcutaneous inguinal depot had the inoculation medium replaced after 48 h and 24 h later (72 h after they were initially inoculated), the cells were thoroughly washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells from both depots were allowed to stay in induction medium for 48 h after which this was replaced by feeding medium. The feeding medium was changed every 48 h.

2.2.5 Collection of conditioned media and cells

Conditioned media was collected by transferring the medium from each well into 2 ml tubes and centrifuging at 200x*g* for 10 min at room temperature in order to pellet any cells or debris. After the centrifugation, the supernatants were transferred into fresh 2 ml tubes and stored at -20°C until further analysis.

Cells were harvested by firstly either collecting or discarding the media, then adding Tri-Reagent to each well. After allowing the plate to sit for 5 min, the Tri-Reagent was pipetted over the surface of the well several times to disrupt the cells. These cell suspensions were transferred to 2 ml tubes and stored at -80°C until further use.

2.2.6 Cell treatment

Time course studies using cells from both the subcutaneous inguinal and gonadal depots were performed by collecting media on the day of induction (day 0), and then on days 1, 2, 3, 4, 6, 8, 10, 12, and 15 post-induction. Cells were harvested in Tri-Reagent at the appropriate time points. In studies on the dose response of treatment agents on adipokine gene expression, canine adipocytes were first deprived of medium containing foetal calf serum (FCS) and indomethacin for 24 h on day 12 after the induction of differentiation and subsequently treated with different concentrations of each agent for 2, 4 or 24 h.

2.2.7 Oil red O staining

2.2.7.1 *Reagents*

Oil red O

haematoxylin

37% formaldehyde

1x PBS

Both stains were filtered through a 0.22 µm membrane to remove any dye not in solution.

2.2.7.2 Method

Conditioned media was either collected or discarded and the remaining cells washed twice with 1x PBS and fixed using a 3.7% solution of formaldehyde for no more than 1 h at room temperature. Once fixed, cells were again washed with 1x PBS and the same volume of Oil red O added to each well. Cells were incubated on a shaking platform at room temperature for 1 h. The washing step was repeated and the same volume of haematoxylin was added to each well. Cells were incubated on a shaking platform at room temperature for a further 5 min. The stained cells were washed with distilled water at 45°C and images captured using a Zeiss Axiovert 200M inverted microscope (Hitachi High-Technologies).

2.3 Total RNA isolation from tissues and cells

2.3.1 Tri-Reagent RNA extraction

2.3.1.1 Reagents

RNase AWAY

Tri-Reagent

Chloroform

Isopropanol

Molecular biology grade ethanol, 75%

Ultra-pure water (0.1 µm filtered, DNase and RNase free)

UV-irradiated distilled water

2.3.1.2 RNA extraction method

Ribonucleic acid (RNA) extraction from both cells and tissues was carried out in a category I chemical fume cabinet. Dressing forceps, dissecting scissors and electric homogenizer blade attachments were treated with RNase AWAY and rinsed with

UV-irradiated distilled water several times prior to use to avoid contamination with RNases. For each group of tissue samples, the homogenizer was rinsed twice in UV-irradiated distilled water.

For RNA extraction from white adipose tissue, 50-150 mg of snap-frozen tissue was cut from the sample using a fresh sterile scalpel, dissecting scissors and forceps, while on dry ice. Alternatively, extraction from denser tissues such as liver and spleen was carried out by placing the sample in a polythene sachet and gently breaking it into approximately 50 mg pieces with a hammer. One ml of Tri-Reagent was added to the 4 ml tube into which the tissue sample was placed, and the sample homogenized using an electric homogenizer (Polytron Ultra-Turrax T25). The tissue homogenate was transferred into a 2 ml tube and centrifuged at 14000x*g* for 10 min at 4°C. The clear supernatant was aspirated and transferred into a fresh 2 ml tube. Care was taken to avoid aspirating any cell debris or any lipid overlying the supernatant. RNA extraction from cells was carried out as described by collecting cells using Tri-Reagent and subsequently homogenizing the sample by repeat aspiration using a sterile 1 ml syringe with a 23 gauge needle.

After leaving the tissue or cell homogenate at room temperature for 5 min, 200 µl of chloroform were added per ml of Tri-Reagent. Each tube was then vortexed for 15 sec and allowed to stand for 10 min at room temperature. This was followed by centrifugation at 14000x*g* for 15 min at 4°C, leading to separation of the solution into three layers; a colourless upper aqueous phase containing the total RNA, a white interphase layer containing DNA and a lower red phenol phase containing protein. The uppermost layer containing the total RNA was transferred into a fresh 2 ml tube.

To minimise any possible contamination of the sample with genomic DNA, a further purifying step was carried out as follows; 50 µl of isopropanol were added to the sample per ml of Tri-Reagent. The sample was vortexed, allowed to stand for 10 min at room temperature and then centrifuged at 14000x*g* for a further 10 min at 4°C. The supernatant was transferred into a 1.5 ml tube, taking care to avoid aspirating the area where any genomic DNA would have been precipitated.

A larger volume of isopropanol was added to the sample (450 µl per ml of Tri-Reagent in the initial sample preparation) to precipitate the total RNA. This was followed by vortexing, incubation of the sample for 10 min at room temperature and centrifugation at 14000x*g* for a further 10 min at 4°C. Once this step was complete, the precipitated RNA pellet could usually be visualised at the bottom of the tube.

The supernatant was discarded and 100 µl of a 75% solution of molecular grade ethanol in ultra-pure water was added to the RNA pellet. This was followed by vortexing and centrifugation at 14000x*g* for 5 min at 4°C. The supernatant was again discarded and the RNA pellet allowed to air dry for 2-3 min at room temperature. The pellet was dissolved by adding 10 µl of ultra-pure water, briefly vortexed and spun. Samples were then either stored briefly at -20°C prior to DNase treatment and quantification or placed at -80°C for long-term storage. All non-disposable equipment used was cleaned of any tissue debris, rinsed and soaked for 1-2 h in a 1% Virkon solution before being further rinsed with distilled water and stored.

2.3.2 DNase treatment of RNA

2.3.2.1 Reagents

Turbo DNA-*free* Kit:

120 µl TURBO DNase (2 Units/µl)
600 µl 10x TURBO DNase Buffer
600 µl DNase Inactivation Reagent
1.75 ml Nuclease-free water

2.3.2.2 Method

RNA samples which were to be analysed by real time polymerase chain reaction (qPCR) were treated to further minimise any genomic DNA contamination. One µl of turbo DNase and the same volume of 10x turbo DNase buffer were added to each 10 µl RNA sample. The sample was then gently vortexed, centrifuged and incubated at 37°C in a hybridisation oven (HB-1000) for 20-30 min. Two µl of DNase inactivation reagent were added and the sample vortexed gently. This was followed by a further incubation of 2 min at room temperature during which the samples were gently agitated to re-disperse the DNase inactivation reagent. The sample was

centrifuged at 10000x*g* for 1.5 min at room temperature to pellet the DNase inactivation reagent. The supernatant containing total RNA was aspirated and placed in a separate tube for further use or storage at -80°C.

2.3.3 RNA quantification

RNA samples were quantified using a spectrophotometer (BioPhotometer). One μl of total RNA was added to 69 μl of distilled water, a 1 in 70 dilution (dilution factor (DF) of 70). The diluted sample was placed in a disposable plastic cuvette (UVette), and the absorbance measured at 260 nm (A_{260}) and 280 nm (A_{280}) using distilled water as a blank.

Nucleic acids have their maximal light absorbance at A_{260}, whilst proteins absorb light maximally at A_{280}. The extinction coefficient of RNA at A_{260} is approximately 40. The concentration of each RNA sample was determined by the spectrophotometer using the Beer-Lambert law:

$$[RNA]\ \mu g/ml = A_{260} \times 40 \times DF / 1000$$

The ratio between A_{260} and A_{280} was calculated as an indication of the purity of the RNA in the sample. A ratio of 1.8 to 2.0 was indicative of a sample of high purity, a low ratio of 1.6 or less was suggestive of protein contamination (increased absorbance at A_{280}), whereas a high ratio above 2.0 was indicative of genomic DNA contamination.

2.3.4 RNA integrity

More than 80% of the total RNA extracted from mammalian cells is comprised of ribosomal RNA (rRNA), mainly consisting of 28S and 18S species. The remainder is made up of the other RNA, of which messenger RNA (mRNA) comprises only 1-3%. The method used to check RNA integrity (denaturing gel electrophoresis) relies on the assumption that rRNA quality and quantity reflect that of other RNA species including that of mRNA (Palmer & Prediger, 2007). There may also be a degree of difference between the integrity of the long-lived and abundant rRNA molecules and that of the mRNA population, which has a much more rapid turnover.

As will be described in section 2.5, RT-PCR (reverse transcription-polymerase chain reaction) was performed for each total RNA sample. Once complementary DNA (cDNA) had been synthesised from existing mRNA by RT, the quality was assessed by performing PCR using primers for β-actin (the housekeeping gene of choice), and running the products on an agarose gel. The intensity of the DNA band observed on the gel, at a specified number of cycles of amplification, was used as an indication of the integrity of the original mRNA template. Faint or undetectable bands were suggestive of degradation of the mRNA or of a failed RT reaction. The former was investigated by checking the integrity of the mRNA directly by running a specified amount (usually 1 μg) of total RNA on a gel, and if found to be significantly degraded the sample was re-extracted from the tissue (see Fig. 2.2).

2.3.4.1 *Reagents*

Ultra-pure water

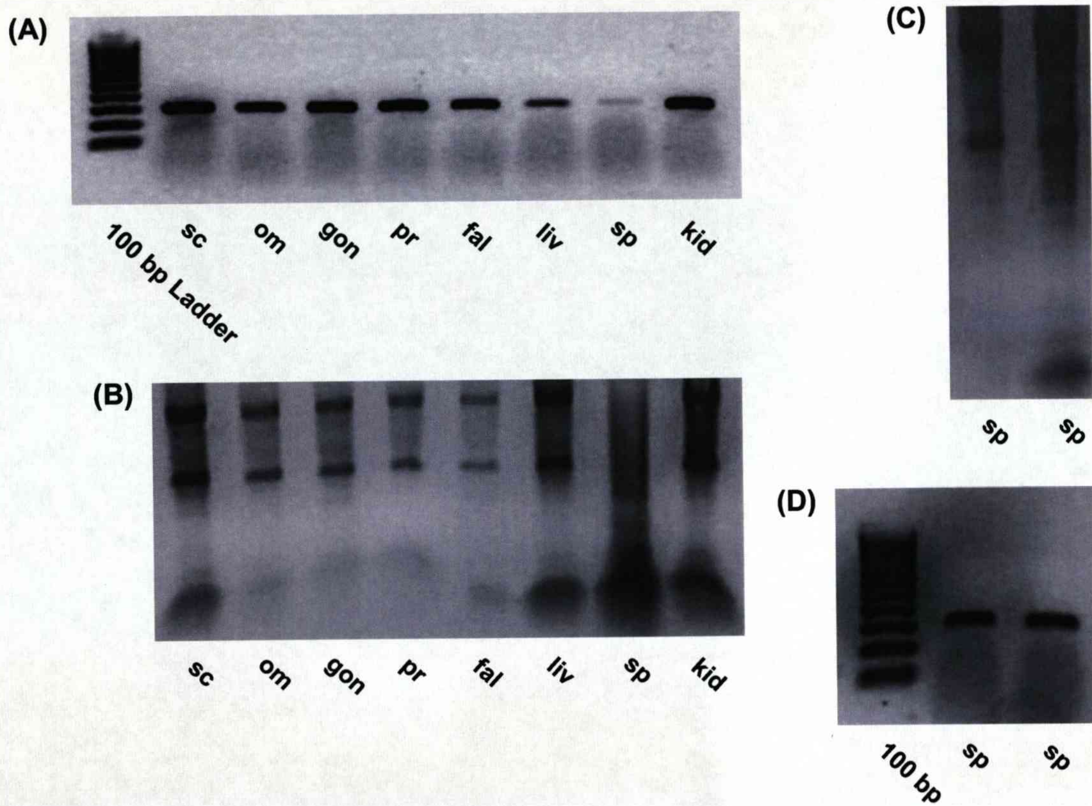
Sample buffer (1 ml 40% glycerol, 40 μl saturated BPB solution

Reagents used for agarose gel electrophoresis (see section 2.5.3)

2.3.4.2 *Method*

One μg of total RNA was diluted with ultra-pure water to a total volume of 5 μl in a 0.2 ml tube and a further 2 μl of sample buffer added. The sample was then briefly vortexed and loaded onto a pre-prepared 1% agarose gel (see section 2.4.3) and run at 80-100 V for 30-40 min. An image of the gel was photographed as described in section 2.4.3.2. An undegraded RNA sample would have two clearly visible and well-defined bands representing 28S and 18S rRNA with little or no visible debris at the base of the gel. Degradation would be inferred from the absence or smudging of the rRNA bands coupled with a strong signal at the base of the gel.

Figure 2.2 Checking mRNA integrity from a sample of total RNA



This figure illustrates how the integrity of the mRNA in a sample of total RNA can be inferred from that of the rRNA present. RT-PCR of total RNA extracted from tissues shows a faint band for spleen (usually more resilient to RNA degradation compared to other tissues such as liver), indicative of degradation (A). When total RNA samples were run on a denaturing gel no clear 28 or 18S bans were visible with a pooling of RNA fragments at the bottom of the gel (B). Spleen samples were re-extracted in duplicate and the RNA run on a gel. Distinct 28 and 18S bands can be seen in approximately a 2:1 ratio as expected (C). Samples were subjected to RT-PCR and a clear bands showing good product amplification were seen (D). sc, subcutaneous inguinal; om, omental; gon, gonadal; pr, peri-renal; fal, falciform ligament; liv, liver; sp, spleen; kid, kidney. β-actin (25 cycles). Amplicon size 289 bp.

The most critical factor in determining the quality of extracted RNA is the physiological state of the tissue at the point of removal (Palmer & Prediger, 2007). Isolating RNA from companion animal tissue presents many challenges which are not always present in laboratory animal work. Factors which may affect the quality of the RNA sample include the physiological state of the tissue prior to death (agonal state), and the post-mortem interval (the delay between time of death and tissue collection). Thus it is rare that RNA extracted from tissues will have 28S:18S ratios of exactly 2.0. The level of degradation has also been found to vary, to some degree, with the tissue of origin. This is likely to be due to tissue-specific responses to physiological stress both prior to and following death. Lower rRNA ratios are characteristic of some tissues such as liver and lung, regardless of whether the tissue is sourced from rodent or human (Palmer & Prediger, 2007). Other tissues such as spleen and kidney are known to be more resilient and if faint bands are seen on RT-PCR, re-extraction will usually yield a good quality product.

2.4 Reverse-transcription polymerase chain reaction

2.4.1 Reverse transcription

Once samples of total RNA had been quantified and treated for any remaining genomic DNA contamination, RT was used to generate single-stranded DNA (ssDNA) strands complementary to those of the mRNA sequences in the total RNA. These ssDNA sequences are referred to as complementary DNA (cDNA). Anchored oligo dT primers were used as they are specifically designed to anneal at the mRNA/poly-A junction rather than at random points along the poly-A tail of the mRNA molecule (as is the case with standard dT primers), or at random points along the mRNA sequence as occurs with random decamers. Commencing transcription at the start of the 3' end of the coding mRNA sequence results in an increased yield of cDNA with fewer truncated sequences, which is an advantage when later using PCR or real-time PCR (qPCR) to study genes which are not highly expressed.

2.4.1.1 Reagents

Ultra-pure water

Reverse-iT 1st Strand Synthesis Kit:

500 ng/μl Anchored oligo dT

5x 1st Strand Synthesis buffer

5 mM each dNTP mix

50 units/μl Reverse-iT RTase Blend

100 mM DTT

2.4.1.2 *Method*

For this procedure, sterile RNase/DNase-free 0.2 ml tubes and filter tips were used. All incubations were performed using a thermal cycler (PCR Express). Reactions were performed in either a 10 or 20 μl reaction volume. For a 20 μl reaction volume, 1 μg of total RNA and 1μl of anchored oligo dT were placed into a 0.2 ml tube, together with ultra-pure water to a total volume of 13 μl. A negative control tube was always used where the total RNA template was replaced with ultra-pure water. The samples were then incubated at 70°C for 5 min to denature the RNA. Samples were then rapidly cooled on ice to minimise the formation of any secondary structures.

A master mix was made containing 4 μl of 5x 1st Strand Synthesis buffer, 2 μl of dNTP mix, 1 μl of Reverse-iT RTase Blend and 1 μl of DDT per sample of total RNA (with a 5-10% excess). Eight μl of this master mix were added to each sample tube and each tube vortexed and centrifuged. The reaction was performed by incubating the tubes at 47°C for 30 min during which the cDNA was synthesised. The reaction was terminated by incubating at 75°C for 10 min to denature the RTase. The final reaction was then diluted 1 in 4 with ultra-pure water to generate cDNA template for PCR use. The cDNA product was stored at -20°C if not used immediately for PCR.

2.4.2 Polymerase chain reaction

PCR uses DNA polymerase and gene-specific primers to amplify the cDNA of interest. The process begins with a denaturation phase, followed by 15-40 cycles of product amplification. Each cycle consists of three steps: denaturation, annealing and extension. The initial denaturation at 94°C for 4 min allows the dissociation of any double-stranded DNA (dsDNA) into ssDNA and the unwinding of the helical structure of DNA. The annealing temperature (T_a) is primer specific, and depends on several factors such as primer length and GC content (the higher the GC content the

lower the annealing temperature a primer will have). The T_a enables the primers to hybridise to their complementary bases on the ssDNA. This is followed by an extension phase during which the DNA polymerase synthesises a complementary DNA strand starting from the site where the primer anneals in a 5'-3' direction.

When assessing the level of gene expression of a particular gene, it is compared with that of a gene whose level of expression is known to be stable; such a gene is referred to as a 'housekeeping' gene. This is one which ideally should be expressed at a constant level in different tissues, and within the same tissue under all conditions. The selection of an appropriate housekeeping gene is critical as it is used to assess the relative degrees of expression of the genes of interest as well as indicate the quality of the template material used. Typical housekeeping genes are β-actin, glyceraldehyde-3-phosphate (GAPDH) 18S rRNA and RNA polymerase IIa. Studies examining gene expression in WAT have successfully used β-actin as the housekeeping gene (Gorzelniak *et al.*, 2001; Eisele *et al.*, 2005; Zhang *et al.*, 2005) and this therefore was the gene of choice for both PCR and real-time PCR (qPCR) assays. However, if its level of expression was affected by experimental conditions, a second gene, RNA polymerase IIa (RNAPolIIa) was used instead.

2.4.2.1 Primer design and optimisation

Primers were designed using Primer Premier 5 software and synthesised commercially (Eurogenetec Romsey, UK). The sequence for the gene of interest was obtained by searching the NCBI PubMed Entrez Nucleotide database (www.ncbi.nlm.nih.gov/entrez) for the most complete sequence available. The protein coding sequence (CDS) in FASTA file format, together with the locations on the CDS or mRNA sequence for exon-exon boundaries were identified.

Identifying exon-exon boundaries was important, as a pair of primers (forward and reverse) could then be designed to span at least one such junction, ideally containing a large intron. This would make any genomic DNA contaminants present in the cDNA template easily identifiable as a much larger band on the agarose gel. The location of introns were established either directly (using NCBI's Map Viewer utility), or calculated from the chromosomal exon locations when the sequence was subjected to a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) against the

genome. Ideally, all primers would be sited so as to generate a product from the CDS. However, if necessary, one of the primers could be sited up or down-stream of this region so as to still span an intron.

The design of primers was carried out according to several parameters. The optimal primer length was chosen to be between 18 and 24 bases. Longer primers were preferred as they are more specific during amplification (allowing a lower T_a, and thus a faster reaction). However, these are more likely to dimerise and form secondary structures. Primers with the least and most unstable secondary structures were always chosen as these structures would tend to disappear at higher T_as. Any secondary structure or false priming at the 3' end of the primer was especially avoided, as these could theoretically be extended and therefore amplified by the DNA polymerase.

The primer melting temperature (T_m) was ideally between 60-65°C (and within 2°C of each other for a primer pair), so that the T_a would not be below 55°C since a low T_a promotes non-specific binding of primers. The guanine-cytosine (GC) content of each primer was in the region of 50-60%, and roughly equal between the two primers, as with increasing GC content the T_m increases. The ideal amplicon size was between 200-600 bp as larger products require longer annealing and extension times. Finally, after designing each primer, both forward and reverse primer sequences as well as that of the expected amplicon were subjected to a BLAST search to ensure specificity.

On receipt, primers were tested by performing PCR with one or more cDNA samples in which a signal for that gene might be expected. A high cycle number (>35), and a low annealing temperature (53-55°C) were used initially to maximise the chances of obtaining a product. If a product was obtained, the primer was then optimised by performing PCR over a range of T_as (5°C below, at the recommended T_a, and 5°C above the T_a), and selecting the highest at which a strong signal was obtained with no non-specific product formation. Finally, the cycle number was optimised by performing PCR over a range of cycle numbers and selecting that at which a distinct signal could be detected. RT-PCR primer sequences and optimal cycling conditions are detailed in Table 2.1.

2.4.2.2 *Reagents*

Ultra-pure water

10 μM each sense and anti-sense primer mix

ReddyMix PCR Master Mix Kit:

1.1x ReddyMix

2.4.2.3 *Method*

As for reverse transcription, this procedure was carried out using sterile 0.2 ml RNA/DNA-free tubes and filter tips. Reactions were performed using a thermal cycler (PCR Express). A master mix containing 11.25 μl of 1.1x ReddyMix, 0.25 μl of primer mix and 0.5 μl of ultra-pure water per sample was prepared (with a 5-10% excess to allow for pipetting inaccuracies). Samples were then vortexed and centrifuged. Twelve μl of this master mix were transferred into each tube and 0.5 μl of the appropriate cDNA template added for a 12.5 μl final reaction volume. All volumes were proportionally scaled up for 25 and 50 μl reactions as needed. Ultra-pure water was used as the non-template control (NTC). The tubes were vortexed, centrifuged and placed into the thermal cycler with the PCR program as follows:

1. 94°C for 2 min (denaturation step)
2. 20-35 cycles (specified by the optimal cycle number for each primer pair) of:
    i. 94°C for 20 sec (denaturation)
    ii. 53-62°C for 30 sec (annealing; T_a optimised for each primer pair)
    iii. 72°C for 30 sec (extension)
3. 72°C for 5 min (final product extension)

Table 2.1 Sequences of RT-PCR primers

Primer	T_a (°C)	Cycles	Product (bp)	Sequence (5'-3')
β-actin s	55.0	25	289	AGGACTCCTACGTGGGCGATGA
β-actin as				CCAGAGGCATACAGGGACAGCA
NGF-β s	56.0	28	368	CCACTGGACTAAACT TCAGCATTC
NGF-β as				TCACCTCCTTGCCCTTGATG
TNFα s	56.0	34	248	AACCCCAAGTGACAAGCCAGTA
TNFα as				TCTGGTAGGAGACGGCGAAG
IL-6 s	55.0	34	237	GCTACTGCTTTCCCTACCCC
IL-6 as				GCATCCATCTTTTCCCTCCA
IL-18 s	54.0	30	345	AGGATATGCCCGATTCTGACTG
IL-18 as				CCCCATTTTCATCCTTGTCTTT
Leptin s	56.0	32	387	CTGTGCCAATCCGAAAAGTC
Leptin as				GTCTGTTCAGAGCCACCACC
Adiponectin s	60.0	26	416	AAGGAGATCCAGGTCTTGTTGG
Adiponectin as				TTCCAGATGAAGGAGCACAGAG
MCP-1 s	51.2	23	234	GCTCACCCAGCCAGATGC
MCP-1 as				GCAGTTTGGGTTTGGCTTTT
MIF s	60.0	32	225	TCATCGTAAACACCAACGTGCCC
MIF as				GCTTGCTGTAGGAGCGGTTCTGC
GLUT4 s	57.0	32	403	TTGGTCTCGGTGTTCCTGGTC
GLUT4 as				CGGGTTTCAGGCACTTTTAGGA
LPL s	59.0	32	193	TTGAGAAAGGGCTTTGCCTGAG
LPL as				GGCCTGGTTGGTCTGTGCATCA
Haptoglobin s	60.0	26	409	GTAGAGCACATGATTCGCTACCAG
Haptoglobin as				GTAGGACCACCTTTTCAACCTCC
Adipsin s	57.1	27	176	CAGCCTCGTGGGCGCATCCT
Adipsin as				AGCACCCGCACCTTCCCGTC
PAI-1 s	56.0	32	325	ATGTTCATCGCCGCTCCCTAT
PAI-1 as				CGATAATGGCAGTAGAGGAGGAC
VEGF s	61.5	32	344, 373, 283	CATCTTCCAGGAGTACCCTGACG
VEGF as				AAACAAATGCTTTCTCCGCTCT
PPARγ1 s	57	25	410	CATCTTTCAGGGGTGTCAGTTCC
PPARγ1 as				TCCAAGGCTTGTAGCAGGTTGT
PPARγ2 s	57.9	25	400	ATCCCTCTTCCATGCTGTTATG
PPARγ2 as				GGCTCTTCGTGAGGCTTATTGT
SAA s	60.4	28	334	AGGGAGAAGCAGCGAACC
SAA as				ACCCAGGACCAAGGAACAG

This table shows the sequences of primers used for RT-PCR reactions together with the expected product sizes and annealing temperatures (T_a). The amplification cycle numbers are those used, unless stated otherwise. All primers were designed based on sequences obtained from the canine genome.

2.4.3 Agarose gel electrophoresis

2.4.3.1 Reagents

Agarose

5 mg/ml EtBr

10x TBE buffer

100 bp DNA ladder (Superladder-Low 100 bp Ladder with ReddyRun)

Distilled water

1% agarose gel (final volume 60/90 ml):

0.6/0.9 mg agarose

60/90 ml 1x TBE buffer

2/3 µl EtBr (final concentration of 0.167 µg/ml)

Distilled water was used to dilute 10x TBE buffer to prepare 1x TBE for use.

2.4.3.2 Method

A gel casting tray of appropriate size was prepared by sealing both ends with a strip of autoclave tape. The agarose was weighed and placed into a 500 ml conical flask containing 1x TBE and the flask heated for ~ 5 min in a microwave. The flask was removed every few seconds and agitated until the agarose was completely dissolved. The solution was placed in a category I chemical safety cabinet and once cool, the EtBr was added and mixed by gentle swirling of the flask. The mixture was poured into the casting tray and any bubbles pushed to the edges of the tray with one of the combs. The combs were then placed into their slots, and the gel allowed to set for at least 30 min. Once set, the autoclave tape and combs were removed and the gel placed into the gel tank. The tank was filled with 1x TBE until the gel was fully submerged.

Ten µl of PCR product and 1.5 µl of ladder were loaded into separate wells. The gel was run at 85-100 V for 30-60 min or until it was judged that the samples had run far enough for adequate separation of the bands on the ladder. Bands were detected by placing the gel under a UV Transilluminator (2011 Macrovue). The images were recorded by a DC120 digital camera (Kodak) and analysed using digital Science ID 1 image analysis software (Kodak).

2.4.4 PCR product purification and sequencing

If PCR primers are designed correctly, the product obtained should be the amplification of the desired mRNA. However, in order to confirm that the target cDNA had indeed been amplified, the product obtained was sequenced. All sequencing was performed commercially by MWG Biotech (Germany).

2.4.4.1 Reagents

ReddyMix PCR Master Mix Kit (1.1x Reddy Mix)

NucleoSpin Extract 2-in-1 Kit:

Buffer NT1

Buffer NT2

Buffer NT3

NucleoSpin columns

NucleoSpin collecting tubes

Ultra-pure water

100 bp ladder GeneRuler

Sample buffer (1ml 40% glycerol, 40 µl saturated BPB solution)

2.4.4.2 Method

Two 50 µl PCR reactions using a single template cDNA previously identified as strongly expressing the gene of interest were performed to obtain a total reaction volume of 100 µl. High cycle numbers were used to generate a large quantity of PCR product. The reaction volumes were pooled and run on a pre-prepared 1% agarose gel using a single composite well (obtained by placing autoclave tape over several teeth of the desired comb in advance), large enough to accommodate both reaction volumes. The gel was then run at 80-100 V or 30 min and the band visualised under UV light. Once visualised, the band was excised with a scalpel, weighed and the entire gel fragment transferred into a pre-weighed 2 ml tube and its mass determined. The PCR product was extracted from the gel according to instructions in the NucleoSpin Extract 2-in-1 kit as follows:

Three hundred µl of NT1 was added to the tube containing the PCR product in the gel per 100 mg of gel fragment. This was incubated at 50°C in a water bath with gentle agitation until the gel had dissolved (~ 10 min). A NucleoSpin column was

placed in a sterile 2 ml tube and 700 μl of the sample loaded. This was centrifuged for 1 min at 8000x*g*. The flow-through the collecting tube was discarded. If the sample volume was >700 μl, the procedure was repeated and the flow-through discarded every time until the total volume had been processed. Once this step was complete, the PCR product (impure) was bound to the membrane of the column. A washing step was undertaken which was designed to remove any inhibitors or contaminants from the agarose gel. This was performed by adding 500 μl of NT2 to the column and centrifuging for 1 min at 11000x*g*. The flow-through was again discarded and a further washing step was carried out by adding 600 μl of NT3 to the column which was centrifuged for a further 60 sec at 11000x*g*. After discarding the flow-through, 200 μl of NT3 was added and the column centrifuged for a further 2 min at 11000x*g* to dry the membrane. The column was transferred into a 1.5 ml tube and 30 μl of ultra-pure water added. The column was allowed to stand for 1 min at room temperature to allow full dissolution of the DNA product. Finally, the column was centrifuged for 1 min at 11000x*g* to collect the eluted DNA within the 1.5 ml tube.

For a gene product to be accurately sequenced, a minimum of 20 ng of DNA product is needed per 100 bp of product. The concentration of the purified DNA in solution was estimated by preparing a sample consisting of 1 μl of the purified DNA, in 5 μl of ultra-pure water and 2 μl of sample buffer. This was loaded into a pre-prepared 1% agarose gel and run as described previously alongside 2 μl of quantifying 100 bp ladder (GeneRuler). The concentration of DNA in the solution could be estimated according to the brightness of the band. If the band was faint or absent, the whole procedure was repeated from the PCR reaction onwards.

The 100 bp GeneRuler was prepared by adding 1 μl (0.5 μg) of ladder DNA, 1 μl of loading dye solution and 4 μl of ultra-pure water. The ladder DNA was therefore diluted 1:6 (working concentration of 0.083 μg/μl). Two μl of ladder, containing 0.17 μg of DNA, were loaded onto the gel alongside the sample to be quantified. According to the manufacturer's protocol, a specific quantity of DNA is present in each band seen on the gel when 1 μg of the ladder DNA is used. Thus, the amount of DNA present in each band when 0.17 μg of ladder were used could be determined.

A set volume (1 µl), of the purified DNA solution was run alongside the ladder and the brightness of the purified DNA band was compared to that of each of the Gene Ruler ladder's bands by eye and that of the nearest intensity identified. The DNA concentration of that ladder band was then taken to be the approximate concentration of DNA in the purified solution. An aliquot (in duplicate), of this solution containing the required amount of DNA for sequencing was then placed in a 1.5 ml tube and dried using a Heptovac VR-1 vacuum centrifuge (Thermo). Both aliquots were then sent for sequencing as well as a solution containing 10 µl of the sense and anti-sense primers. When results from the sequencing were returned, these were subjected to a basic local alignment search tool (BLAST) search to confirm their identity.

2.5 Real-time polymerase chain reaction

Real-time PCR (qPCR) is a technique which allows the absolute or relative quantification of the initial amount of template in a sensitive, specific and reproducible manner. This is possible as qPCR monitors the fluorescence emitted during the reaction as an indicator of product production during each PCR cycle, the fluorescence signal increasing in direct proportion to the amount of PCR product (or amplicon). During the exponential phase of the reaction, there is a marked increase in the amount of product produced. The more of the initial template there is, the earlier during the reaction the florescence signal will begin its exponential rise.

2.5.1 Detection chemistries

There are a range of different detection chemistries available for qPCR; these include not only the hydrolysis or TaqMan Probes system and SYBER Green I, but also molecular beacons, scorpions and hybridisation probes.

2.5.1.1 Hydrolysis or Taqman® system

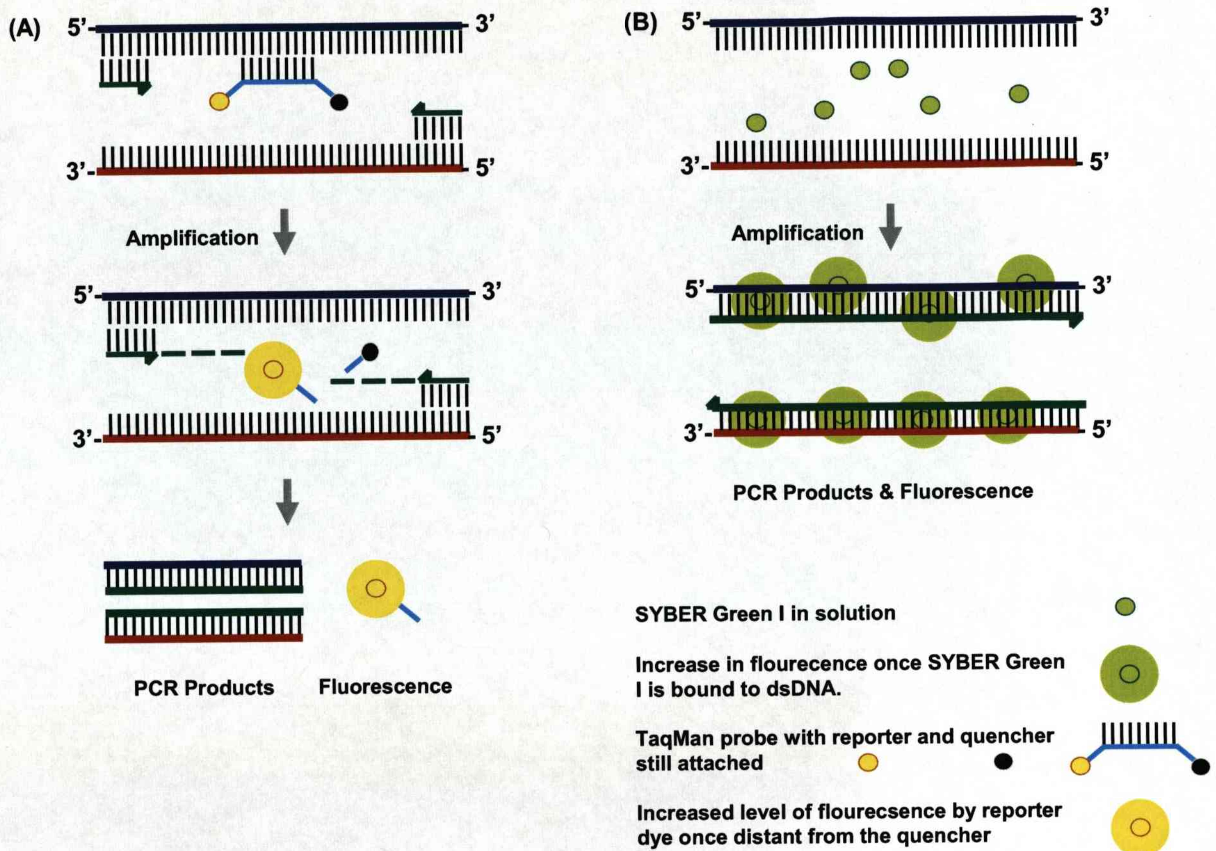
This system involves the use of a pair of primers (forward and reverse) and a Taqman probe (Applied Biosystems) to amplify the mRNA of interest. The probe is an oligonucleotide with a fluorescent (reporter) dye (usually FAM – 6-carboxyfluorescein) on the 5'base, and a quenching dye (usually TAMRA – 6 carboxytetrametheyrhodamine) located on the 3'base. All three oligonucleotides are specific for the target and are able to bind to it. When the probe is intact, energy is

transferred from the reporter dye to the nearby quenching dye by fluorescence resonance energy transfer (FRET), rather than be emitted as fluorescence detectable by the machine (Giulietti *et al.*, 2001). When the Taq polymerase replicates a template onto which the probe is bound, its 5' exonuclease activity cleaves the probe separating the reporter and quenching dyes ending the FRET. The reporter dye is now able to emit fluorescence (at 518 nm for FAM) which the machine can then detect (Fig 2.3 A). The amount of fluorescence increases in each cycle in a manner proportional to DNA amplification. This accumulation of PCR product is detected by monitoring the increase in fluorescence (ΔR) of the reporter dye plotted as ΔR_N after normalisation of the reporter dye signal to a reference dye, usually ROX (carboxy-X-rhodamine). The reference dye is used to correct for differences in reaction mix volumes between wells.

2.5.1.2 SYBER Green I

SYBER Green I is a DNA-binding dye which can be used for the detection of PCR product formation avoiding the need for an expensive, albeit specific probe. Its fluorescence is virtually undetectable when free in solution, but when bound to double-stranded DNA (dsDNA), the fluorescence increases by over 1000-fold (Fig 2.3 B). As DNA is amplified, the amount of dsDNA increases and the more binding sites there are for the dye, so fluorescence increases in proportion with DNA concentration. This property allows the dye to be used to track the accumulation of PCR product as the increasing concentration of dsDNA in the solution can be directly measured by the increase in fluorescence signal. One limitation of such an assay is the non-specificity of this method, as SYBER Green I will lead to an increase in fluorescence when bound to any dsDNA molecule. The specificity of the reaction is determined solely by the primers. Its many uses include optimising primer concentrations prior to optimising that of the probe (see section 2.5.2).

Figure 2.3 Detection chemistries used in qPCR



This figure illustrates the two forms of detection chemistries for qPCR used in this study. The TaqMan assay utilises as specific probe oligonucleotide with a reporter dye at the 5' end (FAM), and a quenching dye at the 3' end (TAMRA). As product is amplified the 5' to 3' exonuclease activity of the polymerase cleaves probe ending the FRET and allowing fluorescence emission from the reporter dye to occur (A). DNA-binding dyes such SYBER Green I emit barely detectable levels of fluorescence in solution. However, once bound to dsDNA product the level of fluorescence is markedly increased and this is then detected allowing the amount of template to be quantified (B).

2.5.2 Design of primers and Taqman probes

Canine primers and probe sequences were designed using Beacon Designer 4.02 software (Premier Biosoft International) and synthesised commercially (Eurogenetec). Gene sequences and intron/exon data were obtained as in section 2.5.2.1. Primers were designed to span an intron and be 20-30 bases in length, with primer T_ms of 58-60°C. The probe was designed to have a T_m 10°C higher than that of the primers. This allows both the hybridisation of the probe and primers, and cleavage of the probe. The product size was designed to be between 60-120 bases, as PCR is more efficient with smaller amplicons. A specific requirement of Taqman probes was that there be no G at the 5' end, as a G adjacent to the reporter dye results in quenching of the reporter fluorescence even once the probe has been cleaved.

Canine primer and TaqMan probe sequences, as well as the optimised final reaction concentrations for each, are detailed in Table 2.2. The optimal concentration for each new pair of primers was determined by performing an assay with final concentrations of sense and anti-sense primers ranging from 100 nM to 600 nM, in all possible combinations, using SYBER Green I. The resulting amplification plots were examined, and the primer concentration providing the lowest C_T (threshold cycle, the cycle at which fluorescence measured by the instrument is at a statistically significant level above that of the background signal) and the highest ΔR_N was selected for use. All products generated during a qPCR amplification reaction are melted at 95°C, then annealed at 55°C and subjected to a gradual increase in temperature to 95°C. The result is a plot of raw fluorescence data versus temperature where one should aim to see a rapid decline in fluorescence between 82°C and 84°C where the main PCR product melts. If this same data is analysed by plotting the negative first derivative of raw fluorescence against increasing temperature, a single peak (indicating one major product) should be observed between 84.5°C and 88°C. TaqMan probes were optimised at concentrations of 100-200 nM using the optimised primer pair concentrations. The amplifications plots were used to select the optimal probe concentrations using the same criteria (lowest C_T and highest ΔR_N) as used for primer concentration selection.

Table 2.2 Sequences of primers and TaqMan probes used for qPCR

Primer	Product (bp)	Final conc. (nM)	Sequence (5'-3')
β-actin s	96	300	GCATCCTGACCCTCAAGTACC
β-actin as		300	AGCTCGTTGTAGAAGGTGTGG
β-actin P		200	CGAGCACGGCATCGTCACCAACTG
RNApolIIa s	122	600	TCAACTTCCTCAGTTACGTGGTC
RNApolIIa as		600	GGCAGCAACCATTGGATAAGC
RNApolIIa P		200	CCCAGCCAGAGAGGAGACGACAACAAT
NGF-β s	99	300	TCTAGGCACCACCCCTCTG
NGF-β as		300	ATCAGAGTGTAGAACAACATGGAC
NGF-β P		200	AGGAATTATCACCCTCCCTGGACAGTGC
TNFα s	78	300	CCGTGAGAAGATGACCCAGATC
TNFα as		300	GACAGCACAGCCTGGATGG
TNFα P		200	CGAGACTTTCAACACCCCAGCCATGTACG
MCP-1 s	128	600	TCCTCTGCCTGCTGCTCATAG
MCP-1 as		600	GGCCAGCCTCTGAATTGAGATC
MCP-1 P		200	CGCCGCCCTCACCACCCAGG
IL-6 s	125	600	AAAGAGCAAGGTAAAGAATCAGGATG
IL-6 as		600	GCAGGATGAGGTGAATTGTTGTG
IL-6 P		200	ACTCCTGACCCAACCACAGACGCCA
Leptin s	99	600	AGGATCAATGACATTTCACACACG
Leptin as		600	GGACAAACTCAGGACTGGTTGG
Leptin P		200	CCAGGAATGAAGTCCAGACCAGCGACC
Adiponectin s	119	600	CCGTGATGGCAGAGATGGC
Adiponectin as		600	AGCCTCGGGGACCTTCAAC
Adiponectin P		200	CCAGTTACTCCAGTTTCACCAGTGTCACCC
11β-HSD1 s	149	600	CAGACCAGAGATGCTCCAAGG
11β-HSD1 as		600	GGGATACCACCTTCTTTAGAGTTTC
11β-HSD1 P		200	CCTTGCTGTCACCACCACATGGGCTC

This table shows the sequences of canine primers and TaqMan probes used in real-time PCR (qPCR) analysis for relative quantification of canine gene expression. P, TaqMan probe.

2.5.3 Preparation of 96 well plates for real-time PCR

2.5.3.1 Reagents

qPCR Core Kit:

- 10x reaction buffer
- 50 mM magnesium chloride
- 5 mM dNTP mix
- 5 U/μl Hot Goldstar enzyme

Sense primer, anti-sense primer and Taqman probe stock solutions

Ultra-pure water

2.5.3.2 Method

A master mix was made up for each gene of interest containing all the necessary components for the qPCR reaction, except for the cDNA template, as follows:

10x reaction buffer	1.25 μl per well
50 mM magnesium chloride	1.25 μl (2.5 mM)
5 mM dNTP	0.5 μl (100 mM)
Sense primer	0.075 μl (see table 2.2)
Anti-sense primer	0.075 μl (see table 2.2)
Taqman probe	0.5 μl (see table 2.2)
5 U/μl Hot Goldstar enzyme	0.063 μl (0.025 U/μl)
Ultra-pure water	7.79 μl

Having made up the master mix, 11.5 μl were aliquoted into each well of a 96-well plate. One μl of the appropriate template cDNA was then added to each well for a total reaction volume of 12.5 μl. The plate was then sealed with an adhesive optical cover. All samples were run in duplicate. NTCs were included on each plate for each set of primers and probes used. The plate was spun at 1000 rpm for 30 sec (to ensure the reaction mix was at the bottom of each well and no air bubbles were present) in an IEC Centra-7R Centrifuge (Damon). If the assay was not to be performed immediately, the plate could be stored for up to 5 days at 4°C. If a set of samples (comprising more than one group) required analysis over several plates, at least one sample (usually from the control group) was present on all plates. Data was

normalised so that the mean ΔC_T for that sample (see section 2.6.5), had the same value for each assay. This enabled the data on the different plates to be directly compared.

2.5.4 Taqman system real-time PCR setup

All reactions were performed using an Mx3005P real-time machine (Stratagene). Once the heat lamp stabilised, the plate was inserted into the heat block. Amplifications were performed as follows:

1. 50°C for 2 min (activation of Hot Goldstar enzyme)
2. 95°C for 10 min (denaturation)
3. 35-40 cycles (dependent on the level of expression of each gene)
   i. 95°C for 15 sec (denaturation)
   ii. 60°C for 60 sec (combined primer annealing and extension)

Data was collected automatically by the software after each cycle of amplification and analysed once the run was complete (total run time was ~ 2 h).

2.5.5 Analysis of qPCR data

The amplification plots were displayed on a log scale using the MxPro-Mx3005P software (Stratagene). The threshold was manually adjusted to ensure that it was clear of any background noise. The software automatically re-calculated the C_T value for each well. The results were exported as a Microsoft Excel file and gene expression analysed by relative quantification with the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). All samples were normalised to values for either β-actin or RNAPolIIa, and results expressed as fold changes of C_T value relative to controls. For example, to compare a treated versus a non-treated control group with IL-6 as the target gene and β-actin as the housekeeping gene, the formulae would be:

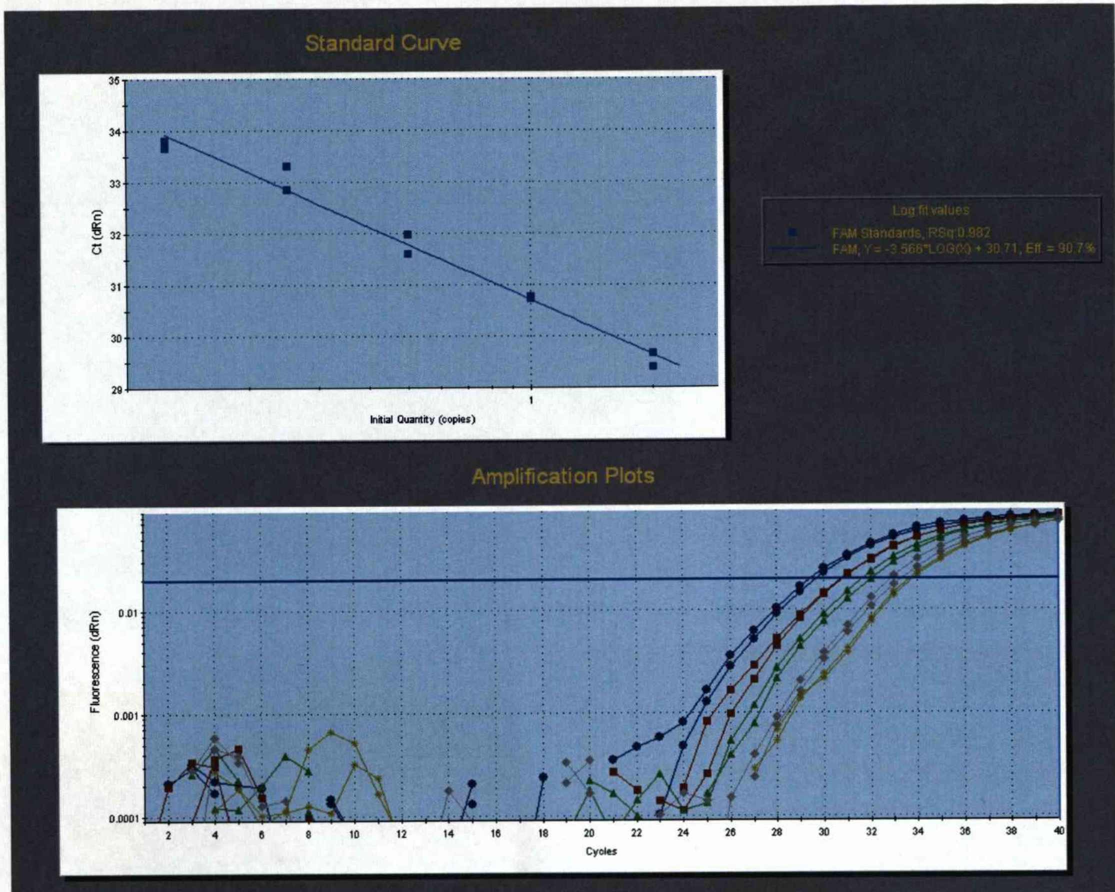
$$\Delta C_T = C_T(\text{IL-6}) - C_T(\beta\text{-actin})$$

$$\Delta\Delta C_T = \text{Mean } \Delta C_T(\text{treated samples}) - \text{Mean } \Delta C_T(\text{control samples})$$

$$\text{Fold Change} = 2^{-\Delta\Delta C_T}$$

In order to ensure that the use of this method was appropriate, a validation experiment was performed for each set of primers and probe to check that the amplification efficiencies were adequate (see Fig. 2.4).

Figure 2.4 Validation of the $2^{-\Delta\Delta C_T}$ method



This figure shows the results of a representative validation experiment for a set of qPCR primers and probe. A 5-fold dilution series was carried out using a positive control template encompassing the expected levels of target within experimental samples. The slope of the standard curve is used to determine the efficiency of the reaction. Since the PCR reaction is based on exponential amplification, if the efficiency is 100%, the amount of template will double with each cycle. This will generate a linear fit with a slope between ~ -3.1/-3.6 (in this example -3.57). The reaction efficiency should be between 90-110%, (here 90.7%). These assays were run at least in duplicate to determine the precision of pipetting, the reproducibility and the overall sensitivity of the assay. If all the data lie perfectly on the line Rsq (the fit of all data to the standard curve plot) will be 1.00 (here 0.982). An Rsq value ≥ 0.985 is acceptable for most assays (Stratagene, 2004).

2.6 Enzyme-linked immunosorbent assay

Secreted adipokine protein levels were measured in conditioned media (collected as described in section 2.3.5) by immunoassay. All assays were performed in Nunc MaxiSorp 96-well microplates.

2.6.1 NGF E_{max} Immunoassay System (Promega)

Secreted NGF protein levels in conditioned media were measured using the NGF E_{max} Immunoassay System (Promega). This is an enzyme-linked immunosorbent assay (ELISA) kit designed for the detection of NGF in an antibody sandwich format. The kit displays cross-reactivity across several species including rat, mouse and human. No cross-reactivity with the dog was known prior to this study. The kit is specific for NGF demonstrating a very low level of cross-reactivity with other neurotrophic factors. The intra-assay coefficient of variation is reported to be in the region of 4.1-4.2%. The level of detection (or sensitivity), is 7.8 pg/ml of NGF.

2.6.1.1 Reagents

(i) NGF E_{max} Immunoassay System:

Anti-NGF Polyclonal Antibody (pAb)
5x Block and Sample Buffer (BSB)
1 μg/ml NGF standard
Anti-NGF Monoclonal Antibody (mAb)
Anti-rat IgG, HRP (horseradish peroxidiase) conjugate
TMB One solution

(ii) Carbonate coating buffer

25 mM sodium bicarbonate
25 mM sodium carbonate
Distilled water

Buffer prepared and pH adjusted to 9.7

(iii) TBST wash buffer

20 mM Tris hydrochloride (pH 7.6)
150 mM sodium chloride

0.05% (v/v) Tween® 20

Distilled water

(iv) 1 N hydrochloric acid (HCl)

2.6.1.2 *Method*

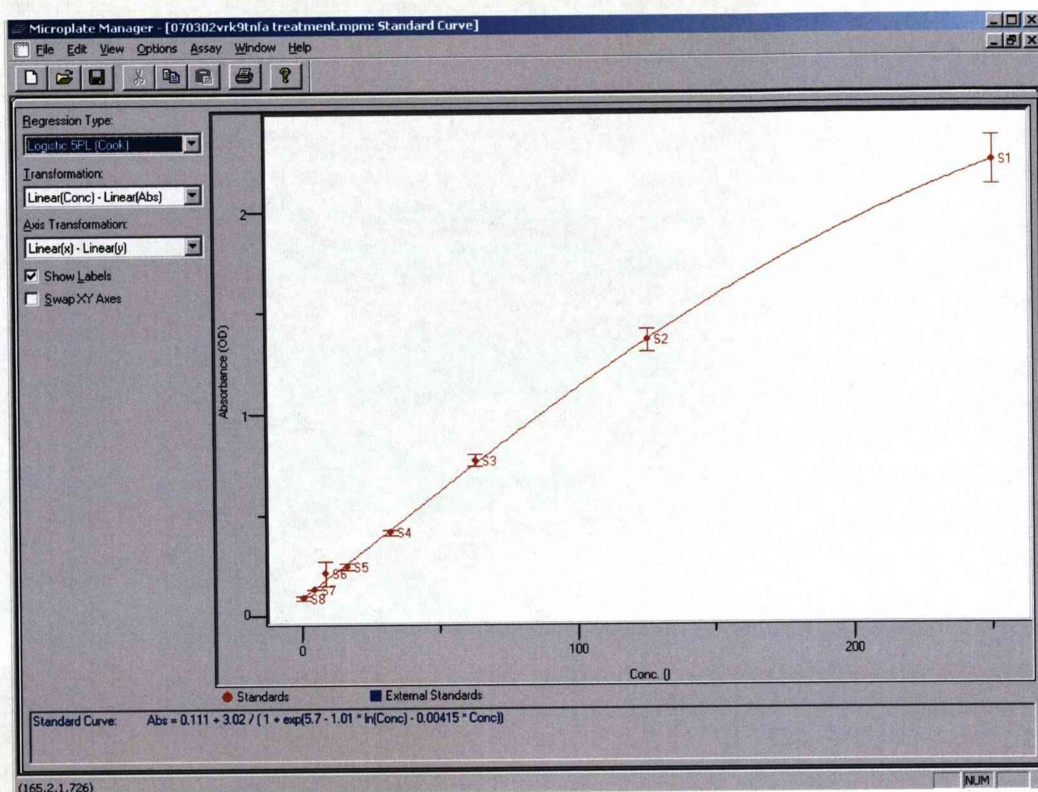
Ten μl of the Anti-NGF pAb were added to 10 ml of carbonate coating buffer to prepare enough reagent for a 96-well plate. 100 μl were then added to each well, the plate sealed, and incubated overnight at 4°C. The following day the plate was allowed to reach room temperature, its contents were discarded and all wells washed with TBST buffer. A 1 in 4 dilution of block and sample buffer (BSB) was made using distilled water, and 200 μl of 1x BSB were added to each well. The plate was incubated at room temperature for 1 h.

The NGF standard was diluted with 1x BSB to a final dilution of 1:4,000. Serial 1:2 dilutions were made using 1x BSB to generate 7 standards with a maximal concentration of 250 pg/ml. An eighth standard contained no NGF. Following blocking, the plate contents were discarded and the wells washed with TBST buffer. 100 μl of each standard and the same volume of conditioned media were added to the plate in duplicate. Samples were diluted in 1x BSB if necessary. In all cases, a preliminary assay was performed with serial dilutions of representative samples of media (conditioned and fresh) to identify the appropriate dilution for each assay. The plate was again sealed and incubated at room temperature on a plate shaker at 500 rpm for 6 h. The plate was washed 5 times with TBST buffer and 10 ml of 1x BSB were mixed with 2.5 μl of Anti-NGF mAb (1:4,000 dilution) and 100 μl were added to each well. The plate was sealed and incubated overnight at 4°C.

Fresh 1x BSB was prepared and 100 μl of Anti-Rat IgG HRP conjugate were mixed with 9.9 ml of 1x BSB. The seal was removed and the plate washed 5 times with 1x BSB and 100 μl of the diluted antibody conjugate added to each well. The plate was sealed and incubated at room temperature for 2.5 h on a plate shaker at 500 rpm. During this incubation step the TMB One solution was allowed to reach room temperature. After the incubation, the wells were washed with TBST buffer and 100 μl of TMB One solution added to each well and the plate incubated for a further 10

min at room temperature to allow colour development. The reaction was then stopped by adding 100 μl of 1 N HCl to each well and the absorbance measured at 450 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad). Data was collected and analysed using Microplate Manager v5.2 software (Bio-Rad) to determine the concentration of NGF protein in each well (see Fig. 2.5)

Figure 2.5 ELISA standard curve



This figure illustrates a typical standard curve obtained with the ELISA kits used in this study. Once the plate had been read by the plate reader, the software was able to calculate the concentration of standard at each serial dilution step. By identifying the concentrations of each of the standards, a curve could be plotted with a line of best fit. This was then used by the software to calculate the protein concentration in each sample well based on its absorbance.

2.6.2 IL-6, TNFα and MCP-1 assays (R&D)

Levels of canine interleukin 6 (IL-6), tumour necrosis factor-alpha (TNFα) and monocyte chemoattractant protein-1 (MCP-1) in conditioned media were measured using DuoSet® ELISA Development System kits (R&D Systems). These are also sandwich ELISA kits but here designed specifically for the detection the canine protein. The kits displayed cross-reactivity across several species including rat, mouse, cat and human to varying degrees. The sensitivity of each were canine IL-6, 1.5 pg/ml ; canine TNFa, 0.9 pg/ml; canine MCP-1, 1.2 pg/ml. Any value below these was considered non-detectable.

2.6.2.1 Reagents

(i) DuoSet® ELISA – Canine CCL2/MCP-1

4.0 μg/ml Capture Antibody mouse anti-canine MCP-1
50 ng/ml Detection Antibody goat anti-canine MCP-1
1000 pg/ml Canine MCP-1 Standard
1 ml diluted 1:200 Streptavidin-HRP

(ii) DuoSet® ELISA – Canine IL-6

0.8 μg/ml Capture Antibody goat anti-canine IL-6
200 ng/ml Detection Antibody goat anti-canine IL-6
4000 pg/ml Canine IL-6 Standard
1 ml diluted 1:200 Streptavidin-HRP

(iii) DuoSet® ELISA – Canine TNFα

1.0 μg/ml Capture Antibody mouse anti-canine TNFα
100 ng/ml Detection Antibody goat anti-canine TNFα
1000 pg/ml Canine TNFα Standard
1.0 ml diluted 1:200 Streptavidin-HRP

(iv) 1x PBS (see section 2.3.1)

(v) Wash Buffer

0.05% (v/v) Tween® 20

1x PBS

(vi) Reagent Diluent

1% BSA Fraction V, Protease free

1x PBS

(vii) Substrate Solution

50% H_2O_2

50% Tetramethylbenzidine

(viii) Stop Solution

2 N H_2SO_4

2.6.2.2 Method

The capture antibody was diluted to the stated working concentration in PBS without carrier protein (0.8 μg/ml goat anti-canine IL-6, 1.0 μg/ml mouse anti-canine TNFα and 4.0 μg/ml mouse anti-canine MCP-1). One hundred μl of the diluted capture antibody was added to each well of a 96-well microplate, the plate was sealed and incubated overnight at room temperature.

The contents of each well were discarded and the wells washed three times with 400 μl of wash buffer. The plates were blocked by adding 300 μl of reagent diluent (RD) to each well, sealed and incubated at room temperature for 1 h. The wash step was then repeated. The standards for each protein were allowed to sit with gentle agitation for 15 min prior to use. The standards were reconstituted using RD and diluted to a high standard of specified concentration for each protein (4000 pg/ml IL-6 and 1000 pg/ml for both TNFα and MCP-1). Serial 1:2 dilutions were made using RD to generate 7 standards with the desired maximal concentration. An eighth standard contained no protein. After washing, 100 μl of sample (diluted in RD if required) or standard was added to each well. The plates were covered and incubated at room temperature for 2 h. A 1 in 200 solution of streptavidin-HRP conjugate was

made using RD and100 µl added to each well. The plates were sealed and incubated at room temperature for 20 min.

After incubation, the plates were washed as described previously and 100 µl of substrate solution added to each well. The plates were sealed and incubated at room temperature for 20 min to allow colour development. 50 µl of stop solution was added to each well and the absorbance determined immediately using a Benchmark Plus microplate spectrophotometer (Bio-Rad), at 450 nm corrected to 540 nm. Data was collected for each protein, analysed and a standard curve generated for each plate as described in section 2.7.1.2.

2.6.3 Leptin and adiponectin assays (LINCO)

Due to the lack of availability of in-house ELISA assays for both canine leptin and adiponectin, levels of these two key adipocyte hormones in cell culture supernatant was assayed externally by LINCO using Beadlyte and LINCOplex Multiplex immunoassays. The technology involves the use of 5.6 µm beads, call microspheres, which are colour coded to distinct sets, each representing a protein of interest. Each bead set is conjugated with a unique monoclonal antibody with high specificity for the protein of interest. Samples are mixed with the beads allowing capture of the specific protein. A complementary secondary antibody is then added, completing the immunological sandwich reaction. The reaction is detected with streptavidin-phycoerythrin. Lasers are used to excite both the internal dye that identifies each microsphere particle and the phycoerythrin bound to the reporter antibody in the assay allowing quantification.

2.7 Statistical analysis

The statistical significance between the means of 2 groups of samples was assessed by Student's t-test (Excel 2003 software, Microsoft). Differences were considered to be significant when $P < 0.05$. All results are presented as means ± standard error of the mean (SE); the group size is indicated in the legends to the figures.

Chapter 3

Adipokine gene expression in canine white adipose tissues

3.1 Introduction

As described in Chapter 1, in mammals the majority of adipose tissues are contained in a multi-depot organ which can be broadly characterised as consisting of both subcutaneous and visceral depots (Cinti, 2005). These can be further classified according to anatomical location (such as abdominal and inguinal subcutaneous) or in terms of association with specific organ systems, such as the kidney in the case of the perirenal depot. In the dog, five main WAT depots are recognised; subcutaneous inguinal, omental, perirenal, gonadal and falciform ligament (the falciform ligament is a 'sickleshaped' saggital fold of peritoneum responsible for the attachment of the liver to the diaphragm, separating the right and left liver lobes).

In the obese state, expansion of white fat mass occurs through both hypertrophy of existing mature adipocytes and increased hyperplastic proliferation of preadipocytes (Hausman *et al.*, 2001). The main function of WAT is as a store for TG providing a long-term fuel reserve (Trayhurn, 2007). In addition, adipose tissue is also an important source of endocrine signals, these include the adipocyte hormone leptin, which is involved in appetite regulation and energy balance (Trayhurn *et al.*, 1999; Rayner & Trayhurn, 2001). Other protein signals, termed adipokines, include several linked to inflammation and the inflammatory response such as TNFα and IL-6. Their levels rise in obesity which is now thought to be characterised by a state of chronic low-grade inflammation (Fantuzzi, 2005; Trayhurn, 2005a).Thus, WAT may be the main site of inflammation in obesity, with increased levels of inflammation related bio-markers reflecting a spillover from an 'inflamed' tissue contributing to obesity-related pathologies (Trayhurn, 2005c).

Previous studies have indicated that genes encoding several adipokines are expressed in canine white adipocytes (Eisele *et al.*, 2005). These include leptin, where *in vivo* studies have demonstrated that in a similar manner to humans and rodents, plasma levels of leptin in the dog increase with obesity (Sagawa *et al.*, 2002). In humans, both mRNA and systemic levels of leptin are raised in women compared to men (Montague *et al.*, 1997b; Castracane *et al.*, 1998). However, in dogs there currently appears to be no association between sex, and systemic or mRNA levels of leptin (Ishioka *et al.*, 2002; Ishioka *et al.*, 2007).

