Mechanisms of Insulin Resistance in Prenatally Programmed Rats

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Declaration

I declare that this thesis was written by me, and that the data presented within are a result of my own work, except where outlined specifically in the text.

This work has not been submitted for any other degree.

Mark E. Cleasby, BVM&S MRCVS
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M.E. Cleasby, P.A.T. Kelly, M.J. Nyirenda, C.J. Kenyon, B.R. Walker and J.R. Seckl. Prenatal programming of rat adipose tissue and skeletal muscle metabolism by glucocorticoids *(in preparation).*

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M.E. Cleasby, M.J. Nyirenda, C.J. Kenyon, B.R. Walker and J.R. Seckl. Glucocorticoid Receptor expression is programmed in skeletal muscle and liver by prenatal dexamethasone and reduced by insulin-sensitising drugs in adult rats. *Pediatric Research (2001) 50(1 Suppl) pp 1A-68A. (1st World congress on the foetal origins of adult disease).*

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Abbreviations

Ab	Antibody
AMP	Ampicillin
ATP	Adenosine triphosphate
ANOVA	Analysis of Variance
BHB	β-Hydroxybutyrate
B_{max}	Maximal binding capacity (quantity of ligand required to saturate receptor)
bp	base pairs (of nucleic acid)
BP	Blood pressure
BSA	Bovine serum albumin
cAMP	cyclic Adenosine Monophosphate
cDNA	Complementary deoxyribose nucleic acid
CNS	Central nervous system
cort	Corticosterone
cpm	Counts per minute
CV	Coefficient of variation
dATP	deoxyadenosine triphosphate
DEPC	Diethylpyrocarbonate
dCTP	deoxycytosine triphosphate
Dex	Dexamethasone
dGDP	deoxyguanosine diphosphate
dH_2O	deionised water
DNA	Deoxyribose Nucleic Acid
dNTP	deoxyNucleotide triphosphate
dpm	disintegrations per minute
DTT	Dithiothreitol
dTTP	deoxythymosine triphosphate

EDL	Extensor digitorum longus muscle
EDTA	Ethylene Diamine Tetra-Acetate
ELISA	Enzyme-linked Immunosorbent Assay
GLUT-4	Glucose Transporter 4
GR	Glucocorticoid Receptor
GTP	Guanosine Triphosphate
HDL	High Density Lipoprotein
HEPES	(N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid])
HSL	Hormone-sensitive lipase
Hsp	Heat-shock protein
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IPTG	Isopropyl-1-thio-β-D-galactoside
i.u.	international units
IUGR	Intrauterine growth retardation
kb	kilobases
K_d	Dissociation constant (concentration of ligand required to achieve half maximal binding of receptor)
kDa	kiloDaltons
LB	Luria-Bertoni / liquid broth
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
MOPS	(3-[N-Morpholino]) propanesulphonic acid
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NEFA	Non-esterified Fatty Acid
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate

PEPCK	Phosphoenolpyruvate carboxykinase
PMSF	Polymethylsulphonyl fluoride
PPAR	Peroxisome Proliferator-Activated Receptor
RACE	Rapid Amplification of cDNA ends
rATP	Adenosine triphosphate
rCTP	Cytosine triphosphate
rGTP	Guanosine triphosphate
RNA	Ribose Nucleic Acid
RPA	Ribonuclease Protection Assay
rpm	Revolutions per minute
RT	Reverse Transcription
RTP	Room temperature and pressure
rUTP	Uridine triphosphate
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
TAG	Triacylglycerol
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Tris[hydroxymethyl]-aminomethane
UCP	Uncoupling Protein
UV	Ultraviolet (light)
VLDL	Very Low Density Lipoprotein
w/v	Weight / volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

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Abstract

Accumulating epidemiological evidence correlates indices of poor growth *in utero* with increased risk of disease in later life, particularly hyperglycaemia, insulin resistance, hypertension, and ischaemic heart disease. This concept of early life events having permanent effects upon health has been termed "programming". The mechanisms linking these effects are not understood, but over-exposure of the foetus to glucocorticoids has been proposed as a potential mediator. Treatment of pregnant rats with dexamethasone (dex) results in offspring of low birth weight, which become hypertensive, glucose intolerant and insulin resistant in adulthood. Permanent tissue-specific changes in the expression of key genes, particularly the glucocorticoid receptor (GR), have been observed in the offspring of dex-treated mothers. In the liver, increased GR is associated with elevated expression of phosphoenolpyruvate carboxykinase, the rate-limiting enzyme of gluconeogenesis, and therefore, increased hepatic glucose output. This thesis addresses the question of whether programming of insulin resistance extends to skeletal muscle and adipose tissue, and whether altered expression of GR in insulin target tissues is important in determining the programmed insulin resistance.

