THE DIFFERENTIAL REGULATION OF THE ADIPONECTIN SYSTEM IN RESPONSE TO LIPOPOLYSACCHARIDE AND SEPSIS

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

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LIST OF ABBREVIATIONS

APACHE - Acute physiology and chronic health evaluation

APPL1 - Adaptor protein containing pleckstrin homologous domains

AdipoR1 - Adiponectin receptor 1

AdipoR2 - Adiponectin receptor 2

AMPK - AMP-activated protein kinase

BCA - Bicinchoninic acid

BSA - Bovine serum albumin

BDBM - Bone marrow derived macrophages

BMI - Body mass index

Bp - Base pair

cAMP - Cyclic Adenosine monophosphate

cDNA - Complimentary deoxy ribonucleic acid

cGMP - Cyclic Guanine monophosphate

CIPM - Critical illness polymyopathy

CLP - Caecal ligation and puncture

CSF - Cerebrospinal fluid

Ct -Cycle at threshold

DEXA - Dual energy x-ray absorbometry

DM - Diabetes Mellitus

DMEM - Dubecco's modified eagle medium

DNA - deoxy ribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

EF - Epididymal fat

ELISA - Enzyme linked immunosorbent assay

Epo - Erythropoietin

FCA - Foetal calf serum

FLA - Full length adiponectin

GA - Globular adiponectin

G6P - Glucose-6-phosphatase

GLUT 1 - glucose transporter 1

GCK - Glucokinase

HFD - High fat diet

HIF-1α - Hypoxia inducible factor -1α

HIV- Human immunodeficiency virus

HRE - Hypoxia responsive elements

HRP - Horseradish peroxidase

HMW - High molecular weight

HPA - Hypothalamic-pituitary axis

IMCL - Intramyocellular lipid

Ip - intraperitoneal

IL-1 RA - Interleukin-1 receptor antagonist

IL - Interleukin

kDa - Kilodaltons

KO - Knockout

LPS - Lipopolysaccharide

MAPK - Mitogen activated protein kinase

mRNA - Messenger ribo-nucleic acid

N_2O - Nitrous oxide

NFκB - Nuclear factor kappa B

Ucp2 - Uncoupling protein 2

PAMPs - Pathogen associated molecular patterns

PBS - Phosphate buffered saline

PBST - Phosphate buffered saline/tween

PCK1 - Phosphoenolpyruvate carboxykinase

PCR - Polymerase chain reaction

PI3K - Phosphoinositol 3-kinase

PKC - Protein kinase C

PO_2 - Partial pressure of oxygen

PPAR - Peroxisome proliferator activated receptor

PRF - Peri-renal fat

PRRs - Pattern recognition receptors

qPCR - Real time PCR

SCF - Subcutaneous fat

SEM - Standard error of the mean

SHE - Sucrose / Hepes /Ethylenediamine-tetraacetic acid

SIRS - Systemic inflammatory response syndrome

Srebf1 - Sterol regulatory element-binding protein 1

TLR- Toll like receptor

TNF-α - Tumour necrosis factor-α

UV - Ultraviolet

VHL - Von Hippel Lindau protein

WAT - White adipose tissue

WT - Wild type

ABSTRACT

Background

Sepsis is a condition characterised by a massive acute inflammatory response and insulin resistance. Several inflammatory mediators involved in the immune response during sepsis have been identified. Recently it has become clear that adipose tissue contributes to the production of pro- and anti-inflammatory mediators, which have been termed adipokines. Adiponectin is an adipokine that has anti-diabetic, anti-atherogenic and anti-inflammatory properties. Its role in chronic inflammatory diseases, such as type II diabetes mellitus (DM) and obesity has been extensively studied. Generally, adiponectin is down-regulated in these conditions which are characterised by insulin resistance. Adiponectin, previously thought to be exclusively expressed in and secreted from adipocytes, has now been shown to be released from other tissues such as skeletal muscle, cardiac muscle and bone. Adiponectin from adipose tissue is down-regulated in mouse models of sepsis, however, no information is available about the role of adiponectin receptors. In chronic insulin resistance, adiponectin receptor gene expression is decreased, suggesting a down-regulation of the 'adiponectin system'. Adiponectin gene expression appears to be partially regulated by NFκB, a transcription factor co-ordinating the release of inflammatory mediators in response to an appropriate stimulus, such as lipopolysaccharide. Other signalling mechanisms may also be involved, in particular the HIF-1α pathway. HIF-1α is another transcription factor with a large number of target genes, many of which are involved in the inflammatory process. Although HIF-1α was initially discovered as a cellular regulator of hypoxia, the pathway has now been shown to be activated by other non-hypoxic mechanisms of up-regulation, including bacterial infection. HIF-1α is expressed in immune cells, however, its role in adipose tissue during sepsis remains unclear.

Methods

Three different lines of experiments used in this thesis. The animal model used high dose LPS injected intra-peritoneally (under general anaesthesia) into 8-10 week old male C57BL/6J mice. Mice were killed at 4 or 24 hours after injection and tissues (Peri-renal, subcutaneous and epididymal fat, liver, muscle, small bowel and spleen) were removed for analysis. Adiponectin and adiponectin receptor gene expression was determined by quantitative real-time PCR (qPCR). The cell culture model used cell lines, 3T3-L1 adipocytes and C2C12 myocytes, grown in culture and then treated with varying concentrations of LPS. Cells were harvested at 4 and 24 hours and qPCR was performed to ascertain adiponectin and adiponectin receptor gene expression. The same animal and cellular models were utilised for the HIF-1α investigations with protein determination carried out using ELISA.

Finally, twenty-one septic patients were recruited from the Intensive care unit at the Royal Liverpool University Hospital, following ethical approval and written consent. Blood samples were taken on

days 1 and 2 and day of discharge and serum levels of total and HMW adiponectin were determined by ELISA.

Results

Alterations in adiponectin and its receptors expression in murine endotoxaemia

Adiponectin receptors were down-regulated following LPS injection. The greatest changes acutely were in muscle, liver and peri-renal fat (adipoR1) and liver, muscle, peri-renal and sub-cutaneous fat (adipoR2). There were no significant changes in the other tissue depots. After 24 hours, there were fewer changes in gene expression with adipoR1 being down-regulated in liver and skeletal muscle and AdipoR2 in skeletal muscle only. Down-regulation of adiponectin gene expression following LPS was confirmed in the adipose tissue depots. We demonstrated that the adiponectin gene was expressed in skeletal muscle and sequencing of the PCR product confirmed a 100% match for adiponectin mRNA. C2C12 myocytes were then used to verify the presence of adiponectin mRNA in skeletal muscle cells. In tissue depots, adiponectin gene expression was significantly reduced in skeletal muscle in both the 4 and 24 hour cohorts respectively.

Alterations in adiponectin and its receptors expression in cell lines

In the cell lines, the inflammatory response to LPS was confirmed using IL-6 as a reference gene. This also confirmed methodological success. Adiponectin gene expression from 3T3-L1 adipocytes was acutely reduced following treatment with high dose LPS but there were no changes in expression in cells treated with lower concentrations of LPS. There were no changes at 24 hours. Adiponectin receptors were down-regulated but not consistently with dose and these changes were only observed in the cells harvested after 4 hours. In C2C12 myocytes, there was a significant reduction in adiponectin gene expression following high doses of LPS but there were minimal changes in adiponectin receptor expression in the C2C12 myocytes.

Human Study

There was a significant increase in both total and HMW adiponectin between day 1 and day of discharge and the ratio of HMW adiponectin to total adiponectin also increased between admission and discharge. There were no changes in total or HMW adiponectin or their ratio between day 1 and day 2 of admission.

HIF-1α

HIF-1α gene expression was up-regulated in liver and spleen 4 hours post LPS injection. The changes persisted 24 hours after LPS treatment with increased expression in liver, small bowel and spleen. Protein levels were elevated in skeletal muscle after 4 hours and liver after 24 hours and spleen.

Discussion

These results increase the evidence that adipose tissue is not an inert storage medium for fatty acids but a sophisticated endocrine organ. The 'adiponectin system', including adiponectin and its two receptors, is down-regulated in-vivo and in-vitro models of sepsis. This may play a role in the metabolic derangements such as hyperglycaemia and insulin resistance. In addition, hypoadiponectinaemia may have a significant role in the disordered inflammatory process known to occur in sepsis, possibly impacting on mortality as shown in some animal studies. Adiponectin is not exclusively adipose tissue derived and interestingly we have demonstrated the presence of adiponectin mRNA in other tissue such as skeletal muscle. The effect of reduced gene expression from extra-adipose tissue depots is yet to be established but may have a paracrine or autocrine effect rather than an endocrine role.

Low total and HMW adiponectin levels during human sepsis have also been identified. Whilst hypoadiponectinaemia in sepsis has been observed in previous studies, increases in HMW adiponectin associated with clinical improvement have not been previously demonstrated. A further signalling pathway investigated in these models was HIF-1α. These results demonstrate a global up-regulation of HIF-1α gene expression across tissue depots and cellular models. This may reflect tissue hypoxia but also may reflect non-hypoxic up-regulation by LPS and inflammatory mediators. HIF-1α is known to play a part in the inflammatory process and, like adiponectin, has links to the NFκB signalling pathways.

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ABSTRACTS ASSOCIATED WITH THIS WORK

Hall A, Leuwer M et al. High molecular weight adiponectin increases with improvement of severe sepsis: *Int Care Med* **2011**; 37: (S1) S140

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CHAPTER 1: INTRODUCTION

1.1 Sepsis

Sepsis is a condition responsible for thousands of deaths annually and a large burden on health budgets throughout the world. Angus et al. estimated the annual incidence to be 750,000 cases resulting in 215,000 deaths, nearly 10% of all deaths in the USA[1]. Although in-hospital costs in the USA are estimated at $16 billion[2], the post-hospital care costs will almost certainly be much more[3].

Until 20 years ago, there were no clear definitions of sepsis. However, the concept of the Systemic inflammatory response syndrome (SIRS) and its wide ranging triggering factors (Infective and non-infective (burns, trauma, acute pancreatitis)) was clearly established in a consensus conference in 1992 (Table 1).

Table 1: Criteria for diagnosis of the Systemic Inflammatory Response syndrome (SIRS)

Two or more of:	
Temperature	>38°C or <36°C
Pulse	>90 beats per minute
Respiratory rate	>20/min or PCO_2 <32mmHg
White Cell Count	>12,000/mm^3or <4000/mm^3

Table 1: SIRS Criteria: A consensus conference (1992) defined sepsis as a SIRS secondary to a confirmed or high suspicion of a pathogen[4]

Severe sepsis was defined as sepsis associated with organ dysfunction, hypoperfusion or hypotension and septic shock as sepsis-induced hypotension despite adequate fluid resuscitation along with evidence of organ malperfusion[4]. These definitions are purely based on clinical symptoms. Quantification of severity is currently difficult with the lack of specific biochemical markers to define the septic pathology[5]. In 2001, a second consensus conference sought to further clarify the conditions of 'infection', 'inflammation' and 'organ dysfunction'[5]. This was aimed at

helping the clinician to distinguish morbidity from the infective process from that of the host response, and target therapy appropriately[5].

Targeted treatment, however, has been difficult to find for a number of reasons: Patients with sepsis and septic shock are a very heterogeneous population with numerous different causative organisms and individual host responses. In addition, many trials use retrospective data set analyses based on notes coding and, despite large numbers, the frequency of known and unknown missing data is high[1 2 6].

Sepsis can be caused by various organisms. Gram-negative organisms were shown to be the most frequently identified organism during the eighties (1979-1987) but thereafter, gram-positive organisms have predominated. In more recent studies, gram-negative and -positive organisms account for 80-90% and polymicrobial and fungal infection for about 5% each of all infections, depending on their source[2 6 7]. Gram-positive infections as a cause for sepsis have increased for several reasons, in particular frequent use of antibiotics causing gram-positive hospital-acquired infections, increased use of indwelling catheters and other invasive devices and a rise in recreational drug use and HIV status allowing more opportunistic infection to occur[2]. Overall, respiratory tract infections are the most common site of infection accounting for 45-58% of cases of sepsis. Other common sources are primary bloodstream, the abdominal cavity and the urinary tract[1].

1.1.2 Innate immunity and inflammation

There are a large number of potential pathogens which may cause sepsis and subsequently activate the immune system, but the host response remains the same irrespective of the invading pathogen. Non-mammalian cells display highly conserved sequences of DNA which are not present in human DNA (pathogen associated molecular patterns, PAMPs)[8-10]. Human immune cells have pattern recognition receptors (PRRs) to identify the PAMPs and instigate a plan of attack against microbial invasion[9].

The subsequent immune response is divided into two parts, the innate and the adaptive response. The innate immune system is the ingrained immediate response whereby host monocytes and macrophages recognise the PAMP of an invading organism as 'foreign'. Host cell PRRs are activated and thus directly stimulate the innate immune system[10]. The end result includes phagocytosis and synthesis of antimicrobial peptides with the ultimate aim of destroying of the invading organism. Also, the expression of inflammatory markers, in particular cytokines and chemokines, is induced to control the recruitment of leucocytes to the infection site and the subsequent inflammatory response[10].

Activation of lymphocytes marks the initiation of the adaptive immune response[11]. Following the destruction of the organism, antigen presenting cells (e.g. macrophages) present the antigen to T-lymphocytes. This requires a co-stimulatory molecule expressed on the antigen presenting cell[10]. The combination of the PAMP and the co-stimulatory signal results in the clonal expansion and activation of specific T-lymphocytes. This also induces the activation and expansion of B-lymphocytes for the production of antibodies to protect against subsequent attack[10].

The inflammatory response to the invasion of pathogenic organisms is present throughout the body and has been shown to occur in other organs including skeletal muscle and adipose tissue[12 13]. White adipose tissue (WAT) is a sophisticated endocrine organ and producer of numerous signalling molecules which interact with many physiological processes including immunity, coagulation and glucose homeostasis[14-20]. It has been shown to release many inflammatory markers in response to stimulation with infective organisms[12].

Quantitatively, fatty acid secretion remains the greatest fraction, but many other molecules are produced from WAT. These include many hormones, particularly adiponectin and leptin, but also a range of proteins with wide diverse biological function. These have been termed adipokines and it

has been recommended that the term is restricted to molecules synthesised and secreted from adipocytes and does not include molecules released from other cell types in adipose tissue such as macrophages[20]. The adipokines released include cytokines such as TNF-α[12 21], IL-6[12] and IL-18[22], leptin [23 24], Plasminogen activator inhibitor-1[25], resistin[26], adipsin[27] and adiponectin[15 21 28-30].

One of these 'adipokines', adiponectin, has been extensively studied since its identification in 1995. It has been shown to be an anti-inflammatory, anti-atherogenic insulin sensitizer, thus promoting glucose utilisation and fatty acid oxidation[28 30-36].

1.1.3 Host recognition and signalling pathways

The host is able to recognise sections of the cell wall of different microbial species. Toll-like receptors (TLRs) are highly conserved transmembrane PRRs of the innate immune system[3 11]. They respond to the presence of bacterial products and alert the host to a potential invader[11 37]. TLRs respond following binding of highly conserved parts of the invading microbe, instigating the innate immune system response [9].

Nine TLRs have been described thus far (TLR1-9)[38-40]. They are integral membrane proteins, spanning the plasma membrane once. The TLR subtypes differ structurally with TLR2 and TLR4 sharing only 24% of identical sequences[11]. This is suggestive that they bind different ligands. In contrast, the intracellular portion contains approximately 200 amino acids and is evolutionarily conserved suggesting they share similar intracellular pathways[11 41]. TLR1 is ubiquitously expressed whereas TLR2 is expressed on blood mononuclear cells and lymphoid tissue. TLR3 is present in lung, muscle, heart, brain and intestine. TLR4 was previously thought be expressed only in lymphocytes, spleen and heart[11], however, it is now evident that in many insulin sensitive tissues (adipose tissue, liver and skeletal muscle), pancreatic cells and vascular tissue [42]. TLR2 and TLR4 are the most intensively studied of this receptor family. They have a number of ligands which include many components of the bacterial cell[11] (Table 2). Although there is some overlap in the ligands for each receptor, TLR2

does not appear to be essential in cells that also express TLR4 but may be an alternative LPS receptor in those that do not[43].

Table 2: Ligands for Toll-like receptors 2 and 4

	Gram negative ligands	Gram positive ligands
TLR 2	LPS (salmonella, shigella and E.Coli only)	Lipoteichoic acid Peptidoglycans Lipopeptides
TLR 4	LPS (Most gram-ve bacteria) Lipid A	Lipoteichoic acid

LPS preferentially activates the innate immune system by binding to TLR4. LPS binding protein (LBP) binds LPS and presents it to CD14, a known PRR on the surface of monocytes[37]. CD14 is an opsonic receptor but is unable to produce a transmembrane signal and therefore must interact with TLR4 for intracellular signalling[8 11]. The binding of TLR4 to LPS is enhanced by MD2, an accessory protein which binds to the extracellular domains of TLR4 for maximal responsiveness[44] (Figure 1).

Post-receptor signalling

Post-receptor signalling in response to LPS has been well documented. Nuclear Factor-κB (NFκB) activation is the final common pathway following stimulation of TLRs (Figure 1). NFκB belongs to a category of rapid acting transcription factors and does not require protein synthesis for activation[45]. It is present in all animal cells and is highly conserved throughout many species except yeasts[45].

Figure 1: Signaling pathway of NFκB following LPS binding to TLR-4

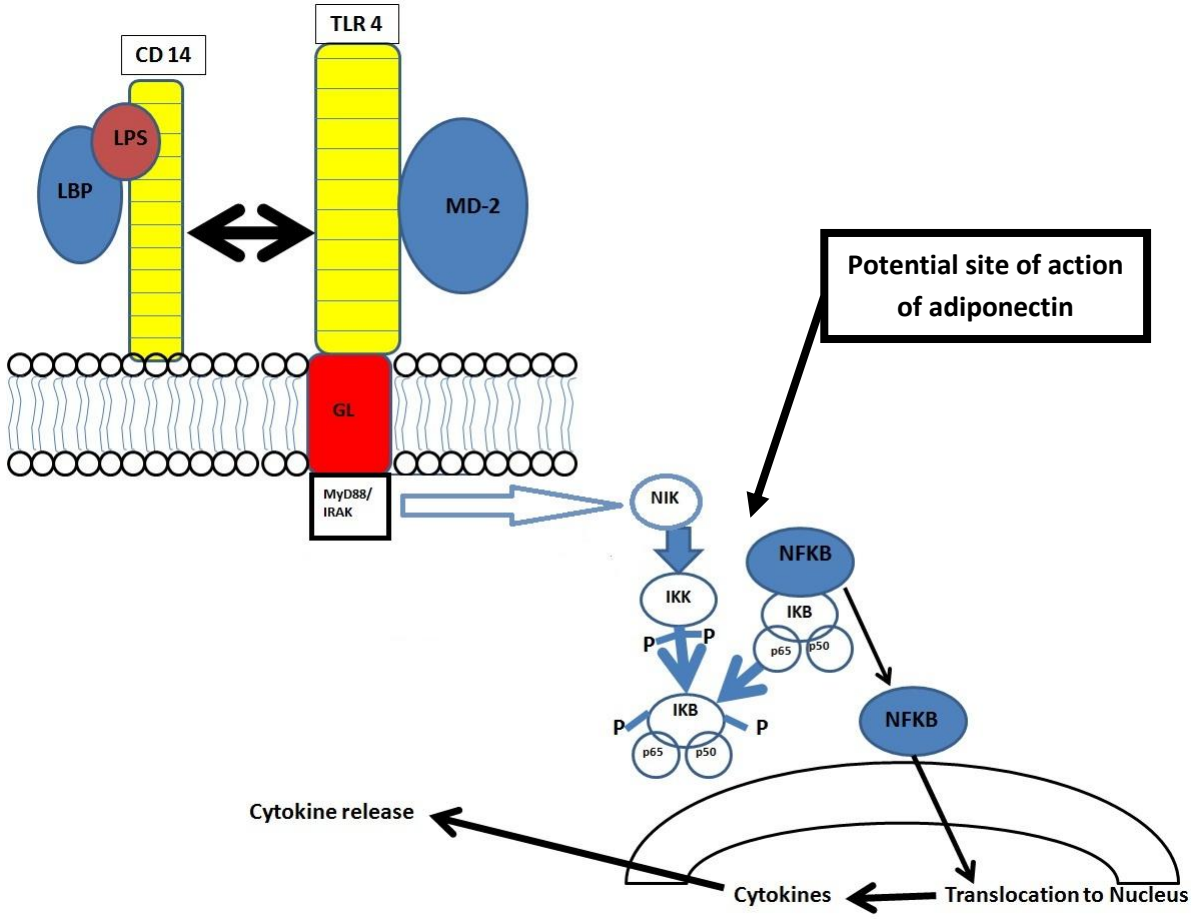


Figure 1: Recognition of LPS on the surface of phagocytes leads to NFκB activation. LPS is opsonised by LBP and the complex is recognized by the opsonic receptor, CD 14, on the macrophage surface. CD 14 associates with the cell surface by means of a glycolipid linkage and is not capable of generating its own transmembrane signal. The complex of LPS/LBP/CD14 activates the TLR 4 complex and signals through the adaptor protein MyD88 and the serine kinase IRAK. NFκB is constitutively expressed in an inactive form, dimerised with Inhibitor-κB (I-κB) inhibitors. They form a complex that is sequestered in the cytoplasm of cells. I-κB has numerous ankyrin repeats which prevent transmission of nuclear localisation signals to NFκB[45 46]. Ligand binding to TLR-4 causes formation of a complex with the intracellular domain MyD88 (an adaptor protein) and IRAK (a kinase). IRAK then phosphorylates the downstream molecule TRAF6, which activates NKKB inducing kinase (NIK) and I-κB which then dissociates. NFκB is then free to translocate to the nucleus and induce expression of NFκB target genes[11]. Upon activation, I-κB is degraded by its own kinase, I-κB kinase (IKK) which phosphorylates 2 serine residues in the I-κB regulatory domain[46-48]. Adapted from Aderem A et al[8 11]. (LPS: Lipopolysaccharide, LBP: Lipopolysaccharide Binding protein, TLR4: toll-like receptor-4)

There are more than 150 known target genes of NFκB, many related to immunity and inflammation (cytokines, chemokines, MHC complexes, Nitric Oxide (NO) and Cyclo-oxygenase-2) thus earning its name as the 'central mediator of the human immune response[49]. NFκB regulates many acute phase proteins and is also induced by non-infective stress such as ischaemia/reperfusion injury, haemorrhagic shock and irradiation.

1.1.4 Cytokines

Following TLR activation, the expression of many pro-inflammatory and anti-inflammatory molecules is increased. They can be broadly divided into pro-inflammatory (e.g. IL-6, TNF-α) and anti-inflammatory (e.g. IL-10, IL-13) cytokines. Following pathogen invasion, the body mounts an inflammatory response, releasing pro-inflammatory cytokines in reaction to the triggering insult. Rapidly thereafter, an anti-inflammatory response occurs to down-regulate the release of inflammatory mediators and to alter their effects, thus attempting to restore homeostasis[50] [51] (Figure 2).

Figure 2: Cytokine response in sepsis

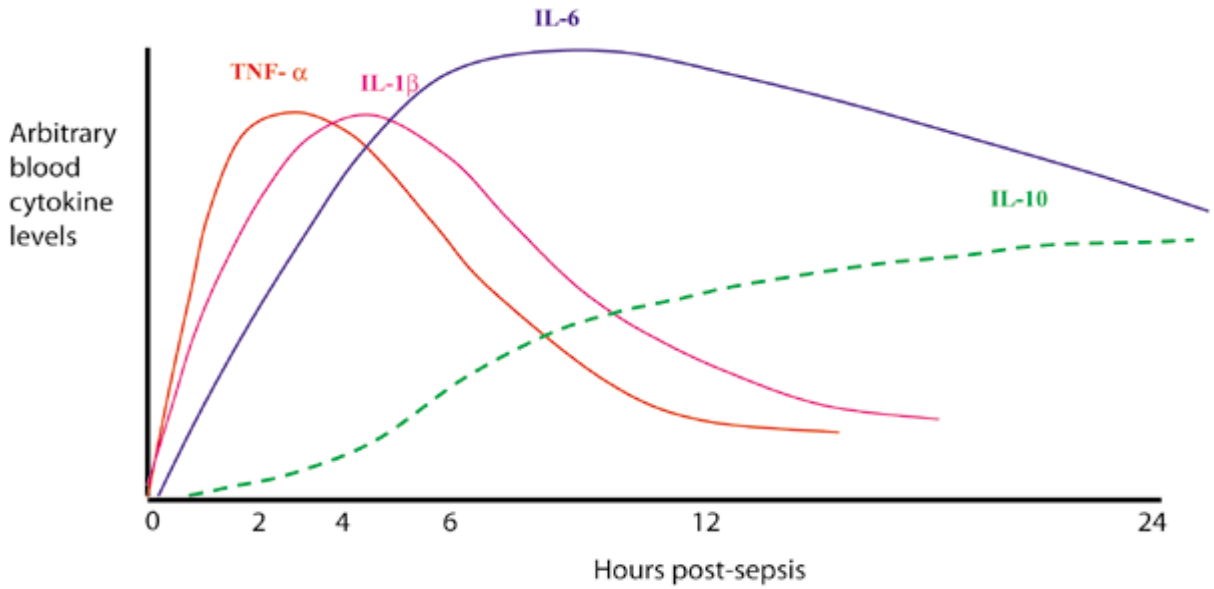


Figure 2: Following pathogen invasion, there is rapid up-regulation of pro-inflammatory cytokines (e.g. IL-6, IL-1β, TNF-α). This can be seen as a response to mobilise immune cells and energy substrates to counteract the infection and represents a transient and highly regulated response[52]. In order to restore homeostasis, anti-inflammatory cytokines are released. In progressing sepsis, this process can become unchecked and out of control. Figure Adapted from Boontham et al.[53]

The severity of this reaction varies depending on the infection and also the host's individual response. It is poorly understood why some infections cause sepsis and others do not, but genetic variations are likely to play a role[54]. The inflammatory response affects many physiological systems which are intricately linked to the inflammatory process. These include coagulation pathways, endothelial function and many metabolic pathways including glucose haemostasis.

1.1.5 Glucose metabolism in sepsis

Hyperglycaemia in sepsis and other forms of severe stress is very common[55-57]. Critical illness or trauma causes activation of hypothalamic-pituitary axis (HPA), resulting in the production of cortisol[58 59]. This is closely linked to the regulation of blood glucose levels. The neuroendocrine response is an essential component of adapting to illness and stress and is crucial for the maintenance of cellular homeostasis[58]. Although activation of the HPA axis is well described[56 57], the mechanisms of dysfunctional glucose metabolism alterations in sepsis are more complex and not yet fully understood (Figure 3).

Figure 3: Contributing factors to hyperglycaemia during sepsis

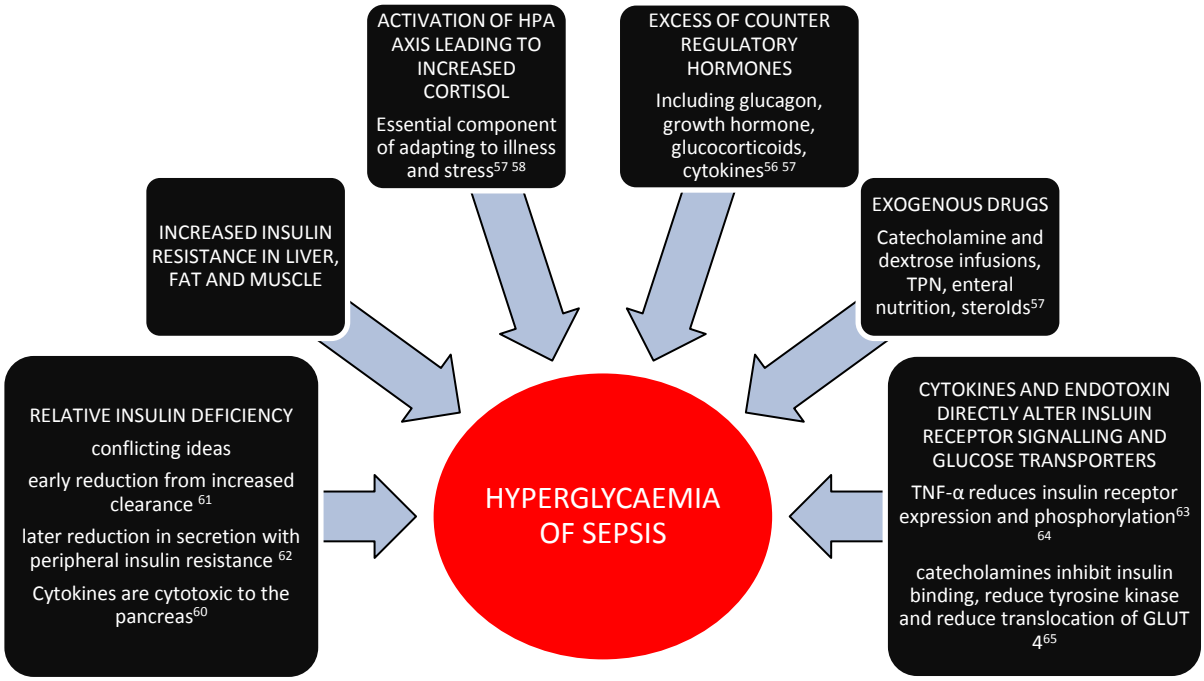


Figure 3: Critical illness or trauma causes activation of the hypothalamic-pituitary axis (HPA), resulting in the production of cortisol[58 59]. This is an essential component of adapting to illness and stress in order to maintain cellular homeostasis[58]. As a result of HPA activation, an orchestrated counter-regulatory hormone release occurs, which includes glucagon, growth hormone, cortisol, glucocorticoids and pro-inflammatory cytokines[57 58]. This results in the 'diabetes of injury', thought to be beneficial in the initial stages of illness by promoting glucose uptake in non-insulin sensitive cells. During septic shock, exogenous drugs are administered including infusions of exogenous catecholamines, dextrose, TPN and enteral nutrition and steroids which aggravate hyperglycaemia[58]. Endogenous and exogenous catecholamines reduce pancreatic beta cell insulin secretion[57 60 61]. In early sepsis, there may also be low circulating insulin concentrations secondary to increased clearance[62]. A reduced peripheral response to insulin[63] as well as increased gluconeogenesis occurs despite high circulating insulin levels due to increased gluconeogenic substrates e.g. lactate, alanine, glycerol but also due to an increase in glucagon[58]. TNF-α, which is released during early sepsis, induces insulin resistance by reducing insulin receptor expression, tyrosine phosphorylation and inhibition of insulin induced phosphorylation of Insulin Receptor Substrate-1[64 65]. Catecholamines also inhibit insulin binding, reduce tyrosine kinase and reduce the availability of membrane bound glucose receptors[66].

1.2 Adiponectin

White adipose tissue (WAT) has long been considered to be purely a storage organ for high energy compounds for release at times of energy depletion. However, more recently, WAT has come under the spotlight following its discovery as a sophisticated endocrine organ. Adiponectin (also previously known as Acrp30[67], adipoQ[68], apM1[69] and GBP28[70]) was discovered independently by four groups, with most demonstrating its adipose specificity using northern blotting techniques. Human adiponectin was shown to have 83% morphology with mouse adiponectin[69]. Adiponectin circulates in nanomolar concentrations in serum at approximately 10-30 μg/ml and has a relatively short half life of approximately 5-6 hours[71]. It accounts for approximately 0.05% of total serum protein[67]. Adiponectin has attractive properties to be a potential signalling molecule involved in hyperglycaemia and insulin resistance commonly seen clinically in sepsis.

1.2.1 Structure

Adiponectin is a 30 kDa, 244 amino acid polypeptide, coded for on chromosome 3q27 and is structurally similar to complement factor C1q and various members of the collagen family, namely Collagen X and VIIIa. The full length protein contains an amino terminal followed by a collagenous structure similar to Collagen X. This is extended by collagen repeats essential for the collagen triple helix formation, and there is a globular head at the C-terminus end [29 68 72]. Complement factor C1q, an oligomer, contains a heterotrimeric unit with a three stranded collagen tail and three globular heads. The globular domain of adiponectin is structurally similar to TNF-α, suggesting a potential evolutionary link between the two molecules[31].

Adiponectin expression in-vitro commences from the intermediate stage (day 2-5 post confluence) of adipogenesis onwards[67 68]. Its expression is increased during adipocyte differentiation (up to 350 – fold) and may therefore be able to act as a marker of adipocyte differentiation[67 73 74].

Figure 4: Structure and hypothesized signalling pathways of adiponectin

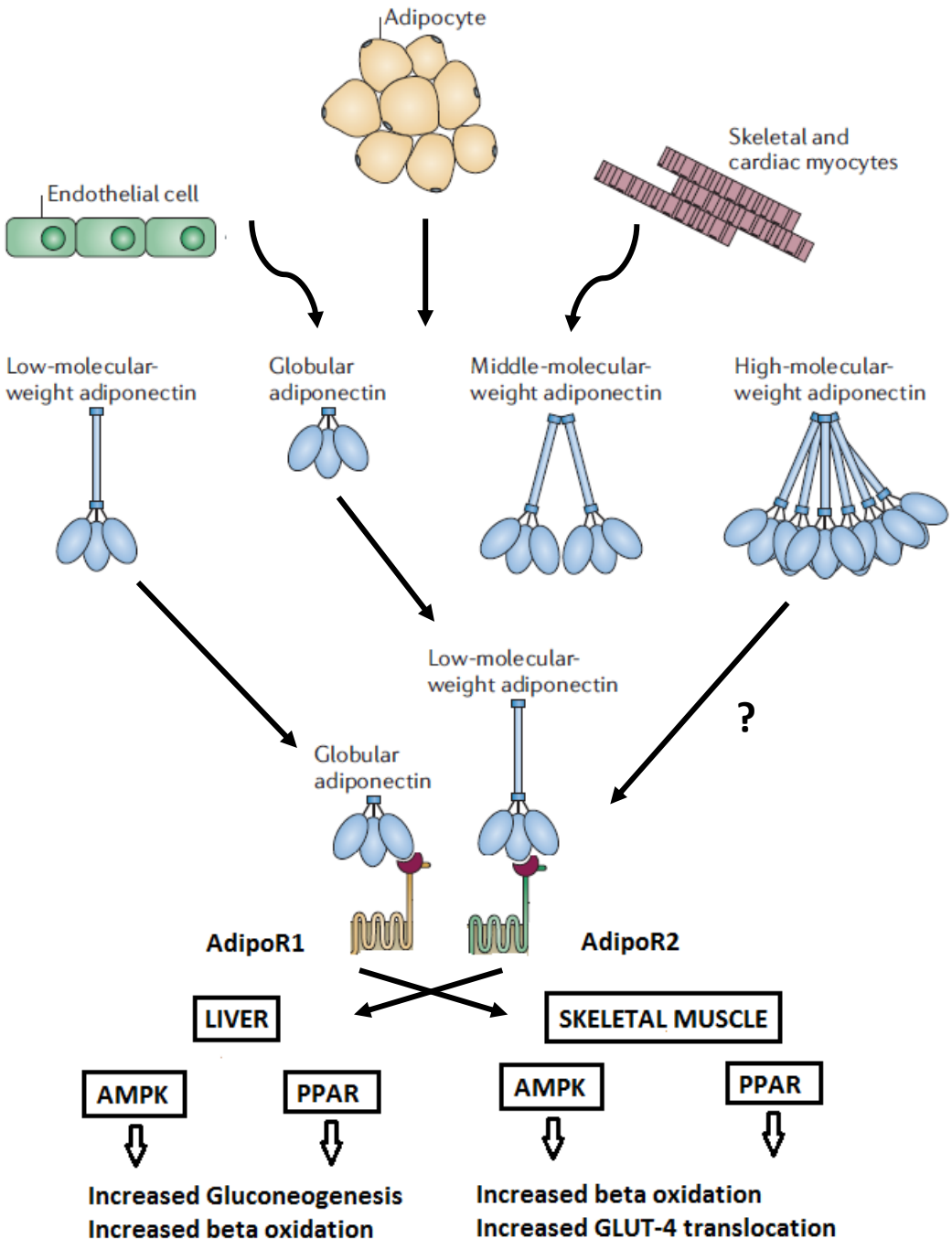


Figure 4: Adiponectin is produced predominantly by adipocytes but also by other cell types including skeletal muscle. The full length molecule undergoes post-translational modifications forming trimers, hexamers and 12-18-mers called low (LMW), middle (MMW) and high-molecular weight (HMW) respectively[71 75 76]. These are thought to be essential to its final function and to the insulin sensitising features of the molecule[77 78]. Adiponectin interacts with its two membrane receptors to initiate changes in glucose and insulin signalling via AMPK and PPARα[72 79]. Hara et al. found that HMW adiponectin binds the most avidly to its adiponectin receptors, thus stimulating AMP activated protein kinase (AMPK)[80]. Modified from Tilg et al. 2006[72].

Adiponectin is produced predominantly by adipocytes but also by other cell types including skeletal muscle and endothelial cells (Figure 4). Once synthesized, it circulates as the full length protein and its proteolytic cleavage product consisting of the globular C-terminal domain. The globular head is thought to be cleaved by leukocyte elastases secreted from activated monocytes or neutrophils[72 81]. Globular adiponectin is present in very small amounts in plasma, if at all[32 79]. Globular adiponectin can trimerise after cleavage but cannot form any larger multimers[81]. The full length adiponectin molecule undergoes post-translational modifications thought to be essential to its final function. Modifications include hydroxylation and glycosylation of highly conserved lysine and proline residues within the collagenous section of the molecule. These are thought to be important to the insulin sensitising features of the molecule[77 78]. In bacterial recombinant non-glycosylated adiponectin where these changes do not occur, the end product is significantly less potent[77]. Within plasma, the full length adiponectin protein is present as homo-oligomers of which there are three distinct types[29 67 70]. Circulating full length adiponectin forms trimers, hexamers and 12-18-mers called low (LMW), middle (MMW) and high-molecular weight (HMW) respectively [71 75 76]. The stability of the larger multimers is maintained by di-sulphide bonds and represents a post-translational modification of the adiponectin[71]. It has been suggested that the HMW adiponectin is the more biologically active molecule[70 80 82]. Adiponectin interacts with its two membrane receptors to initiate changes in glucose and insulin signalling via AMPK and PPARα[72 79]. Hara et al. found that HMW binds the most avidly to its adiponectin receptors, thus stimulating AMP activated protein kinase (AMPK)[80].

1.2.2 Regulation

Much of the work performed investigating adiponectin has utilised different mouse models that are genetically prone to obesity. This includes the following:

Table 3: Common mouse models used

Mouse model	Genetic modification and phenotype
db/db mice	Deletion of leptin receptor gene Mice develop human Type 2 DM, hypertension and obesity [83 84 85].
ob/ob mice	Deletion of the leptin gene Mice develop insulin resistance, obesity and hyperinsulinaemia[86 87].
Obese Zucker rats	Recessive modification of the leptin receptor Mice develop obesity and insulin resistance[88 89]
KKAy	Heterozygotes for the yellow spontaneous mutation Develop severe obesity, hyperlipidemia, and insulin resistance[90]

There are consistent reports of down-regulation of adiponectin in pathologies characterised by chronic insulin resistance. These include type II DM [15 91 92], obesity [36 79 93] and the metabolic syndrome[15 28]. Gender differences in adiponectin expression and secretion have been described with plasma levels being consistently higher in females than males even with similar degrees of body adiposity[77 94]. This indicates that sex hormones may play a role in adiponectin regulation[95 96]. Similar to total adiponectin, HMW adiponectin shows a consistent relationship with gender with females having a higher concentration than males[75 80 97 98].

1.3 Mechanism of action

Adiponectin has numerous actions including anti-inflammatory, anti-diabetic and anti-atherogenic effects[31 32 36]. Its main metabolic effect is to increase fatty acid oxidation, thus reducing intracellular triglyceride concentration in insulin sensitive cells[36]. Secondly, it appears to enhance the suppressive effect of insulin on hepatic gluconeogenesis, via reduction in enzyme expression thus reducing hepatic glucose output[31 99].

1.3.1 Adiponectin, the insulin sensitiser

There is a clear link between adiponectin and insulin sensitivity/resistance which is demonstrated by many studies[15 31 91 94 99 100]. Many conditions that are characterised by insulin resistance e.g. obesity and type II DM display impaired glucose and lipid metabolism. The end result is an increase in lipid stores in insulin sensitive tissues e.g. muscle and liver, and hyperglycaemia[35 101]. Adiponectin attenuates insulin resistance by increasing fatty acid oxidation and thereby reducing the triglyceride concentration of skeletal muscle and liver[35]. This process is dependent on the intracellular activation of 5' adenosine monophosphate-activated protein kinase (AMPK) and peroxisome-proliferator activated receptor (PPARα)[32 35]. In skeletal muscle, adiponectin can directly increase fatty-acid transport, oxidation and dissipation, thus reducing the levels of intramyocellular lipids. Furthermore, treatment with exogenous adiponectin in diabetic mouse models has shown consistent reductions in plasma glucose levels and improvements in insulin resistance[31 36]. After adiponectin treatment, a decrease in circulating glucose without increase in plasma insulin levels is observed which indicates a potential reduction in hepatic glucose production[31]. This is consistent with a reduction in hepatic expression of gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PECK) and Glucose-6-phosphatase (G6Pase) following adiponectin infusion[99], suggesting a reduction in hepatic gluconeogenesis as an additional mechanism.

1.3.2 Adiponectin, the anti-inflammatory hormone

Adiponectin has anti-inflammatory properties[77 102 103]. This is important as it has been demonstrated that adiponectin may be reduced in the acute inflammatory processes[12] and hypoadiponectinaemia in mice carries a higher mortality[104 105]. Numerous studies have attempted to elucidate some of the complexities of the hormone and its role in inflammatory processes.

Adiponectin has been shown to inhibit proliferation of a number of myeloid cell lines, to reduce the phagocytic ability of macrophages and to down-regulate macrophage recruitment to sites of

inflammation[100 103 106]. TNF-α gene expression (adipose tissue) and plasma concentration are significantly higher in adiponectin knock-out mice compared to control animals, a phenomenon almost completely reversed by the addition of viral recombinant adiponectin[28]. A number of studies have demonstrated the role of pre-treatment with adiponectin to reverse the increased inflammatory cytokine release in macrophages following LPS treatment in-vitro[106-108]. Although these results are consistent, these studies clearly demonstrate that adiponectin given concurrently with LPS does not have the same effect. Therefore, adiponectin treatment may not be effective in clinical sepsis as pre-treatment before activation of the immune system by LPS or other bacterial components is practically impossible.

In addition to reducing pro-inflammatory mediator production, adiponectin also appears to increase the production of anti-inflammatory mediators from macrophages and their precursors, as treatment with adiponectin increased IL-10 and IL-1RA in primary human monocytes, macrophages and dendritic cells[107 109].

The effects of adiponectin on white cell function are of interest. Sepsis is characterised by migration of leukocytes to the area of inflammation or damaged tissue in an attempt to remove the invading organism. This can result in further damage to the inflamed tissues. Various studies have demonstrated a role for adiponectin in this process. A reduction in cell adhesion molecules, which mediate adhesion of neutrophils to the endothelium, has been shown after treatment with adiponectin[110 111], which could theoretically prevent endothelial damage during the inflammatory process. Adiponectin may therefore have a key role in sepsis, not only in the metabolic derangements that occur but also in regulation of the inflammatory cascade that accompanies the disease.

Investigations using murine 3T3-L1 adipocytes exposed to various mediators showed a reduction in adiponectin expression by glucocorticoids (Dexamethasone 100 nmol/L), insulin (100 nmol/L) and inflammatory cytokines such as TNF-α (10 and 100 ng/ml) and IL-6 (30 ng/ml) [28] [112] [113]. These hormones resulted in up to 50% reduction in adiponectin gene and protein expression. In addition, removal of the respective mediator for 24 hours reversed these effects[112] [113]. These observations support the concept that pro-inflammatory mediators contribute clinically to sepsis-induced insulin resistance by their interaction with adiponectin.

In-vivo, in severe sepsis, as in obesity, adipose tissue and plasma adiponectin gene expression and secretion are reduced. This coincides with a rise in adipose tissue inflammatory cytokines[12] [103]. This enhanced inflammation may contribute to the higher mortality observed in animals with hypoadiponectinaemia and polymicrobial sepsis than WT animals[104] [105]. Adiponectin KO mice showed vastly elevated inflammatory markers, including cell adhesion molecules. Pharmacological interventions in particular the PPAR-γ agonist, rosiglitazone[105] and recombinant adiponectin[104] improved mortality and ameliorated the rise in inflammatory markers and may therefore possess therapeutic potential for the treatment of sepsis. There is some evidence that recombinant adiponectin binds to and possibly neutralises LPS[103] [114]. This may account for some of the documented reduction in plasma and adipose tissue adiponectin levels in models using LPS.

Few clinical studies have been performed on the role of adiponectin in sepsis and endotoxaemia in humans. Human volunteer studies demonstrated no alteration in adiponectin or its multimer levels, despite rises in inflammatory cytokines, after injection with endotoxin[115] [116]. However, the same study demonstrated the down-regulation of adipoR1 and adipoR2 gene expression in human whole blood (49% and 65% respectively) and monocytes (33% and 28% respectively) 12 hours after an IV injection of endotoxin in to healthy volunteers[116]. A further study, using a small number of samples from critically ill patients with historical controls, showed a much lower mean plasma adiponectin

concentration in the critically ill patients[117]. There was no correlation between plasma adiponectin and APACHE score. Although this study provides further information on adiponectin in the critically ill patient, the study was not initially designed to measure adiponectin and in addition, the group of patients was highly heterogeneous and male dominated. Due to lack of samples, there was only analysis on days 3 and 7 and no baseline samples for analysis.

1.3.3 High molecular weight adiponectin and sepsis

Post translational modification of adiponectin leads to multimerisation forming low, middle and high molecular weight adiponectin[71]. Emerging literature has demonstrated a greater correlation to markers of insulin resistance with High Molecular Weight (HMW) adiponectin rather than the total concentration[80]. Insulin resistance is also associated with alterations in HMW adiponectin and mutations in the adiponectin gene are associated with an increase in insulin resistance and type II DM[76].

The concept of the HMW/total adiponectin ratio has subsequently been established. This compares the concentration of HMW to total adiponectin in plasma. It has been demonstrated that, even without changes in total adiponectin, increases in the ratio demonstrating a rise in the HMW adiponectin multimer, confers favourable effects on insulin sensitivity and other metabolic parameters[82]. It has also been shown to be useful as a monitoring tool to assess response to the thiazolidinediones class of drug, used commonly to treat type II DM[82].

Despite increasing evidence that total adiponectin is reduced in sepsis and experimental endotoxaemia[12 104 105], the role of HMW adiponectin in human sepsis has not been investigated thoroughly. A small study investigated HMW adiponectin in children with sepsis, septic shock and a control group. They found that HMW adiponectin levels were increased on day 1 compared to controls (8 vs 3.3 μg/ml $p<0.05$) and the absolute values correlate with the PRISM score, a score of

severity of illness in children[118]. However, other studies have shown a reduction in total adiponectin in mice with polymicrobial sepsis[104 105] and in LPS induced endotoxaemia[12].

The link between adipose tissue inflammation and chronic insulin resistance may well be relevant in acute inflammatory conditions such as sepsis. It is also likely that adiponectin is not only linked to the metabolic derangements but also to the inflammatory changes seen in sepsis.

Potential links include NFκB [119 120] and its target genes as well as mitochondrial dysfunction which has been postulated as a mechanism underlying changes in insulin signalling in the chronic inflammatory changes in type II DM and obesity[121]. Mitochondrial dysfunction is also a key feature of organ failure and dysfunction in sepsis and may therefore indicate a common process in acute as well as chronic inflammation[122].

1.4 Adiponectin receptors

In the last 10 years, two adiponectin receptors, adipoR1 and adipoR2, have been identified[123]. Both act as receptors for globular adiponectin (gAd) and the full length molecule (FLA), through modulation of the AMP activated protein kinase (AMPK) and peroxisome proliferator-activated α receptors (PPARα) pathways[84]. Adiponectin receptors are expressed in significant quantities in numerous tissues including skeletal muscle, liver, adipose tissue and pancreatic islet and acinar cells[124-127].

Adiponectin receptors are a type IVA protein with 7 trans-membrane domains with the N-terminus located intra-cellular and the C-terminus extra-cellular[123]. The two receptors share 66.7% sequence homology in the mouse[123]. Despite a distant relationship to G proteins, they are not coupled to them as there are no changes in cAMP, cGMP or intracellular calcium following adipoR1 and adipoR2 over-

expression[15]. Table 4 summarises and compares the properties of the two adiponectin receptor subtypes.

Table 4: Characteristics of adiponectin receptors

	AdipoR1	**AdipoR2**
Gene	1q36.13[123]	12p13.31[123]
Size of coding gene	17K base pair[123]	97K base pair[123]
Protein	375 amino acid[123]	311 amino acid[123]
Molecular weight	42.4 kDa[123]	35.4 kDa[123]
Site	Skeletal muscle, adipose tissue, liver, cerebellum, thyroid, colon, pancreas and bone marrow[124 125 127 128]	Liver, adipose tissue, skeletal muscle, testis, pancreas and adipose tissue[124 127]

The regulation of adiponectin receptors has not been clearly established. 3T3-L1 adipocytes increase their expression of adipoR1 and adipoR2 with differentiation from pre-adipocytes to mature adipocytes[129]. However, hormonal manipulation had only minimal effects on the expression of either receptor with adipoR2 expression only being increased following treatment with growth hormone. There was no effect on adipoR1[129].

There have been reports of differing concentrations of receptors in different tissues. A relative abundance of adipoR1 in murine skeletal muscle compared to adipoR2 and that adipoR2 predominated in hepatic tissue[123 126]. Within fat depots, the evidence is less clear. AdipoR1 appears in greater concentrations in subcutaneous adipose tissue (SAT) compared to visceral adipose tissue (VAT) fat in lean individuals (30-50% less)[130 131]. In obese individuals, evidence is conflicting with up to 50% reduction in receptor expression seen in different fat depots[130 131]. AdipoR2 appears to be more consistently reduced in VAT in obese individuals than levels in SAT[130 131].

1.4.1 Post-receptor signalling pathway

Ligand binding to adiponectin receptors activates various intracellular signalling pathways resulting in activation of AMP-activated kinase (AMPK) and PPARα (Figure 5)[15 34 35 123]. Activation of AMPK has been reported to reduce the expression of genes encoding for gluconeogenic enzymes such as glucose-6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PCK1) and the lipogenic enzyme srebf[132]. Tomas et al. showed an AMPK mediated increase in fatty acid oxidation and glucose transport in skeletal muscle[34]. Adiponectin binding to the extracellular C-terminal domain stimulates N-terminal binding to adaptor protein containing pleckstrin homologous domain (APPL1)[133 134]. APPL1 is involved in the regulation of cell proliferation and in the crosstalk between adiponectin signalling and insulin-triggered pathways[133]. Although APPL1 binding is not exclusive to adiponectin receptors, it may represent the missing link between the intracellular domains of adipoR1 and intra cellular signalling leading to activation of AMPK and MAPK[15 133] (Figure 5).