This Chapter aims to further characterise gene expression in the main WAT depots in the dog. The importance of research in this area has been outlined in Chapter 1. Despite the increase in the level of interest in WAT biology, there is still a paucity of information on companion animals. As such, the expression of a number of classical adipocyte markers including PPARγ and LPL are examined in dog WAT. The mRNA levels of the adipocyte hormones leptin and adiponectin were also examined in WAT of both male and female dogs, where quantitative comparisons between sexes was carried out. In addition, expression levels of proinflammatory adipokines including TNFα and IL-6 were also investigated in all depots examined.

Over the past few hundred years dogs have been bred for different phenotypic characteristics including coat colour, coat length and body size. This has led to the formation of distinct genetic isolates referred to as breeds (Sutter & Ostrander, 2004). Several show a high prevalence of specific diseases (Patterson, 2000), many of which are also commonly seen in the human population and the clinical manifestations in the two species are often similar (Sargan, 2004). In terms of choosing a suitable breed for these studies the Staffordshire bull terrier (SBT) was the clear choice in that it is a medium sized breed which has a tendency to weight gain, and is common in the locality.

3.2 Methods

3.2.1 Animals and tissues

Tissues from entire adult male and female SBT dogs were obtained from those euthanased at an animal shelter, for reasons unrelated to the study. Animals were only included if there was no indication of disease, both externally and during gross examination of abdominal organs. In addition, animals suffering from cachexia or emaciation were also excluded. All WAT samples (subcutaneous inguinal, omental, perirenal, gonadal and falciform ligament depots) and samples from liver, spleen and kidney were obtained within thirty minutes of euthanasia and immediately frozen in liquid nitrogen.

3.2.2 RT-PCR and qPCR

RNA was extracted from tissues using Tri-Reagent as described in section 2.3.1. RNA which was to be analysed by qPCR was subsequently treated using a DNA-free kit to minimise levels of genomic DNA contamination (section 2.3.2). Once extracted, the total RNA was quantified using a BioPhotometer and reverse transcribed using the Reverse-iT First Strand Synthesis kit (sections 2.3.3 and 2.4.1). The cDNA was then analysed by PCR using the Reddymix PCR Master Mix kit and PCR Express thermal cyclers (sections 2.4.1 and 2.4.2).

Faint or undetectable bands at high PCR cycle numbers (>35) were suggestive of either very low levels of mRNA, mRNA degradation or a failed RT reaction. In such cases, the integrity of the mRNA was checked by running a specified amount of total RNA on a gel and, if found to be significantly degraded, the sample was re-extracted from the tissue (see section 2.3.4). PCR products were purified using a NucleoSpin Extract 2-in-1 kit and sequenced commercially (see section 2.5.4). Real-time PCR (qPCR) was carried out using the qPCR Core Kit and an Mx3005P real-time machine (section 2.5.3 and 2.5.4). All qPCR data was analysed using MxPro-Mx3005P software.

3.3 Results

3.3.1 Expression of adipocyte-related genes by canine WAT

In initial studies, RT-PCR was used to establish whether dog adipose tissues express genes encoding proteins indicative of WAT; specifically GLUT-4 (insulin sensitive facilitative glucose transporter), LPL and PPARγ (the gamma-2 isoform). PPARγ isoforms, PPARγ1 and γ2, differ in their N-terminal end as a result of alternative promoter usage and differential splicing (Zhu *et al.*, 1995). Whilst PPARγ1 is ubiquitously expressed, PPARγ2 is predominantly expressed in adipose tissue where it regulates adipocyte differentiation and fatty acid storage (Ren *et al.*, 2002; Hammarstedt *et al.*, 2005). Primers were designed specifically to detect expression of the gene coding for PPARγ2 (canine gamma-2, accession number AJ972913), see Fig. 3.1.

To confirm the expected product identity, all products obtained using newly designed primer pairs were subject to sequencing and comparison against the dog genome database. PPARγ2 gene expression was examined in all five separate adipose tissue depots (subcutaneous inguinal, omental, perirenal, gonadal and falciform) as well as in liver, spleen and kidney. As expected, a band consistent with PPARγ2 was detected exclusively in WAT, unlike PPARγ1 where, expression was detected in all tissues examined (Fig. 3.2A). The level of PPARγ2 expression appeared not to vary between depots. Bands observed for all genes examined were found to be fainter in the liver of both animals compared to that of other organs.

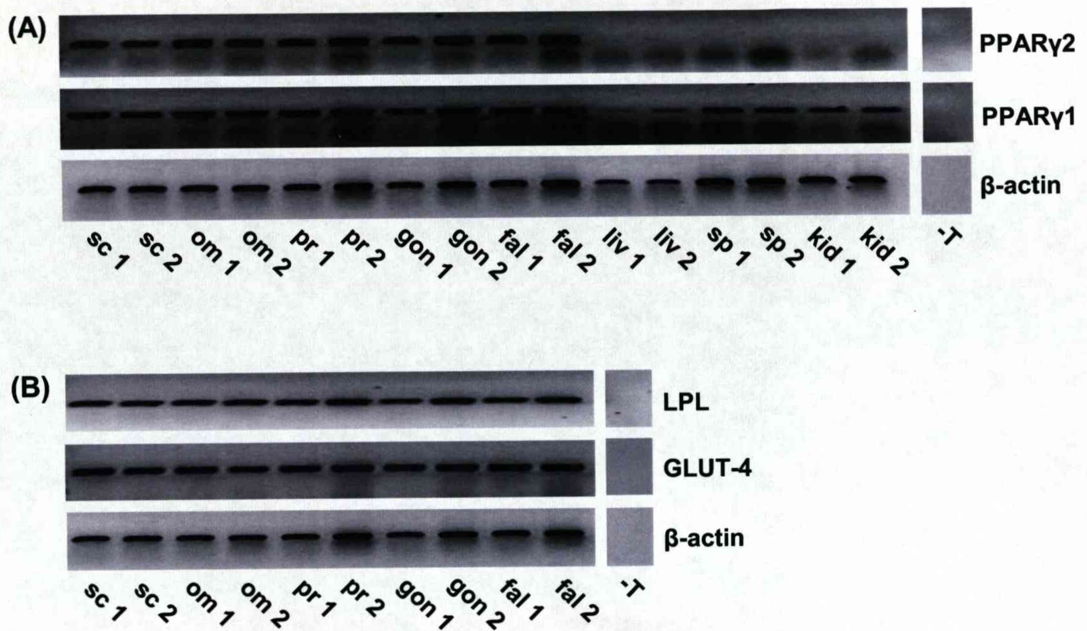
Having identified that PPARγ2 mRNA can be detected in dog WAT, the expression of other adipocyte-related genes was investigated by examining LPL and GLUT4 mRNAs. In a similar manner to PPARγ2, expression was detected in all WAT depots examined with little apparent difference between depots (Fig. 3.2B).

Figure 3.1 Canine PPARγ2 sequence alignment

```
human           AACGGATTGATCTTTTGCTAGATAGAGACAAAATATCAGTGTGAATTACAGCAAACCCCT 120
canine          AATGGATTGGTCTTTTGCAAGAAATAGACAAAATATCAGTGTGAATTACAGAAAATCCCT 75
                ** ****** ******** *** * ************************** *** ****

human           ATTCCATGCTGTTATGGGTGAAACTCTGGGAGATTCTCCTATTGACCCAGAAAGCGATTC 180
canine          CTTCCATGCTGTTATGGGTGAAACTCTGGGAGATTCTCTCATTGACCCAGAAAGCGACTC 135
                 *************************************  ***************** **

human           CTTCACTGATACACTGTCTGCAAACATATCACAAGAAATGACCATGGTTGACACAGAGAT 240
canine          CTTTGCTGATACACTGTCTGCAAGCACTTCACAAGAAACTACCATGGTTGACACAGAGAT 195
                ***  ****************** **  **********  ********************

human           GCCATTCTGGCCCACCAACTTTGGGATCAGCTCCGTGGATCTCTCCGTAATGGAAGACCA 300
canine          GCCATTCTGGCCCACCAACTTTGGAATCAGTTCAGTGGATCTGTCCGTAATGGATGACCA 255
                ************************ ***** ** ******** *********** *****

human           CTCCCACTCCTTTGATATCAAGCCCTTCACTACTGTTGACTTCTCCAGCATTTCTACTCC 360
canine          CTCCCATTCTTTTGACATCAAGCCATTCACCACCGTGGATTTCTCCAGCATTTCCACTCC 315
                ****** ** ***** ******** ***** ** ** ** ************** *****

human           ACATTACGAAGACATTCCATTCACAAGAACAGATCCAGTGGTTGCAGATTACAAGTATGA 420
canine          ACACTATGAAGACATTCCATTCTCAAGAGCGGACCCGATGGTTGCAGATTATAAGTATGA 375
                *** ** *************** ***** * ** **  ************* ********

human           CCTGAAACTTCAAGAGTACCAAAGTGCAATCAAAGTGGAGCCTGCATCTCCACCTTATTA 480
canine          TCTGAAGCTCCAAGAGTATCAAAGTGCAATCAAAGTGGAGCCTGCATCCCCACCTTATTA 435
                 ***** ** ******** ***************************** ***********

human           TTCTGAGAAGACTCAGCTCTACAATAAGCCTCATGAAGAGCCTTCCAACTCCCTCATGGC 540
canine          TTCTGAAAAGACTCAGCTGTACAATAAGCCTCACGAAGAGCCTTCCAACTCCCTCATGGC 495
                ****** *********** ************** **************************

human           AATTGAATGTCGTGTCTGTGGAGATAAAGCTTCTGGATTTCACTATGGAGTTCATGCTTG 600
canine          AATTGAATGCCGAGTCTGTGGAGATAAAGCTTCTGGGTTCCACTATGGAGTTCATGCTTG 555
                ********* ** *********************** ** ********************
```

There are 1830 nucleotides that code for the canine PPARγ sequence, of which 1681 comprise the protein coding region (CDS). By comparing the full sequence for the human PPARγ2 with that of canine PPARγ we were able to identify the canine γ2 region and design primers such that the forward primer (red bold) would span the 5'- untranslated region immediately upstream of the initial 82 nucleotides of the CDS coding specifically for the gamma-2 component of the gene (yellow block). The reverse primer was designed to be immediately downstream of the gamma-2 region (yellow bold). Remaining nucleotides of the gene coding for PPARγ1 are indicated in blue block. Sequences were aligned using ClustalW software (http://www.ebi.ac.uk/clustalw). Percentage similarity between canine and human PPARγ was 91%.

Figure 3.2 Expression of adipocyte-related genes by canine white adipose tissue



Total RNA was extracted from subcutaneous inguinal (sub), omental (om), perirenal (peri), gonadal (gon) and falciform (fal) WAT depots, and from liver (liv), spleen (spl) and kidney (kid) of two entire female SBT dogs (1 and 2). RT-PCR was performed using dog-specific primers. In (A), representative gels for PPARγ1 and γ2 are shown. In (B), representative gels for other adipose tissue-related genes are shown. Amplification cycle numbers were: PPARγ1 and –γ2, 27; LPL and GLUT4, 30; β-actin, 25; -T, no template control.

3.3.2 Expression of adipokine genes in canine WAT

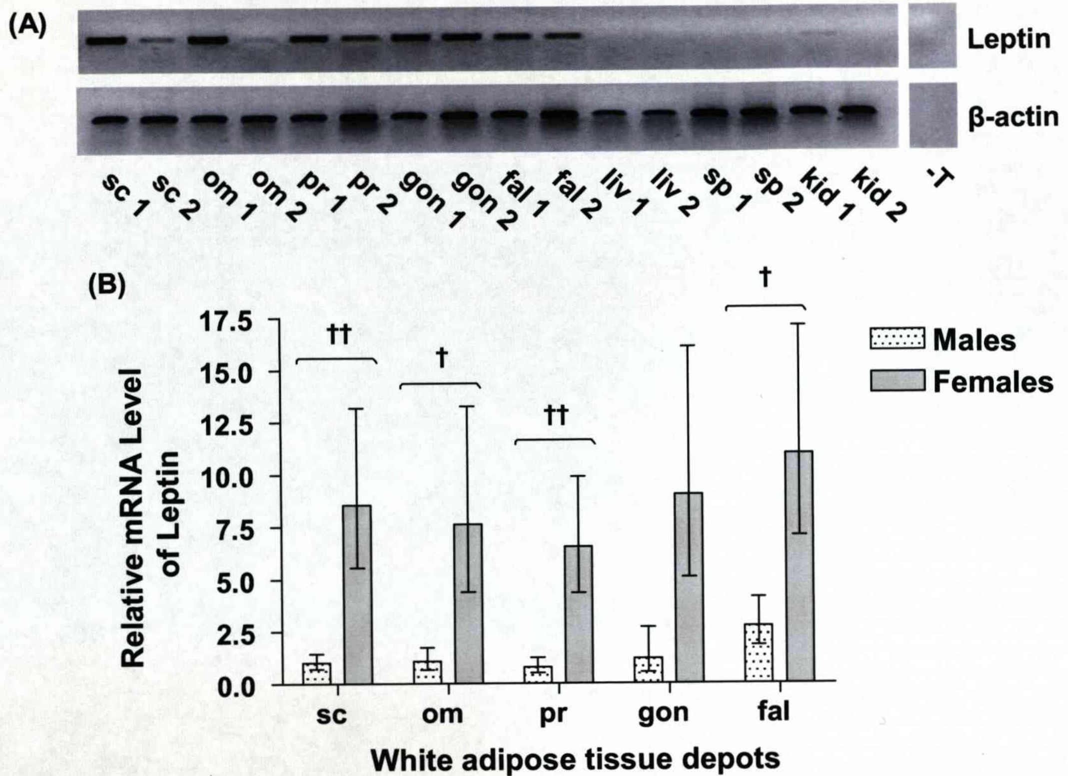
WAT depots were next screened for the expression of key adipokine genes. These included established adipokines such as the hormones leptin and adiponectin. mRNA encoding both leptin and adiponectin were detected in all WAT depots (Fig. 3.3A and Fig. 3.4A). A very faint signal was also detected in kidney tissue of animal 1 (Fig. 3.3A). Although RT-PCR established that both genes were expressed in all WAT depots, it was unclear whether the level of expression varied between depots, or indeed, if there was a difference in the level of expression between male and female dogs.

Therefore, the levels of mRNA encoding leptin and adiponectin were quantified using qPCR. Although there were no differences in the level of leptin gene expression amongst depots in either male of female dogs, when male and female data were directly compared, there was found to be a significant upregulation in the level of leptin mRNA in four out of the five depots examined in female compared to male dogs; these were subcutaneous inguinal, omental, perirenal and falciform ligament depots (Fig. 3.3B). There were no differences in the level of adiponectin gene expression between depots or between genders in dogs (Fig. 3.4B).

3.3.3 Expression of pro-inflammatory adipokine genes in canine tissues

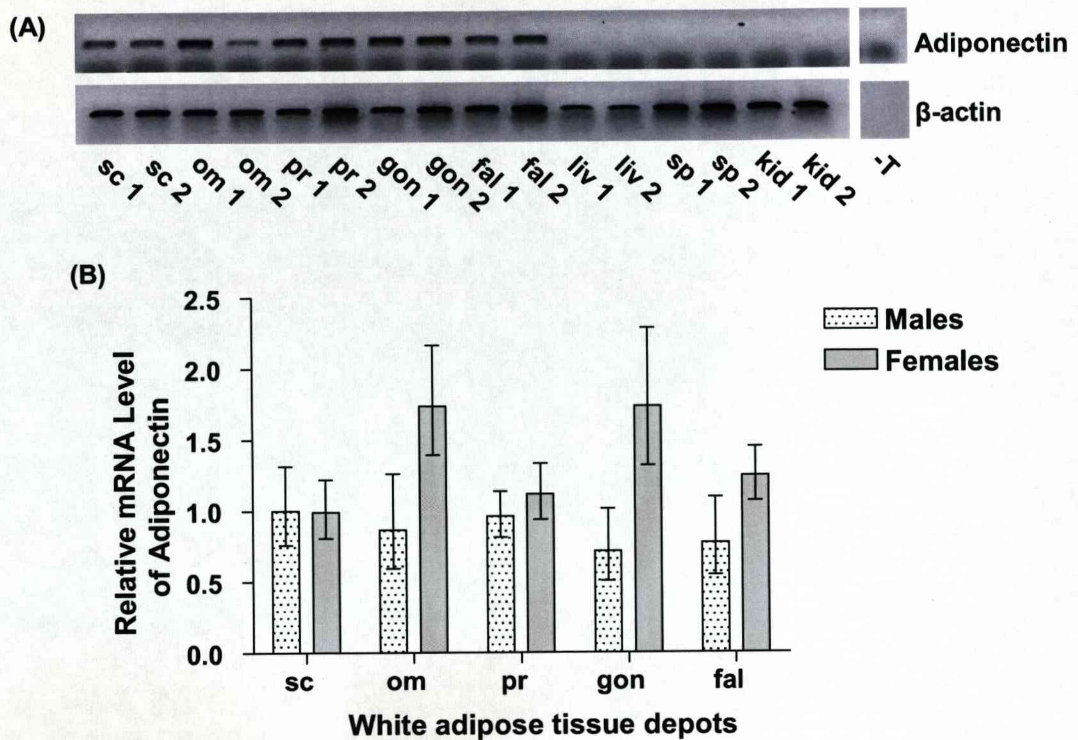
WAT depots were also examined for the expression of proinflammatory adipokine genes. These included the adipokines TNFα, IL-6 and IL-18, and chemokines MCP-1 and MIF, the target-derived neurotrophin NGF, VEGF (an important factor in angiogenesis) and PAI-1 (an adipokine involved in vascular haemostasis). In initial studies, mRNA levels were examined using RT-PCR, where all adipokine mRNAs were detected in all WAT depots (Fig. 3.5). The level of expression of certain adipokines; namely IL-6, IL-18, MCP-1 and NGF, appeared to be similar between different depots. In contrast, the expression of adipokines such as MIF, PAI-1, TNFα and VEGF appeared to differ between depots. In some cases analysis by qPCR was also carried out in order to quantify these changes (Figs. 3.6A, B, C, and D).

Figure 3.3 Expression of leptin by canine white adipose tissue



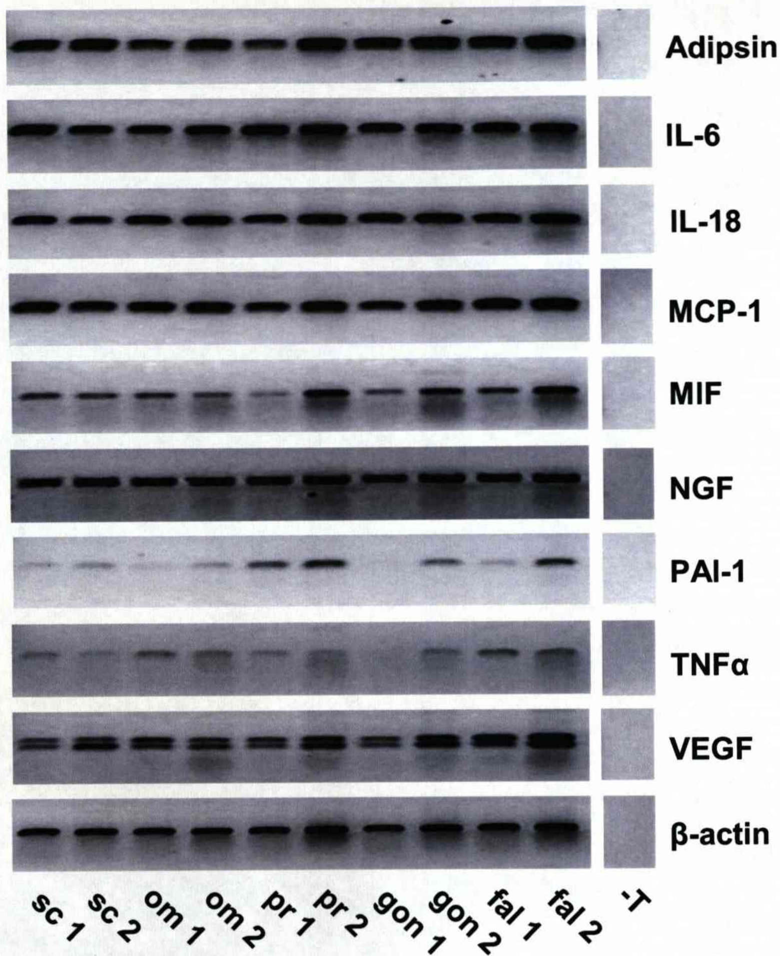
Total RNA was extracted from subcutaneous inguinal (sc), omental (om), perirenal (pr), gonadal (gon) and falciform (fal) WAT depots, and from liver (liv), spleen (sp) and kidney (kid) of two entire female SBT dogs (1 and 2). RT-PCR was performed using dog-specific primers. In (A), representative gels are shown for leptin and β-actin. Amplification cycle numbers were: Leptin, 32; β-actin, 25. In (B), real-time PCR was used for relative quantification of leptin mRNA levels. Results are expressed relative to the expression of β-actin in the subcutaneous inguinal depot for each gender and are presented as means ± SE (bars) 6-7 individuals $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared to male animals.

Figure 3.4 Expression of adiponectin by canine white adipose tissue



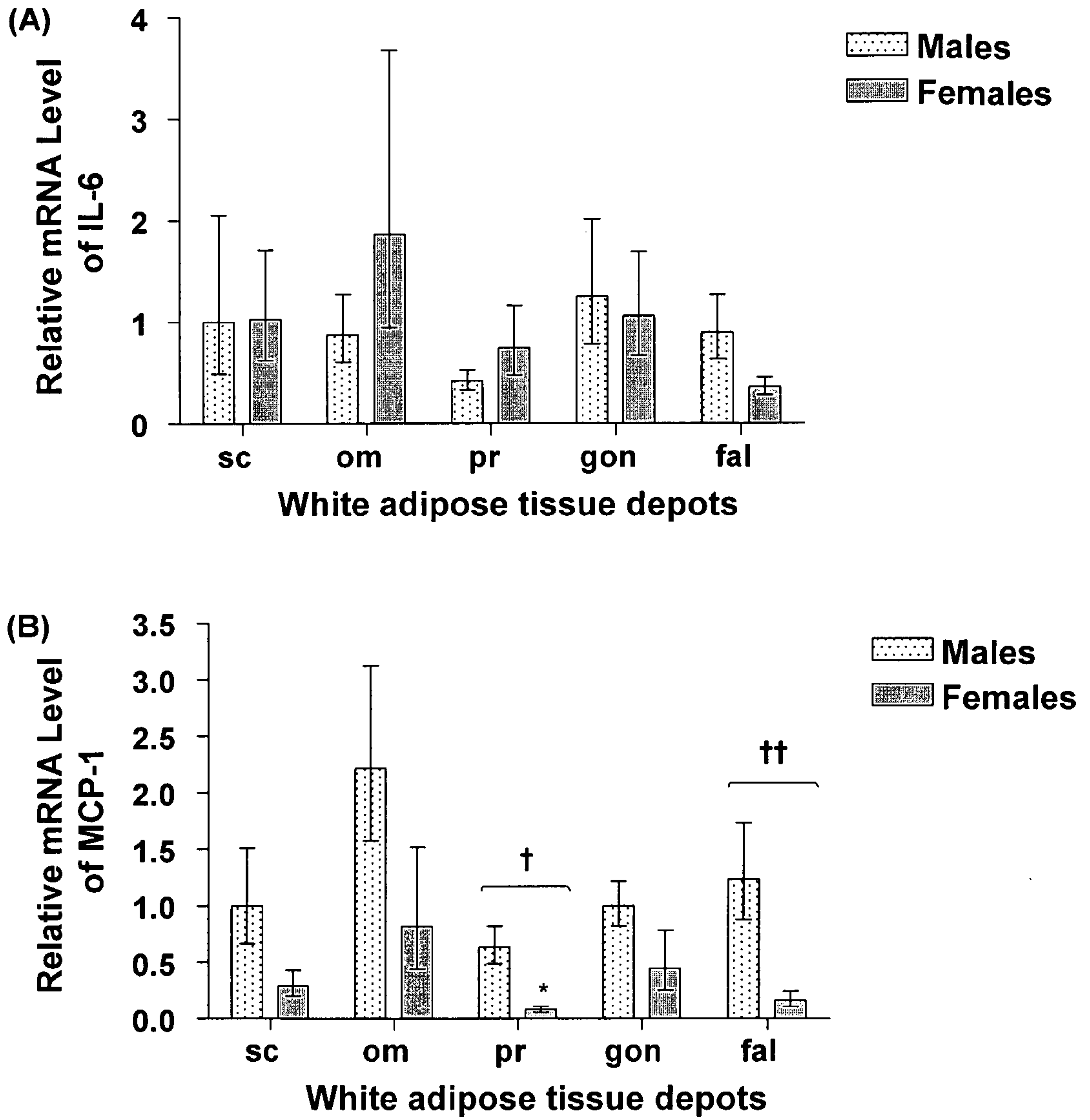
Total RNA was extracted from, subcutaneous inguinal (sc), omental (om), perirenal (pr), gonadal (gon) and falciform (fal) WAT depots, and from liver (liv), spleen (sp) and kidney (kid) of two entire female SBT dogs (1 and 2). RT-PCR was performed using dog-specific primers. In (A), representative gels are shown for adiponectin and β-actin. Amplification cycle numbers were: Adiponectin, 27; β-actin, 25. In (B), real-time PCR was used for relative quantification of adiponectin mRNA levels. Results are expressed relative to the expression β-actin in the subcutaneous inguinal depot for each gender and are presented as means ± SE (bars) 6-7 individuals.

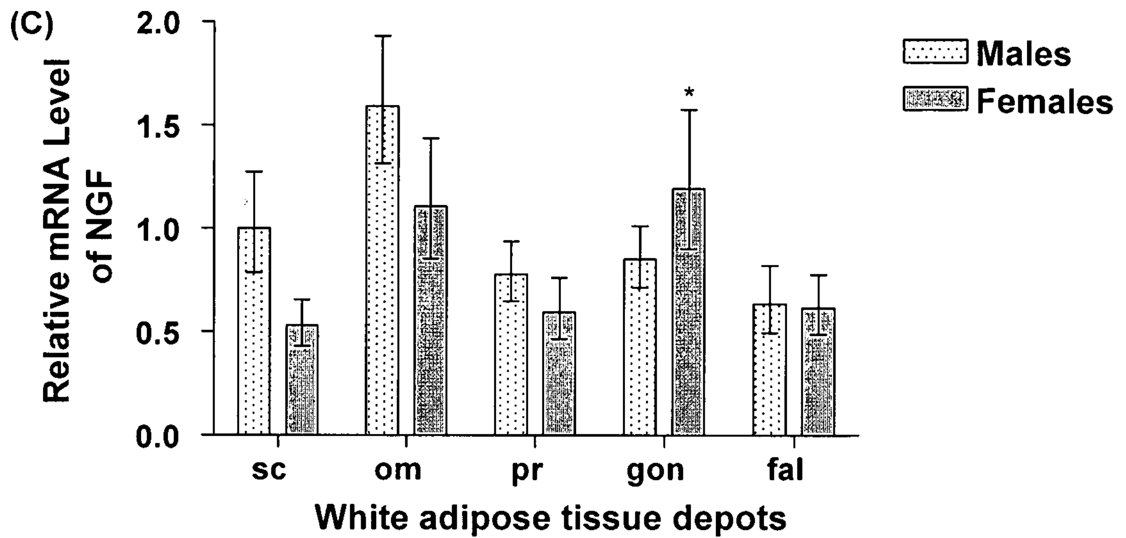
Figure 3.5 Expression of pro-inflammatory adipokine genes by canine white adipose tissue

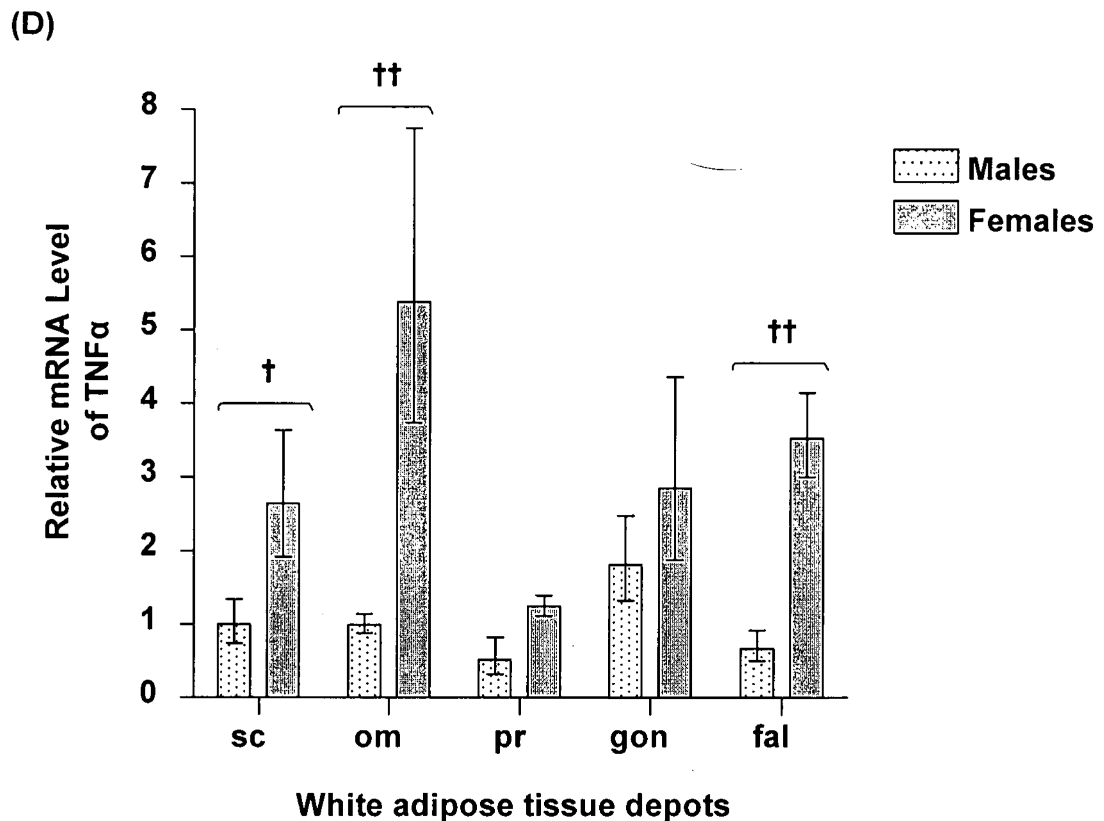


Total RNA was extracted from, subcutaneous inguinal (sc), omental (om), perirenal (pr), gonadal (gon) and falciform (fal) WAT depots of two female entire SBT dogs (1 and 2). RT-PCR was performed using dog-specific primers for different proinflammatory adipokine genes. Amplification cycle numbers were: Adipsin, 27; IL-6, 34; IL-18, 28; MCP-1, 30; MIF, 35; NGF, 32; PAI-1, 34; TNFα, 27; VEGF, 32; β-actin, 25; -T, no template control.

Figure 3.6 Quantification of the level of expression of pro-inflammatory adipokine genes by canine white adipose tissue







Total RNA was extracted from subcutaneous inguinal (sc), omental (om), perirenal (pr), gonadal (gon) and falciform (fal) WAT depots. Real-time PCR was used for relative quantification of (A) IL-6, (B) MCP-1, (C) NGF and (D) TNFα mRNA levels; Results are expressed relative to the expression β-actin in the subcutaneous inguinal depot for each gender and are presented as means ± SE (bars) 6-7 individuals † $P < 0.05$, †† $P < 0.01$, compared to male animals. * $P < 0.05$, compared to same gender depot control. Reference depot used was subcutaneous inguinal.

There was no significant difference in the level of mRNA, encoding IL-6, amongst depots and between genders in the dog (Fig. 3.6A). In contrast, the levels of mRNA encoding MCP-1 were significantly elevated in both perirenal and falciform depots in male compared to female dogs. There was also a small, but statistically significant, down-regulation of MCP-1 mRNA level in the perirenal depot in female dogs compared to the control depot (Fig. 3.6B).

In terms of NGF, there was a small but nevertheless statistically significant upregulation in the mRNA level in the female for the gonadal depot (Fig. 3.6C). TNFα, mRNA levels were significantly elevated in three out of the five depots examined in female compared to male dogs; these were subcutaneous inguinal, omental and falciform ligament. There were however, no significant differences in mRNA level of TNFα between depots for either sex (Fig. 3.6D).

3.4 Discussion

Isolating RNA from the tissue of non-laboratory animals presents many challenges which are not always present in laboratory animal work. In the present work, dog tissues were collected with up to half an hour delay from the time of euthanasia. Thus, there was an initial concern about the quality of the RNA extracted and whether this would be suitable for further analysis. However, despite these initial concerns, RNA quality was consistently good with minimal evidence of degradation. It is of note that, regardless of the species examined liver tissue is known to yield RNA of lower quality than tissues such as spleen and kidney where the mRNA is thought to be more resistant to degradation (Palmer & Prediger, 2007). This was reflected in the present results where, despite tissues being frozen in liquid nitrogen as soon as possible after collection, signals obtained in the liver did appear to be lower (including that for the housekeeping gene, β-actin), when compared to that of other tissues.

The studies described in this chapter demonstrate that the genes encoding several key adipose tissue-related proteins are expressed in canine WAT. One such protein is the adipose tissue-specific transcription factor PPARγ2, whose signal was documented in all depots examined. In contrast, PPARγ1 is ubiquitously expressed, and as such, a signal was obtained in all tissues. Genes whose expression is characteristic of adipocytes (although not exclusively) namely LPL and GLUT4, were shown to be expressed in all depots examined, with little difference in the level of expression between depots.

Signals for both leptin and adiponectin mRNAs were detected in all WAT depots, whilst expression in all other organs examined was minimal to non-existent. Levels of mRNA encoding leptin were found to be higher in the adipose tissues of female, compared with male, dogs for four out of the five depots examined, with only the gonadal depot not reaching statistical significance. All female dogs were entire at the time of sampling, and none were overweight/obese. In humans, mRNA levels of leptin appear to be higher in subcutaneous compared to omental depots (Montague *et al.*, 1997b; Montague *et al.*, 1998). Since in females this is the main depot to expand in obesity, women have been found to have higher levels of circulating leptin

compared to age matched men (Castracane *et al.*, 1998). Studies looking at systemic levels of leptin in the dog have found a clear association between weight and levels of leptin, but no gender association (Ishioka *et al.*, 2002; Ishioka *et al.*, 2007). In dogs, unlike humans, leptin mRNA levels are raised not only in the subcutaneous depot but also in several visceral depots (omental, perirenal and falciform ligament) in the female. Furthermore, despite raised mRNA levels in WAT of female dogs, this does not seem to reflect the situation seen systemically in relation to circulating levels of leptin (Ishioka *et al.*, 2005a).

There were no differences in the level of expression between depots or between genders in the dogs of this study. In contrast, studies in humans have demonstrated an association between the male sex and visceral depots with lower levels of circulating adiponectin (Lihn *et al.*, 2004; Dullaart *et al.*, 2007). It is speculated that this effect may be due to systemic levels of the male hormone testosterone (Andersen *et al.*, 2007). In terms of gene expression in WAT, no such association was evident in the dog.

The current study also demonstrated expression of key proinflammatory adipokine genes in the main WAT depots in the dog, and results are consistent with the exploratory study of Eisele *et al.,* 2005. The adipokines examined include genes encoding adipsin, PAI-1 and VEGF. Adipsin is an important protein required for the synthesis of acylation-stimulating protein (ASP), which is derived from complement C3a (Trayhurn & Beattie, 2001). A signal for adipsin was found in all WAT depots of the dog with little difference in the level of expression between depots. There is a similarity between adipsin and human and murine complement factor D. However, mouse adipsin expression appears to be specific to adipose tissue, whereas human complement factor D is reported to be synthesised mainly by cells of the macrophage/monocyte lineage (Barnum & Volanakis, 1985). With increased infiltration of WAT by macrophages in obesity (Weisberg *et al.*, 2003), ASP may play a key role in adipose tissue metabolism in the obese state.

PAI-1 is an acute phase protein and a key factor in vascular homeostasis, inhibiting the activation of plasminogen, the precursor of plasmin (Sprengers & Kluft, 1987). Increased PAI-1 production occurs in WAT in obese humans, and increased

circulating levels of the protein in obesity may be largely due to contribution by WAT (Mutch *et al.*, 2001). PAI-1 expression was established in all depots examined in the dog.

VEGF is an important angiogenic factor expressed in a variety of cell types including WAT in both humans and rodents (Fain & Madan, 2005; Miyazawa-Hoshimoto *et al.*, 2005). In the dog, as in humans and rodents (Robinson & Stringer, 2001; Miyazawa-Hoshimoto *et al.*, 2005), VEGF is encoded by a single gene; however, several isoforms are produced as a result of alternate splicing (Jingjing *et al.*, 2000). Here we have shown VEGF expression in all main dog WAT depots identifying the expression of both 182 and 164 isoforms. As demonstrated in other dog tissues (Jingjing *et al.*, 2000), VEGF 164 was expressed at a higher level compared to other isoforms. No difference in the level of VEGF expression was evident between depots in the dog. This is unlike rodents where a higher level of VEGF expression has been demonstrated in visceral compared to subcutaneous depots (Miyazawa-Hoshimoto *et al.*, 2005). The identification of VEGF isoforms in dog WAT should prove a useful tool in study of angiogenesis in WAT in obesity.

A signal for several important cytokines particularly TNFα, IL-6 and IL-18, and chemokines MCP-1 and MIF was identified in all five depots examined in the dog. Both IL-6 and TNFα are increased in obesity in humans and rodents, and both have been implicated in the development of insulin resistance in adipocytes (Bastard *et al.*, 2000; Hotamisligil, 2003). Quantitatively, IL-6 mRNA levels were not significantly different amongst depots or between genders in dogs. Conversely, levels of mRNA encoding TNFα were elevated in three out of the five depots examined in female as compared to male dogs. These, depots; namely subcutaneous inguinal, omental and falciform were also found to have significantly raised levels of leptin mRNA. Leptin levels have been shown to rise in response to increased levels of TNFα in humans (Zumbach *et al.*, 1997). Thus raised levels of leptin expression seen in dog WAT depots may have been in response to raised levels of TNFα.

IL-18 is also an important pro-inflammatory cytokine whose expression is elevated in obesity and reduced in response to weight loss (Esposito *et al.*, 2002). Recently, it has been established that IL-18 is expressed in WAT, where a marked upregulation

of expression by human adipocytes is seen with TNFα treatment (Wood *et al.*, 2005). However, there is mixed evidence as to whether it is secreted by adipocytes (Skurk *et al.*, 2005b; Wood *et al.*, 2005). This study has shown that IL-18 is expressed in all WAT depots in the dog. The apparent difficulty experienced in terms of identifying whether IL-18 is in fact secreted by adipocytes, and is thus an adipokine in its own right, clearly raises questions as to the extent to which this cytokine plays a role in the inflammatory and immune response in WAT.

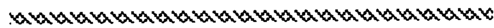
This study has further demonstrated that genes for MCP-1 and MIF, which both play a central role in inflammatory processes by regulating leukocyte migration (in the case of MCP-1), and as a potent inhibitor of monocyte/macrophage migration (in the case of MIF), are expressed in all main WAT depots in the dog. Unlike results for other proinflammatory cytokines, levels of MCP-1 mRNA were found to be raised in perirenal and falciform depots in male compared to female dogs. Furthermore, there was a small but significant down-regulation of MCP-1 mRNA level in female dogs for the perirenal depot when compared to the subcutaneous inguinal depot. Levels of MCP-1 gene expression in endometrial stromal cells in humans are inversely related to levels of oestrogen (Arici *et al.*, 1999). Thus, the results seen in the dog may be representative of a similar picture in canine WAT.

The target-derived neurotrophin, NGF, has recently been shown to be expressed and secreted from mouse and human white adipocytes (Peeraully *et al.*, 2004; Wang *et al.*, 2004; Wang *et al.*, 2005). NGF is not only responsible for the growth and survival of sympathetic neurones, but is involved in both inflammatory and immune responses (Levi-Montalcini *et al.*, 1996). In white adipocytes, expression and secretion are strongly stimulated by the proinflammatory adipokine TNFα (Wang *et al.*, 2004; Wang *et al.*, 2005). NGF expression was observed in all WAT depots of dog (Fig 3.5). Quantification of NGF mRNA levels by qPCR demonstrated a small but significant upregulation of NGF mRNA levels for the gonadal depot relative to the subcutaneous inguinal depot in female dogs. This is similar to what has been documented in rodents, where NGF gene expression is significantly higher in epididymal compared to subcutaneous WAT (Peeraully *et al.*, 2004). NGF gene expression has been established in both subcutaneous and omental depots in humans, where relative levels of expression have been found to be higher in omental

compared to subcutaneous depot (Peeraully *et al.*, 2004). No sexual dimorphism in NGF gene expression has been reported for WAT or other tissues where the gene is expressed, such as the hippocampus of the developing rat brain (Kornack *et al.*, 1991).

This initial study has demonstrated that genes encoding a number of key adipokines are expressed in dog WAT, in a manner similar to humans and rodents. All PCR products were confirmed by sequencing. Genes identified include not only those encoding key adipose tissue proteins LPL and GLUT4, and the transcription factor PPARγ2, but also the adipocyte hormones leptin and adiponectin. In addition, the detection and quantification of these adipokines and others which have a pro-inflammatory role in adipose tissue biology has been established providing a basis for further investigations into adipose tissue and adipokine biology in the dog.

Chapter 4



Establishment of a canine primary cell culture protocol: Adipokine gene expression and protein secretion during canine adipocyte differentiation and development

4.1 Introduction

In Chapter 3, the main WAT depots of the dog were characterised by expression of key molecular markers, including three genes whose expression occurs in white adipocytes namely; PPARγ1, LPL and GLUT4. In addition, the adipose tissue specific expression of the gamma-2 isoform of PPARγ, and the expression of two adipocyte hormones, leptin and adiponectin was demonstrated in dog WAT. Furthermore, the expression profile of key proinflammatory adipokine genes including TNFα and IL-6 was also established in dog WAT depots. Expression of adipokine genes in WAT does not necessarily imply adipocyte-specific expression, thus, in this chapter, studies focused on developing a cell culture protocol for the study of canine adipocytes *in vitro*. Such a system would ultimately allow the investigation of adipokine gene expression in adipocytes themselves and further allow the characterisation of canine adipocyte differentiation and protein secretion by canine adipocytes.

As discussed in Chapter 1, there are currently three main *in vitro* systems in use for investigating adipocyte physiology, clonal cell lines, isolated mature adipocytes and primary culture of preadipocytes. Clonal cells lines such as the murine 3T3-L1 (Chang & Polakis, 1978) and 3T3-F442A cells (Green & Meuth, 1974), undergo spontaneous growth arrest at confluence and can be induced to differentiate after treatment with adipogenic compounds (Spiegelman *et al.*, 1993). Mature adipocytes can be harvested from freshly dissected adipose tissue by collagenase digestion and once isolated; these can be maintained in culture for up to 48 h (Fain *et al.*, 2003). The use of adipose tissue explants is also an important tool for investigating adipose tissue metabolism *in vitro*. These are slightly more robust than mature adipocytes and can be maintained in culture for several days (Smith, 1974). It can be argued that the use of mature adipocytes and or tissue explants are the closest to the *in vivo* situation, as they involve only limited intervention in transferring the cells from the source to the culture system. They do not, however, allow the differentiation process to be studied and in addition, explants involve all cells in a specific organ or tissue, a disadvantage where secretion by a given cell type cannot be studied in detail.

Adipose cells differentiate well in culture, and *in vitro* differentiation leads to activation of most of the same set of genes characteristic of adipose tissue *in vivo* (Spiegelman *et al.*, 1993), this makes preadipocyte conversion an attractive model for the study of adipose cell differentiation. At a set point after inoculation, cells are exposed to an 'adipogenic cocktail' containing several agents responsible for the activation of differentiation. These cells can then be maintained for a period of 2-4 weeks. An advantage of the primary culture system is that it is only one step removed from the culture of mature adipocytes, and as such may be just as reliable (Hauner *et al.*, 2001).

As discussed in Chapter 1, no canine adipose tissue cell lines have been characterised. Thus to date, work on canine adipocytes *in vitro* has involved the use of primary cell culture systems. Two methods currently in the literature describe the culture of primary preadipocytes from various depots, including visceral (omental, perirenal and gonadal) and subcutaneous (abdominal and inguinal-subcutaneous) (Wu *et al.*, 2001; Eisele *et al.*, 2005). These methods make use of different 'adipogenic cocktails' and both appear successful in terms of inducing adipocyte differentiation. This chapter will outline the establishment of a system for the differentiation of canine fibroblastic preadipocytes from both a visceral and subcutaneous depot *in vitro* based on these two techniques. Thus, allowing the characterisation of canine adipocyte differentiation and development through the expression of key molecular markers and immunodetection of adipokines secreted into the cell culture medium by canine adipocytes.

4.2 Methods

4.2.1 Cell culture – Eisele *et al.*, 2005 protocol

This method of canine primary adipocyte cell culture was developed at the Obesity Biology Unit, University of Liverpool, and is largely based on that by Wu *et al.*, 2001 and on the method of culture of human fibroblastic preadipocytes by Hauner *et al.*, 2001. Differences in both reagents used and cell culture methods from those described in section 2.2 are outlined below:

4.2.1.1 Reagents

(i) Transport/Basal Medium

15 mM HEPES
15 mM sodium hydrogen carbonate
33 µM D-biotin
17 µM D-panthothenate
1x DMEM/Ham's F12

(ii) Digestion Medium (pH 7.4)

1 mg/ml Collagenase
4 mg/ml BSA
1x PBS

(iii) Washing Medium

10% FCS
1x DMEM
100 U/ml penicillin G sodium
100 µg/ml streptomycin sulphate

(iv) Erythrocyte lysis buffer (pH 7.4)

155 mM ammonium chloride
5.7 mM potassium dihydrogen orthophosphate
0.1 mM EDTA

(v) Inoculation Medium

10% FCS
15 mM HEPES
15 mM sodium hydrogen carbonate
33 μM D-biotin
17 μM D-panthothenate
1x DMEM/Ham's F12
100 U/ml penicillin G sodium
100 μg/ml streptomycin sulphate

(vi) Induction Medium

2% FCS
15 mM HEPES
15 mM sodium hydrogen carbonate
33 μM D-biotin
17 μM D-panthothenate
10 μg/ml human transferrin
1 nM T_3
10 μg/ml insulin
0.1 μM cortisol
0.5 mM IBMX
1 μg/ml rosiglitazone
1x DMEM/Ham's F12
100 U/ml penicillin G sodium
100 μg/ml streptomycin sulphate

(vii) Feeding Medium

2% FCS
15 mM HEPES
15 mM sodium hydrogen carbonate
33 μM D-biotin
17 μM D-panthothenate
10 μg/ml human transferrin
1 nM T_3

10 μg/ml insulin

0.1 μM cortisol

1x DMEM/Ham's F12

100 U/ml penicillin G sodium

100 μg/ml streptomycin sulphate

4.2.1.2 *Culture method*

Cells were collected and fractionated as described in sections 2.2.2 and 2.2.3 respectively. The cell culture method was as described in section 2.2.4. However, in this case only a single filtration step was carried out. In addition, 24 h after inoculation, preconfluent cells were thoroughly washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells were then exposed to this adipogenic medium for 72 h, after which the induction medium was replaced by feeding medium, which was replaced every 48 h.

4.2.2 Cell culture – Wu *et al.*, 2001 protocol

The fundamental protocol for canine primary adipocyte cell culture adopted in the current study was largely based on that by Wu *et al.*, 2001, and as such is described in detail in section 2.2. Any differences in both reagents used and cell culture methods relating specifically to the Wu *et al.*, 2001 protocol are outlined below:

4.2.2.1 *Reagents*

(i) Digestion Medium

2 mg/ml Collagenase

4 mg/ml BSA

20 mM HEPES

1x DMEM

None of the media used contained fungicide and only inoculation and induction medium contained penicillin/streptomycin.

4.2.2.2 *Culture method*

Cells were collected and fractionated as described in sections 2.2.2 and 2.2.3 respectively. The cell culture method was as described in section 2.2.4, with the

following exceptions: all centrifugation steps were carried out at 1000xg for 5 min. Only a single filtration step was carried out after the tissues were subjected to digestion with collagenase. Cells were inoculated at a density of 0.5 x 10^4 cells/cm^2. Twenty four hours after inoculation, the preconfluent cells were thoroughly washed three times in 1x PBS. Adherent cells were maintained in the same medium for an undisclosed period of time until they reached confluence and once cells were confluent, the inoculation medium replaced by induction medium. Cells were then exposed to this for 48 h, after which the induction medium was replaced by feeding medium, which was replaced every 48 h.

4.2.3 Cell culture – Current method – adapted from Wu *et al.*, 2001 and Eisele *et al.*, 2005

4.2.3.1 Culture method

White adipose tissue from both the subcutaneous inguinal and gonadal depots of SBT dogs were harvested as described in sections 2.1.1 and 2.1.2. Cells were cultured as described in section 2.3 at 37°C in a humidified atmosphere of 5% CO_2/95% air. 24 h after they were inoculated, cells from the gonadal depot were washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells from the subcutaneous inguinal depot had their inoculation medium changed 48 h post-inoculation and 24 h later (at 72 h post-inoculation) were washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells of both depots were exposed to the adipogenic medium for 48 h. This was replaced by feeding medium, which was renewed every 48 h.

To investigate the time course of gene expression and protein secretion during adipocyte differentiation, conditioned media and cells from both depots were collected as described in section 2.2.5 from three individual wells of culture plates when cells were induced (*day 0*) and subsequently at *days 1-4, 6, 8, 10, 12* and *15* post-induction. Cell cultures were carried out in triplicate for each depot.

4.2.4 RT-PCR, qPCR and ELISA.

RNA was extracted from cultured cells using Tri-Reagent as described in section 2.4.1. RNA which was to be analysed by qPCR was subsequently treated using a

DNA-free kit to minimise levels of genomic DNA contamination (section 2.3.2). Once extracted, the total RNA was quantified using a BioPhotometer and reverse transcribed using the Reverse-iT First Strand Synthesis kit (sections 2.3.3 and 2.4.1). The cDNA was then analysed by PCR using the Reddymix PCR Master Mix kit and PCR Express thermal cyclers (sections 2.4.1 and 2.4.2) and by real-time PCR (qPCR) using the qPCR Core Kit and an Mx3005P real-time machine (section 2.5.3 and 2.5.4).

PCR products were purified using a NuceloSpin Extract 2-in-1 kit and sequenced commercially (see section 2.4.4). All qPCR data was analysed using MxPro-Mx3005P software. Conditioned media samples were analysed for NGF protein concentration using the NGF E_{max} Immunoassay System (section 2.6.1). TNFα, IL-6 and MCP-1 protein concentration were analysed using ELISA kits from R&D Systems (see section 2.6.2). Adiponectin protein concentration in conditioned media was assayed commercially by LINCO (section 2.6.3).

4.2.5 Oil red O staining

Oil red O staining of cells at various points during differentiation and maturation was carried out as described in section 2.3.7. Conditioned media was discarded and the remaining cells washed twice with 1x PBS and fixed using a 3.7% solution of formaldehyde. Once fixed, cells were stained with Oil red O and haematoxylin. The stained cells were washed with distilled water and images captured using an inverted microscope. Percentage differentiation was estimated at various stages according to the number of cells displaying evidence of intracellular lipid accumulation.

4.3 Results – Part A

Establishment of a canine primary cell culture protocol

With the success of Eisele *et al.*, 2005 in inducing cells from the gonadal depot to differentiate, initial studies attempted to achieve the same goal, as tissue from this depot could be obtained from routine neutering of dogs at the University of Liverpool Small Animal Practice. However, despite several attempts, differentiation of preadipocytes from the gonadal depot using the Eisele *et al.*, 2005 protocol proved unsuccessful. The only study outlining a comparison of the level of differentiation

capacity of canine adipocytes *in vitro* was that by Wu *et al*, 2001. They reported that according to glycerol-3-phosphate dehydrogenase (GPDH) activity, which has been successfully used as a sensitive marker for adipocyte differentiation (Ailhaud, 1982 940), cells of the subcutaneous inguinal depot exhibited greatest capacity for differentiation compared to other depots *in vitro* (Wu *et al.*, 2001). Thus, tissues from the subcutaneous inguinal depot were used in order to compare the efficacy of the two protocols at inducing preadipocyte differentiation.

4.3.1 Protocol comparison

Tissues were harvested from the subcutaneous inguinal depot of three dogs of different breeds; a female a SBT, a female German shepherd dog and a male Japanese akita as they became available. The protocol comparison was thus carried out in triplicate. Cells were cultured according to both protocols, inoculated onto 6-well plates and induced to differentiate at specified times post-inoculation, namely 24, 48, 72 and 96 h. Cells were inoculated at densities of 40,000-60,000 cells/cm^2 as per Eisele *et al.,* 2005.

Clonal cell lines such as 3T3L-L1 adipocytes must undergo a period of growth arrest before successful differentiation (Chang & Polakis, 1978), one of the ways in which this is achieved is through contact inhibition once cells reach a state of confluence. In contrast, both human and canine preadipocyte cells appear not to need to reach a confluent state to achieve successful differentiation (Hauner *et al.*, 2001) (Eisele *et al.*, 2005). Thus, this first investigation not only compared the efficacy of the two protocols using the ideal depot in different breeds, but also whether rates of differentiation were dependent on the state of cell confluency and at what stage this was achieved. In all cases, successful preadipocyte differentiation was characterised by visual inspection of cells, where a change from a fibroblastic preadipocyte morphology, to one where cells were considerably larger and contained multiple lipid droplets which stained positive for lipid using Oil red O, was used as an indication of terminal differentiation.

Both protocols were successfully able to induce preadipocyte differentiation to mature adipocytes as characterised by intracellular lipid accumulation in cells of the

subcutaneous inguinal depot (Fig. 4.1A and B). Cells cultured according to the protocol established by Wu *et al.,* 2001 were most successful in accumulating lipid when induced 72 h post-inoculation, with a differentiation rate in excess of 85% (Fig. 4.1B). Although cells differentiated using the Eisele *et al.,* 2005 protocol did successfully accumulate intracellular lipid, the highest recorded percentage differentiation was of the order of 55-60% (Fig. 4.1A). Confirmation of lipid accumulation was achieved by staining cells with the lipid specific stain, Oil red O (Fig. 4.2C). Once cells were allowed to reach confluence, approximately 96 h post-inoculation, no intracellular lipid droplets were visible on staining with Oil red O (Fig. 4.1D).

4.3.2 Subcutaneous inguinal and gonadal depot comparison

All subcutaneous inguinal white adipose tissues were obtained from animals euthanased at a local animal shelter for reasons unrelated to the study. This was the preferred depot according to the literature, where cells have been shown to have the greatest capacity to differentiate and accumulate lipid when compared to those of other depots; namely, omental, perirenal and abdominal subcutaneous tissue in the dog (Wu *et al.*, 2001). However, Eisele *et al.,* 2005 had shown success using cells from the gonadal depot obtained during routine neutering (Eisele *et al.*, 2005). Cells from this depot would also have been a reasonable choice due to the freshness of the tissue, as it was obtained during routine surgery.

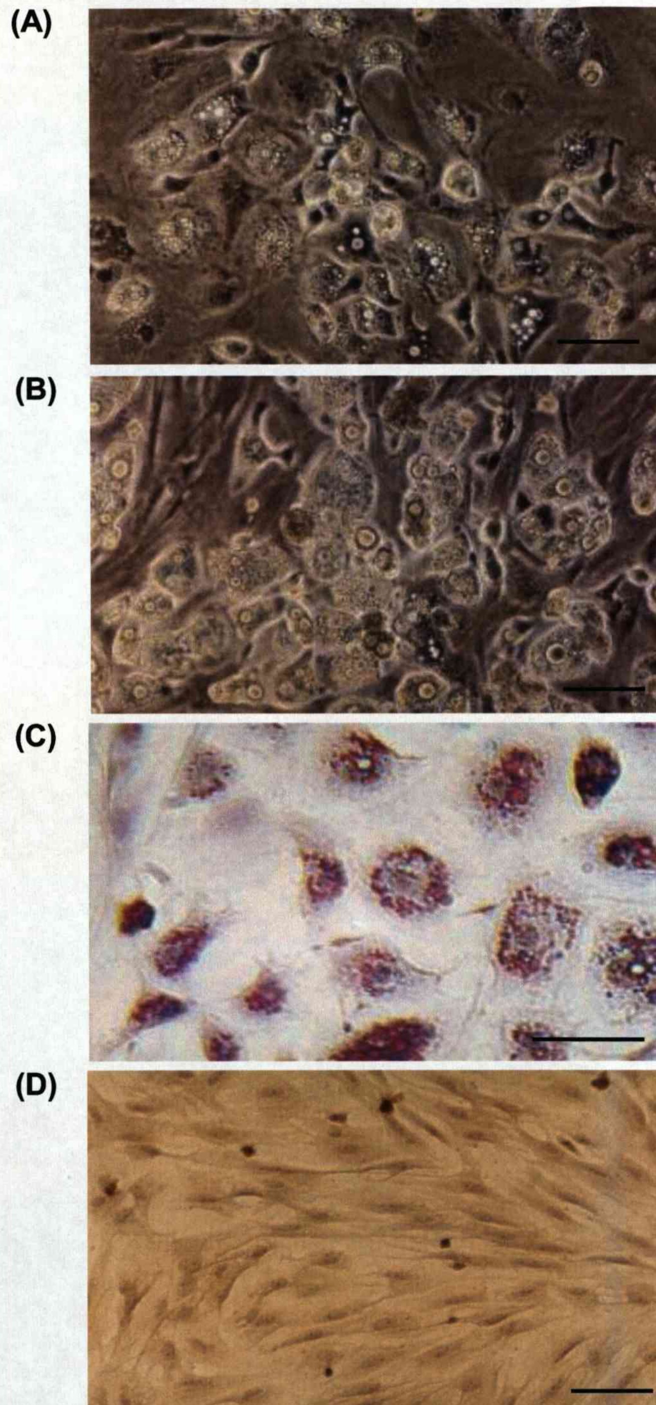
As mentioned previously, attempts to induce gonadal preadipocytes to differentiate using the Eisele *et al.*, 2005 protocol had been unsuccessful. Therefore, in this study cells from both depots were cultured using the Wu *et al.*, 2001 protocol. Cells were again induced to differentiate at a pre-confluent state at 24, 48 and 72 h post-inoculation. Cells from both depots were successfully induced to differentiate using the Wu *et al.*, 2001 protocol. Cells from the subcutaneous inguinal depot induced to differentiate 24 h post-inoculation (Fig. 4.2A) accumulated lipid readily. However, in terms of percent of cells containing multiple lipid droplets, on average as had been shown previously, cells from this depot did better if they were induced to differentiate 72 h post-inoculation (Fig. 4.2B). Cells from the gonadal depot induced to differentiate 24 h post-inoculation accumulated lipid droplets in a comparable manner to those of the subcutaneous inguinal depot induced at 72 h post-inoculation

(Fig. 4.2C). In contrast, cells from the gonadal depot induced at 72 h post-inoculation were unable to accumulate lipid to the same extent. (Fig. 4.3D).

4.3.3 Well size as an indication of stress

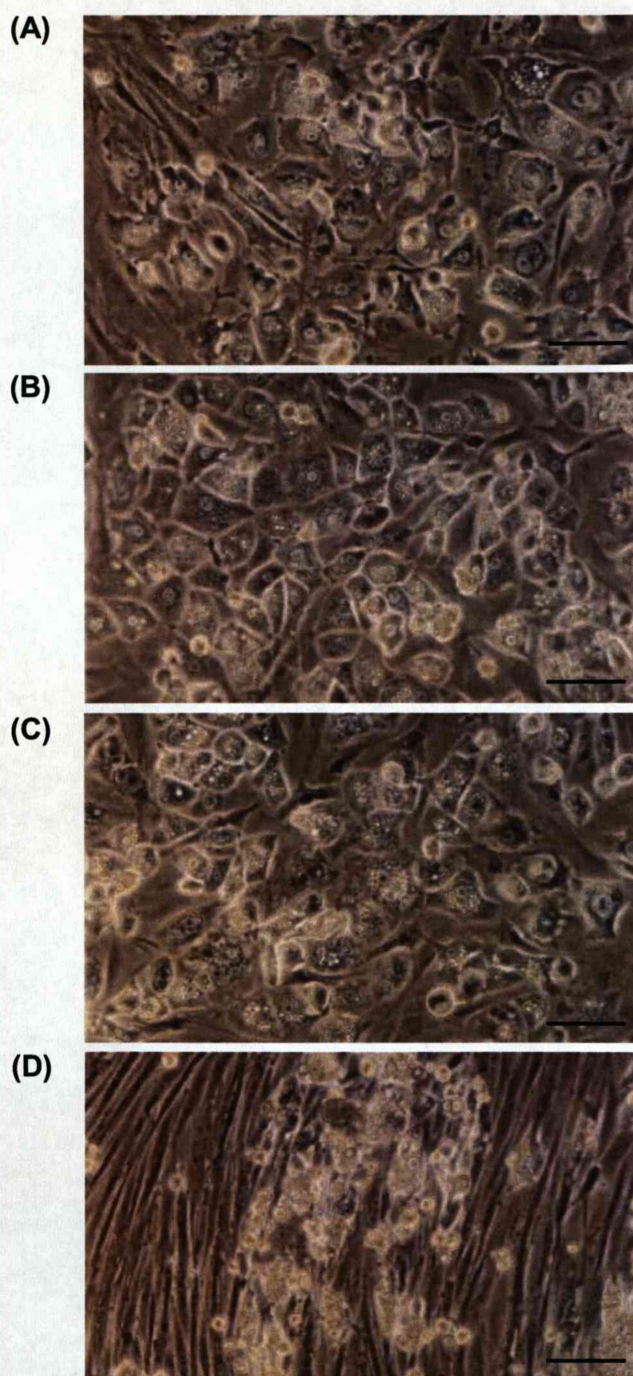
Cells cultured *in vitro* are not only removed from their original physiological environment, but are also exposed to a range of potential stressors. These include, hypoxia, aberrant nutritional conditions such as reduced substrate availability, and in addition, clearance of metabolites is likely to differ in culture from that provided by the vascular system. In order to further optimise culture conditions and investigate levels of possible stressors, cells from the subcutaneous inguinal depot were seeded at the higher densities suggested by the Eisele *et al.,* 2005 protocol in 6, 12 and 24-well plates, where it was hypothesised that constraints in plate area in terms of diffusion of nutrients could potentially affect cell metabolism and hence the ability of cells to differentiate. Cells were otherwise cultured as per Wu *et al.*, 2001 and induced 72 h post-inoculation. As is evident from Figs 4.3 A, B and C, well size did not appear to play a role in the ability of cells to differentiate and it was decided therefore, to use the 6-well plate as the plate of choice for further experiments as a mater of convenience, and so as to be able to extract enough total RNA for future analysis.