GR was elevated by prenatal treatment with dex in retroperitoneal (RP) fat, and reduced in soleus muscle, while its expression was unchanged in two other muscles and subcutaneous fat. In RP fat, this was accompanied a reduction in lipoprotein lipase mRNA and a shift from PPARγ1 to PPARγ2 expression, while leptin and resistin mRNA levels were unchanged. In quadriceps muscle, glycogen storage was reduced, associated with a downward trend in expression of the insulin-regulated glucose transporter. In summary, tissue-specific programming of GR in muscle and fat is associated with impaired lipolytic activity and a shift in PPARγ expression in visceral fat, and evidence of attenuated glucose uptake into skeletal muscle. These findings are consistent with the presence of insulin resistance in muscle and visceral adipose tissue as a result of prenatal dex.

To test whether changes in GR expression were secondary to hypercorticosteronaemia or insulin resistance, the [corticosterone] (cort) was varied in adult offspring, or rats were treated with insulin-sensitising drugs. No effect of varying [cort] was found in muscles or fat depots, while liver GR was increased by both adrenalectomy and supraphysiological cort. Metformin reduced GR, especially in the liver and muscle of dex-treated rats, but rosiglitazone reduced GR only in the liver and independently of prenatal treatment. The implications are that programming of GR in insulin target tissues is independent of circulating hypercorticosteronaemia and insulin resistance *per se*. Moreover, it seems that the mechanism of action of metformin may be in part glucocorticoid-mediated.

Finally, exposure of pregnant rats to noise pollution perinatally resulted in vehicle-treated offspring developing similar hypertension and features of the insulin resistance syndrome as seen in dex-treated offspring, despite normal birth weight. This demonstrates that perinatal stress can induce a similar phenotype to glucocorticoid over-exposure.

These findings add weight to the hypothesis that *in utero* over-exposure to glucocorticoids can programme insulin resistance in later life, and support a role for both skeletal muscle and adipose tissue metabolism in mediating this. Furthermore, it seems that programmed dysregulation of GR in insulin target tissues plays a significant role in determining the offspring phenotype.

1 Chapter One – Introduction

1.1 Background

My thesis is concerned with a possible mechanism for the "Foetal origins of disease" hypothesis, proposed by Professor David Barker as a result of epidemiological studies undertaken in the late 1980s and early 1990s (Barker 1995).

Barker et al obtained collections of detailed records concerning term births made by midwives early in the 20th century, and followed up the individuals concerned in middle age. Strong correlations were discovered between indices of poor growth *in utero* and increased incidence of disease and factors predisposing towards disease in adulthood (Barker *et al.* 1990; Barker *et al.* 1989; Hales *et al.* 1991). Barker's resulting hypothesis stated that "Foetal undernutrition in middle to late gestation, which leads to disproportionate foetal growth, programmes later...disease" (Barker 1995).

There has been much interest in the likely physiological and molecular mechanisms involved in the "programming" of metabolism by early life environment. In our laboratory, we are utilising a rodent model of *in utero* over-exposure of the foetus to glucocorticoids to attempt to explain this phenomenon. The phenotype of the model recapitulates many of the features associated with low birth-weight in humans, including poor glucose tolerance and insulin resistance (Lindsay *et al.* 1996b). In my investigations I have been attempting to determine whether gene expression and metabolism of peripheral insulin-responsive tissues are "programmed" by prenatal treatment, as part of this syndrome.

In this introduction, I describe the pertinent biology associated with these investigations, as well as the epidemiology of, and the progress made towards the understanding of, the "programming" effect.

1.2 Glucose and Lipid Metabolism

In this section I will present a summary of the physiology and biochemistry of the principal insulin-responsive tissues: liver, muscle and fat. Derangement of some or more of these processes may underlie the observed programming effect.

1.2.1 Metabolic substrates in the fed and fasted state

Energy is required by all living tissues in order to carry out their functions. This is provided in animals by the oxidation of complex organic molecules, principally carbohydrates, lipids and proteins, which are ultimately provided by ingestion in food.

Glucose derived from ingested carbohydrates provides the bulk of the body's short-term energy requirements in the fed state. Excess glucose is stored as glycogen in the liver and skeletal muscle (Radziuk & Pye 2001), or used along with lactate, pyruvate and amino acids in lipogenesis (Wakil *et al.* 1983). The resulting lipid, along with the bulk of ingested lipid, is stored in adipose tissue. Proteins not required for other uses are degraded, and the resulting amino acids deaminated and excreted.

In conditions of relative starvation, fatty acids are liberated from adipose tissue and oxidised to provide the bulk of the body's energy requirement. Ketone bodies are also generated by fatty acid oxidation, and are preferentially oxidised (McGarry & Foster 1980). Gluconeogenesis takes place in the liver principally, and produces glucose from other substrate molecules (Radziuk & Pye 2001). Gluconeogenesis and

glycogenolysis in the liver are necessary to provide the CNS and red blood cells with glucose, as these tissues are obligate glucose consumers (Smith 1997).

The energy liberated by the catabolism of any of these compounds is stored in the high-energy phosphoanhydride bonds of ATP molecules, which are generated in these reactions. This chemical energy (7.3 kcal / mole) is released when required by the hydrolysis of ATP, a reaction which is linked to most energy-utilising processes (Olson 1997).