Figure 5: Physiological effects of adiponectin signalling

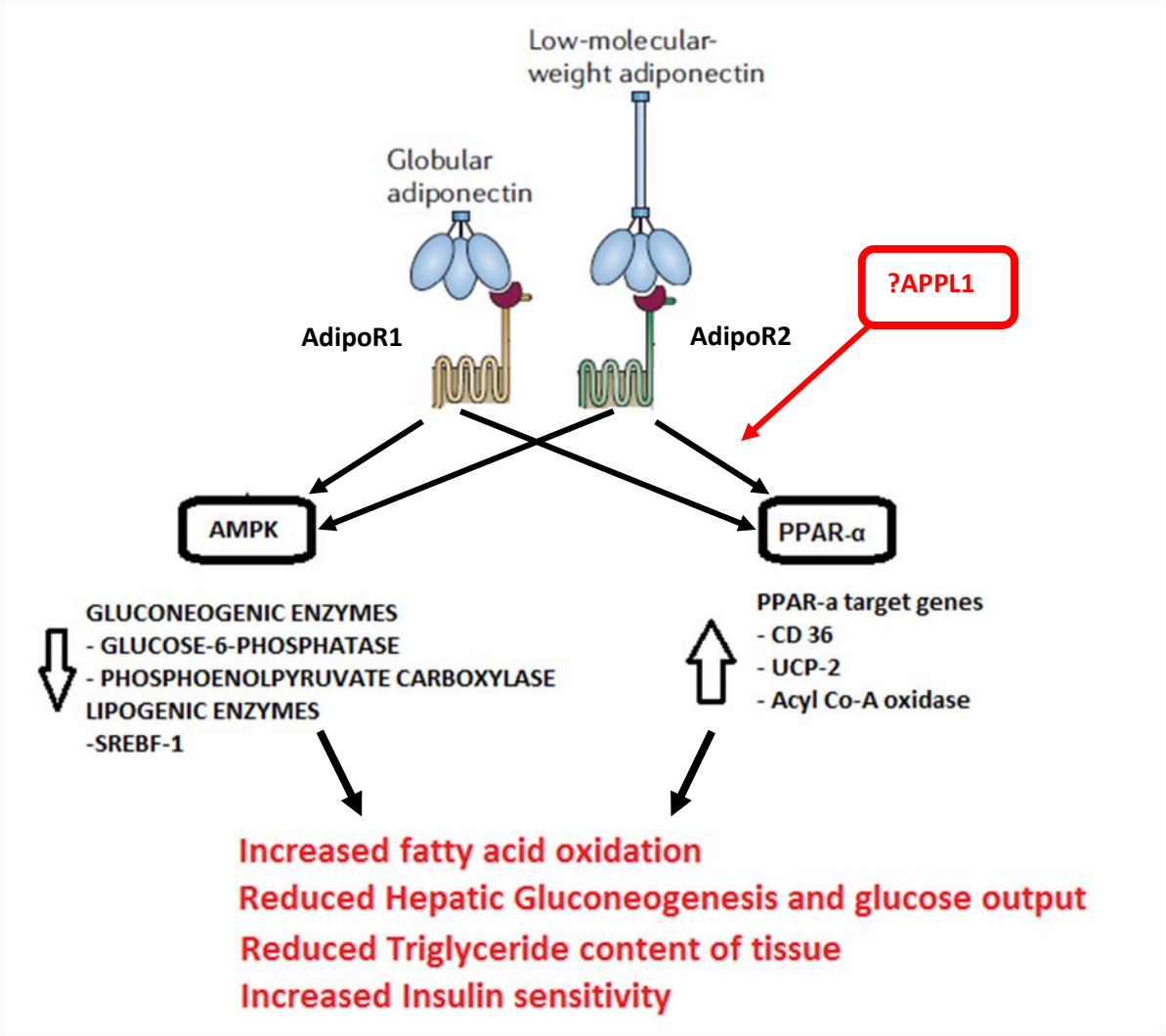


Figure 5: Ligand binding to the adiponectin receptors activates various intracellular signalling pathways resulting in activation of AMPK, PPARα and p38 Mitogen activated protein kinase (MAPK) [15 34 35 123]. This reduces the expression of genes encoding for gluconeogenic enzymes such as glucose-6 phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PCK1) and lipogenic enzymes such as srebf1[132]. In addition, PPAR-α target genes are increased.

Initially, adipoR1 and adipoR2 were thought to have similar intracellular and metabolic effects. However, new evidence may suggest that they may be in fact two distinct entities. Yamauchi et al. found that restoring adipoR1 levels in db/db mice, known to have reduced adipoR1 gene expression, increased activation of AMPK in the liver whereas adipoR2 over-expression did not[84]. This was further confirmed by reduced gene expression of G6P, PCK1 and srebf1 in the liver of adipoR1 restored db/db mice but not following adipoR2 restoration. Conversely, the over-expression of adipoR2 increased the expression of glucokinase (gck) which is involved in glucose uptake, a finding not seen in adipoR1 restored mice. Also, the adipoR2 restored mice exhibited increased expression of the PPARα target genes such as Acox1 and Uncoupling protein-2[84].

As expected, adipoR1 deficient mice have impaired glucose tolerance, insulin resistance and increased endogenous production of glucose[84]. They are obese, glucose-intolerant animals with increased liver triglyceride concentrations, increased plasma cholesterol and show reduced locomotor activity and energy expenditure[14].

AdipoR2 Knockout (KO) mice, conversely, have a different phenotype. Yamauchi et al. showed that these mice had no glucose intolerance or increased endogenous insulin production but who did have increased plasma insulin suggesting the presence of insulin resistance[84]. In addition, Bjursell demonstrated a resistance to diet-induced obesity (DIO), weight gain and hepatic steatosis with reduced plasma cholesterol and lower fasting insulin. However, this study also demonstrated a degree of impaired glucose intolerance[14].

1.4.2 Insulin resistance

Adiponectin expression and secretion is down-regulated in both animal and human models of type II DM and obesity[68 70 92 93 130]. Interestingly, other adipokines e.g. leptin and resistin, tend to increase with increasing adiposity[26 135 136]. Expression of adiponectin receptors is also down-regulated in these mouse models (ob/ob and db/db (Table 3))[79 126 127] and also in humans with obesity with some restoration of levels with weight loss[131 137]. The addition of type II DM in humans increased the down-regulation by a very small amount only[130]. Skeletal muscle adipoR1 levels also correlate with markers of central obesity as defined by waist circumference and truncal fat[138], although this has been disputed[128].

Current hypotheses for insulin resistance in type II DM include a reduction in insulin induced glucose uptake and utilisation, despite a usually normal plasma insulin concentration, and a reduced ability to oxidise lipids, despite increased fatty acid levels and triglyceride concentrations in skeletal muscle[121]. This may be due to impaired insulin signalling possibly at the mitochondrial level[121]. Insulin resistance and obesity are very closely linked and adiponectin may form part of the link between the two pathologies[15]. Similarly sepsis-induced insulin resistance and hyperglycaemia may be partly caused by interaction between adiponectin and insulin signalling. Mitochondrial dysfunction is well established as a cause of cellular hypoxia during sepsis, therefore this may also play a role[122].

In conclusion, insulin resistant states are associated with changes in the adiponectin system, including adiponectin and its receptors. Down-regulation of both modalities indicates an overall system suppression causing hypoadiponectinaemia and adiponectin tissue resistance. So far, adiponectin has been shown to be down-regulated in animal and cellular models of sepsis but there is no information available regarding adiponectin receptor expression in different tissues. There may

also be further links between adiponectin signalling and inflammation, NFκB activation, and mitochondrial dysfunction, which can be elucidated by focussing on adiponectin receptor expression.

To date, there have been no human studies on adiponectin receptor regulation in sepsis or endotoxaemia or human sepsis.

1.5 Hypoxia Inducible Factor-1α.

Several theories exist which link cellular inflammation and metabolic derangement including NFκB activation and mitochondrial dysfunction, both of which are crucial features of inflammation and sepsis. Hypoxia, caused by reduced oxygen delivery as a consequence of impaired tissue perfusion and centralisation of the circulation is also discussed as a key factor in sepsis. Many treatment strategies have been targeted at increasing tissue perfusion and thus oxygen delivery[139].

Hypoxia, defined as a reduction in oxygen tension (PO_2) within tissues and cells, can occur in numerous pathological conditions including ischaemia and inflammation[140]. Cells undergo a hypoxic transformation in order to maintain tissue integrity and to restore tissue oxygenation. This response is especially important for immune cells such as neutrophils that migrate into inflamed tissues characterised by profound hypoxia[140 141].

Obesity as a state of chronic inflammation also induces hypoxic changes. As a consequence of adipose tissue expansion the distance from the supplying capillary to the adipocytes increases, thus rendering them hypoxic[20]. Ye et al. analysed adipose tissue in ob/ob and wild type mice fed on a high fat diet and found a significant reduction in adipose tissue PO_2[142]. In both mice strains, there was significant up-regulation of hypoxia related genes including HIF-1α and glucose transporter-1. Interestingly this was not observed in the skeletal muscle of obese mice. These changes were reversible following caloric restriction and weight loss.

HIF-1α is a transcription factor which is critically involved in the adaptive response of mammalian cells to hypoxia. It was discovered in 1994 during experiments investigating the production of Erythropoietin (Epo). Under hypoxic conditions, red blood cells increase the production of Epo which stimulates the production of haemoglobin to increase the body's oxygen carrying capacity[143]. HIF-1α is known to play a crucial role in angiogenesis, cell invasion, anaerobic energy metabolism, cell survival, inflammation and drug resistance[144 145-148]. It also has a major function in innate immunity, cellular adaption to cellular stress and has therefore been described as a 'master regulator of innate

host defences'[149]. HIF-1α is a global regulator of macrophage and neutrophil function in inflammation and innate immunity[141 150] and is capable of producing a response that it tailored to the needs of the cell by upregulating glycolysis under times of cellular stress[143 151].

1.5.1 Structure

HIF is a heterodimer containing the subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed whereas the α-subunit is unstable under normoxic conditions and rapidly undergoes oxygen and iron dependent breakdown[149 152]. HIF-1α is present in most mammalian cells and co-ordinates the cellular response to reducing oxygen tension[144].

1.5.2 Hypoxic regulation

HIF-1α is post-translationally modified by prolyl hydroxylases (Figure 6) which hydroxylate specific proline residues on the HIF-1α protein. This allows von Hippel Lindau protein (VHLp), a tumour suppressor protein and component of the ubiquitin ligase complex, to form high affinity bonds with the α-subunit. The VHLp tags the complex for destruction by the ubiquitin proteinase mechanism and the protein is destroyed[144 149 153 154]. The half life of HIF-1α is very short due to the activities of the proline hydroxylases enzymes (PHD 1, 2 and 3)[155]. In addition to the prolyl hydroxylases, an asparagine residue is hydroxylated under normoxic conditions thus preventing interaction with HIF transcriptional coactivators, p300/CBP. Under hypoxic conditions, the activity of the proline hydroxylase enzymes is inhibited thus preventing hydroxylation and ubiquination, therefore HIF-1α protein accumulates intracellularly. It then translocates into the nucleus where it forms heterodimers with HIF-1β. These heterodimers are able to bind to the hypoxia responsive elements (HREs) of specific genes, thus increasing their transcription[141 144 156-160]. Genes whose expression is increased by HIF-1α-mediated effects are numerous and have a wide range of functions and include erythropoietin, glucose transporters (GLUT), glycolytic enzymes and vascular endothelial growth factor (VGEF)[152]. HIF-α is essential in foetal development and HIF-1α KO mice are unviable as they develop disordered vasculature not compatible with life[149 161].

Figure 6: Regulation of HIF-1α in hypoxic conditions

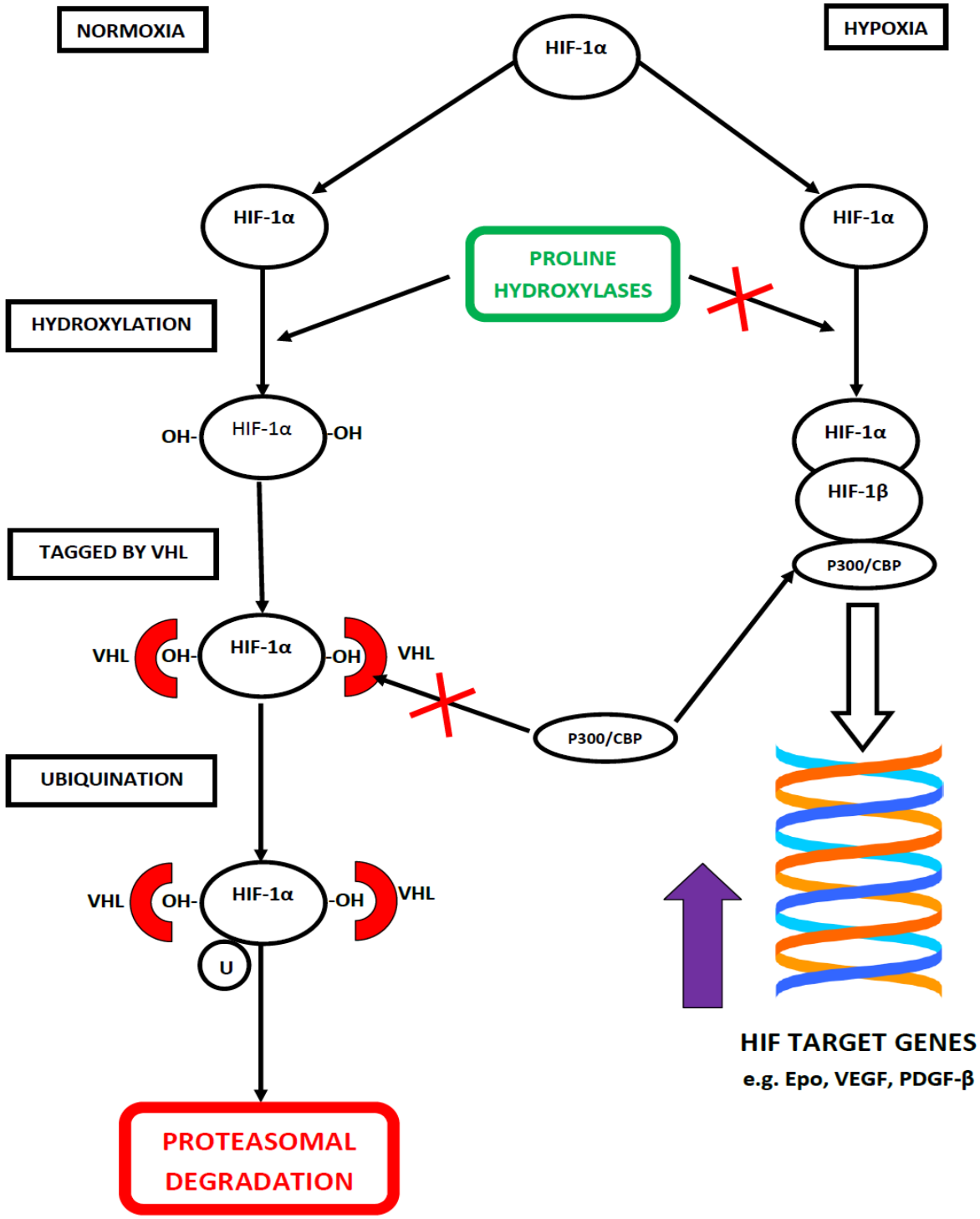


Figure 6: Under normoxic conditions, prolyl hydroxylases hydroxylate specific proline residues on the HIF-1α protein. This allows the von Hippel Lindau protein (VHLp) to form high affinity bonds with the α-subunit, subsequently tagging it for destruction by the ubiquitin proteinase mechanism[144 149 153 154]. Under hypoxic conditions, the activity of the proline hydroxylase enzymes are inhibited thus preventing hydroxylation and ubiquination and the HIF-1α protein accumulates intracellularly. HIF-1α then translocates into the nucleus where it forms heterodimers with HIF-1β. These heterodimers are able to bind to the hypoxia responsive elements (HREs) of specific genes, thus increasing their transcription[141 144 156-160]. Genes include erythropoietin, glucose transporters (GLUT), glycolytic enzymes and vascular endothelial growth factor (VGEF).

1.5.3 Non-hypoxic regulation

For many years, hypoxia was regarded as the main regulator of HIF-1α (Figure 6). However, there is now increasing evidence that non-hypoxic stimuli can promote HIF-1α accumulation, even under normoxic conditions[150]. These stimuli are diverse both in structure and function and include insulin[162 163], angiotensin II[158 164 165], inflammatory cytokines[166] and LPS[153 158 167 168]. Many studies have attempted to elucidate the complex signalling pathways of HIF-1α. It is now known that the mechanisms of action of hypoxic and non-hypoxic stimulation of HIF-1α are different.

Under hypoxic conditions, it has been shown that the relationship between gene expression and protein was unreliable and showed reduced, increased and static changes in gene expression following stimulation with hypoxia [153 158 164 169]. Increasing evidence is now available demonstrating that hypoxia does not increase HIF-1α transcription[170].

Non-hypoxic stimuli, however, may act differently resulting in a transcriptional increase to allow HIF-1α accumulation. Experiments with angiotensin II in vascular smooth muscle cells clearly show differences in time curves and peak concentrations compared to hypoxia[164]. In addition, when these cells are treated with transcriptional inhibitors, non-hypoxic accumulation of HIF-1α is inhibited whereas hypoxic accumulation is not[164]. Also, an increase in transcriptional activity has been demonstrated with a combination of hypoxia and angiotensin II[165].

Numerous studies have now confirmed that transcriptional up-regulation is the predominant factor in LPS-induced HIF-1α activation[153 158]. Interestingly, bacterial exposure appears to be a stronger stimulus to HIF-1α stabilisation than hypoxia[144]. Peysonnaux showed a greater increase in HIF-1α gene and protein expression following treatment of mouse macrophages with *group A streptococcus, MRSA, pseudomonas aeruginosa and salmonella typhimurium*[144], an effect which again was caused by increased transcriptional activity.

In re-oxygenation experiments, Frede found a similar rate of degradation of HIF-1α (half-life approximately 5 minutes) in cells treated with hypoxia or LPS, thus concluding that LPS did not interfere with the ubiquitination process[153]. These results were confirmed by using angiotensin II to induce HIF-1α in vascular smooth muscle cells[164]. Hence, hypoxic and non-hypoxic stimulated accumulation of HIF-1α must follow different pathways

1.5.4 HIF-1α/immunity, myeloid cells and the response to bacterial stimulation

Evidence is accumulating that HIF-1α plays an important role in controlling the inflammatory response in macrophages[141 144 149 152 168 171-174]. HIF-1α Knockout (KO) mice are universally fatal in-utero implicating HIF-1α in numerous pathways and cellular functions[149 161]. However, targeted deletion of HIF-1α in phagocytes provides phenotypically normal animals with significant differences in their response to inflammation[141 171 175 176] (Figure 7).

Infection leads to acidosis and hypoxia within tissues[141]. In healthy tissue, oxygen concentrations are 17.5-63 mmHg (2-9%) whereas in infected tissues this can be as low as <1%. Myeloid cells have adapted to efficiently circulate normally and, when activated, be recruited to damaged/infected cells and perform cellular functions in hypoxic environments[141 152]. This forms part of the innate immune system of the body and is important as many bacteria survive and replicate well under anaerobic conditions. Therefore, immune cells must adapt to control the infective processes. Following discovery of HIF-1α in myeloid cells, this led the way for a potential regulator of hypoxic changes in immune cells[144 149 161].

1.5.5 Phagocyte function

In HIF-1α deficient macrophages, the ability for intracellular killing of gram-negative and gram-positive bacteria is significantly reduced without alteration in phagocyte recruitment[144 150]. In addition, macrophages with a HIF-1α targeted deletion display a 2-fold reduction under normoxic and a 3-fold reduction under hypoxic conditions in intracellular bacterial killing. Compared to normal

cells, there was a 15-fold increase in intra-cellular bacterial colonies present in HIF-1α deleted cells after 120 minutes of incubation with bacteria[144]. Under hypoxic conditions, wild-type macrophages have a greater bactericidal effect on *Group A Streptococcus* than under normoxic conditions[144].

HIF-1α null macrophages showed significant reductions in pro-inflammatory cytokine release following LPS stimulation including TNF-α, IL-1α and β and IL-12[168]. There were no effects on the concentrations of IFN-γ or anti-inflammatory cytokines such as IL-4 or IL-10[168]. Furthermore, a significant reduction in TNF-α gene expression and protein secretion from mouse macrophages with HIF-1α deletions has been demonstrated[144]. It is therefore likely that HIF-1α forms part of a local bactericidal pathway which is stimulated during infection to produce a wide variety of mediators including cytokines and Nitric Oxide (NO) in order to increase bacterial killing. Following bacterial invasion, phagocytes extravasate into oxygen-poor areas within the infected tissue. Under these conditions, HIF-1α production is increased by both direct stimulation from the invading pathogen and by tissue hypoxia, leading to increased phagocytosis and bactericidal activity of immunocytes. As a local process, this mechanism also prevents damage caused by radicals and pro-inflammatory mediators in a normoxic, non-infected parts of the body.

Figure 7: Consequences of HIF-1α activation

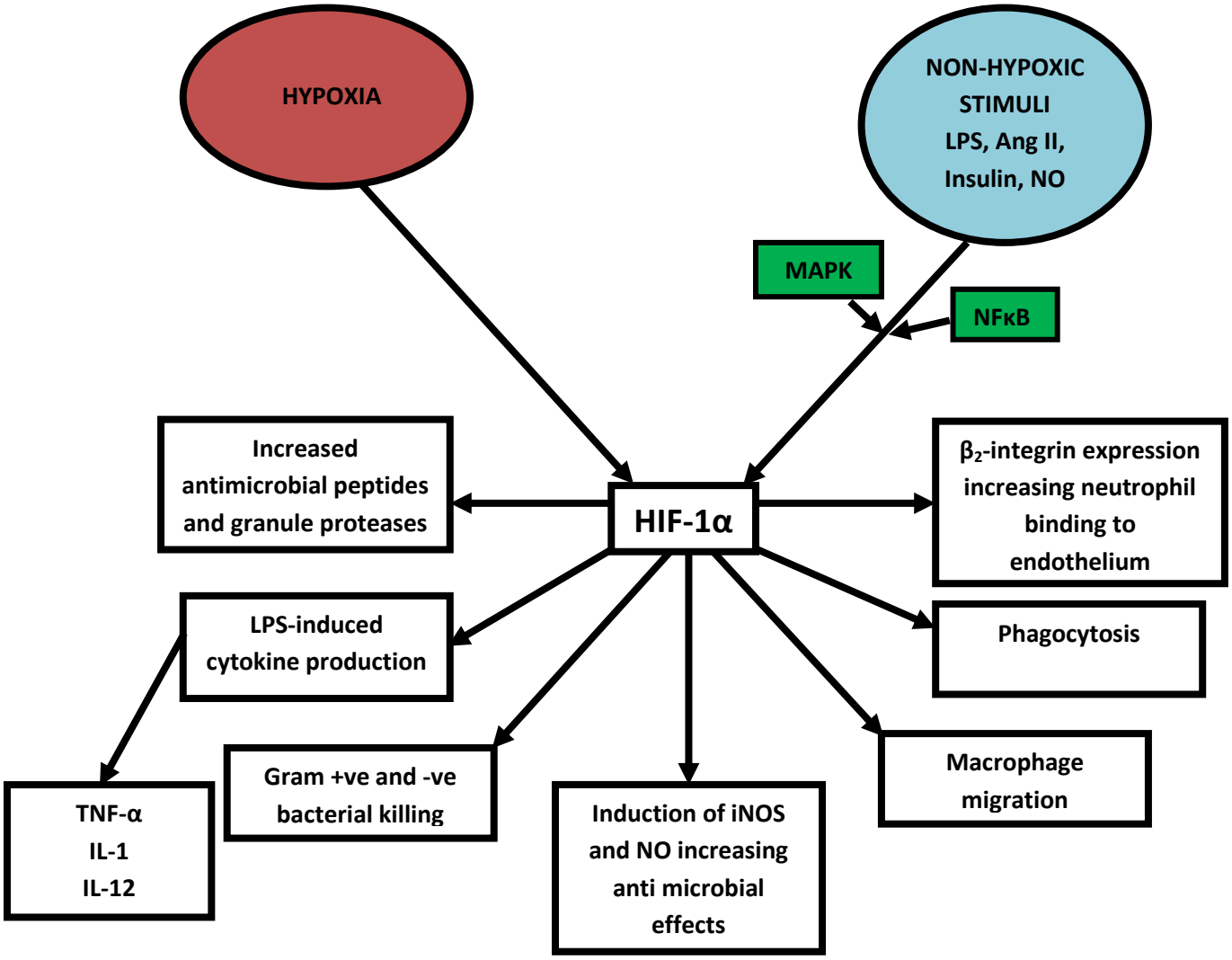


Figure 7: Links of HIF-1α with the immune system: HIF-1α, originally described as a regulator of hypoxia-mediated cell function, now has many links to the immune system. These include increased bacterial killing[141], alterations in phagocytes motility and function[141 149 168 174], regulation of LPS induced cytokine and nitric oxide production[144 168], and the up-regulation of natural antimicrobial peptides including granule proteases and nitric oxide[144]. HIF-1α is therefore evolving as a mediator of immune function, with links to the NFκB pathway.

1.5.6 Potential signalling pathways for non-hypoxic regulation of HIF-1α

HIF-1α activation is intricately involved in the immune response to infection and is not restricted to the mammalian cell's response to altered oxygen tension. Links to numerous signalling pathways have been demonstrated (Figure 8), including p44/42 MAPK, PI3K, PKC and NFκB.

Figure 8: Signalling pathways induced by HIF-1α

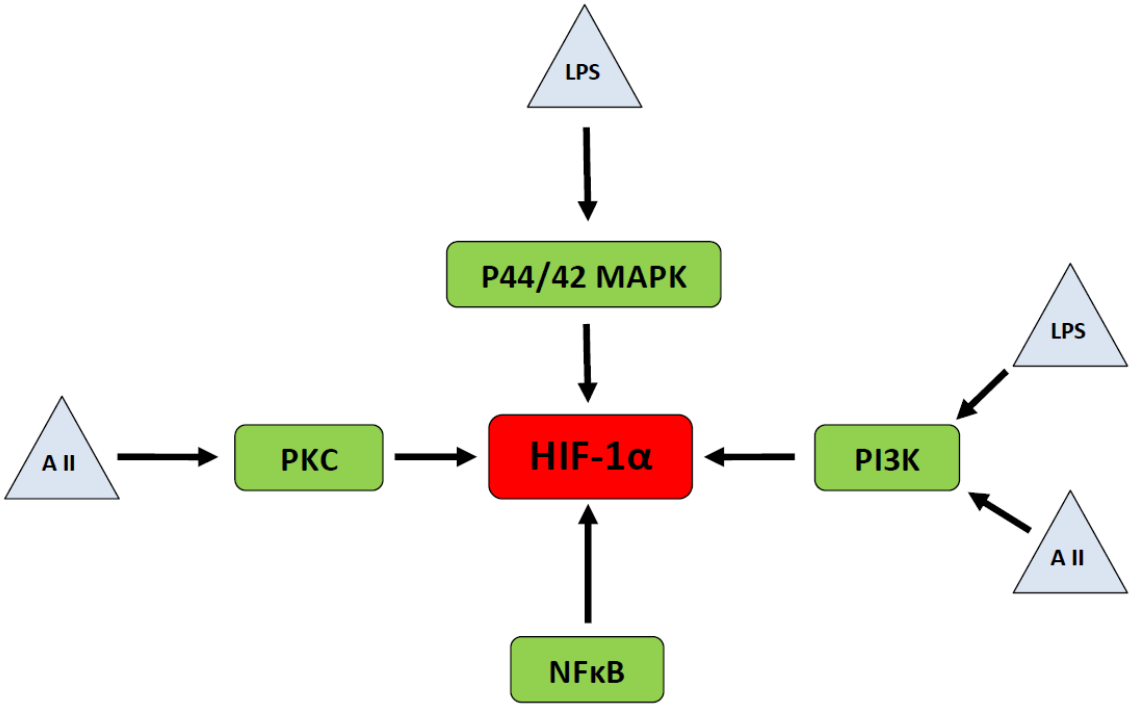


Figure 8: HIF-1α is likely to be a final common pathway for many signalling pathways. These include protein kinase C, PI3K and the p44/42 MAPK pathways[153 164 165 181 182]. These pathways, in turn, may be activated by a number of different ligands including angiotensin II (A II), Lipopolysaccharide (LPS) and other pro-inflammatory mediators.

The p44/42 MAPK pathway can be activated by a wide variety of proliferative and inflammatory stimuli[177 178]. An increase in the phosphorylation of p44/42 MAPK following both LPS and hypoxia resulted in an increase in HIF-1α gene expression and protein accumulation[153]. With LPS stimulation, this pathway appears to be critical as inhibiting the upstream kinase molecules limits the production of HIF-1α[153].

HIF-1α protein levels are clearly inhibited when inhibitors of phosphoinositol 3-kinase (PI3K) are used in angiotensin II treated smooth muscle cells[164 165]. An increase in HIF-1α protein levels following LPS which was dependent on PI3K has also been observed[158]. Interestingly, PI3K inhibition is not associated with an increase in HIF-1α gene expression, thus implying a translational role for this pathway. Page et al. demonstrated a clear link between angiotensin II up-regulation of HIF-1α gene expression and protein kinase C signalling not present in hypoxic cells. This was further confirmed by Blouin et al. who identified that activators and inhibitors of PKC increased and decreased HIF-1α levels via altering gene expression.

1.5.7 NFκB regulates HIF-1α

It is becoming more evident that HIF-1α is dependent on the NFκB pathway. Van Uden et al. demonstrated in cell lines that all NFκB subunits could activate the HIF-1α promoter site under normoxic conditions[170]. Basal HIF-1α gene expression is also significantly downregulated in IKK-β deficient macrophages and p65 deficient fibroblasts prior to stimulation with LPS[140]. Bonello et al. demonstrated that p50 and p65 (Rel A) NFκB subunits directly attach to the HIF-1α promoter, and mutation of this site significantly reduced the induction of HIF-1α expression[173 179]. Following non-hypoxic stimulation, HIF-1α accumulation has been shown to be dependent on NFκB activation. TNF-α stimulation of kidney cells in-vitro resulted in accumulation of NFκB subunits in the nucleus and a significant increase in HIF-1α gene expression and protein accumulation[170]. Further investigation demonstrated that deletion of all NFκB subunits resulted in significantly reduced TNF-α induced HIF-1α expression.

In addition, Rius et al. demonstrated that p65 was recruited to the HIF-1α promoter site bone marrow derived macrophages (BMDM) after treatment for 4 hours with gram-positive and -negative bacterial species[140]. This site contains an NFκB binding site suggesting a further link between NFκB and hypoxia. The fact that TLR-4 KO macrophages have significantly reduced HIF-1α protein and gene expression further emphasizes the role of HIF-1α activation by LPS[168].

In summary, there is now increasing evidence that non-hypoxic stimulation and up-regulation of HIF-1α through increased transcription occurs. This is in contrast to the hypoxia related changes where the prevention of protein breakdown is the primary pathway. It is also becoming clear that the HIF-1α pathways and NFκB activation are mutually dependent and therefore may play a role in the inflammatory process seen both systemically and in individual tissues such as adipose tissue.

There is an emerging link between the role of hypoxia and the inflammatory response. Infected tissues are known to be profoundly hypoxic, with tissue PO_2 being significantly lower than systemic PO_2. Myeloid cells have subsequently evolved to function well in this hypoxic environment as part of the inflammatory response. It is now becoming clearer that these changes may not be restricted to myeloid cells but also occur in other cells such as skeletal muscle and adipose tissue. The systemic response to inflammation includes changes in the metabolic processes, glucose and fatty acid metabolism, and a myriad of other changes. Adiponectin is involved in the metabolic changes and also the response to inflammation. Activation of NFκB appears to be crucial to this process and is linked to inflammatory and adiponectin pathways. It is also important in the response to hypoxia/inflammation via interaction with HIF-1α.

1.6 Summary

In summary, there are a number of changes that occur during the inflammatory process, many of which are interlinked and include NFκB as a central theme. This study will investigate two molecules, adiponectin and HIF-1α. Both molecules initially were thought to merely play a role in metabolic pathways, adiponectin in fatty acid metabolism in adipose tissue and HIF-1α as an up-regulator of glycolysis in hypoxic conditions. It is now becoming more evident that both molecules are intricately involved in the inflammatory process both in acute and chronic inflammation. The inflammatory process is also now digressing from focussing on classical immune cells, such as neutrophils and macrophages, to involving other tissues such as adipose tissue and skeletal muscle. This provides an ideal starting point to investigate the role of both molecules in sepsis and endotoxaemia in other tissues such as adipose tissue, skeletal muscle and liver, and to give further insight into their role in the immune response.

1.7 Hypotheses

1. Adiponectin and its receptors are down-regulated in response to LPS

The hypothesis is that adiponectin and its receptors will be down-regulated in acute inflammation, thus not only contributing to the disordered metabolic state but also to the inflammatory process. Thus, the primary aim is to investigate the expression of adiponectin and its receptors in *in-vivo* and *in-vitro* models of sepsis and endotoxaemia. This will be achieved by experiments to investigate:

- Adiponectin and adiponectin receptor gene expression in tissue depots of endotoxaemic mice.
- Adiponectin and adiponectin receptor expression gene expression in murine cell lines, 3T3-L1 adipocytes and C2C12 myocytes.
- Adiponectin receptor protein levels in tissue depots and cell lines.

2. Adiponectin plasma levels are decreased during human sepsis

We aim to identify whether adiponectin expression in mouse models follows the same pattern as plasma adiponectin levels in human septic patients. This may, in the future, provide a pharmacological option for the management of sepsis. Therefore, high molecular weight and total serum adiponectin and their ratio (HMW/total) were measured in patients with sepsis and compared to post-recovery levels. The hypothesis is that serum total and HMW adiponectin would be down-regulated in patients with sepsis or septic shock and that levels recover with improved clinical condition.

3. Expression of Hypoxia Inducible Factor -1α in experimental endotoxaemia

We investigated the expression of hypoxia-inducible factor 1-α in adipose tissue, skeletal muscle and other organs linked to adiponectin signalling. The hypothesis is that HIF-1α expression is up-regulated in as previously described in response to LPS. Therefore, HIF-1α gene expression and protein levels were investigated in tissue depots of endotoxaemic mice and in murine cell lines treated with LPS.

CHAPTER 2: METHODS

2.1 Animals and tissue collection

All experiments were carried out on 8- to 10-week-old male C57BL/6J mice (Charles River, UK). The care of the mice and all experimental procedures were approved by the UK Home Office and were conducted in accordance with the appropriate Project License (PPL 40/2692). LPS (Escherichia coli O 111:B4, Sigma-Aldrich) was injected intraperitoneally (ip) under general anaesthesia (2% isoflurane in N_2O/O_2) at a dose of 25 mg/kg.

Control animals were administered equivalent volumes of normal saline ip. All animals received 1 ml of normal saline subcutaneously (SC) simultaneously with LPS to compensate for fluid losses. Mice were housed in separate cages post procedures and maintained in the same temperature-controlled conditions ($22\pm2^{o}C$, 12 h light/12 h dark cycle) with free access to standard laboratory chow and water.

All mice were killed at 4 or 24 hours after injection of LPS by cervical dislocation. A 24 hours time point is commonly used in studies on LPS induced endotoxaemia, providing a sufficient period for a severe response to be established, while 4 hours was used to investigate acute effects. The tissue depots were removed and immediately frozen in liquid nitrogen and stored at -80°C until analysis.

2.2 Primer Design

Primers were designed using Beacon Designer 4 computer programme and have been validated in a previous peer review publication.

Primers

Primers used can be seen in Tables 5 and 6.

Table 5: Real time PCR primers

PRIMER	FORWARD	REVERSE	PROBE
β- ACTIN NM-007393	ACGGCCAGGTCATCACTATTG	CAAGAAGGAAGGCTGGAAAAG	ACGAGCGGTTCCGATGCCCTG
ADIPONECTIN AF304466	GGCTCTGTGCTCCTCCATCT	AGAGTCGTTGACGTTATCTGCATAG	CCCATACACCTGGAGCCAGACTTGGT
ADIPO R1	AGATGGAGGAGTTCGTGTATAAGG	GGCCATGTAGCAGGTAGTC	TCAGCCAGTCAGGAAGCACATCATACGG
ADIPO R2	CTTTCGGGCCTGTTTTAAGAGC	ATATTTGGGCGAAACATATAAAAGATCC	TACACACAGAGACGGGCAACATTTGGACAC
HIF-1α NM-010431.1	CAAGTCAGCAACGTGGAAGGT	CTGAGGTTGGTTACTGTTGGTATCA	TTCACTGCACGGGCCATATTCATGTC
IL-6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG	TTCACAGAGGATACCACTCCCAACAGAACCT

Table 6: Standard PCR primers

PRIMER	FORWARD	REVERSE
β-ACTIN	TGCTGTCCCTGTATGCCTCT	AGGTCTTTACGGATGTCAACG
ADIPONECTIN	TTAATCCTGCCCAGTCATGCCG	AGAACTTGCCAGTGCTGCCGTC
ADIPO R1	AAACTGGACTATTCAGGGATTGC	CACCATAGAAGTGGACGAAAGC
ADIPO R2	CCTATGCCTTCCTTTCG	CACTCCTGCTCTGACCC

2.3 Polymerase chain reaction (Standard)

Total RNA was extracted from adipose tissues with Trizol reagent (Invitrogen, UK), and 0.5 µg of DNase I-treated RNA was reverse transcribed using a Reverse-iT™ 1^{ST} Strand Synthesis Kit (Abgene, UK) in the presence of anchored oligo dT in a total volume of 10 µl .

Standard PCR reactions were carried out in a final volume of 12.5 µl. Components were separated on a 1% agarose/ethidium bromide gel at 100-110 mV. A 100bp ladder was used as a reference. The gel was examined with a UV transilluminator and photographed with the geldoc system.

2.4 Polymerase chain reaction (Real time)

Reactions were carried out in a final volume of 12.5 µl consisting of 12.5–50 ng of reverse transcribed cDNA mixed with the appropriate primers and probe and qPCR™ Core kit (Eurogentec, UK) in 96-well plates on a Mx3005P detector (Stratagene, USA). The primer and probe sets were designed using Primer Express software (Applied Biosystems) or Beacon Designer (Biosoft, USA) and synthesized commercially (Eurogentec). Amplification started with 10 minutes at 95°C and then 40 cycles of the following: 15 seconds at 95°C and 1 minute at 60°C. β-actin was used as an endogenous reference. Relative quantification values were expressed using the $2^{-\Delta\Delta CT}$ method[180] as fold changes in the target gene normalized to the reference gene and related to the expression of the controls. The PCR efficiency in all runs was close to 100% and all samples were analysed in duplicate. Statistical significance was determined using paired t-tests and Mann-Whitney U tests.

2.5 Protein extraction

Protein determination by BCA method

Samples were homogenized in SHE buffer (sucrose 250 mM / HEPES 1 mM / EDTA 0.2 mM, pH 7.2) and the concentration of protein in the supernatant determined by the BCA method. A solution of 1/50 dilution of Copper sulphate/Bicinchoninic acid (BCA) was made. Using a protein standard solution (2 mg/ml BSA solution in 1% SDS) a standard curve was constructed in duplicate (0-40 µg/µL). Then 1 µL sample was mixed with 19 µL 1% SDS and 200 µL BCA solution was added to each well. The 96 well plate was then incubated at 37°C for 30 minutes and read on a plate reader at 570 nm.

2.6 Western Blotting

A 10% separating gel and 4% stacking gel were placed between two glass plates. The correct amount of protein added to the loading buffer in a 1:1 dilution and run at a constant voltage for 1-2 hours. All gels were transferred on to a nitrocellulose membrane and presence of protein bands was confirmed using ponceau stain.

Phosphate Buffered Saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.2 - 7.4) with Tween (0.5 ml/L) was used as a washing solution. Skimmed milk or bovine serum albumin (BSA) were used for blocking unspecific binding and as antibody diluents.

Membranes were blocked overnight at 4°C on a rocking platform and then incubated in dilutions of primary antibody (AdipoR1: ADIPOR11-A, AdipoR2: ADIPO21-A, both Alpha Diagnostic, San Antonio, USA) at varying concentrations for 1 hour at room temperature (Appendix 2). Following three washes, the membranes were incubated with the secondary antibody (Goat anti-rabbit) (Serotec, UK) at a concentration of 1/1000 for 1 hour at room temperature. Detection was performed using chemiluminesence detection reagents (GE Healthcare). Membranes were imaged on to X-ray film.

2.7 Optimisation of Western Blots

Membrane fractionation

Tissue was homogenized in SHE buffer with protease inhibitors. Following initial centrifugation, samples were centrifuged at 100,000 g using the ultra-centrifuge and the pellets were resuspended in 1% SDS. Protein concentrations were determined by the BCA method. Following membrane fractionation, an increased concentration was used for the western blotting (20 μg). The same concentrations of 1° (1/1000) and 2° (1/5000) antibodies were used.

Dot blot

In order to assess the function of all antibodies used, small amounts of cell lysates and recombinant protein were placed onto a nitrocellulose membrane. Blocking was carried out with 1% milk and dilution with 0.5% milk. 10, 20 or 40 μg of membrane fractionated protein was incubated with diluted antibodies (1/200 for the primary and 1/2000 for the secondary antibody). Bands were then detected with chemi-illuminescence. Membranes were imaged on to X-ray film.

2.8 HIF-1α ELISA

(R&D Systems DYC 1935)

Samples were homogenized in lysis buffer (50 mM Tris (pH 7.4), 300 mM NaCl, 10% (w/v) Glycerol, 3 mM EDTA, 1 mM $MgCl_2$, 20 mM β-glycerophosphate, 25 mM NaF, 1% Triton X-100, 25 μg/mL Leupeptin, 25 μg/mL Pepstatin, and 3 μg/mL Aprotinin). The capture antibody was diluted to a working concentration of 4 μg/ml with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.2 - 7.4), and immediately was used to coat a 96-well plate which was sealed and incubated overnight at room temperature. After three washes with wash buffer (PBST 0.05% tween), plates were blocked using 5% BSA in PBS and incubated for 2 hours. After a further three washes, the samples and standards were diluted in reagent diluent (5% BSA in PBS) and added to the plate

and incubated for 2 hours. After washing detection antibody, at a concentration of 400 ng/ml, was added to each well and incubated for 2 hours. Streptavadin-HRP was diluted to a 1:200 concentration and was added to each well and incubated for 20 minutes. Following a further the wash, colour substrate solution (1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) was added. The reaction was stopped after 20 minutes with $2NH_2SO_4$. Optical density was determined using a microplate reader at 450 nm with a reference wavelength of 570 nm. To construct the standard curve, standards of 8000, 4000, 2000, 1000, 500, 250, 125 and 0 pg/ml were used.

2.9 Adiponectin ELISA (Mouse)

(R&D systems DY1119)

Samples were homogenized in lysis buffer (sucrose 250 mM / HEPES 1 mM / EDTA 0.2 mM, pH 7.2). The capture antibody was diluted to a working concentration of 2 µg/ml with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.2 - 7.4) and immediately was used to coat a 96-well plate which was sealed and incubated overnight at room temperature. After three washes with wash buffer (PBST 0.05% tween), plates were blocked using 1% BSA in PBS and incubated for 2 hours. After a further three washes, the samples and standards were diluted in reagent diluent (1% BSA in PBS) and added to the plate and incubated for 2 hours. After washing, the detection antibody (concentration 50 ng/ml) was added to each well and incubated for 2 hours. Streptavadin-HRP was diluted to a concentration of 1/200 and was added to each well and incubated for 20 minutes. Following a further the wash, the colour substrate solution (1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine)) was added for a further 20 minutes. The reaction was stopped after 20 minutes with $2NH_2SO_4$. Optical density was determined using a microplate reader at 450 nm with a reference wavelength of 570 nm. To construct the standard curve, standards of 2000, 1000, 500, 250, 125, 62.5, 37.25 and 0 pg/ml were used.

2.10 3T3-L1 adipocyte cell culture

3T3-L1 adipocytes were grown in culture media (Dulbecco's Modified Eagle Medium (DMEM) with 10% Foetal Calf Serum (FCS)) until confluent. Pre-adipocytes were then differentiated with 10 mg/ml insulin, 1 mM dexamethasone and 100 mM 3-isobutyl-1-methylxanthine (IBMX). The feeding medium (feeding media DMEM, 10% FCS and 10 mg/ml insulin) was changed every 48 hours. On day 12, cells were treated with LPS or cytokines. Cells were harvested after the appropriate time point using trizol reagent (Invitrogen, UK) and immediately frozen for RNA extraction. Six wells per time point per treatment/control were used for each experiment.

Plates were stained at intermittent time points (pre-adipocytes, pre-differentiation, day 0, day 3 and day 12) using Oil Red O and Haematoxylin Staining. Cells were washed twice with 1x PBS and fixed using 10% formaldehyde (37% molecular biology grade) in PBS for 1 hour at room temperature. After washing with PBS, Oil Red solution was added and incubated for 1 hour at room temperature. After washing, haematoxylin was added and left for 5 minutes at room temperature. The plate was exhaustively washed with hot water and viewed under phase contrast microscopy.

2.11 C2C12 myocyte culture

C2C12 myocytes were grown in culture media (DMEM with 10% FCS, 1% penicillin/streptomycin and L-glutamine) until 60% confluent. Cells were then differentiated with differentiation media (DMEM with 2% horse serum, 1% penicillin/streptomycin and L-glutamine) 10 mg/ml insulin. On day 7, cells were treated with LPS. Cells were harvested after the appropriate time point using trizol reagent and immediately frozen for RNA extraction. All cell culture incubations were performed at 37°C. Six wells per time point per treatment/control were used for each experiment.

2.12 Sequencing real time PCR product

RT PCR was run as per protocol. 6 duplicate samples had 1 µL dye added to each were run on a medium size 1% agarose gel with ethidium bromide (105 volts). Successful experiments were confirmed by visualisation under UV light.

Purification for sequencing was carried out according to manufacturer's guidelines (Nucleospin PCR clean-up gel extraction, MWG, Germany). Briefly, 6 PCR products (samples in duplicate) were combined and underwent gel lysis, DNA binding, membrane washing and drying and DNA elution. These samples were combined with PCR dye and run on a 1% agarose gel at 105 volts. Band brightness was assessed using UV light to compare to the ladder. The size of the band representing the gene of interest was then estimated according to the size of ladder bands. This sample was then sent for sequencing. A positive control using epididymal fat was also sequenced.

2.2 Clinical study on changes in the adiponectin system in sepsis

A pilot observational prospective pilot study was carried out to determine the plasma concentrations of total and HMW adiponectin in septic patients.

2.2.1 Ethical approval

The study received approval from the Local Research and Ethics committee (06/Q1502/7) and from the NHS trust (no 3258). This allowed witnessed assent from relatives with patients being informed as soon as practical to obtain retrospective consent.

2.2.2 Patient recruitment (Inclusion and exclusion criteria)

All patients between 18 and 85 years admitted to the Intensive Care Unit at the Royal Liverpool University Hospital with sepsis or septic shock according to current consensus guidelines (ACCP/SCCM 2001)[5] were approached for consent.

Patients were divided into two groups:

1. Patients with sepsis or septic shock and BMI <30 kg/m^2 (n=10)

2. Patients with sepsis or septic shock and BMI > 30 kg/m^2 (n=11).

Exclusion Criteria

1. Pregnancy or lactation
2. Insulinoma
3. Immunosuppression due to other causes than sepsis (immunosuppressive treatment after organ transplant, AIDS or ongoing chemotherapy)
4. < 18 yrs of age
5. Unwilling to give consent (or consent not given by an appropriate representative)

2.2.3 Data collected

Demographic (age, sex, weight, height), clinical (Glasgow Coma Score, temperature, heart rate, respiratory rate, oxygen requirements) and laboratory data (baseline haematological and biochemical parameters) were collected. Data relating to ongoing therapies (inotrope and insulin requirements, feeding regime and daily glucose and lactate measurements) were also collected.

2.2.4 Samples

Serum samples (7.5ml EDTA sample) were taken from each patient on admission to the intensive care unit. Further serum samples were taken on day 2 and on day of discharge. For patients who died, only day 1 and day 2 samples were taken. Following centrifugation (10 minutes at 3000 rpm), samples were stored at -80°C until analysis.

2.2.5 Sample analysis

2.2.6 Adiponectin ELISA (Human)

(R&D systems DY1065)

Samples were homogenized in lysis buffer (sucrose 250 mM / HEPES 1 mM / EDTA 0.2 mM, pH 7.2). The capture antibody was diluted to a working concentration of 2 µg/ml with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.2 - 7.4) and immediately was used to coat a 96-well plate which was sealed and incubated overnight at room temperature. After three washes with wash buffer (PBST 0.05% tween), plates were blocked using 1% BSA in PBS and incubated for 2 hours. After a further three washes, the samples and standards were diluted in reagent diluent (1% BSA in PBS) and added to the plate and incubated for 2 hours. After washing, the detection antibody (concentration 2 µg/ml) was added to each well and incubated for 2 hours. Streptavadin-HRP was diluted to a concentration of 1/200 and was added to each well and incubated for 20 minutes. Following a further the wash, colour substrate solution (1/1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine)) was added for a further 20 minutes. The reaction was stopped after 20 minutes with $2NH_2SO_4$. Optical density was determined using a microplate reader at 450nm with a reference wavelength of 570nm. To construct the standard curve, standards of 4000, 2000, 1000, 500, 250, 125, 62.5and 0 pg/ml were used.

2.2.7 HMW Adiponectin ELISA (Human)

(Alpico 47-ADPHU-E01)

Samples were pre-treated with Protease II and the remaining HMW fraction was treated with Sample Pre-treatment Buffer. 100 µL of Protease I was added to 10 µL of sample and incubated for 20 minutes at 37°C. Immediately, 400 µL of Sample Pre-treatment Buffer (sample dilution = 1/51) was added and vortexed. Pre-treated samples were diluted (1/101) as follows and vortexed allowing a final sample dilution of 1/5,151. 50 µL of each standard diluted pre-treated sample was added to the appropriate wells. The plate was incubated for 1 hour at room temperature. All wells were washed using wash buffer 3 times (Phosphate buffer (pH 7.2)). 50 µL of Biotin labelled monoclonal antibody was added to each well and incubated for 1 hour at room temperature. Following a second wash, 50 µL of the Enzyme Labelled Streptavidin were added to each well and incubated for 30 minutes at room temperature. After a further wash, 50 µL of the substrate solution was added to each well and incubated for 10 minutes at room temperature. The reaction was stopped by adding 50 µL of stop reagent (7.7% H_2SO_4) was added to each well. The absorbance of each well was measured between 10-30 minutes after addition of the stop reagent, using a microplate reader set to 492 nm, with a reference wavelength of 600-700 nm.

2.2.8 Statistical analysis

Mouse model and cell line experiments

Relative gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method [180]. Data are presented as mean values ± Standard Error of Mean (SEM). Differences between groups were analysed by Student's unpaired t-test or non -parametric tests when data was non-normally distributed. In the animal model, 4 and 24 hour LPS treatment was compared with its own control group. Results were considered to be statistically significant when $p<0.05$. Where multiple comparisons were performed the significance level was corrected using Bonferroni's method. Fold change was calculated as $1/2^{-\Delta\Delta CT}$.

Clinical study

Patient values were displayed as median and interquartile range and statistical significance between the groups was calculated using the Mann-Whitney U test. Correlations were performed between adiponectin and markers of clinical severity using Spearman's correlation coefficient. Dependent data was analysed using the Wilcoxon test with Bonferroni correction.