Figure 4.1 Comparison between Wu and Eisele protocols



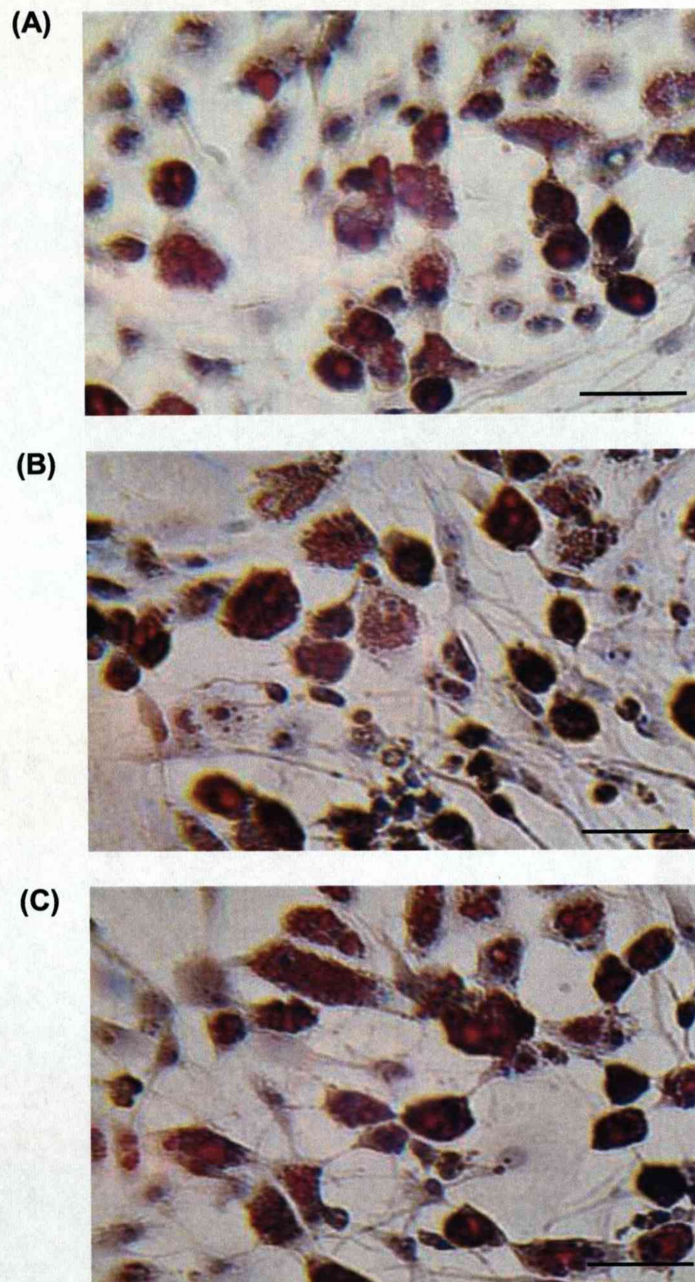
Representative phase contrast images of canine preadipocytes from the subcutaneous inguinal depot induced to differentiate (*day 0*) at different time points post-inoculation and cultured according to both protocols. Images represent adipocytes at *day 14* post-induction. (A) Cells induced 24 h post-inoculation and cultured according to the Eisele *et al.*, 2005 protocol. (B) Cells induced 72 h post-inoculation and cultured according to the Wu *et al.*, 2001 protocol. (C) Cells induced 72 and (D) 96 h post-inoculation and cultured according to the Wu *et al.,* 2001 protocol stained with Oil red O. Cells were cultured in triplicate. The bars represent 100 μm.

Figure 4.2 Depot comparison using the protocol by Wu *et al.,* 2001



Representative phase contrast images of canine preadipocytes from the subcutaneous inguinal and gonadal depots induced to differentiate (*day 0*) into mature adipocytes at different time points and cultured until *day 10* post-induction according to the protocol by Wu *et al.,* 2005. (A) Cells from the subcutaneous inguinal depot induced 24 h post-inoculation. (B) Cells from the subcutaneous inguinal depot induced 72 h post-inoculation. (C) Cells from the gonadal depot induced 24 h post-inoculation. (D) Cells from the gonadal depot induced 72 h post-inoculation. Cells were cultured in triplicate. The bars represent 100 μm.

Figure 4.3 Comparison of well size as a potential stress for cells in culture



Representative phase contrast images of canine preadipocytes from the subcutaneous inguinal depot induced to differentiate (*day 0*) into mature adipocytes 72 h post-inoculation and cultured until *day 14* post-induction according to the protocol by Wu *et al.,* 2005. Cells were cultured in triplicate. (A) Cells cultured in 24 –well plates. (B) Cells cultured in 12 –well plates. (C) Cells cultured in 6 –well plates. Cells were stained with Oil red O. The bars represent 100 μm.

4.4 Results – Part B

Adipokine gene expression and protein secretion during adipocyte differentiation and development

4.4.1 Differentiation and development of canine adipocytes in primary cell culture

Having established a robust system for the differentiation of canine fibroblastic preadipocytes to mature adipocytes in primary culture from both a visceral and subcutaneous depot in the dog, the process of differentiation and maturation of these cells from both depots was then investigated in detail initially through the phenotypic characterisation of the cells in culture. At induction (day 0), cells displayed a typical fibroblastic appearance (Fig. 4.4A). Terminal differentiation, as referred to by the accumulation of intracellular lipid droplets (Ailhaud, 1990), was first observed in cells of both depots by *day 3* post-induction and by *day 6*, several cells could be seen to contain multiple lipid droplets (Fig. 4.4B). These then increased both in number (Fig. 4.4C), and size (Fig. 4.4D), as cells matured. The depot of choice for many of the future investigations using primary cell culture was the subcutaneous inguinal, for reasons of tissue availability. However, where possible, investigations also used cells from the gonadal depot not only to characterise this depot in terms of adipokine expression and secretion, but also to identify any differences between adipocytes from visceral and subcutaneous sources.

4.4.2 Characterisation of canine adipocyte differentiation and development: expression of molecular markers

Adipose cell differentiation is characterised by a series of events during which cells become committed to differentiate and this can be investigated through the expression of key molecular markers and those indicative of terminal differentiation include LPL, GLUT4, PPARγ2 (Ailhaud, 1990). In addition, the gene for haptoglobin has also been found to be expressed late during the differentiation process, with expression being evident from *day 4* post-induction in murine cell lines (do Nascimento *et al.*, 2004). A similar pattern of expression was documented in canine adipocytes where a signal for haptoglobin was also detected at *day 4* post-induction of differentiation (Fig. 4.5).

4.7.3 Expression and secretion of adipocyte hormones in preadipocytes and differentiated adipocytes

Having characterised canine adipocytes through the expression of adipose tissue-related molecular markers, the expression of the key adipocyte hormones leptin and adiponectin was then investigated in canine adipocytes of both depots during differentiation and development *in vitro*. A signal for leptin was detected by RT-PCR from *day 3* post-induction of differentiation in cells of the subcutaneous inguinal depot (Fig. 4.6A). Relative levels of leptin mRNA were quantified by qPCR, where levels were raised from *day 2* post-induction by over 30-fold and were essentially unchanged thereafter (Fig. 4.6B). A signal for leptin was detected at the same time point, from *day 3* post-induction, in gonadal cells by RT-PCR (Fig. 4.7A). Leptin mRNA reached a peak at *day 6* post-induction (11-fold), and subsequently there was little change in relative levels of expression (Fig. 4.7B). Thus, leptin mRNA levels in cells from the gonadal depot peaked later and levels were less than half those seen in cells of the subcutaneous inguinal depot. Furthermore, leptin mRNA levels in cells of the subcutaneous inguinal depot were on average over four times higher from *day 3* post-induction, when compared to cells of the gonadal depot.

In a similar manner to leptin, adiponectin gene expression was only detected post-induction in cells of both depots. A signal was obtained with RT-PCR by *day 4* post-induction in subcutaneous inguinal adipocytes (Fig. 4.8A). Relative levels were quantified by qPCR, where a significant rise in levels of adiponectin mRNA of >25-fold were detected as early as *day 1* post-differentiation (Fig. 4.8B). Adiponectin mRNA levels then continued to rise as cells matured reaching a peak at *day 15* post-induction of >18,000-fold. Active secretion of adiponectin into the cell culture medium by canine adipocytes from the subcutaneous inguinal depot was established by commercial analysis of cell culture supernatant using a canine specific adiponectin ELISA (Fig. 4.8C).

A signal for adiponectin was present from *day 1* post-induction in cells from the gonadal depot. However, a strong signal was only detected, in a similar manner to cells from the subcutaneous inguinal depot, at *day 4* post-induction (Fig. 4.9A). Quantitatively, adiponectin mRNA levels were elevated by 32-fold by *day 2* post-induction. Levels continued to rise reaching >1,700-fold by *day 4* post-induction,

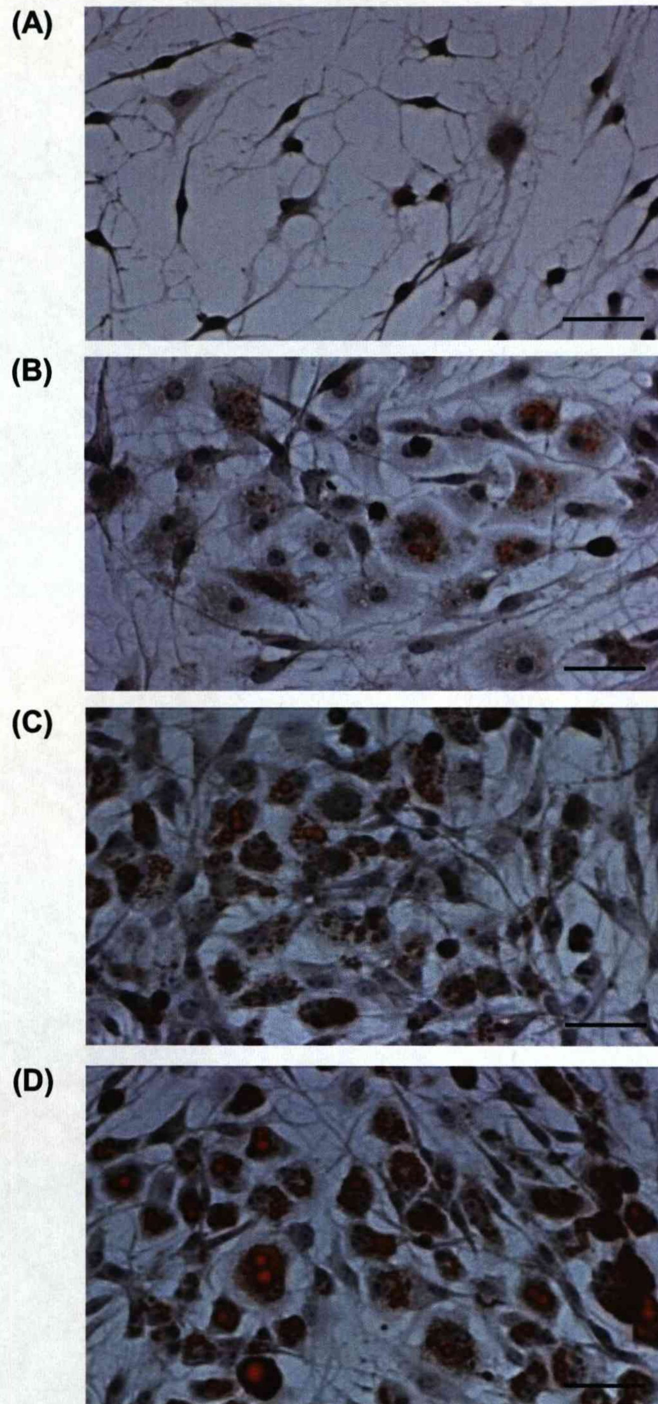
with little change in relative mRNA levels as cells matured (Fig. 4.9B). In a manner similar to leptin, adiponectin mRNA levels were on average four times higher in cells from the subcutaneous inguinal depot compared to gonadal cells.

Adiponectin secretion was also documented from cells of the gonadal depot. As outlined earlier, the main depot used for experimental investigations was the subcutaneous inguinal. In the case of the gonadal depot, the aim was to establish that (in a similar manner to cells of the subcutaneous inguinal depot), the protein was indeed secreted and to detect any differences in the level of secretion between mature cells and cells at the preadipocyte stage. Thus, only two time-points were chosen, in this case cells at *day 0* and mature cells at *day 10* post-induction of differentiation. In a similar manner to cells from the subcutaneous inguinal depot, adiponectin secretion into the medium by cells from the gonadal depot was established in both preadipocytes and mature adipocytes (Fig. 4.9C).

The fact that a reading was obtained for adiponectin protein in preadipocytes of both depots was surprising given the absence of an mRNA signal at *day 0*. In both cases the signal obtained for canine preadipocytes was several times above the reported sensitivity of the assay (22.1 pg/ml). However, readings for mature adipocytes of both depots were approximately 100 times that of preadipocytes allowing a greater degree of confidence in terms of the specificity of the assay, which according to the commercial lab had no cross-reactivity with adiponectin from other species. Furthermore, as the assay was carried out commercially, unlike those for other adipokines, there may be uncertainties in these values which may have been due to cross-reactivity with other canine proteins present in cell culture media, thereby contributing to the detection of adiponectin early during the differentiation process.

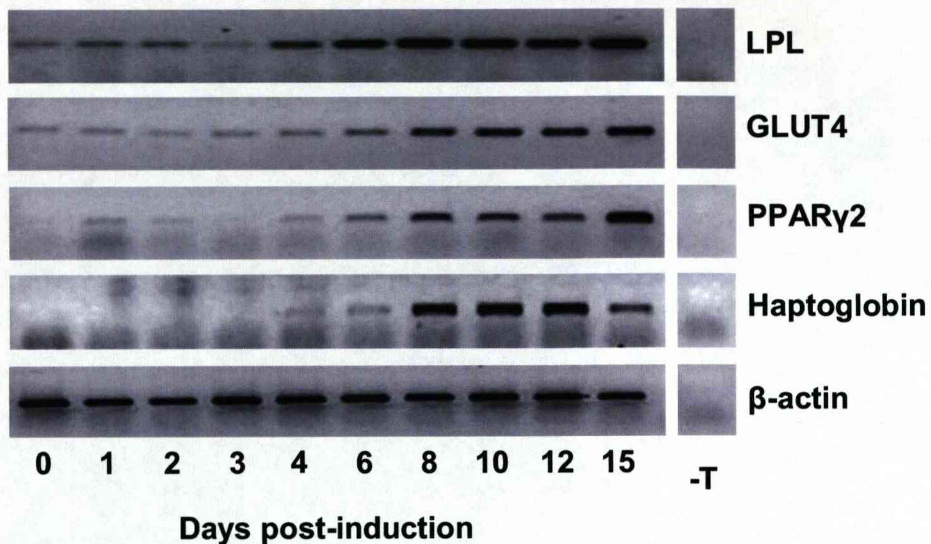
Unlike mRNA levels, adiponectin protein concentrations were >30% higher in cells of the visceral depot (gonadal) compared to those of the subcutaneous inguinal depot at *day 10* post-induction (Fig. 4.9C). However, quantitative protein comparisons between depots should nevertheless be treated with caution as they assume an identical number of cells being present and this was not directly measured.

Figure 4.4 Time course of differentiation and development of canine adipocytes in primary culture



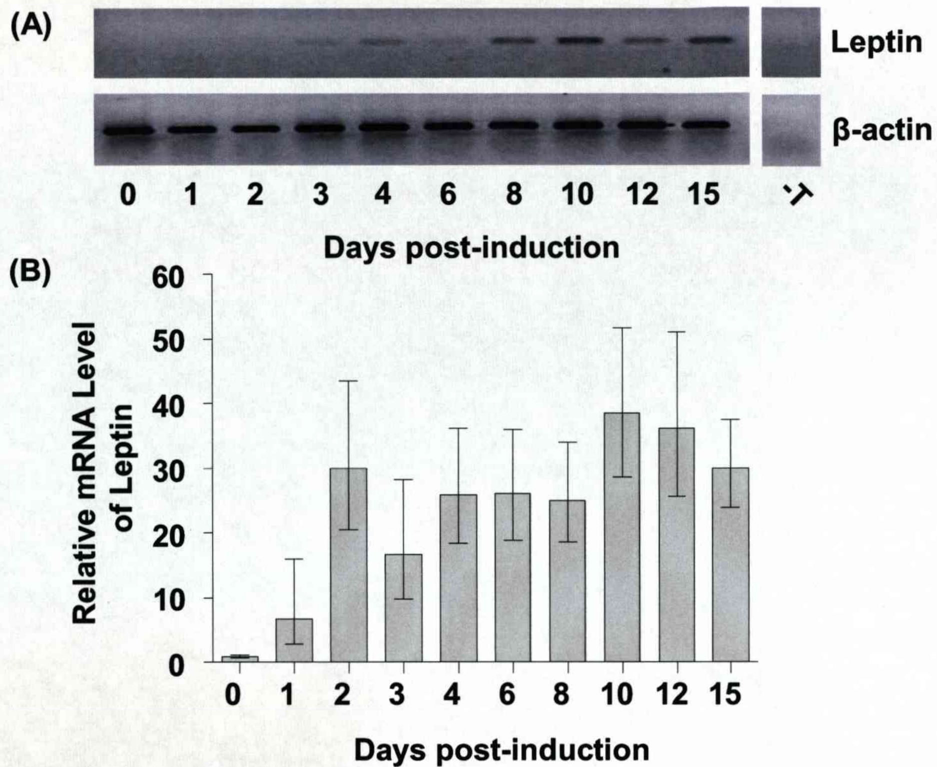
Representative phase contrast images of cells from the subcutaneous inguinal depot. Preadipocytes were harvested, cultured and induced to differentiate (day 0) into adipocytes. Cells were sampled at *day 0* (A), *day 6* (B), *day 10* (C) and *day 12* (D) post-differentiation and stained with the lipid specific stain, Oil red O. Bar represents 100 μm.

Figure 4.5 Time course of gene expression of adipose tissue-related markers in canine adipocytes in primary culture



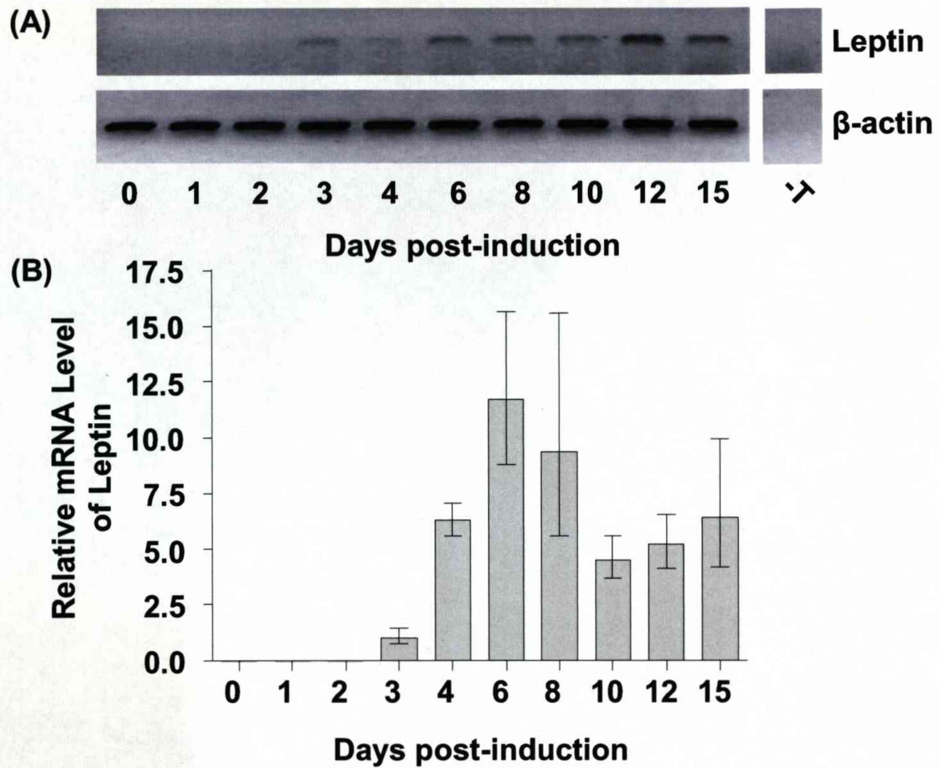
Representative RT-PCR gels of cells from the subcutaneous inguinal depot. Canine adipocytes in culture were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). Total RNA was extracted and examined by RT-PCR using dog-specific primers to determine the expression of adipose tissue-related markers during differentiation and development *in vitro*. Cells represented are from the subcutaneous inguinal depot. Amplification cycle numbers were: LPL, 30; GLUT4, 30; PPARγ2, 27; haptoglobin, 27; β-actin 25; -T, no template control.

Figure 4.6 Time course of leptin gene expression in adipocytes of the subcutaneous inguinal depot differentiated in primary culture



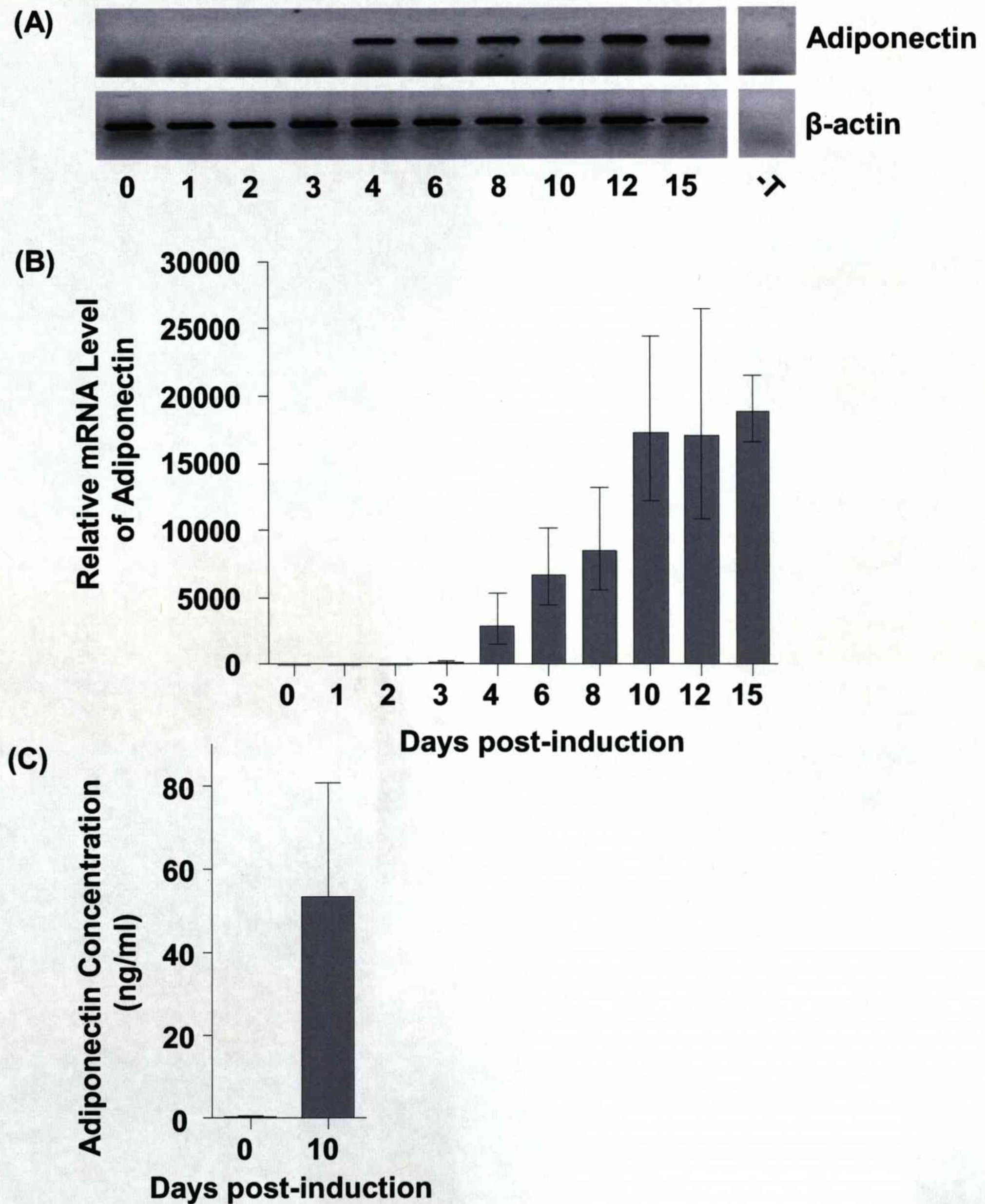
Canine adipocytes and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). In (A), total RNA was extracted and leptin gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: Leptin, 32 and β-actin, 25; -T, no template control. In (B), relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.7 Time course of leptin gene expression in adipocytes of the gonadal depot differentiated in primary culture



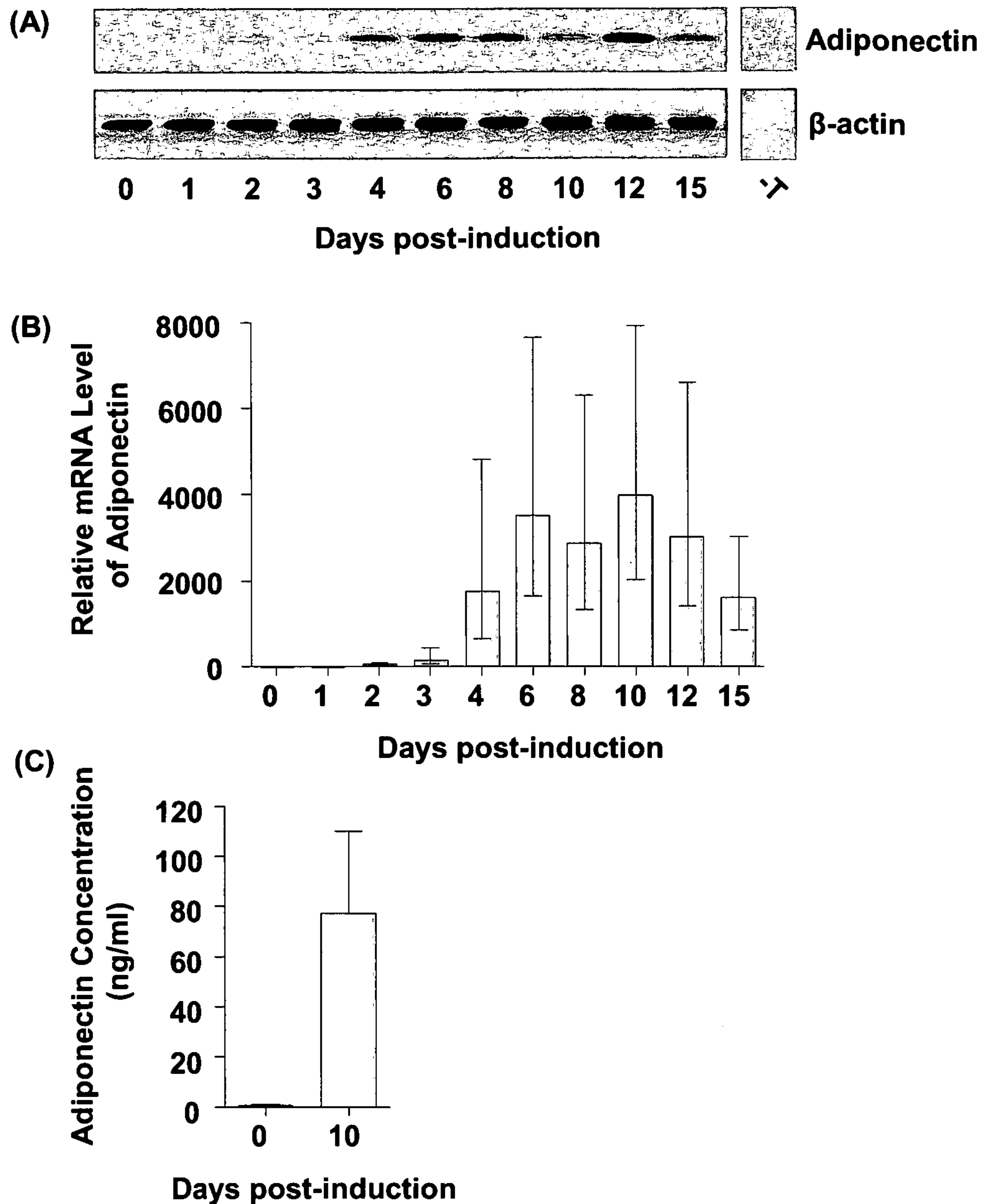
Canine adipocytes and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). In (A), total RNA was extracted and leptin gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: Leptin, 32 and β-actin, 25; -T, no template control. In (B), relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 3*, as no signal was evident at *day 0*. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.8 Time course of adiponectin gene expression and protein secretion in adipocytes of the subcutaneous inguinal depot differentiated in primary culture



Canine adipocytes and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). In (A), total RNA was extracted and adiponectin gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: Adiponectin, 26 and β-actin, 25; -T, no template control. In (B), relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. (C) Adiponectin protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.9 Time course of adiponectin gene expression and protein secretion in adipocytes of the gonadal depot differentiated in primary culture



Canine adipocytes and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). In (A), total RNA was extracted and adiponectin gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: Adiponectin, 26 and β-actin, 25; -T, no template control. In (B), relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. (C) Adiponectin protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

4.7.4 Expression of inflammation-related adipokines in preadipocytes and differentiated adipocytes

The expression of several inflammation-related adipokines during canine adipocyte differentiation and development was investigated by RT-PCR in cells of the subcutaneous inguinal depot; these were PAI-1, SAA and VEGF, where a signal for all three adipokines was detected both pre- and post-induction of differentiation (Fig. 4.10).

4.7.5 Expression and secretion of inflammation-related adipokines in preadipocytes and differentiated adipocytes

Having characterised the expression of several inflammation-related adipokines during the differentiation and development of canine adipocytes from the subcutaneous inguinal depot *in vitro*, the next set of experiments set out to explore this further by investigating the expression and possible secretion of key inflammation-related adipokines including TNFα and IL-6 during the differentiation of canine adipocytes from both the subcutaneous inguinal and gonadal depots.

TNFα gene expression was initially examined by RT-PCR, where a signal was readily detected both pre- and post-differentiation in cells of both depots (Fig. 4.11A and 4.12A). In terms of cells from the subcutaneous inguinal depot, the signal appeared most intense early-on in the differentiation process at around *day 1-2* post-induction (Fig. 4.11A). This inference was substantiated by the quantification of TNFα mRNA levels through qPCR (Fig. 4.11B). In contrast, the signal for TNFα detected by RT-PCR in cells of the gonadal depot suggested a more uniform level of expression during differentiation of cells in culture (Fig. 4.12A). However, when mRNA levels were quantified by qPCR, a similar trend to that observed in cells from the subcutaneous inguinal depot was demonstrated, with a decrease in TNFα mRNA levels detected as cells reached maturity (Fig. 4.12B).

TNFα protein was detected in conditioned media collected from cultures of adipocytes from both depots pre- and post-differentiation using an ELISA specific for canine TNFα (Fig. 4.11C and Fig. 4.12C). Levels of protein secreted by cells of both depots into the media decreased as cells matured, where by *day 10* post-induction, TNFα protein in conditioned media from cells of the subcutaneous

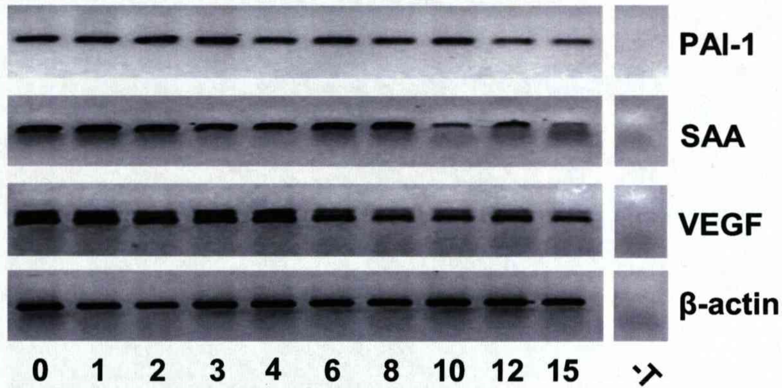
inguinal depot had fallen by nearly 50% (Fig. 4.11C). This fall was even more marked in media collected from cells of the gonadal depot where TNFα levels fell by nearly 80% by *day 10* post-induction (Fig. 4.12C). At the preadipocyte stage, levels of TNFα protein were >80% higher in cells of the gonadal depot compared to those of the subcutaneous inguinal depot. This persisted as cells matured where at *day 10* post-differentiation, where levels of TNFα secreted by cells from the gonadal depot were still >50% higher than those secreted by cells of the subcutaneous inguinal depot. However, as with adiponectin, these comparisons in terms of levels of protein secreted should be treated with caution as they do depend to a large extent on the number of cells present in each sample and this was not directly measured.

The time course of the expression of another important inflammation-related adipokine IL-6, was also examined by RT-PCR for cells of both depots, where a signal was evident both pre- and post-differentiation (Fig. 4.13A and 4.14A). For cells of the subcutaneous inguinal depot, signal intensity appeared higher pre- and during differentiation (Fig. 4.13A). These inferred changes in level of expression were substantiated by quantification of IL-6 expression by qPCR, where levels were raised early on during the differentiation process. IL-6 mRNA levels then decreased markedly by >90% from *day 3* post-induction with levels remaining low as cells matured (Fig. 4.13B). The same trend in IL-6 mRNA level was observed in cells from the gonadal depot (Fig. 4.14B).

IL-6 protein was readily detected in conditioned medium from cells of both depots both pre- and post-differentiation by a canine IL-6 specific ELISA (Fig. 4.13C and 4.14C). In a similar manner to TNFα, levels of IL-6 actively secreted by cells of both depots decreased as cells matured. In terms of cells from the subcutaneous inguinal depot, levels of IL-6 protein in conditioned medium peaked at day 2 post-induction, after which levels fell by >70% by *day 3* (Fig. 4.13C). By *day 10* post-induction, levels of IL-6 protein had fallen by >90 and >80% in media from cells of the subcutaneous inguinal and gonadal depots, respectively. IL-6 protein secreted by cells at the preadipocyte stage were >85% higher in cells of the gonadal depot compared to those of the subcutaneous inguinal depot and despite the decline in IL-6 protein levels for both depots as cells matured, cells from the gonadal depot still had

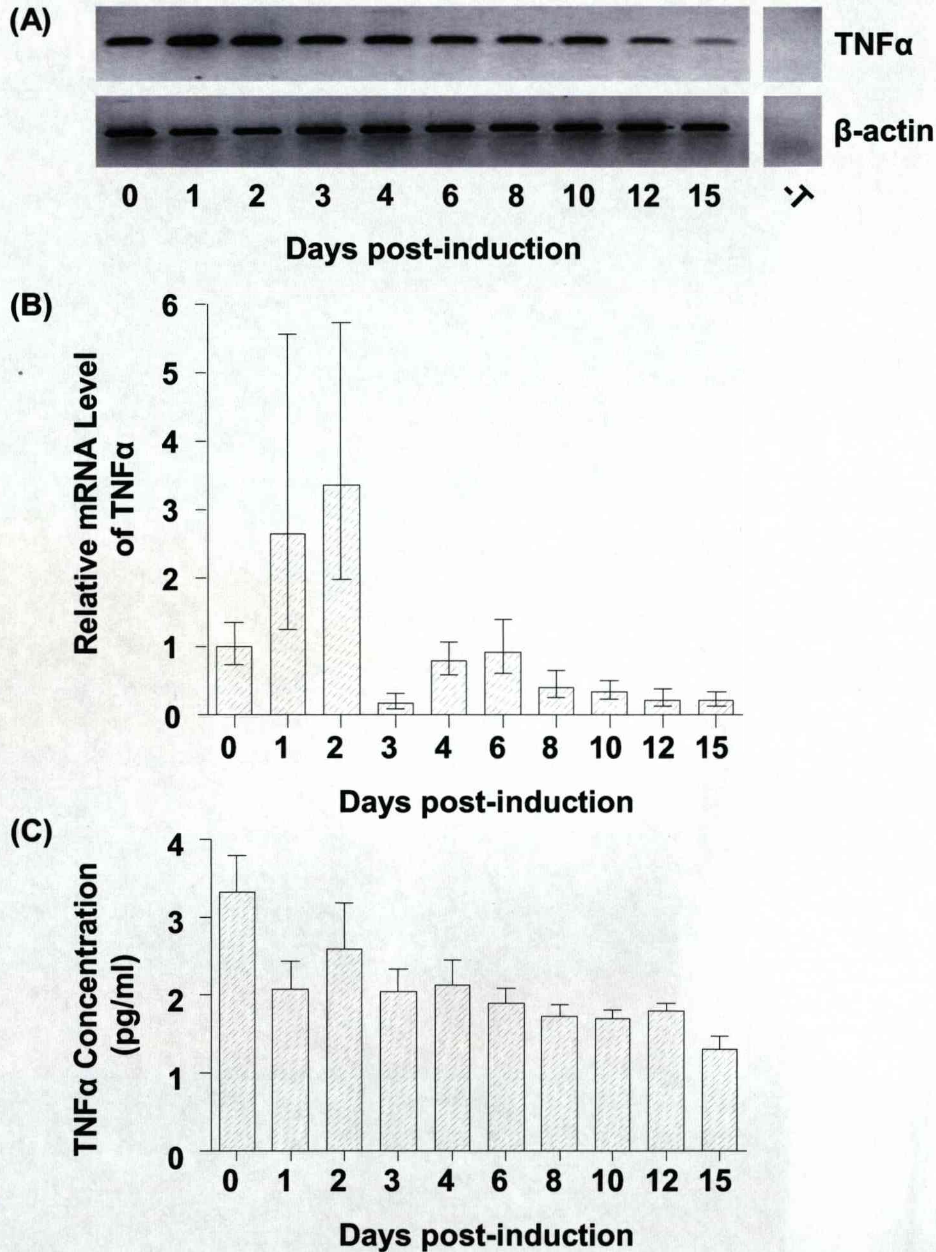
IL-6 levels which were >75% greater than those of the subcutaneous inguinal depot at *day 10* post-induction.

Figure 4.10 Time course of pro-inflammatory adipokine gene expression in adipocytes of the subcutaneous inguinal depot differentiated in primary culture



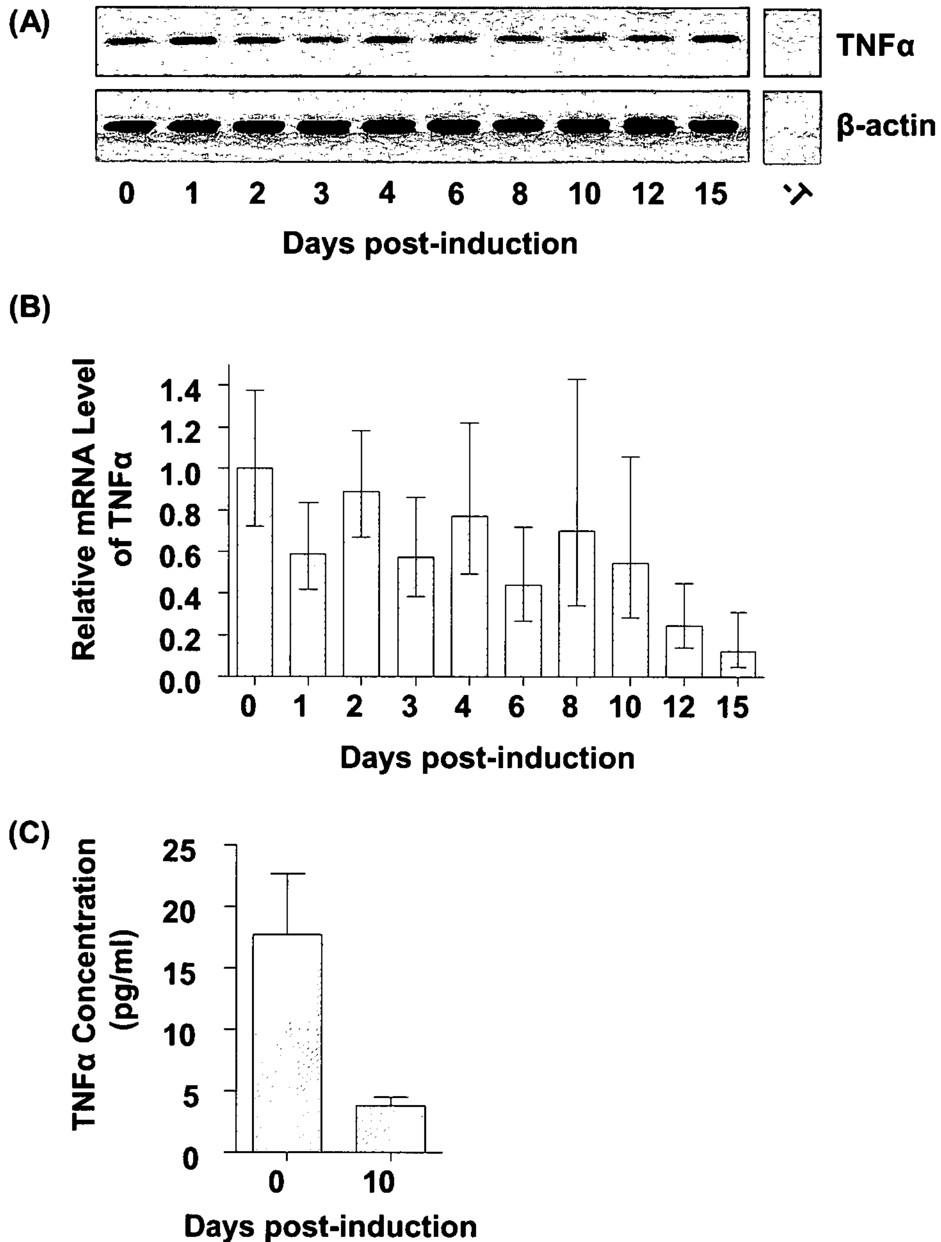
Canine adipocytes in culture were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). Total RNA was extracted and examined by RT-PCR using dog-specific primers to determine the expression of proinflammatory adipokine genes during differentiation and development *in vitro*. Cells represented are from the subcutaneous inguinal depot. Amplification cycle numbers were: PAI-1, 32; SAA, 28; VEGF, 30; β-actin 25; -T, no template control.

Figure 4.11 Time course of TNFα gene expression and protein secretion in canine subcutaneous inguinal adipocytes in primary culture



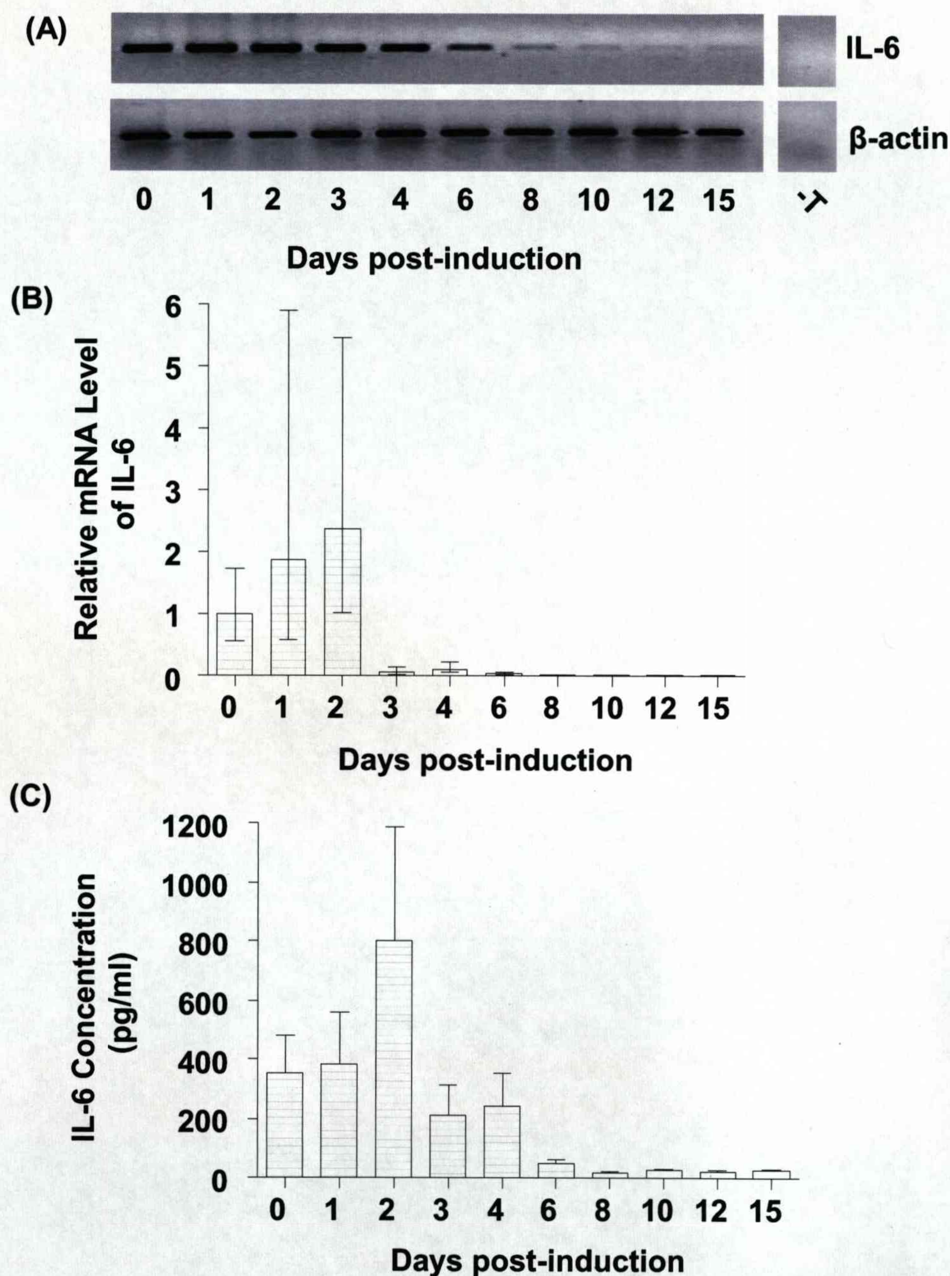
Canine adipocytes from the subcutaneous inguinal depot and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). (A) Total RNA was extracted and TNFα gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: TNFα, 34 and β-actin, 25; -T, no template control. (B) Relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. (C) TNFα protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.12 Time course of TNFα gene expression and protein secretion in canine gonadal adipocytes in primary culture



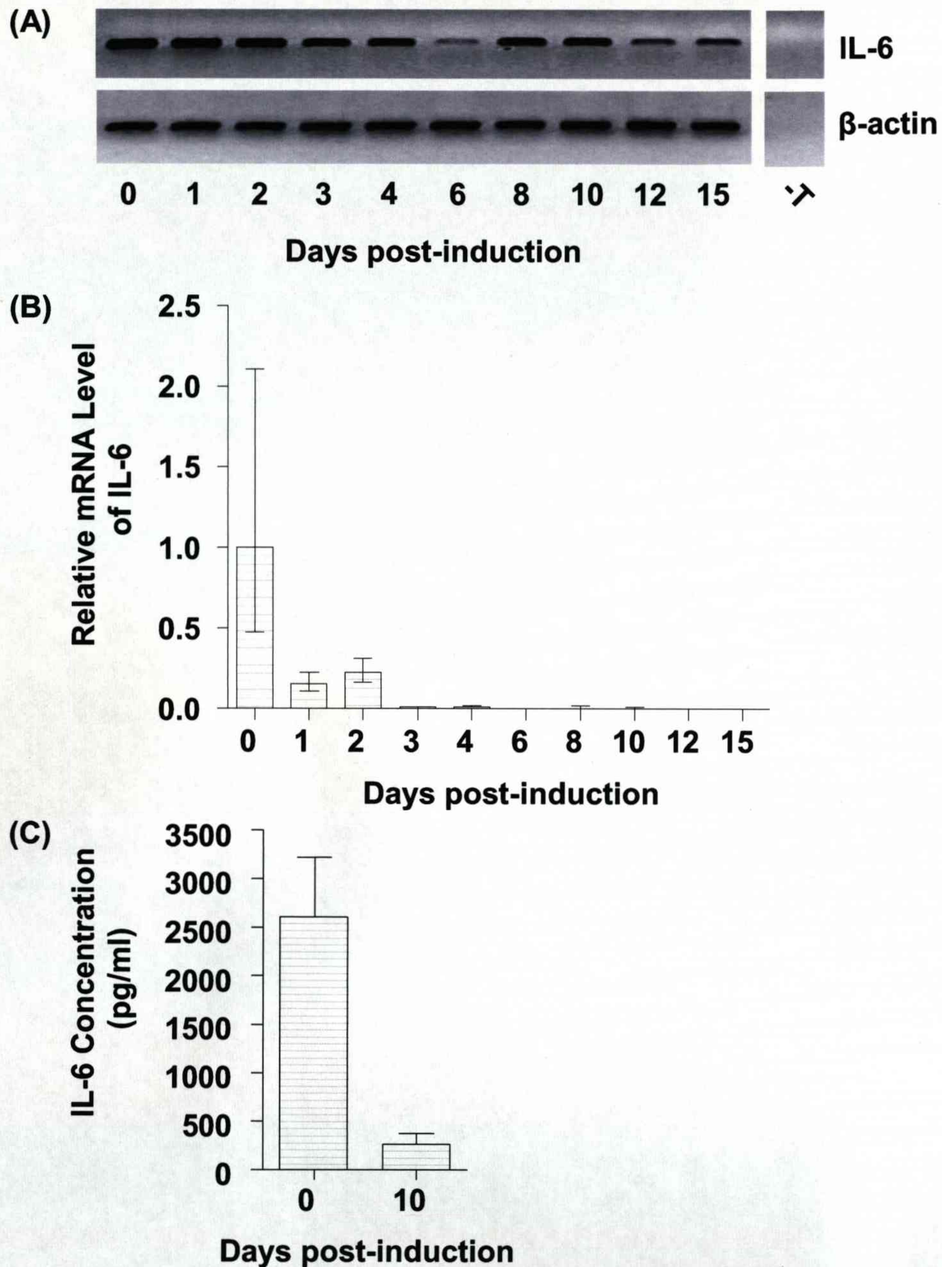
Canine adipocytes from the gonadal depot and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). (A) Total RNA was extracted and TNFα gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: TNFα, 34 and β-actin, 25; -T, no template control. (B) Relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. (C) TNFα protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.13 Time course of IL-6 gene expression and protein secretion in canine subcutaneous inguinal adipocytes in primary culture



Canine adipocytes from the subcutaneous inguinal depot and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). (A) Total RNA was extracted and IL-6 gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: IL-6, 34 and β-actin, 25; -T, no template control. (B) Relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0.* (C) IL-6 protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.14 Time course of IL-6 gene expression and protein secretion in canine gonadal adipocytes in primary culture



Canine adipocytes from the gonadal depot and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). (A) Total RNA was extracted and IL-6 gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: IL-6, 34 and β-actin, 25; -T, no template control. (B) Relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. (C) IL-6 protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

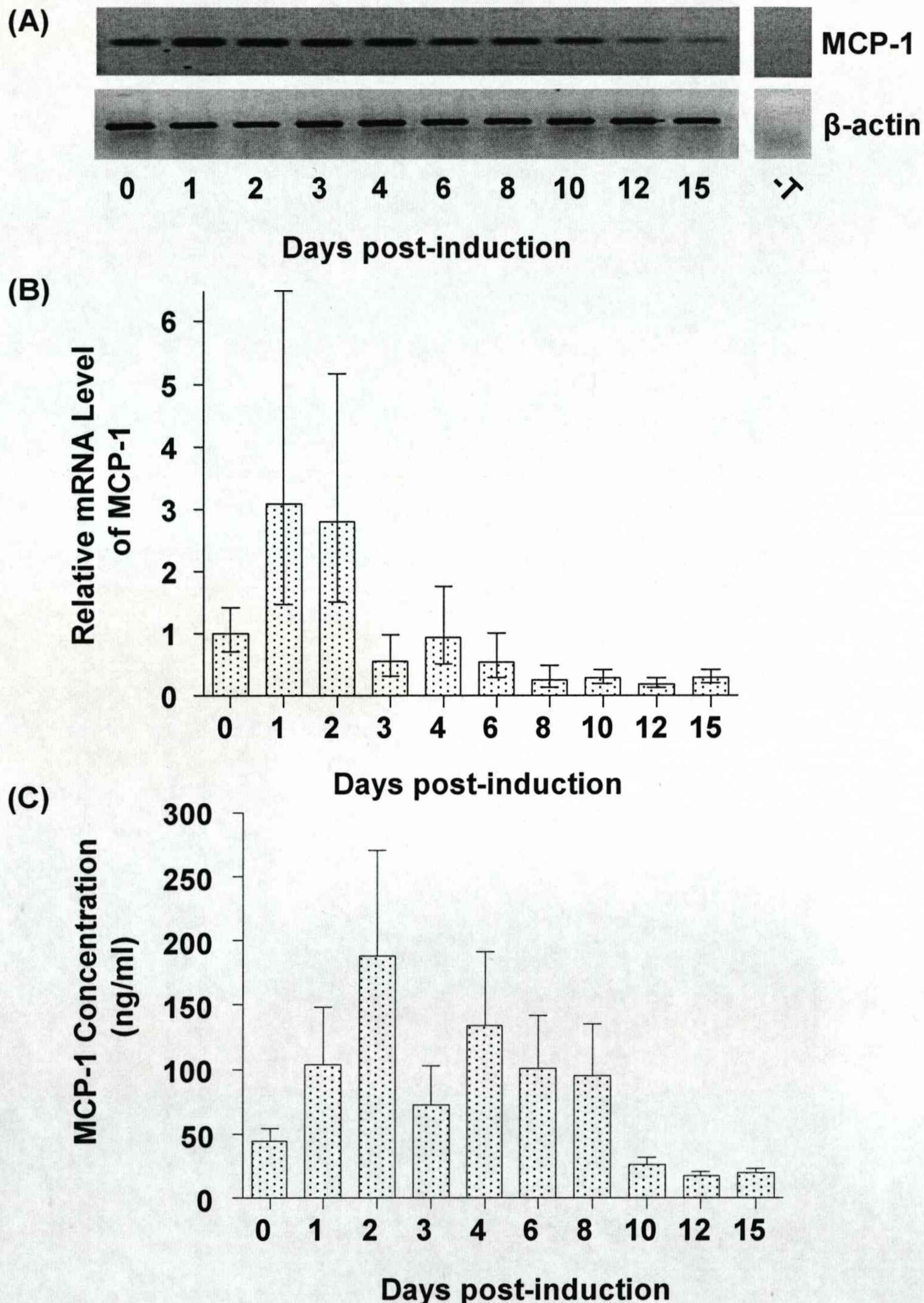
A signal for MCP-1 was detected in cells of both depots pre- and post-differentiation (Fig. 4.15A and 4.16A). Quantitative changes in MCP-1 gene expression in cells of both depots during differentiation were analysed by qPCR. For cells of the subcutaneous inguinal depot, MCP-1 mRNA levels were highest at *day 1* post-induction and in a manner similar to other inflammation-related adipokines, mRNA levels then declined as cells matured (Fig. 4.15B). Quantitatively, MCP-1 mRNA levels in cells of the gonadal depot fell by 70% after the induction of differentiation. There was then little change until *day 4* post-induction were levels recovered to the pre-differentiation value. However, levels of expression then fell by a similar extent and remained low as cells reached maturity (Fig. 4.16B).

MCP-1 protein was detected in conditioned media secreted by canine adipocytes of the subcutaneous inguinal and gonadal depot both pre- and post-differentiation (Fig. 4.15C and 4.16C). Secretion of MCP-1 protein by cells of the subcutaneous inguinal depot reached a peak at *day 2* post-induction (shortly after the peak for MCP-1 mRNA levels). Subsequently, levels of protein secreted into the medium declined as cells reached maturity (Fig. 4.15C). This is unlike what was seen for cells of the gonadal depot, where MCP-1 levels were highest in mature adipocytes compared to preadipocytes (Fig. 4.16C). In a similar manner to other proinflammatory adipokines, levels of MCP-1 protein were raised in cells of the gonadal depot at the preadipocyte stage by >30% compared to those of the subcutaneous inguinal depot. By *day 10* post-differentiation this difference had increased to >80%.

NGF expression was examined in canine adipocytes, in a similar manner to other inflammation-related adipokines by RT-PCR, where a signal was present for cells of both depots both pre- and post-differentiation (Fig. 4.17A and 4.18A). Quantitative changes in NGF gene expression were analysed by qPCR where NGF mRNA levels increased post-induction of differentiation. However by day 3, mRNA levels fell by nearly 60% and changed little thereafter as cells matured (Fig. 4.17B). In contrast, NGF mRNA levels in gonadal cells fell sharply immediately post induction of differentiation by >75%, and in a similar manner to cells from the subcutaneous inguinal depot, levels remained low and relatively unchanged as cells matured (Fig. 4.18B).

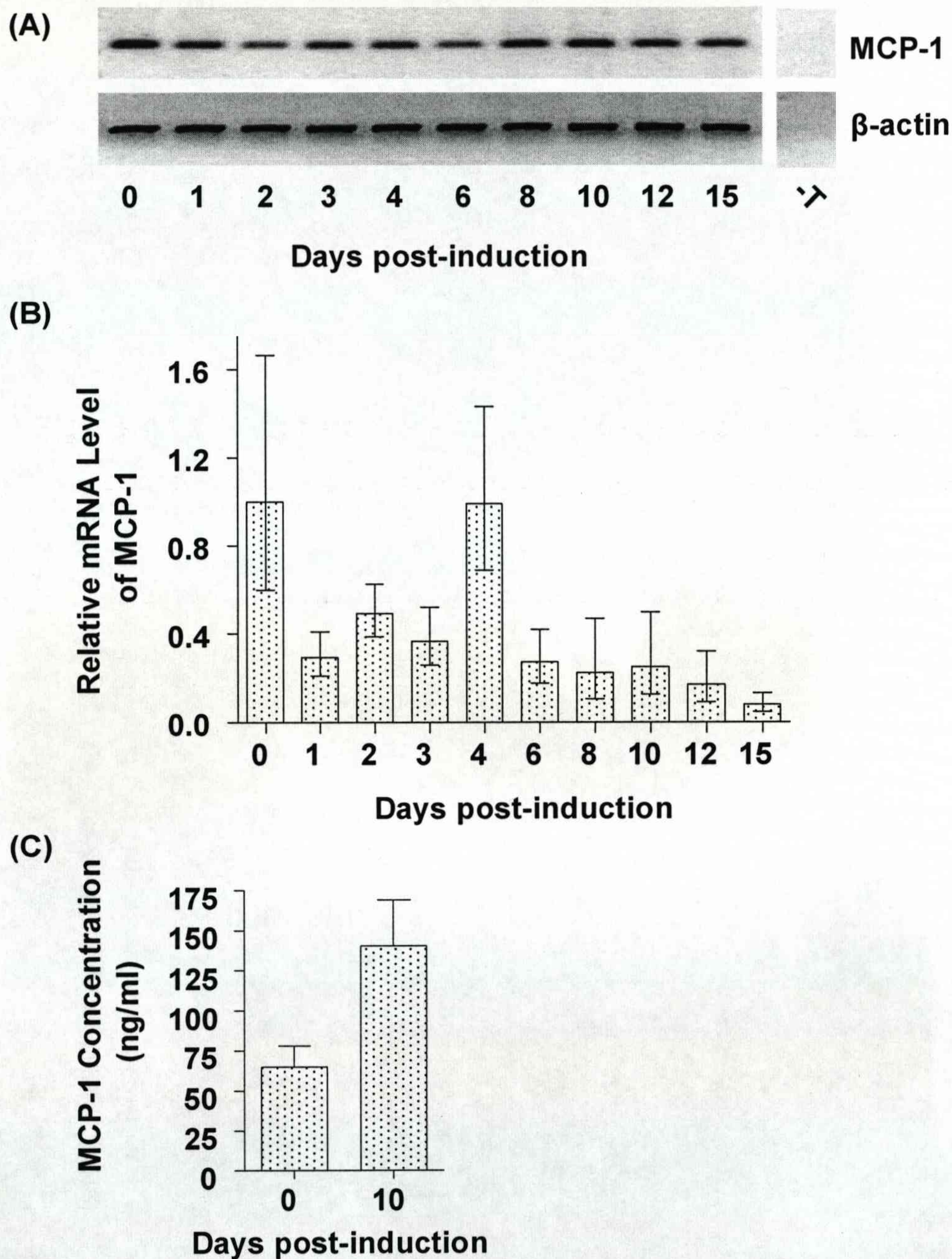
Having established that canine adipocytes express the NGF gene, an anti-murine NGF ELISA which was shown to be cross-reactive to canine NGF was used to determine whether the cells also secrete immunoreactive NGF protein. Cells from the subcutaneous inguinal depot were chosen in this case, as they represent those from the depot which was chosen as that to be used as the main depot for further *in vitro* investigations. NGF was readily detected in the cell culture medium, both before and after the induction of differentiation (Fig. 4.17C). Here, in a similar manner to IL-6, the rate of release reached a peak shortly after the induction of differentiation at *day 2*, and levels of secretion fell as cells reached maturity.