1.2.2 Uptake and storage of glucose

Ingested carbohydrates are digested by enzymes in the alimentary tract to yield glucose and other monosaccharides, which are absorbed in the small intestine. Glucose reaches the liver via the hepatic portal vein, and then passes into the systemic circulation for distribution throughout the body.

When plasma glucose is in excess, glycogenesis occurs in the liver and skeletal muscle, producing a non-osmotically active carbohydrate, which can be stored. Glucokinase in liver, and hexokinase in muscle, catalyse the phosphorylation of glucose, and the glucose 6-phosphate generated is further "activated" by glucose 1-phosphate uridylyltransferase. Glycogen synthase then catalyses the transfer of the activated glucosyl moiety of UDP-glucose to a glycogen molecule. This enzyme, along with 1,4-α-glucan branching enzyme, is responsible for creating the chains of glucose units that comprise glycogen (Harris 1997).

The disposal of glucose into muscle as glycogen is quantitatively the most important means of lowering blood glucose levels after a meal. However, excess pyruvate produced from glucose in glycolysis can be used to synthesise fatty acids in the liver, via the production of acetyl CoA, and hence lipids for deposition in adipose tissue (Wakil *et al.* 1983).

1.2.3 Release and use of glucose

Glucose yields energy as ATP when it is metabolised by the glycolytic pathway and by aerobic oxidation.

Glycolysis occurs in the cytoplasm of all cells, and yields two moles of ATP per mole of glucose metabolised (Harris 1997). Glucose is first phosphorylated by hexokinase, in common with the first step of glycogen synthesis. 6-Phosphofructo-1-kinase is a "rate-limiting" enzyme of the pathway, catalysing the unidirectional ATP-dependent phosphorylation of fructose 6-phosphate. Pyruvate kinase catalyses another irreversible reaction: the conversion of PEP into pyruvate. In the presence of oxygen, pyruvate is final product of the glycolytic pathway, whereas lactate is produced in an anaerobic environment. Tissues containing relatively few mitochondria, including white muscle, are dependent on glycolysis to fulfil their ATP requirements. White (type II, fast twitch) muscle fibres have a much higher capacity for glycogenolysis and glycolysis than red (type I, slow twitch) muscle fibres, permitting short bursts of intense anaerobic activity. Red muscle fibres are rich in mitochondria, and tend aerobically to oxidise glycogen in a slower and more sustained fashion (Gaster *et al.* 2000; Rivero *et al.* 1998).

Glucose can be readily released from stored glycogen. The action of glycogen phosphorylase and α-amylase produces glucose 1-phosphate units, from which glucose 6-phosphate is regenerated by phosphoglucomutase. In the liver, glucose 6-phosphatase hydrolyses the glucose 6-phosphate, releasing glucose for use around the body (Harris 1997). In muscle, however, this enzyme is present in only trace amounts (Lackner *et al.* 1984), so the majority of the glucose 6-phosphate is consumed within the muscle, generating ATP locally as required by activity levels.

Gluconeogenesis is the net synthesis of glucose in the liver and kidney from other substrates; amino acids, lactate, pyruvate, glycerol, and other monosaccharides are used for this purpose. Alanine and lactate generated by skeletal muscle activity are used for gluconeogenesis, as is the glycerol released from fat after lipid hydrolysis

(Harris 1997). The gluconeogenic pathway utilises many of the glycolytic enzymes, catalysing the reverse reactions to those of glycolysis. Additional enzymes are required to bypass the irreversible steps: mitochondrial pyruvate carboxylase catalyses the synthesis of oxaloacetate from pyruvate, and PEPCK catalyses the production of PEP from oxaloacetate (Hanson & Reshef 1997). Fructose 1,6-bisphosphatase and glucose-6-phosphatase are also required to yield glucose as an end product, and all of these enzymes provide critical points for regulation of the process.