CHAPTER 3: RESULTS

3.1 Alterations in adiponectin and its receptors in murine endotoxaemia

3.1.1 Initial experiments

Mouse models

All experiments were carried out on 8- to 10-week-old male C57BL/6J mice (Charles River, UK). Table 7 shows the mean body weights of the animals used in the experiments. As can be seen there were no significant differences between any of the groups, before and after treatment.

Table 7: Mouse groups

				Differences between groups	
Treatment group	Time point	n	Weight (g) (mean +/-sd)	Between time points	Between groups
SALINE	4 hours	6	24.13+/- 1.66	SAL p=0.85	
	24 hours	14	25.06+/- 0.96		4 hour p=0.51
LPS	4 hours	6	24.81+/- 1.80	LPS p=0.47	
	24 hours	14	25.43+/-1.39		24 hours p = 0.41

Mortality Rates

No mice in the 4 hours treatment group died. In the 24 hour group, however, nine out of 23 mice died, an average of 17.44 hours following injection giving an overall mortality rate of 39.1%. This mortality rate is not dissimilar to that of severe sepsis in humans[2]

Confirmation of presence of beta actin and adipoR in tissue samples

In order to confirm the presence of beta actin in the tissue samples, standard PCR was performed using four samples of control liver tissue. Using 0.5 µg cDNA per sample, beta actin was found in all samples (Figure 9).

Figure 9: Standard PCR demonstrating beta actin gene expression in liver

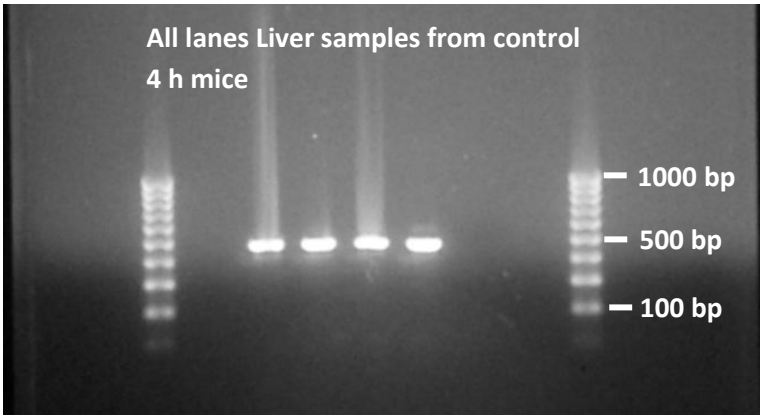


Figure 9: Beta actin expression was confirmed in liver tissue from control (4 h) mice using standard PCR. A PCR product of between 400 and 500 base pairs was identified in all samples using primers for beta actin.

Adiponectin receptors are known to be found in many tissues including liver, skeletal muscle and fat depots[123]. Initial experiments confirmed the expression of both receptor subtypes in mouse liver tissue using standard PCR (0.5 µg cDNA) (Figure 10). Splenic receptor mRNA has not been previously noted in the literature and therefore, standard PCR to identify this was performed (Figure 11).

Figure 10: Standard PCR of adiponectin receptor expression in liver tissue from control mice

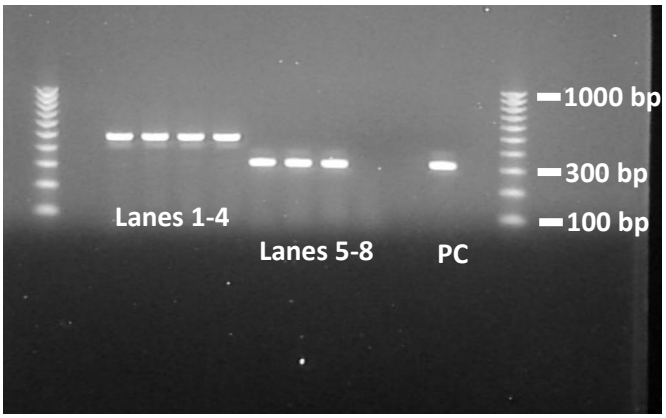


Figure 10: Adiponectin receptors 1 and 2 gene expression was confirmed in liver tissue from control mice using standard PCR. PCR products of adipoR1 (447 base pairs) and adipoR2 (332 base pairs) were identified in all samples using appropriate primers. An Epididymal fat samples was used as a positive control. (Lanes 1-4 adipoR1 (0.5 µg cDNA per sample), Lanes 5-8 adipoR2 (0.5µg cDNA per sample), PC – positive control (Epididymal fat R2), bp: base pairs)

Figure 11: Standard PCR of adiponectin receptor expression in spleen tissue from control mice

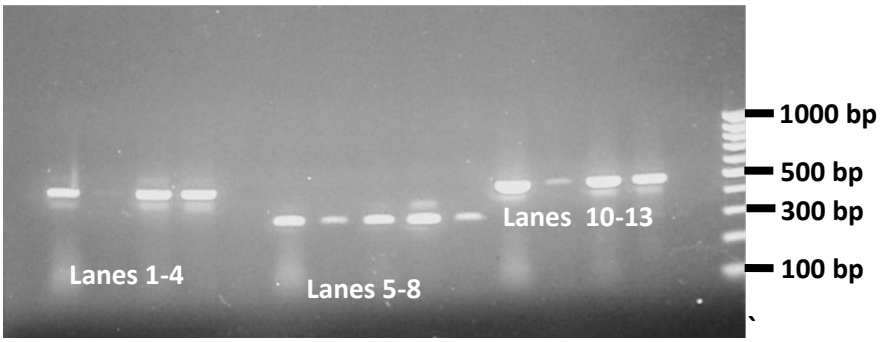


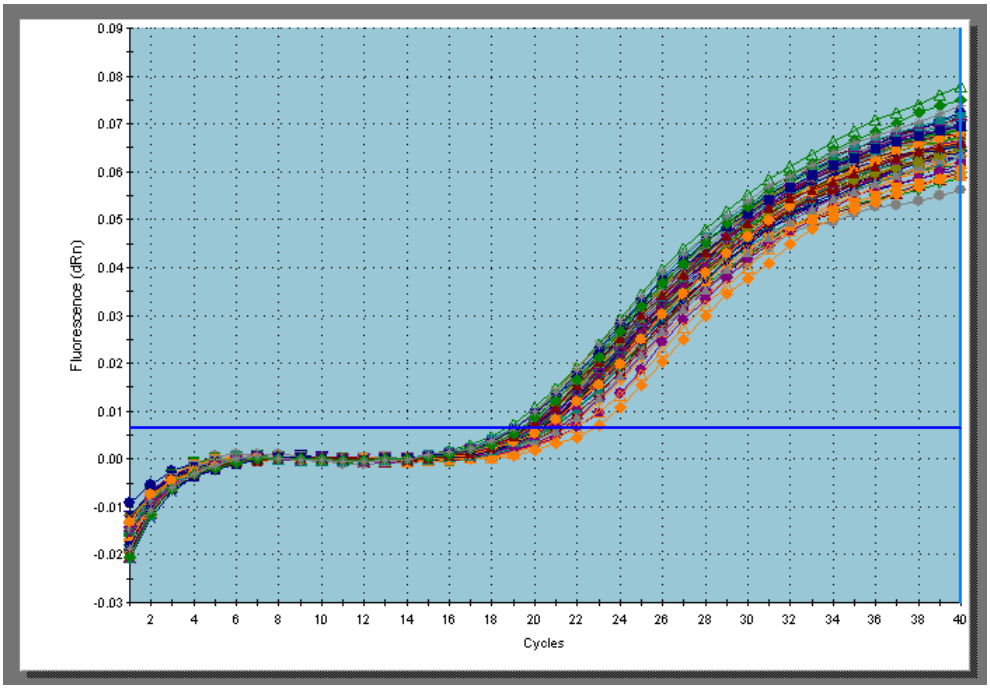
Figure 11: Adiponectin receptors 1 and 2 gene expression was confirmed in spleen tissue from control mice using standard PCR. PCR products of adipoR1 (447 base pairs) and adipoR2 (332 base pairs) were identified in all samples using appropriate primers. Beta actin expression was also confirmed in spleen tissue. (Lanes 1-4 – spleen adipoR1, Lanes 5-8 spleen adipoR2, Lanes 10-13 spleen beta actin, bp: base pairs).

Real Time PCR

Following confirmation of adiponectin receptors in liver and spleen, real-time PCR (qPCR) was then performed to quantify gene expression in all mouse tissue depots including three depots of WAT (epididymal, subcutaneous and peri-renal), skeletal muscle, liver, and spleen. qPCR experiments were run to 40 cycles however, Ct > 35 cycles depict no or very low expression of that gene. Each line on the graph represents one individual sample (Figure 12).

Figure 12: Amplification plots of the real-time PCR products

A: Amplification plots using a Linear scale (myocyte beta actin)



B: Amplification plots using a Logarithmic scale (myocyte beta actin)

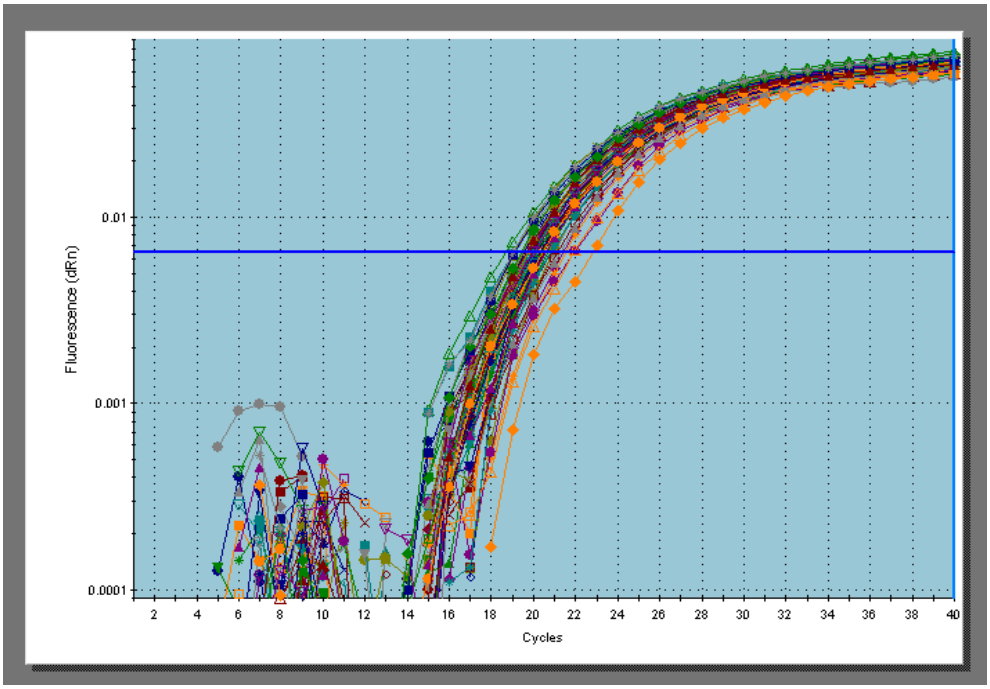


Figure 12: The example amplification plots show the cycle number on the x-axis and the fluorescence concentration on the y-axis. The horizontal blue line depicts the Ct, threshold of detection of fluorescence (on the logarithmic graph). qPCR experiments were run to 40 cycles however, Ct > 35 cycles depicts no or very low expression of that gene. Each line on the graph represents one individual sample. A: Linear scale, B: logarithmic scale

Assessment of primers

This experiment was performed to confirm that the primers used were only identifying a signal from cDNA and not from components of the RNA. Primer design techniques are such that this should be avoided but this experiment confirms this. The cycle number at which the amplification product becomes detectable is known as the $Cycle_{threshold}$ or Ct. With this method, the Ct value identified does not include a signal from the amplification of genomic DNA which will impact on the result.

Exclusion of contamination from genomic DNA contaminants during qPCR

Tissue samples

Real-time PCR primers (beta actin, adipoR1, adipoR2, adiponectin and HIF-1α) were combined with RNA from Epididymal fat and muscle (each with a positive control of cDNA). Using RNA, Ct was not achieved for any gene in either sample whereas cDNA samples reached threshold (Table 8). This confirms that the primers used do not amplify any signal from DNA in the final Ct and therefore DNA contamination is unlikely.

Table 8: Ct values for RNA when combined with appropriate primers for each gene

Gene	Ct (cycle)
β-actin	21.73
AdipoR1	24.16
AdipoR2	21.09
Adiponectin	20.1
HIF-1α	27.9

Assessment of treatment on beta actin Ct values

For correct interpretation of the PCR results, the effect of the treatment on the expression of the housekeeping gene had to be evaluated. Results are expressed in values of relative expression; small changes in beta actin expression may alter the results.

We therefore excluded any effect of LPS on the expression of beta actin mRNA in each tissue (Table 9), 3T3-L1 adipocytes (Table 10) and C2C12 myocytes (Table 11) respectively.

Table 9: Tissue average beta actin values following LPS

	Liver	Muscle	Epi fat	PR fat	SC fat	Small Bowel	Spleen
4 hour Control	23.54	24.62	21.88	23.00	21.38	18.8	18.95
4 hour LPS	21.96	22.63	22.22	22.14	22.87	19.06	19.54
24 hour Control	22.56	24.61	26.97	23.79	21.76	18.78	19.49
24 hour LPS	20.86	22.85	24.98	23.10	20.97	19.36	19.41

Table 10: Adipocyte average beta actin values following LPS

Group	$2^{-\Delta\Delta CT}$
Control	18.425
0.1 µg/ml	19.15667
1 µg/ml	19.76167
5 µg/ml	18.83667
10 µg/ml	18.91

Table 11: Myocyte average beta actin values following LPS

Group	$2^{-\Delta\Delta CT}$
Control	19.75667
0.1 µg/ml	19.97167
1 µg/ml	20.385
5 µg/ml	19.82167
10 µg/ml	19.1667

3.1.2 Discussion: Initial experiments

Mouse models are frequently used in the investigation of alterations in gene expression in endotoxaemia. Animal models of sepsis are complex and there have been significant concerns in the past with animal model research resulting in treatment regimes that are not efficacious in humans[181] [182]. There are a number of fundamental differences between human sepsis and experimental endotoxaemia underlying this lack of translation.

There is no single animal model which replicates the human development of and host response to sepsis[183]. Also, animals used are frequently young, healthy males of the same breed giving a very homogenous population who require a large bacterial innoculum to develop a septic state[183]. There are two types of animal models commonly used:

1. LPS injection models which mimic the early features of sepsis and induce an inflammatory state without bacteraemia[183] [184]. This method is simple to perform and gives generally reproducible results and the injected dose can be altered to alter symptoms and severity of disease[183]. The sharp rise in cytokine concentrations seen following injection is felt to be much earlier and to a greater magnitude than that found in human sepsis[181] [183] [185].

2. Endogenous faecal contamination, usually performed by caecal ligation and needle puncture gives a state of polymicrobial intra-abdominal sepsis[183] [185]. Some authors consider it to be the gold standard animal model for sepsis research[181]. Although not well standardised and therefore not always reproducible, the resulting cytokine profile is similar to that seen in human sepsis, as it relies on growth and replication of bacteria rather than direct innoculum[183] [185] [186].

Both methods have a similar morbidity, mortality and degree of immunosuppression[181 185]. A further limitation of these models is that clinical sepsis affects both genders, predominantly elderly patients with multiple co-morbidities. Hence, the population profile is not correctly represented by young healthy mice. Advantages of murine models of sepsis include that the animals are easy to breed, come at a low cost and the use of rodents carries fewer ethical implications than models involving higher mammals or primates[181]. Turnbull et al. investigated the effect of age in murine models of CLP and found in young, mature and old mice mortality rates of 20, 70 and 75% respectively. This difference in mortality may significantly affect many results. Many clinicians feel that patient heterogeneity contributes to the lack of progression from animal studies to successful human therapeutic trials in anti-inflammatory agents in sepsis[181 183]. To improve correlation between animal and human studies, further studies need to be performed on varying breeds, genders and ages of animals[187].

The animal experiments used in these series of experiments were performed with 25 mg/kg LPS intraperitoneally to induce systemic endotoxaemia. Animals had access to standard laboratory chow and subcutaneous fluids were used to compensate for fluid losses. This model uses a very high dose of LPS, in an attempt to achieve a severe sepsis model, anticipating the same mortality rate as is seen in severe sepsis in other species[2]. As predicted, the mortality rate in this model was high at 39.1%.

There were no differences in body weight between animal groups or after treatment (Table 7). This was important to establish as caloric restriction and weight loss frequently observed in sepsis, are known to have an effect on adiponectin expression and secretion[92 188].

This model used only male mice for two reasons: Firstly, it was an attempt to provide reproducible results from a homogenous mouse population. Secondly, using male animals could overcome the known phenomenon that female individuals (mouse and human) express higher levels of adiponectin than males[77 94 93 104].

The presence of beta actin and adiponectin receptors was confirmed in fat, liver and spleen as described previously. PCR products from murine liver tissue treated with LPS were separated on an agarose gel (Figures 9 & 10), thus confirming previous findings[123]. This method was also successful in spleen (Figure 11). Real-time PCR was the primary experiment used to demonstrate relative changes in gene expression. Figure 12 shows a typical real-time PCR graph demonstrating the threshold at which expression becomes detectable: the earlier the threshold of detection (i.e. the lower the numerical value of Ct), the greater the expression of the gene in that sample. The Ct values observed in the initial experiments were between 18 and 24 cycles for beta actin, adiponectin and its receptors. Those for HIF-1α were higher with a mean of 27.9 (Table 8). This indicates that the expression of beta-actin, adiponectin and its receptors is high in these tissue depots whereas the expression of HIF-1α in these tissues is lower, although no accurate conclusions can be drawn from the raw data as to expression levels.

Prior to performing qPCR on the tissue samples, confirmation that the primers were not identifying genomic DNA was essential. Although the primers were designed to theoretically exclude the replication of genomic DNA, confirmation was required. RNA from adipose tissue and muscle samples with positive and negative controls were used. RNA samples did not reach a threshold for any of the 5 genes tested (beta actin (BA), adipoR1, adipoR2, adiponectin or HIF-1α) whereas the positive controls achieved cycle at threshold (Ct) values of 21.73, 24.16, 21.09, 20.1 and 27.9 respectively (Table 9). Thus extraneous signals from genomic DNA were not detected in tissue, adipocyte and myocyte samples.

Finally, it was important to identify the effect of the treatment on the expression of the housekeeping gene. There was very little effect of LPS on the expression of beta actin, thus making it a suitable housekeeping gene for the subsequent experiments (Table 9-11).

3.2 Alterations in adiponectin and its receptors in murine endotoxaemia

This chapter describes results of the experiments investigating the expression of adiponectin and its receptors in the mouse model. Initially, the investigation of adiponectin in peri-renal (PRF), epididymal (EF) and subcutaneous (SCF) depots of WAT was investigated. PRF and EF are visceral fat depots whereas SCF is not.

Adiponectin is expressed in adipose tissue and its expression and secretion is known to be reduced in sepsis[12]. We investigated three depots of adipose tissue: PRF, EF and SCF as they are different organs with respect to the type of adipose tissue they contain.

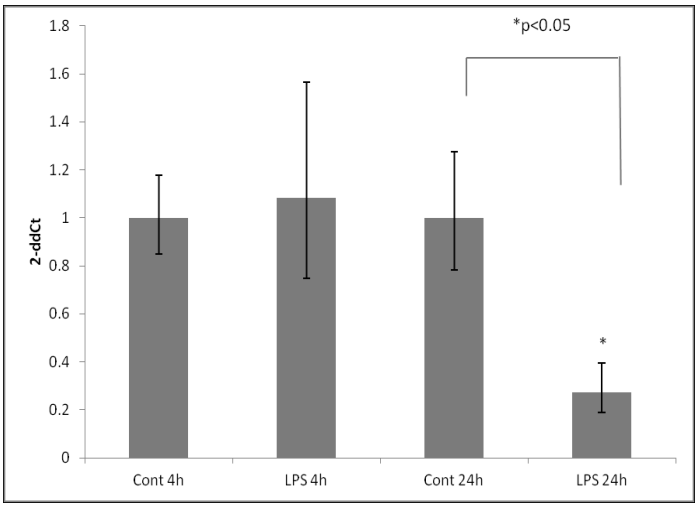
We confirmed down-regulation of adiponectin in all three depots but only in the mice treated for 24 hours. There were no changes at 4 hours (Figure 13). This down-regulation in the adiponectin system may be implicated in the metabolic and inflammatory changes seen in endotoxaemia.

The following graphs individually display the expression of adiponectin in individual tissue depots following treatment with LPS. Each treatment group is compared to its own control only.

3.2.1 Adipose tissue: Adiponectin

Figure 13: Adiponectin expression in white adipose tissue

A: Adiponectin expression in epididymal fat



B: Adiponectin expression in peri-renal fat

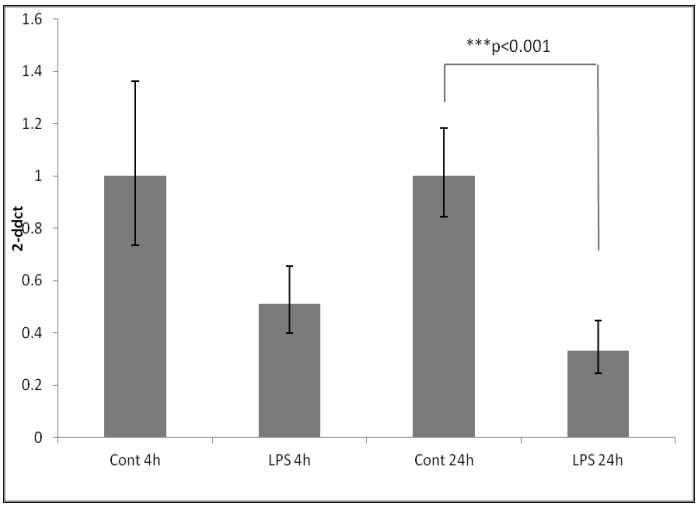
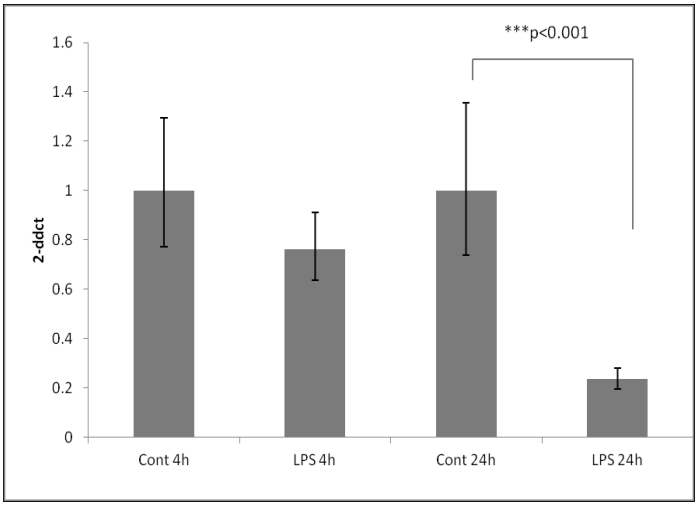


Figure 13: Relative change in adiponectin gene expression in mouse tissue depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and p<0.05 was considered significant. (*p<0.**05**, ** p<0.01). Housekeeping gene was β-actin.

A: Epididymal Fat adiponectin expression, B: Peri-renal fat adiponectin expression, C: Subcutaneous Fat adiponectin expression,

(Cont = Control group, LPS = Treatment group, 4 h 4 hours treatment group, 24 h: 24 hours treatment group, Error bars display SEM)

C: Adiponectin expression in subcutaneous fat



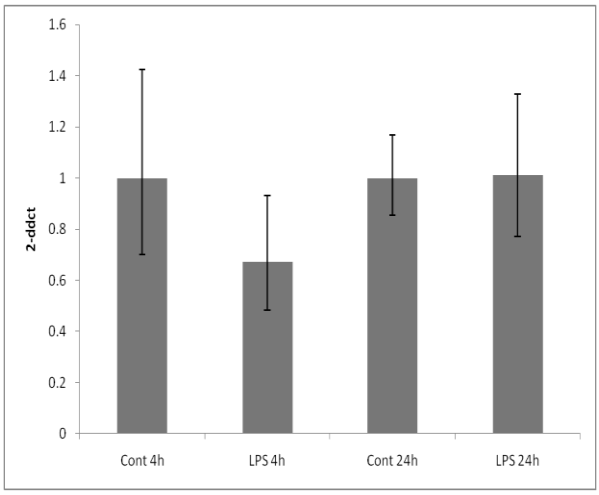
3.3 Adipose tissue: Adiponectin receptor expression

Following the confirmation of changes in adiponectin expression with LPS treatment, the expression of adiponectin receptors in the same three adipose tissue depots was investigated.

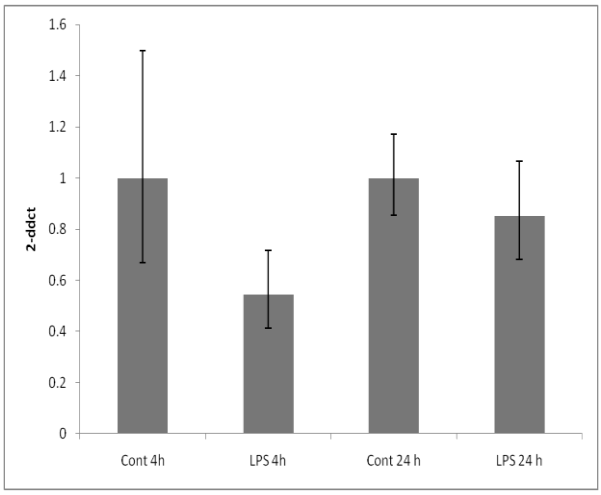
AdipoR1 and adipoR2 gene expression in peri-renal (PRF) is rapidly down-regulated whereas in subcutaneous fat (SCF) only adipoR2 gene expression is reduced (Figure 14 C-F). Epididymal fat (EF) does not display any changes in gene expression at either time point (Figure 14 A&B). Potential hypotheses for these changes will be discussed at length in the discussion. However, they include tissue hypoxia, inflammatory cytokine release and differences between visceral and non-visceral fat depots.

Figure 14: Adiponectin receptor expression in white adipose tissue

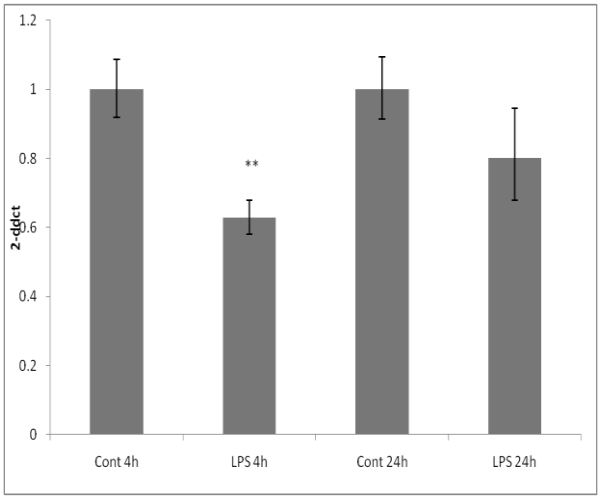
A: AdipoR1 expression in epididymal fat



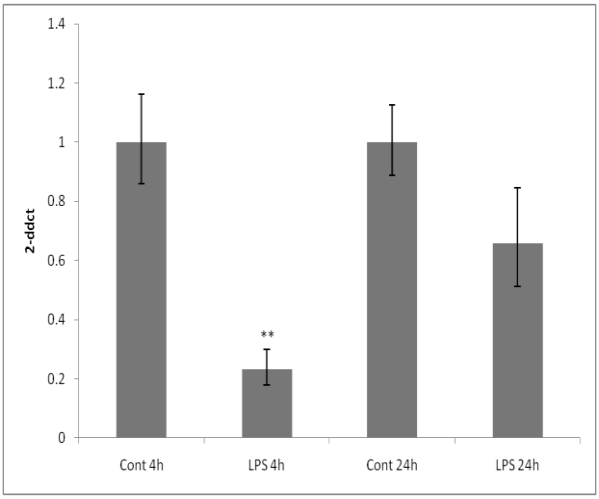
B: AdipoR2 expression in epididymal fat



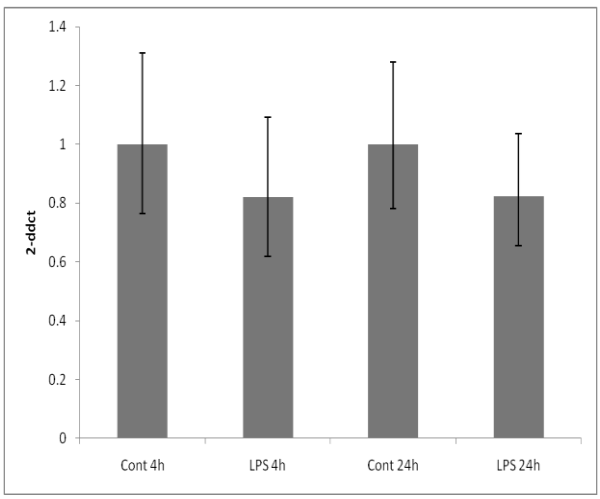
C: AdipoR1 expression in peri-renal fat



D: AdipoR2 expression in peri-renal fat



E: AdipoR1expression in sub-cutaneous fat



F: AdipoR2 expression in sub-cutaneous fat

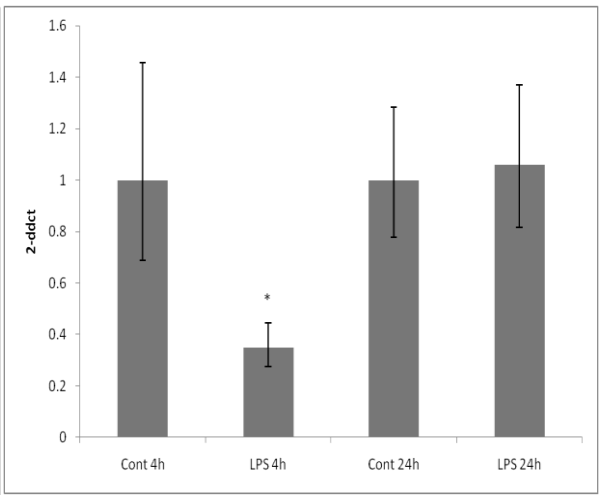


Figure 14: Relative change in adiponectin receptor gene expression in mouse tissue depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR in samples run in duplicate. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. A: Epididymal Fat adipoR1 expression, B: Epididymal Fat adipoR2 expression, C: Peri-renal fat adipoR1 expression, D: Peri-renal fat adipoR2 expression, E: Subcutaneous Fat adipoR1 expression, F: Subcutaneous Fat adipoR2 expression. (Cont = Control group, LPS = Treatment group, 4 h 4 hours treatment group, 24 h: 24 hours treatment group, error bars display SEM). 4 hour mice n=6, 24 hour mice n=14.

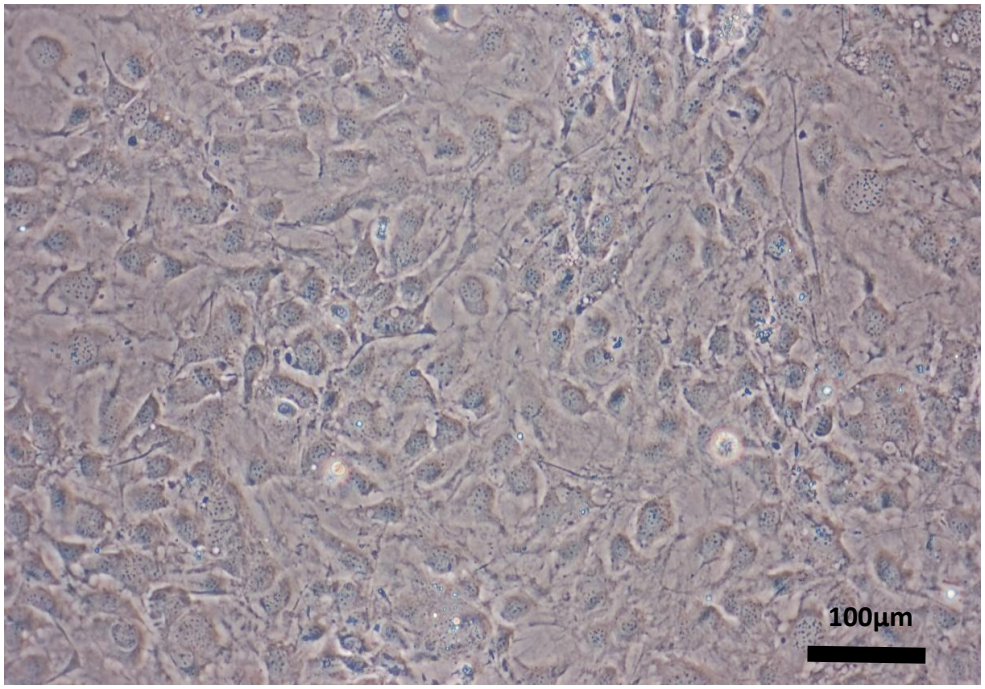
3.4 3T3-L1 Adipocytes – initial experiments

Following the identification of changes in adiponectin and their receptors in the mouse model of endotoxaemia, experiments were subsequently performed using 3T3-L1 adipocytes. Despite cell lines being a lower model for investigation, these experiments were performed in an attempt to elucidate regulation and signalling pathways. 3T3-L1 adipocytes were chosen as they are a murine cell line and are well established in the research of adipokines in chronic disease.

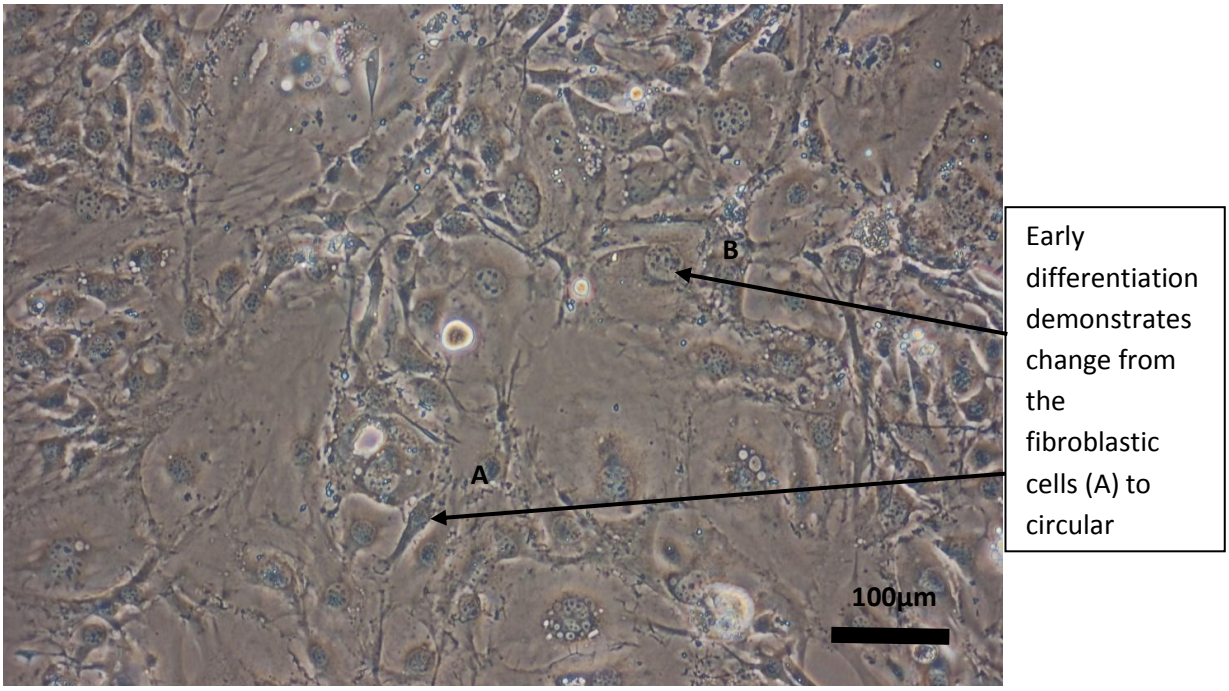
3T3-L1 adipocytes were grown in culture according to standard laboratory protocols and confirmation of growth and differentiation was confirmed by visualisation of cells and staining for fat content. The following pictures demonstrate an example of cells at different stages of differentiation and staining (Figure 15).

Figure 15: Pictures of 3T3-L1 adipocytes in culture at various stages of differentiation

A: Pre-adipocytes - unstained



B: 3T3-L1 Adipocyte day 3 post differentiation



C: 3T3-L1 Adipocyte day 12 post differentiation – Treatment day

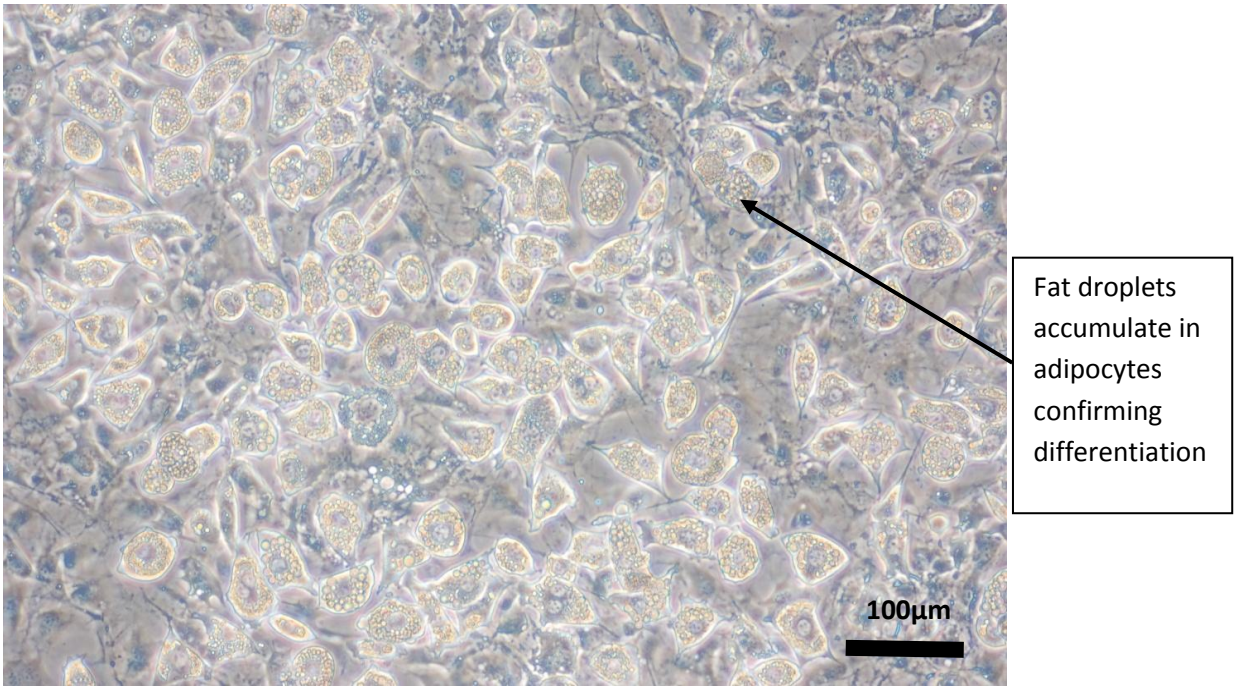


Figure 15: 3T3-L1 adipocytes at different stages of culture from pre-adipocytes to differentiated cells ready for treatment. A: Pre-adipocytes – unstained, B: 3T3-L1 Adipocyte day 3 post differentiation, C: 3T3-L1 Adipocyte day 12 post differentiation H&O stained. All pictures taken at X10 magnification.

3.4.1 3T3-L1 adipocytes: IL-6 gene expression following LPS

IL-6 is a known adipokine with its expression increased following LPS administration[12]. This gene has been used to ensure that the cells are responding to the LPS in an appropriate manner, thus rendering the result of the receptors and adiponectin PCR more reliable and confirming that it is a true result. The time curve seen here (Tables 12 & 13 and Figure 16) is typical for IL-6 as it is released early in the response of adipose tissue to LPS and then decreases over time[53]. In addition, the rise in IL-6 gene expression indicates that adipocytes are becoming inflamed following LPS treatment and releasing inflammatory cytokines, thus confirming our group's previous results in the murine model[12].

Table 12: IL-6 gene expression in 3T3-L1 adipocytes following 0.1 µg/ml LPS treatment for up to 24 hours

Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
1 h Control	1	0.262637	0.208007	
1 h LPS	1.86736	0.559301	0.430392	0.128918
4 h Control	1	0.108668	0.098016	
4 h LPS	25.42778	9.860946	7.105441	0.000478
8 h Control	1	0.471814	0.320566	
8 h LPS	5.010658	1.588566	1.206166	0.000996
24 h Control	1	0.553274	0.356199	
24 h LPS	2.666597	0.652098	0.523966	0.061253

Table 12: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. IL-6 expression increases significantly (25-fold) after 4 hours treatment with 0.1 µg/ml LPS. n=6, samples run in duplicate.

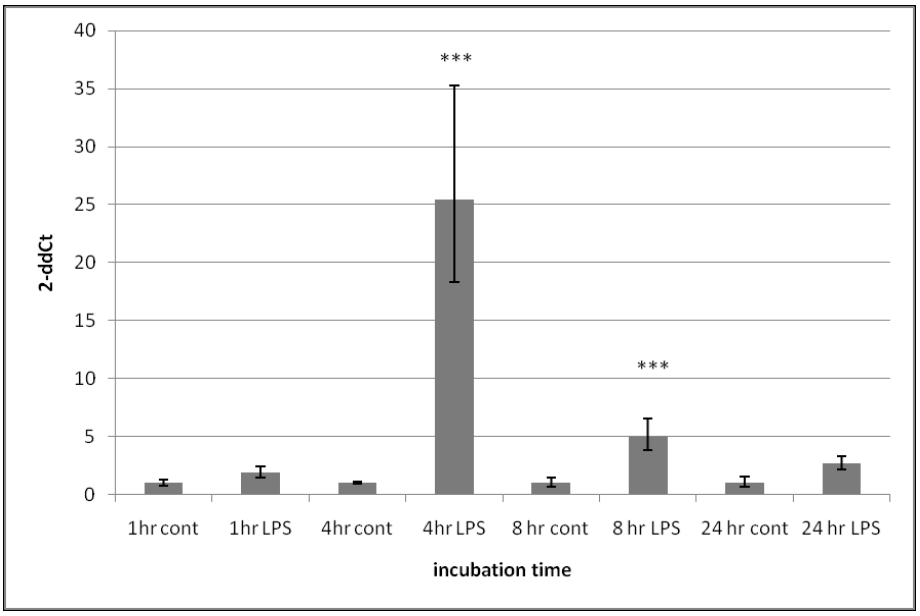
Table 13: IL-6 gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.190989	0.160361	
	1µg/ml	34.29675	7.750036	6.321554	$1.5x10^{-7}$
	5 µg/ml	20.60611	7.156536	5.311755	$2.14x10^{-5}$
	10 µg/ml	22.85862	4.097921	3.474957	$6.81x10^{-7}$
24 hours	Control	1	0.23303	0.18899	
	1 µg/ml	14.6213	4.111382	3.209031	$1.05x10^{-5}$
	5 µg/ml	10.76542	2.299231	1.894593	0.000109
	10 µg/ml	6.611603	1.805666	1.418316	0.000138

Table 13: Results displayed as $2^{-\Delta\Delta CT}$ with each treatment group compared to its own control group only with Bonferroni correction. n=6, samples run in duplicate

Figure 16: IL-6 gene expression in 3T3-L1 adipocytes following LPS treatment

A: IL-6 gene expression in 3T3-L1 adipocytes following 0.1 µg/ml LPS treatment for up to 24 hours



B: IL-6 gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

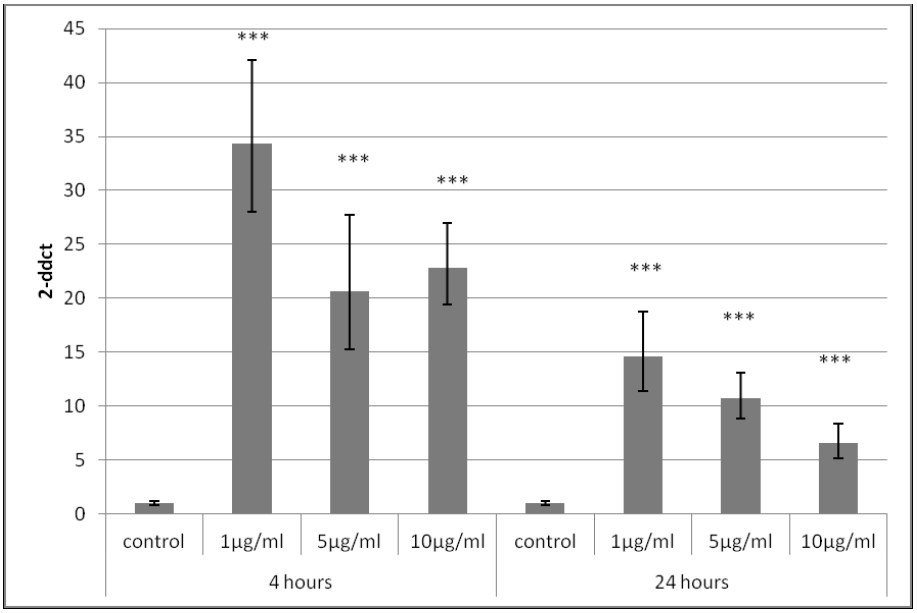


Figure 16: Graphs depicting the fold change of IL-6 gene expression in 3T3-L1 adipocytes following treatment with LPS (1-10 µg/ml) for 4 or 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$, ***$p<0.001$). Housekeeping gene was β-actin. (cont: control cells). n=6, samples run in duplicate

3.4.1 3T3-L1 Adipocytes: Adiponectin gene expression following LPS

Following the successful induction of inflammation confirmed by using IL-6 gene expression, the same experimental technique was used to investigate adiponectin and adiponectin receptor gene expression. Interestingly, compared to control, there was no difference in adiponectin gene expression using low dose LPS (0.1 μg/ml) however, there was a demonstrable dose response with increasing doses of LPS (1-10 μg/ml) (Table 14 & 15, Figure 17 & 18). This result is surprising as 0.1 μg/ml LPS induced a significant rise in IL-6 gene expression. The two main time points of 4 and 24 hours were used to maintain consistency with the animal experiments.

Table 14: Adiponectin gene expression in 3T3-L1 adipocytes following 0.1 µg/ml LPS for up to 24 hours

Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
1 h Control	1	0.364885	0.267337	
1 h LPS	1.351286	0.572911	0.402332	0.560966
4 h Control	1	0.182703	0.154479	
4 h LPS	1.406393	0.26345	0.221886	0.210711
8 h Control	1	0.201604	0.167779	
8 h LPS	1.052145	0.120409	0.108044	0.831424
24 h Control	1	0.316166	0.240217	
24 h LPS	0.669737	0.100179	0.087144	0.186943

Table 14: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. There are no changes in adiponectin gene expression with any time point using 0.1 µg/ml LPS. n=6, samples run in duplicate.

Figure 17: Adiponectin gene expression in 3T3-L1 adipocytes following 0.1 µg/ml LPS treatment for up to 24 hours

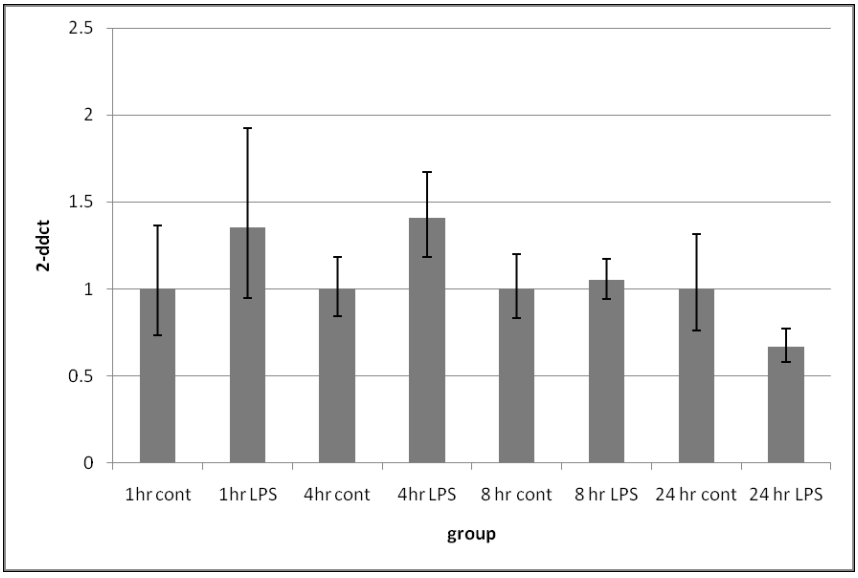


Figure 17: Graph depicting a lack of change in adiponectin gene expression in 3T3-L1 adipocytes following treatment with LPS (0.1 µg/ml) for up to 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. Housekeeping gene was β-actin. (cont: control cells) n=6, samples run in duplicate.

3.4.3 3T3-L1 adipocytes: Adiponectin gene expression following LPS treatment

Table 15: Adiponectin gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	control	1	0.381852	0.276333	
	1 µg/ml	0.406596	0.248782	0.154344	0.153832
	5 µg/ml	0.223498	0.319371	0.131484	0.161381
	10 µg/ml	0.148137	0.04888	0.036753	0.002181
24 hours	control	1	0.725798	0.420558	
	1 µg/ml	0.593231	0.352092	0.220953	0.483881
	5 µg/ml	0.860551	0.349309	0.248457	0.83982
	10 µg/ml	0.565135	0.368188	0.222941	0.419607

Table 15: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only and Bonferroni correction applied. n=6, samples run in duplicate.

Figure 18: Adiponectin gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

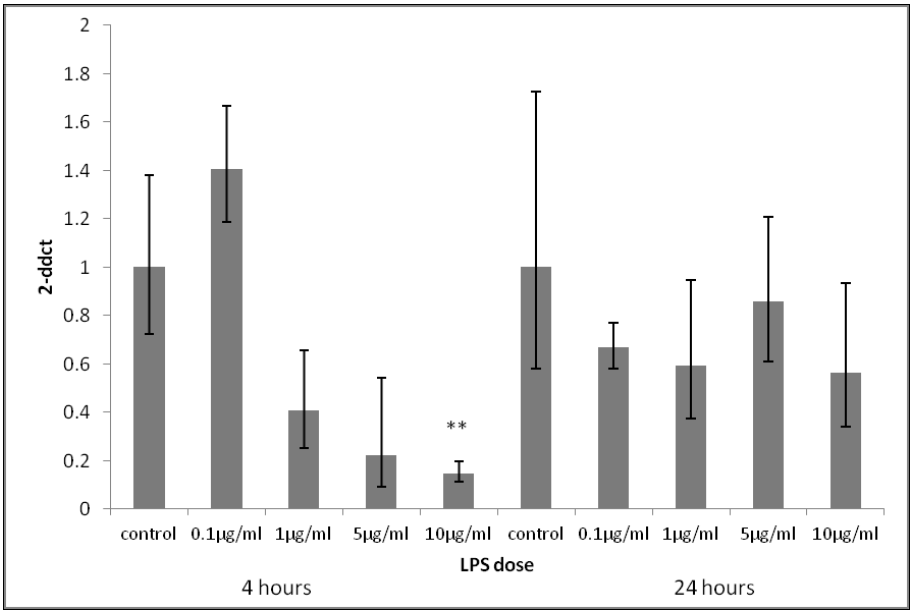


Figure 18: Graph depicting the changes in adiponectin gene expression in 3T3-L1 adipocytes following treatment with LPS (1-10 µg/ml) for 4 and 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. (** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

Increasing concentrations of LPS (1 μg/ml, 5 μg/ml and 10 μg/ml) led to a clear reduction in adiponectin gene expression, with increasing concentration having an increased effect. 10 μg/ml LPS induced a significant 7-fold reduction in gene expression. Again this was a rapid effect, with changes after four hours. After 24 hours, again, there were no significant changes (Figure 18). This indicates that adiponectin gene expression in-vitro responds much quicker than in *in-vivo* murine adipose tissue studies.