Figure 4.15 Time course of MCP-1 gene expression and protein secretion in canine subcutaneous inguinal adipocytes in primary culture



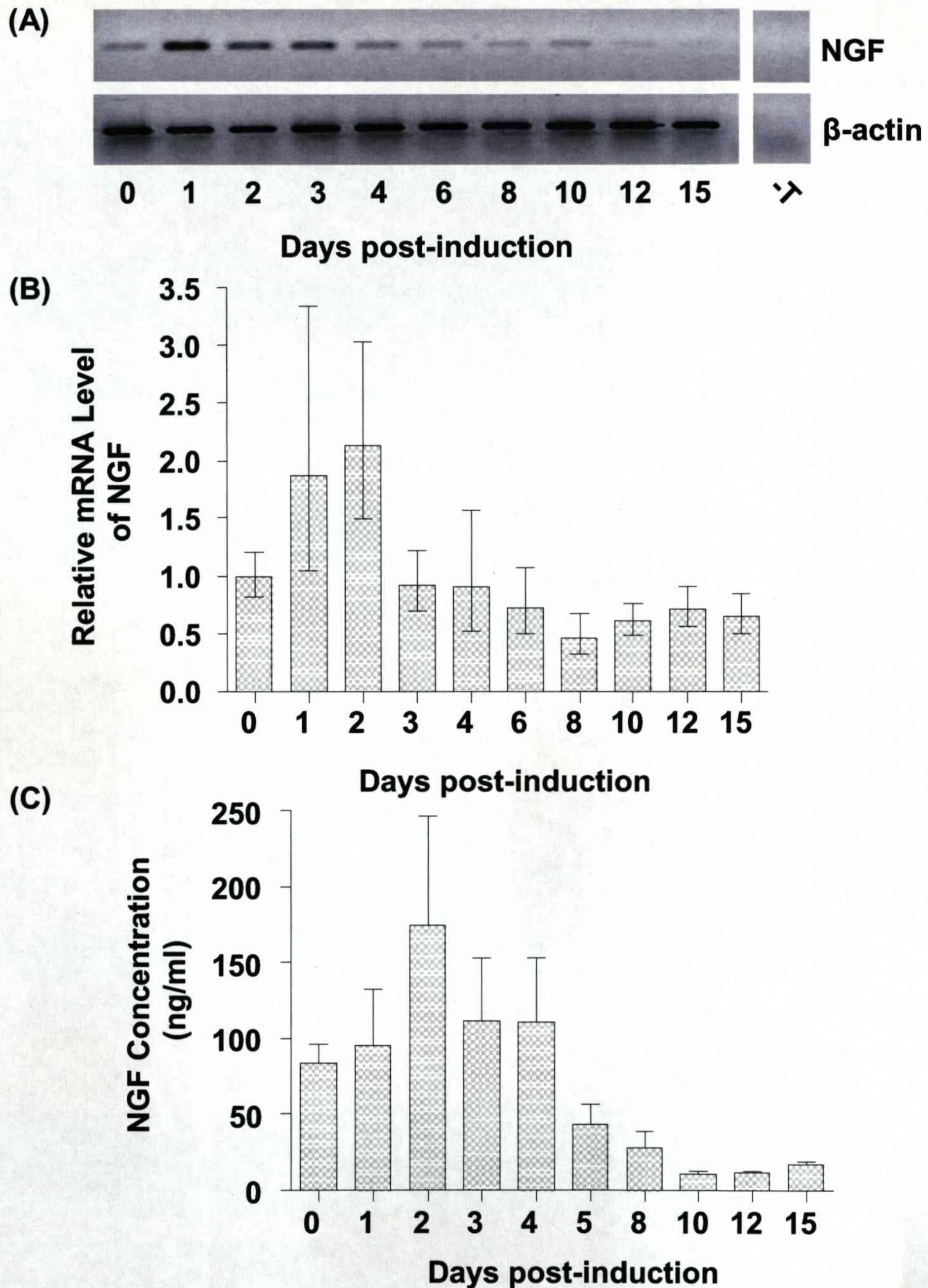
Canine adipocytes from the subcutaneous inguinal depot and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). (A) Total RNA was extracted and MCP-1 gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: MCP-1, 23 and β-actin, 25; -T, no template control. (B) Relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. (C) MCP-1 protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.16 Time course of MCP-1 gene expression and protein secretion in canine gonadal adipocytes in primary culture



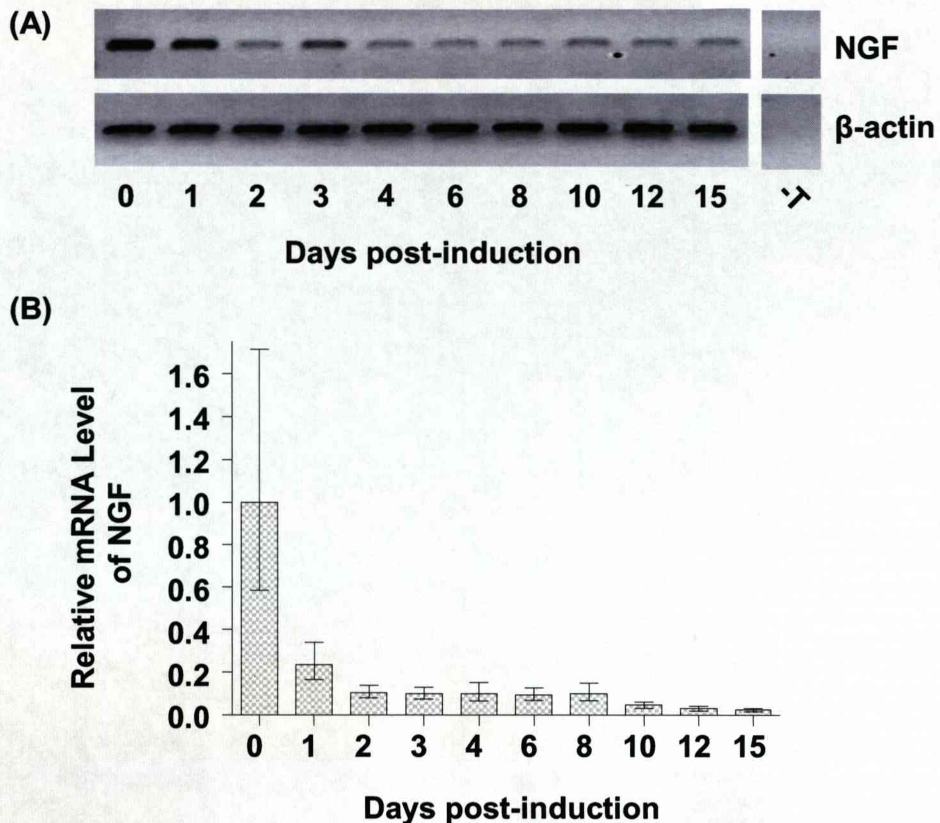
Canine adipocytes from the gonadal depot and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). (A) Total RNA was extracted and MCP-1 gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: MCP-1, 23 and β-actin, 25; -T, no template control. (B) Relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. (C) MCP-1 protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.17 Time course of NGF gene expression and protein secretion in canine subcutaneous inguinal adipocytes in primary culture



Canine adipocytes from the subcutaneous inguinal depot and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). (A) Total RNA was extracted and NGF gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: NGF, 28 and β-actin, 25; -T, no template control. (B) Relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. (C) NGF protein levels in the media were determined by canine-specific ELISA; values shown are actual concentrations in the medium. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 4.18 Time course of NGF gene expression in canine gonadal adipocytes in primary culture



Canine adipocytes from the gonadal depot and media were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). (A) Total RNA was extracted and NGF gene expression examined by RT-PCR using dog-specific primers. Amplification cycle numbers were: NGF, 28 and β-actin, 25; -T, no template control. (B) Relative quantification was carried out by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

4.5 Discussion

In Chapter 3, the expression of key adipose tissue-related genes (LPL and PPARγ2), hormones (leptin and adiponectin) and proinflammatory markers (TNFα and IL-6) was demonstrated in WAT depots in the dog. This chapter outlines investigations into the establishment of a protocol for the development of a primary cell culture system for the differentiation of fibroblastic canine preadipocytes *in vitro*. Once established, this would prove to be a powerful tool for further investigations into adipose tissue differentiation and development.

Of the two published protocols outlining this procedure, the one by Eisele *et al.,* 2005 was the first to be attempted as it had demonstrated success using a depot to which we had access during routine surgery. Furthermore, Eisele had previously attempted to differentiate cells from the gonadal depot unsuccessfully using the Wu *et al.*, 2001 protocol (unpublished observations). Despite initial expectations, attempts to differentiate canine preadipocytes from the gonadal depot using the Eisele *at al.*, 2005 protocol were unsuccessful.

Our efforts were then focused on comparing both protocols using the 'ideal' depot (subcutaneous inguinal) as outlined by Wu *et al.,* 2001. In addition, it has been documented that preadipocytes isolated from different depots in humans, exhibit different capacities to undergo replication and differentiation. Those found in subcutaneous WAT are able to undergo more extensive lipid accumulation and exhibit higher C/EBPα expression than those from the omental depot. Thus, it has been proposed that preadipocytes can be divided into subtypes which contribute to different extents to the overall preadipocyte population in individual depots and these have different capacity to undergo replication and differentiation *in vitro* (Tchkonia *et al.*, 2005).

Investigations comparing both protocols using the subcutaneous inguinal depot were carried out in triplicate using animals of various breeds including the breed of choice, the SBT. Cells were induced at different times post-inoculation to investigate the effect of confluency (in terms of cell-to-cell contact) on the ability of cells to differentiate. Cells were seeded according to Eisele *et al.,* 2005, where seeding

densities were ten-fold higher than those recommended by Wu *et al.,* 2001. Both protocols were successfully able to induce differentiation of canine fibroblastic preadipocytes from the subcutaneous inguinal depot. Although cells cultured using the Eisele *et al.,* 2005 protocol successfully induced cells to accumulate lipid, which was most successful if cells were induced 24 h post-inoculation, this was not to the same extent as that observed with cells induced 72 h post-inoculation cultured using the Wu *et al.,* 2001 protocol. Here in excess of 85% of cells were shown to have intracellular lipid droplets when stained with Oil red O. Unfortunately, due to limited availability of tissue, we were unable to detect an effect of breed or gender on the ability of canine preadipocytes to differentiate in culture.

In terms of cell culture technique, that by Wu *et al.*, 2001 utilises a centrifugation rate which is five times higher than that by Eisele *et al.*, 2005. It was found that at such high rates of centrifugation a much lower cell yield was obtained for the same amount of starting material. Furthermore, the seeding density used in the Wu *et al*, 2001 protocol is ten times less than that in the Eisele *et al.,* 2005 protocol. Cells inoculated at seeding densities of < 40 000 cells/cm^2 did not accumulate lipid and as such did not successfully undergo differentiation. In addition, it was found that in order to remove contamination from other cell types, two filtration steps were required, instead of the single filtration step suggested by both protocols. Finally, the medium used in each protocol was also different, where in the Eisele *et al.* 2005, protocol, in a manner similar to that used in human preadipocyte cell culture, the main medium was DMEM/F12 (Hauner *et al.*, 2001). Amongst other components, DMEM/F12 contains a several more amino acids and inorganic compounds compared to DMEM used by Wu *et al.,* 2001.

There are numerous hormones and other factors which play a role in proliferation and differentiation of adipocytes (Ailhaud, 1990). Insulin and T_3 have both been shown to be important for terminal differentiation in adipocyte cell models (Hauner, 1990). Glucocorticoids are also known to be involved in fat metabolism and adipocyte differentiation through the activation of C/EBPδ (Yeh *et al.*, 1995), although their effect seems to be dependent on the time of addition relative to the cell-cycle and the presence of insulin (Hauner, 1990; Xu & Bjorntorp, 1990). The addition of an agent responsible for the elevation in intracellular cAMP levels such

as 3-isobutyl-1-methylxanthine (IBMX) is also needed for successful induction of adipogenesis. cAMP plays an important role as a second messenger for intracellular signal transduction and is involved in the activation of protein kinases (Ailhaud, 1990; Hauner, 1990). Furthermore, IBMX is also responsible for the activation of C/EBPβ, thus playing a key role in the activation of the cascade of transcription factors which lead to successful adipocyte differentiation (Yeh *et al.*, 1995).

The Eisele *et al.*, 2005 protocol, as described in section 4.2.1, makes use of an adipogenic cocktail based on that used in human primary cell culture, using insulin, cortisol, IBMX, T_3 and human transferrin (as a means for chelating iron, making it unavailable for bacterial use) (Hauner *et al.*, 2001). In addition, it incorporates the PPARγ agonist rosiglitazone, which is a well-characterised regulator of adipose differentiation (Spiegelman, 1998). In contrast, the Wu *et al.,* 2001 protocol lacks a PPARγ agonist, T_3 and transferrin. In addition, instead of a glucocorticoid, it makes use a non-steroidal anti-inflammatory drug (NSAID), indomethacin which functions as a PPARγ ligand thereby playing a key role in the induction of adipogenesis (Lehmann *et al.*, 1997).

Earlier investigations had established that the Wu *et al.,* 2001 protocol, rather than that outlined by Eisele *et al.,* 2005, successfully induced in excess of 85% differentiation in cells of the subcutaneous inguinal depot, with best results if cells were induced to differentiate 72 h post-inoculation. Thus a study comparing the subcutaneous inguinal and gonadal depots was carried out using the Wu *et al.,* 2001 protocol. Here, cells from the subcutaneous inguinal depot displayed successful intracellular lipid accumulation when induced to differentiate 24 and 72 h post-inoculation, with best results being obtained at 72 h (as had been previously demonstrated when the two protocols were compared) see Fig 4.2B. In terms of cells from the gonadal depot, numbers of cells displaying intracellular lipid accumulation were comparable to those of the subcutaneous inguinal depot if cells were induced 24 h post-inoculation. If induced any later, differentiation rates were markedly reduced.

The induction of cells prior to 96 h post-inoculation meant inducing them at a preconfluent stage. Cells from different depots, inoculated at the same density, achieved maximal differentiation when induced at different time points post-

inoculation. Growth arrest at the G_0/G_1 stage of the cell cycle, rather than contact inhibition *per se* amongst arrested cells, is the main requirement for successful adipose differentiation (Ailhaud, 1990). This discrepancy in the time post-inoculation leading to optimal differentiation in the different depots may be explained by the fact that a population of precursor cells may not be at the same stage in the cell cycle at the time of inoculation in culture dishes. Thus, hormonal effects on cell differentiation may depend not only on the hormonal environment these cells are exposed to, but also on exposure happening at a 'critical window' were cells will be more or less susceptible to those effects (Ailhaud, 1990), and this may vary between different depots.

The present work has documented the successful isolation of viable canine preadipocytes from both visceral and subcutaneous depots in the dog. It has further outlined the establishment of a robust method of canine adipocyte cell culture in which fibroblastic preadipocytes were successfully induced to differentiate into adipocytes. The method outlined in section 2.3, was largely based on that by Wu *et al* in terms of reagents used at each of the stages of the cell culture protocol (Wu *et al.*, 2001). However, the experimental technique, including inoculation density and centrifugation speed was as described for Eisele *et al* (Eisele *et al.*, 2005). The validity of the culture system was demonstrated by profiling the expression of differentiation dependent genes, such as; LPL, GLUT4, PPARγ2 and haptoglobin. The expression of these genes has been documented to increase during differentiation and development of adipocytes in both human and murine culture systems (Wabitsch *et al.*, 2001; do Nascimento *et al.*, 2004), and as such their expression increased in canine adipocytes as cells matured.

Subsequent studies used the preadipocyte cell culture system, RT-PCR (to identify the expression of key adipokine genes) and qPCR to examine quantitative changes in the relative levels of expression of these genes, during the differentiation and development of canine adipocytes of both depots *in vitro*. Using RT-PCR, a signal for leptin was only visible post-differentiation in both depots examined. This is in accordance with leptin being a late marker of differentiation in this and other species (Wabitsch *et al.*, 2001; Wang *et al.*, 2004; Eisele *et al.*, 2005). Quantitatively, levels of leptin mRNA from *day 3* post-induction were four times higher in cells of the

subcutaneous inguinal compared to the gonadal depot. Higher levels of leptin gene expression in subcutaneous compared to visceral depots has been documented in humans (Montague *et al.*, 1997b; Schoof *et al.*, 2004). This difference is thought to be due to the fact that adipocytes from visceral and subcutaneous depots differ in their sensitivity to the effects of insulin on lipolysis, those from visceral depots being less sensitive than those from a subcutaneous depot (Engfeldt & Arner, 1988). As insulin promotes leptin gene expression, this mechanism might be an explanation for the reduced levels of leptin mRNA documented in visceral as compared to subcutaneous depots. However, the sympathetic nervous system is the primary regulator of lipolysis (Penn *et al.*, 2006) and leptin increases sympathetic outflow to WAT depots in rodents and thus these differences may also be due to differences in sympathetic tone between depots.

Only in the last few years have canine specific protein assays been available commercially and at the time of writing only one such assay is available for canine leptin. This assay, from LINCO, has a sensitivity of 250 pg/ml. This is over thirty times higher than that reported by human kits, of the order of <10 pg/ml (Wang *et al.*, 2004). A sandwich ELISA assay system has been developed and validated for canine leptin again with sensitivity in the order of 1.4 ng/ml (Iwase *et al.*, 2000a). This, however, is not commercially available. An attempt was made to quantify leptin secreted by canine adipocytes from both depots by LINCO. However, levels in the cell culture supernatant were below the level of detection of the available assay system.

A signal for the adipocyte hormones leptin and adiponectin was documented in canine adipocytes of both depots post-differentiation using RT-PCR. Like leptin, adiponectin is also a marker for terminal differentiation in canine adipocytes (Wang *et al.*, 2004), and as such expression was evident post-differentiation in both depots. In contrast to leptin, the adiponectin gene appears to be highly expressed in dog adipocytes as only 26 cycles of amplification were needed to obtain a strong signal; this is in keeping with evidence from other species. Adiponectin is generally a highly expressed gene, and the protein comprises 0.01% of total serum protein in humans (Wolf, 2003).

There was a marked difference in relative levels of adiponectin mRNA in cells of the two depots, where on average, adiponectin gene expression was more than 4-fold higher in cells of the subcutaneous inguinal compared to those of the gonadal depot. This difference in the level of expression between depots has been also documented in humans (Fisher *et al.*, 2002; Hernandez-Morante *et al.*, 2007). Immunoreactive adiponectin was detected in cell culture supernatant of canine adipocytes from both the subcutaneous inguinal and gonadal depots. Thus, these results indicate that in addition to synthesising adiponectin mRNA, canine adipocytes synthesise and subsequently release adiponectin protein. In accordance with data from other species (Fasshauer *et al.*, 2003b; Degawa-Yamauchi *et al.*, 2005), canine adipocytes are therefore a source of the protein, which can thus be considered an adipokine, and in addition to having a local autocrine/paracrine effect, adiponectin enters the circulation to act in an endocrine manner in the dog (Brunson *et al.*, 2007).

It is now evident that obesity is characterised by chronic low-grade inflammation and adipocytes are active participants in the generation of the inflammatory state (Fruhbeck *et al.*, 2001; Fasshauer & Paschke, 2003). A number of adipokines are linked to inflammation and the inflammatory response (Rajala & Scherer, 2003; Trayhurn & Wood, 2004; Trayhurn & Wood, 2005), and as such the expression profile of a series of adipokine genes linked to inflammation was examined by both RT-PCR and qPCR techniques during the differentiation of canine preadipocytes to adipocytes in primary culture. Initially, the expression of a panel of inflammation-related adipokines was examined in cells of the subcutaneous inguinal depot. A signal was obtained in preadipocytes and also late during the differentiation process for all adipokines examined.

PAI-1, an acute-phase protein whose elevated circulating levels are characteristic of obesity has been studied in WAT in relation to its role in thrombosis (Skurk & Hauner, 2004). PAI-1 was expressed both before and after differentiation in the dog. This is in accordance with studies carried out using human cells (Wang *et al.*, 2004). The expression of another acute-phase protein was also examined, this was SAA. SAA has been established has an important risk factor for coronary artery disease and serum concentrations are correlated with BMI, adiposity and adipose tissue SAA mRNA levels (Poitou *et al.*, 2005). Here, in a similar manner to PAI-1, expression of

SAA was detected both pre and post-differentiation in canine adipocytes of the subcutaneous inguinal depot. The expression of VEGF was also examined by RT-PCR and in a similar manner to both PAI-1 and SAA expression was again detected throughout differentiation and maturation of canine adipocytes *in vitro*.

Next, expression and secretion of two major cytokines, TNFα and IL-6 were investigated in canine adipocytes. TNFα has a well-established role in inflammatory responses (Sethi & Hotamisligil, 1999; Qi & Pekala, 2000) and production of the cytokine by human fat cells has been widely documented (Kern *et al.*, 2001; Wang *et al.*, 2004; Wang *et al.*, 2005). As in the present study, TNFα expression levels have been found to decline during adipogenesis (Gerhardt *et al.*, 2001; Wang *et al.*, 2004; Eisele *et al.*, 2005). Here, this effect was documented for both cells of the subcutaneous inguinal and gonadal depots and quantitatively, changes were comparable to those documented for human adipocytes (Wang *et al.*, 2004).

Immunoreactive TNFα protein was detected in cell culture supernatant of canine adipocytes from both depots both pre- and post-differentiation. These results indicate that in a similar manner to adiponectin in the dog, in addition to synthesising TNFα mRNA, canine adipocytes synthesise and subsequently release TNFα protein. In accordance with data from both humans and rodents (Hotamisligil *et al.*, 1993; Kern *et al.*, 2001), canine adipocytes are therefore a source of the protein, which can thus be considered an adipokine. Higher levels of the protein were secreted from cells of the gonadal as opposed to those of the subcutaneous inguinal depot. There is a strong link between visceral adiposity and increased levels of proinflammatory cytokines, and local expression of TNFα is higher in adipose tissue of subjects with visceral obesity and insulin resistance (Kern *et al.*, 2001). Thus, the insulin resistance seen in obese dogs may be related to TNFα levels produced locally by visceral WAT.

IL-6 is an important cytokine produced by many cell types including white adipose tissue (Fried *et al.*, 1998). Serum IL-6 concentrations have been found to be positively correlated with BMI in humans (Mohamed-Ali *et al.*, 2001). IL-6 levels have been found to decline sharply shortly after differentiation of human adipocytes, with levels subsequently reaching pre-induction levels as cells mature (Wang *et al.*, 2004). In the dog, unlike human cells, expression levels of IL-6 decline during

adipocyte differentiation and development. This was documented for cells of both depots. In addition, the initial decline seen immediately post-induction in humans is seen later in the dog, from *day 3* post-induction, and levels remain low throughout adipocyte differentiation and development.

Immunoreactive IL-6 was found in medium collected from cells of both depots both pre- and post-induction. Thus, this cytokine, in a manner similar to adiponectin and TNFα, can be classified as an adipokine in the dog. Levels of IL-6 protein detected in medium collected from cells of both depots declined significantly during differentiation. For cells of the subcutaneous inguinal depot this decline was slightly later than that detected for IL-6 mRNA, from *day 6* post-induction. Levels of IL-6 detected in media collected from cells of the gonadal depot at both *day 0* and *10* post-induction were substantially higher than in conditioned media from cells of the subcutaneous inguinal depot at the same time-points. This difference in secretion levels by different depots has also been documented for human adipocytes in culture (Fried *et al.*, 1998), and as such, like TNFα may play an important role in the development of metabolic derangements in obesity in the dog.

Obesity has been associated with macrophage infiltration and accumulation in WAT (Weisberg *et al.*, 2003). MCP-1 is responsible for the attraction of macrophages and T-lymphocytes to sites of inflammation and circulating levels of MCP-1 have been shown to be significantly raised in obesity (Kim *et al.*, 2006). Like IL-6, MCP-1 is produced by a variety of cells, including human adipocytes (Rollins, 1997; Gerhardt *et al.*, 2001) and its production has been linked to inflammatory stimuli (Rollins, 1997). In addition, MCP-1 has been implicated in the development of insulin resistance and impaired differentiation of murine adipocytes *in vitro* (Sartipy & Loskutoff, 2003). In dogs, MCP-1 is expressed both before and after differentiation in cells of both the subcutaneous inguinal and gonadal depots. Levels of expression then appear to fall in cells of the subcutaneous inguinal depot as cells mature. This is unlike what is seen in human adipocytes in culture, where MCP-1 gene expression levels either exhibit no change (Gerhardt *et al.*, 2001), or increase, as cells mature (Wang *et al.*, 2004). Cells from the gonadal depot appeared to resemble more closely the situation seen in human cells, were semi-quantitatively there was little evidence

of change in levels of MCP-1 gene expression. However, the decrease in level of MCP-1 gene expression was again documented quantitatively as cells matured.

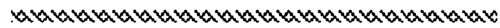
MCP-1 protein was also detected in cell culture supernatant from cells of both depots both pre- and post-differentiation. Thus, like adiponectin, TNFα and IL-6, MCP-1 can also be characterised as an adipokine in the dog and as such may contribute to circulating levels seen in obesity with expansion of WAT mass. Similar to mRNA levels, MCP-1 protein concentration in the subcutaneous inguinal depot appeared to be greater pre- than post-differentiation. However, this is unlike the changes observed in the gonadal depot, and what has been documented in human adipocytes in culture where MCP-1 protein levels in the media increase as cells mature (Wang *et al.*, 2004). Furthermore, the amount of protein secreted by canine adipocytes was considerably greater than that reported by human adipocytes in culture (Gerhardt *et al.*, 2001; Wang *et al.*, 2004), nearly ten-fold higher at their lowest level (*day 10* post-differentiation) in the dog. Thus, although numbers of cells in each cell culture system were not documented, quantitatively this adipokine may play a key role in adipose tissue and whole body metabolism in the dog, again with a strong link to visceral adiposity.

NGF mRNA levels in canine preadipocytes were higher than in mature adipocytes of both depots examined (Fig 4.17B and 4.18B) and this is in accordance with data for both murine and human adipocytes *in vitro* (Peeraully *et al.*, 2004; Wang *et al.*, 2004). Immunoreactive NGF protein was detected in cell culture supernatant of cells of the subcutaneous inguinal depot both pre and post-differentiation. Thus this important target-derived neurotrophin can also be defined as an adipokine in the dog where release appeared to mirror closely changes in mRNA levels and it may therefore play an important part in the development and survival of sympathetic neurones in canine WAT.

In summary, this chapter has examined the differentiation and development of canine adipocytes from two different depots (visceral and subcutaneous) in culture. Cells have been characterised not only histologically but also through the expression of key molecular markers. The expression of adipokine genes has been established both pre- and post-differentiation with proinflammatory adipokines being expressed early

on in the differentiation process in the dog with levels of expression decreasing as cells mature. Importantly, canine adipocytes have been shown to actively secrete key adipokines making them a valuable tool for the investigation of adipocyte biology in terms of responses to the inflammatory stimuli and agents which may improve proinflammatory states and insulin sensitivity.

Chapter 5



Adipokine expression and secretion by canine adipocytes in primary culture: Regulation by the inflammatory mediators TNFα, IL-6 and LPS

5.1 Introduction

In Chapter 4, a robust method of canine primary adipocyte cell culture was outlined where cells from both the subcutaneous inguinal and gonadal depot were successfully induced to differentiate into mature adipocytes *in vitro*. This was characterised by histological changes, including the accumulation of intracellular lipid droplets, which were identified as such using the lipid specific stain Oil red O. The system was further validated by the expression and secretion of key molecular markers, such as the adipocyte hormone adiponectin, by cells of both depots. It was also established that canine adipocytes in culture express and secrete key adipokines including IL-6 and TNFα. Studies in this chapter focused on pro-inflammatory agents and their role in the regulation of adipokine gene expression and secretion by canine adipocytes.

A number of adipokines have been linked to inflammation and the inflammatory response (Coppack, 2001; Rajala & Scherer, 2003; Trayhurn & Wood, 2004), and these include cytokines such as IL-6 and TNFα and chemokines such as MCP-1 and MIF. Leptin itself has also been linked to inflammation (Sarraf *et al.*, 1997; Faggioni *et al.*, 1998), whereas the other major adipocyte hormone, adiponectin, has an anti-inflammatory effect (Ouchi *et al.*, 1999). The pro-inflammatory cytokine TNFα plays an extensive role in adipose tissue function, exerting a range of actions such as the stimulation of lipolysis (Ryden *et al.*, 2004) and the induction of apoptosis (Prins *et al.*, 1997). In addition, TNFα has been reported to increase the expression of several adipokines; for example, IL-6 (Fasshauer *et al.*, 2003a; Wang *et al.*, 2004), and MCP-1 (Gerhardt *et al.*, 2001; Wang *et al.*, 2004), whereas leptin and adiponectin expression is inhibited by TNFα (Wang *et al.*, 2005).

As discussed in Chapter 1, TNFα has been shown to be synthesised and secreted by human adipocytes (Kern *et al.*, 1995) and importantly, TNFα has a substantial effect on its own level of expression in human adipocytes (Wang *et al.*, 2005). Furthermore, in humans, a strong link between TNFα and insulin resistance in obesity has been established (Hotamisligil, 2003) and the production of the cytokine has been shown to increase in rodent models of obesity (Hotamisligil *et al.*, 1993).

IL-6 is another pro-inflammatory cytokine secreted by adipocytes in culture (Fried *et al.*, 1998). Studies have indicated that up to a third of circulating levels of IL-6 are derived from adipose tissue, the production of which increases with obesity (Mohamed-Ali *et al.*, 1997). Few studies have assessed the effect of IL-6 on adipocyte metabolism (Path *et al.*, 2001); however, there is evidence that IL-6 has a strong autocrine effect on its own level of expression and secretion in murine adipocytes (Lagathu *et al.*, 2003) and is also thought to play a key role in the induction of insulin resistance in murine adipocyte cell lines *in vitro* (Lagathu *et al.*, 2003; Rotter *et al.*, 2003). In chapter 4, it was established that IL-6 can also be considered an adipokine in the dog, being actively secreted by adipocytes of both visceral and subcutaneous depots during differentiation. The role of IL-6 in terms of inflammation and regulation of secretion of other adipokines in canine adipocytes remains to be investigated.

Factors which regulate the secretion of cytokines by WAT include inflammatory stimuli such as lipopolysaccharide (LPS) (Coppack, 2001). LPS is a major constituent of the membrane of Gram-negative bacteria, and as such is responsible for the activation of the innate immune pathway via toll-like receptors (TLRs). These are important in terms of antigen recognition allowing rapid and effective response to infection (Kaisho & Akira, 2002). Recent studies have reported the expression of TLR-2 and -4 in 3T3-L1 adipocytes. In those studies, LPS has been shown to signal through TLR-4 and induce both IL-6 and TNFα secretion (Lin *et al.*, 2000). LPS has been shown to strongly upregulate both expression and secretion of adipokines such as NGF (Peeraully *et al.*, 2004) and haptoglobin (Chiellini *et al.*, 2002).

Earlier studies into adipokine expression and secretion in canine adipocytes allowed the time-point of stable expression of key adipokine genes to be identified (between *days 10* and *15* post-differentiation). This formed the basis for subsequent experiments outlined in this chapter where the effects of the proinflammatory agents LPS, TNFα and IL-6 on the expression of adipokines in differentiated canine adipocytes were investigated. The adipokines studied include leptin, adiponectin, IL-6, MCP-1, NGF and TNFα itself. NGF has been described as an adipokine in mice (Peeraully *et al.*, 2004) and humans (Wang *et al.*, 2004) and as outlined in the previous chapter; this important target-derived neurotrophin is also an adipokine in

the dog. Thus, one of the aims of these investigations was to establish whether NGF is also linked to the inflammatory response in canine WAT.

5.2 Methods

5.2.1 Cell culture

White adipose tissue from both the subcutaneous inguinal and gonadal depots of SBT dogs were harvested as described in sections 2.1.1 and 2.1.2. Cells were cultured as described in section 2.2 at 37°C in a humidified atmosphere of 5% CO_2/95% air. Twenty four hours after they were inoculated, cells from the gonadal depot were washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells from the subcutaneous inguinal depot had their inoculation medium changed 48 h post-inoculation and 24 h later (at 72 h post-inoculation) were washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells of both depots were exposed to the adipogenic medium for 48 h. This was replaced by feeding medium, which was renewed every 48 h.

The cells were pre-incubated 12 days after differentiation for 24 h with FCS-free feeding medium. Cells were then treated with FCS-free feeding media containing each of the specific agents; the control cells for each study had their pre-incubation media renewed. In preliminary studies, cells from the subcutaneous inguinal depot were treated with human recombinant TNFα at doses of 5 and 50 ng/ml. Subsequently, cells from both depots were treated with canine recombinant TNFα at the same doses used for human recombinant TNFα treatment studies. Cells were harvested at the time of treatment and at 2 and 24 h post-treatment as described in section 2.3.5. Only cells from the subcutaneous inguinal depot (see section 4.4), were treated with IL-6 at doses of 1 and 25 ng/ml, and LPS at doses of 10 and 100 ng/ml. Cells were harvested at the time of treatment and at 4 and 24 h post-treatment as described in section 2.3.5. Either 7 or 8 individual wells in cell culture plates were treated with each agent, or used as controls.

5.2.2 qPCR and ELISA

RNA was extracted from cultured cells using Tri-Reagent as described in section 2.3.1. RNA, which was to be analysed by qPCR, was subsequently treated using a DNA-free kit to minimise levels of genomic DNA contamination (section 2.3.2). Once extracted, the total RNA was quantified using a BioPhotometer and reverse transcribed using the Reverse-iT First Strand Synthesis kit (sections 2.3.3 and 2.4.1). The cDNA was then analysed by real-time PCR (qPCR) using the qPCR Core Kit and an Mx3005P real-time machine (section 2.5.3 and 2.5.4).

PCR products were purified using a NuceloSpin Extract 2-in-1 kit and sequenced commercially (see section 2.4.4). All qPCR data was analysed using MxPro-Mx3005P software. Conditioned media samples were analysed for NGF protein concentration using the NGF E_{max} Immunoassay System (section 2.6.1). TNFα, IL-6 and MCP-1 protein concentration were analysed using ELISA kits from R&D Systems (see section 2.6.2). Adiponectin protein concentration in conditioned media was assayed commercially by LINCO (section 2.6.3).

5.3 Results

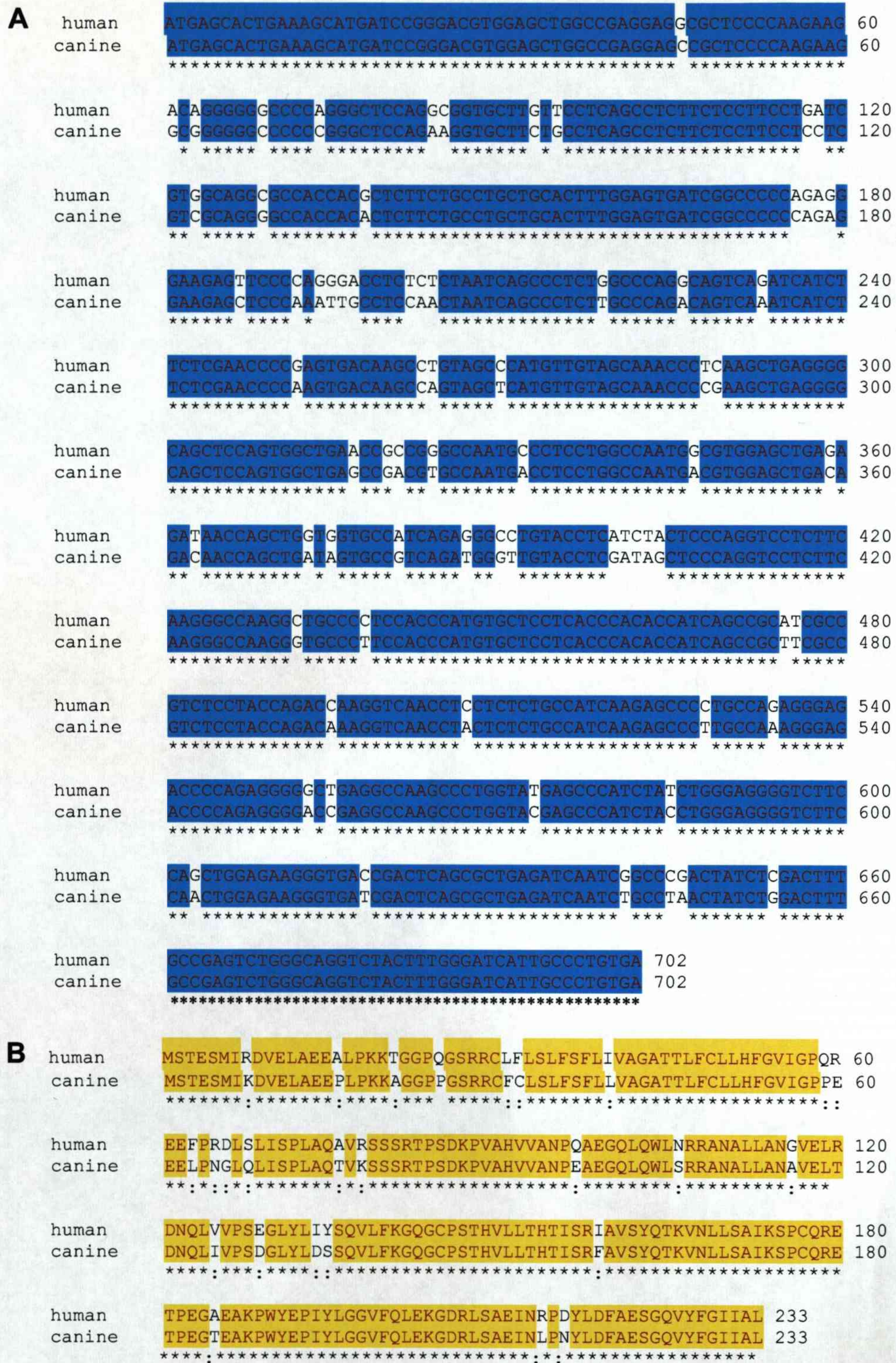
5.3.1 Regulation of adipokine expression in canine adipocytes: Response to human recombinant TNFα

Initial studies examined the integrated effect of the pleiotropic pro-inflammatory cytokine TNFα on the expression of genes encoding inflammation-related adipokines in differentiated canine adipocytes. A dose response experiment was carried out where cells of the subcutaneous inguinal depot were incubated with a low (5 ng/ml) and high (50 ng/ml) dose of human recombinant TNFα for periods of 2 and 24 h. From analysis carried out using NCBI and EMBL-EBI search engines, human and canine TNFα share a high level of homology, both at the mRNA (90%) and protein (88%) level (Fig. 5.1). Thus, as expected, treatment of canine adipocytes with human recombinant TNFα resulted in an increase in mRNA level for inflammation-related adipokines and TNFα itself (Fig. 5.2).

This response, however, was muted in comparison to that reported for TNFα treatment of human adipocytes (Wang *et al.*, 2004; Wang *et al.*, 2005). An example is the effect of human TNFα on its own level of expression, where a response of nearly 2000-fold increase in TNFα expression is reported with TNFα treatment at 2 h post-treatment with 25 ng/ml of human recombinant TNFα (Wang *et al.*, 2005). In the dog, TNFα mRNA levels were significantly raised at both 2 and 24 h post treatment. However, the maximal effect of human recombinant TNFα treatment on canine adipocytes was just over 3-fold at 24 h post-treatment with the high dose of TNFα (Fig. 5.2A).

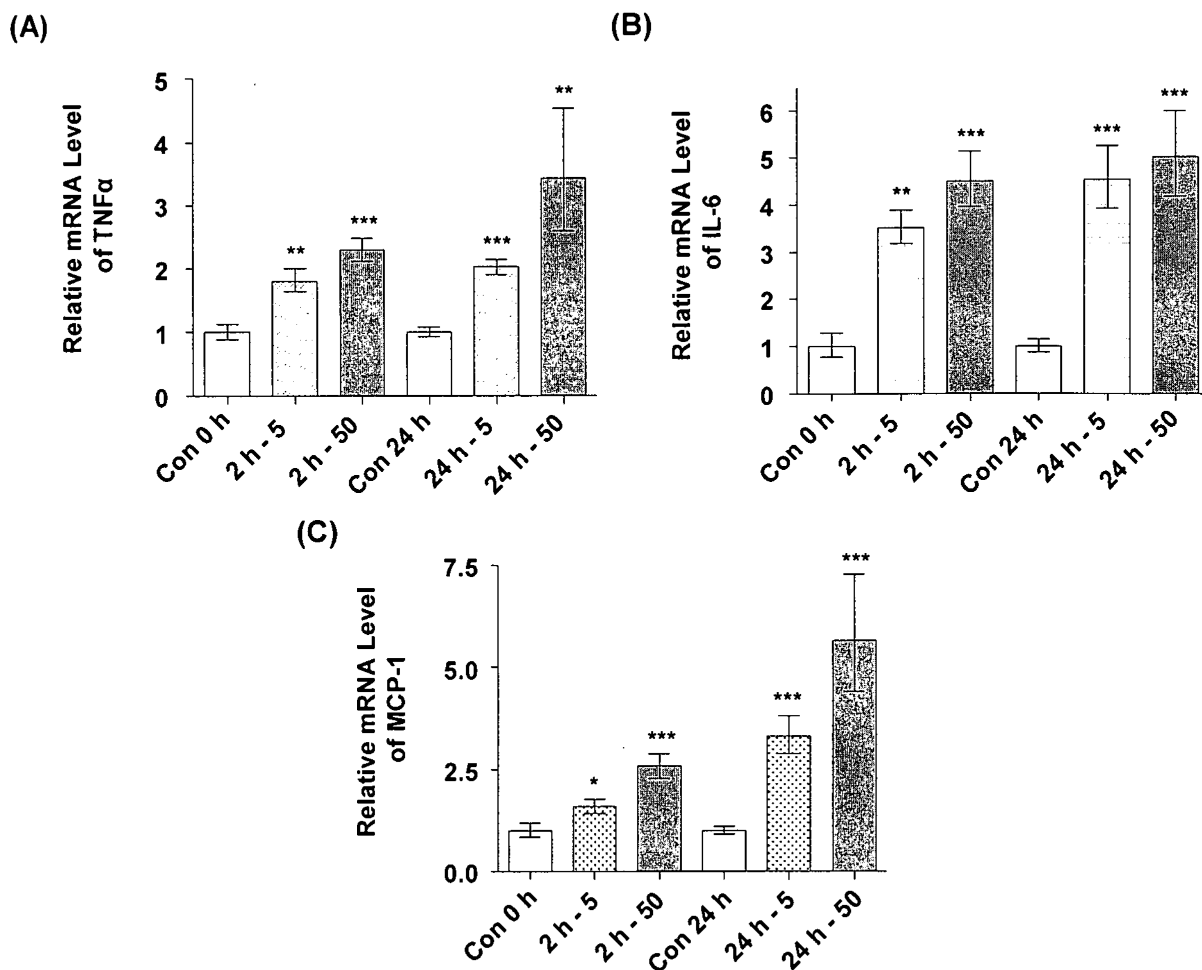
The same muted response was also observed for other adipokines such as IL-6 and MCP-1. IL-6 mRNA levels in human adipocytes were upregulated by nearly 50-fold at 2 h post-treatment (Wang *et al.*, 2005), whereas in the dog, although a significant increase in IL-6 mRNA levels was observed at both 2 and 24 h with low and high dose treatment, mRNA levels only reached a peak of 5-fold at 24 h post-treatment with the high dose of TNFα (Fig. 5.2B). In terms of MCP-1, in human adipocytes, a maximal effect has been reported of close to 25-fold at 24 h post-treatment with 25 ng/ml TNFα (Wang *et al.*, 2005), whereas in the dog, despite a significant upregulation in MCP-1 mRNA levels at 2 and 24 h (with the effect at 2 h being dose dependent), levels peaked at around 5-fold at 24 h post-treatment with the high dose (50 ng/ml) of TNFα (Fig. 5.2B).

Figure 5.1 Human and canine TNFα coding sequence (mRNA) and amino acid sequence alignment



The mRNA coding sequence and amino acid sequence for TNFα of human and dog were obtained from the NCBI database. Sequences were aligned using ClustalW software (http://www.ebi.ac.uk/clustalw). In (A), blue blocks indicate matching nucleotides and in (B), yellow blocks indicate matching amino acids.

Figure 5.2 Regulation of adipokine gene expression in canine adipocytes by human recombinant TNFα



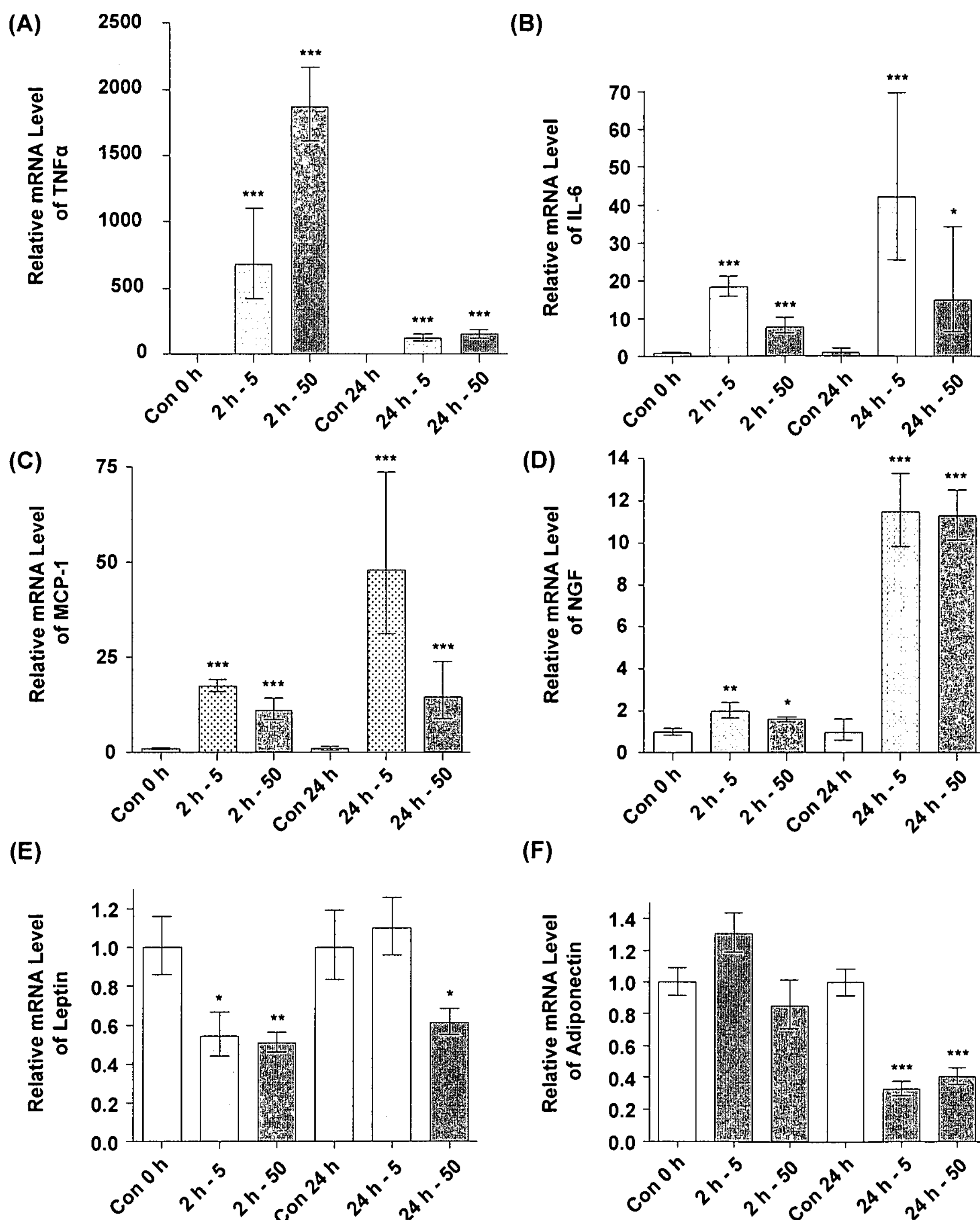
Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing human recombinant TNFα for 2 and 24 h. Adipokine mRNA level was measured by qPCR and normalised to canine β-actin relative to the untreated control group (=1). Results are means ± SE (bars) for groups of 8. (A), TNFα; (B), IL-6; (C), MCP-1. -5, 5 ng/ml TNFα; -50, 50 ng/ml TNFα. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls.

5.3.2 Regulation of adipokine expression in canine adipocytes: Response to canine recombinant TNFα

Due to the muted effects observed when canine adipocytes were treated with human recombinant TNFα, the next set of experiments set out to investigate whether such effects were due to species differences between dogs and humans in the response to TNFα or, due to the potency of the treatment agent itself. Despite the high level of homology between human and canine TNFα, amino acid differences and subsequent effects of this on protein structure may have rendered the human protein less effective at inducing a response in canine adipocytes. The experiment was carried out at same doses and time-points as outlined in section 5.3.1, however, this time using canine recombinant TNFα to treat cells of both depots in order to also identify any depot differences in the effect of TNFα on canine adipocytes.

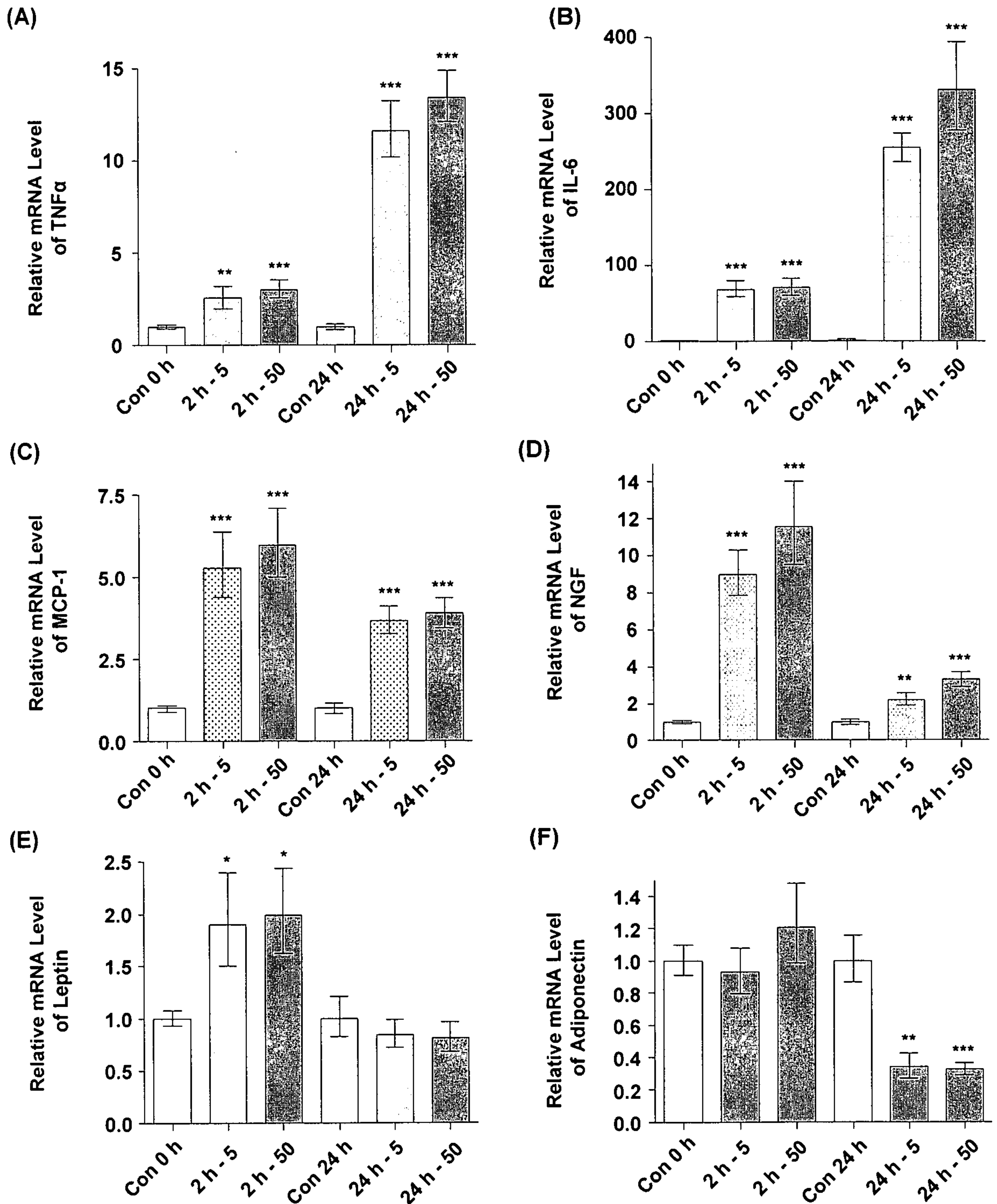
It was evident that the amino acid differences between human and canine TNFα were significant in terms of the biological activity of the protein, where canine recombinant TNFα induced substantial and rapid increases of >600-fold with low dose TNFα (5 ng/ml) treatment on its own level of mRNA as early as 2 h post-treatment in the cells of the subcutaneous inguinal depot (Fig. 5.3A). A peak response of a >1800-fold increase in TNFα mRNA occurred with high dose treatment (50 ng/ml) at 2 h post-treatment. This is similar, in both magnitude and time, to what has been reported in human adipocytes with TNFα treatment (Wang *et al.*, 2005). TNFα mRNA levels then fell precipitously by 24 h post-treatment, but were still, on average 130-fold higher than in the controls (Fig. 5.3A). TNFα also had a potent autocrine effect on its own level of expression in cells of the gonadal depot. However, this was at best 100 times less than that observed for cells of the subcutaneous inguinal depot (Fig. 5.4A).

Figure 5.3 Regulation of adipokine gene expression in canine subcutaneous inguinal adipocytes by canine recombinant TNFα



Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing canine recombinant TNFα for 2 and 24 h at low -5, 5 ng/ml TNFα; and high -50, 50 ng/ml TNFα. Adipokine mRNA level was measured by qPCR and normalised to canine RNAPolIIa relative to the untreated control group (=1). Results are means ± SE (bars) for groups of 8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Leptin; (F), Adiponectin. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with controls.

Figure 5.4 Regulation of adipokine gene expression in canine gonadal adipocytes by canine recombinant TNFα



Differentiated canine adipocytes from the gonadal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing canine recombinant TNFα for 2 and 24 h at low -5, 5 ng/ml TNFα; and high -50, 50 ng/ml TNFα. Adipokine mRNA level was measured by qPCR and normalised to canine RNAPolIIa relative to the untreated control group (=1). Results are means ± SE (bars) for groups of 8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Leptin; (F), Adiponectin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls at the same time-points.

IL-6 mRNA in cells of both depots reached a peak at 24 h post treatment with a 44-fold increase observed in cells of the subcutaneous inguinal depot (Fig. 5.3B). The response by cells of the gonadal depot, however, was even more marked with levels being >80% higher than those observed for cells of the subcutaneous inguinal depot, with a 330-fold increase in mRNA level with the high dose treatment at 24 h (Fig. 5.4B). MCP-1 mRNA levels were substantially higher in cells of the subcutaneous inguinal depot compared to those of the gonadal depot, reaching a peak at 24 h post-treatment of 47-fold (Fig. 5.3C). This is in contrast to cells of the gonadal depot where a modest 6-fold increase in mRNA levels was reached at 2 h post-treatment (Fig. 5.4C). The peak in NGF mRNA level with TNFα treatment was seen later in cells of the subcutaneous inguinal depot than by cells of the gonadal depot. The magnitude of the effect of TNFα in cells of both depots was, however, similar at between 10- and 12-fold (Fig. 5.3D and 5.4D).

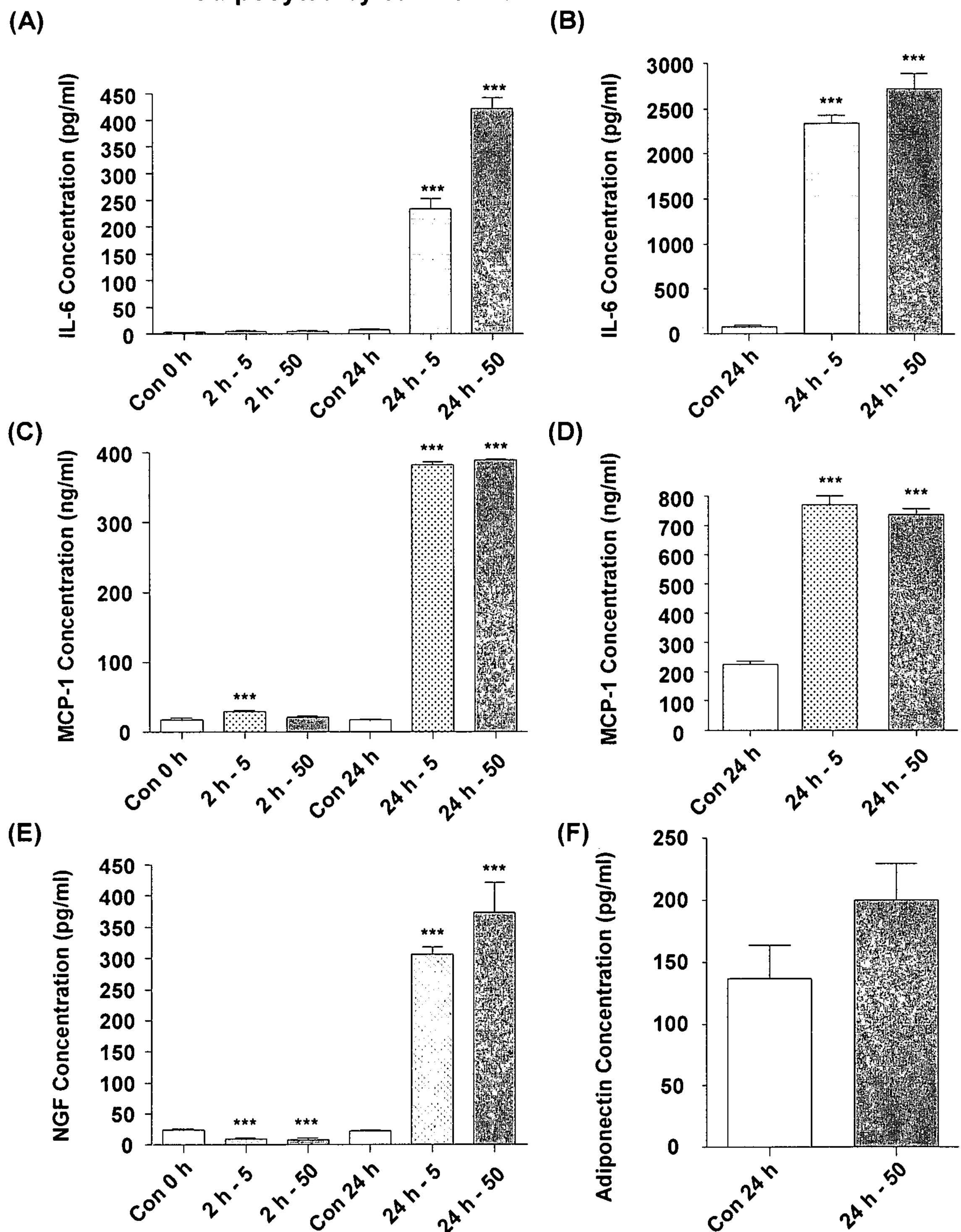
A significant reduction in leptin mRNA levels was observed both at 2 and 24 h of 46 and 39% respectively, post-treatment with canine recombinant TNFα in cells of the subcutaneous inguinal depot (Fig. 5.3E). In contrast, cells of the gonadal depot had a modest but significant increase of nearly 2-fold in leptin mRNA levels at 2 h post-treatment with both low and high dose TNFα. However, this effect was no longer present by 24 h (Fig. 5.4E). Unlike leptin, levels of adiponectin mRNA were significantly reduced to a similar extent (by approximately 60%) in cells of both depots at the end of the 24 h treatment period (Fig. 5.3F and 5.4F).

5.3.3 Regulation of adipokine secretion by canine adipocytes: Response to canine recombinant TNFα

The secretion into the medium of a selected group of adipokines was next examined during the treatment of canine adipocytes with canine recombinant TNFα using ELISA. The secretion of inflammation-related adipokines such as IL-6, MCP-1 and NGF by canine adipocytes was substantially increased by TNFα treatment, the effect being more marked at 24 h post-treatment (Fig. 5.5). The secretion of IL-6 into the medium by cells of the subcutaneous inguinal depot was increased by 33-fold by treatment with low dose TNFα at 24 h. In this case, secretion was dose dependent, with the level of IL-6 secreted into the medium increasing by 60-fold with high dose TNFα treatment at 24 h (Fig. 5.5A). Cells of the gonadal depot also responded in a similar manner, although the effect was not as marked, with a 30-fold increase in the level of protein secreted into the medium at 24 h with the high dose treatment (Fig. 5.5B).

There was a modest but nevertheless significant increase in MCP-1 protein secreted into the medium by canine adipocytes at 2 h post-treatment with the low dose of canine recombinant TNFα (Fig. 5.5C). MCP-1 secretion by cells of both depots was, however, markedly increased by 24 h post-treatment; for cells of the subcutaneous inguinal depot, the concentration of MCP-1 in the medium increased by 23-fold (Fig. 5.5C), whereas for cells of the gonadal depot this effect was more muted at only just over 3-fold (Fig. 5.5D). The effect of TNFα treatment on NGF secretion was documented for cells of the subcutaneous inguinal depot. Here, unlike other adipokines, the level of NGF protein in the medium fell significantly; by on average 38% at 2 h post-treatment with both low and high doses of TNFα (Fig. 5.5E). This effect, however, did not persist with levels rising by 15-fold at 24 h post-treatment. Unlike other adipokines, TNFα treatment had no effect on the amount of adiponectin secreted into the medium (Fig. 5.5F).

Figure 5.5 Regulation of adipokine protein secretion in canine adipocytes by canine recombinant TNFα



Differentiated canine adipocytes from the subcutaneous inguinal and gonadal depots at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing canine recombinant TNFα for 2 and 24 h at low -5, 5 ng/ml TNFα; and high -50, 50 ng/ml TNFα. Adipokine concentration was measured by ELISA normalised to control at the same time-point. Results are means ± SE (bars) for groups of 3-8. (A), IL-6 sc; (B), IL-6 gon; (C), MCP-1 sc; (D), MCP-1 gon; (E), NGF sc; (F), Adiponectin sc. Sc, subcutaneous inguinal depot; gon, gonadal depot. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls.

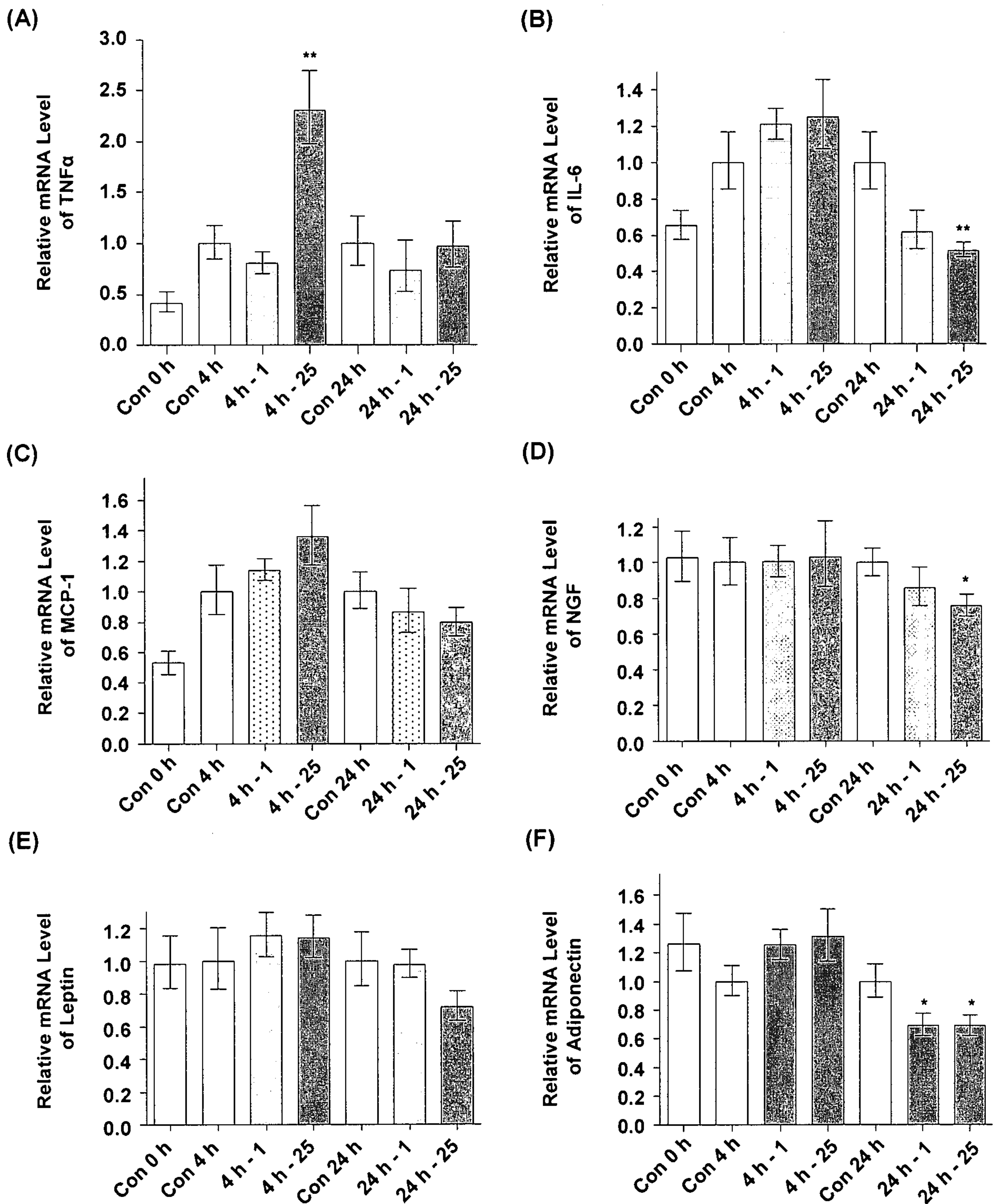
5.3.4 Regulation of adipokine expression and secretion in canine adipocytes: Response to IL-6

In the next experiments, cells from the subcutaneous inguinal depot were treated with the pro-inflammatory cytokine IL-6. Canine recombinant IL-6 was available commercially from R&D Systems and this was the treatment agent of choice. In addition, there is less similarity between human and canine IL-6 (76% CDS similarity but only 57% similarity in terms of amino acid sequence) than between human and canine TNFα. Cells were sampled at 4 and 24 h, so initially 2 h later than cells treated with TNFα as studies have shown that optimaly results in terms of adipocyte metabolism can be detected with IL-6 treatment from this time-point (Greenberg *et al.*, 1992). Logistically, it was possible to obtain a control sample at the first time-point (4 h post-treatment), in addition to that at time of treatment (Con 0 h).