1.2.4 Mitochondrial oxidation and uncoupling

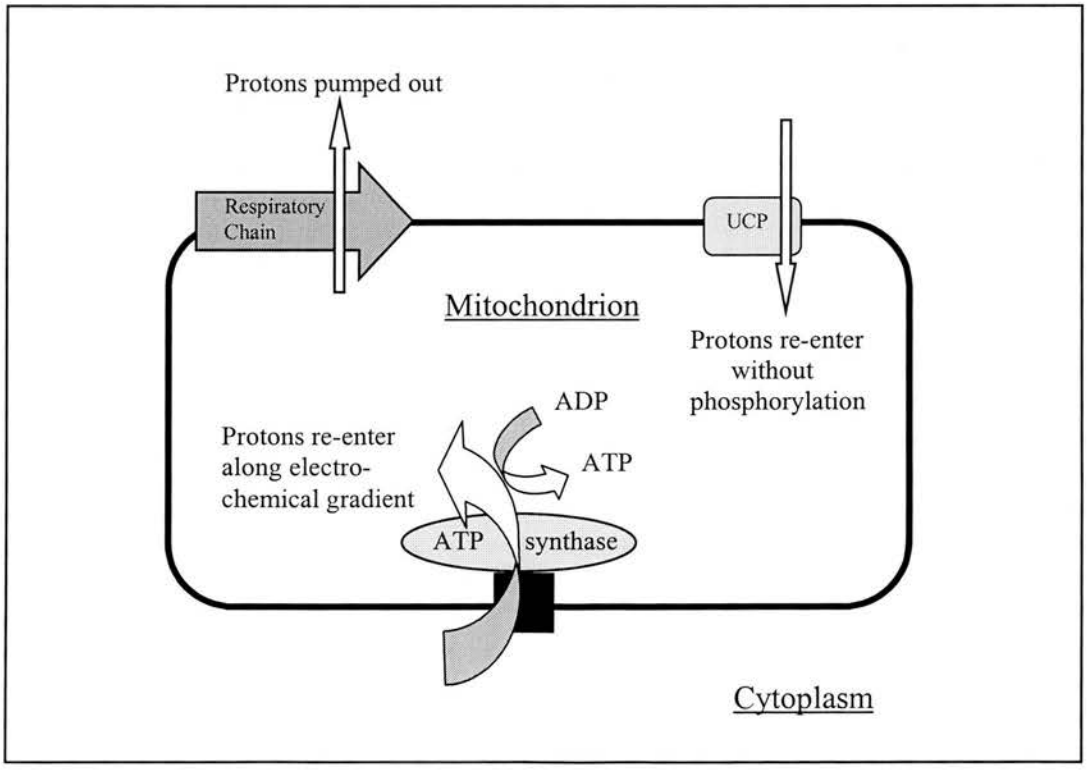
Pyruvate undergoes oxidative decarboxylation in mitochondria to form acetyl CoA, catalysed by the pyruvate dehydrogenase complex. This enzyme is a key point for regulation, as the reaction product can be used for oxidation, ketone body production, or fatty acid synthesis (Hultman 1995; Mandarino *et al.* 1987).

Acetyl CoA is completely oxidised via the tricarboxylic acid cycle in mitochondria. The cycle consists of a series of reactions which generate reducing equivalents, which are then utilised to generate ATP by electron transport / oxidative phosphorylation along the inner mitochondrial membrane. Three molecules of NADH and one of $FADH_2$ are generated per molecule of acetyl CoA entering the cycle. A total of twelve ATPs are produced as a result of these two processes, and during the complete oxidation of glucose, the net yield is 38 ATPs (Olson 1997).

Electron transport occurs through a series of oxidation-reduction reactions, utilising a sequence of electron donating and accepting proteins, with oxygen as the final acceptor. According to Mitchell's chemi-osmotic hypothesis, this process creates an electrochemical gradient across the mitochondrial membrane by the outward pumping of protons. The subsequent dissipation of the gradient is "coupled" to the synthesis of ATP by mitochondrial F_1F_0 ATPase (Mitchell 1979).

"Uncoupling" of respiration and phosphorylation occurs in response to the destruction of this proton gradient, allows the consumption of oxygen without ATP production, and the consequent dissipation of the energy generated as heat. Chemicals such as 2,4-dinitrophenol can achieve this effect, but endogenous uncoupling proteins also trigger it (Olson 1997). See **Figure 1-1** for a summary. The first uncoupling protein discovered (designated UCP-1) is expressed in brown adipose tissue, which occurs principally in small mammals, and is the site for non-shivering thermogenesis. In rodents exposed to the cold, newborns, or animals emerging from hibernation, activation of the sympathetic innervation to brown adipocytes initiates the protonophoric effect of UCP-1 in the numerous mitochondria present therein (Ricquier & Bouillaud 2000). In these scenarios, additional body heat is the useful product.

Figure 1-1 Diagrammatic summary of the coupling and uncoupling of oxidative phosphorylation in mitochondria (after Ricquier & Bouillaud 2000)
An electrochemical gradient is established across the mitochondrial membrane by the outward pumping of protons. Dissipation of this gradient is coupled to ATP synthesis. "Uncoupling" of respiration and phosphorylation occurs in response to the destruction of this proton gradient by chemicals or endogenous uncoupling proteins (UCPs). This allows the consumption of oxygen without ATP production, and the consequent dissipation of the energy generated as heat.



A further three homologous UCPs have now been discovered: UCP2 is widely distributed throughout the body (Fleury *et al.* 1997), while UCP3 is expressed specifically in skeletal muscle and the brown fat of rodents (Vidal-Puig *et al.* 1997a). These proteins have been shown to be involved in the metabolic adaptation to fasting, (Millet *et al.* 1997) weight control and thermogenesis (Fleury *et al.* 1997), through their influence on metabolic rate and glucose homeostasis of the whole animal (Clapham *et al.* 2000).