3.4.4 3T3-L1 Adipocytes: Adiponectin receptor R1 gene expression following LPS

The same experimental technique was used to examine the effects of LPS on adiponectin receptor expression. In a similar manner to previous experiments, a time curve was performed using a concentration known to have had an effect in previous cells in this laboratory, 0.1 μg/ml[189]. The experiments were performed at four time points over a 24 hour period (1, 4, 8 and 24 hours). As can be seen from the following two graphs, there was a small down-regulation in adipoR1 expression after 4 hours treatment (65% control). This was not seen in cells incubated for longer time periods. There were no changes in adipoR2 expression at any time period (Table 16 & 17, Figure 19 & 20).

Table 16: AdipoR1 gene expression in 3T3-L1 adipocytes following 0.1 µg/ml LPS treatment for up to 24 hours

Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
1 h cont	1	0.293573	0.226947	
1 h LPS	0.820362	0.165604	0.137789	0.559855
4 h cont	1	0.105678	0.095577	
4 h LPS	0.651574	0.088875	0.078208	0.044523
8 h cont	1	0.210417	0.173839	
8 h LPS	1.135504	0.26164	0.212643	0.661814
24 h cont	1	0.199722	0.166474	
24 h LPS	0.904379	0.158907	0.135159	0.682949

Table 16: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. Only after 4 hours following treatment with 0.1 µg/ml LPS a reduction in adipoR1 gene expression was observed. n=6, samples run in duplicate.

Figure 19: AdipoR1 gene expression in 3T3-L1 adipocytes following 0.1 µg/ml LPS treatment for up to 24 hours

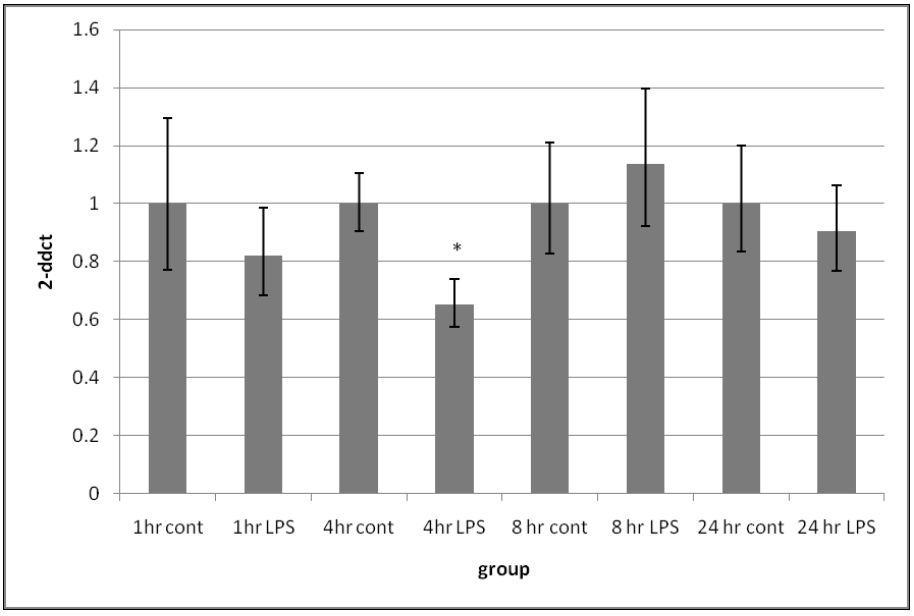


Figure 19: Graph depicting a small change in adiponectin receptor 1 gene expression in 3T3-L1 adipocytes following treatment with LPS (0.1 µg/ml) for up to 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. (*$p<0.05$). Housekeeping gene was β-actin. (cont: control cells). n=6, samples run in duplicate.

3.4.5 3T3-L1 adipocytes: Adiponectin receptor R2 gene expression following LPS

Table 17: AdipoR2 gene expression in 3T3-L1 adipocytes following 0.1 µg/ml LPS treatment for up to 24 hours

Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
1 h Control	1	0.132643	0.117109	
1 h LPS	1.271325	0.136571	0.123323	0.185265
4 h Control	1	0.10078	0.091553	
4 h LPS	0.721798	0.183034	0.146009	0.272215
8 h Control	1	0.188327	0.158481	
8 h LPS	1.324089	0.168915	0.149804	0.172314
24 h Control	1	0.3369	0.252001	
24 h LPS	0.961483	0.131424	0.11562	0.898072

Table 17: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. There are no changes in adipoR2 gene expression following 0.1 µg/ml LPS treatment. n=6, samples run in duplicate.

Figure 20: AdipoR2 gene expression in 3T3-L1 adipocytes following 0.1 µg/ml LPS treatment for up to 24 hours

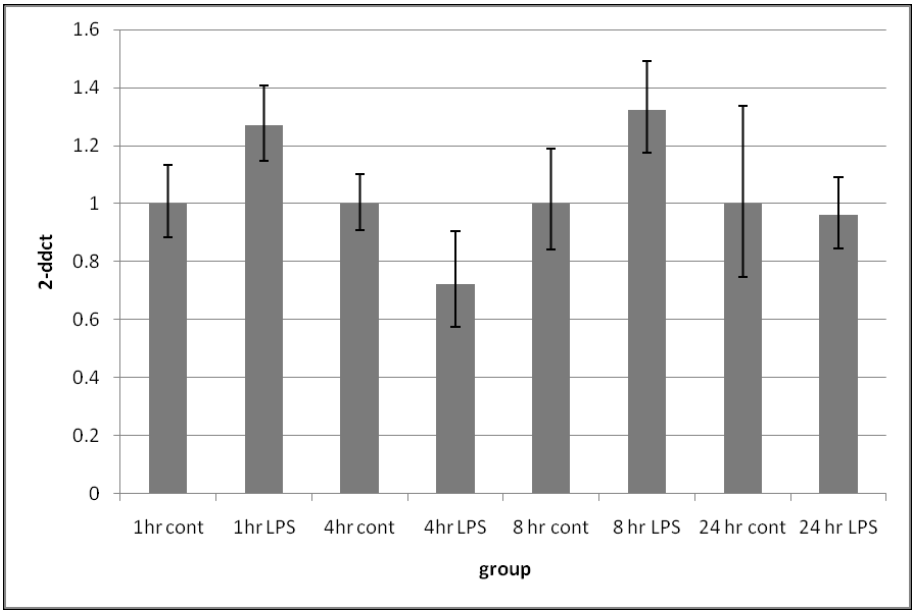


Figure 20: Graph depicting the changes in adiponectin receptor 2 gene expression in 3T3-L1 adipocytes following treatment with LPS (0.1 µg/ml) for up to 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. Housekeeping gene was β-actin (cont: control cells). n=6, samples run in duplicate.

3.4.6 3T3-L1 Adipocytes: Adiponectin receptor gene expression following LPS

In view of the minor changes observed after treatment with 0.1 μg/ml LPS, higher doses of LPS were used to treat the same cell line. A further three concentrations of LPS were used (1 μg/ml, 5 μg/ml and 10 μg/ml). AdipoR1 did not respond to LPS at higher concentrations (Figure 21) but adipoR2 was down-regulated (Figure 22). The response of adipoR2 was greater at higher concentrations of LPS. AdipoR2 responded rapidly with effects being seen at 4 hours. In a similar manner to the tissue experiments, there were no changes seen at 24 hours.

These results demonstrate time-dependent changes in gene expression in response to the varying doses of LPS, which vary between receptor subtype and adiponectin itself. There appears to be few similarities between the three genes, suggesting that there may be differences in regulation and/or response to different doses of LPS.

Table 18: AdipoR1 gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.106533	0.096276	
	1 µg/ml	1.107009	0.171281	0.14833	0.577524
	5 µg/ml	0.83702	0.127975	0.111004	0.334789
	10 µg/ml	0.870752	0.067074	0.062277	0.314486
24 hours	Control	1	12.53022	0.926091	
	1 µg/ml	0.908568	0.176939	0.148098	0.626799
	5 µg/ml	0.99654	0.155679	0.134645	0.981832
	10 µg/ml	0.95705	0.091054	0.083144	0.707906

Table 18: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only with Bonferroni correction. There are no changes in adipoR1 gene expression following 1-10 µg/ml LPS treatment. n=6, samples run in duplicate.

Figure 21: AdipoR1 gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

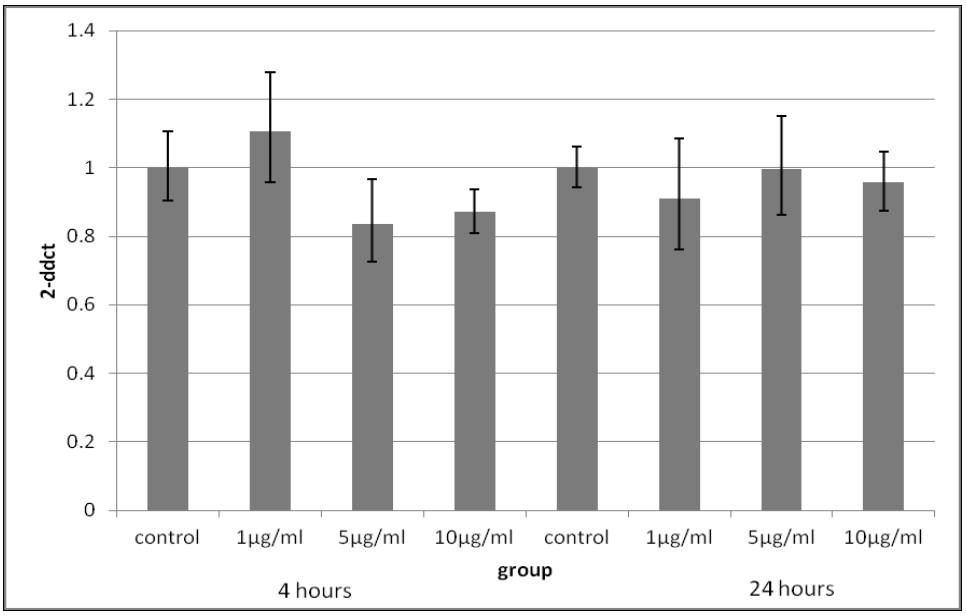


Figure 21: Graph depicting the changes in adiponectin receptor 1 gene expression in 3T3-L1 adipocytes following treatment with LPS (1-10 µg/ml) for 4 and 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.4.7 3T3-L1 Adipocytes: Adiponectin receptor R2 gene expression following LPS

Table 19: AdipoR2 gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	control	1	0.216885	0.17823	
	1 µg/ml	0.387786	0.127606	0.096012	0.02309
	5 µg/ml	0.596668	0.098189	0.084314	0.066087
	10 µg/ml	0.255784	0.104501	0.07419	0.017648
24 hours	control	1	0.247877	0.198639	
	1 µg/ml	0.92445	0.147892	0.127495	0.77511
	5 µg/ml	1.194715	0.237475	0.198099	0.553701
	10 µg/ml	0.907519	0.139032	0.120562	0.698579

Table 19: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only with Bonferroni correction. There are no changes in adipoR1 gene expression following 1-10 µg/ml LPS treatment. n=6, samples run in duplicate.

Figure 22: AdipoR2 gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

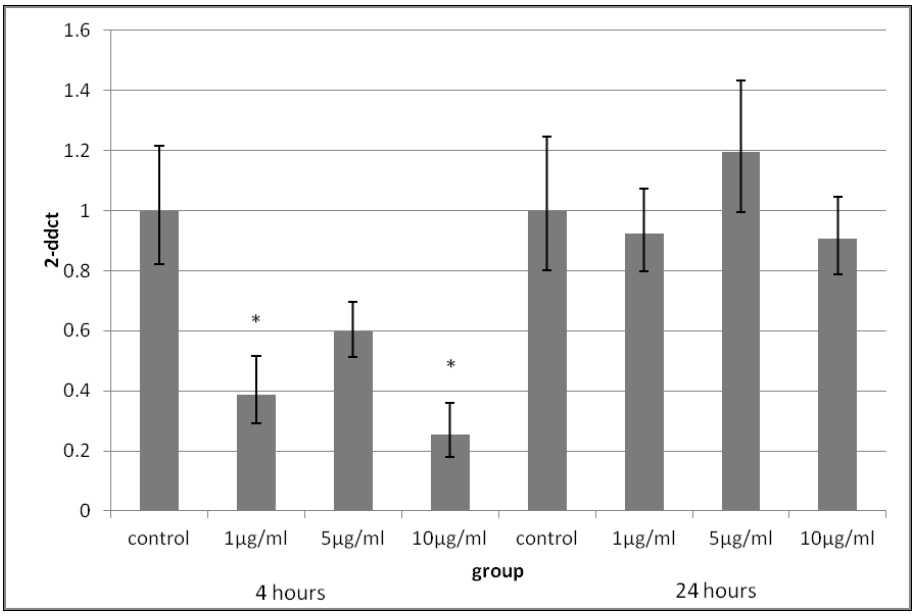


Figure 22: Graph depicting the changes in adiponectin receptor 2 gene expression in 3T3-L1 adipocytes following treatment with LPS (1-10 µg/ml) for 4 and 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. (*$p<0.05$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.5 Cytokine treatment

Sepsis is a multi-factorial process comprising an initial insult followed by a cascade of further physiological and metabolic changes including inflammatory cytokine release. It is therefore important to discriminate whether the changes seen in-vivo represent a direct effect of LPS or whether inflammatory cytokines and other metabolic changes had any influence. Inflammatory cytokines, IL-6 and TNF-α were therefore used to treat the 3T3-L1 cells using the same technique as in previous experiments. 10 ng/ml IL-6 and TNF-α are commonly used doses in cell line experiments and has been demonstrated to have effects on adiponectin expression in 3T3-L1 cells in previous studies[112]. The next series of tables and figures display the effect on 3T3-L1 cells of treatment with inflammatory cytokines for 24 hours.

IL-6 down-regulates its own expression following four hours of treatment. TNF-α, however, significantly up-regulates the expression of IL-6. However, AdipoR1 only was down-regulated significantly by IL-6 at both time points but not by TNF-α and neither cytokine had any effect on adipoR2 or adiponectin gene expression (Tables 20-23, Figures 23-26). Therefore, again a difference in the regulation of adiponectin and its receptors was observed with different cytokines.

3.5.1 3T3-L1 Adipocytes: IL-6 gene expression following cytokine treatment

Table 20: IL-6 gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	control	1	0.226396	0.184603	
	IL-6 10 ng/ml	0.446273	0.021079	0.020128	0.015238
24 hours	Control	1	0.241821	0.194731	
	IL-6 10 ng/ml	1.035026	0.665136	0.404922	0.951551
4 hours	Control	1	0.196829	0.164459	
	TNF-α 10 ng/ml	3.850378	1.087733	0.848135	0.001693
24 hours	Control	1	0.40609	0.288808	
	TNF-α 10 ng/ml	8.291693	5.093624	3.155305	0.000843

Table 20: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. IL-6 down-regulates its own expression and increases the gene expression of TNF-α. n=6, samples run in duplicate.

Figure 23: IL-6 gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

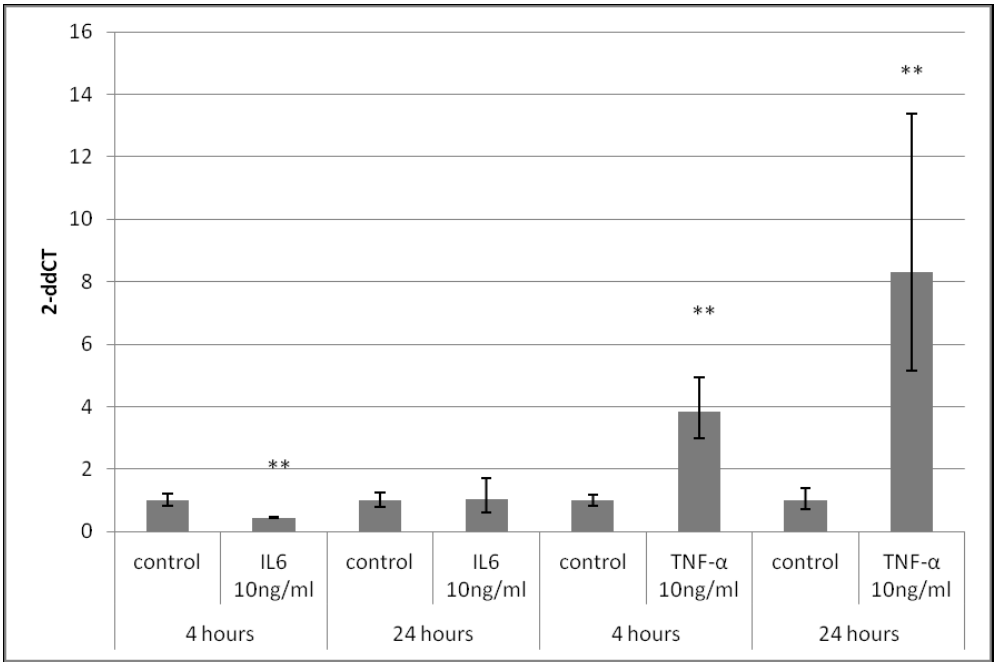


Figure 23: Graph depicting the changes in adiponectin IL-6 gene expression in 3T3-L1 adipocytes following treatment with IL-6 and TNF-α (10 ng/ml) for 4 and 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. (** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.5.2 3T3-L1 adipocytes: Adiponectin gene expression following treatment with inflammatory cytokines

Table 21: Adiponectin gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.352997	0.2609	
	IL-6 10 ng/ml	0.961927	0.241654	0.193135	0.923161
24 hours	Control	1	0.714318	0.416678	
	IL-6 10 ng/ml	0.997462	0.958329	0.488752	0.99772
4 hours	Control	1	0.456027	0.3132	
	TNF-α 10 ng/ml	3.29056	1.595431	1.074472	0.053954
24 hours	Control	1	0.431551	0.301457	
	TNF-α 10 ng/ml	0.593917	0.420729	0.246271	0.0873

Table 21: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. There are no changes in adiponectin gene expression following treatment with inflammatory cytokines. n=6, samples run in duplicate.

Figure 24: Adiponectin gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

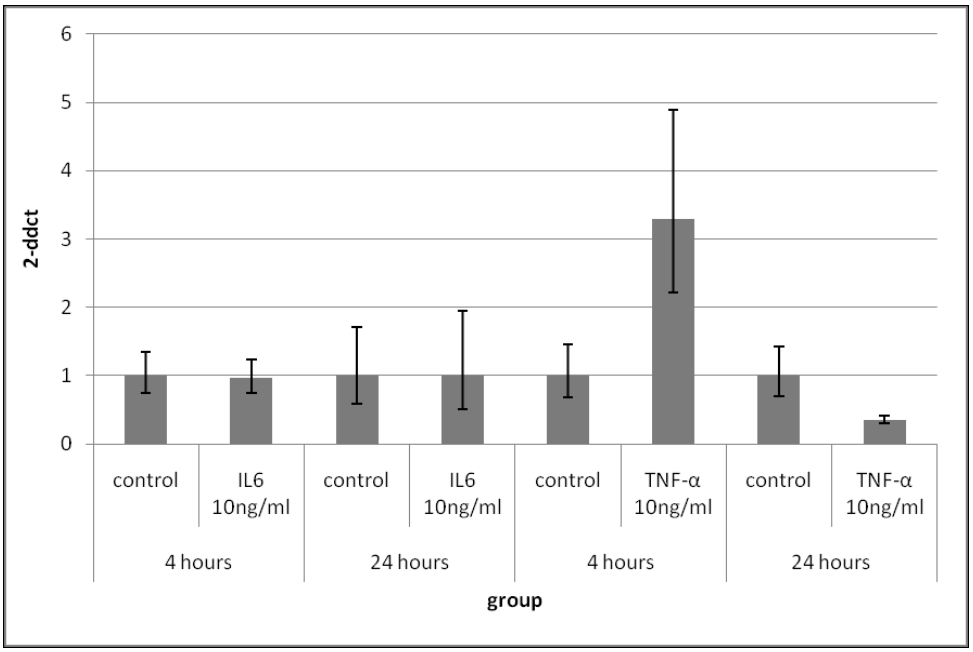


Figure 24: Graph depicting the changes in adiponectin gene expression in 3T3-L1 adipocytes following treatment with IL-6 and TNF-α (10 ng/ml) for 4 and 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.5.3 3T3-L1 Adipocytes: Adiponectin receptor R1 gene expression following treatment with inflammatory cytokines

Table 22: AdipoR1 gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.063686	0.059873	
	IL-6 10 ng/ml	0.386355	0.066346	0.056622	0.003589
24 hours	Control	1	0.245595	0.197171	
	IL-6 10 ng/ml	0.50162	0.085981	0.0734	0.032261
4 hours	Control	1	0.578653	0.366549	
	TNF-α 10 ng/ml	0.737987	0.155018	0.128108	0.090398
24 hours	Control	1	0.214513	0.176625	
	TNF-α 10 ng/ml	0.863539	0.207015	0.166984	0.629802

Table 22: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. IL-6 significantly down-regulates the expression of adipoR1 in 3T3-L1 adipocytes but TNF-α has no effect. n=6, samples run in duplicate.

Figure 25: AdipoR1 gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

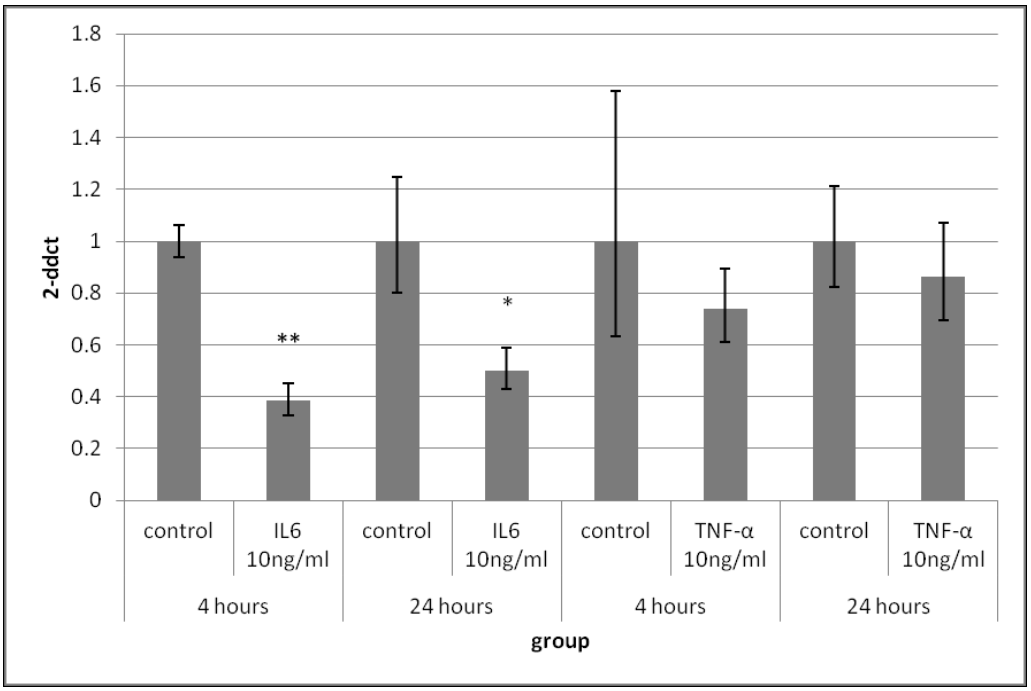


Figure 25: Graph depicting the changes in adipoR1 gene expression in 3T3-L1 adipocytes following treatment with IL-6 and TNF-α (10 ng/ml) for 4 and 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.5.4 3T3-L1 Adipocytes: Adiponectin receptor R2 gene expression following treatment with inflammatory cytokines

Table 23: AdipoR2 gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.212667	0.175371	
	IL-6 10 ng/ml	0.548666	0.209785	0.151759	0.186241
24 hours	Control	1	1.12393	0.529175	
	IL-6 10 ng/ml	0.537375	0.651549	0.29449	0.604379
4 hours	Control	1	0.294139	0.227285	
	TNF-α 10 ng/ml	0.962594	0.204053	0.168363	0.908183
24 hours	Control	1	1.971691	0.663491	
	TNF-α 10 ng/ml	0.566115	0.240198	0.168644	0.586458

Table 23: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. There are no changes in adipoR2 expression following treatment with inflammatory cytokines. n=6, samples run in duplicate.

Figure 26 : AdipoR2 gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

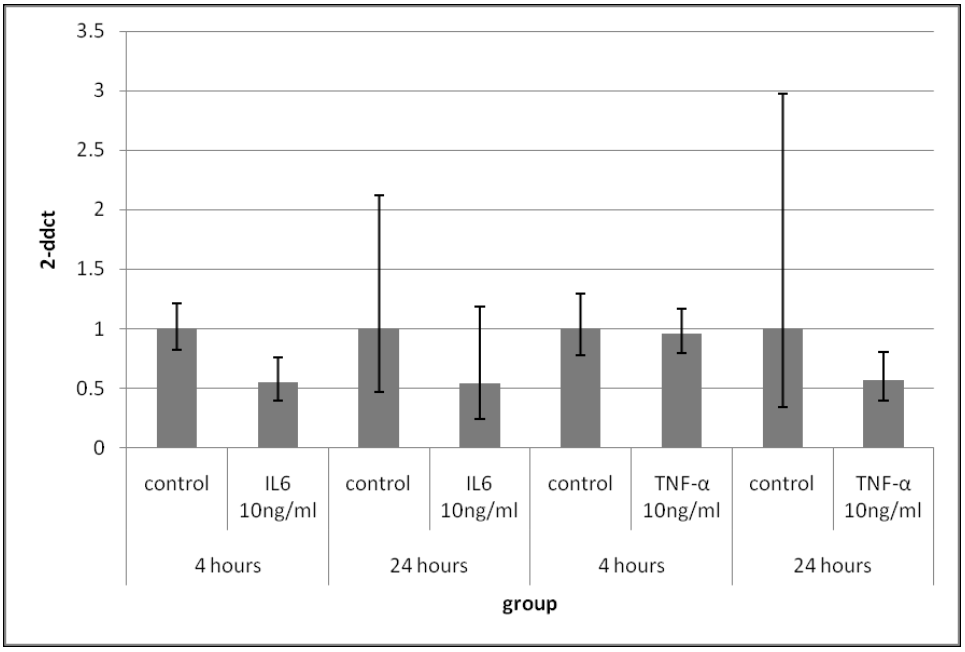


Figure 26: Graph depicting the changes in adipo R2 gene expression in 3T3-L1 adipocytes following treatment with IL-6 and TNF-α (10 ng/ml) for 4 and 24 hours. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and error bars display SEM. $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.6 Skeletal Muscle

3.6.1 Initial experiments to confirm the presence of adiponectin gene expression in murine tissue

Adiponectin is thought to be exclusively expressed from adipocytes. However, a small number of studies now have shown that adiponectin is expressed in other tissues including bone, cardiomyoctes and skeletal muscle[190-195]. Skeletal muscle is a large insulin sensitive organ and plays a major role in glucose metabolism and metabolic control in health[196]. In sepsis, there are well documented changes in skeletal muscle morphology and physiology and myopathies are not uncommon[197]. We therefore investigated adiponectin and receptor gene expression in murine skeletal muscle.

3.6.2 Skeletal muscle: Adiponectin gene expression

A 10-fold reduction in adiponectin gene expression after fours 4 hours following LPS injection was demonstrated. This down-regulation in gene expression persisted and increased further in the animals treated for 24 hours (Figure 27). This is different to adipose tissue where a demonstrable and less marked down-regulation was observed but only in the mice treated for 24 hours (Figure 13). Thus, skeletal muscle seems to respond more rapidly to treatment with LPS in-vivo than adipose tissue.

Figure 27: Adiponectin expression in Murine Skeletal muscle following LPS treatment

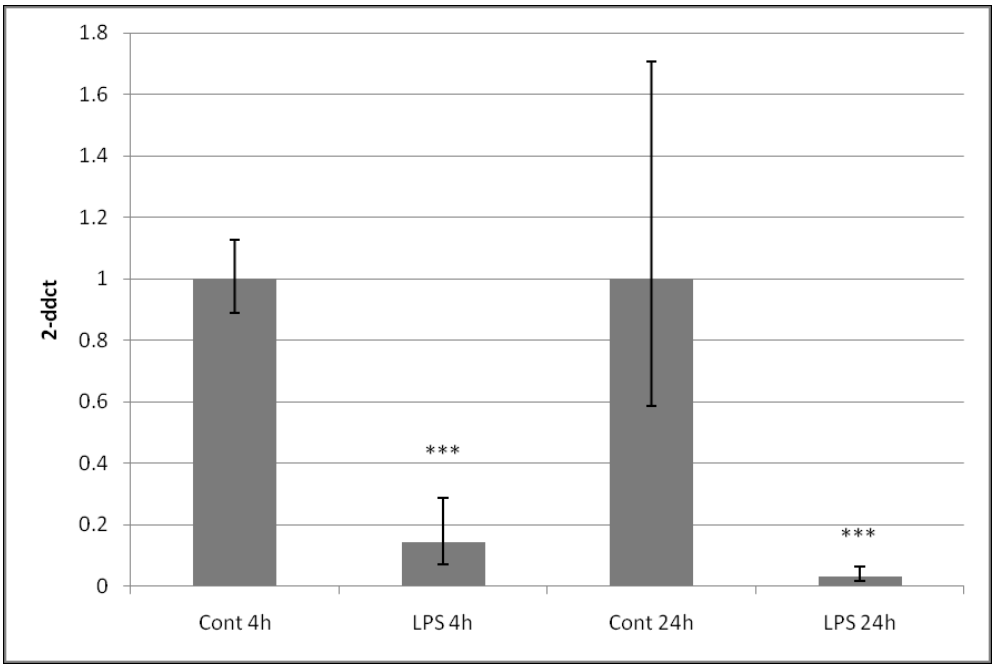


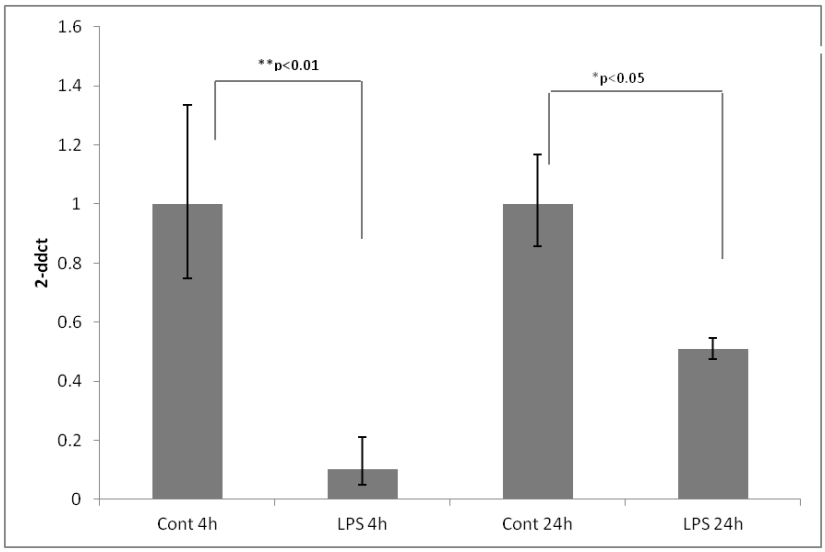
Figure 27: Relative change in adiponectin gene expression in mouse skeletal muscle tissue depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin (Cont = Control group, LPS = Treatment group, 4 h 4 hours treatment group, 24 h: 24 hours treatment group)**.** 4 hour mice, n=6, 24 hour mice n=11, samples run in duplicate.

3.6.3 Skeletal muscle: Adiponectin receptor expression

Murine skeletal muscle tissue was then investigated with respect to adiponectin receptors. Adiponectin receptor expression was significantly down-regulated following treatment with LPS with gene expression in the treated mice being only 10% of that of control mice after 4 hours and 50% after 24 hours (Figure 28). Despite a 10-fold reduction in expression in adipoR1 at 24 hours, there is a large SEM and a small sample size, therefore rendering the result non-significant.

Figure 28: Adiponectin receptor expression in Murine Skeletal muscle

A: Adiponectin receptor 1 expression in skeletal muscle following LPS treatment



B: Adiponectin receptor 2 expression in skeletal muscle following LPS treatment

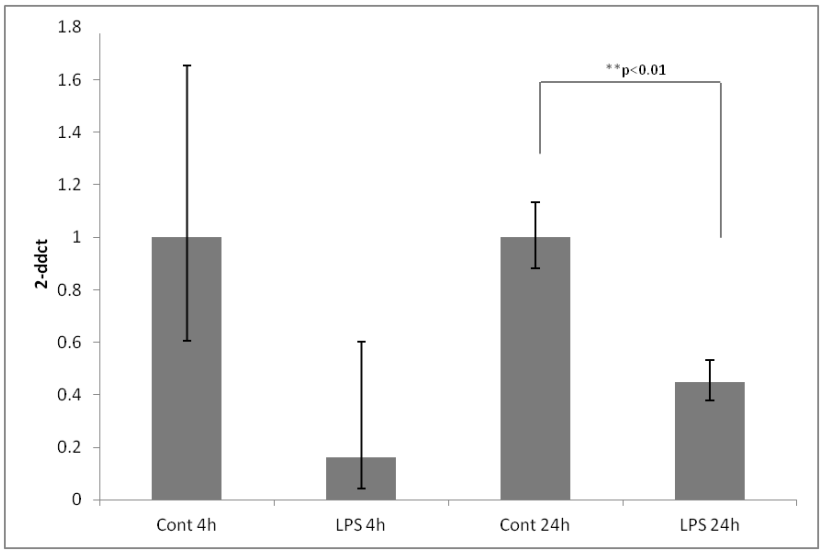


Figure 28: Relative change in adiponectin and adiponectin receptor gene expression in mouse skeletal muscle depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. A: Skeletal Muscle adipoR1 expression, B: skeletal Muscle adipoR2 expression (Cont = Control group, LPS = Treatment group, 4 h 4 hours treatment group, 24 h: 24 hours treatment group). 4 hour mice, n=6, 24 hour mice n=11, samples run in duplicate

Having demonstrated such significant changes in adiponectin receptor expression in response to LPS, it was crucial to investigate this further. Firstly, the PCR product from the animal samples was sequenced to ensure the correct amplification product. Secondly, C2C12 myocytes were grown in culture and baseline experiments including identification of adiponectin by standard and real-time PCR. This is to ensure no contamination from blood or perimuscular fat providing the results observed.

3.6.4 Adiponectin Real-time PCR product sequencing

Epidydimal fat adiponectin sequencing

```
Query  3    CCCATACACCTGGAGCCAGACTTGGTCTCCCACCTCCAGATGGAGGAGCACAGAGCC  59
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  774  CCCATACACCTGGAGCCAGACTTGGTCTCCCACCTCCAGATGGAGGAGCACAGAGCC  718
```

57/57

100% match

Skeletal Muscle adiponectin sequencing (tissue)

```
Query  1    CCCATACACCTGGAGCCAGACTTGGTCTCCCACCTCCAGATGGAGGAGCACAGAGCACAG  60
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  774  CCCATACACCTGGAGCCAGACTTGGTCTCCCACCTCCAGATGGAGGAGCACAGAGC-CAG  716
```

59/60

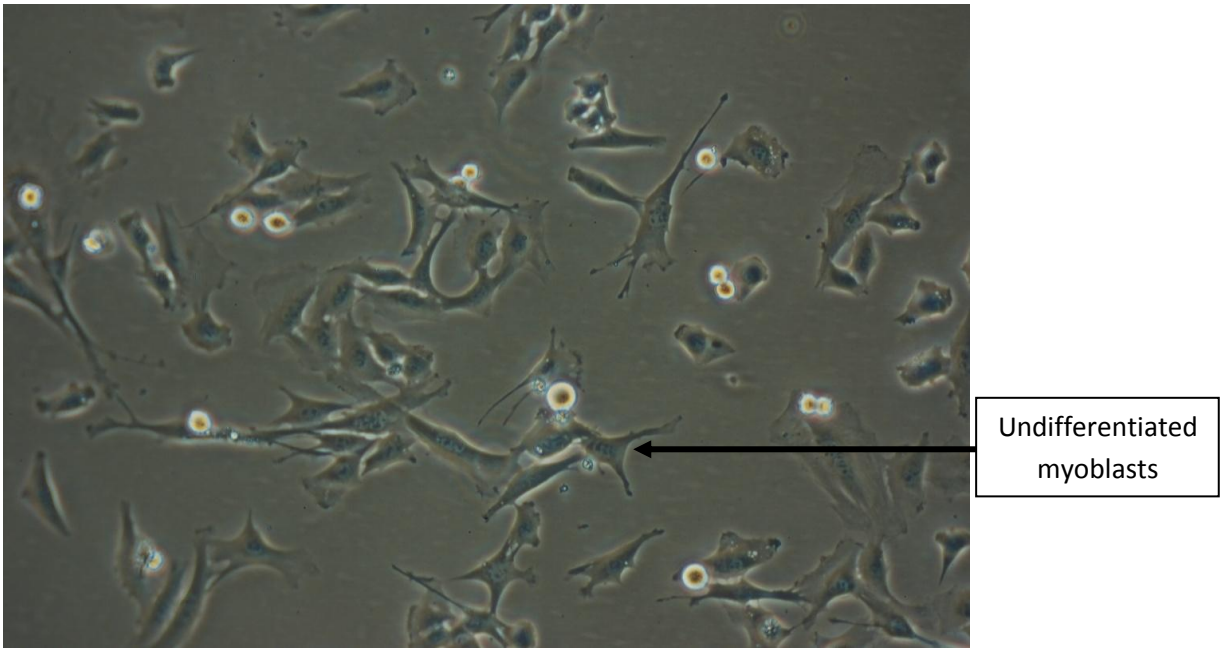
98% match

Examining the electropherogram, the A nucleotide 4 from the end is a misread and should not be there therefore the sequencing process showed a 100% match to adiponectin in both the adipose tissue sample and the muscle sample sequenced. This demonstrates that adiponectin is correctly amplified in the murine skeletal muscle.

3.7 C2C12 Myocyte Culture and differentiation

Cell Pictures

Figure 29: Pictures of C2C12 myocytes at different stages of differentiation
A: C2C12 Myocytes – Myoblasts



B: C2C12 Myocytes – day 6 in differentiation media

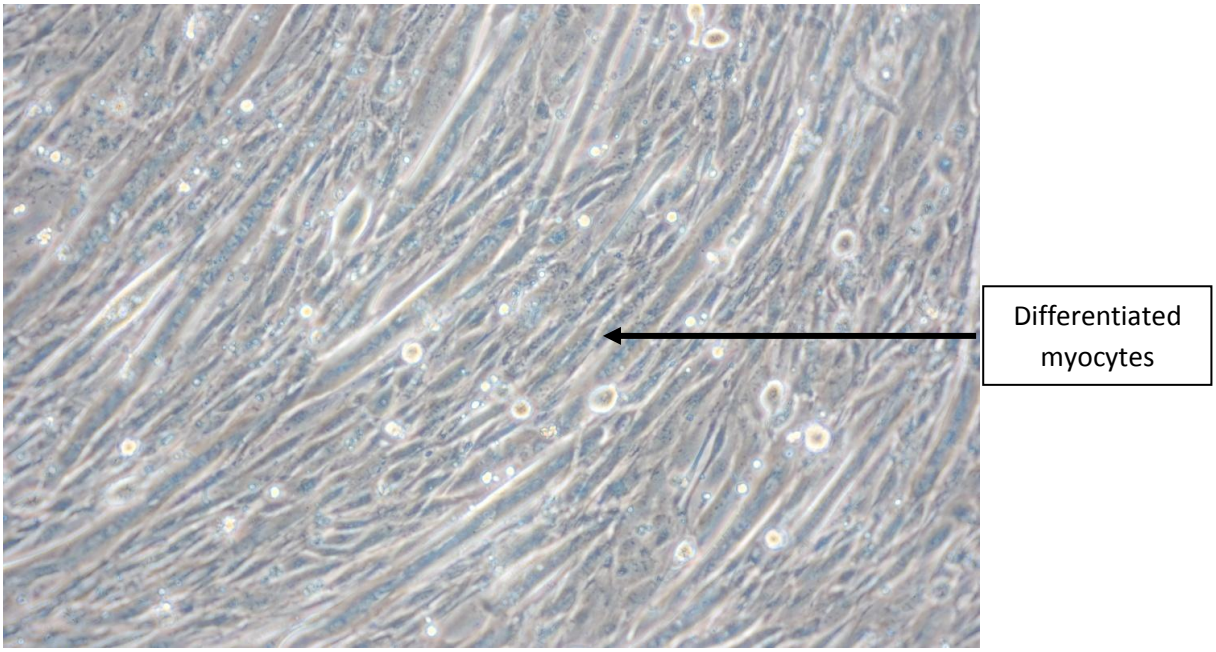


Figure 29: Pictures of C2C12 myocytes at varying stages during the differentiation process. A: C2C12 Myocytes – myoblasts, B: C2C12 myocytes – day 6 in differentiation media

3.7.1 Confirmation of adiponectin mRNA in C2C12 myocytes

Figure 30: Standard PCR displaying the presence of adiponectin mRNA in C2C12 myocytes

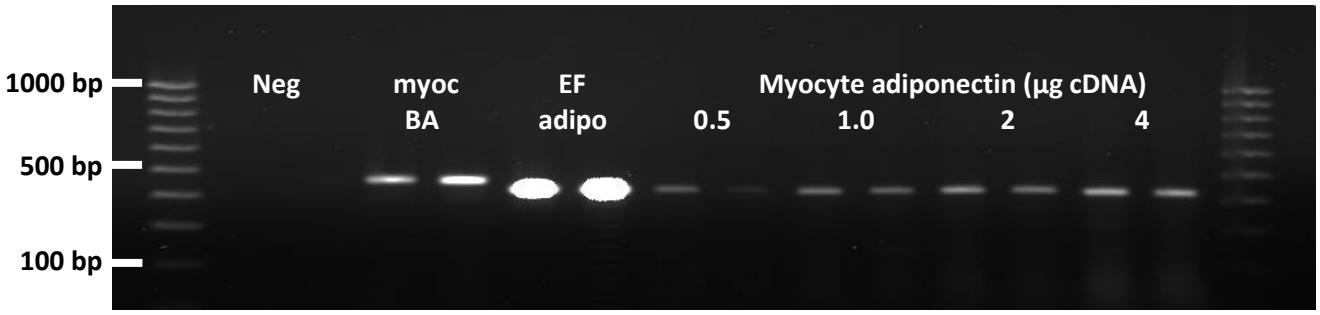


Figure 30: Standard PCR gel of C2C12 Myocyte cDNA (kindly donated by Adam Lightfoot, University of Liverpool) showing a dose response curve (all samples in duplicate) with increasing signal with increasing concentration of cDNA used in the experiment (0.5-4 µg/ml cDNA). Positive (Epididymal fat) and negative (myocyte RNA) controls were used. Signal approximately 400-450bp (actual size 430bp). (neg: negative control, myoc BA: myocyte beta actin, Efat adipo: epididymal fat adiponectin, myocyte adiponectin (µg): increasing concentrations of myocyte cDNA (0.5-4.0µg), bp: base pairs). Adiponectin PCR: 34 cycles, Beta actin PCR: 25 cycles).

At low concentrations of cDNA, there were weak bands only. However, at increased concentrations of cDNA, there were obvious bands, indicating that adiponectin is expressed but at low concentrations. The difference in band signal between the positive control and the myocyte adiponectin band demonstrates that C2C12 myocytes express adiponectin at a lower level than epididymal fat (Figure 30).

Myocyte experiments

Following the observations in whole mouse skeletal muscle, C2C12 myocytes were grown in culture and treated with LPS to ascertain any changes in the gene expression of adiponectin or its receptors. C2C12 myocytes were grown in culture using the standard laboratory protocol and were treated on day 7 with various doses of LPS. This was the same experimental conditions as the 3T3-L1 adipocyte experiments. Confirmation of LPS effects and identification of skeletal muscle inflammation were confirmed using IL-6 expression as IL-6 is known to be released during skeletal muscle contraction (Table 24 & Figure 31).

3.7.2 C2C12 myocytes: IL-6 gene expression as a reference gene

The response to LPS was confirmed in C2C12 myocytes by determination of IL-6 expression which showed a rapid 1000-fold increase in gene expression. Following a personal communication with Adam Lightfoot (pHD student, muscle group), previous results in C2C12 cells indicate that LPS concentrations of 1-10 µg/ml are generally required to elicit a change in gene expression. Therefore, a 0.1 µg/ml treatment time curve (1, 4, 8 and 24 hours with 0.1 µg/ml LPS) was not performed as previously done in 3T3-L1 adipocytes treated with LPS. However, the 0.1 µg/ml dose was included in the concentration curve to achieve consistency between the adipocyte and the myocyte experiments. Thus the same concentrations were used (0.1-10 µg/ml LPS). In contrast, however, we did elicit a response with 0.1 µg/ml LPS with respect to IL-6 gene expression.

Table 24: IL-6 gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.104326	0.094471	
	0.1 µg /ml	849.6157	115.0902	101.3599	$1.83x10^{-11}$
	1 µg/ml	55.63805	8.739577	7.553137	$1.38x10^{-7}$
	5 µg/ml	953.6614	227.2549	183.5221	$8.98x10^{-9}$
	10 µg/ml	486.4002	57.23853	51.21201	$3.23x10^{-10}$
24 hours	Control	1	0.183848	0.155297	
	0.1 µg /ml	1.652901	0.359435	0.295234	0.081948
	1 µg/ml	3.714926	0.783289	0.646893	0.000457
	5 µg/ml	7.077959	0.826607	0.740166	$6.29x10^{-6}$
	10 µg/ml	5.296356	0.393872	0.366609	$4.99x10^{-5}$

Table 24: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only with Bonferroni correction. There is a significant up-regulation in IL-6 gene expression in C2C12 myocytes (approx 1000-fold) after four hours which rapidly reduces, although remains significant (with the exception of the 0.1 µg/ml group), after treatment for 24 hours. n=6, samples run in duplicate.

Figure 31: IL-6 gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

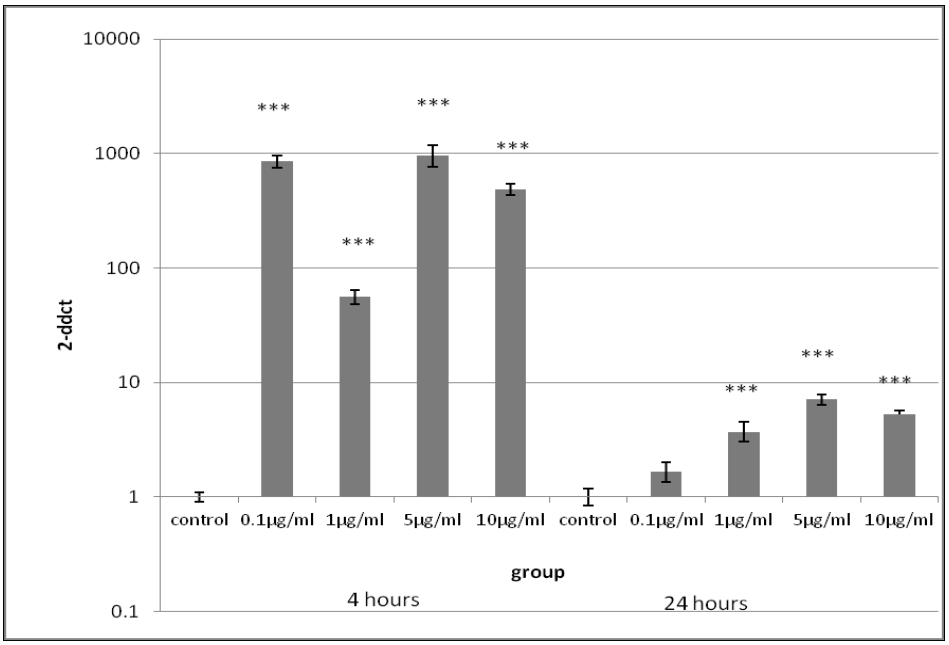


Figure 31: Relative change in IL-6 gene expression in murine C2C12 myocytes. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$, ***$p<0.0001$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.7.3 C2C12 myocytes: Adiponectin gene expression following LPS

Following treatment with LPS (0.1/1/5/10 µg/ml), there was a rapid down-regulation of adiponectin gene expression after only four hours treatment with 0.1, 1 and 10 µg/ml LPS (Table 25 & Figure 32). There was little change in adiponectin receptor gene expression with any concentration of LPS with the exception of a small reduction in expression of adipoR1 after treatment with 5 µg/ml LPS for four hours (Table 26 & Figure 33). AdipoR2, however, showed an increase in expression after treatment with 0.1 µg/ml and 5 µg/ml LPS (Table 27, Figure 34).

Table 25: Adiponectin gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.238	0.192	
	0.1 µg/ml	0.51	0.059	0.05	0.04*
	1 µg/ml	0.77	0.168	0.138	0.43
	5 µg/ml	0.34	0.093	0.07	0.01*
	10 µg/ml	0.46	0.10	0.08	0.04*
24 hours	Control	1	0.317	0.24	
	0.1 µg/ml	0.709	0.192	0.151	0.37
	1 µg/ml	1.19	0.520	0.36	0.71
	5 µg/ml	0.95	0.167	0.142	0.89
	10 µg/ml	0.93	0.040	0.038	0.81

Table 25: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only with Bonferroni correction. There is significant down-regulation in adiponectin gene expression in C2C12 myocytes after four hours which is not present at 24 hours. n=6, samples run in duplicate.

Figure 32: Adiponectin gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

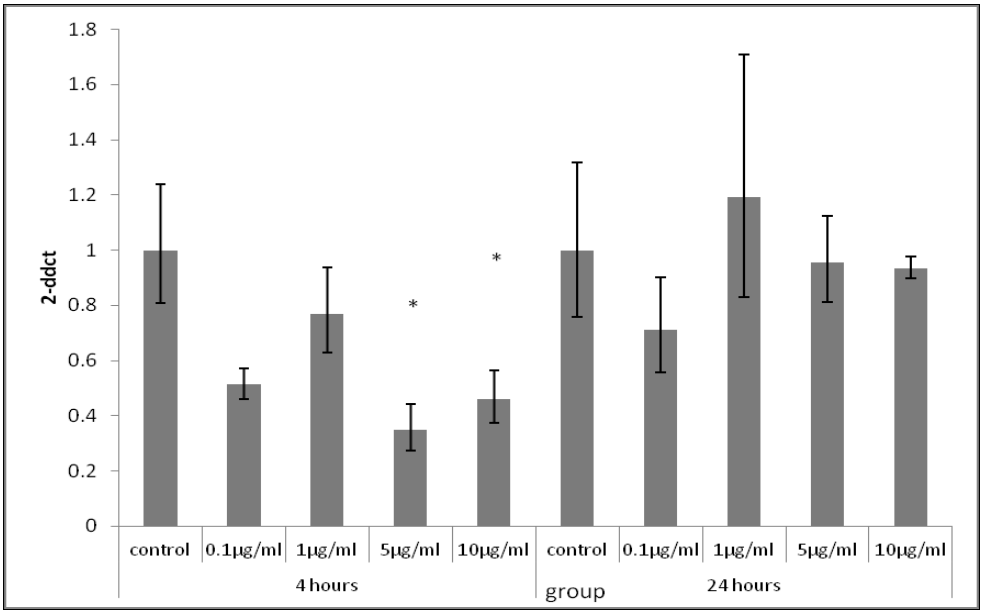


Figure 32: Relative change in adiponectin gene expression in C2C12 myocytes. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.7.4 C2C12 Myocytes: Adiponectin receptor R1 gene expression following LPS

AdipoR1 was down-regulated at moderate doses of LPS (5 µg/ml) after 4 hours whereas there was no significant change after 24 hours of treatment.