In contrast to TNFα, the effect of IL-6 treatment on canine adipocytes in terms of adipokine gene expression was muted (Fig. 5.6). Three distinct responses to the cytokine were observed. There was a moderate, but nevertheless significant, upregulation of TNFα mRNA of 2.3-fold at 4 h post-treatment with the high dose of IL-6 (Fig. 5.6A). However, there was a significant reduction in mRNA of IL-6 itself and NGF, of 48 and 25% respectively, with high dose IL-6 at 24 h post treatment (Fig. 5.6B and D). In addition, adiponectin mRNA was significantly reduced with both low and high dose IL-6 at 24 h post-treatment by just over 30% (Fig. 5.6F). No effect was observed in terms of MCP-1 and leptin gene expression (Fig. 5.6C and E).

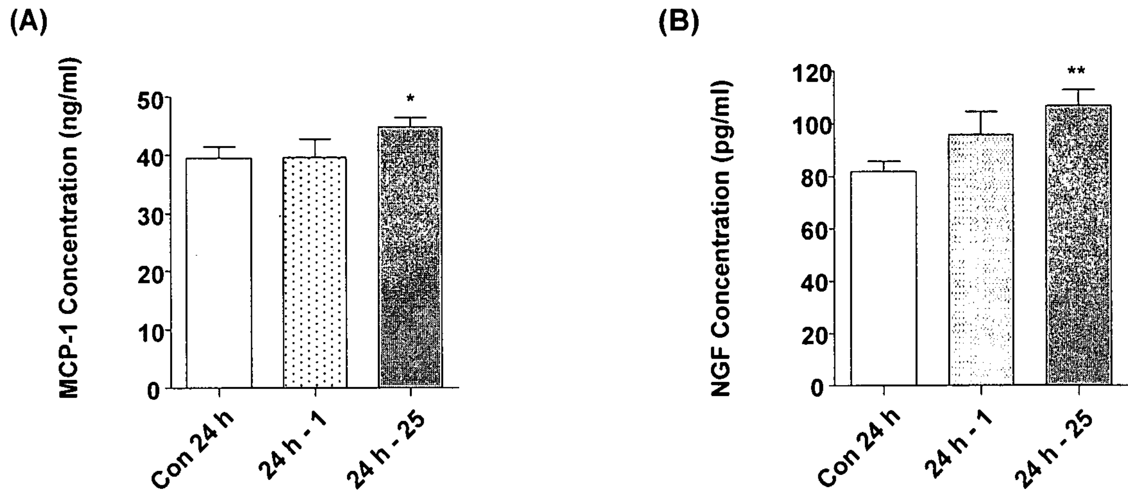
The effect of IL-6 on adipokine secretion by canine adipocytes was also investigated. Due to its muted effects on adipokine expression, only a restricted number of adipokines were investigated in terms of secretion by canine adipocytes in response to treatment with recombinant IL-6. Treatment of cells with IL-6 had a small, but significant, effect on MCP-1 and NGF secretion of 24 and 11% respectively, into the medium at 24 h post-treatment with the high dose of IL-6 (Fig. 5.7).

Figure 5.6 Regulation of adipokine gene expression in canine adipocytes by canine recombinant IL-6



Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing canine recombinant IL-6 for 2 and 24 h at low -1, 1 ng/ml IL-6; and high -25, 25 ng/ml IL-6. Adipokine mRNA level was measured by qPCR and normalised to canine RNAPolIIa relative to the untreated control group (=1). Results are means ± SE (bars) for groups of 8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Leptin; (F), Adiponectin. *$P < 0.05$, **$P < 0.01$ compared with controls at 4 and 24 h.

Figure 5.7 Regulation of adipokine protein secretion in canine adipocytes by canine recombinant IL-6



Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing canine recombinant IL-6 for 4 and 24 h at low -1, 1 ng/ml IL-6; and high -25, 25 ng/ml IL-6. Adipokine concentration was measured by ELISA normalised to control at the same time-point. Results are means ± SE (bars) for groups of 6-7. (A), MCP-1; (B), NGF. $*P < 0.05$, $**P < 0.01$ compared with controls.

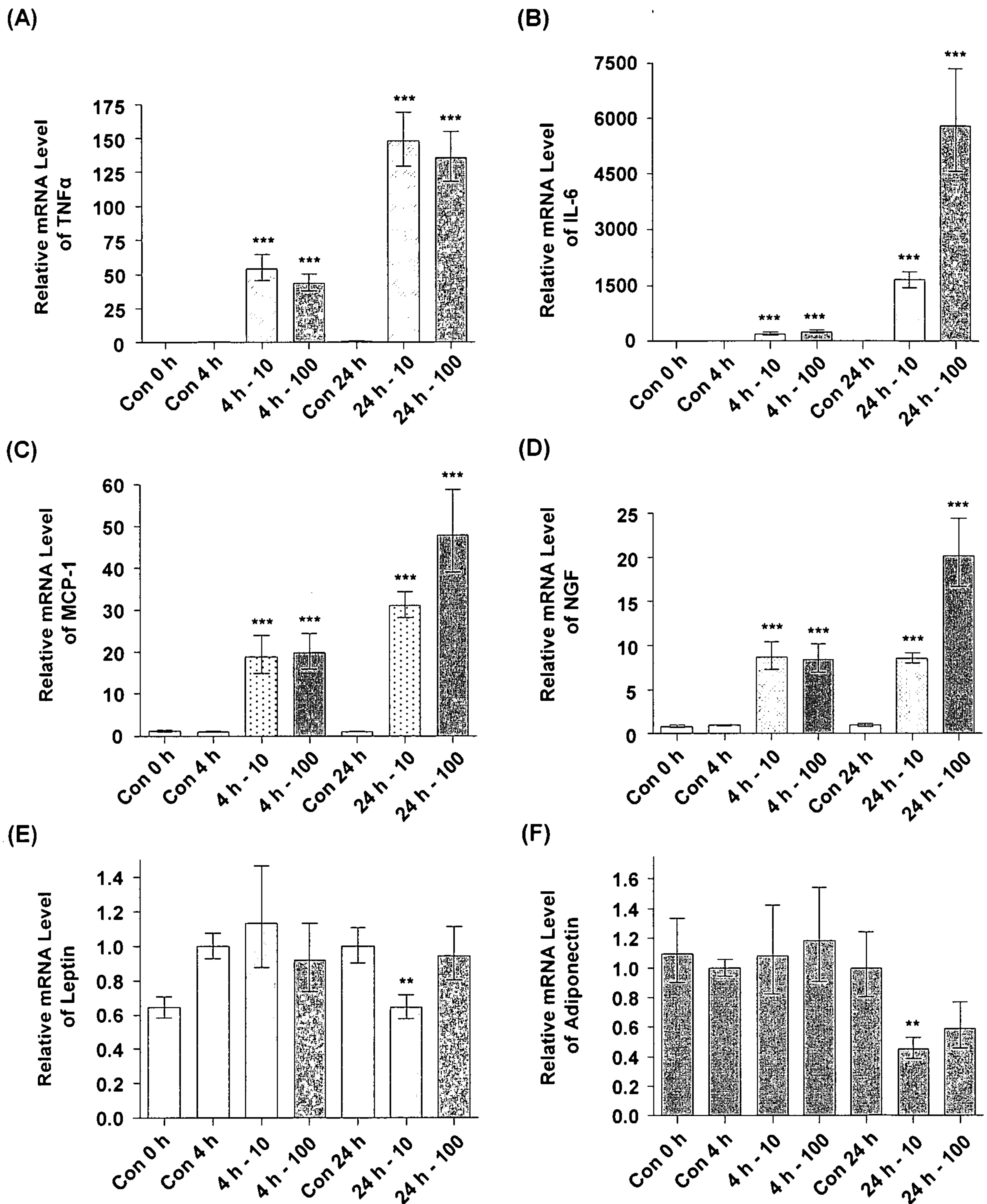
5.3.5 Regulation of adipokine expression and secretion in canine adipocytes: Response to LPS

The next experiments examined the effect of the pro-inflammatory agent LPS on canine adipocytes of the subcutaneous inguinal depot. The response in terms of pro-inflammatory adipokine expression and secretion was much more marked than for IL-6 treatment (Fig. 5.8). Here, two distinct responses to the pro-inflammatory agent were observed. Treatment with LPS resulted in a marked increase in mRNA level for TNFα, IL-6, MCP-1 and NGF. An increase of between 40 and 55-fold was seen in the case of TNFα at 4 h of treatment with LPS. The effect on TNFα mRNA was even more marked at 24 h post treatment reaching >145 and >135-fold with low and high doses of LPS, respectively (Fig. 5.8A).

The effect of LPS on IL-6 mRNA was even more marked than that observed for TNFα, with IL-6 mRNA increasing by 185 and >230-fold by 4 h of treatment with LPS. Remarkably, the effect of LPS treatment on IL-6 mRNA levels was even more substantial at 24 h post-treatment, where levels were raised in a dose dependent manner by nearly 2,000 and 6,000-fold with low and high doses of LPS respectively (Fig. 5.8B). MCP-1 mRNA levels were raised by nearly 20-fold at 4 h of treatment with low and high doses of LPS. The increase in MCP-1 mRNA was even more substantial at 24 h post-treatment with >30-fold and nearly 50-fold upregulation in MCP-1 mRNA with low and high doses of LPS treatment respectively (Fig. 5.8C). The effect of LPS on NGF mRNA was also documented as early as 4 h with an average increase of 8.5-fold. At 24 h, with the low dose of LPS the increase in NGF mRNA was similar to that seen at 4 h. In contrast, a significant dose effect was seen with the high dose of LPS, where NGF mRNA was increased by 20-fold at 24 h post-treatment (Fig. 5.8D).

There was a significant reduction in mRNA level for both leptin (36%) and adiponectin (55%) at 24 h of treatment with the low dose of LPS. No effect was observed for high dose LPS treatment at the same time-point (Fig. 5.8E and F).

Figure 5.8 Regulation of adipokine gene expression in canine adipocytes by LPS

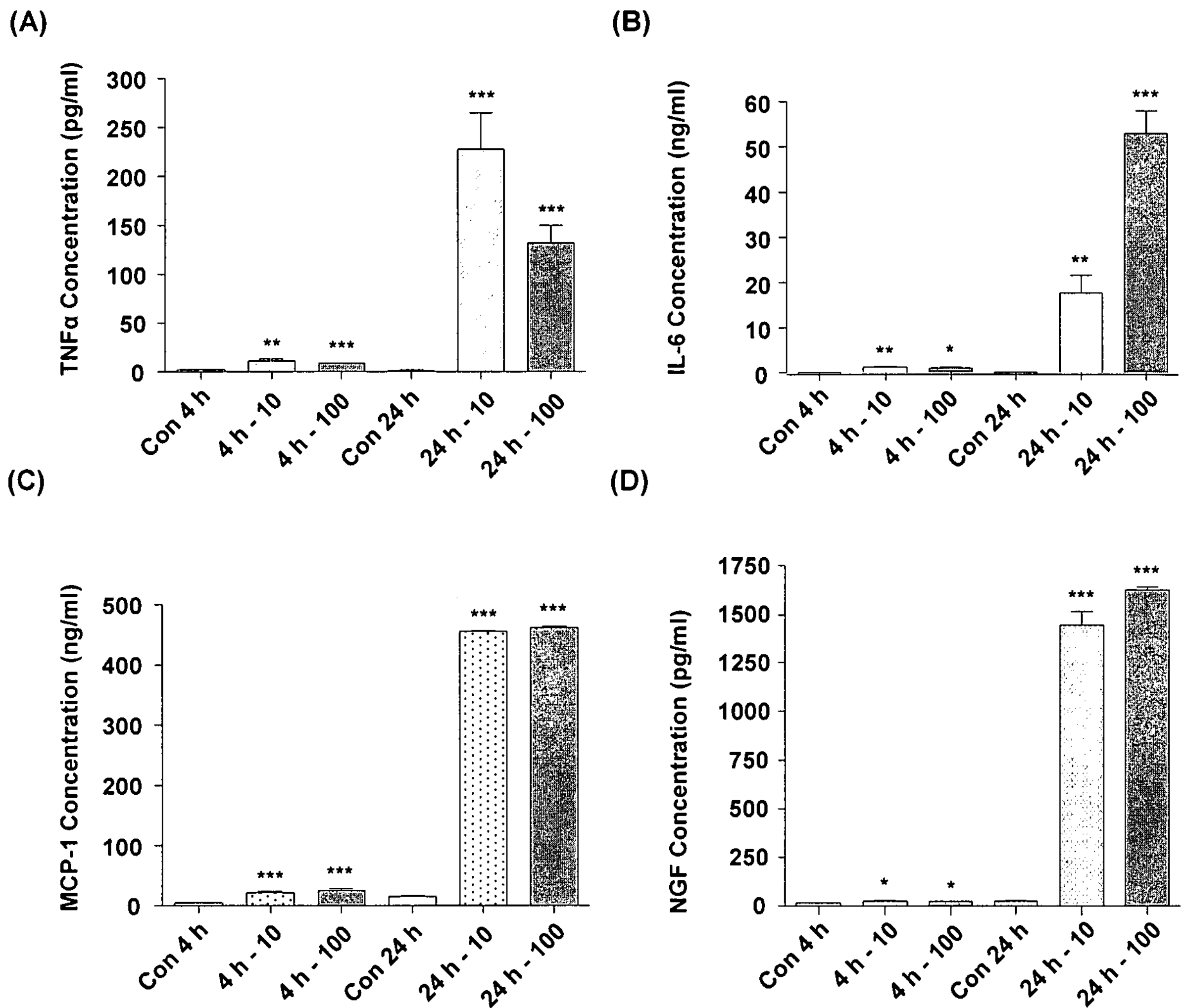


Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing LPS for 4 and 24 h at low -10, 10 ng/ml LPS; and high -100, 100 ng/ml LPS. Adipokine mRNA level was measured by qPCR and normalised to canine RNAPolIIa relative to the untreated control group (=1). Results are means ± SE (bars) for groups of 6-7. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Leptin; (F), Adiponectin. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with controls at 4 and 24 h.

The secretion of a select group of adipokines into the medium during treatment of cells with LPS was next examined using ELISA. Treatment of cells with LPS induced a significant increase in TNFα, IL-6, MCP-1 and NGF secretion with both low and high doses of LPS as early as 4 h of treatment (Fig. 5.9). TNFα secretion was increased by 8 and 6-fold by low and high doses of LPS at 4 h. The effect of LPS on TNFα secretion by canine adipocytes was substantially increased at 24 h post-treatment. A peak of 22-fold in TNFα secretion was observed with the low dose of LPS (Fig. 5.9A).

IL-6 concentrations in the medium were also markedly elevated by 14-fold at 24 h of treatment, and in contrast to TNFα, this effect was dose dependent, with levels rising by >55-fold with the high dose of LPS (Fig. 5.9B). MCP-1 protein levels in cell culture supernatant were also markedly elevated by an average of 40-fold at 24 h of treatment (Fig. 5.9C). In a similar manner to IL-6, NGF protein secretion into the medium by canine adipocytes at 24 h was elevated significantly in a dose dependent manner by 58 and 74-fold with low and high doses of LPS respectively (Fig. 5.9D).

Figure 5.9 Regulation of adipokine protein secretion in canine adipocytes by LPS



Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing LPS for 4 and 24 h at low -10, 10 ng/ml LPS; and high -100, 100 ng/ml LPS. Adipokine concentration was measured by ELISA normalised to control at the same time-point. Results are means ± SE (bars) for groups of 6-7. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls.

5.4 Discussion

The studies described in this chapter have used the canine preadipocyte system outlined in Chapter 4 to examine the effect of the pro-inflammatory agents TNFα, IL-6 and LPS on adipokine expression and secretion by canine adipocytes. A detailed time-course study on the effect of TNFα on human adipocytes demonstrated that substantial effects of TNFα on adipokine expression and secretion can be detected as early as 2 h of treatment with TNFα at doses of 25 ng/ml (Wang *et al.*, 2005). Thus, the 2 h time-point was chosen for the study of the acute effects of TNFα on canine adipocytes *in vitro*, with more long-term effects being examined at 24 h of treatment. Analysis carried out using NCBI and EMBL-EBI search engines, revealed that human and canine TNFα share a high level of homology at both the mRNA and amino acid level. Thus, initial studies investigated the effect of human recombinant TNFα on canine adipocytes. It has been widely documented that TNFα has a strong stimulatory effect on the expression of several inflammatory cytokine genes in human adipocytes, including a substantial stimulation of its own expression (Wang *et al.*, 2004).

Treatment of canine adipocytes with human recombinant TNFα led to a significant upregulation in the level of expression of pro-inflammatory cytokine genes including IL-6, MCP-1 and TNFα itself by cells of the subcutaneous inguinal depot as early as 2 h post-treatment. These effects, although significant, were muted in comparison to the effects of human recombinant TNFα (same source) on human adipocytes in culture (Wang *et al.*, 2005). In contrast, studies using canine recombinant TNFα demonstrated effects which were similar in magnitude to those documented in human adipocytes with close to 2,000-fold upregulation in TNFα expression at 2 h post-treatment by cells of the subcutaneous inguinal depot. Thus, despite the close homology between the human and canine forms of the protein, significant differences in terms of protein structure and function appear to exist between them. Effects of canine TNFα on cells of the gonadal depot were less marked when compared to those of the subcutaneous inguinal depot where, despite levels being significantly elevated both at 2 and 24 h of treatment, the effects were at best 100 times less than that observed for cells of the subcutaneous inguinal depot. Similar results have been documented in human adipocytes in culture, where expression of

TNFα in visceral depots such as the omental depot, have been documented to be significantly less than that for adipocytes of the subcutaneous depot (Hube *et al.*, 1999). This finding highlights the importance of site-specific differences in adipocyte gene expression. One possible explanation may be the higher level of sympathetic innervation in visceral depots, as catecholamines are known to suppress TNFα expression (van der Poll *et al.*, 1996). The autocrine loop by which TNFα increases its own expression serves to amplify the effects of this key pro-inflammatory cytokine on adipocytes (Wang *et al.*, 2005). Thus, with the expansion of WAT mass in obesity, TNFα may also prove to have significant effects in terms of whole body metabolism in the dog.

A similar pattern of expression was observed with IL-6, where an acute and dramatic up-regulation of IL-6 mRNA level was seen in cells of both depots as early as 2 h post-treatment. However, in the case of IL-6 expression, this effect was more marked in cells of the gonadal depot. Here, at 24 h of treatment, IL-6 mRNA levels were nearly 8-fold higher than those of the subcutaneous inguinal depot in response to TNFα treatment. TNFα also stimulated the secretion of IL-6 by cells of both depots. A significant rise in secreted levels of IL-6 only occurred 24 h after treatment, suggesting that the secreted protein involved *de novo* protein synthesis. Overall, levels of IL-6 secreted by cells of the gonadal depot were nearly 10-fold higher than those of the subcutaneous inguinal depot. Isolated human omental adipocytes have been documented to release 2-3 times more IL-6 than subcutaneous inguinal adipose tissue (Fried *et al.*, 1998). The present results demonstrate that canine adipocytes from visceral and subcutaneous depots secrete IL-6 in a manner similar to human adipocytes, and that the response to TNFα treatment is more marked in cells of the visceral compared to the subcutaneous inguinal depot.

In addition to IL-6 and TNFα itself, MCP-1 and NGF was strongly up-regulated in canine adipocytes after treatment with canine recombinant TNFα, at both the gene expression and protein secretion levels. MCP-1 expression was induced by TNFα in cells of both depots by 2 h of treatment. Expression and secretion increased by 24 h, again suggesting *de novo* MCP-1 protein synthesis in response to treatment with TNFα. In addition, TNFα had a greater stimulatory effect on total 24 h MCP-1 release, than on the release of the other adipokines examined by cells of both depots.

In humans, obesity is associated with an infiltration of adipose tissue by macrophages (Weisberg *et al.*, 2003; Xu *et al.*, 2003). MCP-1 is a chemokine involved in the recruitment of monocytes to sites of inflammation and whose circulating levels are elevated in humans with obesity and insulin resistance (Nomura *et al.*, 2000; Sartipy & Loskutoff, 2003), and thus the high level of secretion of MCP-1 documented in canine adipocytes may be significant in terms of macrophage/monocyte recruitment into WAT in obesity.

A substantial increase in NGF gene expression occurred in cells of both depots with TNFα treatment. NGF expression was significantly upregulated in cells of both depots at both 2 and 24 h of treatment, and the magnitude of the response was similar in cells of both depots. However, cells of the gonadal depot had a maximal response earlier (2 h treatment) compared to cells of the subcutaneous inguinal depot. The amount of NGF protein in the medium was substantially elevated following treatment with TNFα for 24 h, suggesting *de novo* NGF protein synthesis in response to treatment with TNFα. A strong stimulation of NGF synthesis and secretion has been observed previously in human and murine adipocytes (Peeraully *et al.*, 2004; Wang *et al.*, 2005). NGF has been recognised as having an important role in immunity and inflammation, including in stimulating TNFα production by macrophages (Levi-Montalcini *et al.*, 1996; Perez-Perez *et al.*, 2003). Overall, the marked response of canine adipocytes to TNFα suggests that NGF is an important inflammatory factor in canine WAT.

There was a significant down-regulation of leptin gene expression by cells of the subcutaneous inguinal depot at both 2 and 24 h treatment with TNFα. In contrast, cells of the gonadal depot displayed a small but nevertheless significant upregulation in levels of leptin mRNA acutely at 2 h treatment with TNFα. This effect was however, no longer present by 24 h. Previous studies have provided conflicting observations, with TNFα being reported to stimulate leptin secretion in short-term suspension culture or ceiling culture of human adipocytes (Zhang *et al.*, 2000), whereas treatment of 3T3-L1 adipocytes has been reported to result in rapid stimulation followed by a decline in leptin to undetectable levels (Kirchgessner *et al.*, 1997). Recent studies on human adipocytes treated with TNFα have reported a down-regulation in both leptin gene expression and protein secretion (Wang &

Trayhurn, 2006). These differences may reflect the impact of varying culture conditions and differences between cell types. Another potential issue is whether discrepancies are introduced through chromosomal changes in clonal cell lines, such as the 3T3-L1 adipocytes.

In accordance with previous data published on the effect of TNFα on human adipocytes (Wang & Trayhurn, 2006), treatment of canine adipocytes with TNFα led to a significant decrease in adiponectin mRNA by 24 h in cells of both depots. TNFα has also been shown to decrease adiponectin gene expression in both human preadipocytes (Kappes & Loffler, 2000) and 3T3-L1 adipocytes (Fasshauer *et al.*, 2002). However, in contrast to human data, TNFα had no effect on adiponectin release by canine adipocytes. This dissociation between adiponectin mRNA and protein levels with TNFα treatment has also been reported in human adipocytes (Wang *et al.*, 2004), and may reflect differences in time course, since mRNA measurements relate to the situation in cells at the end point of treatment, whereas protein level in the medium is the sum of the secretion throughout the preceding 24 h. Thus, as the reduction in mRNA level occurred only towards the end of the incubation period, this would not be expected to affect the aggregated protein secretion over the full 24 h. In both dogs (Ishioka *et al.*, 2005b) and humans (Weyer *et al.*, 2001), adiponectin production is reduced in obesity, and its expression is regulated by agents which improve insulin sensitivity (Maeda *et al.*, 2001). This suggests that increased TNFα levels may lead to the impairment of insulin sensitivity in dogs as well as in humans, through suppression of circulating levels of adiponectin.

Although IL-6 is regarded as a potent pro-inflammatory cytokine (Trujillo *et al.*, 2004), its effects on canine adipocytes were muted compared to those of TNFα both in terms of adipokine gene expression and protein secretion. IL-6 treatment of canine adipocytes resulted in a modest, but nevertheless significant, upregulation in TNFα gene expression at 4 h of treatment. This effect, however, was short-lived and no longer significant after 24 h of treatment. Furthermore, in the dog, there was a significant down-regulation of IL-6 expression by canine adipocytes at 24 h of treatment. This is unlike reports in 3T3-L1 adipocytes, where IL-6 has been shown to have a marked autocrine effect on its own level of expression and secretion

(Fasshauer *et al.*, 2003a). There was also no effect of IL-6 treatment on MCP-1 gene expression, but nevertheless a significant increase in MCP-1 protein secretion by canine adipocytes by 24 h of treatment. The magnitude of this effect was small, and thus was likely to have been due to accumulation of protein secreted over 24 h, rather than a true effect of the treatment agent. In terms of NGF, there was also a down-regulation of expression with IL-6 treatment at 24 h and, despite there being a small but significant upregulation in the amount of secreted NGF in the cell culture medium at 24 h post-treatment, this effect was modest; as with MCP-1, this is likely to represent accumulation of the protein over the 24 h treatment period. A more marked down-regulation of NGF expression and secretion has been documented in 3T3-L1 cells with IL-6 (Peeraully *et al.*, 2004). It has been reported that sympathetic neurones are able to secrete IL-6 (Marz *et al.*, 1998) so the inhibition of NGF by this cytokine may be equivalent to the effect of catecholamines in terms of a sympathetically induced down-regulation.

Both leptin expression and systemic levels of the protein have been shown to be unaffected by IL-6 in humans (Keller *et al.*, 2005), and this was also found to be the case in canine adipocytes *in vitro*. In contrast, IL-6 treatment induced a significant down-regulation in adiponectin expression by 24 h of treatment with both high and low-dose IL-6. This is similar to what has been observed in 3T3-L1 adipocytes, with IL-6 inducing a marked and reversible down-regulation in adiponectin gene expression and secretion (Fasshauer *et al.*, 2003b). Adiponectin is an important adipokine whose expression and secretion is influenced by hormones and drugs affecting insulin sensitivity. Thus, these *in vitro* results suggest that IL-6 may be an important factor contributing to hypoadiponectinaemia and insulin resistance in the obese state in the dog.

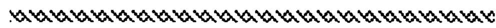
Treatment of canine adipocytes with low and high doses of LPS, led to a marked increase in the level of expression and secretion of TNFα, IL-6, MCP-1 and NGF at both 4 and 24 h of treatment. The effect on IL-6 expression and secretion was particularly significant with levels of expression being elevated by nearly 6,000-fold with the high dose of LPS treatment at 24 h, and levels of secreted protein being nearly 100 times greater than that documented by other treatment agents. Plasma concentrations of IL-6 are increased in human obesity, and secretion of IL-6 from an

expanded WAT mass is the likely explanation (Flower *et al.*, 2003), Increased IL-6 production and release, including from WAT in the obese state, may contribute to the pathogenesis of coronary artery disease (Yudkin *et al.*, 2000) and insulin resistance (Lagathu *et al.*, 2003; Rotter *et al.*, 2003)in humans.

The increase in the level of the expression and secretion of pro-inflammatory adipokines by canine adipocytes with LPS treatment is likely to occur directly via Toll-like receptor (TLR)-4, which is located in adipocytes (Lin *et al.*, 2000). Conversely, treatment of canine adipocytes with low doses of LPS led to a down-regulation of leptin and adiponectin gene expression at 24 h. The fact that the down-regulation was not observed with the high dose of LPS may have been due to a subtle effect of the protein at its receptor with high levels of the agent in the case of these two adipokines. In terms of leptin, recent studies have shown an upregulation of gene expression with LPS treatment of 3T3-L1 adipocytes (Poulain-Godefroy & Froguel, 2007). The anti-adipogenic effect of pro-inflammatory mediators such as LPS has been well documented and, therefore, is it unsurprising to find a down-regulation of adiponectin expression in canine adipocytes by LPS. The same has also been documented in murine 3T3-L1 adipocytes (Poulain-Godefroy & Froguel, 2007).(Poulain-Godefroy & Froguel, 2007).

In summary, canine adipocytes from both visceral and subcutaneous depots appear to be able to respond to inflammatory agents such as TNFα and LPS in a time dependent manner. Pro-inflammatory agents thus appear to be important regulators of adipokine production in canine adipocytes. The neurotrophin NGF, as well as important pro-inflammatory cytokines including IL-6 and TNFα and chemokines such as MCP-1, would each appear to be linked with the inflammatory response of canine WAT.

Chapter 6



Adipokine expression and secretion by canine adipocytes in primary culture: Regulation by PPARγ agonists and dexamethasone

6.1 Introduction

In Chapter 4 it was shown that canine adipocytes from both visceral and subcutaneous depots in primary culture express and secrete key adipokines. Subsequent studies, outlined in Chapter 5, demonstrated that adipocytes from both depots were responsive to the effects of inflammatory mediators such as LPS and the pleiotropic pro-inflammatory cytokine TNFα. In this chapter, studies focused on determining whether in addition to responding to inflammatory mediators, canine adipocytes are also able to respond to agents whose properties include an anti-inflammatory action such as peroxisome proliferator-activated receptor-γ (PPARγ) agonists and dexamethasone.

Changes in morphology and composition of adipose tissue in obesity lead to changes in protein production and secretion. As outlined in Chapter 1, the changes in the inflammatory status of adipose tissue with obesity have led to a growing recognition that obesity represents a state of chronic low-grade inflammation. Various molecular mechanisms have been implicated in obesity-induced inflammation, some of which are thought to be modulated by the peroxisome proliferatior-activated receptors (PPARs), in particular PPARγ which is considered the master regulator of adipogenesis in adipose tissue (Stienstra *et al.*, 2006). In Chapter 3 it was shown that both isoforms of PPARγ (PPARγ1 and 2) are expressed in canine WAT, and that as documented in humans (Lehrke & Lazar, 2005), PPARγ1 is the isoform expressed in non-adipose tissues, whereas PPARγ2 is adipose-tissue specific.

The target genes of PPARγ include those involved in adipocyte differentiation, lipid storage and glucose metabolism and are thus of key importance to adipose tissue homeostasis. PPARγ is also involved in the modulation of the inflammatory response, especially in macrophages (Stienstra *et al.*, 2006). Adipose tissue macrophage numbers have been found to increase in obesity and to participate in inflammatory pathways that are activated in adipose tissue of obese individuals (Weisberg *et al.*, 2003). Endogenous agonists of PPARγ include unsaturated fatty acids and several eicosanoids, while anti-diabetic drugs belonging to the thiazolidinediones (TZDs) act as synthetic agonists of PPARγ (Stienstra *et al.*, 2006).

Studies in this chapter investigated the effect of treatment of canine adipocytes with two PPARγ agonists, rosiglitazone and ciglitazone. Both of these agents have been used, in humans, to treat type II diabetes by improving insulin sensitivity (Hammarstedt *et al.*, 2005). However, all TZDs are subject to liver metabolism and have varying degrees of hepatotoxicity making them less suitable as treatment agents in certain instances (Guo *et al.*, 2006). In addition, canine adipocytes were also treated with the synthetic glucocorticoid dexamethasone which is a potent anti-inflammatory agent and has been shown to have a significant effect on the expression and secretion of several adipokines including leptin (Considine *et al.*, 1997; Mitchell *et al.*, 1997) and NGF (Peeraully *et al.*, 2004).

6.2 Methods

6.2.1 Cell culture

White adipose tissue from both the subcutaneous inguinal and gonadal depots of SBT dogs were harvested as described in sections 2.1.1 and 2.1.2. Cells were cultured as described in section 2.2 at 37°C in a humidified atmosphere of 5% CO_2/95% air. Twenty four hours after they were inoculated, cells from the gonadal depot were washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells from the subcutaneous inguinal depot had their inoculation medium changed 48 h post-inoculation and 24 h later (at 72 h post-inoculation) were washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells of both depots were exposed to the adipogenic medium for 48 h. This was replaced by feeding medium, which was renewed every 48 h.

The cells were pre-incubated 12 days after differentiation for 24 h with FCS-free feeding medium. Cells were then treated with FCS-free feeding media containing each of the specific agents; the control cells for each study had their pre-incubation media renewed. Cells from both depots were treated with the PPARγ agonist, rosiglitazone at doses of 50 nM, 200 nM and 5 μM. Cells were harvested at the time of treatment and at 4 and 24 h post-treatment, as described in section 2.2.5. Only cells from the subcutaneous inguinal depot (see section 4.4), were treated with the PPARγ agonist ciglitazone at doses of 500 nM, 10 μM and 50 μM, and

dexamethasone at doses of 2 and 20 nM. Cells were harvested at the time of treatment for all treatment agents and, in the case of ciglitazone, at 4 and 24 h post-treatment, whereas those treated with dexamethasone were harvested at 2 and 24 h post-treatment. All cells were harvested as described in section 2.2.5. Eight individual wells in cell culture plates were treated with each agent, or used as controls.

6.2.2 qPCR and ELISA

RNA was extracted from cultured cells using Tri-Reagent as described in section 2.3.1. RNA, which was to be analysed by qPCR, was subsequently treated using a DNA-free kit to minimise levels of genomic DNA contamination (section 2.3.2). Once extracted, the total RNA was quantified using a BioPhotometer and reverse transcribed using the Reverse-iT First Strand Synthesis kit (sections 2.3.3 and 2.3.1). The cDNA was then analysed by real-time PCR (qPCR) using the qPCR Core Kit and an Mx3005P real-time machine (section 2.5.3 and 2.5.4).

PCR products were purified using a NuceloSpin Extract 2-in-1 kit and sequenced commercially (see section 2.4.4). All qPCR data was analysed using MxPro-Mx3005P software. Conditioned media samples were analysed for NGF protein concentration using the NGF E_{max} Immunoassay System (section 2.6.1). TNFα, IL-6 and MCP-1 protein concentration were analysed using ELISA kits from R&D Systems (see section 2.6.2). Adiponectin protein concentration in conditioned media was assayed commercially by LINCO (section 2.6.3).

6.3 Results

6.3.1 Regulation of adipokine expression in canine adipocytes: Response to rosiglitazone

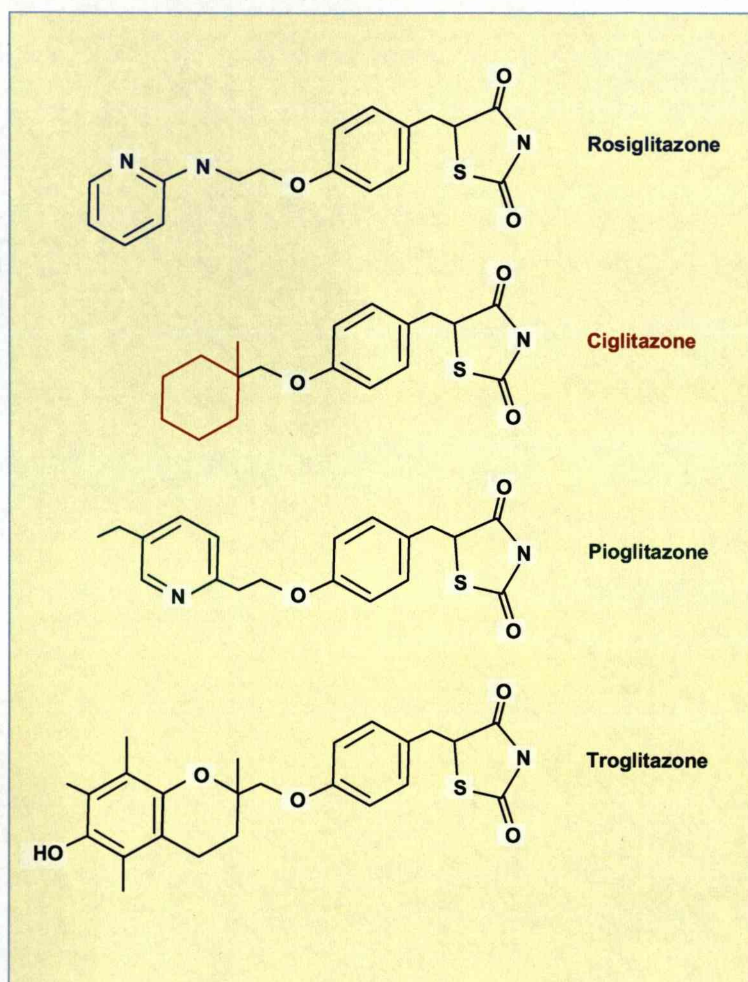
TZDs are PPARγ agonists with a thiazolidinedione ring as the common structural characteristic (Fig. 6.1). The first experiments examined the effect of the PPARγ agonist rosiglitazone on adipokine expression and secretion by canine adipocytes. Data from murine 3T3-L1 cells described the use of rosiglitazone at lower treatment doses of 100 nM and 1 μM (do Nascimento *et al.*, 2004; Peeraully *et al.*, 2004).

However, it was unknown whether canine adipocytes were responsive to exogenous treatment with rosiglitazone and therefore adipocytes from the subcutaneous inguinal depot were treated with rosiglitazone at doses of 50 nM, 200 nM and at 5 μM.

Rosiglitazone significantly reduced the level of expression of key inflammation-related adipokines by canine adipocytes at both 4 and 24 h, while augmenting the expression of key adipocyte hormones leptin and adiponectin by cells of both depots (Fig. 6.2 and 6.3). In cells from the subcutaneous inguinal depot, this effect was clear and dose dependent for TNFα at 4 h with low-, medium- and high-dose rosiglitazone where expression was significantly reduced by 25, 55 and 74%, respectively (Fig. 6.2A). At 24 h, high-dose rosiglitazone treatment reduced TNFα mRNA level to a similar extent as that observed at 4 h. In cells of the gonadal depot, at 4 h treatment, TNFα mRNA levels were reduced by 41% with high doses of rosiglitazone, whereas at 24 h the response to both medium and high doses of rosiglitazone was similar in magnitude to that of cells of the subcutaneous inguinal depot, with a reduction in TNFα mRNA level of approximately 70% (Fig. 6.3A). The effect of rosiglitazone on TNFα secretion was examined in cells of the subcutaneous inguinal depot. Here, there was a modest, but nevertheless significant reduction, of 14% in the level of protein secreted into the medium at 4 h post-treatment with high-dose rosiglitazone (Fig. 6.4A).

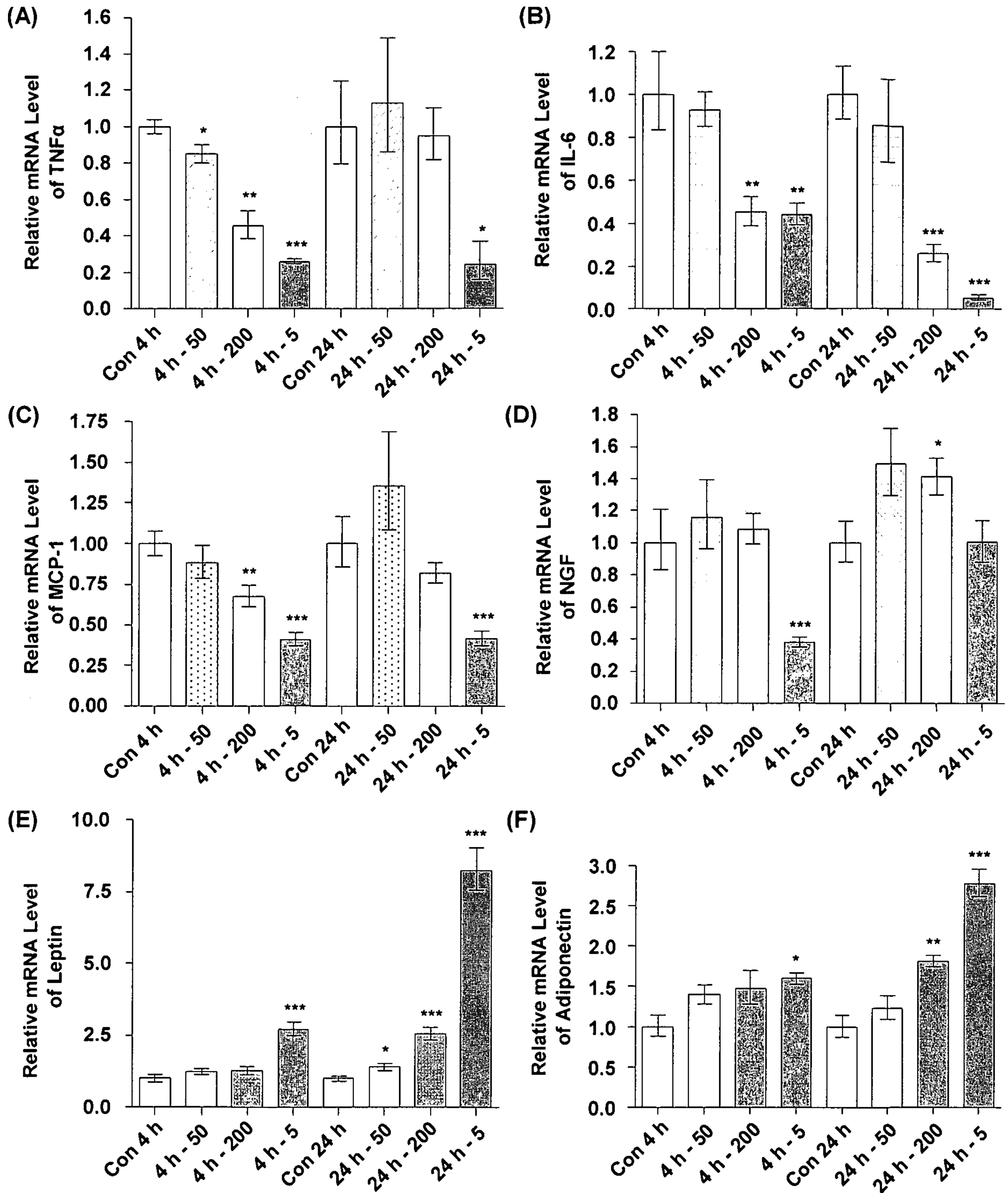
A substantial decrease in IL-6 mRNA level was seen in cells of both depots on treatment with rosiglitazone. In cells of the subcutaneous inguinal depot, at 4 h of treatment, there was a marked reduction in IL-6 mRNA level of approximately 55% with both medium and high doses of rosiglitazone. This effect was even more marked at 24 h, where IL-6 expression was reduced in a dose-dependent manner by 74 and 95% with medium and high doses of rosiglitazone, respectively (Fig. 6.2B). Gonadal adipocytes responded in a similar manner to high dose rosiglitazone treatment with IL-6 mRNA levels falling by 39 and 50% at 4 and 24 h of treatment respectively (Fig. 6.3B). Both low and high doses of rosiglitazone significantly reduced IL-6 secretion by cells of the subcutaneous inguinal depot by an average of 25% at 4 h post-treatment. This effect was not dose dependent (Fig. 6.4B).

Figure 6.1 Chemical structures of four anti-diabetic thiazolinediones



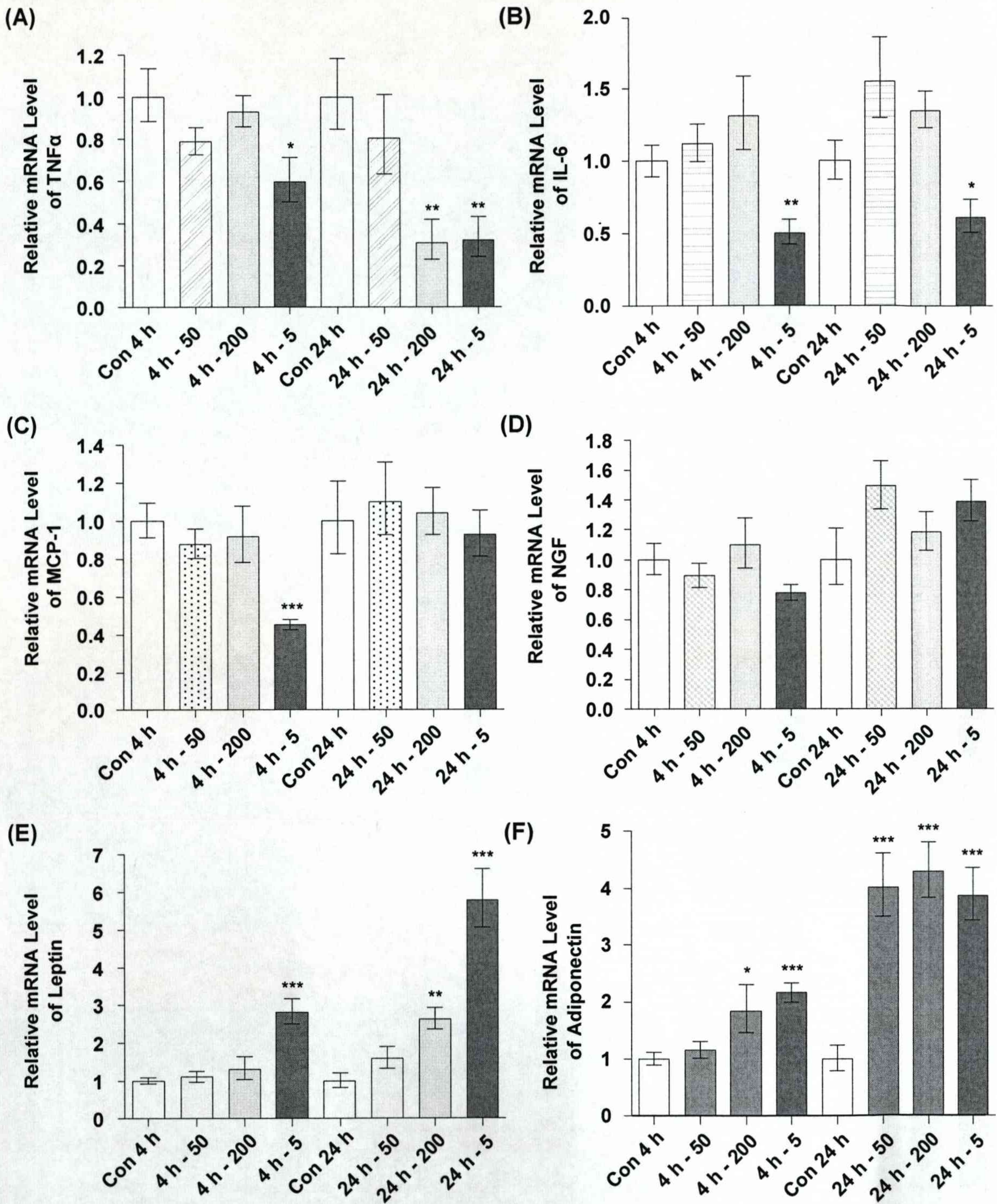
(Guo *et al.*, 2006)

Figure 6.2 Regulation of adipokine gene expression in canine subcutaneous inguinal adipocytes by rosiglitazone



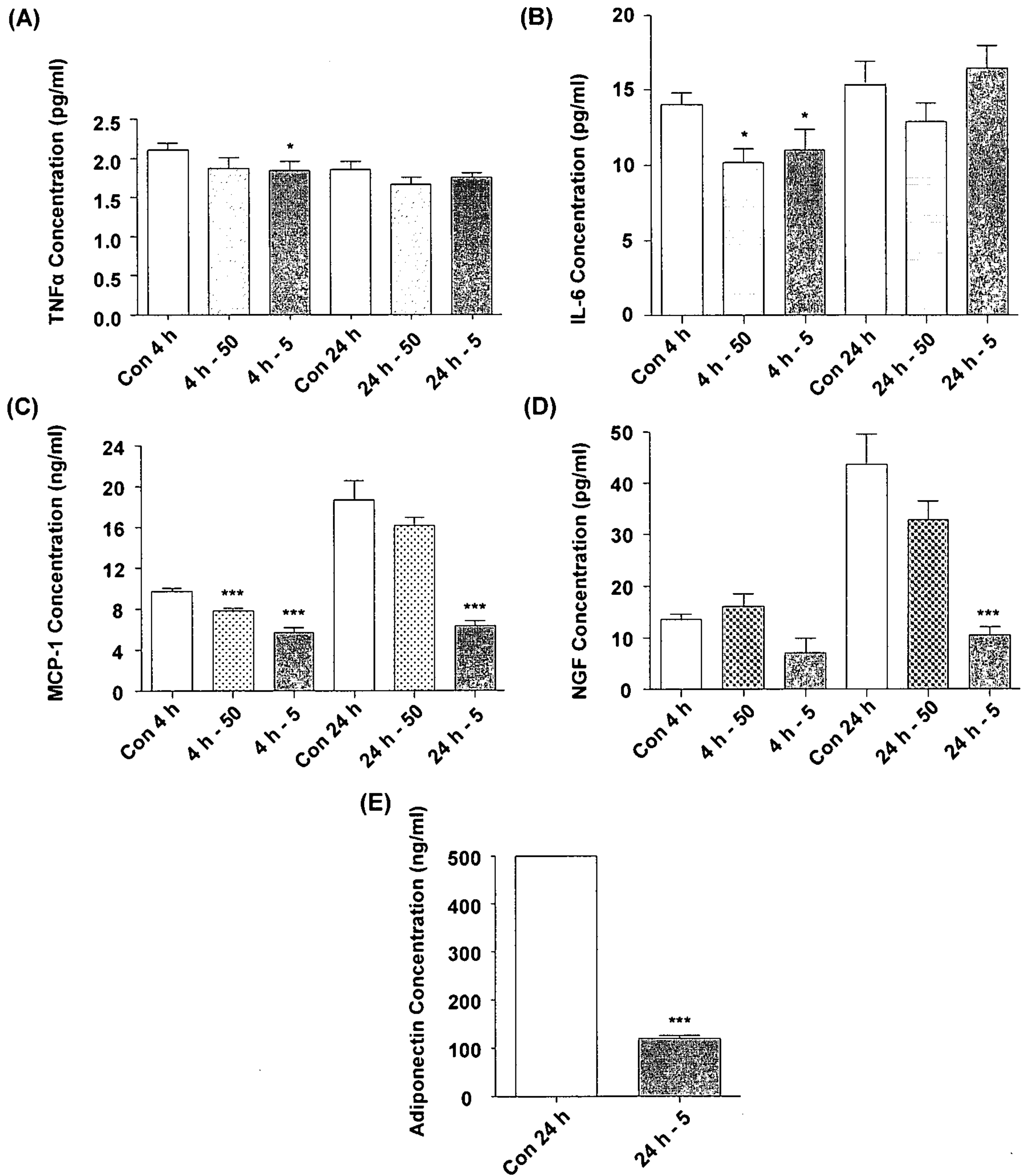
Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing rosiglitazone for 4 and 24 h at low -50, 50 nM; medium -200, 200 nM and high -5, 5 μM rosiglitazone. Adipokine mRNA level was measured by qPCR and normalised to canine β-actin relative to the untreated control group at the same time-point (=1). Results are means ± SE (bars) for groups of 8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Leptin; (F), Adiponectin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls at the same time-point.

Figure 6.3 Regulation of adipokine gene expression in canine gonadal adipocytes by rosiglitazone



Differentiated canine adipocytes from the gonadal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing rosiglitazone for 4 and 24 h at low -50, 50 nM; medium -200, 200 nM and high -5, 5 μM rosiglitazone. Adipokine mRNA level was measured by qPCR and normalised to canine β-actin relative to the untreated control group at the same time-point (=1). Results are means ± SE (bars) for groups of 8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Leptin; (F), Adiponectin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls at the same time-point.

Figure 6.4 Regulation of adipokine protein secretion in canine subcutaneous inguinal adipocytes by rosiglitazone



Differentiated canine adipocytes from the subcutaneous inguinal depot, at day 12 post-induction, were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing rosiglitazone for 4 and 24 h at low -50, 50 nM; medium -200, 200 nM and high -5, 5 μM rosiglitazone. Adipokine concentration was measured by ELISA normalised to control at the same time-point. Results are means ± SE (bars) for groups of 3-8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Adiponectin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls at the same time-point.

The mRNA levels of the inflammation-related chemokine MCP-1 were also reduced by rosiglitazone treatment in a similar manner to TNFα and IL-6. In cells of the subcutaneous inguinal depot, MCP-1 mRNA levels were reduced by 33% with the medium dose of rosiglitazone at 4 h and by 60% with the high dose at both 4 and 24 h of treatment (Fig. 6.2C). High doses of rosiglitazone reduced MCP-1 mRNA levels by a similar extent in gonadal adipocytes at 4 h post-treatment (Fig. 6.3C). In addition, rosiglitazone significantly reduced MCP-1 protein secretion by canine subcutaneous inguinal adipocytes, where levels fell by 20% at 4 h with low doses of rosiglitazone, an effect which doubled with the high dose treatment. Furthermore, at 24 h post-treatment with high dose rosiglitazone MCP-1 protein levels were reduced by more than 66% (Fig. 6.4C).

In a similar manner to other inflammation-related adipokines, treatment with rosiglitazone for 4 h at the higher dose of 5 μM, reduced NGF mRNA level by more than 60% in subcutaneous inguinal adipocytes (Fig. 6.2D). A similar magnitude of effect on NGF protein secretion was observed at 24 h (Fig. 6.4D). In contrast, rosiglitazone had no effect on NGF mRNA level of gonadal adipocytes (Fig. 6.3D), and led to a modest, but nevertheless statistically significant, increase in NGF mRNA level with the medium dose at 24 h post-treatment in subcutaneous inguinal adipocytes (Fig. 6.2D).

Unlike the effect on inflammation-related adipokines, treatment of canine adipocytes of both depots with rosiglitazone led to a significant increase in the level of leptin and adiponectin mRNA. Leptin mRNA levels in cells of both depots were raised by nearly 3-fold with high doses of rosiglitazone at 4 h and an effect of similar magnitude was observed at 24 h with the medium dose of rosiglitazone. Furthermore, the high doses led to an increase in leptin mRNA level of 6-fold in cells of the gonadal depot and of over 8-fold in cells of the subcutaneous inguinal depot (Fig. 6.2E and 6.3E).

Like leptin, adiponectin mRNA levels in cells of both depots were raised by rosiglitazone treatment. A 1.5-fold increase in level of expression was seen at 4 h with high doses of rosiglitazone in cells of the subcutaneous inguinal depot (Fig.

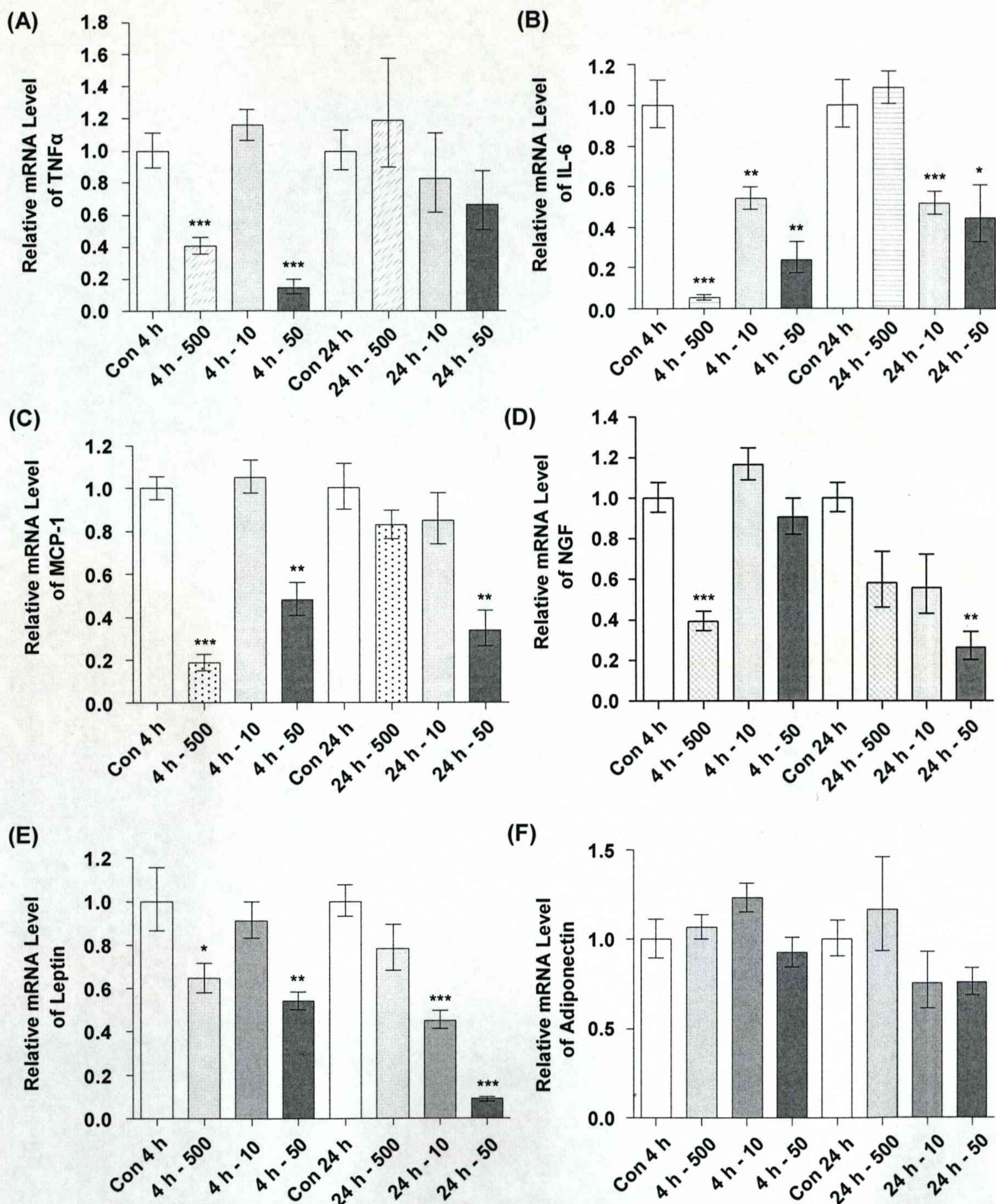
6.2F). Levels were raised to a similar extent at the same time-point in cells of the gonadal depot with both medium and high doses of rosiglitazone (Fig. 6.3F). At 24 h post-treatment, adiponectin mRNA levels were significantly increased by both medium and high dose treatment with a peak response of nearly 3-fold in cells of the subcutaneous inguinal depot (Fig. 6.2F). The response by cells of the gonadal depot was more marked with a nearly 4-fold up-regulation in gene expression by all three treatment doses 24 h (Fig. 6.3F). However, in contrast to changes in gene expression, adiponectin protein secretion fell by over 75% in cells of the subcutaneous inguinal depot with high doses of rosiglitazone (Fig. 6.4E).

6.3.2 Regulation of adipokine expression in canine adipocytes: Response to ciglitazone

The next experiment investigated the effects of a different PPARγ agonist, ciglitazone, on canine subcutaneous inguinal adipocytes. Like rosiglitazone, treatment of canine adipocytes with ciglitazone led to a significant reduction in TNFα mRNA level (60% with the low dose and >85% with the high dose) as compared with the controls at the same time point. In contrast, TNFα mRNA levels were unchanged at 24 h of treatment (Fig. 6.5A). In terms of TNFα protein secretion in response to ciglitazone, a small but nevertheless significant rise in secretion was noted at 24 h with the high dose of ciglitazone (Fig. 6.6A).

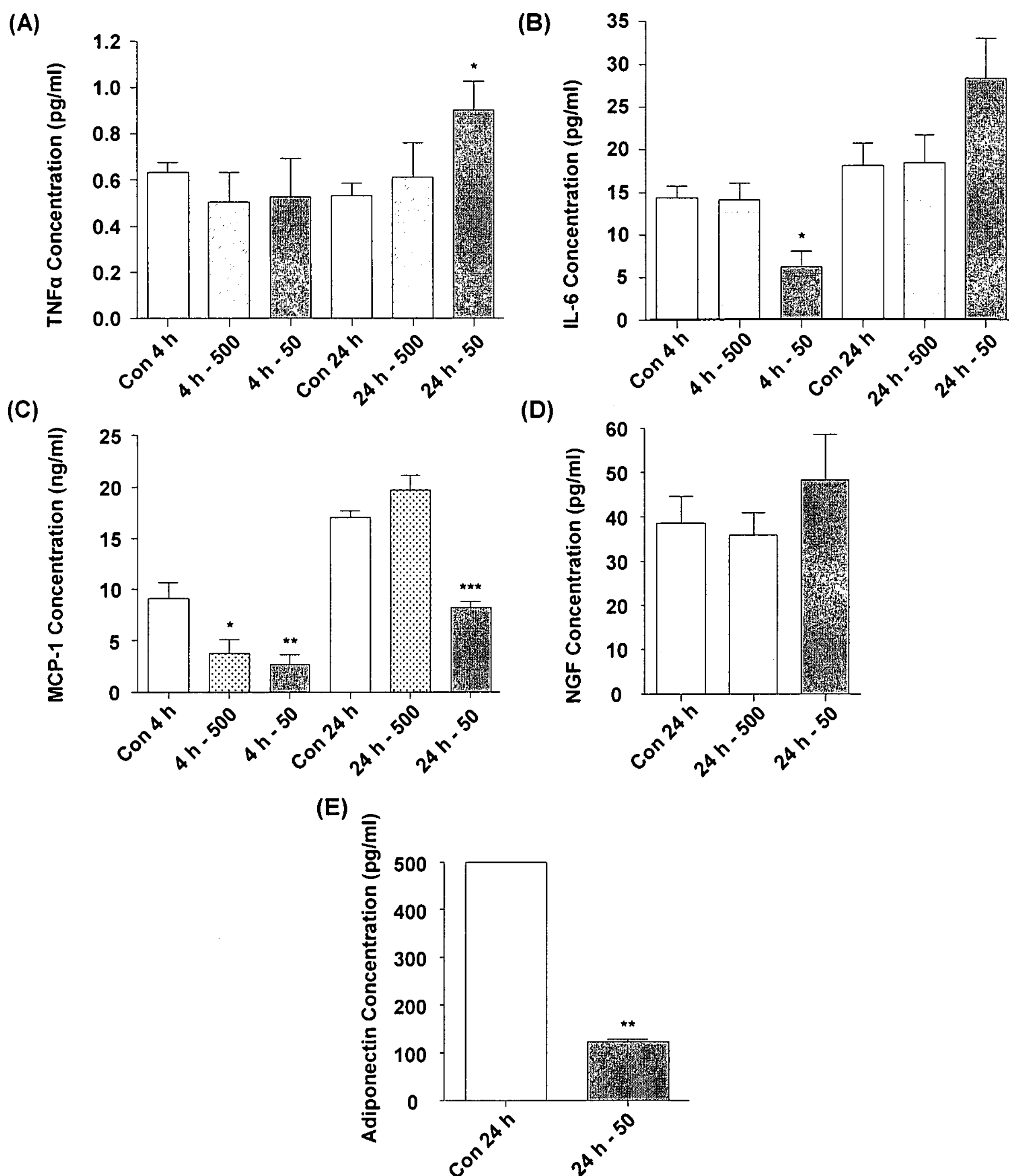
IL-6 mRNA levels were also significantly reduced by treatment with ciglitazone. At 4 h, a substantial decrease in IL-6 mRNA levels of just over 95% with the low dose was observed (Fig. 6.5B). A marked reduction in mRNA level was also seen with the medium and high doses of ciglitazone treatment at 4 h of 47 and 77% respectively. IL-6 protein secretion into the medium by canine adipocytes was reduced by just over 40% at 4 h with the high doses of ciglitazone. At 24 h, IL-6 mRNA levels were significantly reduced by an average of just over 50% with medium- and high-dose ciglitazone. However, at 24 h post-treatment, there were no changes in IL-6 protein secretion with ciglitazone at any of the dose levels (Fig. 6.6B).