1.2.5 Uptake, synthesis and storage of lipids

Ingested lipids are hydolysed to varying extents by digestive enzymes and absorbed through the small intestinal mucosa. Chylomicrons are assembled in the intestine to transport dietary TAG, cholesterol, and fat-soluble vitamins along lacteals, and pass via the lymphatic system into the blood. Useful fatty acids are also derived from the catabolism of sugars, some amino acids, and other fatty acids, via acetyl CoA, which is the source of all carbon atoms for their cytoplasmic *de novo* synthesis (McGarry 1997). Acetyl CoA produced in mitochondria is transported out as citrate, and re-derived in the cytoplasm by ATP-citrate lyase. Carboxylation of acetyl CoA by acetyl CoA carboxylase to form Malonyl CoA is the key initial step in the synthesis (Kim & Tye 1994), then two-carbon units are added progressively by the enzyme fatty acid synthase, yielding palmitoyl CoA. This can then be modified by elongation, desaturation, or hydroxylation in the endoplasmic reticulum to produce the majority of fatty acids required by the body (Cinti *et al.* 1992).

Fatty acids can be converted to triacylglycerol for storage in many tissues of the body, but the liver and adipose tissue largely carry out this process. The liver either utilises the TAG it produces, or incorporates it in VLDL for transport to adipose tissue predominantly, but also to muscle (Ferraro *et al.* 1993; Tan *et al.* 1977), and elsewhere. Lipoprotein lipase is expressed by capillary endothelial cells, especially those within adipose tissue, and is responsible for the hydrolysis of TAG within

chylomicrons and VLDL, allowing the uptake of fatty acids by adjacent cells. (Eckel 1989)

TAG synthesis from fatty acids requires glycerol 3-phosphate or dihydroxyacetone phosphate produced during carbohydrate metabolism. Each hydroxyl group on these molecules is sequentially esterified by acyltransferase to produce TAG, which accumulates as cytoplasmic lipid droplets (McGarry 1997).

1.2.6 Release and use of lipids

Hydrolysis of TAG to form mono- and diacylglycerols by hormone-sensitive lipase is the first step in the recovery of stored fatty acids, and is a key point for control of this pathway (Osterlund *et al.* 1996; Sztalryd & Kraemer 1995). Fatty acids and glycerol are generated by further hydrolysis, and re-circulate from adipose tissue for oxidation elsewhere, and use in carbohydrate metabolism respectively. NEFAs are transported bound to plasma albumin when liberated into the circulation after lipolysis by adipose HSL or LPL (McGarry 1997).

Fatty acids are utilised for energy production by many tissues, principally by the process of β-oxidation, but they are an especially important energy source for the heart, while the appropriate metabolic pathways are absent from the CNS (McGarry & Foster 1980). Fatty acyl CoAs are synthesised and carried into mitochondria as acyl carnitine, produced and de-acylated by carnitine palmitoyltransferases I and II, respectively. β-oxidation by acyl CoA dehydrogenases within the mitochondria produces acetyl CoA, NADH and $FADH_2$ molecules, which are oxidised in the same way as those generated from carbohydrate metabolism, producing up to 129 ATPs per mole of palmitate entering the cell (McGarry 1997).

Ketone bodies are synthesised in the mitochondria of the liver and kidney using acetyl CoA produced by β-oxidation. Acetoacetate and β-Hydroxybutyrate are the final products of a series of reactions, one of which is catalysed by HMG CoA synthase, which is expressed at high levels in liver mitochondria. Ketone bodies are

water-soluble, and hence can circulate around the body dissolved in plasma, to tissues where they are used as fuel alongside fatty acids. They form a significant source of energy in states of starvation, and can also be utilised by the CNS (McGarry & Foster 1980).

1.3 Control of glucose and lipid metabolism

1.3.1 Mechanisms of control of substrate flux through metabolic pathways

Flux through the various interconnected metabolic pathways described above is controlled at several levels, the most basic level being that of substrate availability for each enzyme. For anabolic processes this relies ultimately upon dietary intake, and the quantitative significance of each pathway in the generation of ATPs or the storage of energy depends upon nutritional state. For example, in the well-fed state, oxidation of glucose predominates, while the oxidation of fatty acids becomes a major source of energy when the supply of carbohydrate is reduced, sparing glucose for use in the CNS. This is the basis of the glucose-fatty acid cycle (Randle *et al.* 1963).

Since many reactions, for example in the glycolytic / gluconeogenic pathway, are reversible, the predominant direction of the reaction is determined additionally by product inhibition. Flux of substrates through the pathway in that direction relies on swift removal of the product by further reactions or transport. Additionally, binding of allosteric factors away from the substrate-binding site alters the enzyme conformation such that its affinity for substrate is altered (Harris & Crabb 1997). For example, fructose 2,6-bisphosphate stimulates 6-phosphofructo-1-kinase and inhibits fructose 1,6-bisphosphatase, thereby stimulating glycolysis and inhibiting gluconeogenesis (Pilkis *et al.* 1995).