Table 26: AdipoR1 gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.088	0.080	
	0.1 µg/ml	0.914	0.031	0.029	0.36
	1 µg/ml	0.989	0.039	0.037	0.91
	5 µg/ml	0.65	0.088	0.077	0.02
	10 µg/ml	0.79	0.06	0.056	0.06
24 hours	Control	1	0.10	0.096	
	0.1 µg/ml	1.15	0.083	0.077	0.27
	1 µg/ml	1.34	0.14	0.134	0.08
	5 µg/ml	1.19	0.16	0.147	0.32
	10 µg/ml	0.87	0.05	0.054	0.28

Table 26: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only with Bonferroni correction. There is significant down-regulation in adioR1 gene expression in C2C12 myocytes after four hours which is not present at 24 hours. n=6, samples run in duplicate.

Figure 33: AdipoR1 gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

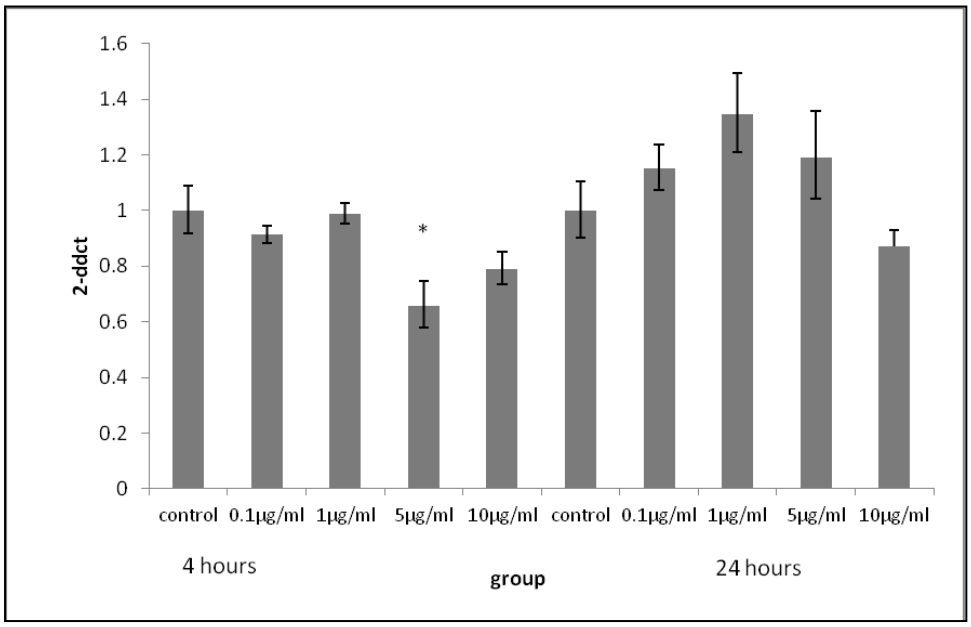


Figure 33: Relative change in adipoR1 gene expression in C2C12 myocytes. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.7.5 C2C12 Myocytes: Adiponectin receptor R2 gene expression following LPS

Table 27: AdipoR2 gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.34	0.25	
	0.1 µg/ml	1.05	0.09	0.08	0.85
	1 µg/ml	1.25	0.12	0.11	0.49
	5 µg/ml	0.76	0.11	0.101	0.44
	10 µg/ml	1.15	0.11	0.1	0.65
24 hours	Control	1	0.07	0.07	
	0.1 µg/ml	1.715	0.28	0.244	0.01
	1 µg/ml	1.14	0.14	0.12	0.36
	5 µg/ml	1.81	0.10	0.09	0.0001
	10 µg/ml	1.185	0.06	0.065	0.106

Table 27: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only with Bonferroni correction. There are no changes in adipoR2 gene expression after 4 hours incubation but there are small increases after 24 hours incubation. n=6, samples run in duplicate.

Figure 34: AdipoR2 gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

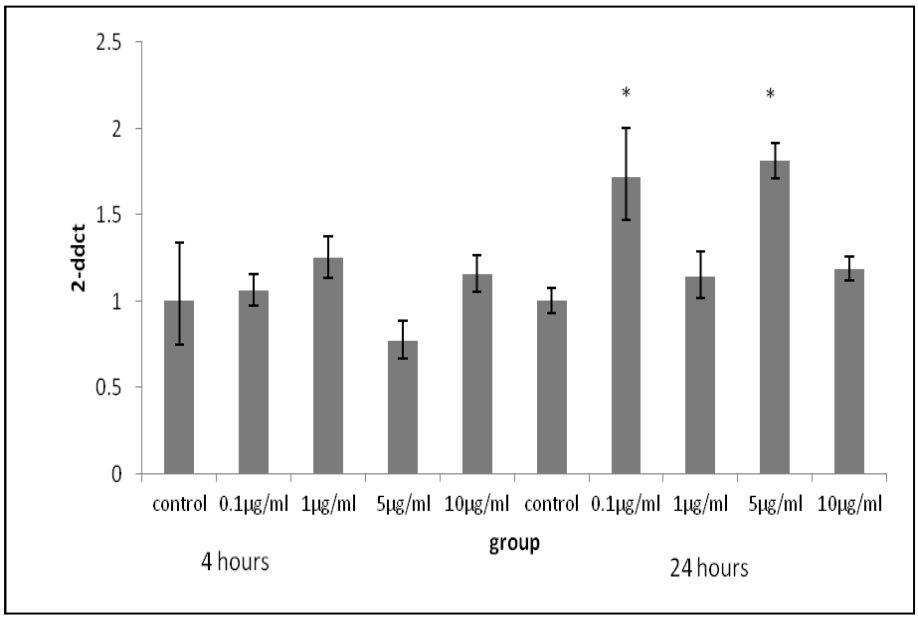


Figure 34: Relative change in adipoR2 gene expression in C2C12 myocytes. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.8 Gene expression of adiponectin and its receptors in other tissues

3.8.1 Liver

In addition to adipose tissue and skeletal muscle, adiponectin receptors are known to be present in other tissues including the liver and spleen. The same experimental technique was subsequently continued investigating liver tissue from the mice injected with LPS. The expression of adiponectin is debated but the current opinion in the literature is that it is not expressed in normal tissue but can be induced in times of disease e.g. hepatic fibrosis models. In this model, there is no change in expression of adiponectin in hepatic tissue subjected to LPS treatment in-vivo.

cDNA from human HUH7 cells, control and treated with LPS 0.1-10 μg/ml was investigated (kindly donated by Professor James Gallagher's group, University of Liverpool). There was no expression of adiponectin mRNA in these cells (Ct values >38 or not obtained). There was also no expression of adiponectin in these cells after treatment with 0.1-10 μg/ml LPS. This is consistent with the current literature. Adiponectin gene expression results must be viewed with caution as the SEMs are very large and the raw data Ct values are high (Figure 35).

AdipoR1 and adipoR2 gene expression, however, are rapidly down-regulated in liver tissue. AdipoR1 expression remains down-regulated at 24 hours but there are no changes at 24 hours in adipoR2 expression (Figure 36).

Figure 35: Adiponectin gene expression in mouse hepatic tissue

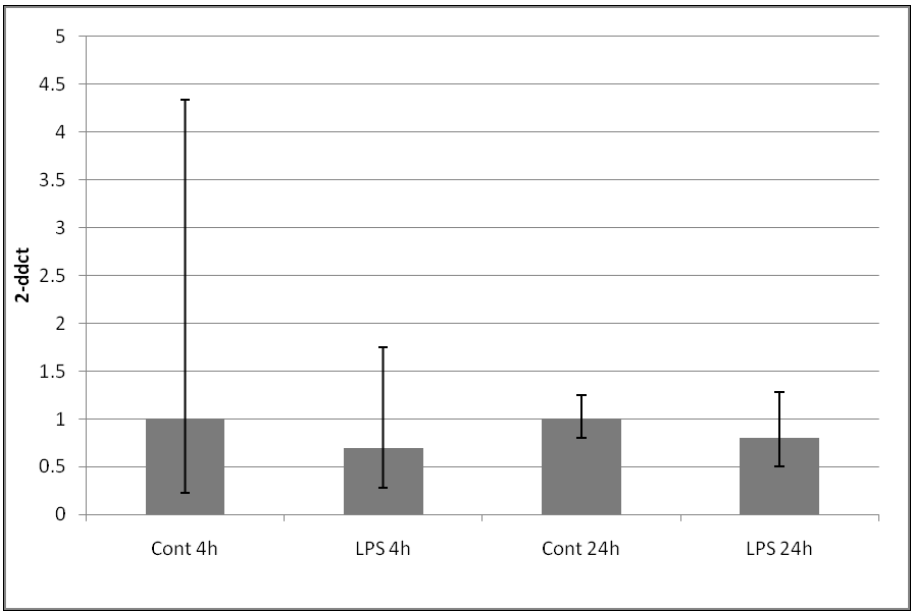
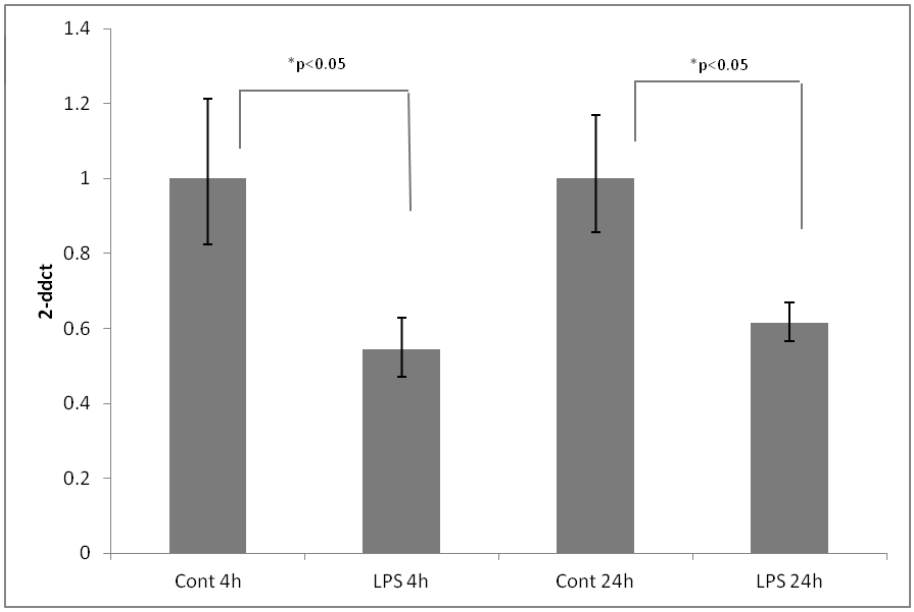


Figure 35: Relative change in adiponectin gene expression in mouse liver tissue depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin (Cont = Control group, LPS = Treatment group, 4 h 4 hours treatment group, 24 h: 24 hours treatment group). 4 hour mice, n=6, 24 hour mice n=11. Samples run in duplicate.

Figure 36: Adiponectin receptor gene expression in mouse hepatic tissue

A: Adiponectin receptor 1 gene expression in liver



B: Adiponectin receptor 2 gene expression in liver

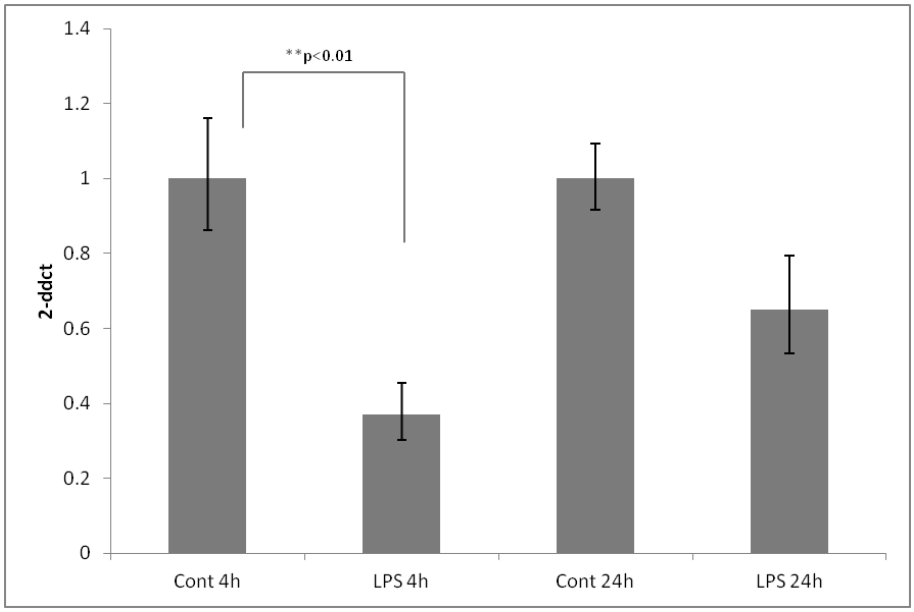


Figure 36: Relative change in adiponectin receptor gene expression in mouse liver depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. A: Liver adipoR1 expression, B: Liver adipoR2 expression. (Cont = Control group, LPS = Treatment group, 4 h: 4 hours treatment group, 24 h: 24 hours treatment group). 4 hour mice, n=6, 24 hour mice n=11. Samples run in duplicate.

3.8.2 Spleen

Adiponectin is a known anti-inflammatory hormone with many roles including reduction in inflammatory cytokines, reduction in neutrophil binding and endothelial dysfunction. The spleen is part of the immune system, making antibodies and removing antibody-coated bacteria and blood cells. The experiments were then continued to include splenic tissue. In the same mouse model, the spleen was investigated with respect to the expression of adiponectin and its receptors. There was a trend towards a down-regulation in adiponectin expression in both the 4 hour (36% of control p=0.2) and 24 hour groups (45% of control p=0.13) (Figure 37). AdipoR1 expression, however, was significantly down-regulated following 24 hours of treatment with LPS. There was no change in the expression of adipoR2 (Figure 38).

Figure 37: Adiponectin gene expression in mouse spleen tissue

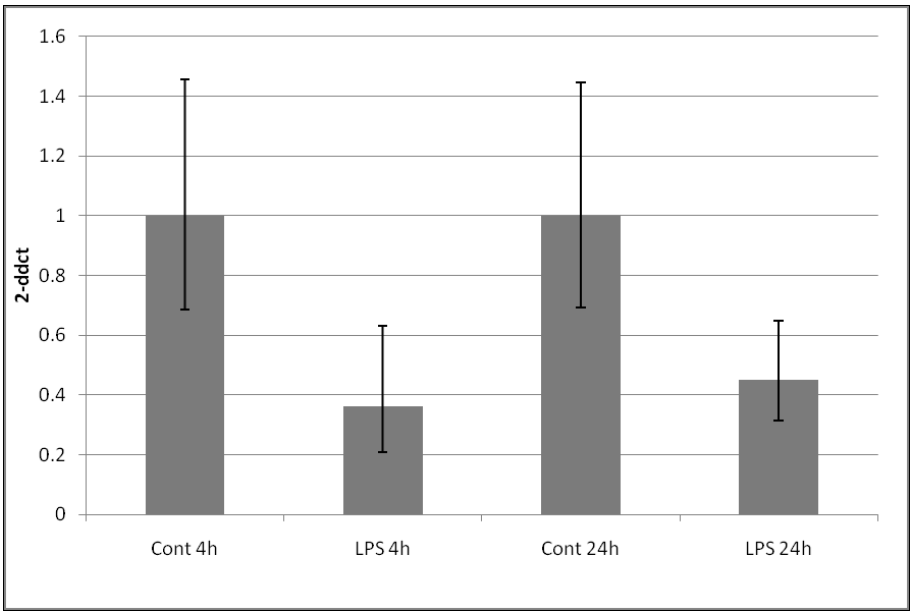
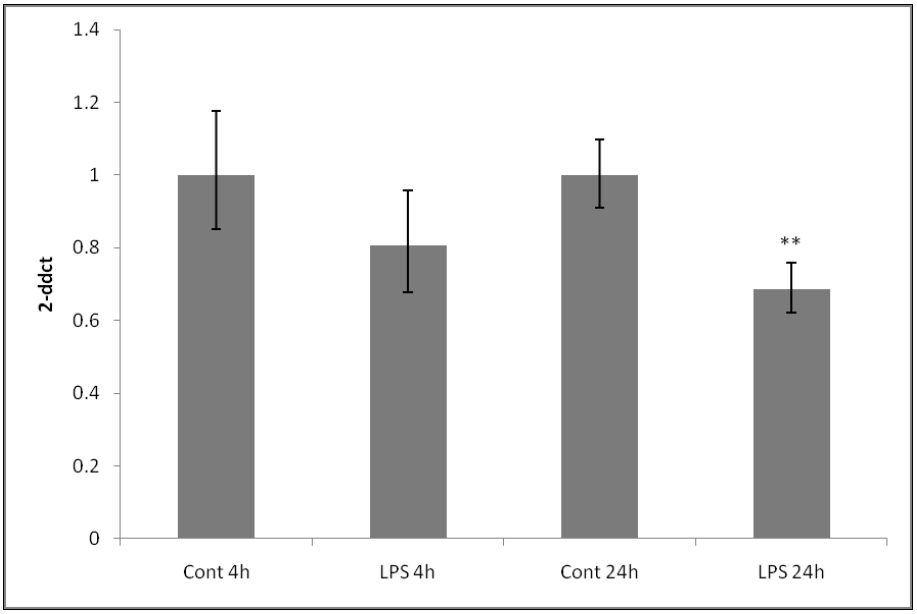


Figure 37: Relative change in adiponectin receptor gene expression in mouse spleen 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. 4 hour mice, n=6, 24 hour mice, n= 14. Samples run in duplicate.

Figure 38: Adiponectin receptor gene expression in mouse spleen tissue

A: Adiponectin receptor 1 expression in spleen



B: Adiponectin receptor 2 expression in spleen

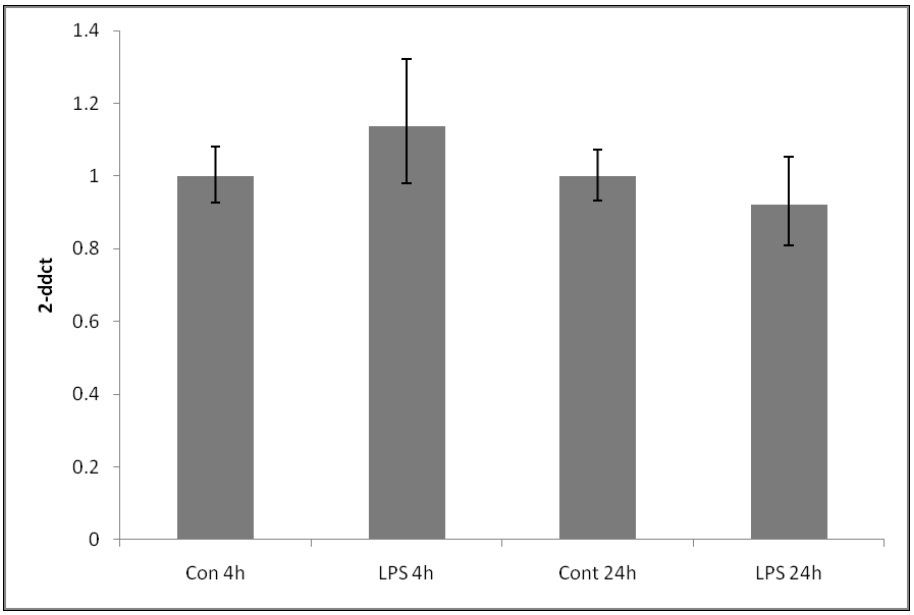


Figure 38: Relative change in adiponectin receptor gene expression in mouse spleen 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. A: Spleen adipoR1 expression, B: Spleen adipoR2 expression. (Cont = Control group, LPS = Treatment group, 4 h: 4 hours treatment group, 24 h: 24 hours treatment group). 4 hour mice, n=6, 24 hour mice, n= 14. Samples run in duplicate.

3.8.3 Small bowel

Small bowel also contains large numbers of immunocytes including lymphocytes but also contains a large number of other different cells including enterocytes, goblet cells, paneth cells and enteroendcrine cells. Interestingly, there were no significant differences in the expression of adiponectin (Figure 39). However, there was a trend towards down-regulation both in adipoR1 expression (58% of control p=0.18) and adipoR2 expression (63% of control p=0.08) in this tissue depot (Figure 40).

Figure 39: Adiponectin gene expression in mouse small bowel tissue

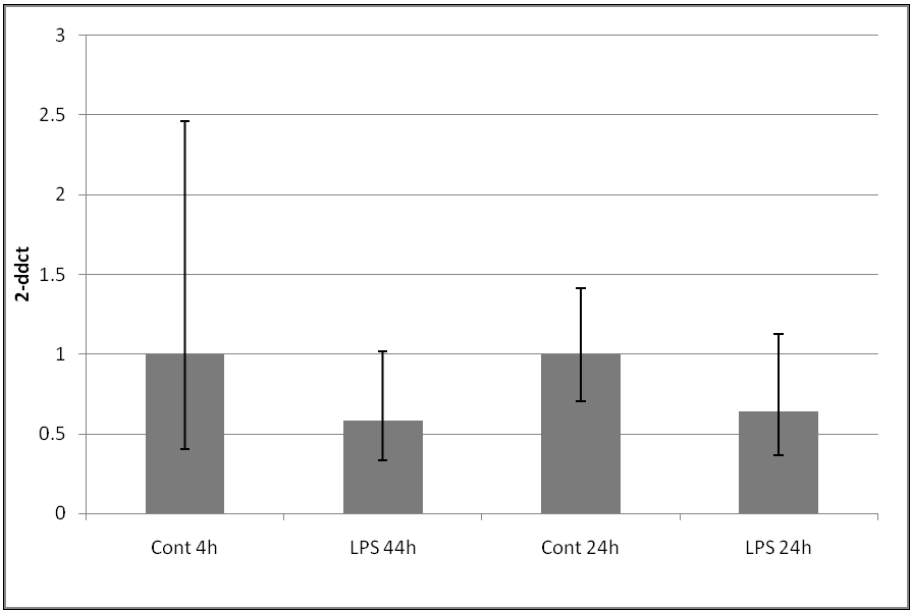
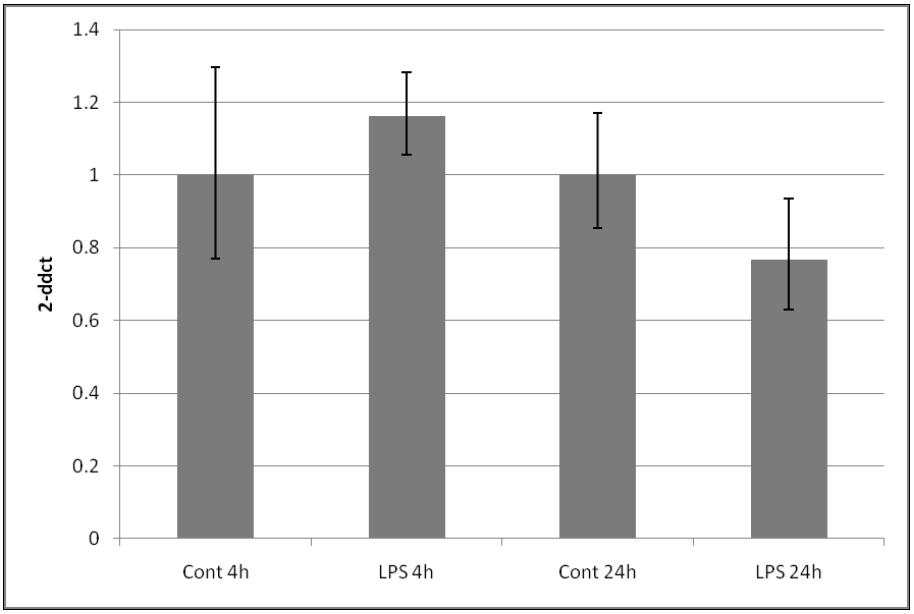


Figure 39: Relative change in adiponectin receptor gene expression in mouse small bowel 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. 4 hour mice, n=6, 24 hour mice, n= 14. Samples run in duplicate.

Figure 40: Adiponectin receptor gene expression in mouse small bowel tissue

A: Adiponectin receptor 1 expression in small bowel



B: Adiponectin receptor 2 expression in small bowel

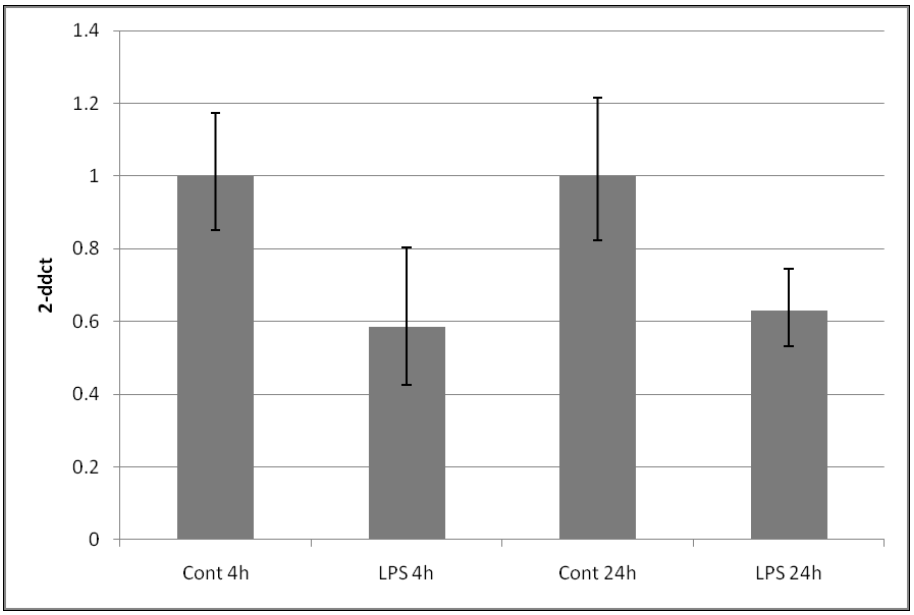


Figure 40: Relative change in adiponectin receptor gene expression in mouse small bowel tissue 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. A: Small Bowel adipoR1 expression, B: Small Bowel adipoR2 expression. (Cont = Control group, LPS = Treatment group, 4 h: 4 hours treatment group, 24 h: 24 hours treatment group). 4 hour mice, n=6, 24 hour mice, n= 14. Samples run in duplicate.

3.9 Protein determination using western blotting

Western blotting was carried out to assess protein levels of adiponectin receptors in the tissue samples. Using a standard laboratory protocol, all blots were incubated overnight at 4°C with a primary antibody and at 1 hour at room temperature with a secondary antibody. Using the standard laboratory protocol (see methods section), phosphate buffered saline with Tween (PBST) with either milk or bovine serum albumin (BSA) as a blocking agent. Following optimisation, BSA was found to be superior. According to the manufacturer's protocol, bands were expected at 43 kDa.

In initial experiments, mouse liver samples were used as they are known to have a high concentration of adiponectin receptors. Initial western blots were unsuccessful as despite adequate membrane blocking, no bands appeared. On subsequent attempts following optimisation (heating and membrane fractionation) and increasing concentrations of 1° (AdipoR1: ADIPOR11-A, AdipoR2: ADIPO21-A, both Alpha Diagnostic, San Antonia, USA) and 2° (Goat anti-rabbit, Serotec, UK) antibodies, no reproducible results were obtained.

Initially, Phosphate Buffered Saline with Tween (PBST) as a wash buffer. Membranes were optimised with different milk and concentrations of Bovine Serum Albumin (BSA) for blocking and dilution. However, bands were not consistent, although membranes appeared well blocked. Altering the blocking solution to BSA further decreased the quality of the blots, therefore milk was used thereafter. Increased concentrations of 1° antibody were used but also had minimal success. Further blots then concentrated on optimisation with differing concentrations of 2° antibody using 1/1000 concentration of 1° antibody and using membrane fractionation. This achieved no real improvement in the blocks.

Interestingly, there were a number of blots with bands at approximately 72 kDa, twice the manufacturer's expected molecular weight, particularly with adiponectin receptor 2. There is no clear explanation for this.

It was then decided that the lack of good results may be due to the quality of the antibody. As it was beyond the scope of this project to raise a non-commercial antibody, it was decided to purchase a second commercially available antibody (AdipoR1: ADIPOR12-A, AdipoR2: ADIPO22-A, both Alpha Diagnostic, San Antonio, USA).

To assess the antibody, a dot blot technique was assessed using the new antibody with its own control peptide and a liver sample. The adipoR1 receptor antibody did not achieve good dots with the control peptide. However, good dots were achieved for all concentrations of sample for both adipoR1 and adipoR2. (Figure 41). The process of western blotting was recommenced using these antibodies at a concentration of 1/200. 1% milk was used for blocking and 0.5% milk for dilution of antibodies.

Figure 41: Dot Blot to assess efficacy of new antibodies

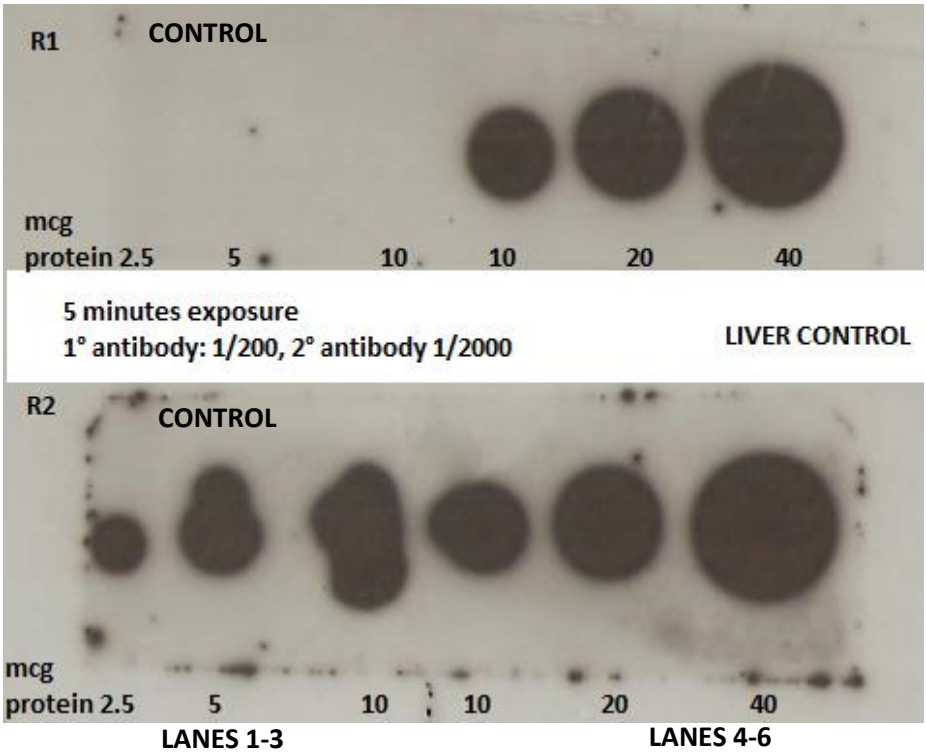


Figure 41: Dot blot using control peptides (lanes 1-3) and the membrane fractionation component of liver tissue from 1 control mouse (lanes 4-6). Concentrations of antibodies were: Primary 1/200, Secondary 1/2000 for both adipoR1 and adipoR2. Increasing concentrations of protein were used in the experiments from 10 to 40 µg. TTBS was used as the wash with milk used for blocking (1%) and dilution solutions (0.5%). Visible dots can be seen at all concentrations for adipoR2 but only at the higher protein concentrations in adipoR1.

The optimisation process then commenced once again using liver and muscle samples (10-15 µg protein). This gave improved blots but with very inconsistent results. There were no bands for adipoR1 or for liver tissue but there were strong bands visible very early for adipoR2 (Figure 42).

Figure 42: Western Blot using Liver and Muscle samples

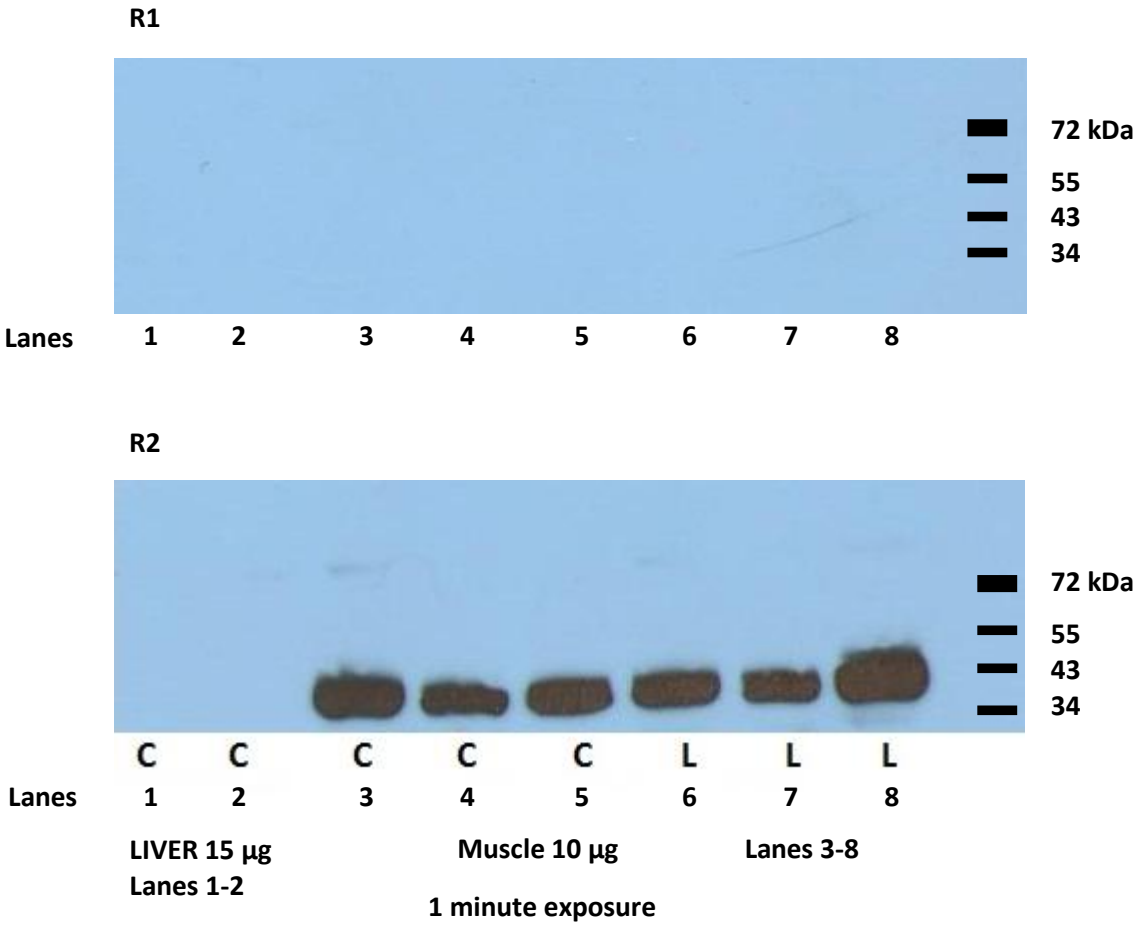


Figure 42: Western blot using whole cell lysate of liver (lanes 1 & 2) and muscle tissue (lanes 3-8) from control and treatment mice (different mice, single experiments). Concentrations of antibodies were: Primary 1/200, Secondary 1/2000 for both adipoR1 and adipoR2. Visible bands can be seen at 43 kDa for adipoR2 protein in the muscle tissue. There are no bands for adipoR1 or for liver tissue (either receptor). TTBS was used as the wash with milk used for blocking (1%) and dilution solutions (0.5%). (C: Control mice, L LPS treated mice). Good bands for muscle R2 were also seen at 5, 20 and 40 minute exposure.

Following more success of the western blot using muscle tissue, this experiment was repeated but did not yield a similar success with difficulties in blocking the membranes and poor end results (Figure 43).

Figure 43: Western Blotting of muscle samples (treatment and control)

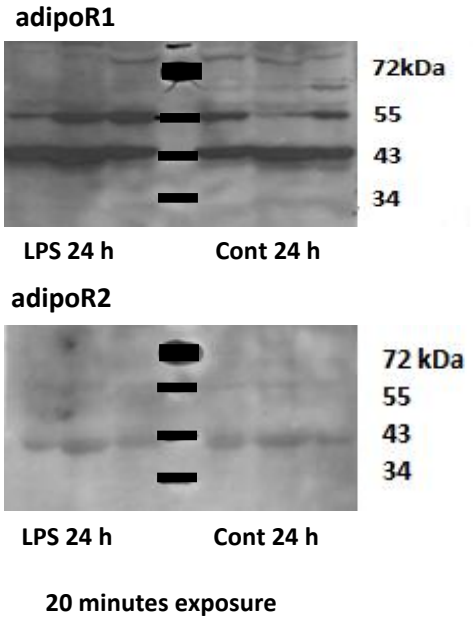


Figure 43: Western blot using 15 µg protein (Whole cell lysate) from muscle (control and treatment 24 hours) samples were used with 1% milk for blocking and 0.5% milk for dilution. Antibody concentrations were : 1°: 1/200, 2°: 1/2000. Bands for adipoR1 visible after 1 minute but at multiple molecular weights including 43 kDa, 55 and 72 kDa. Bands for R2 visible were fainter but the blot was cleaner and visible after 5 minutes. The 20 minute exposure picture demonstrated the clearest bands

Western blotting for adiponectin receptors has therefore been of very limited success, despite numerous optimisation experiments and new antibodies. From the outset, a very high concentration of antibody was required, thus providing evidence of poor antibody efficacy. The commercial antibody options were limited and the generation of new antibodies was beyond the scope of this project. When the western blot was successful, the results were inconsistent and repeat experiments with the same concentrations did not yield the same results. There were a number of membranes that identified bands at approximately 72 kDa in hepatic samples, approximately twice the size of the quoted molecular weight of the receptors. The reason for this is unclear but one postulated theory is that this may have indicated a dimerised receptor. A summary of all successful blots undertaken can be seen in Appendix 2.

3.10 Discussion:

Adiponectin expression in adipose tissue following lipopolysaccharide treatment

Adiponectin is an adipokine which has been extensively studied since its discovery in 1995. Four groups independently identified adiponectin using different techniques including identification of mRNAs induced during adipocyte differentiation and identification of the gene by its ability to bind gelatine, thus naming it gelatine binding protein of 28 kDa or GBP28[67-70] . Using northern blotting several authors demonstrated that it was restricted to adipose tissue in various mouse, rat and human tissues[67-69] . Adiponectin acts via two receptors, identified in 2003 by Yamauchi et al.[123]. Adiponectin receptors have been identified on many tissues with adipoR1 having a higher expression level in skeletal muscle whereas adipoR2 being predominant in hepatic tissue[123]. Adiponectin has been shown to be an anti-inflammatory, anti-atherogenic insulin sensitizer, promoting glucose utilisation and fatty acid oxidation[28 30-36]. This series of experiments investigated the role of adiponectin and its receptors in-vivo and in-vitro models of endotoxaemia. Adiponectin is well known to be down-regulated in chronic inflammatory processes such as obesity, type II DM and cardiovascular disease[30 36 79 198 199]. Our laboratory has previously demonstrated that in a murine model of acute endotoxaemia, WAT adiponectin gene and protein expression decreased 24 hours following ip LPS injection[12]. This series of experiments confirmed this finding at the mRNA level (Figure 13).

Adiponectin receptor mRNA was also confirmed to be present in all tissues and cell types examined by qPCR which is consistent with the current literature[123 124 126 127]. Splenic adiponectin receptors have been identified in many species including the mouse[123] and pig (adipoR2 only)[200] . Changes in adiponectin and its receptor expression following LPS injection varied with tissue type are summarised in the following table:

Table 28: Summary of tissue adiponectin and receptor gene expression results

Gene expressed and time	adipoR1 4 h	adipoR1 24 h	adipoR2 4 h	adipoR2 24 h	Adiponectin 4 h	Adiponectin 24 h
Liver	↓1.5	↓1.4*	↓2.7*	↓1.5	↑0.54	↓1.93
Skeletal Muscle	↓9.8*	↓1.9*	↓6.2	↓2.2*	↓6.94*	↓30*
EF	↓ 1.5	=	↓1.8	↓1.2	=	↓3.66*
PRF	↓1.6*	↓1.2	↓4.3*	↓1.5	↓1.96	↓2.9*
SCF	↓1.2	↓1.2	↓2.9*	=	↓1.31	↓4.27*
Small bowel	=	↓1.3	↓1.7	↓1.5	↓1.71	↓1.56
Spleen	↓1.2	↓1.5*	=	=	↓2.77	↓2.12

Table 28: fold changes of adiponectin and adiponectin receptor gene expression only with direction of change. Mouse tissues treated with 25 mg/kg LPS (* denotes statistical significance ($p<0.05$))

Briefly, there was a significant down-regulation in adiponectin gene expression in the three adipose tissue depots and skeletal muscle (Figures 13 & 27). This was accompanied by a rapid down-regulation (at 4 hours) in adiponectin receptor gene expression which was observed in several tissues: muscle, liver and PRF showed down-regulation of adipoR1 and liver, muscle, PRF and SCF, down-regulation of adipoR2. These changes were accompanied by a sustained decrease (at 24 hours) in liver and skeletal muscle adipoR1 expression and muscle adipoR2 only (Figures 14, 28 & 36). These interesting findings will now be discussed further.

3.10.1 Adipose tissue: Adiponectin and receptor expression

Three depots of WAT were investigated in the mouse model, subcutaneous (SCF), epididymal (EF) and peri-renal fat (PRF). The changes in receptor expression were different in each depot. The greatest changes were observed in PRF with rapid down-regulation of gene expression of both receptors. In SCF, only adipoR2 was down-regulated and EF showed no response. Again the changes seen were only at four hours and gene expression had returned to values close to the control level by 24 hours (Figure 14). Interestingly, adiponectin expression was reduced in all three depots, but this was at the later time point of 24 hours (Figure 13). These depot-dependent changes in receptor expression may reflect a difference in the type of adipose tissue.

The murine adipose organ consists of subcutaneous (anterior and posterior) and visceral (ommental, mesenteric, peri-renal, retroperitoneal, parametrial, periovaric, epididymal and perivesical) depots[201]. In humans, the adipose organ is divided into visceral and subcutaneous adipose tissue. The subcutaneous WAT is a continuous layer under and in continuity with the dermal WAT. Visceral WAT is located in numerous places around organs within the thoracic and abdominal cavities (e.g. omental, mesenteric, peri-renal, epididymal). It accounts for approximately 9-18% of body weight in males and 14-28% in females[202]. This percentage can be increased up to 22 and 32% in obese male and female subjects respectively[202]. Adipocytes structure appears to be similar in the two species but adipocyte size can be up to 30-40% bigger in humans[201].

Studies in animals and humans have shown that increased visceral rather than total adipose tissue plays a greater role in the development of the type II DM and the metabolic syndrome[203]. Interestingly, Nanniperi et al. demonstrated a higher concentration of adiponectin and both receptors in human sub-cutaneous WAT compared to visceral WAT[130]. This suggests that a higher concentration of adiponectin and its receptors in visceral fat per se may not be the most important factor in its role in the metabolic syndrome. The greatest effect of LPS on receptor expression observed in these results was observed in PRF, a visceral depot. This may imply that visceral adipose tissue depot has a greater role in the development of infection-induced metabolic changes, in particular insulin resistance. However, the role of visceral adipose tissue in sepsis has not been extensively studied.

Our results show similar changes to those observed in many mouse models of obesity where the expression of WAT adiponectin and its receptors is significantly reduced[68 79 126]. Plasma and adipose tissue adiponectin are also down-regulated in obese humans[68 93] who display a 60% reduction in adipoR1 gene expression in SCF and ommental fat compared to those with a normal BMI[131]. In agreement with the results presented this thesis, expression differences between different fat

depots have already been observed. Nannipieri et al. demonstrated a down-regulation of adipoR1 and adiponectin only in visceral WAT whereas adiponectin and both receptors were down-regulated in SCF[130]. Therefore, as in obesity, it can be hypothesised that different WAT depots may play different roles in the pathogenesis of hyperglycaemia of sepsis.

A further hypothesis is that the depot differences observed may reflect changes in blood supply to different organs as is frequently observed in sepsis. The peripheral circulation becomes profoundly vasodilated as a result of a multifactorial process involving molecules such as cytokines, endotoxin and nitric oxide[204]. The vasodilation is not uniform accross all tissue beds. Vasodilation can occur in capacitance vessels leading to blood pooling and relative hypovolaemia. Also, local vasodilation can cause shunting of blood past capillary exchange beds causing focal hypoperfusion despite a normal cardiac output[205]. The resulting hypotension leads to organ malperfusion, often of non-essential organs such as the skin and splanchnic areas. This can disturb of the delicate balance between oxygen delivery and consumption, causing hypoxia and the resulting anaerobic cellular metabolism, which, if not corrected quickly, proceeds to organ failure[205]. This may contribute to alterations of adiponectin and its receptor expression as central blood supply to visceral fat depots, may be conserved more than to peripheral tissues. Perfusion to extra-peritoneal tissues, such as EF and SCF depots, therefore, may become compromised as the circulation is centralised to improve essential organ (brain and heart) perfusion. Therefore an improved blood supply leading to enhanced delivery of inflammatory cytokines may be a reason that changes in visceral fat depots are greater than the peripheral tissues.

In contrast to the differing responses in receptor regulation in the various fat depots, adiponectin expression was comparable in all types of WAT (Figure 13). There were significant reductions in all depots after 24 hours but not in the acute phase. The reasons for this are unclear but it is possible that adiponectin is regulated differently to its receptors. So far, there has been no previous

examination of tissue receptor gene expression following LPS stimulation. It would be anticipated that a reduction in ligand expression should be countered by an up-regulation in receptor expression. However, the down-regulation of adiponectin in observed in this thesis appear to parallel the changes in adiponectin receptors with a time-lag. This indicates an *in-vivo* down-regulation of the entire adiponectin system rather than just individual components.

3.10.2 Adipocytes – adiponectin

LPS treatment in-vitro allows the investigator to appreciate whether the changes observed are a phenomenon of LPS per se or whether they are secondary to the inflammatory response in-vivo. The next set of experiments investigated isolated 3T3-L1 adipocytes. The 3T3-L1 mouse fibroblastic cell line rapidly differentiates to an adipocyte phenotype when treated with dexamethasone, 3-Isobutyl-1-methylxanthine (IBMX) and insulin[206] and has been extensively used for the investigation of adipokine expression in different experimental models. We used LPS (E-Coli O 111:B4, Sigma-Aldrich) in varying concentrations to assess the change in expression of the components of the adiponectin system with time (24 hours).

Changes observed following LPS and cytokine treatment of 3T3-L1 adipocytes are summarised in the following table:

Table 29: Summary of 3T3-L1 adipocyte gene expression results

Gene expressed and time	adipoR1 4 h	adipoR1 24 h	adipoR2 4 h	adipoR2 24 h	adiponectin 4 h	adiponectin 24 h	IL-6 4 h	IL-6 24 h
LPS								
0.1 µg/ml	↓1.53*	=	↓1.38	=	↑1.4	↓1.5	↑25.42	↑2.66
1 µg/ml	=	=	↓2.63	=	↓2.46	↓1.69	↑34.29	↑14.62
5 µg/ml	↓1.19	=	↓1.67	↑1.19	↓4.54	↓1.16	↑20.60	↑10.76
10 µg/ml	↓1.14	=	↓3.9	=	↓6.75*	↓1.78	↑22.85	↑6.61
Cytokines								
TNF-α 10 ng/ml	↓1.35	↓1.15	=	↓1.78	↑3.29	↓1.68	↑3.85	↑8.29
IL-6 10 ng/ml	↓2.59*	↓1.99*	↓1.82	↑1.12	=	=	↓2.27	=

Table 29: Fold changes of IL-6, adiponectin and adiponectin receptor gene expression with direction of change. 3T3-L1 cells treated with various concentrations of LPS and inflammatory cytokines. (* denotes statistical significance ($p<0.05$))

As frequently done for cell line experiments, a reference gene was used as a marker of successful experimental conditions. IL-6 is well known to be secreted from adipocytes and myocytes[12 13] and adipose tissue IL-6 may account for up to 15-35% of circulating levels[207 208]. LPS stimulation produced a 25-fold up-regulation in IL-6 gene expression (Figure 16), consistent with previous experiments[12].

IL-6 expression in adipocytes also demonstrates that these cells respond to LPS by increasing expression of inflammatory markers. This further confirms adipocytes as a producer of IL-6 and a contributor to the inflammatory process.

Adiponectin expression in isolated 3T3-L1 adipocytes showed a clear reduction after LPS treatment (Tables 14 & 15, Figures 17 & 18). There is a greater reduction in expression with increasing doses of LPS with 10 μg/ml LPS being statistically significant. Again, there were no changes at 24 hours. This is a much more rapid time frame than the down-regulation observed in the tissue depots. Hence, the direct LPS effect on 3T3-L1 adipocytes in culture may be quicker than the one seen in a whole animal model, in which absorption and circulation of LPS is required. However, these experiments clearly show that there is an effect of LPS per se.

3.10.3 Adipocytes – Adiponectin receptors

In 3T3-L1 adipocytes treated with LPS, there was a concentration effect resulting in a difference in expression of the two receptors. Treatment with 0.1 μg/ml LPS reduced the expression of adipoR1 at four hours with no corresponding change by 24 hours (Table 16 & Figure 19). At higher doses of LPS, there was no change in adipoR1 gene expression (Table 18 & Figure 21). AdipoR2 expression, however, was unchanged following treatment with 0.1 μg/ml LPS (Table 17 & Figure 20). At higher doses of LPS (1 μg/ml and 10 μg/ml) there were significant reductions in adipoR2 gene expression. Again, no changes were observed at 24 hours (Table 19 & Figure 22).

This implies that a similar phenomenon occurs in isolated adipocytes as in whole visceral tissue depots. However, it may be that the two receptor subtypes respond to differing concentrations of LPS with adipoR1 responding to much lower concentrations than adipoR2. It is difficult to compare doses of LPS in-vivo and in-vitro because the systemic component of the response to LPS may potentiate its effects in-vivo.

The differences observed in receptor subtype regulation in this study may reflect the emerging idea that the two receptors may have different modes of action and/or regulation[14 84 209] . With regard to glucose metabolism, adipoR1 deficient mice have been shown to have impaired glucose tolerance, insulin resistance and increased endogenous production of glucose[84]. AdipoR2 KO mice, however, are lean, resistant to diet induced obesity, weight gain and hepatic steatosis, and display reduced plasma cholesterol and fasting insulin levels. Their glucose tolerance may be impaired as demonstrated by increased plasma insulin concentrations[14 84].