Figure 6.5 Regulation of adipokine gene expression in canine subcutaneous inguinal adipocytes by ciglitazone



Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing ciglitazone for 4 and 24 h at low -500, 500 nM; medium -10, 10 μM and high -50, 50 μM ciglitazone. Adipokine mRNA level was measured by qPCR and normalised to canine β-actin relative to the untreated control group at the same time-point (=1). Results are means ± SE (bars) for groups of 8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Leptin; (F), Adiponectin. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls at the same time-point.

Figure 6.6 Regulation of adipokine protein secretion in canine subcutaneous inguinal adipocytes by ciglitazone



Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing ciglitazone for 4 and 24 h at low -500, 500 nM; medium -10, 10 μM and high -50, 50 μM ciglitazone. Adipokine concentration was measured by ELISA normalised to control at the same time-point. Results are means ± SE (bars) for groups of 3-8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Adiponectin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls at the same time-point.

In a similar manner to both IL-6 and TNFα, MCP-1 mRNA levels were also reduced by treatment with ciglitazone. At 4 h, levels were reduced by as much as 82% with the low dose. In contrast, treatment with the medium dose had no effect on mRNA levels. However, treatment with the high dose of ciglitazone reduced MCP-1 mRNA levels significantly by just over 50% at 4 h (Fig. 6.5C). MCP-1 protein secretion by canine adipocytes was significantly reduced at 4 h, by an average of 65% with both the low and high dose of ciglitazone (Fig. 6.6C). In contrast, at 24 h, only the high dose of ciglitazone affected MCP-1 mRNA and protein levels, where a reduction of just over 50% was seen (Fig. 6.5C and 6.6C).

Treatment of canine adipocytes with ciglitazone also led to a significant decrease in NGF mRNA levels. At 4 h with the low dose of ciglitazone, a fall of approximately 60% in mRNA levels was observed. This effect was even more marked at 24 h with the high dose of ciglitazone where a decrease of over 70% in NGF mRNA level was observed (Fig. 6.5D). In contrast to other inflammation-related adipokines, ciglitazone had no effect on NGF protein secretion into the medium by canine adipocytes (Fig. 6.6D).

Unlike rosiglitazone, treatment of canine subcutaneous inguinal adipocytes with ciglitazone led to a reduction in the cellular mRNA levels of leptin at both 4 and 24 h. Initially, at 4 h, leptin mRNA levels fell by an average of 40% with the low and high doses of ciglitazone. At 24 h, this effect was even more marked, with a reduction of 55% in leptin mRNA level with the medium dose of ciglitazone. The effect of high dose was even greater, with mRNA levels falling by as much as 91% (Fig. 6.5E).

In contrast to rosiglitazone, treatment of canine adipocytes with ciglitazone had no effect on cellular adiponectin mRNA levels (Fig. 6.5F). However, levels of protein secreted into the medium by cells treated with the high dose of ciglitazone were reduced by just over 75% in a similar manner to cells treated with the high dose of rosiglitazone (Fig. 6.6E).

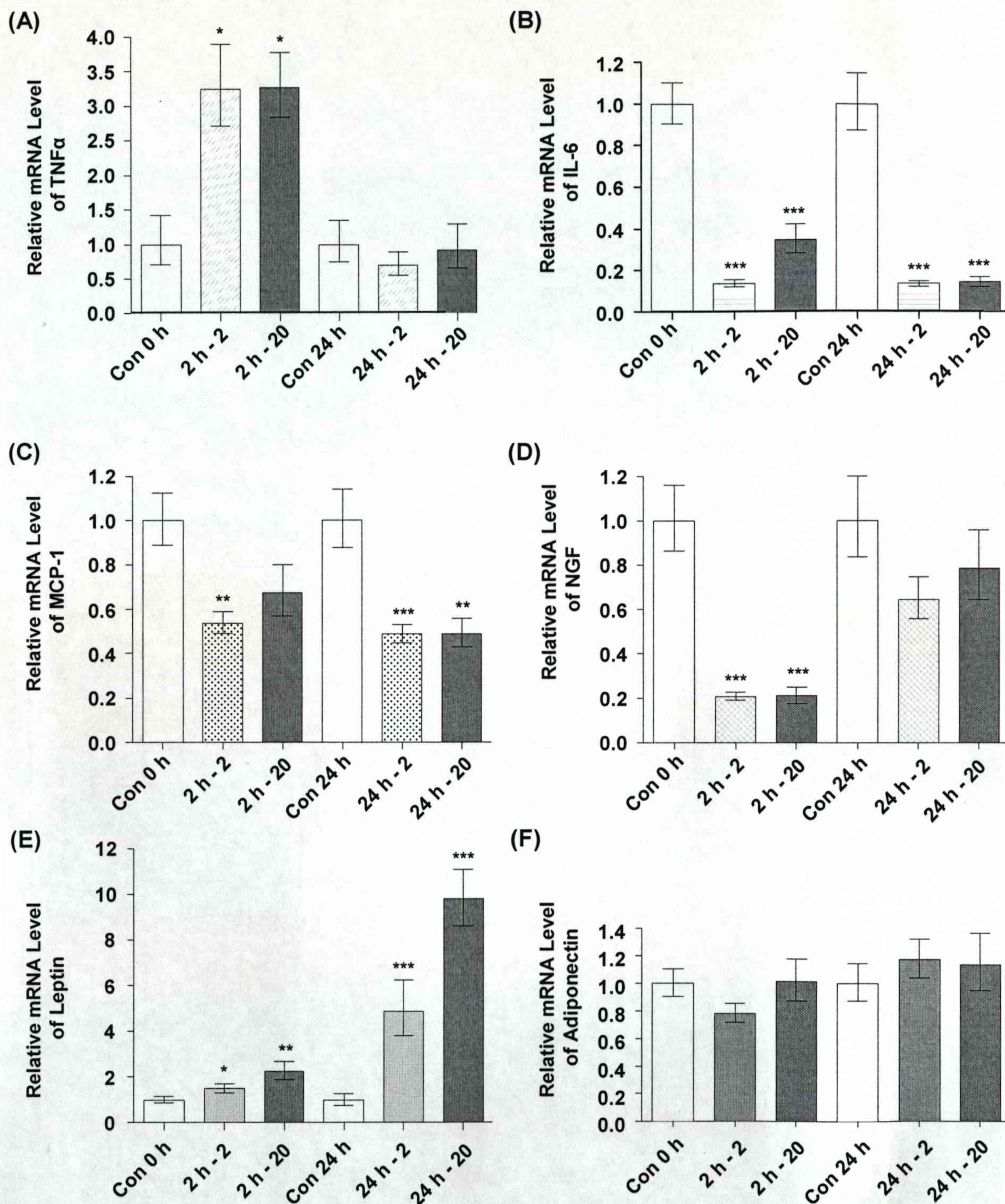
6.3.3 Regulation of adipokine expression in canine adipocytes: Response to dexamethasone

The next experiment examined the effect of dexamethasone treatment on canine subcutaneous inguinal adipocytes. Here, unlike treatment with PPARγ, both high and low doses of dexamethasone led to a small, but significant increase in TNFα mRNA level at 2 h of just over 3-fold (Fig. 6.7A). In contrast, levels of TNFα protein secreted into the medium by canine adipocytes were significantly reduced at 2 h with the low dose of dexamethasone by approximately 16% (Fig. 6.8A). No significant changes in either cell TNFα mRNA level or protein secretion were detected with either low or high doses of dexamethasone at 24 h (Fig. 6.7 and 6.8A).

A substantial decrease in both IL-6 mRNA and protein occurred when the adipocytes were treated with dexamethasone (Fig. 6.7B and 6.8B). With the lower dose at 2 h, there was a reduction of 87% in IL-6 mRNA level in the cells and just over a 55% fall in IL-6 protein in the medium. With the high dose of dexamethasone at 2 h, IL-6 gene expression fell by 66% and protein secretion by approximately 53%. At 24 h, mRNA level at both low and high doses was reduced on average by 85% (Fig. 6.7B); while protein secretion was reduced by 30% at both doses (Fig. 6.8B).

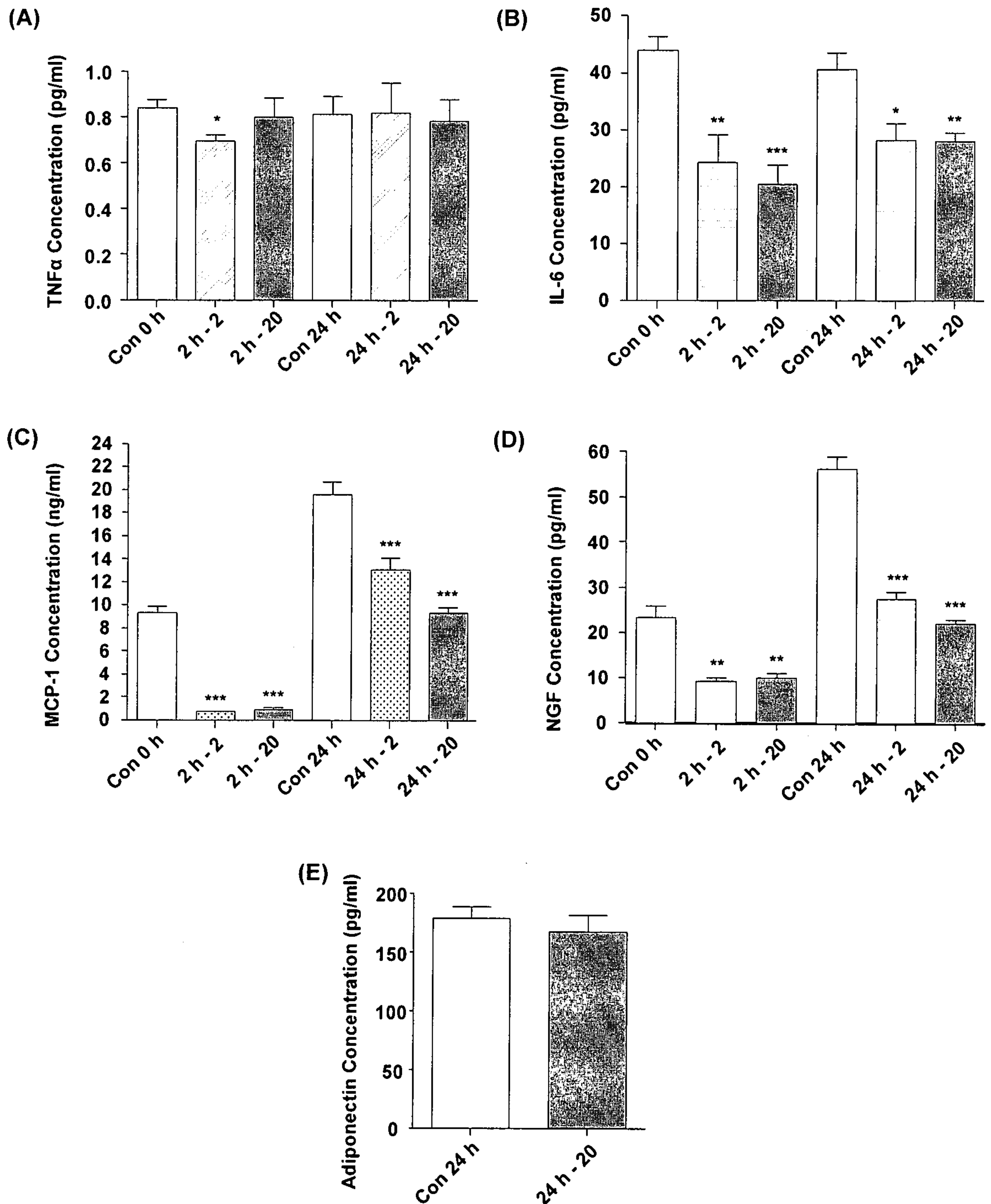
In a similar manner to IL-6, treatment of canine adipocytes with dexamethasone led to a significant reduction in both MCP-1 mRNA and protein levels at 2 and 24 h. At 2 h with the low dose of dexamethasone, MCP-1 mRNA levels were reduced by 47% (Fig. 6.7C). The effect of dexamethasone treatment at 2 h on MCP-1 protein secretion was more dramatic, with the amount being reduced by an average of 91% (Fig. 6.8C). At 24 h, MCP-1 mRNA levels were reduced by 52% with low and high dose treatment, while protein secretion fell in a dose-dependent manner by 31 and 52% for the low and high doses respectively (Fig. 6.7 and 6.8C).

Figure 6.7 Regulation of adipokine gene expression in canine subcutaneous inguinal adipocytes by dexamethasone



Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing dexamethasone for 2 and 24 h at low -2, 2 nM and high -20, 20 nM dexamethasone. Adipokine mRNA level was measured by qPCR and normalised to canine β-actin relative to the untreated control group at the same time-point (=1). Results are means ± SE (bars) for groups of 8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Leptin; (F), Adiponectin. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls.

Figure 6.8 Regulation of adipokine protein secretion in canine subcutaneous inguinal adipocytes by dexamethasone



Differentiated canine adipocytes from the subcutaneous inguinal depot at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing dexamethasone for 2 and 24 h at low -2, 2 nM and high -20, 20 nM dexamethasone. Adipokine concentration was measured by ELISA normalised to control at the same time-point. Results are means ± SE (bars) for groups of 3-8. (A), TNFα; (B), IL-6; (C), MCP-1; (D), NGF; (E), Adiponectin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls.

Treatment of canine adipocytes with dexamethasone led to a marked reduction of approximately 80% in NGF mRNA levels at 2 h (Fig. 6.7D). This was coupled with a reduction in NGF protein secretion by adipocytes into the medium, of 60% with both the low and high doses (Fig. 6.8D). In contrast, at 24 h no significant change in cellular NGF mRNA level was detected with qPCR (Fig 6.7D). However, NGF protein levels fell in a dose-dependent manner by 52 and 61% with the low and high doses respectively (Fig. 6.8D).

Unlike the effect on mRNA level of inflammation-related adipokines such as MCP-1 and NGF, dexamethasone treatment led to an increase in canine adipocyte leptin mRNA level in both a time- and dose-dependent manner. Levels were raised by 1.5 and 2.3-fold respectively with low and high doses at 2 h, whereas at 24 h, the effects were more marked with a nearly 5-fold up-regulation with the low dose and a 10-fold up-regulation with the high dose (Fig. 6.7E).

Treatment of canine adipocytes with dexamethasone had no effect on either adiponectin mRNA level or protein secretion at 2 or 24 h of treatment (Fig. 6.7F and 6.8E).

6.4 Discussion

In Chapter 5, it was demonstrated that adipokine expression and secretion, by canine adipocytes, is modulated by proinflammatory cytokines and LPS. The results in this Chapter indicate, using the robust method of adipocyte primary cell culture outlined in Chapter 4, that in addition to responding to a proinflammatory stimulus, canine adipocytes from both visceral and subcutaneous depots are also strongly influenced by agents such as TZDs and dexamethasone.

As outlined in Chapter 1, PPARγ is a member of the nuclear receptor family that includes 48 human transcription factors whose activity is regulated by direct binding of ligands, including fatty acids, eicosanoids and steroid hormones. (Lehrke & Lazar, 2005). TZDs are a class of oral anti-diabetic drugs with a high affinity for PPARγ used to treat type II diabetes in humans. TZDs activate gene expression, leading to a net increase in lipid partitioning into adipocytes from the circulation (Lehrke & Lazar, 2005). However, this increase in fat storage does not appear to lead to adipocyte hypertrophy as there is also an increase in adipocyte differentiation and increased mitochondrial biogenesis leading to an increase in fatty acid oxidation.

Ciglitazone, the first PPARγ agonist tested in clinical trials, was not marketed because of hepatotoxic effects. Troglitazone, was the first PPARγ agonist approved for treatment of type II diabetes, but was subsequently withdrawn after reports of severe liver toxicity and death (Isley, 2003). Currently, two TZD compounds, rosiglitazone and pioglitazone, are prescribed for the treatment of type II diabetes in humans (Lehrke & Lazar, 2005), and although hepatotoxicity has been observed with these agents, this is much less frequent and severe (Farley-Hills *et al.*, 2004).

Natural and synthetic PPARγ ligands have been shown to exert potent anti-inflammatory effects. Several mechanisms have been suggested for this effect, including interference with proinflammatory transcription factors such as NF-κB and STAT (Welch *et al.*, 2003). Furthermore, PPARγ ligands have been shown to prevent the removal of co-repressor complexes from gene promoter regions resulting in suppression of inflammatory gene transcription, thus retaining inflammatory genes in a suppressed state (Pascual *et al.*, 2005). PPARγ nuclear receptor agonists have

been shown to suppress expression and secretion of inflammation-related adipokines by both murine and human adipocytes (do Nascimento *et al.*, 2004; Peeraully *et al.*, 2004; Hammarstedt *et al.*, 2005). The results outlined in this Chapter demonstrate a distinct inhibition of gene expression and protein secretion of several inflammation-related adipokines with both synthetic PPARγ agonists in the dog.

In terms of treatment with rosiglitazone, a clear inhibition at both the level of gene expression and protein secretion was seen in cells of both depots. Expression levels of several pro-inflammatory adipokines, including TNFα, IL-6 and MCP-1 were reduced. Both PPARγ agonists effectively reduced NGF expression and secretion, in cells of the subcutaneous inguinal depot, with rosiglitazone also having a significant effect on the level of protein secretion by canine adipocytes from the subcutaneous inguinal depot. However, there was no significant effect on NGF expression by cells of the visceral depot with rosiglitazone treatment. This discrepancy may be associated with the fact that visceral adiposity is associated with worsening pro-inflammatory metabolic profile (Stienstra *et al.*, 2006); thus in the case of NGF, rosiglitazone treatment at the doses used, may not have had a significant effect on NGF gene expression.

Treatment of canine adipocytes derived from both subcutaneous and visceral depots with rosiglitazone, led to a significant rise in levels of leptin gene expression. This effect was significant in cells of both depots by 4 h of treatment. The levels of expression continued to rise over the 24 h, at times in a dose-dependent manner in cells of both depots. This is in contrast to data from both rodents and humans, where treatment of adipocytes with rosiglitazone has been shown to lead to a reduction in leptin expression (De Vos *et al.*, 1996; Kallen & Lazar, 1996; Rieusset *et al.*, 1999). PPARγ agonists are thought to suppress leptin expression in humans and rodents thereby limiting lipolysis and fatty acid oxidation both of which are stimulated by leptin. Thus, decreased PPARγ expression may lead to increased leptin levels and lower food intake and weight gain in humans (Wang *et al.*, 1999). This difference between these two species and the dog is very intriguing and presumably reflects a major species difference and a possible route for further investigations into leptin signalling and metabolism in the dog.

In contrast to rosiglitazone, treatment of canine adipocytes of the subcutaneous inguinal depot with ciglitazone led to a clear dose-dependent decrease in leptin gene expression at both 4 and 24 h. Data on the effects of ciglitazone on leptin expression in either human or murine WAT tissue is limited, but this effect may be specific to this agent and to the dog. The fact that the two PPARγ activators had different effects on leptin production in the dog may suggest that the mode of action of rosiglitazone is different in this species, at least in some respects.

In a similar manner to leptin, adiponectin expression increased in cells of both depots with rosiglitazone treatment. In both cases, effects were significant as early as 4 h of treatment and at 24 h, significant up-regulation in adiponectin gene expression was evident at several doses. This is in accordance with data from human studies, where adiponectin expression has been shown to be up-regulated with rosiglitazone treatment in both visceral and subcutaneous WAT (Motoshima *et al.*, 2002). Furthermore, the results in this chapter suggest that canine adipocytes respond in a similar manner to human adipocytes in terms of the response to rosiglitazone treatment with a greater effect seen in adipocytes from the visceral compared to the subcutaneous depot (Motoshima *et al.*, 2002). Thus, in the dog as in humans, enhanced expression of adiponectin from visceral adipocytes in response to rosiglitazone may play a role in the systemic insulin-sensitization and improvement of the inflammatory metabolic profile.

Unlike rosiglitazone, treatment of canine subcutaneous inguinal adipocytes with the PPARγ agonist ciglitazone had no effect on adiponectin expression in canine adipocytes at any of the treatment doses used. Furthermore, in contrast to gene expression data, adiponectin secretion by subcutaneous inguinal adipocytes declined by a similar extent (75%), in response to both treatment with high doses of rosiglitazone and ciglitazone for 24 h. This may have been due to either a delayed response in terms of protein secretion or to an increased protein turnover/degradation of adiponectin in the cell culture medium.

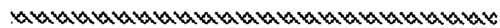
Adipokine expression and secretion has been known to be strongly influenced by glucocorticoids, with the expression of several pro-inflammatory adipokines including IL-6 and NGF, for example, being inhibited by dexamethasone (Fasshauer

et al., 2003a; Peeraully *et al.*, 2004). The results in this chapter indicate that, in a manner similar to rodents and humans, the synthetic glucocorticoid dexamethasone is a potent inhibitor of the expression and secretion of inflammation-related adipokines in the dog. A substantial fall in the expression and release of IL-6, MCP-1 and NGF could be seen as early as 2 h of treatment with a low dose of dexamethasone in cells of the subcutaneous inguinal depot. The effect of dexamethasone treatment on TNFα expression was unlike that seen with other pro-inflammatory adipokines in that a small but nevertheless significant up-regulation in gene expression was seen at 2 h of treatment, an effect which was later undetectable at 24 h. There was, however, a modest but nevertheless significant reduction in adiponectin protein secretion at the same time-point with low dose dexamethasone treatment.

The production of several adipokines has been shown to be strongly stimulated by dexamethasone, and these include leptin, resistin and haptoglobin (De Vos *et al.*, 1995; Haugen *et al.*, 2001; do Nascimento *et al.*, 2004). As such, leptin expression by canine subcutaneous inguinal adipocytes was augmented in both a time- and dose-dependent manner with exogenous dexamethasone treatment. Dexamethasone has been found to reduce significantly adiponectin secretion from human subcutaneous adipocytes (Degawa-Yamauchi *et al.*, 2005). However, the synthetic glucocorticoid appeared to have no effect on adiponectin expression or secretion in canine adipocytes. Thus, while glucocorticoids appear to be important in the regulation of leptin production by canine adipocytes, they appear to have little effect on adiponectin.

In summary, the expression and secretion of several inflammation-related adipokines is down-regulated by TZDs and synthetic glucocorticoids in the dog and as such, they appear to be important regulators of adipokine production in canine visceral and subcutaneous WAT. In contrast, the expression of adipokines involved in insulin-sensitivity and appetite regulation, appear to be augmented by PPARγ agonists and glucocorticoids and this effect may relate in both cases to their anti-inflammatory effects.

Chapter 7



11β-HSD-1 expression by canine adipocytes in primary culture: Regulation by inflammatory mediators, PPARγ agonists and dexamethasone

7.1 Introduction

Glucocorticoids exert potent immunomodulatory effects. At pharmacological levels they are powerful immunosuppressive and anti-inflammatory agents. Clinically, they are therefore, widely used to treat inflammation where often they are the most effective therapy (Bevier, 1990; Padrid & Amis, 1992). Glucocorticoids have been shown to potentiate the adipogenic process and anabolic lipid metabolism in adipocytes (Hauner *et al.*, 1989). Both humans and dogs suffering from hypercortisolism due to hyperadrenocorticism or those undergoing corticosteroid therapy demonstrate specific increases in visceral adiposity and suffer metabolic and signal transduction disturbances mirroring those observed in type II diabetes mellitus, including insulin resistance, hyperglycaemia and hyperlipidaemia (Kelly & Darke, 1976; Rebuffe-Scrive *et al.*, 1988; Walker, 2006).

As outlined in Chapter 1, corticosteroid activity is regulated, in part, by the enzyme 11β-hydroxysteroid dehydrogenase (11-β-HSD-1) which exists as two isoenzymes; 11β-HSD-1 which is predominantly expressed in adipose tissue, liver, gonadal tissue and the central nervous system where it mainly serves as a reductase responsible for the conversion of inactive cortisone to the active glucocorticoid receptor agonist cortisol and 11β-HSD-2, which is expressed primarily in aldosterone target tissues such as kidney and colon where it inactivates cortisol via its dehydrogenase activity, thereby preventing excessive activation of the mineralocorticoid receptor and sequelae including hypertension (Stewart & Krozowski, 1999).

Studies have shown that expression and activity of 11β-HSD-1 is higher in omental compared to subcutaneous sites in humans (Bujalska *et al.*, 1997), and it is possible that the autocrine generation of active cortisol may be a crucial factor in explaining the differential effects of glucocorticoids on different adipose tissue depots and, in turn, the pathogenesis of visceral obesity (Stewart *et al.*, 1999). Furthermore, 11β-HSD-1 knockout mice have been shown to resist diet-induced insulin resistance and hyperglycaemia (Kotelevtsev *et al.*, 1997). Such observations have led to the suggestion that 11β-HSD-1 may play a key role in the dysregulation of metabolism observed in type II diabetes mellitus and other metabolic disorders linked to central obesity in humans (Andrews & Walker, 1999).

Thus far, there are no published studies into the role of 11β-HSD-1 in canine adipose tissue. In this chapter, 11β-HSD-1 expression was investigated in different WAT depots in the dog. Furthermore, expression was also examined in cells of both visceral and subcutaneous depots during differentiation *in vitro*. Since 11β-HSD-1 is recognised as having an important role in inflammation (Chapman *et al.*, 2006), the effects of the pro-inflammatory agents lipopolysaccharide, TNF-α and IL-6 on 11β-HSD-1 were examined in canine adipocytes. Finally, 11β-HSD-1 expression was also investigated in relation to the response to treatment with key agents involved in the regulation of adipokine expression, including dexamethasone and the PPARγ agonists rosiglitazone and ciglitazone.

7.2 Methods

7.2.1 Animals and tissues

Tissues from entire adult male and female SBT dogs were obtained from those euthanased at an animal shelter, for reasons unrelated to the study. Animals were only included if there was no indication of disease, both externally and during gross examination of abdominal organs. All WAT samples (subcutaneous inguinal, omental, perirenal, gonadal and falciform ligament depots) and samples from liver, spleen and kidney were obtained within thirty minutes of euthanasia and immediately frozen in liquid nitrogen.

7.2.2 Cell culture

White adipose tissue from both the subcutaneous inguinal and gonadal depots of SBT dogs were harvested as described in sections 2.1.1 and 2.1.2. Cells were cultured as described in section 2.2 at 37°C in a humidified atmosphere of 5% CO_2/95% air. Twenty four hours after they were inoculated, cells from the gonadal depot were washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells from the subcutaneous inguinal depot had their inoculation medium changed 48 h post-inoculation and 24 h later (at 72 h post-inoculation) were washed three times in 1x PBS and the inoculation medium replaced by induction medium. Cells of both depots were exposed to the adipogenic medium for 48 h. This was replaced by feeding medium, which was renewed every 48 h.

7.2.2.1 Time-course of expression during differentiation

To investigate the time course of 11β-HSD-1 gene expression during adipocyte differentiation, cells from both depots were collected as described in section 2.2.5 from three individual wells of culture plates when cells were induced (*day 0*) and subsequently at *days 1-4, 6, 8, 10, 12* and *15* post-induction. Cell cultures were carried out in triplicate for each depot.

7.2.2.2 Studies on the regulation of 11β-HSD-1 expression

Cells were pre-incubated 12 days after differentiation for 24 h with FCS-free feeding medium. Cells were then treated with FCS-free feeding media containing each of the specific agents; the control cells for each study had their pre-incubation media renewed. Cells from both depots were treated with canine recombinant TNFα at doses of 5 and 50 ng/ml. Only cells from the subcutaneous inguinal depot (see section 4.4), were treated with dexamethasone and this was with doses of 2 and 20 nM.

Cells from the subcutaneous inguinal depot (see section 4.4), were treated with IL-6 (1 and 25 ng/ml), LPS (10 and 100 ng/ml), rosiglitazone (50 nM, 200 nM and 5 μM) and ciglitazone (500 nM, 10 μM and 50 μM). Cells were harvested at the time of treatment and at 4 and 24 h post-treatment as described in section 2.2.5. All cells were harvested as described in section 2.2.5. Either 7 or 8 individual wells in cell culture plates were treated with each agent, or used as controls.

7.2.3 qPCR

RNA was extracted from tissues and cultured cells using Tri-Reagent as described in section 2.3.1. RNA, which was to be analysed by qPCR, was subsequently treated using a DNA-free kit to minimise levels of genomic DNA contamination (section 2.3.2). Once extracted, the total RNA was quantified using a BioPhotometer and reverse transcribed using the Reverse-iT First Strand Synthesis kit (sections 2.3.3 and 2.5.1). The cDNA was then analysed by real-time PCR (qPCR) using the qPCR Core Kit and an Mx3005P real-time machine (section 2.5.3 and 2.5.4).

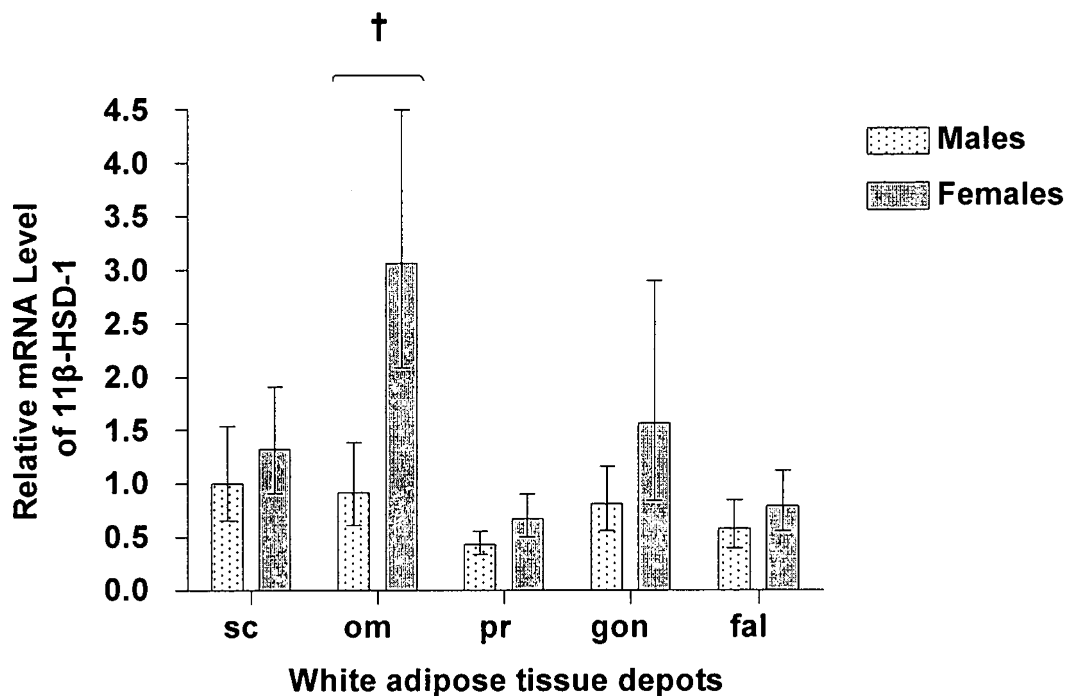
PCR products were purified using a NuceloSpin Extract 2-in-1 kit and sequenced commercially (see section 2.4.4). All qPCR data was analysed using MxPro-Mx3005P software.

7.3 Results

7.3.1 Expression of 11β-HSD-1 in canine white adipose tissue

11β-HSD-1 expression was identified in all of the five main WAT depots of dog, with a clear signal for 11β-HSD-1 mRNA being obtained in all cases using qPCR. Although there were no differences in the level of 11β-HSD-1 expression in different depots in either male or female dogs, when male and female data were directly compared, a significant up-regulation in the level of 11β-HSD-1 mRNA was found in the omental depot in female compared to male dogs (Fig. 7.1).

Figure 7.1 Expression of 11β-HDS-1 by canine white adipose tissue



Total RNA was extracted from subcutaneous inguinal (sc), omental (om), perirenal (pr), gonadal (gon) and falciform (fal) WAT depots from SBT dogs. Once extracted, total RNA was quantified and reverse transcribed to cDNA. Real-time PCR was used for relative quantification of 11β-HSD-1 mRNA levels. Results are expressed relative to the expression of β-actin in the subcutaneous inguinal depot for each gender and are presented as means ± SE (bars) 6-7 individuals † $P < 0.05$, compared to male animals.

7.3.2 Time course of 11β-HSD-1 gene expression in canine adipocytes of the gonadal and subcutaneous depots differentiated in primary culture

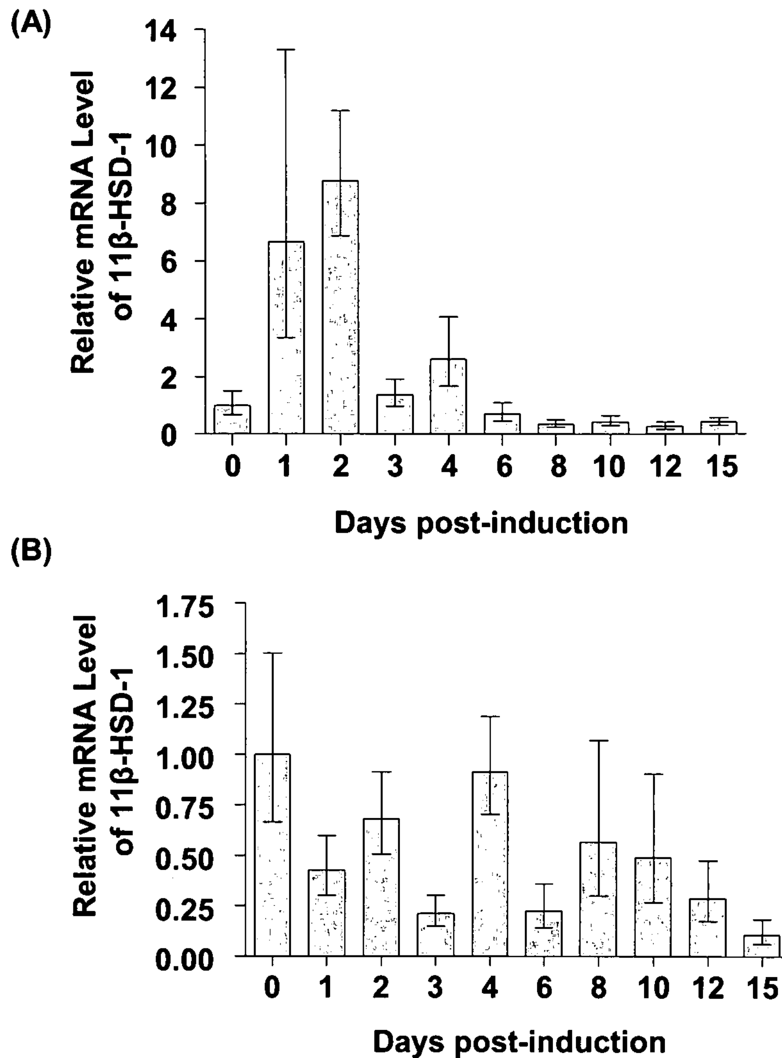
A signal for 11β-HSD-1 mRNA was detected in cells both pre- and post-induction of differentiation. Relative levels of 11β-HSD-1 mRNA were raised from *day 1* post-induction by nearly 7-fold in cells of the subcutaneous inguinal depot (Fig. 7.2A). Peak expression occurred at *day 2* post-induction of differentiation, with 11β-HSD-1 mRNA levels reaching 9-fold up-regulation. Levels of 11β-HSD-1 fell thereafter and remained essentially unchanged as cells matured. In contrast to cells from the subcutaneous inguinal depot, levels of 11β-HSD-1 in cells of the gonadal depot fell immediately post-induction of differentiation and in a similar manner to cells of the subcutaneous inguinal depot, this decrease in mRNA level persisted as cells matured (Fig. 7.2B).

7.3.3 Regulation of 11β-HSD-1 expression in canine adipocytes: Response to inflammatory mediators

Since the time-course study indicated that 11β-HSD-1 is expressed in canine adipocytes, subsequent studies examined the factors which might regulate this expression. The first experiments examined the response of inflammatory mediators. Treatment of canine subcutaneous inguinal adipocytes with canine recombinant TNFα resulted in significant increases in 11β-HSD-1 mRNA level in a time-dependent manner of on average 4-fold at 2 h and of approximately 80-fold at 24 h (Fig. 7.3A). However, there seemed to be no effect in terms of the two dose regimes used. Treatment of gonadal adipocytes with canine recombinant TNFα, in a similar manner to subcutaneous inguinal adipocytes, resulted in a significant increase in 11β-HSD-1 mRNA level at both 2 and 24 h. This effect, however, was more muted with increases of on average 2.5 and 12.5-fold at 2 and 24 h respectively (Fig. 7.3B).

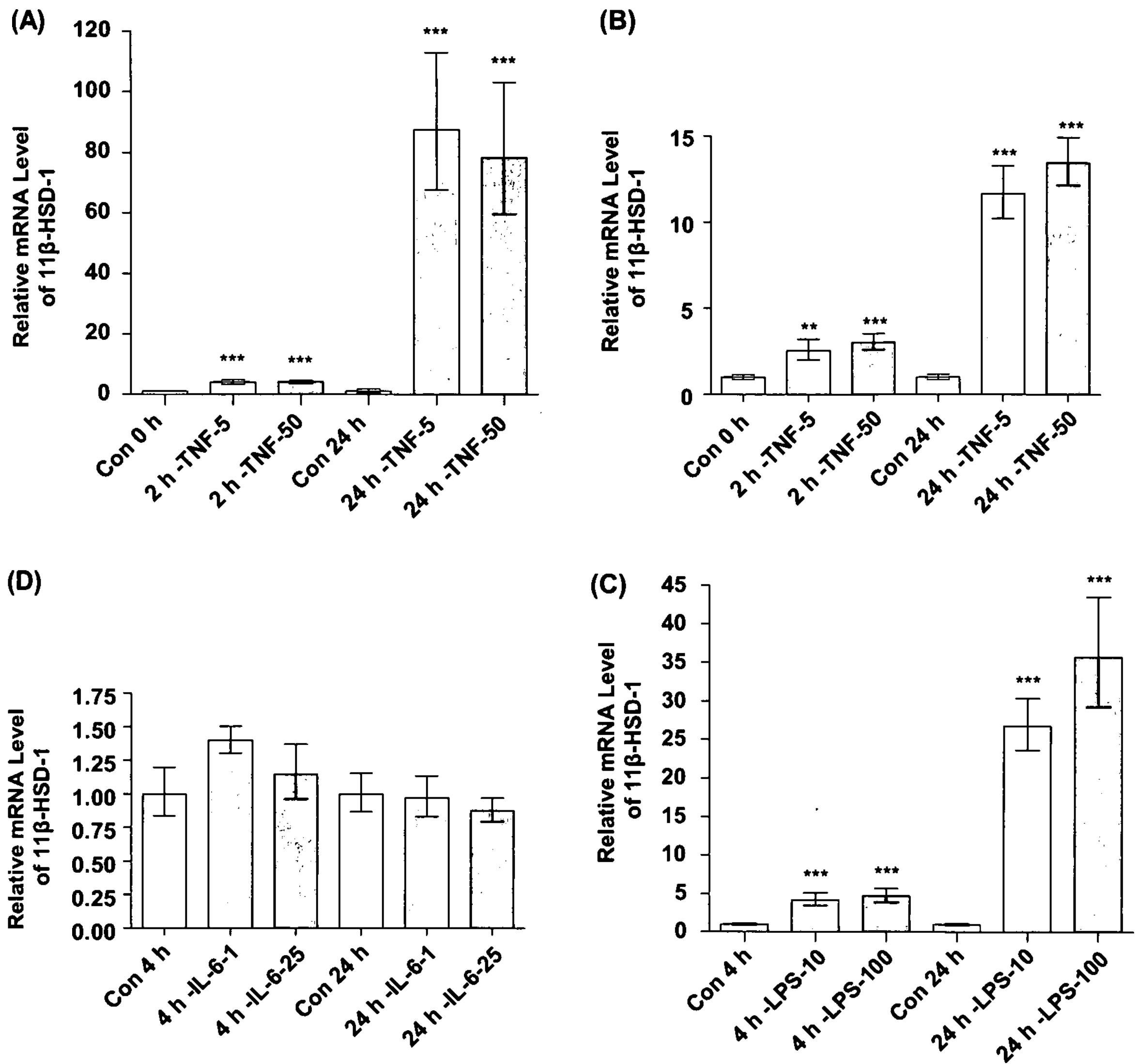
In the next experiments, cells from the subcutaneous inguinal depot were treated with the pro-inflammatory cytokine IL-6. In contrast to TNFα, canine recombinant IL-6 treatment had no effect on 11β-HSD-1 gene expression by canine adipocytes (Fig. 7.3C).

Figure 7.2 Time course of gene expression of 11β-HSD-1 in canine adipocytes in primary culture



Canine adipocytes from both subcutaneous inguinal (A) and gonadal depots (B) were collected at various time points both before (*day 0*) and after the induction of differentiation into adipocytes (*days 1-4, 6, 8, 10, 12* and *15*). Total RNA was extracted and 11β-HSD-1 gene expression examined by qPCR. The mRNA levels were normalised to canine β-actin relative to *day 0*. Results are ± SE (bars) for groups of 3 at each time point. N = 3 individuals.

Figure 7.3 Regulation of 11β-HSD-1 gene expression in canine adipocytes by inflammatory mediators



Differentiated canine adipocytes at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing different inflammatory mediators. (A), Adipocytes from the subcutaneous inguinal depot and (B), adipocytes from the gonadal depot treated with canine recombinant TNFα for 2 and 24 h at low -5, 5 ng/ml TNFα; and high -50, 50 ng/ml TNFα. (C), Adipocytes from the subcutaneous inguinal depot treated with canine recombinant IL-6 for 4 and 24 h at low -1, 1 ng/ml IL-6; and high -25, 25 ng/ml IL-6. (D), Adipocytes from the subcutaneous inguinal depot treated with LPS for 4 and 24 h at low -10, 10 ng/ml LPS; and high -100, 100 ng/ml LPS. 11β-HSD-1 mRNA level was measured by qPCR and normalised to canine RNAPolIIa relative to the untreated control group (=1). Results are means ± SE (bars) for groups of 7 or 8. ***$P < 0.001$ compared with controls at the same time-points.

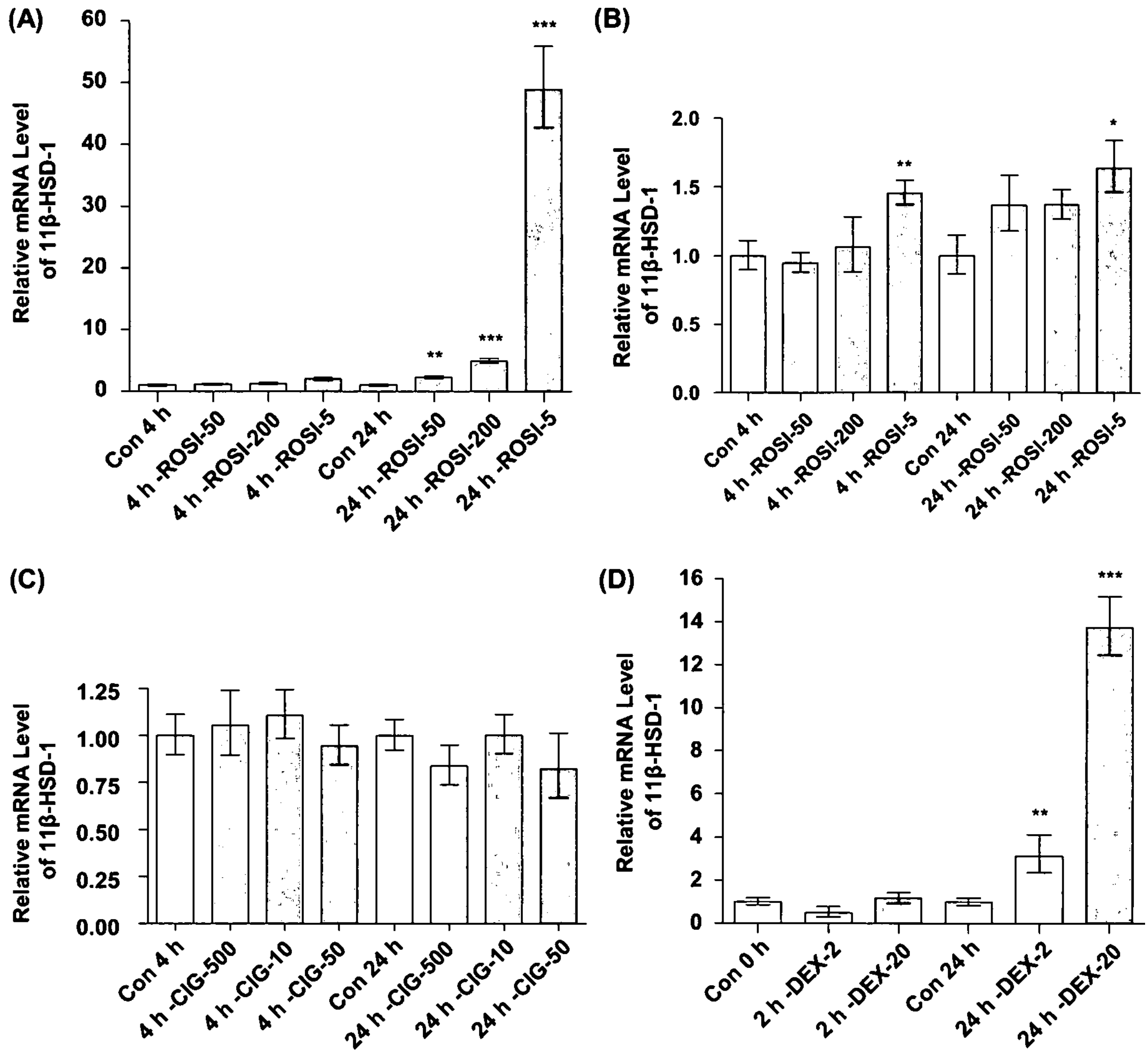
The next experiments examined the effect of the pro-inflammatory agent LPS on 11β-HSD-1 expression in canine adipocytes of the subcutaneous inguinal depot. The response was similar in both manner and magnitude to treatment of adipocytes from the same depot with TNFα, where LPS resulted in an average of 4-fold increase in 11β-HSD-1 mRNA level at 2 h. At 24 h, levels of 11β-HSD-1 mRNA rose to nearly 27- and 36-fold with low and high dose treatment respectively (Fig. 7.3D).

7.3.4 Regulation of 11β-HSD-1 expression in canine adipocytes: Regulation by PPARγ agonists and dexamethasone

Next, the effect of PPARγ agonists and dexamethasone on the regulation of 11β-HSD-1 expression in canine adipocytes was examined. Treatment of canine subcutaneous inguinal adipocytes with rosiglitazone had no effect on 11β-HSD-1 expression at 4 h. However, at 24 h, there was a significant and dose-dependent increase in the level of 11β-HSD-1 expression where mRNA levels reached a peak of nearly 50-fold with high-dose treatment (Fig. 7.4A). The effect of rosiglitazone treatment on 11β-HSD-1 mRNA levels was more muted in cells of the gonadal depot. Here, a modest but nevertheless significant increase of approximately 1.5-fold in mRNA levels was seen at both 4 and 24 h with high-dose treatment (Fig. 7.4B). In contrast to rosiglitazone, treatment of subcutaneous inguinal adipocytes with ciglitazone had no effect on 11β-HSD-1 mRNA levels (Fig. 7.4C).

Treatment of canine subcutaneous inguinal adipocytes with dexamethasone, in a similar manner to rosiglitazone treatment of the same cell type, resulted in a significant time- and dose-dependent increase in 11β-HSD-1 mRNA levels. Levels were increased by 3- and just over 13-fold at 24 h with low and high dose treatment respectively (Fig. 7.4D).

Figure 7.4 Regulation of 11β-HSD-1 gene expression in canine adipocytes by PPARγ agonists and dexamethasone



Differentiated canine adipocytes at day 12 post-induction were pre-treated with FCS-free feeding medium for 24 h and then incubated in medium containing different inflammatory mediators. (A), Adipocytes from the subcutaneous inguinal depot and (B), adipocytes from the gonadal depot treated with rosiglitazone for 4 and 24 h at low -50, 50 nM; medium -500, 500 nM and high -5, 5 μM rosiglitazone. (C), Adipocytes from the subcutaneous inguinal depot treated with ciglitazone for 4 and 24 h at low -500, 500 nM; medium -10, 10 μM and high -50, 50 μM ciglitazone. (D), Adipocytes from the subcutaneous inguinal depot treated with dexamethasone for 2 and 24 h at low -2, 2 nM; and high -20, 20 nM dexamethasone. 11β-HSD-1 mRNA level was measured by qPCR and normalised to canine β-actin or RNAPolIIa relative to the untreated control group (=1). Results are means ± SE (bars) for groups of 7 or 8. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls at the same time-points.

7.4 Discussion

The studies described in this chapter are the first to investigate the expression of the enzyme 11β-HSD-1 in both canine adipose tissue and adipocytes. 11β-HSD-1 is a key enzyme involved in the conversion of inactive cortisone to active cortisol locally in adipose tissues and thereby plays an important role in regulating the actions of corticosteroids within WAT (Wake & Walker, 2004). A signal for 11β-HSD-1 was detected in all WAT depots examined for both male and female SBT dogs; thus, the gene is expressed in each of the depots.

In humans, it has been shown that omental adipocytes contain significantly more 11β-HSD-1 activity than those from the subcutaneous depot (Bujalska *et al.*, 1997), with higher levels of 11β-HSD-1 mRNA also being reported in omental compared to subcutaneous adipocytes (Tomlinson *et al.*, 2002). In women, unlike men, 17β-oestradiol has been reported to strongly stimulate 11β-HSD-1 expression in visceral preadipocytes (Dieudonne *et al.*, 2006). Studies reported here show levels of mRNA encoding 11β-HSD-1 to be significantly higher in the omental adipose tissue of female compared to male dogs. These findings suggest, in a similar manner to humans, that factors such as sex steroids may play a significant role in the control of local cortisol production in adipose tissues of dogs.

Subsequent studies utilised the preadipocyte cell culture system to examine whether 11β-HSD-1 is expressed in canine adipocytes and the expression pattern during differentiation in both subcutaneous inguinal and gonadal adipocytes. Quantitatively, levels of 11β-HSD-1 mRNA were found to be higher in preadipocytes of both depots compared to differentiated adipocytes with levels declining as cells matured. In humans, 11β-HSD-1 has been found to be highly expressed in preadipocytes and it is thought that once differentiation is initiated, 11β-HSD-1 activity is vital to the production of cortisol, and the promotion of adipogenesis (De Sousa Peixoto *et al.*, 2008). Conversely, in the murine cell lines 3T3-L1 and 3T3-F442A, 11β-HSD-1 is characterised as a late marker of differentiation with levels peaking at around day 6-8 post-induction of differentiation (Napolitano *et al.*, 1998). These differences, and the fact that in absolute terms expression of 11β-HSD-1 was greater in cells of the

subcutaneous depot compared to the visceral depot in the dog throughout differentiation, suggest key differences in the role of 11β-HSD-1 and steroid metabolism between different species.

Treatment of canine subcutaneous inguinal and gonadal adipocytes with canine recombinant TNFα led to a significant increase in 11β-HSD-1 expression at both 2 and 24 h. This is in accordance with data from human adipocytes treated with TNFα where similar increases in levels of 11β-HSD-1 expression have been documented with treatment with this pro-inflammatory cytokine (Tomlinson *et al.*, 2001; Friedberg *et al.*, 2003). In addition, the average fold increase in mRNA level was higher in cells of the subcutaneous inguinal depot in dogs compared to the visceral gonadal depot. This again has been observed in human adipocytes treated with TNFα (Tomlinson *et al.*, 2001). However, TNFα has potent direct effects on developing adipocytes to inhibit differentiation and proliferation and to promote apoptosis and de-differentiation (Patton *et al.*, 1986; Petruschke & Hauner, 1993; Prins *et al.*, 1997). It is possible that enhanced expression of 11β-HSD-1 and the resulting increased local production of cortisol may serve as a regulatory feedback loop to balance these direct effects of TNFα.

Unlike TNFα, treatment of canine adipocytes with canine recombinant IL-6 had no effect on 11β-HSD-1 expression in canine adipocytes. This was unexpected as pro-inflammatory cytokines such as IL-1β and IL-6 have been found to significantly up-regulate 11β-HSD-1 expression in human adipocytes (Tomlinson *et al.*, 2001; Friedberg *et al.*, 2003). Thus, this apparent lack of effect to this pro-inflammatory cytokine may reflect a species specific difference in the response of canine adipocytes to this pro-inflammatory cytokine.

Treatment of canine adipocytes with LPS induced a significant up-regulation of 11β-HSD-1 expression at both 4 and 24 h. Studies examining the effect of LPS treatment on adipose tissue expression of 11β-HSD-1 are scarce. However, an investigation into the effect of LPS treatment on murine macrophages has documented a significant increase in the level of 11β-HSD-1 expression with LPS treatment (Ishii *et al.*, 2007). Macrophage infiltration into adipose tissue in obesity provokes local inflammation and insulin resistance. The fact that LPS leads to an increase in the

level of expression of 11β-HSD-1 in canine adipocytes and that a similar effect has been observed in rodent macrophages, highlights a possible role for this enzyme in local cortisol metabolism in canine obesity, where the conversion of inactive cortisone to active cortisol may be augmented not only by production by adipocytes but also by infiltrating macrophages.

Treatment of canine adipocytes with the PPARγ agonist rosiglitazone led to a significant up-regulation of 11β-HSD-1 expression in cells of both subcutaneous inguinal and gonadal depots. This effect was much more marked in cells of the subcutaneous inguinal depot with nearly a 50-fold up-regulation in expression at 24 h with high-dose treatment. Such an effect has also been documented in human adipocytes (Tomlinson *et al.*, 2001). However, there have been several reports to the contrary in both rodents and humans (Berger *et al.*, 2001) (Wake *et al.*, 2007) (Mai *et al.*, 2007). Thus, despite contradictory results, in the dog it is evident that the PPARγ agonist rosiglitazone has a significant effect on 11β-HSD-1 expression in adipocytes of both subcutaneous and visceral depots and some of the effects of PPARγ agonists on canine adipocyte differentiation may be mediated indirectly via glucocorticoids through this action on 11β-HSD-1.

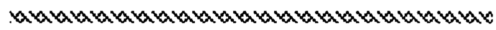
However, unlike rosiglitazone, treatment of canine subcutaneous inguinal adipocytes with another TZD, ciglitazone, had no effect on 11β-HSD-1 expression. There are currently no published studies into the effect of ciglitazone treatment on 11β-HSD-1 expression in adipocytes. Therefore, this lack of effect on 11β-HSD-1 expression may be due to structural differences between the two agents. In order to clarify these differences investigations using further agents such as pioglitazone may help clarify the response of canine adipocytes to PPARγ agonists and their effect on 11β-HSD-1 expression.

In a similar manner to rosiglitazone, treatment of canine adipocytes from the subcutaneous inguinal depot with dexamethasone led to a significant up-regulation in 11β-HSD-1 expression at 24 h in a dose-dependent manner. While exogenous cortisone has been found to stimulate preadipocyte proliferation in humans, dexamethasone has been shown to have a positive effect on adipocyte maturation (Bader *et al.*, 2002). Thus, the increase in 11β-HSD-1 expression seen in canine

adipocytes with dexamethasone treatment may reflect an increased conversion of cortisone to the active metabolite cortisol in mature adipocytes and increased adipocyte maturation and lipid accumulation.

In summary, studies in this chapter have outlined differential and tissue specific regulation of 11β-HSD-1 expression by pro-inflammatory regulators as well as PPARγ agonists and dexamethasone in the dog. The induction of expression by pro-inflammatory cytokines such as TNFα and agents such as LPS has important implications for the pathogenesis of obesity in the dog. Furthermore, regulation of expression by PPARγ agonists and dexamethasone point towards a more complex interaction in terms of inflammation and cortisol metabolism regulation.

Chapter 8



Discussion

8.1 Introduction

White adipose tissue, previously regarded as a site for passive lipid storage, is now widely viewed as a highly dynamic organ (Wang *et al.*, 2008). With the discovery of leptin (Zhang *et al.*, 1994), there resulted a critical shift in perspectives on the role of WAT in the regulation of energy balance and characterised it as a complex secretory and endocrine organ (Trayhurn & Beattie, 2001). Over the past few decades, although the concern with obesity as a health problem has inevitably focused on humans, the disorder and the diseases with which it is associated are now also recognised as a growing problem in companion animals (German, 2006).

With this in mind, over the last few years there has been increasing interest in obesity biology in the dog (Eisele *et al.*, 2005; Gayet *et al.*, 2007). Prospective studies have investigated the levels of key adipokines such as the hormone leptin in dogs under controlled dietary conditions of weight gain and subsequent weight loss (Ishioka *et al.*, 2005a; Ishioka *et al.*, 2007). However, despite these recent advances into the understanding of canine obesity biology, there is still a paucity of information on the endocrine and secretory role of the canine WAT.

With the sequencing of the canine genome (Lindblad-Toh *et al.*, 2005) it became possible to identify key sequences encoding genes of interest in canine adipose tissue and to more easily construct oligonucleotide primers and probes to search for the expression of these genes using known molecular biology techniques. Inasmuch, the hypothesis was proposed that canine, like human and rodent WAT was also responsible for the synthesis and active secretion of protein factors, namely adipokines. Thus, the main focus of the work described in this thesis was the expression and secretion of adipokines by canine white adipocytes. The results presented in Chapters 4, 5, 6 and 7 demonstrate that adipokine genes are indeed expressed by canine white adipocytes and several factors, including the pleiotropic pro-inflammatory adipokine TNFα, are involved in the regulation of adipokine gene expression in canine adipocytes *in vitro*.

Four techniques were employed in the course of these investigations, namely conventional RT-PCR and real-time PCR (qPCR) for the analysis of gene

expression, primary cell culture, and ELISA for the analysis of protein secretion. During this project other studies were carried out using canine lipomatous tissue. Although the data obtained from these studies have not been presented, some consideration will be given to them in the following sections.

8.2 Adipokine gene expression in canine white adipose tissues

Expression of key adipokine genes including leptin and adiponectin were detected and levels of expression quantitated in each of the major white adipose tissue depots of dog. In addition, it was further demonstrated that key adipose tissue-related proteins are also expressed in canine WAT. One such protein is the adipose tissue-specific transcription factor PPARγ2, whose mRNA was documented in all depots examined. In contrast, PPARγ1 was found to be ubiquitously expressed, and as such, a signal was obtained in all tissues. Genes whose expression is characteristic of adipocytes (although not exclusively to these cells) namely GLUT4 and LPS, were also shown to be expressed in all depots examined.

Quantitatively, there were no clear-cut trends in the level of adipokine expression between depots in dog in terms of visceral and subcutaneous tissues. However, when levels of expression were compared between male and female dogs, levels of mRNA encoding leptin were found to be higher in the adipose tissues of female, compared with male dogs for four out of the five depots examined. In humans, mRNA levels of leptin appear to be higher in subcutaneous compared to omental depots (Montague *et al.*, 1997b; Montague *et al.*, 1998). Since in females this is the main depot to expand in obesity, women have been found to have higher levels of circulating leptin compared to age matched men (Castracane *et al.*, 1998). This is unlike the dog were there is no correlation between sex and fat depot distribution (gynoid/android obesity). Thus, the work described in this thesis demonstrates that the major visceral and subcutaneous WAT depots in dogs express key adipokines and that interesting species differences exist between dogs and humans in terms of the possible increased overall expression of the adipocyte hormone leptin in the adipose tissue of female as compared to male dogs.

8.3 Adipokine gene expression and secretion in canine visceral and subcutaneous adipocytes during differentiation *in vitro*

Expression of adipokine genes in WAT does not necessarily imply adipocyte-specific expression, thus, studies were focused on the development of a cell culture protocol for the study of canine adipocytes *in vitro*. Studies outlined in this thesis demonstrate the successful isolation of viable canine preadipocytes from both visceral and subcutaneous depots. It has also outlined the establishment of a robust method of canine adipocyte cell culture in which fibroblastic preadipocytes were successfully induced to differentiate into adipocytes. The validity of the culture system was confirmed not only by profiling the expression of differentiation-dependent genes, such as; LPL, GLUT4, PPARγ2 and haptoglobin (Wabitsch *et al.*, 2001; do Nascimento *et al.*, 2004), but also by examining the expression of key adipokines including leptin and adiponectin whose level of expression increased in canine adipocytes of both visceral and subcutaneous depots during differentiation.

Importantly, it was also demonstrated that canine adipocytes both express and actively secrete inflammation-related cytokines such as TNFα, IL-6 and MCP-1 during differentiation and development. Therefore, it is possible that adipocytes in the dog may contribute to increases in systemic levels of these cytokines in obesity. In addition, by establishing that these biologically-active molecules are expressed and actively secreted by canine adipocytes, the system can then be used as a valuable tool for the investigation of adipocyte biology; thus, responses to various inflammatory stimuli and agents which may improve proinflammatory states and insulin sensitivity can then be assessed.

8.4 Effect of inflammation-related agents and pro-inflammatory cytokines on adipokine expression and secretion

Obesity is characterised by chronic mild inflammation, and as outlined in Chapter 1, WAT may be the main site of the inflammatory state, being responsible for the production of cytokines, chemokines and acute-phase proteins, all of which have been linked to inflammation and the inflammatory response (Rajala & Scherer, 2003;

Trayhurn, 2005b). It is, therefore, possible that adipose tissue is a significant source of the increase in inflammatory markers seen in obesity (Trayhurn & Wood, 2005). The system of primary adipocyte cell culture outlined in Chapter 4 was used to investigate the effects of such agents on adipocytes from both visceral and subcutaneous depots in the dog.

A substantial increase in both the level of expression and secretion of key inflammation-related adipokines such as MCP-1 and NGF was seen in response to treatment of cells with the pleiotropic pro-inflammatory cytokine TNFα. This is in accordance with other *in vitro* data from similar experiments carried out investigating the effect of TNFα on human and rodent adipocytes (Peeraully *et al.*, 2004; Wang *et al.*, 2004). The magnitude of the effect of TNFα treatment on its own level of expression in the dog was considerable with levels of expression increasing by nearly 2,000-fold at 24 h. This mirrors data obtained from human adipocytes treated with exogenous TNFα (Wang & Trayhurn, 2006).