Covalent modification is frequently used to activate or deactivate enzymes through conformational changes, and is under the control of hormones. Phosphorylation by protein kinases and dephosphorylation by protein phosphatases is a common form of this, and is utilised for the control of glycogen synthase activity. Various kinases deactivate the enzyme by phosphorylation, while the active form is regenerated through dephosphorylation by phosphoprotein phosphatase (Hunter 1995).

Changes in the expression of enzymes provide a mechanism for longer term regulation of activity, through induction or repression by hormones or nutritional factors. When over-fed, for example, an individual's liver will show reduced levels of enzymes favouring glucose synthesis, such as PEPCK, while showing increased levels of enzymes concerned with fat synthesis, such as acetyl CoA carboxylase (Gurney *et al.* 1994).

1.3.2 Insulin

Insulin is the hormone secreted by the β cells of the pancreas, responsible for the lowering of blood glucose and triglycerides in situations of plenty- its action is predominant after a meal. It is also significant in the longer term in protein synthesis and growth (Newsholme & Dimitriadis 2001). Control of its secretion is mediated by amino acids and gastro-intestinal hormones, but the main signal is hyperglycaemia, believed to be detected through the activity of pancreatic glucokinase, in a "glucose sensor" mechanism (Matschinsky *et al.* 1993). It is synthesised as an 86 amino acid precursor, proinsulin, which undergoes proteolytic cleavage to form the active peptide prior to secretion.

Insulin achieves its activity through binding to insulin receptor, situated in the plasma membrane of target cells. Binding to the external α-subunit activates the tyrosine kinase activity of the internal β-subunit, which is responsible for the initiation of intracellular second messenger systems. Phosphorylation of insulin-receptor substrates (IRSs) and other proteins can lead to activation of the MAP kinase or inositol triphosphate (IP_3) pathways, resulting in cell division /

differentiation and metabolic effects respectively (Combettes-Souverain & Issad 1998).

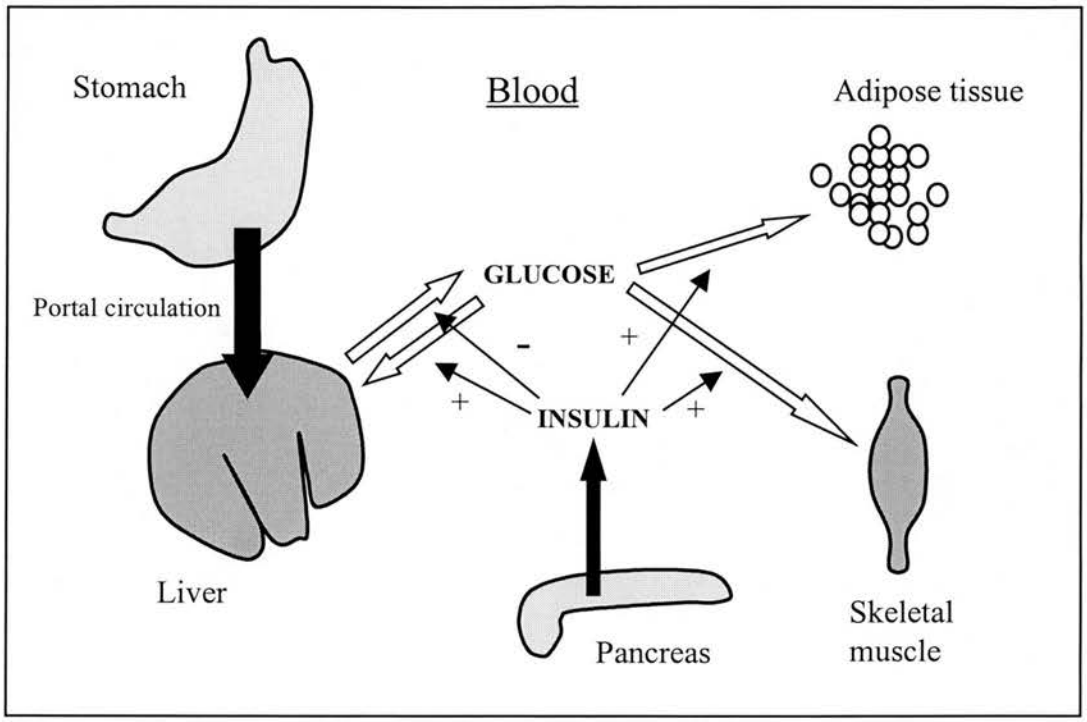
Through these mechanisms, insulin exerts control at many points in the pathways of carbohydrate and lipid metabolism. Please see **Figure 1-2** for a summary. Within seconds, translocation of the insulin-regulated glucose transporter (GLUT-4) from intracellular vesicles to the plasma membrane occurs (Gibbs *et al.* 1988), increasing the rate of glucose uptake, especially in skeletal muscle and adipose tissue. Shortly afterwards, phosphorylation-dephosphorylation cascades are responsible for activation or de-activation of rate-limiting enzymes, for example the activation of glycogen synthase (Shulman *et al.* 1995). Longer term effects are exerted through altered transcription rate of genes, for example glucokinase gene transcription is up-regulated (Vaulont & Kahn 1994), and altered translation rate of mRNA species. The net effects of insulin on carbohydrate metabolism are to increase glucose uptake into cells, to increase the rate of glycolysis and glycogenesis, and to reduce the rate of glycogenolysis and gluconeogenesis.