Yamauchi et al. demonstrated that db/db mice (leptin receptor deficient, prone to type II DM, hypertension and obesity (Table 3)) have reduced expression of both adipoR1 and adipoR2. Over-expression of both receptors in this mouse strain improved the handling of an oral glucose load however, each receptor behaved differently: Restoration of adipoR1 levels significantly reduced endogenous glucose production and improved insulin resistance. Over-expression of adipoR2 had little effect, although, both experimental groups displayed improved insulin sensitivity[84]. A lack of adipoR2 binding leading to an increase in adipoR1 signalling and improved insulin sensitivity may explain the resistance of these animals to diet induced obesity[14]. Therefore, adipoR1 and adipoR2 may not only differ in structure and tissue distribution[123] but also in their signalling pathway and ultimate actions. It is entirely plausible that the response to LPS is different for each receptor within various tissues.

3.10.4 Response to cytokine treatment in 3T3-L1 adipocytes

Cell culture experiments are an appropriate model to assess the cellular response to pro-inflammatory cytokines. Hence, effect of inflammatory cytokines IL-6 and TNF-α on adiponectin receptor gene expression in 3T3-L1 adipocytes was investigated.

IL-6

IL-6 treatment of 3T3-L1 adipocytes resulted in down-regulation of IL-6 whereas TNF-α treatment up-regulated IL-6 gene expression (Table 20 & Figure 23). Treatment with IL-6 (10 ng/ml) also resulted in a 2-fold reduction in adipoR1, both at 4 and 24 hours (Table 22 & Figure 25). This indicates that IL-6 may contribute to the in-vivo response initiated by LPS. There was no effect on adiponectin or adipoR2 gene expression following IL-6 treatment (Tables 21 & 23, Figures 24 & 26). A handful of other studies have investigated the role of IL-6 in mouse and human adipocytes. Fasshauer et al. investigated 3T3-L1 cells extensively with regard to adiponectin and its receptors[112] [129] [210]. In contrast to these results demonstrating no change in adiponectin expression, they found that IL-6 treatment for 16 hours (30 ng/ml) reduced adiponectin expression and secretion by 75% and 25-45% respectively. This was a fully reversible effect following removal of IL-6 for 24 hours. This does not represent a dosing phenomenon as they also demonstrated reduced expression at lower doses of IL-6. Interestingly, the same group found no changes in adiponectin receptor expression.

TNF-α

TNF-α treatment resulted in a significant increase in IL-6 gene expression supporting the concept of adipose tissue contributing to the overall inflammatory process. TNF-α had no effects on adiponectin receptor gene expression in 3T3-L1 cells in this series of experiments (Tables 22 & 23, Figures 25 & 26). This is in agreement with a previous study[129]. In this series, adiponectin gene expression, however, increased initially (3.2-fold at 4 hours p=0.05) supporting previous studies in human

adipocytes[211]. Following the initial rise, adiponectin gene expression then showed a downward trend at 24 hours (60% of control p=0.08) (Table 21 & Figure 24).

This could be secondary to the following hypotheses:

1. The initial rise could represent an initial counter-regulatory anti-inflammatory response to TNF-α treatment. The cells were directly treated with TNF-α. Therefore, the time required for TNF-α to be expressed after LPS treatment in-vivo is not required.

2. Alternatively, this may reflect a delayed response as the experiments demonstrate an up-regulation of IL-6. The IL-6 surge may cause a further down-regulation in adiponectin receptors but a longer observation period would have been required to investigate this hypothesis.

Following LPS and cytokine treatment, many depots of adipose tissue demonstrate a down-regulation of adiponectin and its receptors over varying time periods. This supports the concept that adipose tissue, once inflamed, is an endocrine organ, contributing to insulin resistance. Most importantly, the inflammatory response of WAT may not be confined to chronic disease but may also occur in acute immune activation such as sepsis.

Despite a clear decrease in adiponectin receptor expression, the effect of LPS on adiponectin protein levels remains unclear. Despite experiment optimisation of experimental technique, the protein levels could not be effectively determined by western blotting. Therefore, it was not possible to determine the changes in protein translation. Interestingly, several papers have described immunoblotting for adiponectin receptors, although pictures of western blot bands in the literature are of limited quality. Although both receptor sub-types have been demonstrated at the protein level in tissues other than fat such as placenta[212 213], prostate cancer cells[214] and skeletal muscle[85], only a few studies have identified the adiponectin receptor protein in fat tissue.

3.10.5 Skeletal muscle

Adiponectin has long been thought of as an adipose specific molecule, being secreted only from adipose tissue depots, particularly WAT. Evidence is now accumulating that adiponectin may actually be produced in other tissues including skeletal muscle, cardiac muscle and placental tissues[190-195 212 213]. Skeletal muscle metabolism becomes very disordered in sepsis therefore the next line of investigation examined mouse skeletal muscle tissue and cell lines with respect to components of the adiponectin system.

C2C12 myocytes are differentiated from a myoblast cell line originally obtained through serial passage of myoblasts cultured from the thigh muscle of C3H mice after crush injury[215]. These were used for all experiments. The present study confirmed the presence of adiponectin in depots of whole skeletal muscle and ruled out contamination from blood or peri-muscular fat by identifying adiponectin mRNA in isolated C2C12 myocytes (Figure 30). However, there was a clear concentration effect with more pronounced band intensity with higher concentrations of cDNA (0.5-4 μg) in the standard PCR. This suggests a reduced expression in muscle compared to EF tissue which served as a positive control. For confirmation, the PCR product was sequenced and a 100% match for adiponectin could be demonstrated.

The results for myocyte and skeletal muscle adiponectin are summarised in the following table.

Table 30: Summary of C2C12 myocyte and skeletal muscle adiponectin and receptor gene expression results

Cells: C2C12	adipoR1 4 h	adipoR1 24 h	adipoR2 4 h	adipoR2 24 h	Adiponectin 4 h	Adiponectin 24 h	IL-6 4 h	IL-6 24 h
LPS								
0.1 µg/ml	=	↑1.15	=	↑1.71	↓1.96*	↓1.41	↑849*	↑1.65*
1 µg/ml	=	↑1.34	↑1.25	↑1.15	↓1.29	↑1.19	↑55*	↑3.71*
5 µg/ml	↓1.53*	↑1.19	↓1.31	↑1.81	↓2.94*	=	↑953*	↑7.07*
10 µg/ml	↓1.26	↓1.14	↑1.15	↑1.18	↓2.17*	=	↑486*	↑5.29*
Sk muscle tissue (25 mg/kg)	↓9.8*	↓1.9*	↓6.2	↓2.2*	↓6.94*	↓30*		

Table 30: Fold changes of Il-6, adiponectin and adiponectin receptor gene expression with direction of change. 3T3-L1 cells treated with various concentrations of LPS and inflammatory cytokines. adipoR1: adiponectin receptor 1, adipoR2: adiponectin receptor 2, Sk Muscle: skeletal muscle. (* denotes statistical significance ($p<0.05$)).

In tissue depots, there was a clear sustained down-regulation of both adiponectin and both receptors following LPS treatment (Figures 27 & 28). These were sustained for both time periods, indicating a rapid and prolonged effect on the skeletal muscle, which was not observed in adipose tissue. The reduction in adipoR2 after four hours failed to reach statistical significance due to the large SEM, which most likely represents a type two statistical error due to small sample size. This was greater in magnitude and more rapid than changes observed in adipose tissue.

For the cell line experiments, experimental conditions were again confirmed using IL-6 gene expression where LPS treatment resulted in approximately 1000-fold increase in gene expression (Table 24, Figure 31). IL-6 is known to be expressed from myocytes following LPS treatment[13 216 217]

Adiponectin expression following LPS administration in C2C12 myocytes was significantly down-regulated at all concentrations with the exception of 1 µg/ml LPS (Table 25 & Figure 32). This occurred after four hours of treatment but was not replicated in the cells treated for 24 hours.

LPS treatment had minimal effects on adiponectin receptor expression in isolated murine myocytes. There was a small reduction in expression of adipoR1 mRNA (5 μg/ml) after 4 hours and similar fold increase in adipoR2 by 24 hours but no other changes (Tables 26 & 27, Figures 33 & 34). This, therefore, does not mirror the tissue results. This is also different to the effect of LPS observed in the 3T3-L1 adipocytes, where there were minimal changes in adipoR1 but a dose dependent reduction of adipoR2 (Figures 21 & 22). However, similar to the adipocyte experiments, there is a change in time frame compared to the tissue results which may be a reflection of the in-vitro/in-vivo differences and the time taken to absorb LPS systemically in-vivo.

Our results demonstrate a clear down-regulation of the adiponectin system in-vivo in skeletal muscle in systemic endotoxaemia. This change is quicker than similar changes observed in the WAT depots, and down-regulation is potentiated with a longer period of sepsis. The changes are not so marked in the cell line experiments. It could be expected that a reduction in the ligand would result in an increase in the number of receptors. This is clearly not the case in the results presented in this thesis which demonstrate down-regulation of the adiponectin system. It could be postulated that the ongoing inflammatory stimulus may override the normal homeostatic function of adipocytes, causing further dysregulation. In this and previous series of experiments[12], we have demonstrated an increase in IL-6 expression following LPS administration both in-vivo and in-vitro. This shows that there is an ongoing release of cytokines in the adipose tissue and other organs which may potentiate the inflammatory response.

In skeletal muscle as systemic inflammation has been shown to produce a local release of pro-inflammatory cytokines (IL-6, IL-8, and IL-18)[13 216] which may account for the continuing response in whole skeletal muscle. The down-regulation of the adiponectin system by pro-inflammatory mediators (IL-6, TNF-α) may represent a further aspect of the inflammatory dysregulation.

Skeletal muscle is an insulin sensitive organ which undergoes significant changes during sepsis[218]. Therefore, it is tempting to speculate that adiponectin receptor down-regulation significantly contributes to sepsis-induced insulin resistance in these animals. In order to investigate the role of adiponectin in sepsis-induced insulin resistance, measures of insulin resistance including euglycaemic clamp studies, would be required. Alternatively, septic adiponectin KO mice could be utilised and insulin sensitivity measured in a similar fashion.

Several authors have demonstrated the presence of adiponectin in skeletal muscle[192 194 195] with levels in the gastrocnemius muscle being approximately equivalent to its expression in WAT in mice on a normal diet[195]. The presence of all mono and multimeric forms was confirmed by western blotting[192] but the location of the adiponectin within the muscle cell is not clearly defined. Using immunohistochemical staining, adiponectin has been located in whole muscle samples within the vascular endothelium[194 195], in cellular structures including white adipocytes surrounding the muscle cells[192 195] and in the sarcolemma of skeletal muscle fibres[194]. Most authors currently are of the opinion that adiponectin is not expressed from the myocyte (cytoplasm or nucleus) itself. However, Krause et al. have confirmed the presence of adiponectin in L6 myotubes[192 194 195].

The role of adiponectin in skeletal muscle has been investigated very little. However, one study did show that both long and short term high fat diets resulted in a significantly reduced adiponectin concentration in both WAT and skeletal muscle in rats[195]. There are few reports of adiponectin in septic skeletal muscle, but one study investigated the levels of adiponectin in both whole mouse muscle and in isolated myocytes grown in culture[219]. They found that the intra-peritoneal injection of 25 µg LPS/animal produced an *increase* in adiponectin gene expression (10-fold) and protein level (70%) from skeletal muscle and cells at 24 hours post injection.

The same study assessed the effects of inflammatory cytokines in cultured murine and human myotubes[219]. They used combinations of various cytokines to mimic LPS effects on adiponectin expression and found that only the combination of IFN-γ and TNF-α increased adiponectin expression by over 20-fold. In contrast, none of these cytokines alone had any effect, indicating a synergistic effect of IFN-γ and TNF-α [219 220] . They did not perform the same cell line experiments with LPS.

This is in contrast to the results presented in this thesis, which showed a significant and sustained reduction in tissue adiponectin gene expression after LPS stimulation in-vivo. Two key differences in the experiments may explain these opposing results: Delaigle and colleagues used 25 µg LPS per animal which is approximately 1/25 of the dose used in the experiments I undertook, therefore this may result in a milder form of the disease[219]. Human volunteer studies investigating milder spectrums of endotoxaemia have demonstrated no change in the components of the adiponectin system[115 116].

In addition, Delaigle et al. used murine tibialis anterior muscle and primary culture of human quadriceps muscle. These muscle types both contain predominantly fast twitch (type II) fibres[221 222] whereas in my experiments soleus muscle was used, which contains predominantly slow twitch (type I) fibres. This may be important as adiponectin expression from skeletal muscle has been shown to be associated with type IIA and IID fast twitch oxidative muscle fibre types. Slow (type I) and fast glycolytic muscle fibres (IIB) showed only a low level of adiponectin expression[192]. The fast oxidative fibre type also showed an increase in intramyocellular lipid (IMCL) concentration. Their experiments of adiponectin KO mice demonstrated an increase in IMCL and an increase in type IIB fibre size which is consistent with muscle biopsies from obese humans[223]. It is unexpected that adiponectin expression increases in inflammation particularly since a down-regulation in low-grade chronic inflammatory states is observed. Type II DM, obesity and the metabolic syndrome are all

associated with reductions in adiponectin gene expression in WAT and plasma adiponectin protein levels. Adiponectin expression is increased after caloric restriction or weight loss. Unfortunately, this study did not comment on the feeding situation of their animals or anorexia following LPS. Therefore, weight loss cannot be ruled out as a cause[219].

3.10.6 Liver

Following the identification of adiponectin mRNA in skeletal muscle, hepatic tissue was investigated. Mouse liver depots demonstrated a positive finding for adiponectin mRNA but with little change in either of the treatment groups (Figure 35). However, the majority of the Ct values for liver adiponectin were over 30 cycles and frequently over 35. This implies that adiponectin may not be expressed and if expressed, at very low concentrations only. Furthermore, the standard error of the mean from the hepatic tissue was very high thus producing inconsistent results. Contamination from peri-hepatic fat tissue or blood could also not be ruled out as liver is a very vascular tissue.

We then investigated a hepatocyte cell line (cDNA kindly donated by Professor James Gallagher's group). There was no expression of adiponectin mRNA from these cells implying that the positive finding in the mouse liver tissue may reflect contamination from leucocytes or immunocytes within the liver and does not stem from hepatocytes per se. This would be consistent with the current literature as there are no reports of human studies of normal livers/hepatocytes expressing adiponectin mRNA[224]. Neumeier et al. demonstrated the presence of liver adiponectin in steatic rodent livers, but not in human liver samples, primary human hepatocytes or rat liver. This also suggests either contamination from fat cells or from the circulating blood cells[225]. Kaser et al. demonstrated liver adiponectin protein expression was mainly stemmed from endothelial cells of portal vessels and liver sinusoids and not from hepatocytes. This was confirmed by incubating primary human hepatocytes with adiponectin which resulted in a dose dependent increase in adiponectin protein culture medium. There are also some reports of hepatic mRNA expression following carbon tetrachloride liver injury an experimental model of hepatic fibrosis, in animal and

cell studies[226 227]. Therefore, it appears that hepatic expression of adiponectin mRNA is not present in the normal liver but may be induced following liver injury.

Adiponectin receptor expression has been clearly demonstrated in hepatocytes with hepatic adipoR2 expression being the highest in the body[123]. Expression levels in the liver follow the pattern of plasma and adipose tissue receptors in that expression is down-regulated in mouse models of obesity and diabetes[99 106]. Interestingly, a high fat diet in lean rats has been shown to down-regulate hepatic adipoR2 expression only[228]. The results presented in this thesis demonstrate a down-regulation of both receptors following an LPS challenge in mice after 4 hours (Figure 36). Down-regulation of adipoR1 is sustained, whereas the changes in adipoR2 are not significant after 24 hours.

Liver and muscle appear to have the most widespread change in receptor expression with changes at both time points and in both receptors. Both organs are known to be highly involved in glucose homeostasis and represent insulin responsive tissues[229]. One of the mechanisms of action of adiponectin is to improve insulin sensitivity, partly by reducing tissue stores of fatty acids in insulin sensitive tissues, such as adipose tissue and skeletal muscle, and to reduce hepatic gluconeogenesis. Thus a down-regulation of adiponectin receptors in skeletal muscle and liver may oppose this in sepsis and endotoxaemia may contribute to the observed insulin resistance. This also may imply a regulation through insulin signalling or hyperinsulinaemia which has been demonstrated in 3T3-L1 adipocytes where insulin in concentrations as low as 10 nmol/L can down-regulate adiponectin gene expression[112]. To the best of my knowledge, insulin-dependent down-regulation has not been demonstrated for adiponectin receptors.

To date, adiponectin receptors in skeletal muscle have only been investigated in the context of chronic pathologies characterised by insulin resistance such as obesity and type II DM. Several

mouse models have been used extensively to investigate chronic insulin resistance. Ob/ob and db/db mice are the most commonly used strains which are deplete in the leptin gene and leptin receptor gene respectively (Table 3). These mice develop obesity, type II DM and severe insulin resistance therefore provide a useful model to investigate the adiponectin system. Compared to control mice, there were significant reductions in adipoR1 (db/db) and both receptors (ob/ob) mRNA [21 26 79 126]. In humans, significant negative correlations between skeletal muscle adipoR1 (not adipoR2) and waist circumference, waist-hip ratio and truncal fat measured by DEXA scan in middle aged men have been demonstrated but surprisingly no correlation with BMI could be established[138]. Interestingly, plasma adiponectin was also negatively correlated with BMI, waist circumference and skeletal muscle insulin resistance as measured by a euglycaemic clamp[138]. In-vivo, adipoR1 but not adipoR2 is positively correlated with in-vivo insulin and C-peptide concentrations, first phase insulin secretion after adjustments for age, sex and body fat, and plasma triglyceride and cholesterol concentrations[128].

LPS, per se, may not be implicated in the down-regulation of adiponectin receptors in isolated mouse myocyte cell lines grown in culture. This is different to cultured adipocytes where low dose LPS resulted in a significant down-regulation of adipoR1 and higher doses affected adipoR2, in a similar fashion to systemic endotoxaemia.

IL-6 may not always induce insulin resistance in myocytes. Pedersen et al. describe a phenomenon where IL-6 is released after exercise from the contracting myocyte secondary to metabolic changes within the muscle cell[217]. This leads to induction of the AMP-activated Kinase (a similar signalling pathway to adiponectin) within the myocyte in an autocrine/paracrine fashion. Subsequently fatty acid oxidation and glucose uptake is increased and insulin sensitivity improves. As a consequence, changes in adiponectin and its receptor gene expression may be less pronounced. Systemically, IL-6, however, increases hepatic glucose production during exercise and increases adipocyte lipolysis. This

is more in keeping and in agreement with traditional teaching that IL-6 produces insulin resistance in inflammatory conditions[230] and in 3T3-L1 adipocytes[208]. Therefore there may be different roles of IL-6 depending on the mechanism of secretion. This observation is made after exercise (muscular contraction) induced release of IL-6 and may not be due to the inflammatory process but due to metabolic changes in glycogen stores.

3.10.7 Time course of adiponectin expression in sepsis

Changes in adiponectin and adiponectin receptor gene expression in most tissue depots were seen early, by four hours post LPS injection but are not sustained in many tissues to 24 hours. This leads to the following hypotheses to consider:

1. Endotoxaemia has been shown in previous studies to produce early rises in inflammatory cytokines from adipose tissue including IL-6 and TNF-α[12]. This animal model is known to produce sharp, early rises in inflammatory cytokines[181 183 185] and could account for the early changes observed. In humans, the peak of inflammatory cytokines is 2-8 hours (two hours for TNF-α) and 6-8 hours for IL-6) following pathogen entry into the host[53]. Thereafter, the initial pro-inflammatory stimulus reduces and anti-inflammatory cytokines prevail[53]. This change of plasma cytokine levels may impact on the temporal relationship of adiponectin receptor gene expression in adipose tissue.

2. The 24 hour group of mice are the surviving mice and data from animals that died was not included in the experiments. Thus, the four hour group will contain animals with a full spectrum of severity of sepsis. This will include a wide variety in clinical symptoms, as is the case in human sepsis. The 24 hour group however, may only indicate those with a lesser severity of illness and therefore may not have as marked changes. This may contribute to the lack of changes seen in many tissue depots at 24 hours.

3. Receptor half life may be short and therefore the possibility of receptor destruction must be considered although there is currently little evidence to support this.

4. Skeletal muscle cells produce myokines (e.g. IL-6 and IL-15) in response to an inflammatory stimulus[13 231] which could lead to a paracrine effect on the muscle cells and thus potentiate the stimulus to down-regulate receptor gene expression. This may account for the continuing effects seen in-vivo but not in-vitro.

3.10.8 Adiponectin and adiponectin receptor gene expression in other tissues

The gene expression results from spleen and small bowel are more difficult to interpret.

Spleen

Splenic adiponectin receptor mRNA expression was documented in the initial paper by Yamauchi et al. in 2003. Subsequently, receptors have also been demonstrated in splenic tissue of pigs[200] and chickens[232]. Also, two studies have clearly demonstrated the presence of both receptors at the mRNA and protein level in mononuclear cells including macrophages[233 234]. Significant down-regulation of adiponectin and both receptors in lymphocytes of obese subjects[137] and monocytes of type II diabetics[235] have been demonstrated but there is no literature on the effect of LPS on the spleen. However, these findings may be influenced by different types of immunocytes, in particular lymphocytes, which are present in the spleen. These results demonstrate an isolated reduction in adipoR1 gene expression at 24 hours following LPS injection but no other changes (Figure 37 & 38). This could be interpreted, as with the other results, secondary to tissue hypoxia and/or inflammatory response to cytokines released. This will require further investigation and should include the effect of LPS on different immune cells.

Small bowel

Adiponectin receptor mRNA has also been identified in small bowel of mice as in a previous study[236] . There is an emerging link between hypoadiponectinaemia and disorders of the large intestine such as colon carcinoma[237] [238]. However, there are no studies relating to the expression or regulation of adiponectin following acute or chronic inflammatory stimuli.

The results from this study, showing a trend towards a down-regulation of adipoR2 should, however, be viewed with caution for two reasons:

1. There are a number of different cell types found in small bowel tissue any of which may contribute to the results for adiponectin receptor expression. These include, in the epithelium, enterocytes, goblet cells, paneth cells and enteroendocrine cells.
2. There could be contamination by bowel contents.

Without other confirming data (e.g. cellular data and sequencing), this data is hard to interpret.

3.11 Clinical studies on adiponectin changes in sepsis

3.11.1 Baseline results

The background work in this thesis has clearly demonstrated changes in the adiponectin system in response to LPS (in-vitro and in-vivo) and inflammatory cytokines (in-vitro). In the majority of tissue depots and cell lines examined, a down-regulation of adiponectin and its receptor expression was observed. This allowed us to proceed to an investigation of septic patients on the Intensive Care Unit (ICU) at the Royal Liverpool University Hospital. This hospital is a tertiary referral centre with approximately 8-900 in-patient beds and 15 level three beds.

For the clinical study, 21 patients with severe sepsis were recruited from the ICU. All patients fulfilled the criteria for sepsis according to the 2001 consensus conference[5]. In order to fulfil the criteria for sepsis, patients must have two out of four of the SIRS criteria (Table 1) **AND** a positive culture or a high clinical index of suspicion of infection. Patients with sepsis had multiple sources of infection as shown in Table 31.

Table 31: Sources of infection in patients with sepsis

Source	Patients (n)
Abdominal	8
Chest	9
Necrotising Fasciitis	1
Bone	2
Renal tract	1

Tables 32-34 show baseline characteristics and laboratory results of the recruited patients. These include age, BMI and the APACHE II score[239] (which scores severity of illness on admission to ICU) and markers of organ function. Markers of organ system function include cardiovascular (heart rate (HR)), Respiratory (Respiratory rate (RR), fraction of inspired gases that is oxygen (FiO_2) and partial pressure of oxygen (PO_2)) and baseline biochemical and haematological parameters (white cell count

(WCC), C-reactive protein (CRP), haemoglobin (Hb), urea, creatinine (creat) and bilirubin (Bili)). Normal values can be seen in Appendix 3. These values are routinely measured in all patients to assess severity of illness on admission but also to guide treatment strategies and assess response to treatment. Data was subjected to statistical analysis using Mann-Whitney U test as the data was predominantly non-parametric with small numbers.

In all patients, the median age was 63 year (55-71yr) with a median APACHE II score of 20 (Table 32). The APACHE II scoring system gives an integer score from 0-71, computed based on acute physiological derangements and a previous chronic health assessment[239]. Higher scores correspond to higher disease severity and increased calculated risk of morbidity and mortality. Scores of more than 20 equate to a moderate to severe presentation of sepsis.

The Glasgow coma score is a score from 3-15 to indicate deteriorations in conscious level, median score was 15/15. Clinically, patients were not universally pyrexial but were tachycardic and tachypneoic, all requiring more than 50% supplemental oxygen. Biochemical and haematological markers (WCC / CRP) were universally elevated and most had a degree of renal impairment (Tables 33 & 34)

Table 32: All patients: Biometric data

	n	Median	CI	IQR
Age (years)	21	63	[55.84; 68.64]	[55 ; 71]
APACHE	21	20	[17.86; 23.76]	[18 ; 24]
GCS	21	15	[12.65; 14.78]	[14 ; 15]
Height (cm)	21	167	[163.5;172.29]	[162 ; 176]
Weight (kg)	21	84.4	[74.08; 88.94]	[71 ; 93]
BMI (kg/m^2)	21	30	[26.1; 32.13]	[24 ;32.08]
LOS (days)	21	7	[5.46 ; 13.12]	[3 ; 15]

Table 32: Biometric data for all patients including age, severity of illness scoring (APACHE), height, weight and length of stay (LOS) in the ICU. Parameters displayed as Median, inter-quartile range (IQR) and 95% confidence intervals (CI) (APACHE: acute physiology and chronic health evaluation, GCS: Glasgow Coma score, BMI: Body mass index)

Table 33 demonstrates the baseline cardiorespiratory values and inotrope requirements for the patients on admission to the ICU. This confirms that patients were exhibiting signs of sepsis (tachycardia, tachypnoea and increased oxygen requirements). Interestingly, temperature was near normal which is unusual as a higher temperature would be expected as a result of infection. Inotropes are frequently used to augment the sympathetic nervous system in sepsis. Most commonly used are α-agonists, predominantly noradrenaline in the UK, which cause vasoconstriction and increased systemic vascular resistance. This is to counteract the profound vasodilation seen in sepsis which improves blood pressure and organ perfusion. The amount of inotrope required is a surrogate marker of disease severity and with clinical improvement, this should reduce. Less frequently β- agonists are used to improve cardiac contractility but these are often second line drugs. For the purposes of this table, if two inotropes were used, their volumes infused have been combined.

Table 33: All patients: Cardiorespiratory variables

	n	Median	CI	IQR
Temp (°C)	19	37.1	[36.57 ; 37.91]	[36.95 ; 38.15]
HR (bpm)	21	116	[109.85 ; 128.91]	[110 ; 123]
RR (bpm)	21	20	[17.18 ; 24.34]	[14 ; 26]
FiO_2	21	0.6	[0.53 ; 0.75]	[0.5 ; 0.8]
PO_2 (kPa)	21	10.19	[9.68 ; 13.95]	[9.54 ; 12.2]
Total dose inotropes day 1 (ml)	19	241	[174.71 ; 495.39]	[116 ; 488.5]
Total dose inotrope day 2 (ml)	18	192	[133.16 ; 538.84]	[60.5 ; 469.75]
Mean hrly dose inotrope day 1 (ml/hr)	19	11	[8.9 ; 22.66]	[6.08 ; 22.5]
Mean hrly dose inotrope day 2 (ml/hr)	18	8	[5.33 ; 33.76]	[2.52 ; 19.86]

Table 33: All patients Cardiorespiratory variables on admission to ICU and inotrope requirements. Temp: Temperature, HR: Heart rate, RR: respiratory rate, FiO_2: Fraction of inspired oxygen, PO_2: Partial pressure of oxygen. Parameters displayed as Median, inter-quartile range (IQR) and 95% confidence intervals (CI)

Table 34 shows the baseline laboratory values of the recruited patients. As expected, markers of the inflammatory process (WCC and CRP) are both elevated and there is a degree of mild renal and hepatic impairment not uncommonly seen in patients with sepsis.

Table 34: All patients: Laboratory results

	n	Median	CI	IQR
WCC (x10/L)	21	23	[16.14 ; 27.31]	[11.7 ; 31.9]
CRP (mg/L)	18	198	[152.76 ; 244.46]	[150.25 ; 240.5]
Hb (g/dL)	21	10.3	[9.2 ; 11.18]	[8.5 ; 11.2]
Creat (µmol/L)	21	114	[96.89 ; 209.01]	[76 ; 183]
Urea (mmol/L)	21	9.2	[8.51 ; 14.31]	[7.4 ; 13.1]
Bili (µmol/L)	20	15	[14.55 ; 42.85]	[8 ; 34.75]

Table 34: All patients Laboratory results on admission to ICU. WCC: white cell count, CRP: C-Reactive protein, Hb: Haemoglobin, Creat: creatinine, Bili: Bilirubin, LOS: Length of stay). Parameters displayed as Median, inter-quartile range (IQR) and 95% confidence intervals (CI)

Table 35 displays the metabolic parameters in the recruited patients that are important in sepsis. Serum glucose is measured to assess the degree of insulin resistance and the requirement of the patient for exogenous insulin. Insulin is frequently used in an attempt to maintain normoglycaemia in patients with sepsis. Serum lactate is measured for two reasons: Firstly to assess global organ perfusion as lactate is a by-product of anaerobic respiration. Serial lactate values can help to assess response to initial treatment, frequently aggressive fluid resuscitation and inotropes. Secondly, lactate is used as a surrogate marker of liver function as it is metabolised by the liver. Hyperlactataemia may, therefore indicate a deterioration in liver function.

Table 35: All Patients: Metabolic parameters

	n	Median	CI	IQR
Mean plasma glucose day 1	21	6.65	[6.37 ; 8.19]	[5.86 ; 8.3]
Mean plasma glucose day 2	21	7.67	[7.1 ; 8.06]	[6.95 ; 8.23]
Mean plasma glucose discharge	16	6.64	[5.96 ; 7.06]	[5.96 ; 7.21]
Mean plasma lactate day 1	21	1.75	[1.63 ; 2.67]	[1.49 ; 2.72]
Mean plasma lactate day 2	21	1.65	[1.45 ; 2.63]	[1.44 ; 1.8]
Mean plasma lactate discharge	15	1.32	[0.61 ; 5.2]	[1.21 ; 1.82]
Total insulin required day 1	6	28.5	[15.53 ; 40.8]	[20 ; 36.25]
Total insulin required day 2	9	57	[25.9 ; 69.21]	[24 ; 70]
Mean hrly insulin dose (units/hr) day 1	6	1.49	[0.72 ; 1.97]	[0.91 ; 1.77]
Mean hrly insulin dose (units/hr) day 2	9	2.38	[1.08 ; 2.88]	[1.04 ; 2.91]

Table 35: All patients: metabolic parameters on admission, day1 and day of discharge to ICU. Discharge data are missing who died (SD: standard deviation, CI: 95% confidence interval

A BMI of 30 kg/m^2 is the separation between overweight and obese according to the WHO classification[240] (Table 36).

Table 36: BMI category according to the World Health Organisation

BMI (kg/m^2)	Category
<18	Underweight
18 - 25	Normal
>25	Overweight
25 - 30	Pre-obese
30.1 - 35	Obese Class I
35.1 - 40	Obese Class II
>40	Obese Class III

Table 36: Body Mass index (BMI) is calculated by weight (kg) divided height^2 (m)

When the recruited patients were divided into groups dependent on BMI, there were very few differences, clinically or metabolically, between them. There was a small increase in temperature in the overweight group (37.9°C vs 36.5°C, p=0.023), however, this is unlikely to be clinically significant. The only other difference was in the requirements of insulin on day two of admission. It is routine protocol to correct hyperglycaemia with exogenous insulin. The patients in the higher BMI group required more insulin that their lighter counterparts (60.8 units/day vs 21 units/day p=0.014).

Plasma adiponectin was subsequently measured (µg/ml) using a commercially available ELISA kit. Total and HMW adiponectin were measured and their values compared. Table 37-39 show plasma adiponectin values for all patients, patients BMI<30 kg/m^2 and patients BMI >30 kg/m^2 respectively.

Table 37: All patients: Total and High molecular weight adiponectin and HMW/total ratio

	n	CI	Median (µg/ml)	Interquartile Range	p-value
Total adiponectin day 1	21	[3.1 ; 4.58]	3.78	[2.86 ; 4.25]	
Total adiponectin day 2	21	[2.93 ; 4.74]	3.48	[2.52 ; 4.31]	0.733$^{\$}$
Total adiponectin discharge	11	[3.66 ; 9.52]	4.96	[4.41 ; 8.16]	<0.01**
HMW adiponectin day 1	21	[2.03 ; 3.13]	2.5	[1.7 ; 3.5]	
HMW adiponectin day 2	21	[2.14 ; 3.16]	2.5	[2 ; 3.2]	0.459$^{\$}$
HMW adiponectin discharge	11	[2.89 ; 6.8]	3.8	[2.45 ; 7.8]	<0.001***
HMW/Total Ratio day 1	21	[0.58 ; 0.81]	0.64	[0.59 ; 0.86]	
HMW/Total Ratio day 2	21	[0.61 ; 0.86]	0.74	[0.65 ; 0.78]	0.215$^{\$}$
HMW/Total Ratio discharge	11	[0.53 ; 1.23]	0.75	[0.58 ; 1.05]	<0.01**

Table 37: Total and high molecular weight adiponectin in plasma measured by ELISA (µg/ml) in all patients. (SD: standard deviation, CI: 95% confidence interval). Discharge data are missing for patients who died. Statistically significant increases compared to day 1 ** p<0.01, ***p<0.001. (HMW: high molecular weight). $: non-significant changes comparing day 1 to day 2.

Table 38: Patients BMI <30 kg/m^2: Total and High molecular weight Adiponectin

	n	CI	Median (µg/ml)	Interquartile range
Total adiponectin day 1	10	[2.53 ; 4.68]	3.29	[2.82 ; 4.19]
Total adiponectin day 2	10	[2.57 ; 4.13]	3.4	[2.6 ; 4.1]
Total adiponectin discharge	5	[3.42 ; 8.72]	5.37	[4.39 ; 6.93]
HMW adiponectin day 1	10	[1.22 ; 3.14]	1.75	[1.27 ; 2.65]
HMW adiponectin day 2	10	[1.43 ; 2.87]	2.3	[1.75 ; 2.65]
HMW adiponectin discharge	5	[0.72 ; 7.24]	3.8	[2.2 ; 4]
HMW/Total Ratio day1	10	[0.42 ; 0.79]	0.63	[0.4 ; 0.72]
HMW/Total Ratio day2	10	[0.48 ; 0.78]	0.72	[0.57 ; 0.75]
HMW/Total Ratio discharge	5	[0.2 ; 1.17]	0.71	[0.37 ; 0.91]

Table 38: Total and high molecular weight adiponectin in plasma measured by ELISA (µg/ml) in patients BMI <30 kg/m^2. (SD: standard deviation, CI: 95% confidence interval). Discharge data are missing for patients who died.

Table 39: Patients BMI >30 kg/m^2: Total and High molecular weight adiponectin

	n	CI	Median (µg/ml)	range	p-value
Total adiponectin day1	11	[2.85 ; 5.25]	3.8	[3.08 ; 4.3]	0.582
Total adiponectin day2	11	[2.57 ; 5.98]	3.48	[2.61 ; 5.09]	0.287
Total adiponectin discharge	6	[0.92;13.13]	4.8	[4.48 ; 8.37]	0.721
HMW adiponectin day1	11	[2.27 ; 3.62]	2.9	[2.2 ; 3.7]	0.161
HMW adiponectin day2	11	[2.39 ; 3.81]	3.2	[2.25 ; 3.65]	0.049
HMW adiponectin discharge	6	[2.25 ; 8.88]	5.6	[2.9 ; 7.85]	0.387
HMW/Total Ratio day1	11	[0.63 ; 0.92]	0.71	[0.62 ; 0.94]	0.127
HMW/Total Ratio day2	11	[0.63 ; 1.04]	0.75	[0.69 ; 0.81]	0.089
HMW/Total Ratio discharge	6	[0.43 ; 1.66]	0.78	[0.65 ; 1.4]	0.257

Table 39: Total and high molecular weight adiponectin in plasma measured by ELISA (µg/ml) in patients BMI >30 kg/m^2. (SD: standard deviation, CI: 95% confidence interval). Discharge data are missing for patients who died. Statistical significance of values compared to patients with a BMI of <30 kg/m^2.

Table 40 shows the correlation of adiponectin (HMW and total) and its ratio to other well recognised markers of disease severity, such as APACHE score, CRP and white cell count. There is a positive correlation between APACHE score and total adiponectin at all 3 times points.

Table 40: Correlation of adiponectin values and their ratios to clinical markers of sepsis

Parameter	APACHE	n	CRP	n	WCC	n
Total adiponectin day 1	0.503**	21	-0.126	18	0.042	21
Total adiponectin day 2	0.415**	21	-0.229	18	0.084	21
Total adiponectin discharge	0.356*	11	0.511*	10	-0.118	11
HMW adiponectin day 1	0.371	21	-0.565	18	0.049	21
HMW adiponectin day 2	0.415*	21	-0.370	18	0.173	21
HMW adiponectin discharge	0.151	11	0.201	10	0.178	11
HMW/Total Ratio day 1	-0.142	21	-0.447	18	-0.064	21
HMW/Total Ratio day 2	-0.267	21	-0.272	18	-0.023	21
HMW/Total Ratio discharge	-0.534	11	-0.158	10	0.515	11

Table 40: Correlation of all adiponectin values to markers of disease severity. There is a positive correlation between APACHE score and total adiponectin at all 3 times points. *p<0.05,** p<0.01, ***p<0.001 (APACHE: acute physiology and chronic health evaluation , WCC: White cell count, CRP: C-reactive protein)

In contrast to previous studies[93 94], there were no significant differences between the obese and the non-obese groups of patients with respect to adiponectin and HMW adiponectin. Therefore, the following graphs display the values for all patients. The most striking finding is a significant increase in plasma total and HMW adiponectin from day 1 (admission) to day of discharge when a clinical improvement has been made (Figures 44, 45 & 46).

Figure 44: Total Plasma Adiponectin in all patients on day 1, 2 and discharge

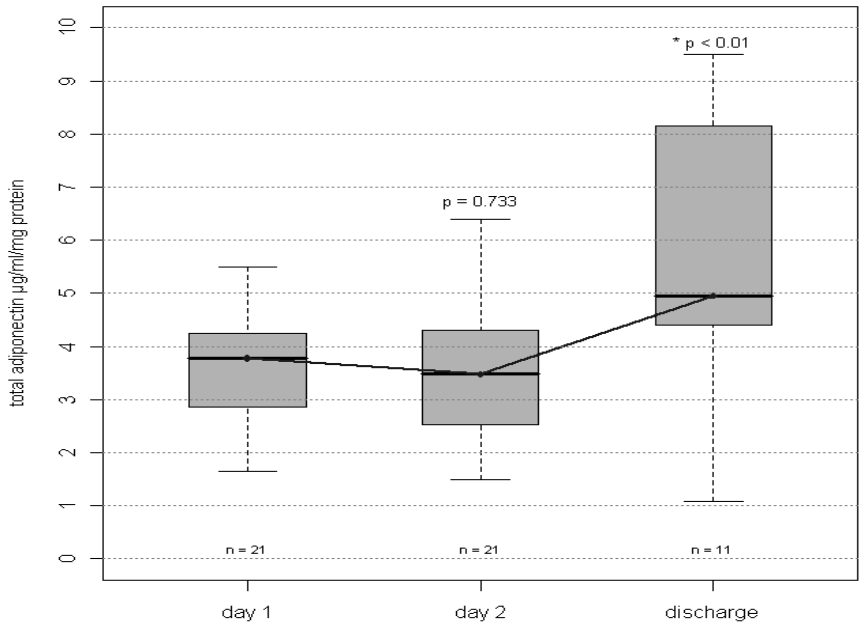


Figure 44: Total plasma adiponectin as measured by ELISA. There is a significant increase from Day 1 to day of discharge when patients have clinically improved and no longer fulfil the criteria for sepsis. Figure displayed as median (dark line), range (whisker plots) and Interquartile range (dark grey box). n=21 for day 1 and 2, n=11 for day of discharge.

Figure 45: High molecular weight Plasma Adiponectin in all patients on day 1, 2 and discharge

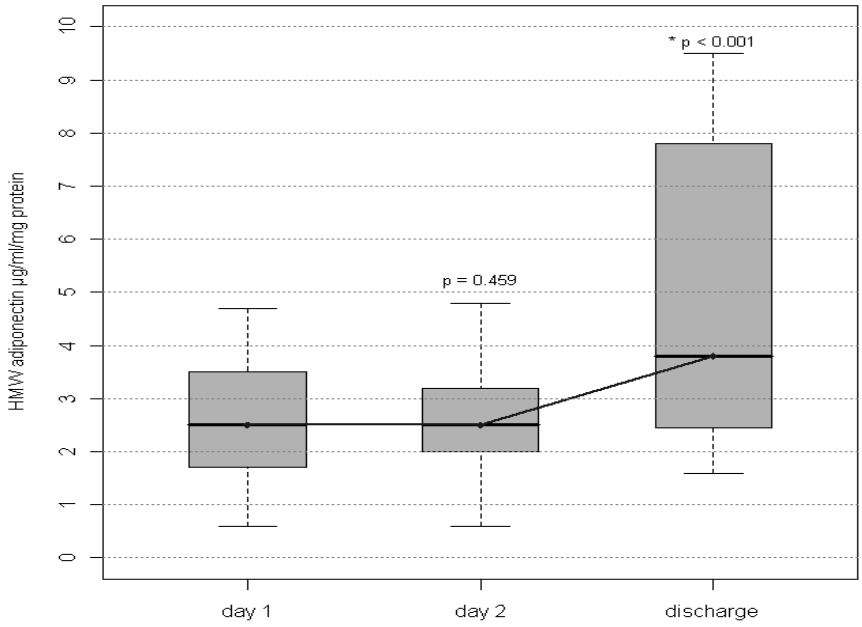


Figure 45: High molecular weight (HMW) plasma adiponectin as measured by ELISA. There is a significant increase from Day 1 to day of discharge when patients have clinically improved and no longer fulfil the criteria for sepsis. Figure displayed as median (dark line), range (whisker plots) and Interquartile range (dark grey box). n=21 for day 1 and 2, n=11 for day of discharge.

Figure 46: Ratio of HMW: Total plasma Adiponectin in all patients on day 1, 2 and discharge

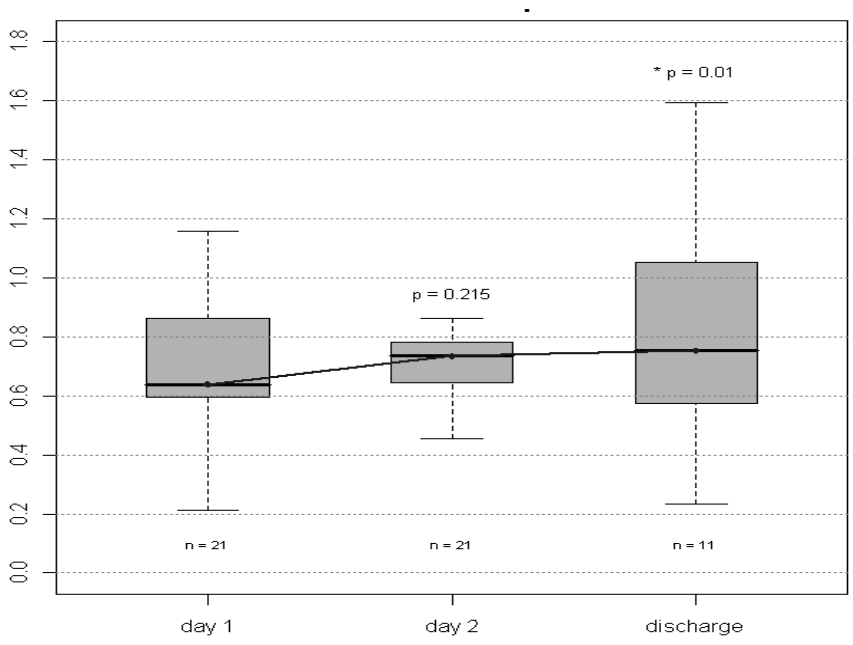


Figure 46: Ratio between High molecular weight (HMW) to total plasma adiponectin as measured by ELISA. There is a significant increase from Day 1 to day of discharge when patients have clinically improved and no longer fulfil the criteria for sepsis. Figure displayed as median (dark line), range (whisker plots) and Interquartile range (dark grey box). n=21 for day 1 and 2, n=11 for day of discharge.

Total and high molecular weight (HMW) adiponectin was measured in all patients (Table 37). There were no significant differences between the two different BMI groups (< and >30 kg/m^2). As there were no differences between the groups, the groups were combined for the purposes of analysis.

There were no differences in total and HMW adiponectin concentrations between day one and two of the sepsis episode. On day of discharge, however, there was a significant increase in both total and high molecular weight adiponectin compared to the admission concentrations (Table 37).

The ratio between HMW and total adiponectin was subsequently measured. This was performed to investigate the contribution of high molecular adiponectin to the total and to assess the change in the ratio with clinical improvement of sepsis. There was an increase in the HMW/total ratio, suggesting that the contribution from HMW adiponectin is greater in health than in patients fulfilling sepsis criteria.

3.12 Discussion

Clinical study

Following the background work in-vivo and in-vitro, the clinical study was performed. The rationale was to investigate plasma adiponectin and HMW adiponectin in septic patients. Adiponectin has been shown to be down-regulated in experimental endotoxaemia. HMW adiponectin, however, has been investigated less.

HMW adiponectin is deemed to be the more active of the different mulitmers of adiponectin. Adiponectin monomers polymerise post-translationally to form larger molecular weight molecules linked via disulphide bonds[98] (Figure 4). In human plasma, adiponectin circulates predominantly as low and high molecular weight monomers (190 kDa and >300 kDa respectively)[71 98]. HMW adiponectin has been shown to more avidly than other oligomers to its receptors[76 80]. Genetic mutations resulting in impaired multimerisation or reduced plasma HMW adiponectin can result in increased type II DM and insulin resistance[76].

Total and HMW adiponectin and their ratio have been intensively studied as potential biomarkers for the development of the type II DM and the metabolic syndrome[80 82 97 98 241 242]. Interestingly, the significance of the correlation between HMW and total adiponectin and markers of insulin resistance appears to be stronger in female patients[97].

Following the identification of tissue hypoadiponectinaemia in the mouse model, the response to clinical sepsis in humans was investigated. Twenty-one patients (BMI<30 kg/m^2 n=10, BMI>30 kg/m^2 n=11) who fulfilled the criteria for sepsis were recruited[4]. Samples were taken in sequence on days 1 and 2 following admission and day of discharge from ICU. This allowed the patients to act as their own control, thus rendering a healthy volunteer control group unnecessary. Median age was 63 years which is in keeping with recent sepsis epidemiological studies[1 2]. All patients fulfilled the

criteria for sepsis and displayed many clinical and laboratory signs on infection and ongoing inflammation (Table 33 and 34). Severity of illness scores were calculated and the median APACHE score was 20, indicative of a moderate to severe severity.

When the groups were divided into BMI categories, with the exception of admission temperature, there were no statistical differences between the groups (36.5°C (BMI<30 kg/m^2) vs 37.9°C (BMI>30 kg/m^2), p=0.023). The clinical significance of this is unknown and is likely to be small. Mean admission glucose levels were all <8 mmol/L with no significant differences between the groups (Table 35) and thus are lower than expected both from anecdotal practice and from the current literature where an estimated 50% of critically ill patients develop hyperglycaemia[243]. This is despite a relatively high severity of illness score (APACHE score median: 20 (18-24)). It is standard to treat hyperglycaemia in all patients to achieve normoglycaemia. Only 6 out of 21 patients required insulin on day 1 of sepsis, five of these were in the obese group. The non-obese and obese groups had average insulin requirements of 0.5 and 1.51 units of insulin per hour respectively. Although numbers are small, this suggests a greater degree of insulin resistance in the obese group. The differences in insulin requirements may either reflect long-standing insulin resistance or an acute change secondary to the activation of pathways associated with insulin resistance such as release of IL-6 or TNF-α. Due to small numbers, we were not able to separate out patients with different sources of infection which may have impacted on the metabolic changes.

Unexpectedly, there were no differences between obese and non-obese patients with respect to total or HMW adiponectin. This is contrary to the current literature associating obesity with hypoadiponectinaemia[15 93 94 100]. However, there is no literature investigating adiponectin in obese septic patients, which renders a direct comparison to current literature difficult as other factors including inflammatory cytokines, endogenous and exogenous insulin infusions may influence

adiponectin levels. However, this study was not powered to investigate differences in adiponectin in obese patients, therefore a negative results is not unexpected.

We did not separate the groups based on gender however, the results confirm the finding that HMW adiponectin is higher in women than men (6 vs 2.82 µg/ml, p=0.03)[75 80 97]. Interestingly, there was no difference in total adiponectin between the sexes despite a statistically significant increase in BMI in the female patients (31.8 vs 26.05 kg/m^2, p=0.03).

HMW adiponectin, total adiponectin and the HMW/total ratio all show a significant increase between day 1 and day of discharge (2.5 vs 3.8 µg/ml (p<0.001), 3.78 vs 4.96 µg/ml (p<0.01) and 0.64 vs 0.75 (p<0.01) respectively) This increase in adiponectin is accompanied by a significant clinical improvement in condition, such that intensive care was no longer required. This is likely to reflect resolution of the pro-inflammatory process or a metabolic change indicating recovery. Sepsis commences with an overwhelming pro-inflammatory response followed by a counter-regulatory anti-inflammatory reaction to maintain homeostasis. This study demonstrated an increase levels of the 'anti-inflammatory' adiponectin corresponding to improved clinical condition. This suggests that the increase in adiponectin plasma level reflects improvement in the clinical condition and normalisation of the initially suppressed immune response.

Although the significance of the different mulitmers is still disputed, it is becoming clear that visceral adiposity[75 98] and pathologies characterised by insulin resistance and known hypoadiponectinaemia, also display lower levels of HMW adiponectin and this may be contributing to their insulin resistance.

Our results have identified a similar phenomenon in septic patients demonstrating lower total and HMW adiponectin. The observation that the ratio also improves significantly with improvement of clinical condition, i.e. the proportion of HMW becomes greater suggests that HMW adiponectin may have a key role in the inflammatory process and also the insulin resistance seen in sepsis.

Correlations of adiponectin with clinical data

We anticipated that an increase in disease severity and markers of the inflammatory response would be inversely correlated with adiponectin and HMW adiponectin. Unexpectedly, the opposite was demonstrated (Table 40). There was a direct correlation of adiponectin with APACHE score on day 1, 2 and discharge and with CRP on day of discharge. The reasons for this are unclear

One interesting finding is that there is a trend towards an inverse correlation between HMW/total adiponectin ratio and APACHE score and to a lesser extent with CRP (Table 40). This would support literature from diabetic patients where differences in the HMW/total adiponectin ratio without correlates to be a better predictor of insulin resistance[80].