The level of similarity, both in terms of effect and magnitude, between canine and human adipocytes is suggestive of a close metabolic link in terms of TNFα signalling pathways in adipose tissue between dogs and humans. Although the precise mechanism by which TNFα up-regulates the expression and secretion of inflammation-related adipokines such as MCP-1 and TNFα itself in the dog has not been determined, it would be likely to be through induction of NF-Kb activity, this being the key signalling pathway through which TNFα stimulates the expression of many other pro-inflammatory genes (MacEwan, 2002).

Alongside TNFα, IL-6 has been shown to be another adipokine strongly implicated in the development of insulin resistance in humans (Bastard *et al.*, 2002) and rodents (Lagathu *et al.*, 2003; Rotter *et al.*, 2003). Therefore, the impact of IL-6 on canine adipocytes *in vitro* was investigated as part of studies into the effects of pro-inflammatory cytokines on canine adipocytes. However, unlike TNFα, IL-6 had only muted effects, if any, on the expression and secretion of adipokines by canine adipocytes of both subcutaneous and visceral depots. IL-6 signal transduction involves the JAK (janus-activated kinase)/STAT (signal transducers and activators of transcription) signalling pathway, and further work would be needed to reveal

possible differences in these signalling pathways which may be responsible for the differences in regulation of cytokine expression and secretion in response to IL-6 treatment in the dog.

The work presented in this thesis also demonstrates significant effects of the pro-inflammatory agent LPS on adipokine expression and secretion by canine adipocytes. LPS is responsible for the activation of the innate immune pathway which represents the first site of defence against infection, and as outlined in Chapter 5, is known to act via toll-like receptors (TLRs) (Kaisho & Akira, 2002). Studies have reported the expression of TLR-2 and -4 in 3T3-L1 adipocytes where LPS has been shown to signal through TLR-4 and induce both IL-6 and TNFα secretion (Lin *et al.*, 2000). The response of canine adipocytes to LPS treatment, characterised by a significant up-regulation in the level of expression and secretion of inflammation-related adipokines, is in accordance with data published for human and rodents (Peeraully *et al.*, 2004; Creely *et al.*, 2007). Thus, canine adipocytes are also likely to respond to LPS in a similar manner to those of other species, via TLR4, and follow-up work to that effect would be needed to show expression of the receptor protein by canine adipocytes and activation of the receptor-linked signalling cascade.

8.5 Effect of PPARγ agonists and dexamethasone on adipokine expression and secretion

Agents such as the PPARγ agonists rosiglitazone and ciglitazone and the synthetic steroid dexamethasone have well-recognised anti-inflammatory properties as well as having other key properties such as improvement of insulin sensitivity in the case of TZDs. Unlike pro-inflammatory agents, these strongly down-regulate both the expression and secretion of inflammation related adipokines such as NGF and MCP-1 by canine adipocytes. This inhibitory effect clearly suggests that these inflammation-related adipokines may play a significant role in the inflammatory response of adipose tissue in the dog.

In contrast, treatment of canine adipocytes from both subcutaneous and visceral depots with rosiglitazone, led to a significant rise in levels of leptin gene expression. As outlined in Chapter 6, this is in contrast to data from both rodents and humans,

where treatment of adipocytes with rosiglitazone has been shown to lead to a reduction in leptin expression (De Vos *et al.*, 1996; Kallen & Lazar, 1996; Rieusset *et al.*, 1999). This difference between these two species and the dog may reflect a major species difference and a possible route for further investigations into leptin signalling and metabolism in the dog.

However, unlike rosiglitazone, treatment of canine adipocytes with the PPARγ agonist ciglitazone led to a dose-dependent decrease in leptin gene expression. Unlike rosiglitazone, ciglitazone is mainly used as an experimental tool due to severe hepatotoxicity during clinical trials in humans (Guo *et al.*, 2006; Masubuchi *et al.*, 2006). Therefore, data on the effects of this agent on leptin expression in either human or murine WAT tissue are limited and it is possible that this effect may be specific to this agent and to the dog.

Studies presented in this thesis have also described how, in accordance with human studies (Motoshima *et al.*, 2002), adiponectin expression increased in cells of both depots with rosiglitazone treatment. These results suggest that canine adipocytes respond to rosiglitazone in a similar manner to human adipocytes, with a greater effect seen in adipocytes from the visceral compared to the subcutaneous depot. Unlike rosiglitazone, ciglitazone had no effect on adiponectin expression in canine adipocytes at any of the treatment doses used. The fact that these two PPARγ activators had different effects on leptin and adiponectin production in the dog is suggestive of a different mechanism of action and it would be tempting to suggest that this may have been due to inherent biochemical differences in the structure of the agents themselves, or that they might differ in their site of action on specific steps of the regulatory pathway in this species.

Dexamethasone has been shown to strongly stimulate leptin production by adipocytes (De Vos *et al.*, 1995). In a similar manner, exogenous dexamethasone treatment augmented leptin expression in canine adipocytes. In contrast, dexamethasone has been found to reduce adiponectin secretion from human subcutaneous adipocytes (Degawa-Yamauchi *et al.*, 2005). Studies presented here have shown that the synthetic glucocorticoid had no effect on adiponectin expression or secretion in canine adipocytes. Thus, whilst glucocorticoids appear to be important

in the regulation of leptin production by canine adipocytes, they appear to be less so in terms of adiponectin.

8.6 Regulation of adipokine expression in canine lipomatous tissue by treatment with dexamethasone and TNFα

One of the initial aims of the thesis was to compare adipokine expression and secretion by canine adipocytes from dogs of different breeds. As outlined in Chapter 1, over the past hundred years, due to extensive human intervention, dogs have been bred to meet specific human requirements, be it for coat colour or body shape, and have evolved distinct genetic traits (Ostrander & Comstock, 2004). Despite our best efforts, some breed comparisons were not possible due to the lack of a consistent supply of dogs from breeds other than the SBT. However, we were able to obtain lipomatous and perilipomatous (subcutaneous adipose tissue) tissue from a dog of the Labrador retriever breed undergoing routine surgery for the removal of the lipoma. Lipomas are adipose tumours that are slow-growing, nearly always benign and are often located in the subcutaneous tissues of the head, neck, shoulders and back of both dogs and humans (Reimann *et al.*, 1999; Salam, 2002). It was hypothesised that lipomatous tissue would respond more acutely and with greater magnitude to the effects of treatment agents than lipomatous tissue due to increased rates of cell turn-over and metabolism.

Preadipocytes from both tissue-types were successfully harvested and induced to differentiate *in vitro* using the same method outlined in Chapter 2. Cells were treated with agents such as TNFα (low- 5 ng/ml and high 50 ng/ml) and dexamethasone (high- 20 nM) in a similar manner to SBT subcutaneous inguinal and gonadal cells, in order to examine their effect on adipokine expression on both tissue types. Treatment of both cell-types with canine recombinant TNFα, in a similar manner to cells from the SBT dog of both visceral and subcutaneous depots, resulted in the up-regulation of mRNA levels of all inflammation-related adipokine genes, namely IL-6, MCP-1, NGF and TNFα itself. Adiponectin mRNA levels were reduced at 24 h for both cell types, and leptin levels were unaffected (data not shown). Dexamethasone treatment led to a significant reduction in mRNA levels of several inflammation-

related adipokine genes in both tissues. There was no effect on adiponectin mRNA levels and, in a similar manner to the SBT, levels of leptin were significantly raised in both lipomatous and perilipomatous tissues at 24 h (data not shown).

There were no significant trends in the levels of expression of any adipokines examined between lipomatous and perilipomatous adipose tissues. 11β-HSD-1 mRNA levels were significantly increased by both treatment with TNFα and dexamethasone in a similar manner to SBT subcutaneous inguinal and gonadal adipocytes (data not shown). Despite this being a small study in which tissues from only one dog were used, the results suggest that that adipocytes from other dog breeds can be successfully extracted and induced to differentiate in culture using the method outlined in Chapter 2. Further, it is likely that canine adipocytes from different breeds respond in a similar manner to treatment with exogenous TNFα and dexamethasone and finally, there appears to be no difference between the response of canine lipomatous and perilipomatous adipocytes to TNFα and dexamethasone. It must be emphasised, however, that although interesting, this data would need to be refined both in terms of numbers of animals used and adipose tissue collection sites.

8.7 11β-HSD-1 expression in canine adipose tissue and adipocytes in primary culture

Thus far, there is little insight into the local control of cortisol metabolism in adipose tissue in the dog. Therefore, the work carried out in this thesis is, to date, the first to examine such a process in this species. As outlined in Chapter 7, both the dog and human develop clinical signs associated with naturally-occurring hyperadrenocorticism, or iatrogenic signs when treated with glucocorticoid therapy; both species demonstrate specific increases in visceral adiposity and suffer metabolic disturbances mirroring those observed in type II diabetes mellitus, including insulin resistance, hyperglycaemia and hyperlipidaemia (Kelly & Darke, 1976; Rebuffe-Scrive *et al.*, 1988). In simple obesity in humans, plasma cortisol levels are not elevated,; however, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1) is elevated in mature adipocytes, where it is responsible for the conversion of inactive cortisone to cortisol, thereby amplifying local glucocorticoid action in those tissues (Seckl *et al.*, 2004).

11β-HSD-1 expression was documented in all of the five main WAT depots in the dog. Expression was also shown in other canine tissues where, in a similar manner to humans and rodents (Morton *et al.*, 2004; Seckl *et al.*, 2004), the liver was the main site of expression in both sexes with mRNA levels being up-regulated by nearly 140-fold (data not shown). However, the other important site of action of this enzyme is WAT, and in obesity 11β-HSD-1 levels are reduced in the liver but elevated in adipose tissue (Wake & Walker, 2006). With increasing size of the adipose organ in obesity, increased activation of cortisol is thought to contribute to the metabolic syndrome.

Studies reported in this thesis show levels of mRNA encoding 11β-HSD-1 to be higher in the omental adipose tissue of female compared to male dogs. Human omental adipose tissue contains significantly higher levels of 11β-HSD-1 mRNA and activity than subcutaneous adipocytes (Bujalska *et al.*, 1997; Tomlinson *et al.*, 2002). In women, unlike men, 17β-oestradiol has been reported to stimulate strongly the expression of 11β-HSD-1 in visceral preadipocytes (Dieudonne *et al.*, 2006). Therefore, these findings demonstrate that in a similar manner to humans, sex steroids may play an important role in the control of local cortisol production in adipose tissues of dogs.

Studies on the regulation of 11β-HSD-1 in canine adipose tissue examined the effect of pro-inflammatory mediators such as TNFα and LPS, which in accordance with human data (Tomlinson *et al.*, 2001), led to increased levels of 11β-HSD-1 mRNA in cells of both depots. However, in contrast to most data presented for human and rodent adipocytes treated with PPARγ agonists, treatment of canine adipocytes with rosiglitazone led to an increase in levels of 11β-HSD-1 expression in the dog. It seems, therefore, that the regulation of this key enzyme in dog WAT is influenced not only by pro-inflammatory mediators, but that agents with proven anti-inflammatory effects may also allow an increased conversion of cortisone to cortisol. It is, therefore, possible to speculate that a delicate balance in terms of 11β-HSD-1 expression may exist in canine adipocytes allowing the control of adipocyte maturation, lipid accumulation and cytokine production.

8.8 Concluding comments

The studies described in this thesis have examined the expression of key adipose tissue-related proteins and adipokines in canine WAT and isolated adipocytes in primary culture. The establishment of a robust system for isolation and culture of preadipocytes, from both visceral and subcutaneous depots, allowed the active secretion of several key adipokines including the adipocyte hormone adiponectin to be documented. Several inflammation-related cytokines, including TNFα, IL-6 and MCP-1, have clearly been shown to be actively secreted by adipocytes, and therefore fit the definition of adipokines in the dog.

Some of the factors and mechanisms regulating the expression and secretion of adipokines from canine white adipocytes have also been investigated. Therefore, this work lays the foundation for future work which, in the first instance, could involve detailed examination of the role of TNFα in canine WAT using a multiplicity of approaches including the use of NFκB inhibitors. Clearly other hormonal factors such as insulin are also likely to be involved, and there is a need to examine this in future studies.

Furthermore, the relationship between obesity and levels of inflammation-related adipokines and insulin resistance remains to be clarified in the dog. Additional studies could be aimed at determining whether WAT is a significant source of these adipokines and the contribution that these make to plasma levels in obesity. The receptors and signalling mechanisms underlying the stimulation of production and secretion of these adipokines by pro-inflammatory cytokines such as TNFα and IL-6 also require detailed investigation.

Obesity is associated with macrophage accumulation in adipose tissue (Weisberg *et al.*, 2003). These have a significant role in contributing to cytokine secretion and metabolic complications associated with the metabolic syndrome in humans (Bouloumie *et al.*, 2005). As such, the role of resident tissue macrophages and active macrophage recruitment in canine adipose tissue in the obese state, and their contribution to cytokine secretion and inflammation would also be an interesting topic for future research in the dog.

Appendix

Appendix I Publications and presentations associated with the thesis

Part of the work in this thesis has been presented in the following publications, oral communications and posters:

Publications

1. Ryan VR, German AJ, Wood IS, Hunter L, Morris P and Trayhurn P. (2008). NGF gene expression and secretion by canine adipocytes in primary culture: Upregulation by the inflammatory mediators LPS and TNFα. Hormone and Metabolic Research (Accepted for publication).

Oral Presentations

1. Development and optimisation of a primary cell culture system for canine adipocytes (2006) *British Small Animal Veterinary Association Congress, Birmingham, UK.*
2. Expression of inflammation-related adipokines by canine adipocytes differentiated in primary cell culture: Response to TNFα (2007) *British Small Animal Veterinary Association Congress, Birmingham, UK* – ***Prize for best abstract in internal medicine.***
3. Obesity in companion animals (2007) *Integrative Physiology Postgraduate Conference, University of Aberdeen, UK.*
4. Expression of inflammation-related adipokines by canine white adipose tissue and adipocytes differentiated in primary cell culture (2007) *Euriscon Conference, London, UK.*

Poster presentations

1. Expression of inflammation-related adipokines by canine adipocytes differentiated in primary cell culture: Response to TNFα (2006) *European Congress on Obesity, Budapest, Hungary.*

Appendix II Reagents and equipment

Chemical Reagents

Reagent	*Source*
Ammonium chloride	Sigma
Agarose	Preprotech
D-Biotin	Sigma
Bovine serum albumin (BSA) - Fraction V	Qbiogene
Bromophenol blue	Sigma
Chloroform	Fisher
Ciglitazone	Tocris
Collagenase – Type VIII	Sigma
Cortisol	Sigma
Dexamethasone	Sigma
DMEM	Invitrogen
D-MEM:F12	Invitrogen
D-Panthothenic acid	Sigma
Ethylenediaminetetraacetic acid	Sigma
Ethanol 100% molecular biology grade	VWR
Ethidium bromide	Sigma
Foetal calf serum	Biosera
Fungizone – amphotericin B	Invitrogen
GeneRuler™ 100 bp DNA ladder	Fermentas
Glycerol	Sigma
Haematoxylin	HD Supplies
HEPES	Sigma
Hydrochloric acid	Fisher
Hydrogen peroxide	R&D Systems
1-methyl-3-isobutylxanthine	Sigma
IL-6 - Canine recombinant	R&D Systems
Indomethacin	Sigma
Insulin	Sigma
Isopropanol	Sigma

Lipopolysaccharide	Sigma
Liquid nitrogen	BOC
Oil red O	HD Supplies
Penicillin G sodium/streptomycin sulphate	Invitrogen
Di-Potassium hydrogen orthophosphate	BHD Lab Supplies
Potassium chloride	Sigma
Potassium dihydrogen orthophosphate	BHD Lab Supplies
Potassium hydrogen carbonate	BHD Lab Supplies
RNase AWAY™	Molecular BioProd
Rosiglitazone	GlaxoSmithKline
Sodium chloride	Fisher
Di-Sodium hydrogen orthophosphate	BHD Lab Supplies
Sodium dihydrogen orthophosphate	BHD Lab Supplies
Sodium hydrogen carbonate	BHD Lab Supplies
2 N Sulphuric acid	R&D Systems
Superladder-Low 100 bp Ladder with ReddyRun™	ABgene
Tetramethylbenzidine	R&D Systems
Transferrin	Sigma
3,3',5-triiodo-L-thyronine (T_3)	Sigma
Tri-Reagent™	Sigma
Tris-Borate-EDTA 10x	Fisher
TNFα - Canine recombinant	R&D Systems
TNFα – Human recombinant	Sigma
Tween 20™	DakoCytomation
Trypan Blue	Sigma
Ultra-pure water	Sigma
Virkon	Antec International

Kits

Turbo DNA-*free*	Ambion
DuoSet® ELISA – Canine CCL2/MCP-1	R&D Systems
DuoSet® ELISA – Canine IL-6	R&D Systems
DuoSet® ELISA – Canine TNFα	R&D Systems
NGF E_{max}® ImmunoAssay System	Promega

NuceloSpin® Extract 2 in 1	Machery-Nagel
qPCR Core Kit	Eurogentec
Reverse-iT™ 1st Strand Synthesis Kit	ABgene
ReddyMix™ Master PCR Master Mix Kit	ABgene

Equipment

Beckman Preparative Ultracentrifuge	Beckman
Benchmark™ Plus microplate spectrophotometer	Bio-Rad
BioPhotometer	Eppendorf
Centrifuge 5415D and 5471R	Eppendorf
Gallenkamp shaking water bath	Sanyo
HB-1000 hybridisation oven	Ultra-Violet Products
Heptovac VR-1 vacuum centrifuge	Thermo
IEC Centra-7R Centrifuge	Damon
Kodak Digital Science DC120 digital camera	Kodak
2011 Macrovue™ UV Transilluminator	Ultra-Violet Products
Mk II incubator	LEEC
MxPro-Mx3005P® real-time PCR machine	Stratagene
PCR Express® thermal cycler	Hybaid
Polytron Ultra-Turrax® T25 electric homogenizer	Janke & Kunkel
PRODIGY Advance Lunar Densitometer	GE Healthcare
Rocker	Bibby Sterilin
Sequence Detector v1.7a	Applied Biosystems
Vari-Shaker plate shaker	Dynatech
Water bath	Grant Instruments
Zeiss Axiovert 200M inverted microscope	Hitachi

Software

Beacon Designer 4.02 software	Premier Biosoft
enCORE 2004 software	GE Healthcare
Excel 2003	Microsoft
Graph Pad Prism 5 for Windows	Microsoft
Kodak digital Science ID 1 image analysis software	Kodak
Microplate Manager v5.2 software	Bio-Rad

MxPro-Mx3005P software	Stratagene
Primer Express® software	Applied Biosystems
Primer Premier 5 software	Premier Biosoft
Word 2003	Microsoft

Consumables

Corning filter system (0.22 µm membrane)	Fisher
Nescofilm® membrane	Fisher
Nunc Maxisorp™ 96-well EIA microplates	Fisher
Real-time PCR plates	ABgene
Uvette® plastic disposable cuvettes	Eppendorf
Eppendorfs	StarLab
Pipette tips	Anachem
100 µm filter membrane	Fisher
Universals white topped	Fisher

Addresses & URLs

ABgene - ABgene House, Blenheim Road, Epsom UK http://www.abgene.com

Ambion Ltd – Spitfire close, Ermine Business Park, Huntingdon, Cambridgeshire UK www.ambion.com

Anachem Ltd – Anachem House, Luton, Bedfordshire, UK http://www.anachem.co.uk

Antec International - DuPont Animal Health Solutions, Chilton Industrial Estate, Sudbury, Suffolk, UK http://www.antecint.co.uk

Applied Biosystems UK Ltd – Kelvin Close, Birchwood Science Park North, Warrington, Cheshire UK http://www.appliedbiosystems.com

Beckman Instruments Inc – 4300 North Harbour Blvd Fullerton, CA, USA http://www.beckmancoulter.com

BDH Lab Supplies – Poole, UK http://www.uk.vwr.com

Bibby Sterilin Ltd – Stone, Staffordshire, UK

Bio-Rad Laboratories Ltd – Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, UK http://www.bio-rad.com

Biosera - 1 Acorn House, The Broyle, Ringmer East Sussex, UK http://www.biosera.com

BOC gases - The Priestley Centre, 10 Priestley Road, Surrey Research Park, Guildford, Surrey UK http://www.boc-gases.com

Dako UK Ltd - Denmark House Angel Drove, Ely Cambridgeshire, UK www.dako.co.uk

Damon – Global Medical Instrumentation Inc Minnesota, USA http://www.gmi-inc.com/index.htm

Dynatech – Guernsey, UK. http://www.dynatechlaboratories.com

Eppendorf UK Ltd – Endurance House, Chivers Way, Histon, Cambridge, UK http://www.eppendorf.co.uk

Eurogentec Ltd – P.C. House, 2 Sough Street, Hythe, Southampton, Hampshire, UK http://uk.eurogentec.com

Fermentas Inc – 789 Cromwell Park Drive, Suites R-S Glen Burnie, MD USA http://www.fermentas.com

Fisher Scientific – Bishop Meadow Road, Loughborough, Leicestershire, UK http://www.fisher.co.uk

GE Healthcare – Pollards Wood, Nightingales Lane, Chalfont St. Giles BUCKS, UK http://www.gehealthcare.com

GlaxoSmithKline – Glaxo Welcome UK Ltd., Stockely Park West, Uxbridge, Middlesex, UK http://www.gsk.com

Grant Instruments (Cambridge) Ltd – Shepreth, Royston, Hertfordshire, UK http://www.applegate.co.uk

HD Supplies - 44 Rabans Close Rabans Lane Industrial Estate Aylesbury, Bucks UK http://www.hdsupplies.net

Hitachi High-Technologies - 7 Ivanhoe Road, Hogwood Lane Ind Estate, Finchampstead, Wokingham, Berkshire UK http://www.hitachi-hitech.co.uk

Hybaid - Medical Foundation Building 92-94 Parramatta Road Camperdown NSW http://www.bosch.org.au

Invitrogen Ltd – 3 Fountain Drive, Inchinnan Business Park, Paisley, UK http://www.invitrogen.com

Janke & Kunkel - Janke-&-Kunkel-Str. 10 Germany http://www.ika.net

Kodak Ltd – P.O. Box 66, Station Road, Hemel Hempstead, Hertfordshire, UK http://www.kodak.com

LEEC – Private Road No. 7, Colwick Industrial Estate, Nottingham, UK http://www.leec.co.uk

Macherey-Nagel GmbH & Co. KG – P.O. Box 10 13 P52, 52313 Düren, Germany. http//www.mn-net.com

Microsoft Corporation – One Microsoft Way, Redmond, WA, USA http://www.microsoft.com

Molecular BioProd Inc – San Diego, CA USA.

Pepro Tech EC – PeproTech House, 29 Margravine Road, London UK http://www.peprotechec.com

Premire Biosoft International – 3786 Corina Way, Palo Alto, CA, USA http://www.PremierBiosoft.com

Promega - Promega, Delta House Southampton Science Park Southampton, UK http://www.promega.com

Qbiogene - MP Biomedicals Europe Parc d'Innovation, Ilkirch France http://www.mpbio.com

R&D Systems – 4-10 The Quadrant, Barton Lane, Abingdon, UK http://www.rndsystems.com

Sanyo - 9 The Office Village, North Road, Loughborough Leicestershire http://www.sanyo-biomedical.co.uk

Sigma-Aldrich Company Ltd – Fancy Road, Poole, Dorset, UK http://www.sigmaaldrich.com

StarLab – 9 Tanners Drive, Blakelands, Milton Keynes, UK http://www.starlab.de/en/

Stratagene – 11011 N. Torrey Pines Road La Jolla, CA. http://www.stratagene.com

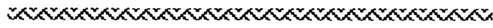
Thermo Electron Corporation – Unit 5, The Ringway Centre, Edison Road, Basingstoke, Hampshire, UK http://www.thermo.com

Tocris Cookson Ltd – Tocris Bioscience Tocris House Hung Road, Bristol UK http://www.tocris.com

Ultra-Violet Products Ltd – Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge, UK http://www.applegate.co.uk

VWR International Ltd – Poole, UK http://uk.vwr.com

References



Abbasi F, Chu JW, Lamendola C, McLaughlin T, Hayden J, Reaven GM & Reaven PD (2004) Discrimination between obesity and insulin resistance in the relationship with adiponectin. *Diabetes* **53**, 585-590.

Ahima RS (2006) Adipose tissue as an endocrine organ. *Obesity (Silver Spring)* **14 Suppl 5**, 242S-249S.

Ahima RS & Flier JS (2000) Adipose tissue as an endocrine organ. *Trends Endocrinol Metab* **11**, 327-332.

Ahima RS & Osei SY (2004) Leptin signaling. *Physiol Behav* **81**, 223-241.

Ailhaud G (1982) Adipose cell differentiation in culture. *Mol Cell Biochem* **49**, 17-31.

Ailhaud G (1990) Extracellular factors, signalling pathways and differentiation of adipose precursor cells. *Curr Opin Cell Biol* **2**, 1043-1049.

Alberti L, Girola A, Gilardini L, Conti A, Cattaldo S, Micheletto G & Invitti C (2007) Type 2 diabetes and metabolic syndrome are associated with increased expression of 11beta-hydroxysteroid dehydrogenase 1 in obese subjects. *Int J Obes (Lond)*.

Andersen KK, Frystyk J, Wolthers OD, Heuck C & Flyvbjerg A (2007) Gender differences of oligomers and total adiponectin during puberty: a cross-sectional study of 859 Danish school children. *J Clin Endocrinol Metab* **92**, 1857-1862.

Andrews RC & Walker BR (1999) Glucocorticoids and insulin resistance: old hormones, new targets. *Clin Sci (Lond)* **96**, 513-523.

Appleton DJ, Rand JS & Sunvold GD (2000) Plasma leptin concentrations in cats: reference range, effect of weight gain and relationship with adiposity as measured by dual energy X-ray absorptiometry. *J Feline Med Surg* **2**, 191-199.

Arch JR, Stock MJ & Trayhurn P (1998) Leptin resistance in obese humans: does it exist and what does it mean? *Int J Obes Relat Metab Disord* **22**, 1159-1163.

Arici A, Senturk LM, Seli E, Bahtiyar MO & Kim G (1999) Regulation of monocyte chemotactic protein-1 expression in human endometrial stromal cells by estrogen and progesterone. *Biol Reprod* **61**, 85-90.

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T & Matsuzawa Y (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* **257**, 79-83.

Aronne LJ (2007) Therapeutic options for modifying cardiometabolic risk factors. *Am J Med* **120**, S26-34.

Aubert J, Darimont C, Safonova I, Ailhaud G & Negrel R (1997) Regulation by glucocorticoids of angiotensinogen gene expression and secretion in adipose cells. *Biochem J* **328 (Pt 2)**, 701-706.

Aubert J, Safonova I, Negrel R & Ailhaud G (1998) Insulin down-regulates angiotensinogen gene expression and angiotensinogen secretion in cultured adipose cells. *Biochem Biophys Res Commun* **250**, 77-82.

Avram AS, Avram MM & James WD (2005) Subcutaneous fat in normal and diseased states: 2. Anatomy and physiology of white and brown adipose tissue. *J Am Acad Dermatol* **53**, 671-683.

Ayachi SE, Paulmyer-Lacroix O, Verdier M, Alessi MC, Dutour A & Grino M (2006) 11beta-Hydroxysteroid dehydrogenase type 1-driven cortisone reactivation regulates plasminogen activator inhibitor type 1 in adipose tissue of obese women. *J Thromb Haemost* **4**, 621-627.

Ayata E, Yumuk V, Gurbu U, Izmir S, Samanci T, Osar Z, Damci T, Ozyazar M, Gorpe U (2000) Obesity: A heavy burden on type 2 diabetes mellitus. *Diabetes Res and Clin Prac* **50**, 123-123(1)

Bach JF, Rozanski EA, Bedenice D, Chan DL, Freeman LM, Lofgren JL, Oura TJ & Hoffman AM (2007) Association of expiratory airway dysfunction with marked obesity in healthy adult dogs. *Am J Vet Res* **68**, 670-675.

Backhed F, Ding H, Wang T, Hooper L, Young Koh G, Nagy A, Semenkovich C & Gordon J (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A* **101**, 15718-15723.

Bader T, Zoumakis E, Friedberg M, Hiroi N, Chrousos GP & Hochberg Z (2002) Human adipose tissue under in vitro inhibition of 11beta-hydroxysteroid dehydrogenase type 1: differentiation and metabolism changes. *Horm Metab Res* **34**, 752-757.

Bailhache E, Nguyen P, Krempf M, Siliart B, Magot T & Ouguerram K (2003) Lipoproteins abnormalities in obese insulin-resistant dogs. *Metabolism* **52**, 559-564.

Banerjee D & Misra A (2007) Does using ethnic specific criteria improve the usefulness of the term metabolic syndrome? controversies and suggestions. *Int J Obes (Lond)* **31**, 1340-1349.

Banks WA, DiPalma CR & Farrell CL (1999) Impaired transport of leptin across the blood-brain barrier in obesity. *Peptides* **20**, 1341-1345.

Banks WA, Kastin AJ, Huang W, Jaspan JB & Maness LM (1996) Leptin enters the brain by a saturable system independent of insulin. *Peptides* **17**, 305-311.

Barnum SR & Volanakis JE (1985) In vitro biosynthesis of complement protein D by U937 cells. *J Immunol* **134**, 1799-1803.

Bartness TJ & Bamshad M (1998) Innervation of mammalian white adipose tissue: implications for the regulation of total body fat. *Am J Physiol* **275**, R1399-1411.

Barzilai N, Wang J, Massilon D, Vuguin P, Hawkins M & Rossetti L (1997) Leptin selectively decreases visceral adiposity and enhances insulin action. *J Clin Invest* **100**, 3105-3110.

Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, Vidal H & Hainque B (2000) Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab* **85**, 3338-3342.

Bastard JP, Maachi M, Van Nhieu JT, Jardel C, Bruckert E, Grimaldi A, Robert JJ, Capeau J & Hainque B (2002) Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. *J Clin Endocrinol Metab* **87**, 2084-2089.

Bauche IB, El Mkadem SA, Pottier AM, Senou M, Many MC, Rezsohazy R, Penicaud L, Maeda N, Funahashi T & Brichard SM (2007) Overexpression of adiponectin targeted to adipose tissue in transgenic mice: impaired adipocyte differentiation. *Endocrinology* **148**, 1539-1549.

Beattie JH, Wood AM, Trayhurn P, Jasani B, Vincent A, McCormack G & West AK (2000) Metallothionein is expressed in adipocytes of brown fat and is induced by catecholamines and zinc. *Am J Physiol Regul Integr Comp Physiol* **278**, R1082-1089.

Beck B (2000) Neuropeptides and obesity. *Nutrition* **16**, 916-923.

Berger J, Tanen M, Elbrecht A, Hermanowski-Vosatka A, Moller DE, Wright SD & Thieringer R (2001) Peroxisome proliferator-activated receptor-gamma ligands inhibit adipocyte 11beta -hydroxysteroid dehydrogenase type 1 expression and activity. *J Biol Chem* **276**, 12629-12635.

Bergman RN, Kim SP, Hsu IR, Catalano KJ, Chiu JD, Kabir M, Richey JM & Ader M (2007) Abdominal obesity: role in the pathophysiology of metabolic disease and cardiovascular risk. *Am J Med* **120**, S3-8; discussion S29-32.

Berndt J, Kloting N, Kralisch S, Kovacs P, Fasshauer M, Schon MR, Stumvoll M & Bluher M (2005) Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. *Diabetes* **54**, 2911-2916.

Bevier DE (1990) Long-term management of atopic disease in the dog. *Vet Clin North Am Small Anim Pract* **20**, 1487-1507.

Bing C, Bao Y, Jenkins J, Sanders P, Manieri M, Cinti S, Tisdale MJ & Trayhurn P (2004) Zinc-alpha2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia. *Proc Natl Acad Sci U S A* **101**, 2500-2505.

Blum WF, Englaro P, Hanitsch S, Juul A, Hertel NT, Muller J, Skakkebaek NE, Heiman ML, Birkett M, Attanasio AM, Kiess W & Rascher W (1997) Plasma leptin levels in healthy children and adolescents: dependence on body mass index, body fat mass, gender, pubertal stage, and testosterone. *J Clin Endocrinol Metab* **82**, 2904-2910.

Bouloumie A, Curat CA, Sengenes C, Lolmede K, Miranville A & Busse R (2005) Role of macrophage tissue infiltration in metabolic diseases. *Curr Opin Clin Nutr Metab Care* **8**, 347-354.

Brakenhielm E, Veitonmaki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, Funahashi T & Cao Y (2004) Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. *Proc Natl Acad Sci U S A* **101**, 2476-2481.

Braun B, Zimmermann MB & Kretchmer N (1996) Relationships between glucose metabolism and thermogenesis with and without prior exercise in obese women with non-insulin-dependent diabetes mellitus. *Metabolism* **45**, 747-752.

Bray GA (2004) Obesity is a chronic, relapsing neurochemical disease. *Int J Obes Relat Metab Disord* **28**, 34-38.

Bremner I & Beattie JH (1990) Metallothionein and the trace minerals. *Annu Rev Nutr* **10**, 63-83.

Brockmann GA & Bevova MR (2002) Using mouse models to dissect the genetics of obesity. *Trends Genet* **18**, 367-376.

Brunson BL, Zhong Q, Clarke KJ, Bedi D, Braden TD, van Santen E & Judd RL (2007) Serum concentrations of adiponectin and characterization of adiponectin protein complexes in dogs. *Am J Vet Res* **68**, 57-62.

Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A & Richelsen B (2003a) Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *Am J Physiol Endocrinol Metab* **285**, E527-533.

Bruun JM, Pedersen SB & Richelsen B (2001) Regulation of interleukin 8 production and gene expression in human adipose tissue in vitro. *J Clin Endocrinol Metab* **86**, 1267-1273.

Bruun JM, Verdich C, Toubro S, Astrup A & Richelsen B (2003b) Association between measures of insulin sensitivity and circulating levels of interleukin-

8, interleukin-6 and tumor necrosis factor-alpha. Effect of weight loss in obese men. *Eur J Endocrinol* **148**, 535-542.

Buffington CA (1994) Management of obesity--the clinical nutritionist's experience. *Int J Obes Relat Metab Disord* **18 Suppl 1**, S29-35.

Bujalska IJ, Kumar S, Hewison M & Stewart PM (1999) Differentiation of adipose stromal cells: the roles of glucocorticoids and 11beta-hydroxysteroid dehydrogenase. *Endocrinology* **140**, 3188-3196.

Bujalska IJ, Kumar S & Stewart PM (1997) Does central obesity reflect "Cushing's disease of the omentum"? *Lancet* **349**, 1210-1213.

Bullo M, Peeraully MR & Trayhurn P (2005) Stimulation of NGF expression and secretion in 3T3-L1 adipocytes by prostaglandins PGD2, PGJ2, and {Delta}12-PGJ2. *Am J Physiol Endocrinol Metab* **289**, E62-67.

Calle EE & Thun MJ (2004) Obesity and cancer. *Oncogene* **23**, 6365-6378.

Campfield LA, Smith FJ, Guisez Y, Devos R & Burn P (1995) Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* **269**, 546-549.

Cannon B & Nedergaard J (2004) Brown adipose tissue: function and physiological significance. *Physiol Rev* **84**, 277-359.

Cao YL, Wang YX, Wang DF, Meng X & Zhang J (2007) Correlation between omental TNF-alpha protein and plasma PAI-1 in obesity subjects. *Int J Cardiol.*

Carr MC & Brunzell JD (2004) Abdominal obesity and dyslipidemia in the metabolic syndrome: importance of type 2 diabetes and familial combined hyperlipidemia in coronary artery disease risk. *J Clin Endocrinol Metab* **89**, 2601-2607.

Carriere A, Carmona MC, Fernandez Y, Rigoulet M, Wenger RH, Penicaud L & Casteilla L (2004) Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: a mechanism for hypoxia-dependent effect. *J Biol Chem* **279**, 40462-40469.

Carroll K, Gomez C & Shapiro L (2004) Tubby proteins: the plot thickens. *Nat Rev Mol Cell Biol* **5**, 55-63.

Carulli L, Ferrari S, Bertolini M, Tagliafico E & Del Rio G (1999) Regulation of ob gene expression: evidence for epinephrine-induced suppression in human obesity. *J Clin Endocrinol Metab* **84**, 3309-3312.

Castracane VD, Kraemer RR, Franken MA, Kraemer GR & Gimpel T (1998) Serum leptin concentration in women: effect of age, obesity, and estrogen administration. *Fertil Steril* **70**, 472-477.

Champigny O, Ricquier D, Blondel O, Mayers RM, Briscoe MG & Holloway BR (1991) Beta 3-adrenergic receptor stimulation restores message and expression of brown-fat mitochondrial uncoupling protein in adult dogs. *Proc Natl Acad Sci U S A* **88**, 10774-10777.

Chandran M, Phillips SA, Ciaraldi T & Henry RR (2003) Adiponectin: more than just another fat cell hormone? *Diabetes Care* **26**, 2442-2450.

Chang TH & Polakis SE (1978) Differentiation of 3T3-L1 fibroblasts to adipocytes. Effect of insulin and indomethacin on the levels of insulin receptors. *J Biol Chem* **253**, 4693-4696.

Chao MV & Hempstead BL (1995) p75 and Trk: a two-receptor system. *Trends Neurosci* **18**, 321-326.

Chapman KE, Gilmour JS, Coutinho AE, Savill JS & Seckl JR (2006) 11Beta-hydroxysteroid dehydrogenase type 1--a role in inflammation? *Mol Cell Endocrinol* **248**, 3-8.

Chase K, Carrier DR, Adler FR, Jarvik T, Ostrander EA, Lorentzen TD & Lark KG (2002) Genetic basis for systems of skeletal quantitative traits: principal component analysis of the canid skeleton. *Proc Natl Acad Sci U S A* **99**, 9930-9935.

Chehab FF, Lim ME & Lu R (1996) Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* **12**, 318-320.

Chen A, Mumick S, Zhang C, Lamb J, Dai H, Weingarth D, Mudgett J, Chen H, MacNeil DJ, Reitman ML & Qian S (2005) Diet induction of monocyte chemoattractant protein-1 and its impact on obesity. *Obes Res* **13**, 1311-1320.

Chen B, Lam KS, Wang Y, Wu D, Lam MC, Shen J, Wong L, Hoo RL, Zhang J & Xu A (2006) Hypoxia dysregulates the production of adiponectin and plasminogen activator inhibitor-1 independent of reactive oxygen species in adipocytes. *Biochem Biophys Res Commun* **341**, 549-556.

Chiellini C, Bertacca A, Novelli SE, Gorgun CZ, Ciccarone A, Giordano A, Xu H, Soukas A, Costa M, Gandini D, Dimitri R, Bottone P, Cecchetti P, Pardini E, Perego L, Navalesi R, Folli F, Benzi L, Cinti S, Friedman JM, Hotamisligil GS & Maffei M (2002) Obesity modulates the expression of haptoglobin in the white adipose tissue via TNFalpha. *J Cell Physiol* **190**, 251-258.

Chua SC, Jr., Chung WK, Wu-Peng XS, Zhang Y, Liu SM, Tartaglia L & Leibel RL (1996) Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* **271**, 994-996.

Church TS, Willis MS, Priest EL, Lamonte MJ, Earnest CP, Wilkinson WJ, Wilson DA & Giroir BP (2005) Obesity, macrophage migration inhibitory factor, and weight loss. *Int J Obes Relat Metab Disord* **29**, 675-681.

Cianflone K, Maslowska M & Sniderman AD (1999) Acylation stimulating protein (ASP), an adipocyte autocrine: new directions. *Semin Cell Dev Biol* **10**, 31-41.

Cigolini M, Tonoli M, Borgato L, Frigotto L, Manzato F, Zeminian S, Cardinale C, Camin M, Chiaramonte E, De Sandre G & Lunardi C (1999) Expression of plasminogen activator inhibitor-1 in human adipose tissue: a role for TNF-alpha? *Atherosclerosis* **143**, 81-90.

Cinti S (2001) *Morphology of the Adipose Organ*, 1st ed. Texas: Eurekah.com / Landes Bioscience.

Cinti S (2005) The adipose organ. *Prostaglandins Leukot Essent Fatty Acids* **73**, 9-15.

Cinti S, Matteis RD, Pico C, Ceresi E, Obrador A, Maffeis C, Oliver J & Palou A (2000) Secretory granules of endocrine and chief cells of human stomach mucosa contain leptin. *Int J Obes Relat Metab Disord* **24**, 789-793.

Clegg DJ, Brown LM, Woods SC & Benoit SC (2006) Gonadal hormones determine sensitivity to central leptin and insulin. *Diabetes* **55**, 978-987.

Clement K, Garner C, Hager J, Philippi A, LeDuc C, Carey A, Harris TJ, Jury C, Cardon LR, Basdevant A, Demenais F, Guy-Grand B, North M & Froguel P (1996) Indication for linkage of the human OB gene region with extreme obesity. *Diabetes* **45**, 687-690.

Colliard L, Ancel J, Benet JJ, Paragon BM & Blanchard G (2006) Risk factors for obesity in dogs in France. *J Nutr* **136**, 1951S-1954S.

Considine RV, Nyce MR, Kolaczynski JW, Zhang PL, Ohannesian JP, Moore JH, Jr., Fox JW & Caro JF (1997) Dexamethasone stimulates leptin release from human adipocytes: unexpected inhibition by insulin. *J Cell Biochem* **65**, 254-258.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL & et al. (1996) Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* **334**, 292-295.

Coppack SW (2001) Pro-inflammatory cytokines and adipose tissue. *Proc Nutr Soc* **60**, 349-356.

Cousin B, Munoz O, Andre M, Fontanilles AM, Dani C, Cousin JL, Laharrague P, Casteilla L & Penicaud L (1999) A role for preadipocytes as macrophage-like cells. *Faseb J* **13**, 305-312.

Creeley SJ, McTernan PG, Kusminski CM, Fisher M, Da Silva NF, Khanolkar M, Evans M, Harte AL & Kumar S (2007) Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab* **292**, E740-747.

Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R & Bouloumie A (2004) From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* **53**, 1285-1292.

Dahlman I, Kaaman M, Olsson T, Tan GD, Bickerton AS, Wahlen K, Andersson J, Nordstrom EA, Blomqvist L, Sjogren A, Forsgren M, Attersand A & Arner P (2005) A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects. *J Clin Endocrinol Metab* **90**, 5834-5840.

Dandona P, Aljada A, Ghanim H, Mohanty P, Tripathy C, Hofmeyer D & Chaudhuri A (2004) Increased plasma concentration of macrophage migration inhibitory factor (MIF) and MIF mRNA in mononuclear cells in the obese and the suppressive action of metformin. *J Clin Endocrinol Metab* **89**, 5043-5047.

Dani C (2006) [Stem cells from human adipose tissue: a new tool for pharmacological studies and for clinical applications]. *J Soc Biol* **200**, 45-50.

Das UN (2001) Is obesity an inflammatory condition? *Nutrition* **17**, 953-966.

de Romo AC (2007) Chance, creativity, and the discovery of the nerve growth factor. *J Hist Neurosci* **16**, 268-287.

De Sousa Peixoto RA, Turban S, Battle JH, Chapman KE, Seckl JR & Morton NM (2008) Preadipocyte 11{beta}-hydroxysteroid dehydrogenase type 1 is a keto-reductase and contributes to diet-induced visceral obesity in vivo. *Endocrinology*.

De Vos P, Lefebvre AM, Miller SG, Guerre-Millo M, Wong K, Saladin R, Hamann LG, Staels B, Briggs MR & Auwerx J (1996) Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma. *J Clin Invest* **98**, 1004-1009.

De Vos P, Saladin R, Auwerx J & Staels B (1995) Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. *J Biol Chem* **270**, 15958-15961.

Degawa-Yamauchi M, Moss KA, Bovenkerk JE, Shankar SS, Morrison CL, Lelliott CJ, Vidal-Puig A, Jones R & Considine RV (2005) Regulation of adiponectin expression in human adipocytes: effects of adiposity, glucocorticoids, and tumor necrosis factor alpha. *Obes Res* **13**, 662-669.

Di Gregorio GB, Yao-Borengasser A, Rasouli N, Varma V, Lu T, Miles LM, Ranganathan G, Peterson CA, McGehee RE & Kern PA (2005) Expression of CD68 and macrophage chemoattractant protein-1 genes in human adipose and muscle tissues: association with cytokine expression, insulin resistance, and reduction by pioglitazone. *Diabetes* **54**, 2305-2313.

Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T & Kunos G (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* **410**, 822-825.

Dieudonne MN, Sammari A, Dos Santos E, Leneveu MC, Giudicelli Y & Pecquery R (2006) Sex steroids and leptin regulate 11beta-hydroxysteroid dehydrogenase I and P450 aromatase expressions in human preadipocytes: Sex specificities. *J Steroid Biochem Mol Biol* **99**, 189-196.

Diez JJ & Iglesias P (2003) The role of the novel adipocyte-derived hormone adiponectin in human disease. *Eur J Endocrinol* **148**, 293-300.

Do MS, Nam SY, Hong SE, Kim KW, Duncan JS, Beattie JH & Trayhurn P (2002) Metallothionein gene expression in human adipose tissue from lean and obese subjects. *Horm Metab Res* **34**, 348-351.

do Nascimento CO, Hunter L & Trayhurn P (2004) Regulation of haptoglobin gene expression in 3T3-L1 adipocytes by cytokines, catecholamines, and PPARgamma. *Biochem Biophys Res Commun* **313**, 702-708.

Doerrler W, Feingold KR & Grunfeld C (1994) Cytokines induce catabolic effects in cultured adipocytes by multiple mechanisms. *Cytokine* **6**, 478-484.

Donoghue S, Khoo L, Glickman LT & Kronfeld DS (1991) Body condition and diet of relatively healthy older dogs. *J Nutr* **121**, S58-59.

Dullaart RP, de Vries R, van Tol A & Sluiter WJ (2007) Lower plasma adiponectin is a marker of increased intima-media thickness associated with type 2 diabetes mellitus and with male gender. *Eur J Endocrinol* **156**, 387-394.

Dulloo AG & Samec S (2000) Uncoupling Proteins: Do They Have a Role in Body Weight Regulation? *News Physiol Sci* **15**, 313-318.

Ebihara K, Ogawa Y, Masuzaki H, Shintani M, Miyanaga F, Aizawa-Abe M, Hayashi T, Hosoda K, Inoue G, Yoshimasa Y, Gavrilova O, Reitman ML & Nakao K (2001) Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. *Diabetes* **50**, 1440-1448.

Edney AT & Smith PM (1986) Study of obesity in dogs visiting veterinary practices in the United Kingdom. *Vet Rec* **118**, 391-396.

Eisele I, Wood IS, German AJ, Hunter L & Trayhurn P (2005) Adipokine gene expression in dog adipose tissues and dog white adipocytes differentiated in primary culture. *Horm Metab Res* **37**, 474-481.

EMEA (2007) European public assessment report for autorrised medicinal products for veterinary use.

Emilsson V, Liu YL, Cawthorne MA, Morton NM & Davenport M (1997) Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion. *Diabetes* **46**, 313-316.

Engeli S, Bohnke J, Gorzelniak K, Janke J, Schling P, Bader M, Luft FC & Sharma AM (2005) Weight loss and the renin-angiotensin-aldosterone system. *Hypertension* **45**, 356-362.

Engeli S, Feldpausch M, Gorzelniak K, Hartwig F, Heintze U, Janke J, Mohlig M, Pfeiffer AF, Luft FC & Sharma AM (2003a) Association between adiponectin and mediators of inflammation in obese women. *Diabetes* **52**, 942-947.

Engeli S, Schling P, Gorzelniak K, Boschmann M, Janke J, Ailhaud G, Teboul M, Massiera F & Sharma AM (2003b) The adipose-tissue renin-angiotensin-aldosterone system: role in the metabolic syndrome? *Int J Biochem Cell Biol* **35**, 807-825.

Engfeldt P & Arner P (1988) Lipolysis in human adipocytes, effects of cell size, age and of regional differences. *Horm Metab Res Suppl* **19**, 26-29.

Espelund U, Hansen TK, Hojlund K, Beck-Nielsen H, Clausen JT, Hansen BS, Orskov H, Jorgensen JO & Frystyk J (2005) Fasting Unmasks a Strong Inverse Association between Ghrelin and Cortisol in Serum: Studies in Obese and Normal-Weight Subjects. *J Clin Endocrinol Metab* **90**, 741-746.

Esposito K, Pontillo A, Ciotola M, Di Palo C, Grella E, Nicoletti G & Giugliano D (2002) Weight loss reduces interleukin-18 levels in obese women. *J Clin Endocrinol Metab* **87**, 3864-3866.

Ettinger SJ & Feldman EC (2000) *Textbook of Veterinary Internal Medicine*, 5th ed: WB Saunders.

Faggioni R, Fantuzzi G, Fuller J, Dinarello CA, Feingold KR & Grunfeld C (1998) IL-1 beta mediates leptin induction during inflammation. *Am J Physiol* **274**, R204-208.

Fain JN, Cheema PS, Bahouth SW & Lloyd Hiler M (2003) Resistin release by human adipose tissue explants in primary culture. *Biochem Biophys Res Commun* **300**, 674-678.

Fain JN & Madan AK (2005) Insulin enhances vascular endothelial growth factor, interleukin-8, and plasminogen activator inhibitor 1 but not interleukin-6 release by human adipocytes. *Metabolism* **54**, 220-226.

Fain JN, Madan AK, Hiler ML, Cheema P & Bahouth SW (2004) Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* **145**, 2273-2282.

Fain JN, Tichansky DS & Madan AK (2005) Transforming growth factor beta1 release by human adipose tissue is enhanced in obesity. *Metabolism* **54**, 1546-1551.

Fantuzzi G (2005) Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol* **115**, 911-919; quiz 920.

Farley-Hills E, Sivasankar R & Martin M (2004) Fatal liver failure associated with pioglitazone. *Bmj* **329**, 429.

Farmer SR (2005) Regulation of PPARgamma activity during adipogenesis. *Int J Obes Relat Metab Disord* **29 Suppl 1**, S13-16.

Fasshauer M, Klein J, Lossner U & Paschke R (2003a) Interleukin (IL)-6 mRNA expression is stimulated by insulin, isoproterenol, tumour necrosis factor alpha, growth hormone, and IL-6 in 3T3-L1 adipocytes. *Horm Metab Res* **35**, 147-152.

Fasshauer M, Klein J, Neumann S, Eszlinger M & Paschke R (2002) Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* **290**, 1084-1089.

Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J & Paschke R (2003b) Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* **301**, 1045-1050.

Fasshauer M & Paschke R (2003) Regulation of adipocytokines and insulin resistance. *Diabetologia* **46**, 1594-1603.

Feldhahn JR, Rand JS & Martin G (1999) Insulin sensitivity in normal and diabetic cats. *J Feline Med Surg* **1**, 107-115.

Filozof C, Gonzalez C, Sereday M, Mazza C & Braguinsky J (2001) Obesity prevalence and trends in Latin-American countries. *Obes Rev* **2**, 99-106.

Fisher FM, McTernan PG, Valsamakis G, Chetty R, Harte AL, Anwar AJ, Starcynski J, Crocker J, Barnett AH, McTernan CL & Kumar S (2002) Differences in adiponectin protein expression: effect of fat depots and type 2 diabetic status. *Horm Metab Res* **34**, 650-654.

Flower L, Gray R, Pinkney J & Mohamed-Ali V (2003) Stimulation of interleukin-6 release by interleukin-1beta from isolated human adipocytes. *Cytokine* **21**, 32-37.

Fontana L & Klein S (2007) Aging, adiposity, and calorie restriction. *Jama* **297**, 986-994.

Frade JM & Barde YA (1998) Nerve growth factor: two receptors, multiple functions. *Bioessays* **20**, 137-145.

Frayn KN (2003) *Metabolic Regulation A Human Perspective*, 2nd ed: Blackwell Publishing.

Fried SK, Bunkin DA & Greenberg AS (1998) Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab* **83**, 847-850.

Fried SK, Russell CD, Grauso NL & Brolin RE (1993) Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. *J Clin Invest* **92**, 2191-2198.

Friedberg M, Zoumakis E, Hiroi N, Bader T, Chrousos GP & Hochberg Z (2003) Modulation of 11 beta-hydroxysteroid dehydrogenase type 1 in mature human subcutaneous adipocytes by hypothalamic messengers. *J Clin Endocrinol Metab* **88**, 385-393.

Fruhbeck G, Aguado M & Martinez JA (1997) In vitro lipolytic effect of leptin on mouse adipocytes: evidence for a possible autocrine/paracrine role of leptin. *Biochem Biophys Res Commun* **240**, 590-594.

Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ & Burrell MA (2001) The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* **280**, E827-847.

Fruhbeck G, Jebb SA & Prentice AM (1998) Leptin: physiology and pathophysiology. *Clin Physiol* **18**, 399-419.

Fuenzalida KM, Aguilera MC, Piderit DG, Ramos PC, Contador D, Quinones V, Rigotti A, Bronfman FC & Bronfman M (2005) Peroxisome proliferator-activated receptor gamma is a novel target of the nerve growth factor signaling pathway in PC12 cells. *J Biol Chem* **280**, 9604-9609.

Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y & Shimomura I (2005) Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* **307**, 426-430.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M & Shimomura I (2004) Increased

oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* **114**, 1752-1761.

Gabay C & Kushner I (1999) Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* **340**, 448-454.

Gadient RA, Cron KC & Otten U (1990) Interleukin-1 beta and tumor necrosis factor-alpha synergistically stimulate nerve growth factor (NGF) release from cultured rat astrocytes. *Neurosci Lett* **117**, 335-340.

Galitzky J, Reverte M, Portillo M, Carpene C, Lafontan M & Berlan M (1993) Coexistence of beta 1-, beta 2-, and beta 3-adrenoceptors in dog fat cells and their differential activation by catecholamines. *Am J Physiol* **264**, E403-412.

Ganda OP (2000) Lipoatrophy, lipodystrophy, and insulin resistance. *Ann Intern Med* **133**, 304-306.

Gayet C, Bailhache E, Dumon H, Martin L, Siliart B & Nguyen P (2004) Insulin resistance and changes in plasma concentration of TNFalpha, IGF1, and NEFA in dogs during weight gain and obesity. *J Anim Physiol Anim Nutr (Berl)* **88**, 157-165.

Gayet C, Leray V, Saito M, Siliart B & Nguyen P (2007) The effects of obesity-associated insulin resistance on mRNA expression of peroxisome proliferator-activated receptor-gamma target genes, in dogs. *Br J Nutr* **98**, 497-503.

Gelfand EV & Cannon CP (2006) Rimonabant: a cannabinoid receptor type 1 blocker for management of multiple cardiometabolic risk factors. *J Am Coll Cardiol* **47**, 1919-1926.

Gerhardt CC, Romero IA, Cancello R, Camoin L & Strosberg AD (2001) Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. *Mol Cell Endocrinol* **175**, 81-92.

German AJ (2006) The growing problem of obesity in dogs and cats. *J Nutr* **136**, 1940S-1946S.

German AJ, Holden SL, Moxham GL, Holmes KL, Hackett RM & Rawlings JM (2006) A simple, reliable tool for owners to assess the body condition of their dog or cat. *J Nutr* **136**, 2031S-2033S.

Gettys TW, Harkness PJ & Watson PM (1996) The beta 3-adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat adipocytes. *Endocrinology* **137**, 4054-4057.

Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P & Dandona P (2004) Circulating mononuclear cells in the obese are in a proinflammatory state. *Circulation* **110**, 1564-1571.

Ghanim H, Dhindsa S, Aljada A, Chaudhuri A, Viswanathan P & Dandona P (2006) Low-dose rosiglitazone exerts an antiinflammatory effect with an increase in adiponectin independently of free fatty acid fall and insulin sensitization in obese type 2 diabetics. *J Clin Endocrinol Metab* **91**, 3553-3558.

Gillard ER, Khan AM, Ahsan ul H, Grewal RS, Mouradi B & Stanley BG (1997) Stimulation of eating by the second messenger cAMP in the perifornical and lateral hypothalamus. *Am J Physiol* **273**, R107-112.

Giordano A, Song CK, Bowers RR, Ehlen JC, Frontini A, Cinti S & Bartness TJ (2006) White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation. *Am J Physiol Regul Integr Comp Physiol* **291**, R1243-1255.

Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R & Mathieu C (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25**, 386-401.

Gorzelniak K, Janke J, Engeli S & Sharma AM (2001) Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. *Horm Metab Res* **33**, 625-627.

Gossellin J, Wren JA & Sunderland SJ (2007) Canine obesity: an overview. *J Vet Pharmacol Ther* **30 Suppl 1**, 1-10.

Green H & Meuth M (1974) An established pre-adipose cell line and its differentiation in culture. *Cell* **3**, 127-133.

Greenberg AS, Nordan RP, McIntosh J, Calvo JC, Scow RO & Jablons D (1992) Interleukin 6 reduces lipoprotein lipase activity in adipose tissue of mice in vivo and in 3T3-L1 adipocytes: a possible role for interleukin 6 in cancer cachexia. *Cancer Res* **52**, 4113-4116.

Gregoire FM (2001) Adipocyte differentiation: from fibroblast to endocrine cell. *Exp Biol Med (Maywood)* **226**, 997-1002.

Gregoire FM, Smas CM & Sul HS (1998) Understanding adipocyte differentiation. *Physiol Rev* **78**, 783-809.

Gualillo O, Eiras S, Lago F, Dieguez C & Casanueva FF (2000) Elevated serum leptin concentrations induced by experimental acute inflammation. *Life Sci* **67**, 2433-2441.

Guan XM, Hess JF, Yu H, Hey PJ & van der Ploeg LH (1997) Differential expression of mRNA for leptin receptor isoforms in the rat brain. *Mol Cell Endocrinol* **133**, 1-7.

Guo L, Zhang L, Sun Y, Muskhelishvili L, Blann E, Dial S, Shi L, Schroth G & Dragan YP (2006) Differences in hepatotoxicity and gene expression profiles

by anti-diabetic PPAR gamma agonists on rat primary hepatocytes and human HepG2 cells. *Mol Divers* **10**, 349-360.

Hakansson ML, Brown H, Ghilardi N, Skoda RC & Meister B (1998) Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. *J Neurosci* **18**, 559-572.

Hammarstedt A, Andersson CX, Rotter Sopasakis V & Smith U (2005) The effect of PPARgamma ligands on the adipose tissue in insulin resistance. *Prostaglandins Leukot Essent Fatty Acids* **73**, 65-75.

Hanif MW & Kumar S (2002) Pharmacological management of obesity. *Expert Opin Pharmacother* **3**, 1711-1718.

Harte A, McTernan P, Chetty R, Coppack S, Katz J, Smith S & Kumar S (2005) Insulin-mediated upregulation of the renin angiotensin system in human subcutaneous adipocytes is reduced by rosiglitazone. *Circulation* **111**, 1954-1961.

Haugen F, Jorgensen A, Drevon CA & Trayhurn P (2001) Inhibition by insulin of resistin gene expression in 3T3-L1 adipocytes. *FEBS Lett* **507**, 105-108.

Hauner H (1990) Complete adipose differentiation of 3T3 L1 cells in a chemically defined medium: comparison to serum-containing culture conditions. *Endocrinology* **127**, 865-872.

Hauner H, Entenmann G, Wabitsch M, Gaillard D, Ailhaud G, Negrel R & Pfeiffer EF (1989) Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J Clin Invest* **84**, 1663-1670.

Hauner H, Skurk T & Wabitsch M (2001) Cultures of human adipose precursor cells. *Methods Mol Biol* **155**, 239-247.

Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ & Martin RJ (2001) The biology of white adipocyte proliferation. *Obes Rev* **2**, 239-254.

Havel PJ (2001) Peripheral signals conveying metabolic information to the brain: short-term and long-term regulation of food intake and energy homeostasis. *Exp Biol Med (Maywood)* **226**, 963-977.

Hennigan A, O'Callaghan RM & Kelly AM (2007) Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. *Biochem Soc Trans* **35**, 424-427.

Hernandez-Morante JJ, Milagro FI, Larque E, Lujan J, Martinez JA, Zamora S & Garaulet M (2007) Relationship among adiponectin, adiponectin gene expression and fatty acids composition in morbidly obese patients. *Obes Surg* **17**, 516-524.

Hess RS, Kass PH, Shofer FS, Van Winkle TJ & Washabau RJ (1999) Evaluation of risk factors for fatal acute pancreatitis in dogs. *J Am Vet Med Assoc* **214**, 46-51.

Himms-Hagen J (1981) Obesity, thermogenesis and the sodium pump. *Nutr Rev* **39**, 30-31.

Himms-Hagen J (2001) Does brown adipose tissue (BAT) have a role in the physiology or treatment of human obesity? *Rev Endocr Metab Disord* **2**, 395-401.

Hirai K, Hussey HJ, Barber MD, Price SA & Tisdale MJ (1998) Biological evaluation of a lipid-mobilizing factor isolated from the urine of cancer patients. *Cancer Res* **58**, 2359-2365.

Hirokawa J, Sakaue S, Tagami S, Kawakami Y, Sakai M, Nishi S & Nishihira J (1997) Identification of macrophage migration inhibitory factor in adipose tissue and its induction by tumor necrosis factor-alpha. *Biochem Biophys Res Commun* **235**, 94-98.

Hoenig M (2002) Comparative aspects of diabetes mellitus in dogs and cats. *Mol Cell Endocrinol* **197**, 221-229.

Hoggard N, Hunter L, Duncan JS, Williams LM, Trayhurn P & Mercer JG (1997a) Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta. *Proc Natl Acad Sci U S A* **94**, 11073-11078.

Hoggard N, Mercer JG, Rayner DV, Moar K, Trayhurn P & Williams LM (1997b) Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and in situ hybridization. *Biochem Biophys Res Commun* **232**, 383-387.

Holmes KL, Morris PJ, Abdulla Z, Hackett R & Rawlings JM (2007) Risk factors associated with excess body weight in dogs in the UK *J Anim Physiol Anim Nutr (Berl)*.

Hossain P, Kawar B & El Nahas M (2007) Obesity and diabetes in the developing world--a growing challenge. *N Engl J Med* **356**, 213-215.

Hotamisligil GS (2003) Inflammatory pathways and insulin action. *Int J Obes Relat Metab Disord* **27 Suppl 3**, S53-55.

Hotamisligil GS (2006) Inflammation and metabolic disorders. *Nature* **444**, 860-867.

Hotamisligil GS, Arner P, Atkinson RL & Spiegelman BM (1997) Differential regulation of the p80 tumor necrosis factor receptor in human obesity and insulin resistance. *Diabetes* **46**, 451-455.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL & Spiegelman BM (1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* **95**, 2409-2415.

Hotamisligil GS, Murray DL, Choy LN & Spiegelman BM (1994) Tumor necrosis factor alpha inhibits signaling from the insulin receptor. *Proc Natl Acad Sci U S A* **91**, 4854-4858.

Hotamisligil GS, Shargill NS & Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91.

Howard JK & Flier JS (2006) Attenuation of leptin and insulin signaling by SOCS proteins. *Trends Endocrinol Metab* **17**, 365-371.