Insulin also increases the rate of lipolysis of chylomicrons and VLDL through increased LPL expression and activation (Eckel 1989), and the subsequent uptake of triglycerides into cells. Conversely, the rate of intracellular lipolysis in adipocytes by HSL (Holm *et al.* 1997) is reduced, and hence the release of fatty acids into the circulation from fat. Most energy is derived from carbohydrates under the action of insulin, while triglyceride synthesis predominates over β-oxidation.

Figure 1-2 Post-prandial effects of insulin on glucose disposal and metabolism.

Increased plasma glucose resulting from intestinal absorption of digested carbohydrates causes insulin secretion by the pancreatic islets. Insulin acts to return plasma glucose to the normal range by discouraging hepatic glycogenolysis and gluconeogenesis, and increasing glucose uptake and synthesis of glycogen and triglycerides by liver, muscle and adipose tissue.

Key: + up-regulation; - down-regulation.



1.3.3 Glucagon

Glucagon is a polypeptide hormone secreted by the α cells of the pancreatic islets in response to falling concentrations of blood glucose, occurring some time after a meal, or during exercise. Its effects on metabolism are broadly antagonistic to those of insulin: liver glycogenolysis and gluconeogenesis are potently induced, resulting in release of glucose into the blood (Exton 1987), and HSL is activated, such that fatty acids are released into the circulation from adipose tissue for oxidation elsewhere. Its activity is mediated through binding of glucagon to cell membrane receptors, which enhances the conversion of ATP to cAMP by adenylate cyclase. cAMP acts through phosphorylation / dephosphorylation cascades, resulting in

deactivation of glycogen synthase, for example (Exton 1987), and also exerts transcriptional effects, e.g. enhancing expression of PEPCK (Hanson & Reshef 1997).

1.3.4 Sympatho-adrenal

Catecholamines (mainly adrenaline) are secreted by chromaffin cells of the adrenal medulla as a result of stress-induced neural activation and glucocorticoid secretion. These hormones prepare the body for activity by increasing blood glucose, amongst other activities. Binding of adrenaline to β-adrenergic receptors on the plasma membrane of hepatocytes results in cAMP production, and hence achieves the same effects as glucagon (Exton 1987). β-adrenergic effects are also exerted on muscle, where glycogenolysis is stimulated. Binding to α-adrenergic receptors on hepatocytes additionally results in glycogen synthase inactivation through the IP_3 signalling system.

β receptors on adipocytes permit catecholamine- and autonomic-induced lipolysis in white adipose tissue, releasing fatty acids for oxidation in other tissues, and uncoupling of mitochondrial oxidation and phosphorylation in brown adipose tissue (Silva & Rabelo 1997).

1.3.5 Hormones secreted by adipose tissue

Several substances have now been identified that are secreted from adipocytes and have an impact on lipid metabolism and energy balance through paracrine or endocrine mechanisms. Adipsin / acylation stimulating protein is a serine protease homologue secreted by adipocytes (Cook *et al.* 1987), which is believed to regulate triglyceride synthesis in a paracrine fashion. It achieves this by increasing adipocyte glucose uptake and the activity of diacylglycerol acyltransferase within (Sniderman & Cianflone 1994). Resistin is a recently-identified signalling molecule secreted by adipose tissue of rodents (Steppan *et al.* 2001), but perhaps not significantly in man (Nagaev & Smith 2001). Its function has not yet been characterised, but its significance will be discussed further later.

Leptin is the hormone which has attracted by far the most attention. It has been identified as the secreted protein produced by the "ob" gene, which is mutated in the obese ob/ob mouse strain. Administration of recombinant leptin reversed the phenotype of this model (Pelleymounter *et al.* 1995), implying a role for this hormone in reducing fat mass: it acts as a fat mass sensor. It is secreted in proportion to adipose mass in many species, including man (Lonnqvist *et al.* 1995), and acts upon specific receptors in the hypothalamus to exert negative neuronal control over appetite (Campfield *et al.* 1995). The gene encoding the leptin receptor protein was identified as the site of mutation in the obese db/db mouse, which could not be rescued by administration of recombinant leptin (Maffei *et al.* 1995). More recently, peripheral direct effects of leptin on white adipose tissue mass have been identified, involving UCP1 (Commins *et al.* 2001), presumably tying-in with the effect of cold in reducing leptin expression (Hardie *et al.* 1996). Additionally, leptin has been shown to induce LPL, UCP1, 2 and 3 expression in adipose tissue (Scarpace *et al.* 1998; Zhou *et al.* 1997), presumably encouraging weight loss. Furthermore, leptin modulates the hypothalamic-pituitary-adrenal (HPA) axis, through inhibition of corticotrophin releasing hormone (CRH) (Heiman *et al.* 1997) and cortisol (Bornstein *et al.* 1997) secretion.