3.13 Summary

The experiments performed in this chapter clearly demonstrate a change in the adiponectin system in response to LPS and pro-inflammatory cytokines in-vitro and in-vivo and in response to sepsis in mice and humans. There is down-regulation of both adiponectin and adiponectin receptors from adipose tissue and skeletal muscle in mice but this is not consistently replicated in cellular studies, which may indicate a response to the ongoing systemic inflammatory process rather than direct cellular LPS effects. We have also demonstrated a down-regulation of adiponectin receptors in splenic tissue, however, the importance of this remains unclear. In septic patients, we have demonstrated an increase in total and HMW adiponectin plasma concentrations with clinical improvement and recovery from sepsis. Although the signalling pathways and clinical importance of these phenomena are not fully understood, this represents a consistent change in the adiponectin system with acute inflammation.

3.14 Role of HIF-1α in sepsis

The signalling pathways involved in the down-regulation of the adiponectin system in sepsis or acute inflammation are unknown. Each tissue depot may have regulatory pathways for different parts of the cellular cascade. Activation of various pathways including NFκB and inflammatory cytokines may be implicated but downstream signalling could be triggered by cellular hypoxia. We have looked at the expression of HIF-1α in tissues, adipocytes and myocytes. HIF-1α is a transcription factor known to be heavily involved in the cellular response to hypoxia. More recently, its role in non-hypoxic regulation of cellular function has been identified. The next line of investigation focussed on HIF-1α expression and secretion in response to LPS treatment.

Using the same experimental model as in previous experiments, mice were given an intraperitoneal injection of 25 mg/kg LPS and killed at 4 or 24 hours. Tissues were then dissected and investigated for HIF-1α expression and protein accumulation.

3.14.1 Murine tissue HIF-1α gene expression

HIF-1α gene expression is upregulated in liver, all 3 depots of adipose tissue and spleen at four hours and liver, small bowel, PR and SC fat and spleen at 24 hours post LPS injection. There was a trend towards a rise in HIF-1α gene expression in epididymal fat at 24 hours. There were no changes in skeletal muscle (Table 41 & Figure 47).

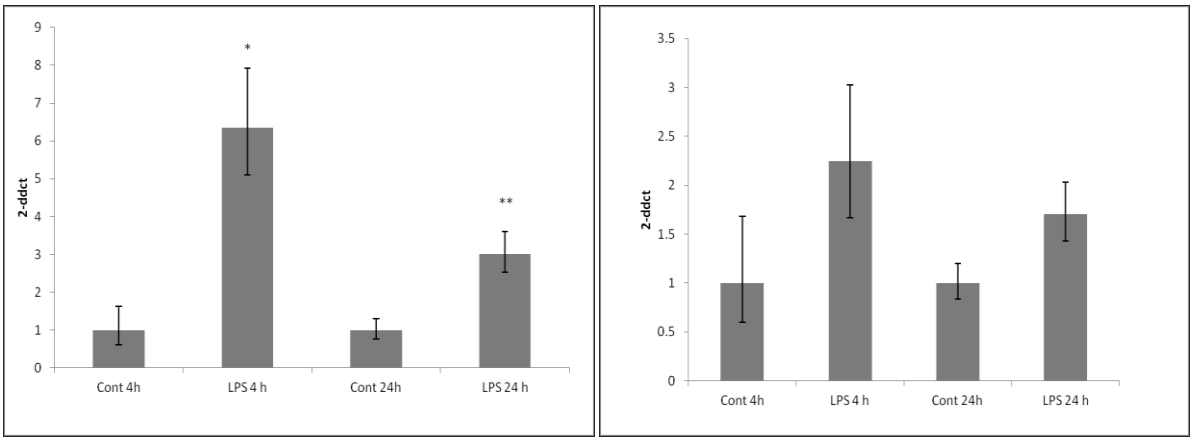
Table 41: HIF-1α expression in Mouse tissue depots

4 hours					24 hours			
	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
Liver	6.355	1.57	1.26	0.022	3.01	0.587	0.497	0.01
Muscle	2.246	0.77	0.577	0.274	1.702	0.33	0.276	0.101
Epi fat	5.211	2.16	1.53	0.005	2.11	0.82	0.595	0.09
PR fat	4.372	0.711	0.611	0.00073	3.103	0.81	0.64	0.0003
SC fat	2.126	0.179	0.15	0.015	2.239	0.529	0.428	0.005
Spleen	2.799	0.535	0.634	0.008	3.239	0.655	0.545	3.23x10-5
SB	3.714	0.377	0.273	0.07	3.08	1.288	0.906	0.02

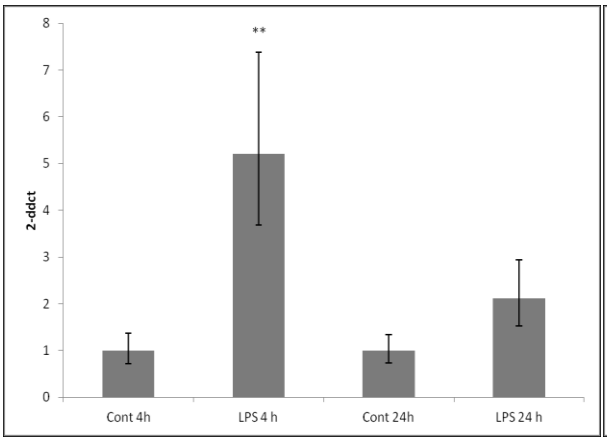
Table 41: Relative change in HIF-1α gene expression in mouse tissue depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. Each treatment group was compared to its own control group only.

Figure 47: HIF-1α gene expression in mouse tissue depots

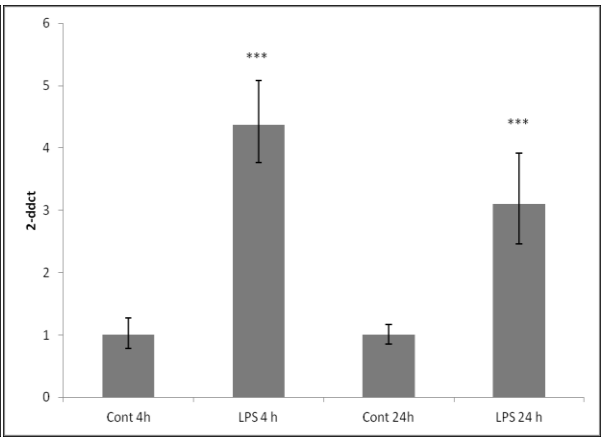
A: Liver **B: Skeletal muscle**



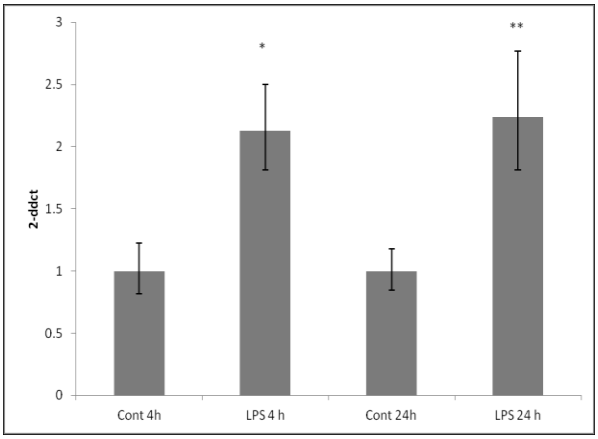
C: Epididymal fat



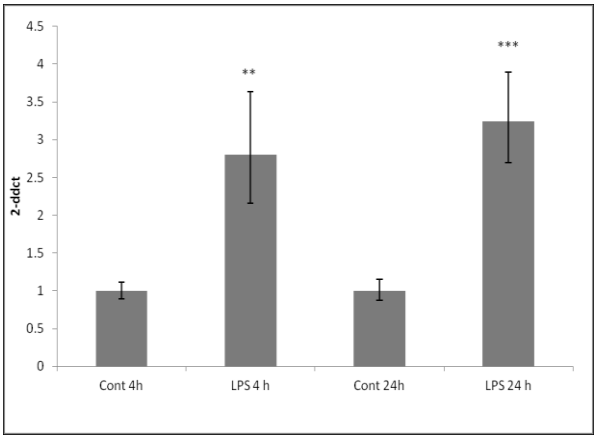
D: Peri-renal fat



E: Subcutaneous fat



F: Spleen



G: Small bowel

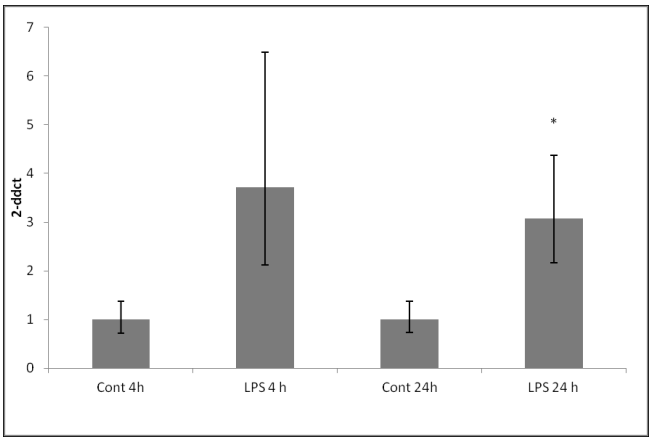


Figure 47: Relative change in HIF-1α gene expression in mouse tissue depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$, ** $p<0.01$). Housekeeping gene was β-actin. Each treatment group was compared to its own control group only. A: Liver, B: Skeletal Muscle, C: Epididymal Fat, D: Peri-renal fat, E: Subcutaneous Fat, F: Spleen G: small bowel (Cont = Control group, LPS = Treatment group, 4 h 4 hours treatment group, 24 h: 24 hours treatment group). 4 hour mice n=6, 24 hour mice n=14 (fat depots, spleen and small bowel) n=11 (muscle and liver)

3.14.2 HIF-1α protein expression

Following the determination of HIF-1α gene expression, we wished to establish the impact of LPS on HIF-1α protein accumulation. This is particularly relevant as hypoxic and non-hypoxic stimulation of HIF-1α have different signalling pathways. Non-hypoxic up-regulation stimulates transcription, whereas hypoxic stimulation prevents the protein breakdown without any effect on transcription.

HIF-1α ELISA was performed on 3 samples from liver, muscle and spleen (in duplicate). Our group has already demonstrated and up-regulation of HIF-1α protein in adipose tissue in the same mouse model[12]. Numerically, liver secretes the most HIF-1α protein (approximately equivalent to adipose tissue[12]) with smaller amounts produced by muscle and spleen. There were increases in muscle expression at 4 hours (2.5-fold) and liver at 24 hours (2-fold). There was only adequate tissue for a very small sample size (n=3 in duplicate). Thus, these results were not subjected to statistical analysis (Figure 48)).

Table 42: HIF-1α protein levels following 25 mg/kg LPS ip injection: 4 hour samples

Sample number	Liver 4 h cont	Liver 4 h LPS	Muscle 4 h cont	Muscle 4 h LPS	Spleen 4 h cont	Spleen 4 h LPS
1	927.504	937.698	92.495	35.638	55.18	66.812
2	568.054	1044.947	207.755	512.565	66.585	65.28
3	1124.55	738.438	160.847	597.549	133.14	76.82
Mean	873.3693	907.0277	153.699	381.9173	84.968	69.637
sd	282.1699	155.5392	57.962	302.882	42.105	6.26

Table 42: HIF-1α protein levels measured in pg HIF-1α/mg protein. (Cont = Control group, LPS = Treatment group, 4 h 4 hours treatment group, 24 h: 24 hours treatment group).

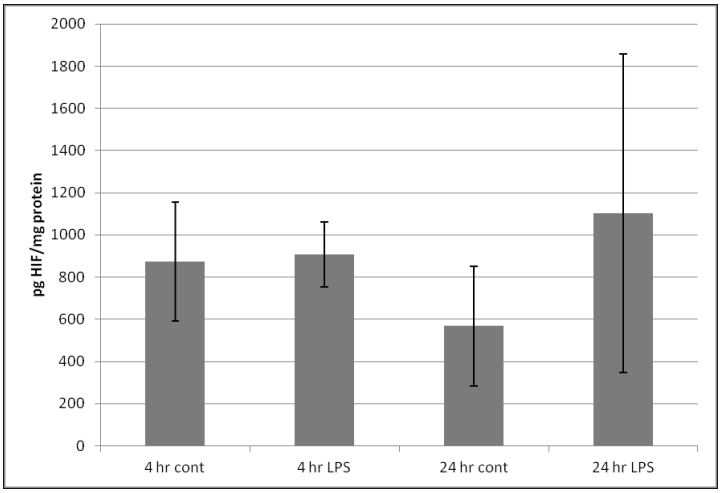
Table 43: HIF-1α protein levels following 25 mg/kg LPS ip injection: 24 hour samples

Sample number	Liver 24 h cont	Liver 24 h LPS	Muscle 24 h cont	Muscle 24 h LPS	Spleen 24 h cont	Spleen 24 h LPS
1	241.409	345.391	24.65	51.101	58.587	28.11
2	767.151	1104.18	84.19	52.614	44.27	23.87
3	694.22	1856.326	55.301	56.85	51.315	43.77
Mean	567.593	1101.966	54.713	53.52	51.39	31.91
sd	284.827	755.469	29.77	2.98	7.15	10.48

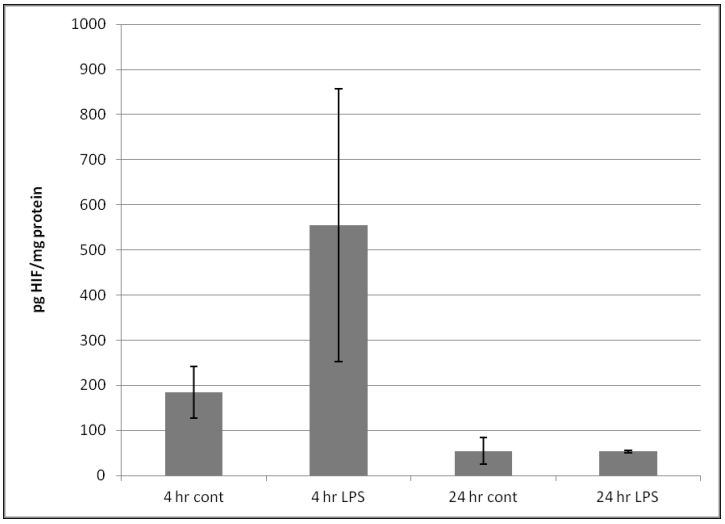
Table 43: HIF-1α protein levels measured in pg HIF-1α/mg protein. (Cont = Control group, LPS = Treatment group, 4 h 4 hours treatment group, 24 h: 24 hours treatment group).

Figure 48: HIF-1α protein levels in murine tissue depots

A: Liver



B: Skeletal Muscle



C: Spleen

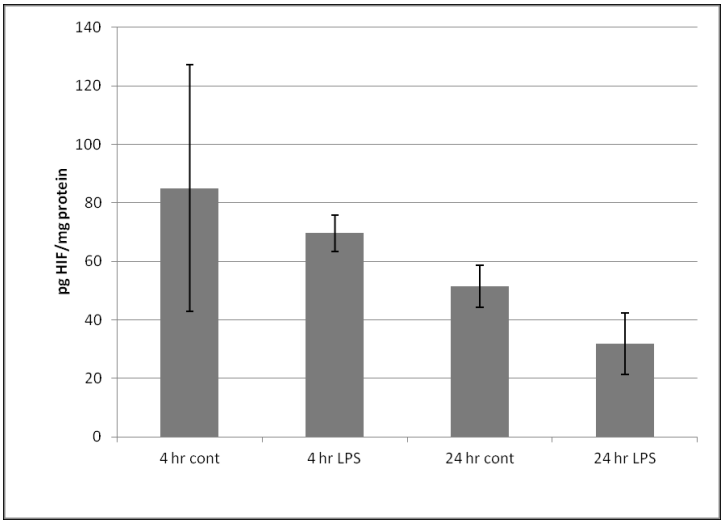


Figure 48: HIF-1α protein expression in mouse tissue depots 4 and 24 hours following 25 mg/kg intra-peritoneal injection of LPS as measured by ELISA. Values are expressed as pg HIF/mg protein. n=3 in duplicate. A: Liver, B: Skeletal Muscle, C: Spleen.

3.14.3 HIF-1α gene expression in 3T3-L1 adipocytes following LPS

Using the same experimental conditions as previous experiments, HIF-1α gene expression was then investigated in 3T3-L1 adipocytes and C2C12 myocytes. HIF-1α gene expression in 3T3-L1 adipocytes was affected very little by LPS treatment. There was a significant increase in HIF-1α gene expression after 24 hours incubation with 5 μg/ml LPS but only a trend towards an up-regulation after four hours and only with a single dose of LPS (1 μg/ml) (Table 44 & Figure 49).

Table 44: HIF-1α gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.171042	0.14606	
	1 µg/ml	1.709214	0.355834	0.294519	0.055476
	5 µg/ml	1.543993	0.314098	0.261002	0.105337
	10 µg/ml	1.435613	0.216339	0.188008	0.135532
24 hours	Control	1	0.126061	0.111948	
	1 µg/ml	1.666321	0.459584	0.36023	0.100067
	5 µg/ml	2.370186	0.212457	0.19498	0.002345
	10 µg/ml	1.183724	0.262426	0.214805	0.518405

Table 44: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only with Bonferroni correction. There is a significant up-regulation in HIF-1α gene expression in 3T3-L1 adipocytes only after 24 hours treatment with 5µg/ml LPS. There were no other changes. n=6, samples run in duplicate

Figure 49: HIF-1α gene expression in 3T3-L1 adipocytes following 1-10 µg/ml LPS treatment for 4 and 24 hours

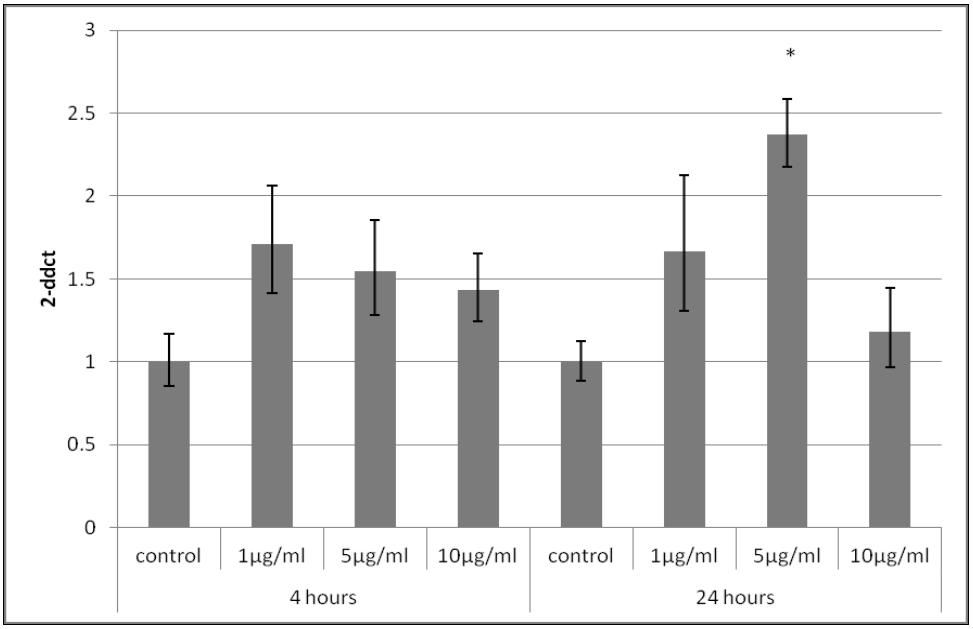


Figure 49: Relative change in HIF-1α gene expression in 3T3-L1 adipocytes. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (*$p<0.05$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.14.4 HIF-1α gene expression in 3T3-L1 adipocytes following cytokine treatment

Following the results with LPS treatment, I wished to ascertain whether the effect was also present following treatment with pro-inflammatory cytokines. 3T3-L1 adipocytes were therefore treated with 10 ng/ml IL-6 and TNF-α as in previous experiments. The results show that the up-regulation of HIF-1α is a phenomenon isolated to TNF-α treatment as is not observed following treatment with IL-6. Interestingly, this was only in the cells observed for 4 hours and not in the 24 hour group.

Table 45: HIF-1α gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.214744	0.176782	
	IL-6 10 ng/ml	0.711039	0.234329	0.176246	0.385882
24 hours	Control	1	0.322187	0.243677	
	IL-6 10 ng/ml	0.545758	0.244219	0.168719	0.228395
4 hours	Control	1	0.144709	0.126416	
	TNF-α 10 ng/ml	1.95432	0.22664	0.203088	0.003473
24 hours	Control	1	0.45698	0.313649	
	TNF-α 10 ng/ml	1.652901	0.631541	0.45695	0.320447

Table 45: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only. n=6, samples run in duplicate.

Figure 50: HIF-1α gene expression in 3T3-L1 adipocytes following IL-6 and TNF-α treatment for 4 and 24 hours

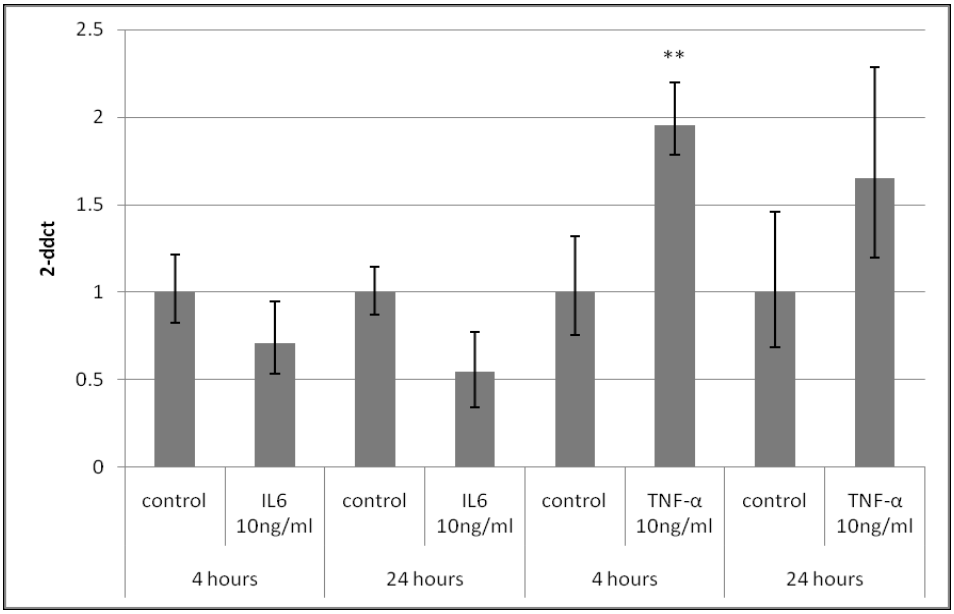


Figure 50: Relative change in HIF-1α gene expression in 3T3-L1 adipocytes. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (** $p<0.01$). Housekeeping gene was β-actin. n=6, samples run in duplicate.

3.14.5 HIF-1α gene expression in C2C12 myocytes following LPS

Following the investigations in 3T3-L1 adipocytes, we performed similar experiments on C2C12 myocytes. There were highly significant increases in all cells and at all time points and concentrations of LPS (Table 46, Figure 51). There was no consistent change with increasing or decreasing concentration.

Table 46: HIF-1α gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

Incubation time	Group	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
4 hours	Control	1	0.13	0.11	
	0.1 µg/ml	3.19	0.28	0.26	$3.89x10^{-5}$
	1 µg/ml	1.74	0.22	0.20	0.015
	5 µg/ml	1.79	0.24	0.21	0.008
	10 µg/ml	2.69	0.28	0.25	0.0001
24 hours	Control	1	0.07	0.06	
	0.1 µg/ml	2.47	0.25	0.23	$3.33x10^{-5}$
	1 µg/ml	2.1	0.28	0.25	0.001
	5 µg/ml	3.41	0.46	0.40	$3.51x10^{-5}$
	10 µg/ml	2.31	0.22	0.20	$4.1x10^{-5}$

Table 46: Results displayed as $2^{-\Delta\Delta CT}$ with SEM, with each treatment group compared to its own control group only with Bonferroni correction. There is a significant up-regulation in HIF-1α gene expression in C2C12 myocytes at all doses of LPS and both time points. n=6, samples run in duplicate.

Figure 51: HIF-1α gene expression in C2C12 myocytes following 0.1-10 µg/ml LPS treatment for 4 and 24 hours

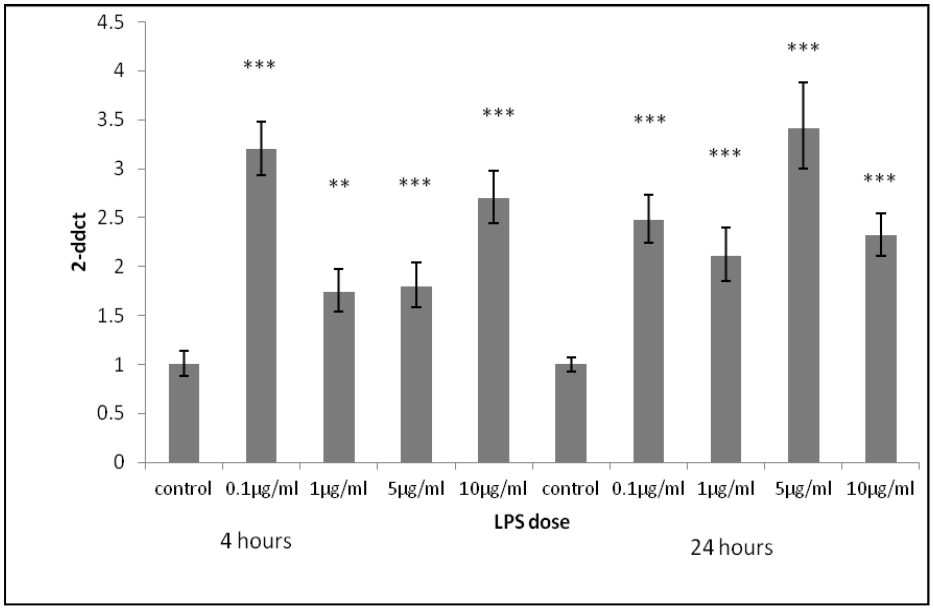


Figure 51: Relative change in HIF-1α gene expression in 3T3-L1 adipocytes. Gene expression was determined by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and $p<0.05$ was considered significant. (** $p<0.01$, ***$p<0.0001$). Housekeeping gene was β-actin.

3.15 Discussion

The role of HIF-1α in sepsis

HIF-1α is a transcription factor which is up-regulated by both hypoxic and by non-hypoxic stimuli such as LPS. Upon hypoxic stimulation, the HIF-1α molecule is prevented from breakdown by inhibition of proline hydroxylases. It has been recently shown that the non-hypoxic stimulation of HIF-1α is more likely to be a transcriptional up-regulation rather than a prevention of breakdown[164].

A summary of results is shown in the following table:

Table 47: Summary of HIF-1α gene expression

	HIF-1α 4 hours	HIF-1α 24 hours
Mouse tissue: LPS 25 mg/kg		
Liver	↑6.355*	↑3.01**
Muscle	↑2.246	↑1.702
EF	↑5.211**	↑2.11
PRF	↑4.372***	↑3.103***
SCF	↑2.126*	↑2.239**
Small Bowel	↑3.714	↑3.08*
Spleen	↑2.799**	↑3.239***
Cells - 3T3-L1: LPS		
1 µg/ml	↑1.70	↑1.66
5 µg/ml	↑1.54	↑2.37*
10 µg/ml	↑1.43	↑1.18
Cells - 3T3-L1: Cytokines		
TNF-α 10 ng/ml	↑1.95*	↑1.65
IL-6 10 ng/ml	↓1.4	↓1.85
Cells C2C12: LPS		
0.1 µg/ml	↑3.19***	↑2.47***
1 µg/ml	↑1.74**	↑2.1**
5 µg/ml	↑1.79***	↑3.41**
10 µg/ml	↑2.69***	↑2.31***

Table 47: Fold changes of HIF-1α receptor gene expression only with direction of change in all samples (Mouse tissues, 3T3-L1 adipocytes and C2C12 myocytes) (* denotes statistical significance (*p<0.05, **p<0.01, ***p<0.0001))

Our results clearly show an up-regulation of HIF-1α gene expression in liver, SCF, PRF and spleen tissue depots at both 4 and 24 hour time points (Figure 47). There is up-regulation in EF at 4 hours, small bowel at 24 hours only but no significant up-regulation in muscle tissue at either time point. It is difficult to assess whether this is due to hypoxic or non-hypoxic up-regulation. Richard et al. have successfully demonstrated that a transcriptional up-regulation represents non-hypoxic accumulation of HIF-1α[165]. Therefore, it can be hypothesized that non-hypoxic up-regulation may play a major role in the response of adipose tissue and adipocytes but also liver and splenic tissue to LPS. In addition to LPS, a number of other mediators involved in the inflammatory process, such as Insulin, thrombin, cytokines and NO, can have the same effect on HIF-1α and therefore may also be implicated (Table 48).

Table 48: Known non-hypoxic stimuli of HIF-1α in different cell types

Stimuli	**Cell type**
Insulin	Retinal epithelial cell[162] human hepatoma cells (Hep G2)[163]
Insulin like growth factor (ILGF) 1 and 2	Human colon carcinoma cells[244] Mouse embryonic fibroblast cells[245]
Thrombin	Vascular smooth muscle cells[165 246]
Angiotensin II	Vascular smooth muscle cells[164 165],
PGE2	Human prostate cancer cell line[247]
Cytokines	rat enterocytes[166], human hepatoma cell (Hep G2)[248]
Nitric oxide (NO)	Proximal tubular LLC-PK1[249 250], human embryonic kidney cells (HEK293)[251]
TNFα	Proximal tubular LLC-PK1[250],
LPS	Mouse Macrophages[158 168], human monocytes[153], hepatocytes[167]

Our group has previously demonstrated increased HIF-1α gene expression in SCF and EF, 4 hours after LPS treatment in the same mouse model, with a prolonged effect seen after 24 hours[12]. Protein concentrations were elevated after 4 hours by 2-3-fold, but there were no changes at 24 hours. Transcriptional up-regulation followed by increases in protein levels as demonstrated in this paper is the classically described non-hypoxic activation pattern[153 164] (Table 49). NFκB activation has been demonstrated as a potential signalling pathway for non-hypoxic HIF-α up-regulation and its activation can explain the increase in gene expression after LPS treatment[153 173 179].

Table 49: Changes in HIF-1α mRNA and protein under hypoxic and non-hypoxic conditions

HIF-1α	mRNA	Protein
Hypoxic effects	↔ or ↓	↑2° reduced breakdown
Non-hypoxic effects	↑	↑ 2° increased transcription

In continuation of these experiments, protein estimation using ELISA was undertaken. Numerically, hepatic tissue produced the greatest amount of HIF-1α (Figure 48). In hepatic tissue, at four hours, despite a 6-fold increase in HIF-1 α gene expression, there was no subsequent rise in HIF-1α protein. By 24 hours, there was a rise in both protein and mRNA. The increase in both gene expression and protein concentrations may represent a transcriptional up-regulation in the liver consistent with non-hypoxic up-regulation of HIF-1α. In skeletal muscle, no statistically significant changes in gene expression were observed but there was an up-regulation of protein levels at 4 hours post LPS treatment. This may imply that skeletal muscle expression of HIF-1α is unaffected by LPS as demonstrated by a lack of increased transcription. Peripheral skeletal muscle is prone to malperfusion during sepsis, while perfusion to central organs is maintained. The results presented in this thesis indicate that skeletal muscle may be sensitive to the resulting hypoxia, which is reflected by a rise in protein concentrations without increased gene expression, (i.e. by prevention of the breakdown of HIF-1α protein). In splenic tissue, despite statistically significant rises in HIF-1α gene expression, there was no change in protein concentration. This result supports previous

observations in murine macrophages where HIF-1α mRNA but not protein, accumulates following bacterial infection, suggesting that an additional hypoxic stimulus, in addition to the inflammatory stimulus, is required[140]. These results should be interpreted with caution in view of the small numbers within each group.

HIF-1α gene expression was also investigated in isolated 3T3-L1 adipocytes treated with varying concentrations of LPS and pro-inflammatory cytokines (Table 44 & Figure 49). The results varied between the different concentrations of LPS and the two time points. LPS had little effect on adipocytes with a small isolated up-regulation after 24 hours treatment with 5 μg/ml LPS. Interestingly, there was significant up-regulation after treatment with TNF-α but not with IL-6. Thus, in isolated 3T3-L1 adipocytes, LPS may not activate HIF-1α but adipocytes may still respond to other inflammatory mediators, such as TNF-α.

This may indicate a difference between the in-vivo and in-vitro experiments. In-vivo, other inflammatory stimuli, such as hypoxia, LPS and/or hyperinsulinaemia may contribute to increased HIF-1α gene expression. There are reports of hypoxic stabilisation of HIF-1α which is augmented by the addition of LPS[153] . Cells kept under normoxic conditions may not respond with increased expression. It remains unclear why IL-6 does not cause an up-regulation in 3T3-L1 adipocytes whereas TNF-α does.

Macrophage infiltration into adipose tissue during inflammation has been previously demonstrated[252 253]. It is possible that the increased in HIF-1α gene expression observed in the presented experiments, is due to macrophage infiltration of the tissues. Leuwer et al. however, demonstrated no increase in macrophage markers in adipose tissue in the same murine model of

endotoxaemia as used in the present study[12]. Therefore, macrophage infiltration and subsequent contribution to the HIF-1α gene transcription appears unlikely.

HIF-1α may represent a critical pathway within the inflammatory response. Interestingly, whilst having a favourable effect on bacterial killing and phagocytic function, increased cytokine production and endothelial binding may have detrimental effects systemically on the whole animal by prolonging and augmenting the inflammatory response. This adds further evidence to the overall complexity of the inflammatory process. HIF-1α may represent a further signalling pathway where different tissues respond in different ways to LPS treatment and systemic endotoxaemia. The combination of pro and anti-inflammatory effects of individual mediators may be one reason why previous attempts at treating sepsis by targeting a single pathway have failed despite promising animal studies[254-256].

CHAPTER 4: GENERAL DISCUSSION

Adiponectin is an adipokine which has been extensively studied since its discovery in 1995. In chronic inflammatory states, e.g. obesity, type II DM and the metabolic syndrome, adiponectin is well documented to be down-regulated[15 28 36 79 91-93]. This series of experiments has demonstrated the down-regulation of the adiponectin system, particularly adiponectin and adipoR1 in an acute inflammatory state.

It has also been hypothesized that adiponectin regulation is related more to the amount of and the changes in visceral fat. In sepsis, however, the role of visceral fat has not been studied and thus the results presented here represent first evidence that it plays an important role in the host response to life threatening infection. This may suggest that adipose tissue and adipokines such as adiponectin may play key roles in the derangement of glucose metabolism and insulin resistance associated with infection. It is also possible that different types of WAT may have differing endocrine roles within the inflammatory state[203].

A difference in the regulation of adipoR1 and adipoR2 also appears to be also emerging. The two receptors respond differently to different stimuli in obese, diabetic mouse models and in human models[84]. This has not been studied in acute inflammatory states such as sepsis and endotoxaemia but it is likely that there is a link between adiponectin regulation and insulin sensitivity. The results presented here, however, show similar differences between the two receptors, further fuelling the hypothesis of the receptors having different roles. It is possible that the changes demonstrated in this work are a reflection of changes in insulin sensitivity in insulin-sensitive tissues rather than an adipose-specific effect. These experiments are not able to show this conclusively as no measure of insulin sensitivity was performed.

4.1 The role of the adiponectin system in sepsis

Our results indicate that the adiponectin system as a whole is altered and predominantly down-regulated in acute endotoxaemia in mice and humans. The impact on the whole animal is likely to be manifold and the most important ones will be discussed here.

1. Hyperglycaemia and insulin resistance

Hyperglycaemia has been intensively studied in the realms of sepsis and increases mortality following sepsis and septic shock[257]. Whilst criticised by some as not being reproducible, the study by Van den Berghe et al. demonstrated clear reductions in mortality from treating hyperglycaemia, and resulted in a worldwide change in practice[257]. Hypoadiponectinaemia could contribute to hyperglycaemia and insulin resistance and potentially increase mortality (Figure 52)), as has been demonstrated in animal studies[104 105]. The results from our clinical study clearly demonstrate that an improvement in clinical condition is associated with increased plasma adiponectin levels. Whilst no causation can be attributed, in clinical practice insulin resistance is known to improve with resolution of sepsis and raising adiponectin levels may facilitate normalisation of glucose metabolism. This process, however, is likely to be multi-factorial with changes in catecholamines, inflammatory cytokines and glucocorticoids as contributing mechanisms.

Figure 52: Hypothetical overview of the role of cytokines and LPS in insulin resistance in adipose tissue

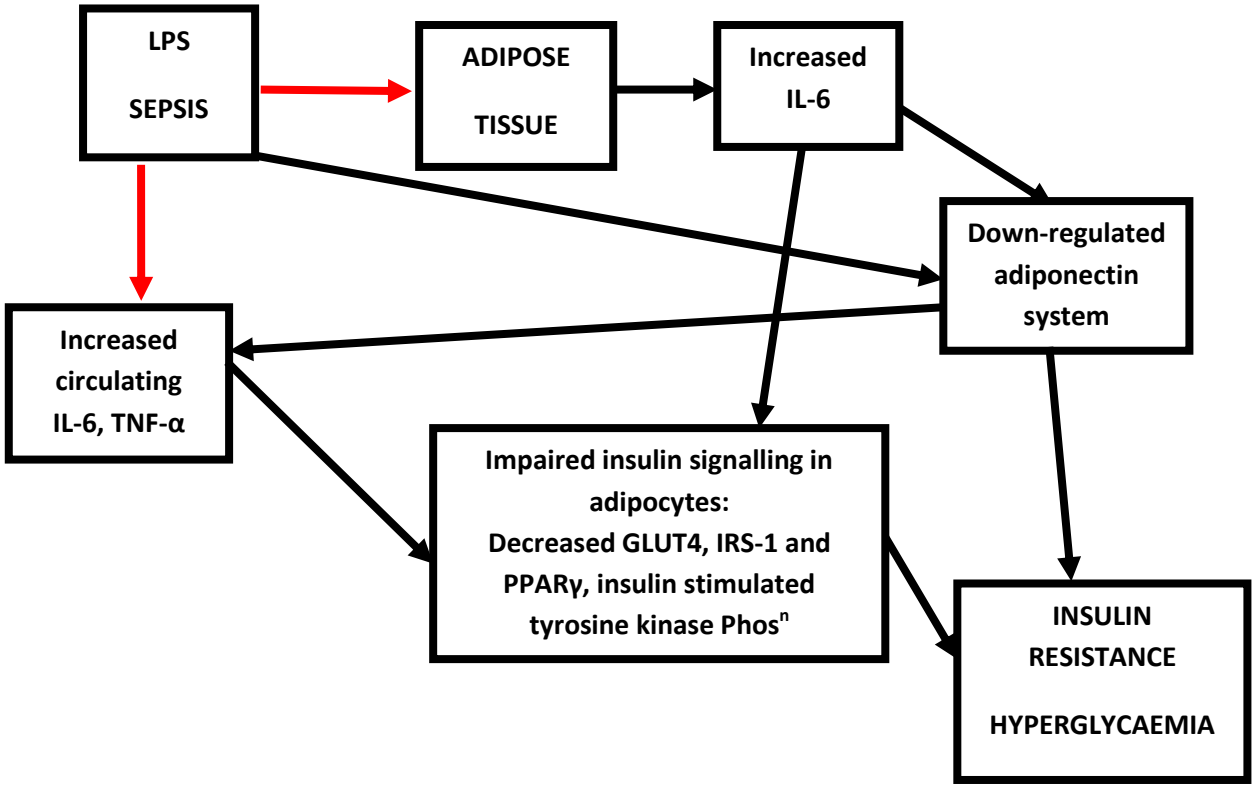


Figure 52: LPS instigates a bi-directional pathway. It stimulates the inflammatory response and subsequent release of pro-inflammatory cytokines from immune cells e.g. macrophages[53]. These cytokines cause impaired insulin signalling in many tissues including adipose tissue[208]. LPS also triggers the inflammatory response in adipose tissue causing a release of inflammatory markers[12] which further fuel this process. In addition, LPS causes down-regulation of the adiponectin system in adipose tissue, thereby reducing fatty acid metabolism and increasing hepatic glucose output. All of these factors contribute to hyperglycaemia and insulin resistance. Hypoadiponectinaemia also contributes to the increased cytokine release and potentiates of the inflammatory response further.

2. Inflammation

Reductions in adipose and plasma adiponectin with sepsis also are associated with a concurrent increase in IL-6[12 105 116], TNF-α[12 103 105 116] and endotoxin levels[103 105] in humans and animals with varied models of endotoxaemia. Interestingly, plasma IL-6 levels, which would normally peak around 4-6 hours in a polymicrobial sepsis mouse model (CLP) and then decline (Figure 53) remained elevated in adiponectin KO mice but not in control mice[105]. This suggests that adiponectin has a role in dampening the pro-inflammatory and promoting the anti-inflammatory response, eventually leading to a much higher mortality in KO mice compared to control animals.

IL-6 is also known to induce insulin resistance in adipocytes and impair insulin signalling[208 258-261]. Thus, the vastly increased IL-6 production from macrophages, lymphocytes, neutrophils and adipose tissue in endotoxaemia may contribute to impaired insulin signalling and alterations in adiponectin receptor expression. In addition, adiponectin is known to reduce the release of inflammatory cytokines from inflammatory cells and therefore, its down-regulation may aggravate or at least maintain the inflammatory response[100 106 108].

Figure 53 shows the well documented time course of cytokine release following a septic innoculum with the results from this thesis superimposed on the graph.

Figure 53: Hypothetical overview of the temporal relationship between pro and anti-inflammatory cytokines and adiponectin and its receptor levels

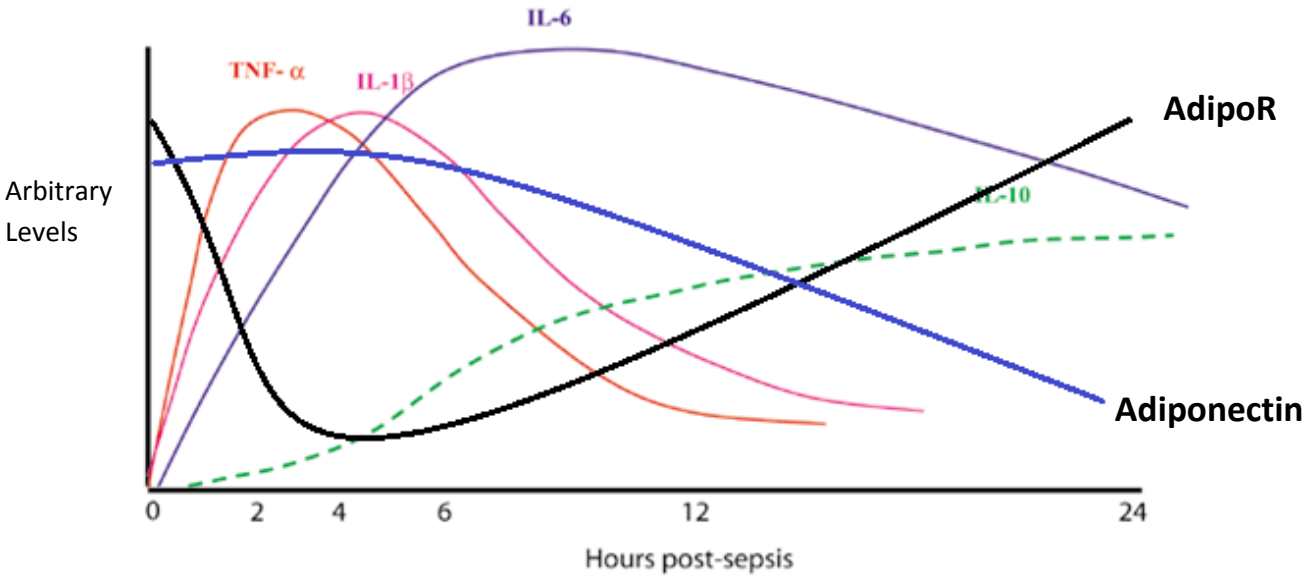


Figure 53: Overview of the temporal relationship between pro- and anti- inflammatory cytokines and adiponectin as suggested by this thesis. The early peak of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) may coincide with reduced levels of adipoR in adipose tissue, and skeletal muscle. In adipose tissue the decline in adiponectin appears later. (figure adapted from Boontham et al. 2003)[53]

In skeletal muscle, systemic inflammation has been shown to produce a local release of pro-inflammatory cytokines[218] . This paracrine effect may account for the continuing response observed in skeletal muscle.

Other known anti-inflammatory properties of adiponectin include inhibition of TLR and NFκB signalling[120 262] suppression of phagocytosis and neutrophil aggregation, inhibition of macrophage recruitment[106] and suppression of cell adhesion molecules[104 110 111].

Systemic hypoadiponectinaemia has been shown to be associated with increased plasma endotoxin levels[103]. This, perhaps contributed to by reduced endotoxin neutralisation[103 114], will likely augment the inflammatory response. The combination of increased pro-inflammatory and reduced anti-inflammatory effects secondary to hypoadiponectinaemia could further contribute to the higher mortality and increased inflammatory response observed with hypoadiponectinaemia in animal studies[104 105].

4.2 Clinical studies

Few clinical studies have been performed on the role of adiponectin in sepsis and endotoxaemia in humans. Two volunteer studies used intravenous injections of endotoxin or LPS to investigate the change in adiponectin multimers[115 116]. Keller et al demonstrated rises in core body temperature, large increases of TNF-α and IL-6 but no corresponding decrease in plasma adiponectin levels[115]. Anderson also confirmed rises in inflammatory cytokines in WAT and plasma and found no change in the multimer composition of plasma adiponectin[116]. Interestingly, Anderson et al demonstrated reductions in both adipoR1 and adipoR2 gene expression by 33% and 28% in monocytes at 24 hours, suggesting a change in adiponectin receptor expression earlier than seen with adiponectin.

The volunteer human studies do not show changes in adiponectin with endotoxaemia possibly because severe sepsis is impossible to mimic in volunteers. The subjects display signs of mild systemic endotoxaemia and therefore represent the clinically milder end of the sepsis spectrum, which may not be associated with disordered glucose metabolism and overwhelming infection. Adiponectin receptor gene expression is, however, reduced in these individuals indicating that either early changes in the adiponectin system or that lower doses of LPS or endotoxin affect receptor expression. This would be consistent with the results of this study where more rapid changes in adipose tissue receptor gene expression were observed compared to adiponectin (Figures 13 & 14).

A further study, using a small number of samples from a heterogenous cohort of critically ill patients, showed a much lower mean plasma adiponectin concentration in critically ill patients and showed a strong positive correlation with plasma cortisol on day 3 and 7[117]. There was no correlation between plasma adiponectin with severity of illness scores, similar to from the clinical study. This may represent a link to glucocorticoid regulation pathways which are fundamental for maintenance of vascular reactivity in sepsis. Although this study provides further information on adiponectin in the critically ill patient, the study was not initially designed to measure adiponectin and in addition, the group of patients was highly heterogeneous and male dominated. Due to lack of samples, there was only analysis on days 3 and 7 and no baseline samples for analysis.

4.3 High molecular weight adiponectin

High molecular weight (HMW) adiponectin is a known biomarker for metabolic diseases and potentially more active than full length adiponectin in promoting insulin sensitivity[76 80]. Reductions in HMW adiponectin levels have been demonstrated in patients with hypertension, hypercholesterolaemia, BMI>25 kg/m^2, abdominal obesity and hyperglycaemia[75]. There is little data regarding HMW adiponectin in human septic patients. Volunteer studies utilising low dose endotoxin failed to demonstrate changes in HMW adiponectin. Hillenbrand et al., however,

demonstrated a significant reduction in septic patients compared to healthy blood donors[263]. This was inversely correlated to APACHE II score and BMI (although weight was estimated). Hypoadiponectinaemia was associated with significant increases in the plasma levels of other adipokines and cytokines such as IL-6, IL-8 and IL-10, PAI, MCP-1 and TNF-α.

In contrast with the results from the clinical study, *increases* in HMW adiponectin levels on day 1 of sepsis compared to controls have been demonstrated in children with sepsis and septic shock[118]. This is unexpected as it contradicts much of the work investigating adiponectin. Potential confounding factors are that there is no gender data for the subjects and both adiponectin and HMW adiponectin are known to be higher in female adults[75 80 97 98]. The control group consisted of children who underwent cardiac catheterisation. This cohort may have underlying alterations in the adiponectin system, as chronic cardiac disease in adults is known to alter adiponectin levels[100]. This, however, has not been established in paediatric cardiac disease.

The increases in plasma adiponectin levels demonstrated were accompanied by an improvement in clinical condition such that intensive care was no longer necessary (Figures 44-46).

In this study, patients acted as their own controls, therefore allowing meaningful comparisons to be made between critical illness and recovery. HMW adiponectin, therefore, may be implicated in the inflammatory process by contributing to decreased cytokine production, as is observed in obese patients[263].

All these results taken together provide further evidence of the down-regulation of anti-inflammatory signalling pathways in early sepsis.

For many years, the signalling pathways of the anti-inflammatory effects of adiponectin have remained largely unknown. In recent studies it has become clear that two intracellular signalling

pathways, NFκB and HIF-1α, both intricately linked to inflammation and to adiponectin receptor regulation and are crucial in mediating the downstream effects of receptor activation. They will be discussed here:

4.4 The potential role of NFκB

NFκB is one of the master regulators of inflammation. As the role of adiponectin in the inflammatory pathway is becoming more defined, a number of studies have investigated the role of adiponectin on the NFκB pathways. Predominantly this has been studied in the context of endothelial cells and endothelial dysfunction in vascular disease[262 264-266] and macrophages[120]. These studies have also demonstrated that the NFκB stimulated production of pro-inflammatory cytokines (IL-8, IL-18) was inhibited by adiponectin via the AMPK signalling pathway[264-266]. In differentiated macrophages, a target molecule has been proposed that works between NIK and IKB (Figure 54)[120].

In adipocytes, the signalling pathway of NFκB has also been investigated. Ajuwon et al. demonstrated that LPS induces nuclear translocation of NFκB in pig adipocytes[267]. In addition, the same group demonstrated that adiponectin over-expression reduced the LPS stimulated NFκB nuclear translocation, both in primary cell culture (porcine adipocytes) and in the cell lines used in this thesis, murine 3T3-L1 adipocytes[268]. This was accompanied by a reduction in pro-inflammatory cytokine release.

It is therefore possible that hypoadiponectinaemia, seen in many models of sepsis and acute infection, may result in reduced suppression of the NFκB pathway, leading to increased endothelial dysfunction, increased and sustained cytokine production and the potential for increased mortality. Increased pro-inflammatory cytokines, particularly IL-6, are known to be associated with increased mortality in human[269 270] and animal studies[271].