Hube F, Birgel M, Lee YM & Hauner H (1999) Expression pattern of tumour necrosis factor receptors in subcutaneous and omental human adipose tissue: role of obesity and non-insulin-dependent diabetes mellitus. *Eur J Clin Invest* **29**, 672-678.

Iguchi M, Aiba S, Yoshino Y & Tagami H (2001) Human follicular papilla cells carry out nonadipose tissue production of leptin. *J Invest Dermatol* **117**, 1349-1356.

Impellizeri JA, Tetrick MA & Muir P (2000) Effect of weight reduction on clinical signs of lameness in dogs with hip osteoarthritis. *J Am Vet Med Assoc* **216**, 1089-1091.

Ishii T, Masuzaki H, Tanaka T, Arai N, Yasue S, Kobayashi N, Tomita T, Noguchi M, Fujikura J, Ebihara K, Hosoda K & Nakao K (2007) Augmentation of 11beta-hydroxysteroid dehydrogenase type 1 in LPS-activated J774.1 macrophages--role of 11beta-HSD1 in pro-inflammatory properties in macrophages. *FEBS Lett* **581**, 349-354.

Ishioka K, Hatai H, Komabayashi K, Soliman MM, Shibata H, Honjoh T, Kimura K & Saito M (2005a) Diurnal variations of serum leptin in dogs: effects of fasting and re-feeding. *Vet J* **169**, 85-90.

Ishioka K, Hosoya K, Kitagawa H, Shibata H, Honjoh T, Kimura K & Saito M (2007) Plasma leptin concentration in dogs: Effects of body condition score, age, gender and breeds. *Res Vet Sci* **82**, 11-15.

Ishioka K, Omachi A, Sagawa M, Shibata H, Honjoh T, Kimura K & Saito M (2005b) Canine adiponectin: cDNA structure, mRNA expression in adipose tissues and reduced plasma levels in obesity. *Res Vet Sci.*

Ishioka K, Soliman MM, Sagawa M, Nakadomo F, Shibata H, Honjoh T, Hashimoto A, Kitamura H, Kimura K & Saito M (2002) Experimental and clinical studies on plasma leptin in obese dogs. *J Vet Med Sci* **64**, 349-353.

Isley WL (2003) Hepatotoxicity of thiazolidinediones. *Expert Opin Drug Saf* **2**, 581-586.

Iwase M, Kimura K, Komagome R, Sasaki N, Ishioka K, Honjoh T & Saito M (2000a) Sandwich enzyme-linked immunosorbent assay of canine leptin. *J Vet Med Sci* **62**, 207-209.

Iwase M, Kimura K, Sasaki N, Komagome R, Ishioka K, Morimatsu M, Murakami T & Saito M (2000b) Canine leptin: cDNA cloning, expression and activity of recombinant protein. *Res Vet Sci* **68**, 109-114.

Jager J, Gremeaux T, Cormont M, Le Marchand-Brustel Y & Tanti JF (2007) Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology* **148**, 241-251.

Jeusette I, Daminet S, Nguyen P, Shibata H, Saito M, Honjoh T, Istasse L & Diez M (2006) Effect of ovariectomy and ad libitum feeding on body composition, thyroid status, ghrelin and leptin plasma concentrations in female dogs. *J Anim Physiol Anim Nutr (Berl)* **90**, 12-18.

Jingjing L, Srinivasan B, Bian X, Downey HF & Roque RS (2000) Vascular endothelial growth factor is increased following coronary artery occlusion in the dog heart. *Mol Cell Biochem* **214**, 23-30.

Juge-Aubry CE, Somm E, Pernin A, Alizadeh N, Giusti V, Dayer JM & Meier CA (2005) Adipose tissue is a regulated source of interleukin-10. *Cytokine* **29**, 270-274.

Juhan-Vague I, Alessi MC & Vague P (1991) Increased plasma plasminogen activator inhibitor 1 levels. A possible link between insulin resistance and atherothrombosis. *Diabetologia* **34**, 457-462.

Kaisho T & Akira S (2002) Toll-like receptors as adjuvant receptors. *Biochim Biophys Acta* **1589**, 1-13.

Kallen CB & Lazar MA (1996) Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A* **93**, 5793-5796.

Kamohara S, Burcelin R, Halaas JL, Friedman JM & Charron MJ (1997) Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* **389**, 374-377.

Kappes A & Loffler G (2000) Influences of ionomycin, dibutyryl-cycloAMP and tumour necrosis factor-alpha on intracellular amount and secretion of apM1 in differentiating primary human preadipocytes. *Horm Metab Res* **32**, 548-554.

Kealy RD, Lawler DF, Ballam JM, Lust G, Biery DN, Smith GK & Mantz SL (2000) Evaluation of the effect of limited food consumption on radiographic evidence of osteoarthritis in dogs. *J Am Vet Med Assoc* **217**, 1678-1680.

Kealy RD, Lawler DF, Ballam JM, Mantz SL, Biery DN, Greeley EH, Lust G, Segre M, Smith GK & Stowe HD (2002) Effects of diet restriction on life span and age-related changes in dogs. *J Am Vet Med Assoc* **220**, 1315-1320.

Kearns CF, McKeever KH, Roegner V, Brady SM & Malinowski K (2006) Adiponectin and leptin are related to fat mass in horses. *Vet J* **172**, 460-465.

Keller P, Keller C, Steensberg A, Robinson LE & Pedersen BK (2005) Leptin gene expression and systemic levels in healthy men: effect of exercise, carbohydrate, interleukin-6, and epinephrine. *J Appl Physiol* **98**, 1805-1812.

Kelly DF & Darke PG (1976) Cushing's syndrome in the dog. *Vet Rec* **98**, 28-30.

Kennedy GC (1953) The role of depot fat in the hypothalamic control of food intake in the rat. *Proc R Soc Lond B Biol Sci* **140**, 578-596.

Kern PA, Ranganathan S, Li C, Wood L & Ranganathan G (2001) Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* **280**, E745-751.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R & Simsolo RB (1995) The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. *J Clin Invest* **95**, 2111-2119.

Kersten S, Desvergne B & Wahli W (2000a) Roles of PPARs in health and disease. *Nature* **405**, 421-424.

Kersten S, Mandard S, Tan NS, Escher P, Metzger D, Chambon P, Gonzalez FJ, Desvergne B & Wahli W (2000b) Characterization of the fasting-induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. *J Biol Chem* **275**, 28488-28493.

Kim CS, Park HS, Kawada T, Kim JH, Lim D, Hubbard NE, Kwon BS, Erickson KL & Yu R (2006) Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *Int J Obes (Lond)* **30**, 1347-1355.

Kim SP, Ellmerer M, Van Citters GW & Bergman RN (2003) Primacy of hepatic insulin resistance in the development of the metabolic syndrome induced by an isocaloric moderate-fat diet in the dog. *Diabetes* **52**, 2453-2460.

Kirchgessner TG, Uysal KT, Wiesbrock SM, Marino MW & Hotamisligil GS (1997) Tumor necrosis factor-alpha contributes to obesity-related hyperleptinemia by regulating leptin release from adipocytes. *J Clin Invest* **100**, 2777-2782.

Kirkness EF, Bafna V, Halpern AL, Levy S, Remington K, Rusch DB, Delcher AL, Pop M, Wang W, Fraser CM & Venter JC (2003) The dog genome: survey sequencing and comparative analysis. *Science* **301**, 1898-1903.

Klaus S (2001a) *Brown Adipocyte Differentiation and Function in Energy Metabolism*, 1st ed. Texas: Eurekah.com / Landes Bioscience.

Klaus S (2001b) *Overview: Biological Significance of Fat and Adipose Tissues*, 1st ed. Texas: Eurekah.com / Landes Bioscience.

Klinkenberg H, Sallander MH & Hedhammar A (2006) Feeding, exercise, and weight identified as risk factors in canine diabetes mellitus. *J Nutr* **136**, 1985S-1987S.

Koistinen HA, Dusserre E, Ebeling P, Vallier P, Koivisto VA & Vidal H (2000) Subcutaneous adipose tissue expression of plasminogen activator inhibitor-1 (PAI-1) in nondiabetic and Type 2 diabetic subjects. *Diabetes Metab Res Rev* **16**, 364-369.

Kornack DR, Lu B & Black IB (1991) Sexually dimorphic expression of the NGF receptor gene in the developing rat brain. *Brain Res* **542**, 171-174.

Kos K, Harte A, James S, Snead DR, O'Hare J P, McTernan PG & Kumar S (2007) Secretion of Neuropeptide Y (NPY) in human adipose tissue and its role in maintenance of adipose tissue mass. *Am J Physiol Endocrinol Metab*.

Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CR, Seckl JR & Mullins JJ (1997) 11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc Natl Acad Sci U S A* **94**, 14924-14929.

Koumenis C, Naczki C, Koritzinsky M, Rastani S, Diehl A, Sonenberg N, Koromilas A & Wouters BG (2002) Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha. *Mol Cell Biol* **22**, 7405-7416.

Kreier F, Fliers E, Voshol PJ, Van Eden CG, Havekes LM, Kalsbeek A, Van Heijningen CL, Sluiter AA, Mettenleiter TC, Romijn JA, Sauerwein HP & Buijs RM (2002) Selective parasympathetic innervation of subcutaneous and intra-abdominal fat--functional implications. *J Clin Invest* **110**, 1243-1250.

Kuczmarski RJ & Flegal KM (2000) Criteria for definition of overweight in transition: background and recommendations for the United States. *Am J Clin Nutr* **72**, 1074-1081.

Lacey LA, Wolf A, O'Shea D, Erny S & Ruof J (2005) Cost-effectiveness of orlistat for the treatment of overweight and obese patients in Ireland. *Int J Obes Relat Metab Disord* **29**, 975-982.

Laflamme DP & Kuhlman G (1995) The effect of weight loss regimen on subsequent weight maintenance in dogs. *Nutrition Research* **15**, 1019-1028.

Lafontan M, Bousquet-Melou A, Galitzky J, Barbe P, Carpene C, Langin D, Berlan M, Valet P, Castan I, Bouloumie A & et al. (1995) Adrenergic receptors and fat cells: differential recruitment by physiological amines and homologous regulation. *Obes Res* **3 Suppl 4**, 507S-514S.

Lagathu C, Bastard JP, Auclair M, Maachi M, Capeau J & Caron M (2003) Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. *Biochem Biophys Res Commun* **311**, 372-379.

Lagathu C, Yvan-Charvet L, Bastard JP, Maachi M, Quignard-Boulange A, Capeau J & Caron M (2006) Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. *Diabetologia* **49**, 2162-2173.

Lane MD, Tang Q & Jiang M (1999) Role of the CCAAT Enhancer Binding Proteins (C/EBPs) in Adipocyte Differentiation. *Biochem Biophys Res Commun* **266**, 677-683.

Larson BT, Lawler DF, Spitznagel EL, Jr. & Kealy RD (2003) Improved glucose tolerance with lifetime diet restriction favorably affects disease and survival in dogs. *J Nutr* **133**, 2887-2892.

Lawler DF, Evans RH, Larson BT, Spitznagel EL, Ellersieck MR & Kealy RD (2005) Influence of lifetime food restriction on causes, time, and predictors of death in dogs. *J Am Vet Med Assoc* **226**, 225-231.

le Roux CW & Bloom SR (2005) Peptide YY, appetite and food intake. *Proc Nutr Soc* **64**, 213-216.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI & Friedman JM (1996) Abnormal splicing of the leptin receptor in diabetic mice. *Nature* **379**, 632-635.

Lehmann JM, Lenhard JM, Oliver BB, Ringold GM & Kliewer SA (1997) Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* **272**, 3406-3410.

Lehrke M & Lazar MA (2005) The many faces of PPARgamma. *Cell* **123**, 993-999.

Lehrke M, Reilly MP, Millington SC, Iqbal N, Rader DJ & Lazar MA (2004) An inflammatory cascade leading to hyperresistinemia in humans. *PLoS Med* **1**, e45.

Levi-Montalcini R, Skaper SD, Dal Toso R, Petrelli L & Leon A (1996) Nerve growth factor: from neurotrophin to neurokine. *Trends Neurosci* **19**, 514-520.

Levine JA (2004) Nonexercise activity thermogenesis (NEAT): environment and biology. *Am J Physiol Endocrinol Metab* **286**, E675-685.

Lihn AS, Bruun JM, He G, Pedersen SB, Jensen PF & Richelsen B (2004) Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. *Mol Cell Endocrinol* **219**, 9-15.

Lin SC & Li P (2004) CIDE-A, a novel link between brown adipose tissue and obesity. *Trends Mol Med* **10**, 434-439.

Lin Y, Hyangkyu L, Berg AH, Lisanti MP, Shapiro L & Scherer PE (2000) The Lipopolysaccharide-activated Toll-Like Receptor (TLR)-4 Induces Synthesis of the Closely Related Receptor TLR-2 in Adipocytes. *J Biol Chem* **275**, 24255-24263.

Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas EJ, 3rd, Zody MC, Mauceli E, Xie X, Breen M, Wayne RK, Ostrander EA, Ponting CP, Galibert F, Smith DR, DeJong PJ, Kirkness E, Alvarez P, Biagi T, Brockman W, Butler J, Chin CW, Cook A, Cuff J, Daly MJ, DeCaprio D, Gnerre S, Grabherr M, Kellis M, Kleber M, Bardeleben C, Goodstadt L, Heger A, Hitte C, Kim L, Koepfli KP, Parker HG, Pollinger JP, Searle SM, Sutter NB, Thomas R, Webber C, Baldwin J, Abebe A, Abouelleil A, Aftuck L, Ait-Zahra M, Aldredge T, Allen N, An P, Anderson S, Antoine C, Arachchi H, Aslam A, Ayotte L, Bachantsang P, Barry A, Bayul T, Benamara M, Berlin A, Bessette D, Blitshteyn B, Bloom T, Blye J, Boguslavskiy L, Bonnet C, Boukhgalter B, Brown A, Cahill P, Calixte N, Camarata J, Cheshatsang Y, Chu J, Citroen M, Collymore A, Cooke P, Dawoe T, Daza R, Decktor K, DeGray S, Dhargay N, Dooley K, Dooley K, Dorje P, Dorjee K, Dorris L, Duffey N, Dupes A, Egbiremolen O, Elong R, Falk J, Farina A, Faro S, Ferguson D, Ferreira P, Fisher S, FitzGerald M, Foley K, Foley C, Franke A, Friedrich D, Gage D, Garber M, Gearin G, Giannoukos G, Goode T, Goyette A, Graham J, Grandbois E, Gyaltsen K, Hafez N, Hagopian D, Hagos B, Hall J, Healy C, Hegarty R, Honan T, Horn A, Houde N, Hughes L, Hunnicutt L, Husby M, Jester B, Jones C, Kamat A, Kanga B, Kells C, Khazanovich D, Kieu AC, Kisner P, Kumar M, Lance K, Landers T, Lara M, Lee W, Leger JP, Lennon N, Leuper L, LeVine S, Liu J, Liu X, Lokyitsang Y, Lokyitsang T, Lui A, Macdonald J, Major J, Marabella R, Maru K, Matthews C, McDonough S, Mehta T, Meldrim J, Melnikov A, Meneus L, Mihalev A, Mihova T, Miller K, Mittelman R, Mlenga V, Mulrain L, Munson G, Navidi A, Naylor J, Nguyen T, Nguyen N, Nguyen C, Nguyen T, Nicol R, Norbu N, Norbu C, Novod N, Nyima T, Olandt P, O'Neill B, O'Neill K, Osman S, Oyono L, Patti C, Perrin D, Phunkhang P, Pierre F, Priest M, Rachupka A, Raghuraman S, Rameau R, Ray V, Raymond C, Rege F, Rise C, Rogers J, Rogov P, Sahalie J, Settipalli S, Sharpe T, Shea T, Sheehan M, Sherpa N, Shi J, Shih D, Sloan J, Smith C, Sparrow T, Stalker J, Stange-Thomann N, Stavropoulos S, Stone C, Stone S, Sykes S, Tchuinga P, Tenzing P, Tesfaye S, Thoulutsang D, Thoulutsang Y, Topham K, Topping I, Tsamla T, Vassiliev H, Venkataraman V, Vo A, Wangchuk T, Wangdi T, Weiand M, Wilkinson J, Wilson A, Yadav S, Yang S, Yang X, Young G, Yu Q, Zainoun J, Zembek L, Zimmer A & Lander ES (2005) Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* **438**, 803-819.

Liu Y, Sun WL, Sun Y, Hu G & Ding GX (2006) Role of 11-beta-hydroxysteroid dehydrogenase type 1 in differentiation of 3T3-L1 cells and in rats with diet-induced obesity. *Acta Pharmacol Sin* **27**, 588-596.

Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408.

Livingstone DE, Jones GC, Smith K, Jamieson PM, Andrew R, Kenyon CJ & Walker BR (2000) Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. *Endocrinology* **141**, 560-563.

Loftus TM & Lane MD (1997) Modulating the transcriptional control of adipogenesis. *Curr Opin Genet Dev* **7**, 603-608.

Lolmede K, Durand de Saint Front V, Galitzky J, Lafontan M & Bouloumie A (2003) Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *Int J Obes Relat Metab Disord* **27**, 1187-1195.

Lue H, Kleemann R, Calandra T, Roger T & Bernhagen J (2002) Macrophage migration inhibitory factor (MIF): mechanisms of action and role in disease. *Microbes Infect* **4**, 449-460.

Lumeng CN, Bodzin JL & Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* **117**, 175-184.

Lund EM, Armstrong PJ, Kirk CA & Klausner JS (2006) Prevalence and risk factos for obesity in adult dogs from private US veterinary practices. *Int J Appl Res Vet Med* **4**, 177-186.

MacEwan DJ (2002) TNF receptor subtype signalling: differences and cellular consequences. *Cell Signal* **14**, 477-492.

Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I & Matsuzawa Y (2001) PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* **50**, 2094-2099.

Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S & et al. (1995) Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* **1**, 1155-1161.

Mai K, Andres J, Bobbert T, Maser-Gluth C, Mohlig M, Bahr V, Pfeiffer AF, Spranger J & Diederich S (2007) Rosiglitazone decreases 11beta-hydroxysteroid dehydrogenase type 1 in subcutaneous adipose tissue. *Clin Endocrinol (Oxf)* **67**, 419-425.

Major GC, Doucet E, Trayhurn P, Astrup A & Tremblay A (2007) Clinical significance of adaptive thermogenesis. *Int J Obes (Lond)* **31**, 204-212.

Manco M, Fernandez-Real JM, Equitani F, Vendrell J, Valera Mora ME, Nanni G, Tondolo V, Calvani M, Ricart W, Castagneto M & Mingrone G (2007) Effect of massive weight loss on inflammatory adipocytokines and the innate immune system in morbidly obese women. *J Clin Endocrinol Metab* **92**, 483-490.

Marcason W (2007) What are the latest figures for state-specific prevalence of obesity in the United States? *J Am Diet Assoc* **107**, 168.

Mark AL, Rahmouni K, Correia M & Haynes WG (2003) A leptin-sympathetic-leptin feedback loop: potential implications for regulation of arterial pressure and body fat. *Acta Physiol Scand* **177**, 345-349.

Markwell P, van Erk W, Parkin G, Sloth C & Shantz-Christienson T (1990) Obesity in the dog. *Journal of Small Animal Practice* **31**, 533-537.

Marz P, Cheng JG, Gadient RA, Patterson PH, Stoyan T, Otten U & Rose-John S (1998) Sympathetic neurons can produce and respond to interleukin 6. *Proc Natl Acad Sci U S A* **95**, 3251-3256.

Mason E (1970) Obesity in pet dogs. *Vet Rec* **86**, 612-616.

Masubuchi Y, Kano S & Horie T (2006) Mitochondrial permeability transition as a potential determinant of hepatotoxicity of antidiabetic thiazolidinediones. *Toxicology* **222**, 233-239.

Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR & Flier JS (2001) A transgenic model of visceral obesity and the metabolic syndrome. *Science* **294**, 2166-2170.

Mawby DI, Bartges JW, A DA, Laflamme DP, Moyers TD & Cottrell T (2004) Comparison of Various Methods for Estimating Body Fat in Dogs. *J Am Anim Hosp Assoc* **40**, 109-114.

Mayes JS & Watson GH (2004) Direct effects of sex steroid hormones on adipose tissues and obesity. *Obes Rev* **5**, 197-216.

McClain D (1998) Further insights into leptin action. *Endocrinology* **139**, 3679-3680.

McGee DL (2005) Body mass index and mortality: a meta-analysis based on person-level data from twenty-six observational studies. *Ann Epidemiol* **15**, 87-97.

McGreevy PD, Thomson PC, Pride C, Fawcett A, Grassi T & Jones B (2005) Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. *Vet Rec* **156**, 695-702.

McMinn JE, Baskin DG & Schwartz MW (2000) Neuroendocrine mechanisms regulating food intake and body weight. *Obes Rev* **1**, 37-46.

McTernan CL, McTernan PG, Harte AL, Levick PL, Barnett AH & Kumar S (2002a) Resistin, central obesity, and type 2 diabetes. *Lancet* **359**, 46-47.

McTernan PG, Harte AL, Anderson LA, Green A, Smith SA, Holder JC, Barnett AH, Eggo MC & Kumar S (2002b) Insulin and rosiglitazone regulation of lipolysis and lipogenesis in human adipose tissue in vitro. *Diabetes* **51**, 1493-1498.

McTernan PG, McTernan CL, Chetty R, Jenner K, Fisher FM, Lauer MN, Crocker J, Barnett AH & Kumar S (2002c) Increased resistin gene and protein expression in human abdominal adipose tissue. *J Clin Endocrinol Metab* **87**, 2407.

Mellersh C (2007) Give a dog a genome. *Vet J.*

Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Morgan PJ & Trayhurn P (1996) Coexpression of leptin receptor and preproneuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus. *J Neuroendocrinol* **8**, 733-735.

Mercer JG, Moar KM, Rayner DV, Trayhurn P & Hoggard N (1997) Regulation of leptin receptor and NPY gene expression in hypothalamus of leptin-treated obese (ob/ob) and cold-exposed lean mice. *FEBS Lett* **402**, 185-188.

Miles PD, Romeo OM, Higo K, Cohen A, Rafaat K & Olefsky JM (1997) TNF-alpha-induced insulin resistance in vivo and its prevention by troglitazone. *Diabetes* **46**, 1678-1683.

Misra A (2003) We need ethnic-specific criteria for classification of BMI. *Progress in Obesity Research* **9**, 547-553.

Mitchell SE, Rees WD, Hardie LJ, Hoggard N, Tadayyon M, Arch JR & Trayhurn P (1997) ob gene expression and secretion of leptin following differentiation of rat preadipocytes to adipocytes in primary culture. *Biochem Biophys Res Commun* **230**, 360-364.

Miyazawa-Hoshimoto S, Takahashi K, Bujo H, Hashimoto N, Yagui K & Saito Y (2005) Roles of degree of fat deposition and its localization on VEGF expression in adipocytes. *Am J Physiol Endocrinol Metab* **288**, E1128-1136.

Mohamed-Ali V, Flower L, Sethi J, Hotamisligil G, Gray R, Humphries SE, York DA & Pinkney J (2001) beta-Adrenergic regulation of IL-6 release from adipose tissue: in vivo and in vitro studies. *J Clin Endocrinol Metab* **86**, 5864-5869.

Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S & Coppack SW (1997) Subcutaneous adipose tissue releases interleukin-6, but

not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* **82**, 4196-4200.

Mohamed-Ali V, Pinkney JH & Coppack SW (1998) Adipose tissue as an endocrine and paracrine organ. *Int J Obes Relat Metab Disord* **22**, 1145-1158.

Moinat M, Deng C, Muzzin P, Assimacopoulos-Jeannet F, Seydoux J, Dulloo AG & Giacobino JP (1995) Modulation of obese gene expression in rat brown and white adipose tissues. *FEBS Lett* **373**, 131-134.

Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB & O'Rahilly S (1997a) Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* **387**, 903-908.

Montague CT, Prins JB, Sanders L, Digby JE & O'Rahilly S (1997b) Depot- and sex-specific differences in human leptin mRNA expression: implications for the control of regional fat distribution. *Diabetes* **46**, 342-347.

Montague CT, Prins JB, Sanders L, Zhang J, Sewter CP, Digby J, Byrne CD & O'Rahilly S (1998) Depot-related gene expression in human subcutaneous and omental adipocytes. *Diabetes* **47**, 1384-1391.

Morooka T, Niiyama M, Uchida E, Uemura M, Miyoshi K & Saito M (2001) Measurement of the back fat layer in beagles for estimation of obesity using two-dimensional ultrasonography. *J Small Anim Pract* **42**, 56-59.

Morton NM, Paterson JM, Masuzaki H, Holmes MC, Staels B, Fievet C, Walker BR, Flier JS, Mullins JJ & Seckl JR (2004) Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes* **53**, 931-938.

Motoshima H, Wu X, Sinha MK, Hardy VE, Rosato EL, Barbot DJ, Rosato FE & Goldstein BJ (2002) Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone. *J Clin Endocrinol Metab* **87**, 5662-5667.

Munday HS (1994) Assessment of body composition in cats and dogs. *Int J Obes Relat Metab Disord* **18 Suppl 1**, S14-21.

Munday HS, Booles D, Anderson P, Poore DW & Earle KE (1994) The repeatability of body composition measurements in dogs and cats using dual energy X-ray absorptiometry. *J Nutr* **124**, 2619S-2621S.

Mutch NJ, Wilson HM & Booth NA (2001) Plasminogen activator inhibitor-1 and haemostasis in obesity. *Proc Nutr Soc* **60**, 341-347.

Nagaev I & Smith U (2001) Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. *Biochem Biophys Res Commun* **285**, 561-564.

Napolitano A, Voice MW, Edwards CR, Seckl JR & Chapman KE (1998) 11Beta-hydroxysteroid dehydrogenase 1 in adipocytes: expression is differentiation-dependent and hormonally regulated. *J Steroid Biochem Mol Biol* **64**, 251-260.

Narayan KM, Boyle JP, Thompson TJ, Sorensen SW & Williamson DF (2003) Lifetime risk for diabetes mellitus in the United States. *Jama* **290**, 1884-1890.

Nechad M, Ruka E & Thibault J (1994) Production of nerve growth factor by brown fat in culture: relation with the in vivo developmental stage of the tissue. *Comp Biochem Physiol Comp Physiol* **107**, 381-388.

Nedergaard J, Bengtsson T & Cannon B (2007) Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab* **293**, E444-452.

Nelson RW, Himsel CA, Feldman EC & Bottoms GD (1990) Glucose tolerance and insulin response in normal-weight and obese cats. *Am J Vet Res* **51**, 1357-1362.

Nishii N, Nodake H, Takasu M, Soe O, Ohba Y, Maeda S, Ohtsuka Y, Honjo T, Saito M & Kitagawa H (2006) Postprandial changes in leptin concentrations of cerebrospinal fluid in dogs during development of obesity. *Am J Vet Res* **67**, 2006-2011.

Nisoli E, Tonello C, Benarese M, Liberini P & Carruba MO (1996) Expression of nerve growth factor in brown adipose tissue: implications for thermogenesis and obesity. *Endocrinology* **137**, 495-503.

Nomura S, Shouzu A, Omoto S, Nishikawa M & Fukuhara S (2000) Significance of chemokines and activated platelets in patients with diabetes. *Clin Exp Immunol* **121**, 437-443.

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL & Petersen CM (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature* **427**, 843-848.

O'Neill JL, McCarthy SN, Burke SJ, Hannon EM, Kiely M, Flynn A, Flynn MA & Gibney MJ (2007) Prevalence of overweight and obesity in Irish school children, using four different definitions. *Eur J Clin Nutr* **61**, 743-751.

O'Rahilly S & Farooqi IS (2006) Genetics of obesity. *Philos Trans R Soc Lond B Biol Sci* **361**, 1095-1105.

Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ & Flegal KM (2006) Prevalence of overweight and obesity in the United States, 1999-2004. *Jama* **295**, 1549-1555.

Ostrander EA & Comstock KE (2004) The domestic dog genome. *Curr Biol* **14**, R98-99.

Ostrander EA & Kruglyak L (2000) Unleashing the canine genome. *Genome Res* **10**, 1271-1274.

Otero M, Lago R, Lago F, Casanueva FF, Dieguez C, Gomez-Reino JJ & Gualillo O (2005) Leptin, from fat to inflammation: old questions and new insights. *FEBS Lett* **579**, 295-301.

Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T & Matsuzawa Y (1999) Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* **100**, 2473-2476.

Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T & Matsuzawa Y (2001) Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* **103**, 1057-1063.

Ouchi N, Ohishi M, Kihara S, Funahashi T, Nakamura T, Nagaretani H, Kumada M, Ohashi K, Okamoto Y, Nishizawa H, Kishida K, Maeda N, Nagasawa A, Kobayashi H, Hiraoka H, Komai N, Kaibe M, Rakugi H, Ogihara T & Matsuzawa Y (2003) Association of hypoadiponectinemia with impaired vasoreactivity. *Hypertension* **42**, 231-234.

Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH & Hotamisligil GS (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* **306**, 457-461.

Padrid P & Amis TC (1992) Chronic tracheobronchial disease in the dog. *Vet Clin North Am Small Anim Pract* **22**, 1203-1229.

Padwal RS & Majumdar SR (2007) Drug treatments for obesity: orlistat, sibutramine, and rimonabant. *Lancet* **369**, 71-77.

Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M & Scherer PE (2003) Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. *J Biol Chem* **278**, 9073-9085.

Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA & Scherer PE (2004) Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem* **279**, 12152-12162.

Palmer M & Prediger E (2007) Assessing RNA Quality. In *Ambion TechNotes*.

Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG & Glass CK (2005) A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* **437**, 759-763.

Path G, Bornstein SR, Gurniak M, Chrousos GP, Scherbaum WA & Hauner H (2001) Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. *J Clin Endocrinol Metab* **86**, 2281-2288.

Patterson DF (2000) Companion animal medicine in the age of medical genetics. *J Vet Intern Med* **14**, 1-9.

Patton JS, Shepard HM, Wilking H, Lewis G, Aggarwal BB, Eessalu TE, Gavin LA & Grunfeld C (1986) Interferons and tumor necrosis factors have similar catabolic effects on 3T3 L1 cells. *Proc Natl Acad Sci U S A* **83**, 8313-8317.

Paulmyer-Lacroix O, Boullu S, Oliver C, Alessi MC & Grino M (2002) Expression of the mRNA coding for 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue from obese patients: an in situ hybridization study. *J Clin Endocrinol Metab* **87**, 2701-2705.

Peeraully MR, Jenkins JR & Trayhurn P (2004) NGF gene expression and secretion in white adipose tissue: regulation in 3T3-L1 adipocytes by hormones and inflammatory cytokines. *Am J Physiol Endocrinol Metab* **287**, E331-339.

Penn DM, Jordan LC, Kelso EW, Davenport JE & Harris RB (2006) Effects of central or peripheral leptin administration on norepinephrine turnover in defined fat depots. *Am J Physiol Regul Integr Comp Physiol* **291**, R1613-1621.

Pennisi E (2007) Genetics. The geneticist's best friend. *Science* **317**, 1668-1671.

Perez-Perez M, Garcia-Suarez O, Esteban I, Germana A, Farinas I, Naves FJ & Vega JA (2003) p75NTR in the spleen: age-dependent changes, effect of NGF and 4-methylcatechol treatment, and structural changes in p75NTR-deficient mice. *Anat Rec A Discov Mol Cell Evol Biol* **270**, 117-128.

Permana PA, Menge C & Reaven PD (2006) Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. *Biochem Biophys Res Commun* **341**, 507-514.

Petruschke T & Hauner H (1993) Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. *J Clin Endocrinol Metab* **76**, 742-747.

Peytremann-Bridevaux I, Faeh D & Santos-Eggimann B (2007) Prevalence of overweight and obesity in rural and urban settings of 10 European countries. *Prev Med* **44**, 442-446.

Phelan S, Wadden TA, Berkowitz RI, Sarwer DB, Womble LG, Cato RK & Rothman R (2007) Impact of weight loss on the metabolic syndrome. *Int J Obes (Lond)* **31**, 1442-1448.

Pinkney JH, Coppack SW & Mohamed-Ali V (1998) Effect of isoprenaline on plasma leptin and lipolysis in humans. *Clin Endocrinol (Oxf)* **48**, 407-411.

Pittas AG, Joseph NA & Greenberg AS (2004) Adipocytokines and insulin resistance. *J Clin Endocrinol Metab* **89**, 447-452.

Poitou C, Viguerie N, Cancello R, De Matteis R, Cinti S, Stich V, Coussieu C, Gauthier E, Courtine M, Zucker JD, Barsh GS, Saris W, Bruneval P, Basdevant A, Langin D & Clement K (2005) Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. *Diabetologia* **48**, 519-528.

Pons F, Freund V, Kuissu H, Mathieu E, Olgart C & Frossard N (2001) Nerve growth factor secretion by human lung epithelial A549 cells in pro- and anti-inflammatory conditions. *Eur J Pharmacol* **428**, 365-369.

Pottie P, Presle N, Terlain B, Netter P, Mainard D & Berenbaum F (2006) Obesity and osteoarthritis: more complex than predicted! *Ann Rheum Dis* **65**, 1403-1405.

Poulain-Godefroy O & Froguel P (2007) Preadipocyte response and impairment of differentiation in an inflammatory environment. *Biochem Biophys Res Commun* **356**, 662-667.

Prins JB, Niesler CU, Winterford CM, Bright NA, Siddle K, O'Rahilly S, Walker NI & Cameron DP (1997) Tumor necrosis factor-alpha induces apoptosis of human adipose cells. *Diabetes* **46**, 1939-1944.

Putz DM, Goldner WS, Bar RS, Haynes WG & Sivitz WI (2004) Adiponectin and C-reactive protein in obesity, type 2 diabetes, and monodrug therapy. *Metabolism* **53**, 1454-1461.

Qi C & Pekala PH (2000) Tumor necrosis factor-alpha-induced insulin resistance in adipocytes. *Proc Soc Exp Biol Med* **223**, 128-135.

Qi Y, Takahashi N, Hileman SM, Patel HR, Berg AH, Pajvani UB, Scherer PE & Ahima RS (2004) Adiponectin acts in the brain to decrease body weight. *Nat Med* **10**, 524-529.

Rahimi N, Tremblay E, McAdam L, Roberts A & Elliott B (1998) Autocrine secretion of TGF-beta 1 and TGF-beta 2 by pre-adipocytes and adipocytes: a potent negative regulator of adipocyte differentiation and proliferation of mammary carcinoma cells. *In Vitro Cell Dev Biol Anim* **34**, 412-420.

Rajala MW & Scherer PE (2003) Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* **144**, 3765-3773.

Ramsay TG (1996) Fat cells. *Endocrinol Metab Clin North Am* **25**, 847-870.

Rask E, Walker BR, Soderberg S, Livingstone DE, Eliasson M, Johnson O, Andrew R & Olsson T (2002) Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity. *J Clin Endocrinol Metab* **87**, 3330-3336.

Rasmussen MS, Lihn AS, Pedersen SB, Bruun JM, Rasmussen M & Richelsen B (2006) Adiponectin receptors in human adipose tissue: effects of obesity, weight loss, and fat depots. *Obesity (Silver Spring)* **14**, 28-35.

Rayner DV (2001) The sympathetic nervous system in white adipose tissue regulation. *Proc Nutr Soc* **60**, 357-364.

Rayner DV, Simon E, Duncan JS & Trayhurn P (1998) Hyperleptinaemia in mice induced by administration of the tyrosine hydroxylase inhibitor alpha-methyl-p-tyrosine. *FEBS Lett* **429**, 395-398.

Rayner DV & Trayhurn P (2001) Regulation of leptin production: sympathetic nervous system interactions. *J Mol Med* **79**, 8-20.

Razak F, Anand SS, Shannon H, Vuksan V, Davis B, Jacobs R, Teo KK, McQueen M & Yusuf S (2007) Defining obesity cut points in a multiethnic population. *Circulation* **115**, 2111-2118.

Rea R & Donnelly R (2004) Resistin: an adipocyte-derived hormone. Has it a role in diabetes and obesity? *Diabetes Obes Metab* **6**, 163-170.

Rebuffe-Scrive M, Bronnegard M, Nilsson A, Eldh J, Gustafsson JA & Bjorntorp P (1990) Steroid hormone receptors in human adipose tissues. *J Clin Endocrinol Metab* **71**, 1215-1219.

Rebuffe-Scrive M, Krotkiewski M, Elfverson J & Bjorntorp P (1988) Muscle and adipose tissue morphology and metabolism in Cushing's syndrome. *J Clin Endocrinol Metab* **67**, 1122-1128.

Reimann N, Nolte I, Bonk U, Bartnitzke S & Bullerdiek J (1999) Cytogenetic investigation of canine lipomas. *Cancer Genet Cytogenet* **111**, 172-174.

Reisin E & Alpert MA (2005) Definition of the metabolic syndrome: current proposals and controversies. *Am J Med Sci* **330**, 269-272.

Ren D, Collingwood TN, Rebar EJ, Wolffe AP & Camp HS (2002) PPARgamma knockdown by engineered transcription factors: exogenous PPARgamma2 but not PPARgamma1 reactivates adipogenesis. *Genes Dev* **16**, 27-32.

Rennie KL & Jebb SA (2005) Prevalence of obesity in Great Britain. *Obes Rev* **6**, 11-12.

Reseland JE, Syversen U, Bakke I, Qvigstad G, Eide LG, Hjertner O, Gordeladze JO & Drevon CA (2001) Leptin is expressed in and secreted from primary cultures of human osteoblasts and promotes bone mineralization. *J Bone Miner Res* **16**, 1426-1433.

Ricquier D & Bouillaud F (2000) Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. *J Physiol* **529 Pt 1**, 3-10.

Rieusset J, Auwerx J & Vidal H (1999) Regulation of gene expression by activation of the peroxisome proliferator-activated receptor gamma with rosiglitazone (BRL 49653) in human adipocytes. *Biochem Biophys Res Commun* **265**, 265-271.

Robinson CJ & Stringer SE (2001) The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J Cell Sci* **114**, 853-865.

Rodriguez AM, Elabd C, Delteil F, Astier J, Vernochet C, Saint-Marc P, Guesnet J, Guezennec A, Amri EZ, Dani C & Ailhaud G (2004) Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochem Biophys Res Commun* **315**, 255-263.

Rollins BJ (1997) Chemokines. *Blood* **90**, 909-928.

Rolls ET (2007) Understanding the mechanisms of food intake and obesity. *Obes Rev* **8 Suppl 1**, 67-72.

Romijn JA & Fliers E (2005) Sympathetic and parasympathetic innervation of adipose tissue: metabolic implications. *Curr Opin Clin Nutr Metab Care* **8**, 440-444.

Ronti T, Lupattelli G & Mannarino E (2006) The endocrine function of adipose tissue: an update. *Clin Endocrinol (Oxf)* **64**, 355-365.

Rost B, Hanf G, Ohnemus U, Otto-Knapp R, Groneberg DA, Kunkel G & Noga O (2005) Monocytes of allergics and non-allergics produce, store and release the neurotrophins NGF, BDNF and NT-3. *Regul Pept* **124**, 19-25.

Rothwell NJ & Stock MJ (1983) Effects of age on diet-induced thermogenesis and brown adipose tissue metabolism in the rat. *Int J Obes* **7**, 583-589.

Rothwell NJ & Stock MJ (1997) A role for brown adipose tissue in diet-induced thermogenesis. *Obes Res* **5**, 650-656.

Rotter V, Nagaev I & Smith U (2003) Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *J Biol Chem* **278**, 45777-45784.

Ruan H, Hacohen N, Golub TR, Van Parijs L & Lodish HF (2002) Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. *Diabetes* **51**, 1319-1336.

Ruan H, Zarnowski MJ, Cushman SW & Lodish HF (2003) Standard isolation of primary adipose cells from mouse epididymal fat pads induces inflammatory mediators and down-regulates adipocyte genes. *J Biol Chem* **278**, 47585-47593.

Rutkowski P, Klassen A, Sebekova K, Bahner U & Heidland A (2006) Renal disease in obesity: the need for greater attention. *J Ren Nutr* **16**, 216-223.

Ryden M, Arvidsson E, Blomqvist L, Perbeck L, Dicker A & Arner P (2004) Targets for TNF-alpha-induced lipolysis in human adipocytes. *Biochem Biophys Res Commun* **318**, 168-175.

Sagawa M, Nakadomo F, Honjoh T, Ishioka K & Saito M (2002) Correlation between plasma leptin concentration and body fat content in dogs. *Am J Vet Res* **63**, 7-10.

Sahu A (2004) Minireview: A hypothalamic role in energy balance with special emphasis on leptin. *Endocrinology* **145**, 2613-2620.

Sakaue S, Nishihira J, Hirokawa J, Yoshimura H, Honda T, Aoki K, Tagami S & Kawakami Y (1999) Regulation of macrophage migration inhibitory factor (MIF) expression by glucose and insulin in adipocytes in vitro. *Mol Med* **5**, 361-371.

Salam GA (2002) Lipoma excision. *Am Fam Physician* **65**, 901-904.

Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ & Murphy WJ (2000) Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* **96**, 34-40.

Salton SR (2003) Neurotrophins, growth-factor-regulated genes and the control of energy balance. *Mt Sinai J Med* **70**, 93-100.

Samad F, Pandey M & Loskutoff DJ (1998) Tissue factor gene expression in the adipose tissues of obese mice. *Proc Natl Acad Sci U S A* **95**, 7591-7596.

Sandeep TC, Andrew R, Homer NZ, Andrews RC, Smith K & Walker BR (2005) Increased in vivo regeneration of cortisol in adipose tissue in human obesity and effects of the 11beta-hydroxysteroid dehydrogenase type 1 inhibitor carbenoxolone. *Diabetes* **54**, 872-879.

Sargan DR (2004) IDID: inherited diseases in dogs: web-based information for canine inherited disease genetics. *Mamm Genome* **15**, 503-506.

Sarraf P, Frederich RC, Turner EM, Ma G, Jaskowiak NT, Rivet DJ, 3rd, Flier JS, Lowell BB, Fraker DL & Alexander HR (1997) Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. *J Exp Med* **185**, 171-175.

Sartipy P & Loskutoff DJ (2003) Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* **100**, 7265-7270.

Sasaki N, Shibata H, Honjoh T, Kimura K, Saito M & Ohishi I (2001) CDNA cloning of feline leptin and its mRNA expression in adipose tissue. *J Vet Med Sci* **63**, 1115-1120.

Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV & O'Rahilly S (2001) Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes* **50**, 2199-2202.

Schoof E, Stuppy A, Harig F, Carbon R, Horbach T, Stohr W, Rascher W & Dotsch J (2004) Comparison of leptin gene expression in different adipose tissues in children and adults. *Eur J Endocrinol* **150**, 579-584.

Schwartz MW & Porte D, Jr. (2005) Diabetes, obesity, and the brain. *Science* **307**, 375-379.

Scully JL & Otten U (1995) NGF: not just for neurons. *Cell Biol Int* **19**, 459-469.

Seckl JR, Morton NM, Chapman KE & Walker BR (2004) Glucocorticoids and 11beta-hydroxysteroid dehydrogenase in adipose tissue. *Recent Prog Horm Res* **59**, 359-393.

Semb H, Peterson J, Tavernier J & Olivecrona T (1987) Multiple effects of tumor necrosis factor on lipoprotein lipase in vivo. *J Biol Chem* **262**, 8390-8394.

Senn JJ, Klover PJ, Nowak IA & Mooney RA (2002) Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes* **51**, 3391-3399.

Senn JJ, Klover PJ, Nowak IA, Zimmers TA, Koniaris LG, Furlanetto RW & Mooney RA (2003) Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. *J Biol Chem* **278**, 13740-13746.

Sethi JK & Hotamisligil GS (1999) The role of TNF alpha in adipocyte metabolism. *Semin Cell Dev Biol* **10**, 19-29.

Shaw DI, Hall WL & Williams CM (2005) Metabolic syndrome: what is it and what are the implications? *Proc Nutr Soc* **64**, 349-357.

Shibata H, Sasaki N, Honjoh T, Ohishi I, Takiguchi M, Ishioka K, Ahmed M, Soliman M, Kimura K & Saito M (2003) Feline leptin: immunogenic and

biological activities of the recombinant protein, and its measurement by ELISA. *J Vet Med Sci* **65**, 1207-1211.

Shoelson SE, Lee J & Yuan M (2003) Inflammation and the IKK beta/I kappa B/NF-kappa B axis in obesity- and diet-induced insulin resistance. *Int J Obes Relat Metab Disord* **27** **Suppl 3**, S49-52.

Shooter EM (2001) Early days of the nerve growth factor proteins. *Annu Rev Neurosci* **24**, 601-629.

Shulman GI (2000) Cellular mechanisms of insulin resistance. *J Clin Invest* **106**, 171-176.

Sjoholm K, Palming J, Olofsson LE, Gummesson A, Svensson PA, Lystig TC, Jennische E, Brandberg J, Torgerson JS, Carlsson B & Carlsson LM (2005) A microarray search for genes predominantly expressed in human omental adipocytes: adipose tissue as a major production site of serum amyloid A. *J Clin Endocrinol Metab* **90**, 2233-2239.

Skurk T & Hauner H (2004) Obesity and impaired fibrinolysis: role of adipose production of plasminogen activator inhibitor-1. *Int J Obes Relat Metab Disord* **28**, 1357-1364.

Skurk T, Herder C, Kraft I, Muller-Scholze S, Hauner H & Kolb H (2005a) Production and release of macrophage migration inhibitory factor from human adipocytes. *Endocrinology* **146**, 1006-1011.

Skurk T, Kolb H, Muller-Scholze S, Rohrig K, Hauner H & Herder C (2005b) The proatherogenic cytokine interleukin-18 is secreted by human adipocytes. *Eur J Endocrinol* **152**, 863-868.

Smith GK, Mayhew PD, Kapatkin AS, McKelvie PJ, Shofer FS & Gregor TP (2001) Evaluation of risk factors for degenerative joint disease associated with hip dysplasia in German Shepherd Dogs, Golden Retrievers, Labrador Retrievers, and Rottweilers. *J Am Vet Med Assoc* **219**, 1719-1724.

Smith U (1974) Studies of human adipose tissue in culture. 3. Influence of insulin and medium glucose concentration on cellular metabolism. *J Clin Invest* **53**, 91-98.

Sonnenschein EG, Glickman LT, Goldschmidt MH & McKee LJ (1991) Body conformation, diet, and risk of breast cancer in pet dogs: a case-control study. *Am J Epidemiol* **133**, 694-703.

Speakman J, Hambly C, Mitchell S & Krol E (2007) Animal models of obesity. *Obes Rev* **8** **Suppl 1**, 55-61.

Speakman JR, Booles D & Butterwick R (2001) Validation of dual energy X-ray absorptiometry (DXA) by comparison with chemical analysis of dogs and cats. *Int J Obes Relat Metab Disord* **25**, 439-447.

Spiegelman BM (1998) PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. *Diabetes* **47**, 507-514.

Spiegelman BM, Choy L, Hotamisligil GS, Graves RA & Tontonoz P (1993) Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes. *J Biol Chem* **268**, 6823-6826.

Spiegelman BM, Frank M & Green H (1983) Molecular cloning of mRNA from 3T3 adipocytes. Regulation of mRNA content for glycerophosphate dehydrogenase and other differentiation-dependent proteins during adipocyte development. *J Biol Chem* **258**, 10083-10089.

Spranger J, Kroke A, Mohlig M, Hoffmann K, Bergmann MM, Ristow M, Boeing H & Pfeiffer AF (2003) Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* **52**, 812-817.

Sprengers ED & Kluft C (1987) Plasminogen activator inhibitors. *Blood* **69**, 381-387.

Stanley S, Wynne K, McGowan B & Bloom S (2005) Hormonal regulation of food intake. *Physiol Rev* **85**, 1131-1158.

Steiner P, Pfeilschifter J, Boeckh C, Radeke H & Otten U (1991) Interleukin-1 beta and tumor necrosis factor-alpha synergistically stimulate nerve growth factor synthesis in rat mesangial cells. *Am J Physiol* **261**, F792-798.

Stephens JM, Lee J & Pilch PF (1997) Tumour Necrosis Factor-alpha-induced insulin resistance in 3T3-L1 Adipocytes Is Accompanied by a Loss of Insulin Receptor Substrate-1 and GLUT4 Expression without a Loss of Insulin Receptor-mediated Signal Transduction. *J Biol Chem* **272**, 971-976.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS & Lazar MA (2001) The hormone resistin links obesity to diabetes. *Nature* **409**, 307-312.

Stewart PM, Boulton A, Kumar S, Clark PM & Shackleton CH (1999) Cortisol metabolism in human obesity: impaired cortisone-->cortisol conversion in subjects with central adiposity. *J Clin Endocrinol Metab* **84**, 1022-1027.

Stewart PM & Krozowski ZS (1999) 11 beta-Hydroxysteroid dehydrogenase. *Vitam Horm* **57**, 249-324.

Stienstra R, Duval C, M ML & Kersten S (2006) PPARs, Obesity, and Inflammation. *PPAR Res* **2007**, 95974.

Straczkowski M, Kowalska I, Nikolajuk A, Otziomek E, Adamska A, Karolczuk-Zarachowicz M & Gorska M (2007) Increased serum interleukin-18

concentration is associated with hypoadiponectinemia in obesity, independently of insulin resistance. *Int J Obes (Lond)* **31**, 221-225.

Stratagene (2004) Introduction to Quantitative PCR Methods and Application Guide, pp. 1-48: Stratagene.

Sutter NB & Ostrander EA (2004) Dog star rising: the canine genetic system. *Nat Rev Genet* **5**, 900-910.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA & Tepper RI (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**, 1263-1271.

Tchkonia T, Tchoukalova YD, Giorgadze N, Pirtskhalava T, Karagiannides I, Forse RA, Koo A, Stevenson M, Chinnappan D, Cartwright A, Jensen MD & Kirkland JL (2005) Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and apoptosis varies among fat depots. *Am J Physiol Endocrinol Metab* **288**, E267-277.

Tilg H & Wolf AM (2005) Adiponectin: a key fat-derived molecule regulating inflammation. *Expert Opin Ther Targets* **9**, 245-251.

Toll PW, Gross KL, Berryhill SA & Jewell DE (1994) Usefulness of dual energy X-ray absorptiometry for body composition measurement in adult dogs. *J Nutr* **124**, 2601S-2603S.

Tomlinson JW, Moore J, Cooper MS, Bujalska I, Shahmanesh M, Burt C, Strain A, Hewison M & Stewart PM (2001) Regulation of expression of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue: tissue-specific induction by cytokines. *Endocrinology* **142**, 1982-1989.

Tomlinson JW, Sinha B, Bujalska I, Hewison M & Stewart PM (2002) Expression of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue is not increased in human obesity. *J Clin Endocrinol Metab* **87**, 5630-5635.

Tomlinson JW & Stewart PM (2005) Mechanisms of disease: Selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 as a novel treatment for the metabolic syndrome. *Nat Clin Pract Endocrinol Metab* **1**, 92-99.

Trayhurn P (2005a) Adipose Tissue in Obesity--An Inflammatory Issue. *Endocrinology* **146**, 1003-1005.

Trayhurn P (2005b) The biology of obesity. *Proceedings of the Nutrition Society* **64**, 31-38.

Trayhurn P (2005c) Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* **184**, 285-293.

Trayhurn P (2007) Adipocyte biology. *Obes Rev* **8 Suppl 1**, 41-44.

Trayhurn P & Beattie JH (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* **60**, 329-339.

Trayhurn P & Bing C (2006) Appetite and energy balance signals from adipocytes. *Philos Trans R Soc Lond B Biol Sci* **361**, 1237-1249.

Trayhurn P, Duncan JS, Rayner DV & Hardie LJ (1996) Rapid inhibition of ob gene expression and circulating leptin levels in lean mice by the beta 3-adrenoceptor agonists BRL 35135A and ZD2079. *Biochem Biophys Res Commun* **228**, 605-610.

Trayhurn P, Duncan JS, Wood AM & Beattie JH (2000) Metallothionein gene expression and secretion in white adipose tissue. *Am J Physiol Regul Integr Comp Physiol* **279**, R2329-2335.

Trayhurn P, Hoggard N, Mercer JG & Rayner DV (1999) Leptin: fundamental aspects. *Int J Obes Relat Metab Disord* **23 Suppl 1**, 22-28.

Trayhurn P, Thomas ME, Duncan JS & Rayner DV (1995) Effects of fasting and refeeding on ob gene expression in white adipose tissue of lean and obese (oblob) mice. *FEBS Lett* **368**, 488-490.

Trayhurn P, Wang B & Wood IS (2008) Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity? *Br J Nutr*, 1-9.

Trayhurn P & Wood IS (2004) Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* **92**, 347-355.

Trayhurn P & Wood IS (2005) Signalling role of adipose tissue: adipokines and inflammation in obesity. *Biochem Soc Trans* **33**, 1078-1081.

Trujillo ME, Sullivan S, Harten I, Schneider SH, Greenberg AS & Fried SK (2004) Interleukin-6 regulates human adipose tissue lipid metabolism and leptin production in vitro. *J Clin Endocrinol Metab* **89**, 5577-5582.

Tsuchida A, Yamauchi T, Takekawa S, Hada Y, Ito Y, Maki T & Kadowaki T (2005) Peroxisome Proliferator-Activated Receptor (PPAR){alpha} Activation Increases Adiponectin Receptors and Reduces Obesity-Related Inflammation in Adipose Tissue: Comparison of Activation of PPAR{alpha}, PPAR{gamma}, and Their Combination. *Diabetes* **54**, 3358-3370.

Um JY, Chung HS, Song MY, Shin HD & Kim HM (2004) Association of interleukin-1beta gene polymorphism with body mass index in women. *Clin Chem* **50**, 647-650.

Uysal KT, Wiesbrock SM, Marino MW & Hotamisligil GS (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* **389**, 610-614.

Vaisse C, Halaas JL, Horvath CM, Darnell JE, Jr., Stoffel M & Friedman JM (1996) Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat Genet* **14**, 95-97.

van der Poll T, Coyle SM, Barbosa K, Braxton CC & Lowry SF (1996) Epinephrine inhibits tumor necrosis factor-alpha and potentiates interleukin 10 production during human endotoxemia. *J Clin Invest* **97**, 713-719.

Vgontzas AN, Bixler EO & Chrousos GP (2003) Metabolic disturbances in obesity versus sleep apnoea: the importance of visceral obesity and insulin resistance. *J Intern Med* **254**, 32-44.

Vilcek J & Lee TH (1991) Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J Biol Chem* **266**, 7313-7316.

Wabitsch M, Brenner RE, Melzner I, Braun M, Moller P, Heinze E, Debatin KM & Hauner H (2001) Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes Relat Metab Disord* **25**, 8-15.

Wabitsch M, Fischer-Posovszky P & Debatin K (2003) Human adipocytes: differentiation and apoptosis. *Progress in Obesity Research* **9**, 96-100.

Waeber G, Calandra T, Roduit R, Haefliger JA, Bonny C, Thompson N, Thorens B, Temler E, Meinhardt A, Bacher M, Metz CN, Nicod P & Bucala R (1997) Insulin secretion is regulated by the glucose-dependent production of islet beta cell macrophage migration inhibitory factor. *Proc Natl Acad Sci U S A* **94**, 4782-4787.

Wajchenberg B, Lerario A, Giannella-Neto D & Rocha M (2003) Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Progress in Obesity Research* **9**, 49-53.

Wake DJ, Stimson RH, Tan GD, Homer NZ, Andrew R, Karpe F & Walker BR (2007) Effects of peroxisome proliferator-activated receptor-alpha and -gamma agonists on 11beta-hydroxysteroid dehydrogenase type 1 in subcutaneous adipose tissue in men. *J Clin Endocrinol Metab* **92**, 1848-1856.

Wake DJ & Walker BR (2004) 11 beta-hydroxysteroid dehydrogenase type 1 in obesity and the metabolic syndrome. *Mol Cell Endocrinol* **215**, 45-54.

Wake DJ & Walker BR (2006) Inhibition of 11beta-hydroxysteroid dehydrogenase type 1 in obesity. *Endocrine* **29**, 101-108.

Walker BR (2006) Cortisol--cause and cure for metabolic syndrome? *Diabet Med* **23**, 1281-1288.

Wang B, Jenkins JR & Trayhurn P (2004) Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: Integrated response to TNF-{alpha}. *Am J Physiol Endocrinol Metab*.

Wang B, Jenkins JR & Trayhurn P (2005) Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. *Am J Physiol Endocrinol Metab* **288**, E731-740.

Wang B & Trayhurn P (2006) Acute and prolonged effects of TNF-alpha on the expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture. *Pflugers Arch* **452**, 418-427.

Wang J, Liu R, Hawkins M, Barzilai N & Rossetti L (1998) A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature* **393**, 684-688.

Wang MY, Lee Y & Unger RH (1999) Novel form of lipolysis induced by leptin. *J Biol Chem* **274**, 17541-17544.

Wang P, Mariman E, Renes J & Keijer J (2008) The secretory function of adipocytes in the physiology of white adipose tissue. *J Cell Physiol*.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL & Ferrante AW, Jr. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* **112**, 1796-1808.

Welch JS, Ricote M, Akiyama TE, Gonzalez FJ & Glass CK (2003) PPARgamma and PPARdelta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages. *Proc Natl Acad Sci U S A* **100**, 6712-6717.

Wells JC, Cole TJ, Bruner D & Treleaven P (2007) Body shape in American and British adults: between-country and inter-ethnic comparisons. *Int J Obes (Lond)*.

Wenger RH (2002) Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *Faseb J* **16**, 1151-1162.

Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE & Tataranni PA (2001) Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* **86**, 1930-1935.

White RAS & Williams JM (1994) Tracheal collapse in the dog - is there really a role for surgery? A survey of 100 cases. *Journal of Small Animal Practice* **35**, 191-196.

WHO (2007) BMI Classification.

Wiesner G, Morash BA, Ur E & Wilkinson M (2004) Food restriction regulates adipose-specific cytokines in pituitary gland but not in hypothalamus. *J Endocrinol* **180**, R1-6.

Wijesinghe PR (2006) Obesity is a wake up call for developing world too. *Bmj* **333**, 809.

Wilding JP (2002) Neuropeptides and appetite control. *Diabet Med* **19**, 619-627.

Wilding JP (2007) Treatment strategies for obesity. *Obes Rev* **8 Suppl 1**, 137-144.

Wilkinson MJ & McEwan NA (1991) Use of ultrasound in the measurement of subcutaneous fat and prediction of total body fat in dogs. *J Nutr* **121**, S47-50.

Williams PM, Chang DJ, Danesch U, Ringold GM & Heller RA (1992) CCAAT/enhancer binding protein expression is rapidly extinguished in TA1 adipocyte cells treated with tumor necrosis factor. *Mol Endocrinol* **6**, 1135-1141.

Wolf G (2003) Adiponectin: a regulator of energy homeostasis. *Nutr Rev* **61**, 290-292.

Wood IS, Wang B, Jenkins JR & Trayhurn P (2005) The pro-inflammatory cytokine IL-18 is expressed in human adipose tissue and strongly upregulated by TNFalpha in human adipocytes. *Biochem Biophys Res Commun* **337**, 422-429.

Wood IS, Wang B, Lorente-Cebrian S & Trayhurn P (2007) Hypoxia increases expression of selective facilitative glucose transporters (GLUT) and 2-deoxy-D-glucose uptake in human adipocytes. *Biochem Biophys Res Commun* **361**, 468-473.

Wren JA, Gossellin J & Sunderland SJ (2007) Dirlotapide: a review of its properties and role in the management of obesity in dogs. *J Vet Pharmacol Ther* **30 Suppl 1**, 11-16.

Wu P, Sato K, Yukawa S, Hikasa Y & Kagota K (2001) Differentiation of stromal-vascular cells isolated from canine adipose tissues in primary culture. *J Vet Med Sci* **63**, 17-23.

Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA & Chen H (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* **112**, 1821-1830.

Xu XF & Bjorntorp P (1990) Effects of dexamethasone on multiplication and differentiation of rat adipose precursor cells. *Exp Cell Res* **189**, 247-252.

Yach D, Stuckler D & Brownell KD (2006) Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. *Nat Med* **12**, 62-66.

Yaissle JE, Holloway C & Buffington CA (2004) Evaluation of owner education as a component of obesity treatment programs for dogs. *J Am Vet Med Assoc* **224**, 1932-1935.

Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R & Kadowaki T (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423**, 762-769.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB & Kadowaki T (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* **8**, 1288-1295.

Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P & Kadowaki T (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* **7**, 941-946.

Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L & Kahn BB (2005) Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* **436**, 356-362.

Yeh WC, Cao Z, Classon M & McKnight SL (1995) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev* **9**, 168-181.

Youngstrom TG & Bartness TJ (1998) White adipose tissue sympathetic nervous system denervation increases fat pad mass and fat cell number. *Am J Physiol* **275**, R1488-1493.

Yudkin JS, Kumari M, Humphries SE & Mohamed-Ali V (2000) Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* **148**, 209-214.

Yudkin JS, Stehouwer CD, Emeis JJ & Coppack SW (1999) C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* **19**, 972-978.

Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G, Zlabinger GJ & Stulnig TM (2007) Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *Int J Obes (Lond)* **31**, 1420-1428.

Zhang HH, Kumar S, Barnett AH & Eggo MC (1999) Intrinsic site-specific differences in the expression of leptin in human adipocytes and its autocrine effects on glucose uptake. *J Clin Endocrinol Metab* **84**, 2550-2556.

Zhang HH, Kumar S, Barnett AH & Eggo MC (2000) Tumour necrosis factor-alpha exerts dual effects on human adipose leptin synthesis and release. *Mol Cell Endocrinol* **159**, 79-88.

Zhang HH, Kumar S, Barnett AH & Eggo MC (2001) Dexamethasone inhibits tumor necrosis factor-alpha-induced apoptosis and interleukin-1 beta release in human subcutaneous adipocytes and preadipocytes. *J Clin Endocrinol Metab* **86**, 2817-2825.

Zhang J, Fu M, Cui T, Xiong C, Xu K, Zhong W, Xiao Y, Floyd D, Liang J, Li E, Song Q & Chen YE (2004) Selective disruption of PPARgamma 2 impairs the development of adipose tissue and insulin sensitivity. *Proc Natl Acad Sci U S A* **101**, 10703-10708.

Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C & Hsueh AJ (2005) Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* **310**, 996-999.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425-432.

Zhang Y & Scarpace PJ (2006) The role of leptin in leptin resistance and obesity. *Physiol Behav* **88**, 249-256.

Zhu Y, Qi C, Korenberg JR, Chen XN, Noya D, Rao MS & Reddy JK (1995) Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene: alternative promoter use and different splicing yield two mPPAR gamma isoforms. *Proc Natl Acad Sci U S A* **92**, 7921-7925.

Ziccardi P, Nappo F, Giugliano G, Esposito K, Marfella R, Cioffi M, D'Andrea F, Molinari AM & Giugliano D (2002) Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year. *Circulation* **105**, 804-809.

Zlotnik A, Morales J & Hedrick JA (1999) Recent advances in chemokines and chemokine receptors. *Crit Rev Immunol* **19**, 1-47.

Zumbach MS, Boehme MW, Wahl P, Stremmel W, Ziegler R & Nawroth PP (1997) Tumor necrosis factor increases serum leptin levels in humans. *J Clin Endocrinol Metab* **82**, 4080-4082.