Figure 54: The NFκB Pathway with the postulated site of action of adiponectin

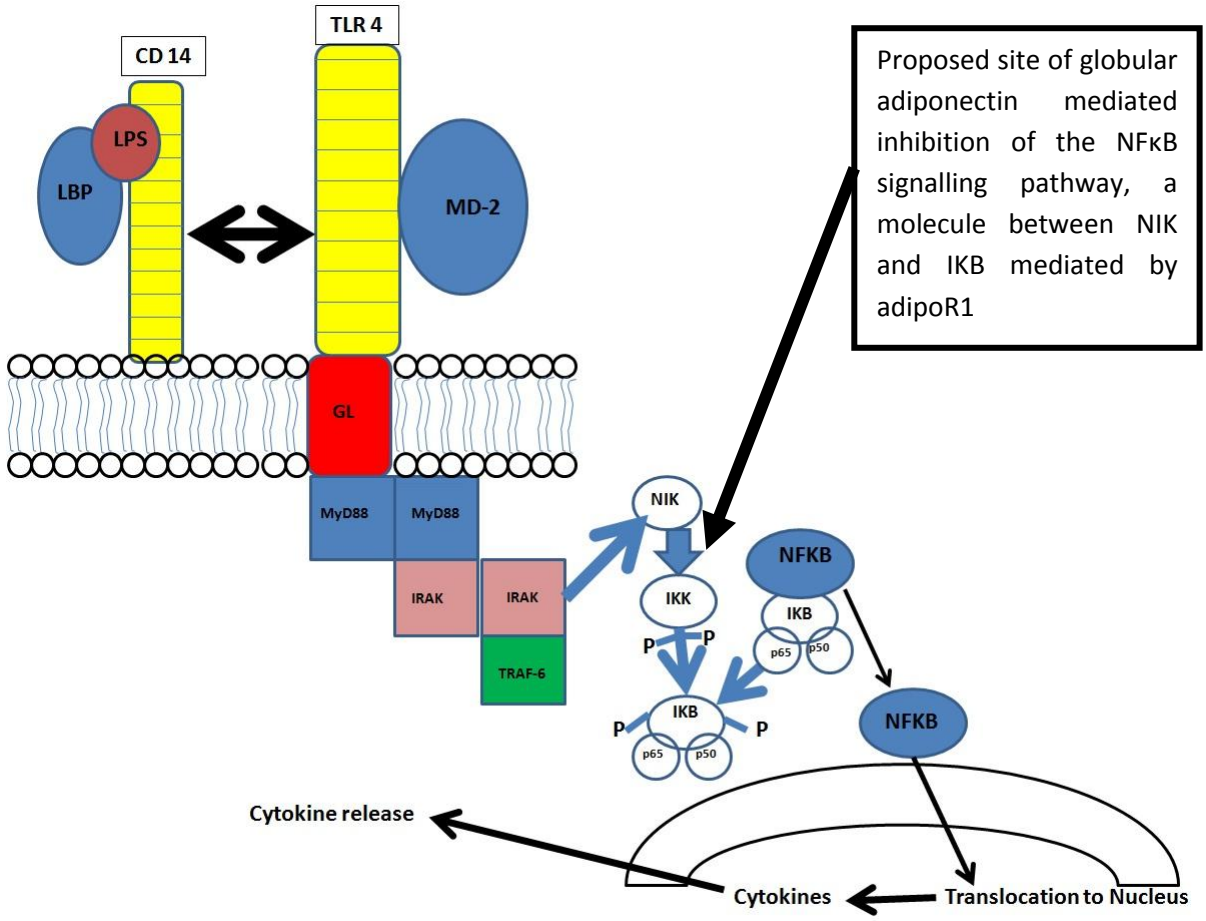


Figure 54: The NFκB Pathway. Globular adiponectin, known for its anti-inflammatory properties, inhibits LPS-induced TLR signalling in mouse differentiated macrophages (RAW264). The proposed site of adiponectin is between NIK and IKB. This phenomenon is mediated by adipoR1[120].

4.5 The potential role of HIF-1α

The results presented here demonstrate elements of an emerging complex interaction between the roles of LPS, inflammation and hypoxia and the HIF-1α signalling pathway. The transcriptional up-regulation of HIF-1α has been demonstrated in the models of systemic and in-vitro endotoxaemia, thus implying a non-hypoxic stimulation of HIF-1α.

Hypoxia was described as the predominant up-regulator of HIF-1α in the original studies investigating the significance of erythropoietin[143]. HIF-1α has subsequently been demonstrated to be up-regulated by non-hypoxic stimuli of different types[153 158 164 165 167 168]. In addition, there is now a well established link to the NFκB pathway, which in turn is intricately associated with inflammation[272 273].

4.5 HIF-1α and inflammation

HIF-1α is involved in the inflammatory process in many ways including immune cell extravasation into hypoxic tissues during inflammation[171]. This is an essential part of the inflammatory cascade as inflamed tissues are profoundly hypoxic; therefore the survival of immune cells in these tissues relies on HIF-1α to increase glycolysis and thus provide energy substrates.

Numerous studies have identified the role of HIF-1α in inflammation utilising HIF-1α null macrophages. In these cells, myeloid cell bactericidal activity is reduced[144 171]. Granule proteases from neutrophils, an important component of cell anti-microbial activity, are reduced in the absence of HIF-1α[144]. Mice with HIF-1α null macrophages showed significantly larger necrotic skin lesions, greater weight loss and much larger bacterial colonies within the skin (area of inoculation), blood and spleen following a subcutaneous innoculum known to result in necrotising soft tissue infection[144]. A further study by the same group also showed that mice with a targeted deletion of

HIF-1α showed significant protection against LPS induced hypothermia and hypotension which corresponded to a greater survival rate[168]. The effects of HIF-1α deletion are summarised below (Figure 55):

Figure 55: Effects of targeted deletion of HIF-1α in macrophages

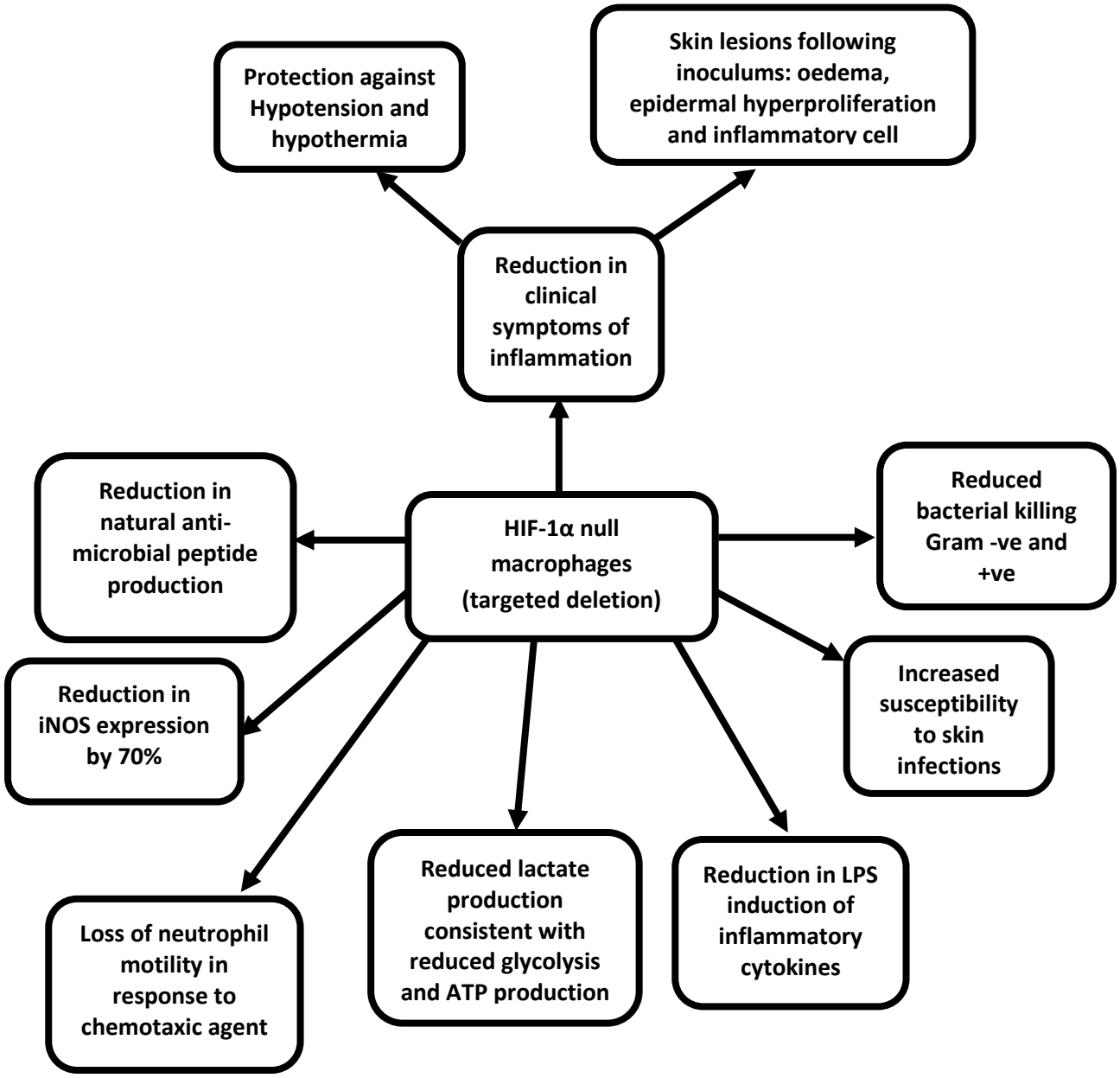


Figure 55: Targeted deletion of HIF-1α macrophages allow identification of the numerous ways HIF-1α interacts with the inflammatory process. These include protection from clinical symptoms[144 168 171], Reduction in antibacterial compound and iNOS production[144], reduced bacterial killing [141 171], loss of neutrophil motility[171], reduced lactate production[171], and reduction in inflammatory cytokines[168 171]

Sepsis is characterised by global tissue hypoxia[139 274]. The HIF-1α pathway stimulated by hypoxia promotes bactericidal killing and protection from clinical symptoms of inflammation. It is also evident that the HIF-1α pathway is up-regulated by inflammatory mediators. This is in agreement with the results presented in this thesis, demonstrating a transcriptional upregulation in HIF-1α which suggests that the likely stimulus to be inflammatory rather than hypoxia.

Interestingly a link between HIF-1α and NFκB activation has also been described. Gram-negative and -positive bacterial species, in addition to LPS, induce HIF-1α mRNA in immune cells in normoxic and hypoxic conditions via NFκB mediated pathways[140 153]. This has also been demonstrated in human embryonic kidney cells exposed to TNF-α[170]. Further immunoprecipitation experiments demonstrated that Rel A is recruited to the HIF-1α promoter, which contains an NFκB binding site.

NFκB may form a link between inflammation, sepsis with its associated cellular hypoxia and HIF-1α, particularly as non-immune cells are also known to induce HIF-1α under inflammatory conditions[170]. HIF-1α could therefore provide a link between two of the inherent survival mechanisms of the body: the response to hypoxia and bacterial invasion. It is likely that the two mechanisms are in some way combined in acute inflammation. There are reports in the literature of hypoxic stabilisation of HIF-1α which is further increased by the addition of LPS[153]. It may be that hypoxia, in-vivo, is the stimulus for increased gene expression and thus cells kept at normoxia may not respond with increased expression. HIF-1α is therefore no longer, merely a response to cellular hypoxia, but is intricately involved in the inflammatory process. It is clearly involved in phagocytic mobility and function and the cytokine response to bacterial invasion. Interestingly, this has also been correlated to clinical symptoms and mortality in mice[168].

The implications of increased HIF-1α in adipose tissue are complex. We have shown that HIF-1α can be up-regulated under normoxic conditions in response to LPS. This may imply a contribution of adipose tissue to the inflammatory process which previously has not been identified.

It is known that in hypoxic adipose tissue, insulin signalling pathways in adipocytes are inhibited by reducing insulin receptor phosphorylation[275] and HIF-1α is activated, leading to the expression of many hypoxia responsive genes (VEGF, GLUT-1 and leptin)[276]. It could be hypothesized that this will have major implications on metabolic pathways, inducing insulin resistance, and on inflammatory pathways. Hypoxia alone induces the release of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) in adipocytes grown in culture[142 277]. In addition to the widely described reduction in adiponectin in chronic inflammation, Ye et al. described reduction in adiponectin mRNA and protein in response to hypoxia [277].

It therefore appears that these interrelated pathways may ultimately control inflammation and the associated metabolic changes. Hypoxia-induced and bacterial stimulation of HIF-1α together with the concomitant reduction in adiponectin have a major effect on the stimulation of the inflammatory process, as the combination of both may contribute to the overwhelming inflammatory process commonly observed in sepsis.

4.6 Potential clinical consequences of adiponectin in sepsis

4.6.1 Potential pharmacological agents modulating adiponectin

Numerous anti inflammatory agents have been used in the treatment of sepsis and septic shock. These include anti-TNF-α antibodies, IL-1 receptor antagonist and anti-endotoxin antibodies[254 256 278 279]. Success rates have been poor, despite reasonable success in animal models, with some showing increases in mortality. Postulated reasons for this include patient heterogeneity with respect to age and gender but also infection source and disease severity.

There are several potential therapeutic targets for adiponectin pathways:

Exogenous Adiponectin

Exogenous adiponectin has been shown to reverse many of the known metabolic effects on hypoadiponectinaemia[31 32 36 99] and is therefore is an attractive therapeutic target. Administration of exogenous adiponectin may be limited by the tertiary and quaternary structure of adiponectin having differing effects on different tissues[100]. One study demonstrated that pre-treatment with recombinant adiponectin in adiponectin KO mice with chemical peritonitis demonstrated reversal of the increases in inflammatory markers, cell adhesion molecules and neutrophil aggregation[104]. Interestingly, low molecular weight adiponectin (trimeric adiponectin) had no effect on the marker levels.

The use of pre-treatment with exogenous adiponectin in acute inflammatory conditions, such as sepsis, may be limited as it would require treatment prior to the infective insult. There may a role in reducing cases of recurrent sepsis in specific conditions however, these are difficult to predict.

Increasing endogenous production via lifestyle changes such as weight loss through caloric restriction may also be an option in the chronic disease population[280]. However, this will not be feasible in patients with acute inflammatory conditions such as sepsis.

Thiazolidinediones

A number of drugs have been shown to enhance adiponectin secretion. The most widely studied are the thiazolidinediones but angiotensin converting enzyme inhibitors (ACEI) and angiotensin 2 receptor blockers (ARB) also lead to increased adiponectin levels[281 282]. Thiazolidinediones are frequently used orally in the treatment of diabetes and are PPARγ agonists[15 283 284]. It is well documented that they increase the expression and secretion of adiponectin in-vivo[285 286]. They have also been shown to increase or normalise adiponectin expression and secretion in 3T3-L1 adipocytes and adipose tissue from obese mice by activating the adiponectin promoter site[33]. Similar findings have been demonstrated in obese and diabetic humans after three to six months treatment[286-289]. These drugs have not been investigated in human endotoxaemia. However, their use prior to the septic insult (CLP) has demonstrated a significantly improved mortality in wild-type mice[105].

4.6.2 Potential pharmacological agents modulating HIF-1α

It could be an attractive therapeutic strategy to improve antibacterial efficiency and increase the immune response to invading micro-organisms. This could theoretically be achieved using a HIF-1α agonist and could be beneficial in localised controlled infection or inflammation, e.g. skin lesions or arthritic lesions. However, the pathology of early sepsis is characterised by disordered and excessive inflammation. Therefore, it can be anticipated that increasing the immune response in systemic sepsis, with the subsequent increase in the inflammatory activity actually worsen the clinical picture.

4.6.3 Critical illness polymyopathy

It is well known that skeletal muscle plays a significant role in whole body glucose metabolism and is dysfunctional in other diseases characterised by insulin resistance e.g. type II DM. Acquired sepsis-related myopathy is very common clinically and is termed the Critical Illness Polymyopathy (CIPM). It is a multi-factorial pathology comprising mitochondrial dysfunction, alterations in the sarcoplasmic reticulum and reductions in contractile proteins[197 218]. It is likely that inflammatory cytokines play a role as the systemic inflammatory response appears to produce a release of pro-inflammatory cytokines from muscle[218]. Recent studies also suggest a causative role for NFκB and its associated pathways[290] in addition to the known link to hyperglycaemia[257 291 292].

It is an attractive theory that adiponectin could be involved in this process. Krause et al. found that the adiponectin KO mice showed a significant reduction (50%) in peak tetanic force (relative to mass) but no change in rate of muscle fatigue during a 2 minute low frequency stimulation protocol[192]. When the skeletal muscle depots (gastrocnemius/plantaris/soleus complex) in both adiponectin KO and control groups were examined for changes in fibre composition and size, no changes in fibre type were found but there were significant increases in fibre area in adiponectin KO IIB fibres. Staining of the muscle did not show any increase in muscle capillary density. Unsurprisingly as adiponectin is known to metabolise lipids in muscle, there was a significantly greater ICML content in the adiponectin KO mice compared to their WT controls in all muscle fibre types.

Skeletal muscle mitochondrial content is also reduced in adiponectin KO mice[293]. In addition, inhibition of adiponectin signalling blunts the induction of mitochondrial function in human skeletal muscle[293]. This indicates a degree of mitochondrial dysfunction in skeletal muscle associated with hypoadiponectinaemia.

The results presented in this thesis demonstrate the presence of adiponectin in skeletal myocytes and adiponectin down-regulation in the murine model of endotoxaemia. This could provide a link between sepsis and CIPM. Both pathologies are similar with hyperglycaemia, increased inflammatory cytokines and mitochondrial dysfunction being associated with a decreased concentration of contractile proteins, reduced peak force and absence of fatigueability. It poses the question as to whether a lack of adiponectin could be involved in the pathogenesis of critical illness polymyopathy (Table 50).

Table 50: Physical characteristics of CIPM and adiponectin KO mice

Critical illness polymyopathy	**Adiponectin KO Mice skeletal muscle**
Reduced peak force	Reduced peak force
No fatigueability	No fatigueability
Reduced involuntary isometric peak force	
Reduced compound muscle action potential	Increased intramyocellular lipid
Muscle atrophy	No change in muscle length
Mitochondrial dysfunction	Mitochondrial dysfunction
Friedrich et al. 2008[197]	**Krause et al. 2008**[192]

Table 50: Physical characteristics of whole muscle in patients with critical illness polymyopathy and adiponectin KO mice. There are a number of similar characteristics which may link the two.

In addition to this postulated hypothesis, there may be an association with the nutritional changes observed in critical illness such as malabsorption of nutrients and calories and anorexia. The literature however suggests that weight loss increases adiponectin levels in children[294] and adults[295]. In most parts, this is correlated not only with body mass index but also with reduced adiposity and markers of inflammation. It is the norm for critically ill patients to lose fat and muscle mass during their illness. Therefore, it would be interesting to ascertain whether adiponectin levels would rise with weight loss or whether they would remain reduced with the ongoing inflammatory process and whether there is any correlation between adiponectin levels and BMI in septic patients.

4.7 A final hypothesis: The role of sphingolipids

Sphingolipids are bioactive lipids which are produced by way of a condensation reaction between palmitoyl-CoA and serine[296]. Ceramide and glucosylceramide are precursors to complex sphingolipids. Accumulation of sphingolipids is associated with disordered metabolism and results in insulin resistance via alterations in insulin signalling (protein kinase C pathway)[297] [298]. Phosphorylation of ceramide produces sphingosine-1-phosphate which has opposing actions to ceramide[297].

As demonstrated by euglycaemic clamp studies in mice, adiponectin reduces cellular ceramide which results in improved insulin resistance[297], a process which is mediated by adipoR1 and adipoR2[297]. Both receptors possess ceramidase activity and over- and under-expression of adiponectin receptors have been demonstrated to increase and reduce ceramidase activity respectively, which is reversed by the addition of recombinant adiponectin[297].

There are many stimuli causing increased ceramide production and accumulation. These include FFA[42], LPS[299], inflammatory cytokines and components of the NFκB signalling pathway[300]. Hence, adiponectin and its receptors may mediate their effects via LPS-induced NFκB activation with increased ceramide production as a final common pathway to disordered metabolism and insulin resistance. A hypothetical overview can be seen in Figure 56:

Figure 56: Hypothetical interaction between sphingolipids and adiponectin in sepsis and acute inflammation

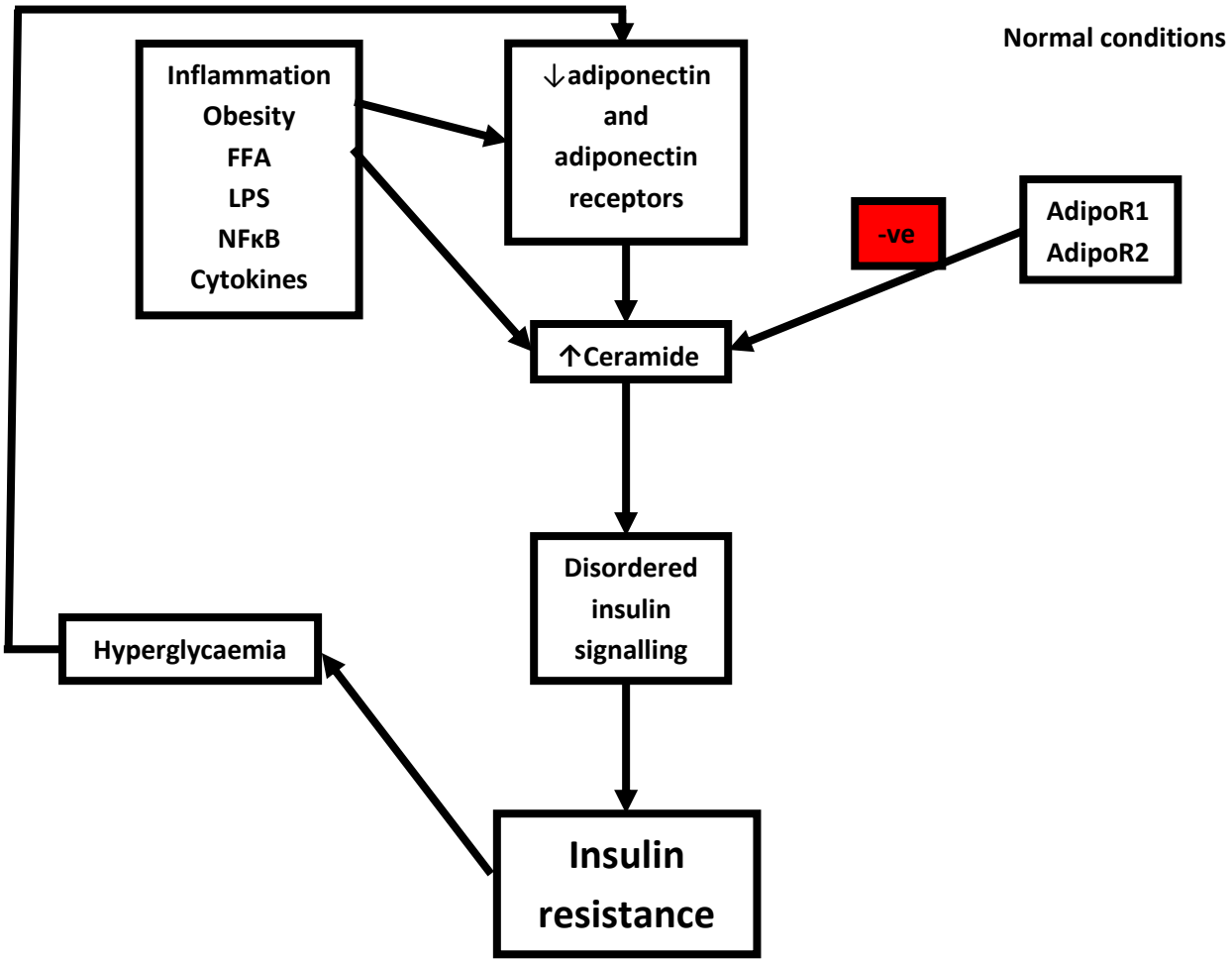


Figure 56: Sphingolipids have been suggested as a link between adiponectin and its numerous metabolic effects in inflammation. Increased ceramide, caused by substances including free fatty acids, LPS, cytokines and components of the NFκB pathway, is known to be associated with disordered metabolism and insulin resistance. Adiponectin, mediated by adipoR1 and R2, reduces ceramide.

:

4.8 Limitations

Mouse model

LPS injection as a model of sepsis is simple to perform and gives generally reproducible results [183]. However, the sharp rise in cytokine concentrations seen following injection occurs much earlier and to a greater magnitude than that found in human sepsis and therefore multiple methods to induce sepsis, including more complex models e.g. caecal ligation and puncture, may have been preferable[181 183 185].

No blood results were taken in the mouse experiments. This was because there was a limited amount of blood available. In addition, samples would only have been available after the death of the animal when tissue harvesting was a priority. Useful results would include measures of glucose and insulin sensitivity (although these would be expected to be initially high in this model)[181]. Cytokine levels would have also been interesting data to collect.

A single high dose (25 mg/kg) of LPS was used to mimic severe sepsis. There are studies that have no or negligible changes in adiponectin with mild endotoxaemia[115 116]. These studies, however, do show changes in adiponectin receptors. Therefore it would have been interesting to investigate a range of doses of LPS in the mouse model as this may have produced differing results.

Adiponectin is known to be regulated by the sex hormones, particularly with testosterone being an inhibitor of adiponectin[77 93-96]. As a result, females are documented to have higher circulating levels of adiponectin which was confirmed in this study. A limitation of the murine model is therefore that only male mice were used. These experiments should therefore be replicated with female mice.

Samples sizes in the mouse model were small to comply with animal protection principles requiring the minimal number of animals to be used. Six mice is a standard number for these experiments, although a larger statistical variation is to be expected. The protein experiments were performed with smaller numbers due to problems in experimental technique; ideally these experiments would have been repeated.

Cell lines

The major limitation of this model is the lack of ability to reproduce the intricacies of different environments within intact mammals[301]. In-vitro experiments only give information about a specific cell type's response to one treatment. For example, LPS was used in both the in-vivo and in-vitro experiments. The in-vivo response may be due to direct cellular LPS effects or alternatively due to systemic derangements such as hypoxia, hyperglycaemia or hyperinsulinaemia. Furthermore, LPS-induced changes in circulating substrate concentrations or inflammatory cytokines may affect the results. Therefore, a negative in-vitro response may not reflect the systemic response to an insult.

There is a lack of data regarding responses of adipocytes and myocytes to other metabolic derangements of sepsis such as hyperglycaemia, hyperlactataemia, hyperinsulinaemia and hypoxia. Further research is warranted to identify the effects of those metabolic disturbances in myocytes and adipocytes.

Clinical study

Obvious limitations include the lack of discharge data from patients who died. This could not be avoided for ethical reasons. However, it limits the sample size for comparisons of discharge data. These samples may have given valuable data regarding the ongoing inflammatory process in patients who did not improve.

In addition, there was no insulin sensitivity data. Whilst giving invaluable information, this is difficult to assess as the fasting status at presentation of septic patients is variable and would therefore not have given consistent results. Patients with type II DM were not excluded and assessment of baseline insulin resistance on admission was not feasible.

A potential criticism of the study was the lack of a true control group to assess the effect of sepsis per se on adiponectin levels. However, the aim of the study was to assess adiponectin levels through the course of the critical illness to compare differences in the acute phase compared to the recovery phase of sepsis.

In a continuation of the in-vitro studies, it would have been beneficial to measure cytokine levels in the septic patients and correlate them to changes in adiponectin levels. This would have clarified whether the changes observed in the animal and cellular models were replicated in the human study.

4.9 Future research

Our main results indicate significant changes in the adiponectin system with the onset of sepsis and endotoxaemia. There are many further research questions that now exist within this field which merit investigation.

For confirmation of the results demonstrated in this thesis, different animal models of sepsis and endotoxaemia should be used. Replication of the experiments using a second model of intra-abdominal sepsis, such as Caecal ligation and puncture which would give data regarding poly-microbial sepsis, would be ideal. It would be essential to obtain blood samples from these animals to assess the systemic inflammatory response and also to assess plasma adiponectin levels. Insulin resistance data would also be very beneficial.

The translation of adiponectin and its receptor gene expression to protein levels now requires to be investigated. This is fundamental to ongoing research as gene expression alone is not adequate to fully ascertain the behaviour of a protein and its receptor. This may require formation of antibodies due to the limited commercially available products.

The signalling pathways of adiponectin remain to be fully elucidated. There is clear evidence that the NFκB signalling pathway is intricately linked to adiponectin in chronic disease and it is an attractive theory that this 'master regulator' of the inflammatory process is also involved in changes observed in the adiponectin system in acute inflammation and infection. This should be further investigated in adipocytes in order to investigate fully the effect of NFκB on adiponectin and its receptors. Experiments should include the use of NFκB and/or TLR receptor antagonists to assess their role in the signalling cascade.

The role of hypoxia, HIF-1α and the adiponectin system in acute inflammation needs further investigation. HIF-1α clearly has a role in the inflammatory process and hypoxic adipocytes show a clear up-regulation in HIF-1α, very similar to the results demonstrated in this thesis. Non-hypoxic up-

regulation of HIF-1α via transcriptional up-regulation is now a recognised signalling pathway. Both activation patterns, hypoxic and inflammatory, need to be further explored to provide insights into intracellular signalling of the adiponectin system.

Acute inflammation is a syndrome comprising of many metabolic disturbances, such as hyperglycaemia, hyperlactataemia, hyperinsulinaemia and hypoxia, all of which may play a role in the regulation of adipokines. A full evaluation of these derangements in conjunction with the increase in inflammatory cytokines and their impact on the adiponectin system is the next step in elucidating the role of the adiponectin system further. Also, the role of any potential therapeutic agents that act upon the adiponectin system needs to be investigated. Therapeutic evaluation should include PPARγ agonists and exogenous adiponectin. There is already preliminary data in mice that PPARγ agonists affect not only adiponectin levels but also mortality, therefore further investigation for its use in humans is required.

The preliminary data presented in this thesis has identified skeletal muscle adiponectin in-vivo and in-vitro and therefore the next step would be to establish the exact location and functional role of adiponectin within the skeletal myocyte.

The subsequent down-regulation of adiponectin and its receptors in skeletal muscle during endotoxaemia also requires further investigation. It would be prudent to repeat the animal experiments in various models of sepsis to assess the changes in the adiponectin system. This will confirm the observed findings and further investigate the hypothesis that the adiponectin system is down-regulated in sepsis. In a translational approach, this should be confirmed using human muscle biopsies.

Speculation to an association of the adiponectin system with critical illness polymyopathy has been highlighted in this work. It would be an interesting line of investigation for future experiments which would need to include investigation of many inflammatory mediators with respect to their effects on

the adiponectin system. These include hyperglycaemia, pro-inflammatory cytokines and the NFκB signalling pathways in skeletal muscle. Further investigation should include skeletal muscle biopsies from patients with critical illness, with and without CIMP. This would allow identification of adiponectin and their receptors within the skeletal muscle and investigation of any differences between those with and without CIMP.

The data presented in this thesis also demonstrate changes in the adiponectin system in septic patients. The clear increase with improvement of clinical condition may represent resolution of the inflammatory process but may also represent a prerequisite for recovery. One of the major limitations of this study is a small patient group with a heterogenous spectrum of sources of infection. A study adequately powered to assess mortality as related to plasma adiponectin levels would be the next step.

A further question that would require investigation is the temporal relationship between the well documented inflammatory process, involving the rise and fall of pro- and anti-inflammatory cytokines, and the adiponectin system. It is possible that the effects we are observing in the animal and cell work is secondary to a sharp peak in pro-inflammatory cytokines. It is therefore essential to measure concomitant plasma pro- and anti-inflammatory cytokines at the same time points. Other known adipokines should also be investigated. Markers of insulin resistance would significantly add to this data but this is appreciated to be difficult in septic patients.

We observed an increase in plasma adiponectin with improved clinical condition. It is likely that this may reflect resolution of the inflammatory cascade. However, it can also be hypothesized that this may reflect recovery of different tissues, including adipose tissue, skeletal muscle, improved nutritional status and resolution of other metabolic derangements, such as hyperinsulinaemia. Adiponectin levels will have to be correlated with other markers of patient recovery, such as acute phase proteins, pre-albumin and albumin levels and vitamin and nutrient levels. This may give important data as to the requirement of adiponectin for the recovery process.

4.10 Conclusions

Adipokines have come to the forefront of metabolic disease and obesity research in the last ten years. They have been widely studied in this context, with the expression and secretion of most increasing directly with increasing adipose mass. Adiponectin is the exception to this rule, with its expression decreasing with increased adiposity and increased markers of inflammation. Adiponectin has many roles including being anti-diabetogenic, anti-inflammatory and anti-atherogenic.

Until recently, adiponectin research has focussed on chronic inflammatory disorders such as obesity and diabetes. However, there is now increasing interest in the investigation of adiponectin in the acute inflammatory setting. Adiponectin, with its multifaceted roles, may play a role in the metabolic disturbances and the inflammatory changes observed in clinical sepsis. The primary research objective in this thesis was to investigate the role of the adiponectin system in various models of sepsis and endotoxaemia. The overall hypothesis, based on background work from acute and chronic inflammation, was that tissue adiponectin and adiponectin receptors were down-regulated in this model.

This thesis contained three main hypotheses: The first was that adiponectin and its receptors are down-regulated in acute inflammation, thus contributing to the disordered metabolic state and to the inflammatory process. Hence, the primary aim was to investigate adiponectin and its receptors in *in-vivo* and *in-vitro* models of sepsis and endotoxaemia.

The second hypothesis aimed to assess plasma adiponectin levels in human septic patients, to identify whether adiponectin expression in cell lines and in mouse models corresponds to changes in human patients. Finally, signalling pathways, in particular, the role of Hypoxia Inducible Factor -1α (HIF-1α) in response to LPS was investigated. Numerous links between adiponectin and NF-κB signalling have been demonstrated. NF-κB is an important regulator of hypoxia-inducible factor 1-α,

a transcription factor up-regulated by non-hypoxic stimuli such as LPS in addition to hypoxia. It was therefore mandatory to further elucidate the link between inflammation, adiponectin and HIF-1α in adipose tissue and other organs linked to adiponectin signalling.

Following treatment with LPS, adiponectin expression, adiponectin expression was down regulated in all depots adipose tissue. This was replicated in adiponectin receptor expression in skeletal muscle, liver and peri-renal fat, however, changes in receptor expression were faster and were not sustained to the second time-point investigated. This may represent changes in perfusion of the tissues or differences in visceral and non-visceral fat. Interestingly, adiponectin gene expression was also down-regulated in skeletal muscle, both acutely and sustained to the second time-point. The presence of skeletal muscle adiponectin was subsequently confirmed in isolated C2C12 myocytes using standard PCR and gene product sequencing.

In the second part of the study, isolated 3T3-L1 adipocytes and C2C12 myocytes were grown in culture and adiponectin and adiponectin receptor gene expression was investigated. Cells were treated with LPS and inflammatory cytokines. Following LPS treatment, there was a clear down-regulation of adiponectin but the receptor expression only showed a consistent response with adipoR2. C2C12 myocytes, again, responded with a down-regulation of adiponectin but minimal significant changes in receptor expression. Interestingly, the response to individual cytokines was different again with no change with adiponectin or adipoR2 gene expression with either IL-6 or TNF-α, however, IL-6 down-regulated adipoR1 gene expression.

In the third part of the study we investigated the adiponectin system in septic patients. As previously described adiponectin levels were higher in women. We further demonstrated that adiponectin and HMW adiponectin significantly increased with clinical improvement of sepsis. Interestingly, the ratio between HMW adiponectin and total plasma adiponectin increased also. HMW adiponectin and the

HMW/total ratio have been identified as potential biomarkers in the development of chronic inflammatory disease such as type II DM. This has not previously been demonstrated in human septic patients.

In an initial attempt to elucidate one of the signalling pathways associated with adiponectin regulation, we investigated the role of HIF-1α. HIF-1α is known to be a transcription factor that regulates many of the intracellular changes occurring secondary to hypoxia. More recently it has been identified as a regulator of the inflammatory cascade. We demonstrated that HIF-1α gene expression was up-regulated in liver and spleen, in addition to the adipose tissue depots but not in skeletal muscle. This was accompanied by up-regulation in protein levels in liver and muscle. Hypoxia-induced up-regulation of protein is not usually accompanied by a transcriptional up-regulation, as observed in the skeletal muscle, whereas both transcription and translation were increased in liver tissue, thus implying a non-hypoxic mechanism of regulation.

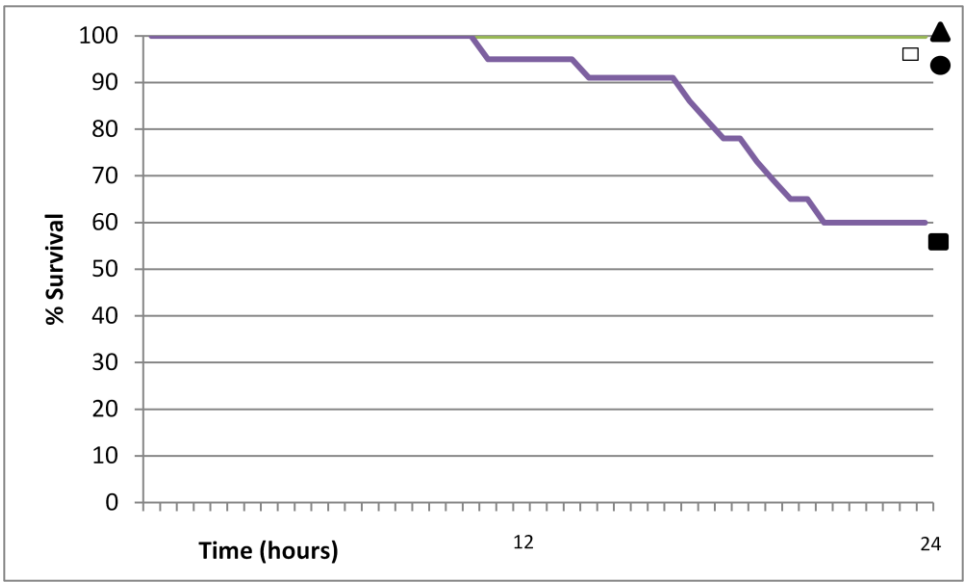
This series of experiments has improved knowledge of adiponectin and its receptors in acute inflammation. There is a paucity of data currently on adipokines in sepsis and this is an interesting field which should be investigated further. Future research should include therapeutic targets which could be used to augment the adiponectin effects, which are potentially beneficial in acute inflammatory conditions including human sepsis.

CHAPTER 5: APPENDICES

Appendix 1: Baseline Mouse data and survival curve

Mouse no	Weight	Exp Length (h)	Group	Survival	Other Information
1	24g	24	LPS	Y	
2	25.4g	24	LPS	Y	
3	25.5g	24	LPS	Y	
4	24.6g	24	Control	Y	
5	24.6g	24	Control	Y	
6	25g	24	Control	Y	
13	27.5g	24	LPS	Y	
14	23g	24	LPS	Y	
15	24.5g	24	LPS	N	Died 11 hours
16	26.5g	24	Control	Y	
17	24g	24	Control	Y	
18	23g	24	Control	Y	
25	26.6g	24	LPS	Y	
26	23.9g	24	LPS	N	Died 21 hours
27	23.5g	24	LPS	Y	
28	25.4g	24	LPS	N	Died 19 hours
29	24.5g	24	Control	Y	
30	26g	24	Control	Y	
35	27g	4	LPS	Y	
36	22.4g	4	LPS	Y	
37	25.4g	4	LPS	Y	
38	24.9g	4	Control	Y	
41	23.5g	4	LPS	Y	
42	26.5g	4	LPS	Y	
43	21.5g	4	Control	Y	
44	24g	4	Control	Y	
47	25.5g	4	LPS	Y	
48	24g	4	LPS	Y	
49	23g	4	Control	Y	
50	26g	4	Control	Y	
53	24.5g	24	LPS	N	Died 20 hours
54	26.7g	24	LPS	Y	
55	24.8g	24	LPS	Y	
56	24.8g	24	LPS	N	Died 17.5 hours
57	26.4g	24	LPS	Y	
58	24.5g	24	LPS	N	Died 18 hours
59	28.7g	24	Control	Y	No samples taken: Large cystic tumour found
60	25g	24	Control	Y	
61	25.1g	24	Control	Y	
62	26g	24	Control	Y	
63	26.4g	24	LPS	N	Died 14 hours
64	26.4g	24	LPS	Y	
65	25.3g	24	LPS	Y	
66	24.1g	24	LPS	Y	
67	26.4g	24	LPS	N	Died 19.5 hours
68	26.9g	24	LPS	Y	
69	26.5g	24	LPS	N	Died 17 hours
70	25g	24	Control	Y	
71	26.5g	24	Control	Y	
72	25.1g	24	Control	Y	

Survival curve for mouse treatment groups



Survival curve for mice treatment groups ●: 4 hour control, ▲: 4 hour LPS □: 24 hour Control, ■: 24 hour LPS

Appendix 2: Raw Data from Adiponectin and receptor experiments

Raw Data: Adiponectin gene expression in all tissue samples

4 h	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value	**24 h**	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value
Liver	1.82	1.849	0.618	0.618		0.516	0.428	0.234	0.473
Muscle	0.144	0.125	0.007	0.04*		0.033	0.022	0.013	0.0009***
SB	0.584	0.434	0.249	0.625		0.641	0.486	0.276	0.509
Epi fat	1.08	0.48	0.33	0.854		0.273	0.120	0.08	0.01**
PR fat	0.51	0.144	0.112	0.122		0.344	0.119	0.08	0.05*
SC fat	0.761	0.148	0.124	0.40		0.234	0.04	0.03	0.0007***
Spleen	0.361	0.268	0.154	0.208		0.452	0.196	0.137	0.136

Raw data for adiponectin receptor gene expression in all tissue samples

4 hrs treatment (control value =1)										
AdipoR1						**AdipoR2**				
	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value	Fold	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value	Fold
Liver	0.544	0.084	0.073	0.05	↓1.5	0.371	0.084	0.069	0.008*	↓2.7
Muscle	0.102	0.109	0.052	0.017*	↓9.8	0.162	0.43	0.11	0.39	↓6.2
SB	1.163	0.12	0.109	0.521	↑1.16	0.584	0.220	0.159	0.309	↓1.7
Epi fat	0.671	0.260	0.187	0.48	↓1.5	0.543	0.173	0.132	0.24	↓1.8
PR fat	0.627	0.005	0.004	0.0087*	↓1.6	0.231	0.067	0.052	0.0043**	↓4.3
SC fat	0.821	0.270	0.203	0.81	↓1.2	0.348	0.095	0.074	0.041*	↓2.9
Spleen	0.807	0.152	0.128	0.48	↓1.2	1.138	0.184	0.159	0.48	↑1.1

24 hour treatment (control value =1)										
AdipoR1						**AdipoR2**				
	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value	fold	$2^{-\Delta\Delta CT}$	+SEM	-SEM	p-value	fold
Liver	0.614	0.053	0.049	0.09	↓1.4	0.650	0.142	0.117	0.148	↓1.53
Muscle	0.509	0.038	0.036	0.01*	↓1.9	0.448	0.083	0.069	0.05*	↓2.2
SB	0.766	0.168	0.137	0.28	↓1.3	0.629	0.114	0.097	0.05	↓1.58
Epi fat	1.01	0.316	0.241	0.47	=	0.852	0.213	0.17	0.55	↓1.2
PR fat	0.801	0.145	0.122	0.27	↓1.2	0.657	0.187	0.145	0.198	↓1.5
SC fat	0.824	0.211	0.168	0.47	↓1.2	1.058	0.312	0.241	0.98	=
Spleen	0.687	0.072	0.06	0.01*	↓1.5	0.922	0.129	0.113	0.58	↓1.1

Appendix 3: Summary of Western Blotting

Summary of western blots and optimisation

No	Tissue depot	Sample	Protein conc	Block	Dilut^{n}	WCL / MF	heat	1° conc	2° conc	Adipo R1 bands	Description	Adipo R2 bands	Description
4 & 5	Liver	LPS	20 µg	5% milk	1% milk	WCL	N	1/ 1000	1/ 1000	No bands		No bands	
	Liver	LPS	20 µg	5% milk	1% milk	WCL	N	1/ 1000	1/ 2000	No bands		No bands	
	Liver	LPS	20 µg	5% milk	1% milk	WCL	N	1/ 1000	1/ 5000	No bands		No bands	
	Liver	LPS	20 µg	2% BSA	2% BSA	WCL	N	1/ 1000	1/ 1000	No bands		No bands	
	Liver	LPS	20 µg	10% milk	3% milk	WCL	N	1/ 1000	1/ 1000	No bands		No bands	
6	Liver	Cont	20 µg	0.5% milk	0.5% milk	WCL	N	1/ 1000	1/1000	No bands		No bands	
	Liver	Cont	20 µg	0.5% milk	0.5% milk	WCL	N	1/ 1000	1/ 2000	No bands		No bands	
	Liver	Cont	20 µg	0.5% milk	0.5% milk	WCL	N	1/ 1000	1/ 5000	No bands		No bands	
	Liver	Cont	20 µg	2% BSA	2% BSA	WCL	N	1/ 1000	1/ 2000	No bands		No bands	
8	Liver	Cont	20 µg	1% BSA	1% BSA	WCL	N	1/ 1000	1/ 2000		No Block		No Block
	Liver	Cont	20 µg	1% BSA	1% BSA	WCL	N	1/ 1000	1/ 5000		No Block		No Block
	Liver	Cont	20 µg	2% BSA	2% BSA	WCL	N	1/ 1000	1/ 2000		No Block		No Block

No	Tissue depot	Sample	Protein conc	Block	Dilut^{n}	WCL/ MF	heat	1° conc	2° conc	Adipo R1 bands	Description	Adipo R2 bands	Description
9	Liver	Cont x3	20 µg	1% BSA	1% BSA	MF	N	1/ 1000	1/ 5000		No Block		No Block
	Liver	LPS x3	20 µg	1% BSA	1% BSA	MF	N	1/ 1000	1/ 5000		No Block		No Block
	Liver	Cont x3	20 µg	1% BSA	1% BSA	MF	N	1/ 1000	1/ 5000		No Block		No Block
	Muscle	Cont x3	20 µg	1% BSA	1% BSA	MF	N	1/ 1000	1/ 5000		No Block		No Block
10	Liver	Cont x3	20 µg	5% milk	1% milk	MF	N	1/ 1000	1/ 5000	72 kDa	Clean Blot 5 FBs 10 VFBs	72 kDa	Clean Blot 5 FBs 10 VFBs
10	Muscle	Cont x3	20 µg	5% milk	1% milk	MF	N	1/ 1000	1/ 5000	No Bands	Clean blot	43 kDa	Clean Blot 5 VFBs 10VFBs
11	Liver	Cont x3	20 µg	5% milk	1% milk	MF	N	1/ 1000	1/ 5000	No bands	Clean blot	72 kDa	Clean Blot 5 SBs 10MBs
11	Muscle	Cont x3	20 µg	5% milk	1% milk	MF	N	1/ 1000	1/ 5000	No bands	Clean blot	43 kDa	CB 5 SBs 10 SBs

No	Tissue depot	Sample	Protein conc	Block	Dilut^{n}	WCL/ MF	heat	1° conc	2° conc	Adipo R1 bands	Description	Adipo R2 bands	Description
12	Liver	Cont	20 µg	5% milk	1% milk	MF	N	1/ 1000	1/ 5000	No Bands	Clean blot	72 kDa	5 SBs 10 SBs
	Liver	Cont	20 µg	5% milk	1% milk	MF	Y	1/ 1000	1/ 5000	No bands	Clean blot	72 kDa	5MBs 10 MBs
	Liver	Cont	20 µg	3% milk	0.5% milk	MF	N	1/ 1000	1/ 5000	No Bands	Clean blot	72 kDa	5 MBs 10 MBs
	Liver	Cont	20 µg	3% milk	0.5% milk	MF	Y	1/ 1000	1/ 5000	No Bands	Clean blot	72 kDa	5 MBs 10 MBs
	Liver	Cont	20 µg	0.5% milk	0.5% milk	MF	N	1/ 1000	1/ 5000	No Bands	Clean blot	72 kDa	5 SBs 10 VSBs
	Liver	Cont	20 µg	0.5% milk	0.5% milk	MF	Y	1/ 1000	1/ 5000	No Bands	Clean blot	72 kDa	5 VSBs 10 VSBs
	Liver	Cont	20 µg	3% milk	1% milk	MF	N	1/ 1000	1/ 5000	No Bands	Clean blot	72 kDa	5 VSBs 10 VSBs
	Liver	Cont	20 µg	3% milk	1% milk	MF	Y	1/ 1000	1/ 5000	No Bands	Clean blot	72 kDa	5 VSBs 10 VSBs
13	Liver	Cont	20 µg	5% milk	1% milk	MF	N	1/ 500	1/ 2000	No Bands	Clean blot	72 kDa	5 VSBs 10 VSBs
	Muscle	Cont	20 µg	5% milk	1% milk	MF	N	1/ 500	1/ 2000	No Bands	Clean blot	72 kDa 43 kDa	5 VSBs 10 VSBs 5 VSBs 10 VSBs
13.5	Liver	Cont	20 µg	1% BSA	1% BSA	MF	N	1/ 500	1/ 2000		PB		PB
	Muscle	Cont	20 µg	1% BSA	1% BSA	MF	N	1/ 500	1/ 2000		PB		PB

No	Tissue depot	Sample	Protein conc	Block	Dilut[n]	WCL/ MF	heat	1° conc	2° conc	Adipo R1 bands	Description	Adipo R2 bands	Description
			WASH	**SOLU**	**TION**	**CHA**	**NGE**	**D TO**	**TTBS**				
14	Muscle	LPS x3	15 µg	1% milk	0.5% milk	WCL	N	1/ 200	1/ 2000	43 kDa	Poor Blot 5 MBs 10 SBs 20 VSBs 40 VSBs	43 kDa	Clean Blot 5 NBs 10-40 MBs
	Muscle	Cont x3	15 µg	1% milk	0.5% milk	WCL	N	1/ 200	1/ 2000	43 kDa	Poor Blot 5 MBs 10 SBs 20 VSBs 40 VSBs	43 kDa	Clean Blot 5 NBs 10-40 FBs
15					**EXPE**	**RIME**	**NT**	**DID**	**NOT**	**WORK**			
16	Muscle	LPS x3	15 µg	1% milk	0.5% milk	WCL	N	1/ 200	1/ 2000	No Bands	Clean Blot	43 kDa	1, 5 20 VSBs
	Muscle	Cont x3	15 µg	1% milk	0.5% milk	WCL	N	1/ 200	1/ 2000	No Bands	Clean Blot	43 kDa	1, 5 20 VSBs
17	Muscle	LPS x3	15 µg	1% milk	0.5% milk	WCL	N	1/ 200	1/ 2000	No Bands	Clean Blot	43 kDa	1, 5 20 VSBs
	Muscle	Cont x3	15 µg	1% milk	0.5% milk	WCL	N	1/ 200	1/2000	No Bands	Clean Blot	43 kDa	1, 5 20 VSBs
	Liver	Cont x3	15 µg	1% milk	0.5% milk	WCL	N	1/ 200	1/2000	No Bands	Clean Blot	CB	No Bands

Abbreviations 1: 1 minute exposure, 5: 5 minute exposure, 10: 10 minute exposure, 20: 20 minute exposure, 40: 40 minute exposure, CB : clean blot, FBs: faint bands. MBs: medium bands, SBs: strong bands, VSBs: very strong bands, kDa: Kilodaltons, WCL: whole cell lysate, MF: Membrane fractionation

Appendix 4: Human biochemical and haematological test normal values

White cell count (WCC): 4.5-10 x 10^9 cells/L

C-reactive protein (CRP): <5 mg/L

Haemoglobin (Hb): Male: 13.8-18 g/dL, Female: 12-15 g/dL

Creatinine (Creat): 50-130 µmol/L

Urea: 2.5-7 mmol/L

Bilirubin (Bili): 2-17 µmol/L

Glucose (gluc): 3.5-5.0 mmol/L

Lactate: 0.5-2.2 mmol/L

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