1.3.6 Transcription Factors

A series of orphan nuclear receptors also have an influence on carbohydrate and lipid metabolism, including the Hepatocyte Nuclear Factors (HNFs), the CCAAT-enhancer binding proteins (C/EBPs), and the peroxisome proliferator-activated receptors (PPARs). These transcription factors bind to response elements on target genes, and exert effects at the level of transcription. C/EBPs are highly expressed in lipid-metabolising tissues, especially liver and fat (Birkenmeier *et al.* 1989), and are important in development (Darlington *et al.* 1995) and metabolic gene regulation in these tissues (Park *et al.* 1990; Kaestner *et al.* 1990). The HNFs are primarily expressed in the liver, and similarly mediate lipid and carbohydrate metabolism (Kaestner 2000; Sladek 1993).

PPARα is predominantly expressed in liver, and PPARγ in adipose tissue, and the putative ligands for each are fatty acids and lipid derivatives, such as prostaglandins (Braissant *et al.* 1996). PPARγ is thought to be important in adipocyte differentiation and metabolism (Chawla *et al.* 1994; Tontonoz *et al.* 1994), and exists in two isoforms, PPARγ1 and 2, which differ by 28 amino acids at the amino terminus (Zhu *et al.* 1995; Yanase *et al.* 1997). PPARγ has been shown to exert transcriptional control over leptin, antagonising the effect of C/EBPα (Hollenberg *et al.* 1997), and over adipogenic genes, such as PEPCK (Tontonoz *et al.* 1995), while PPARα has equivalent functions in the liver (Marcus *et al.* 1993; Keller *et al.* 1993).

1.4 Glucocorticoids

1.4.1 Synthesis, transport and metabolism

Glucocorticoids are steroid hormones, which are synthesised in the mitochondria and endoplasmic reticulum of cells of the zona fasciculata and the zona reticularis in the adrenal cortex. The molecular structures of the principal active hormones, cortisol in man, and corticosterone in rodents, are depicted in **Figure 1-3**. They are synthesised from cholesterol using a common pathway, shown in **Figure 1-4**. Hormonal control over this process is exerted through elevation of intracellular cAMP and calcium ions, which induce the transfer of cholesterol to the mitochondrial inner membrane, where it can be metabolised to form pregnenolone by the cytochrome p450 cholesterol side chain cleavage enzyme, and which affect the transcription of other enzymes of the cascade. The formation of cortisol requires the presence of endoplasmic reticulum cytochrome p450-linked 17α-hydroxylase (17αH) and 21-hydroxylase (21H), and mitochondrial 11β-hydroxylase (11βH), enzymes which are only present in the zona fasciculata and the zona reticularis of the adrenal, hence glucocorticoids cannot be synthesised in other steroidogenic tissues, such as the gonads (Litwack & Schmidt 1997).

Figure 1-3 Molecular structure of the principal active glucocorticoid in man (cortisol) and rodents (corticosterone)

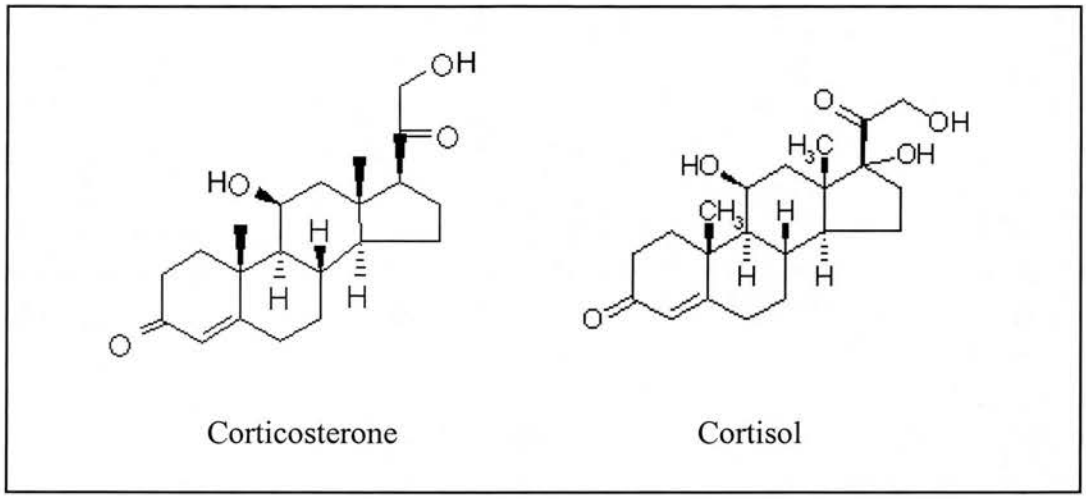
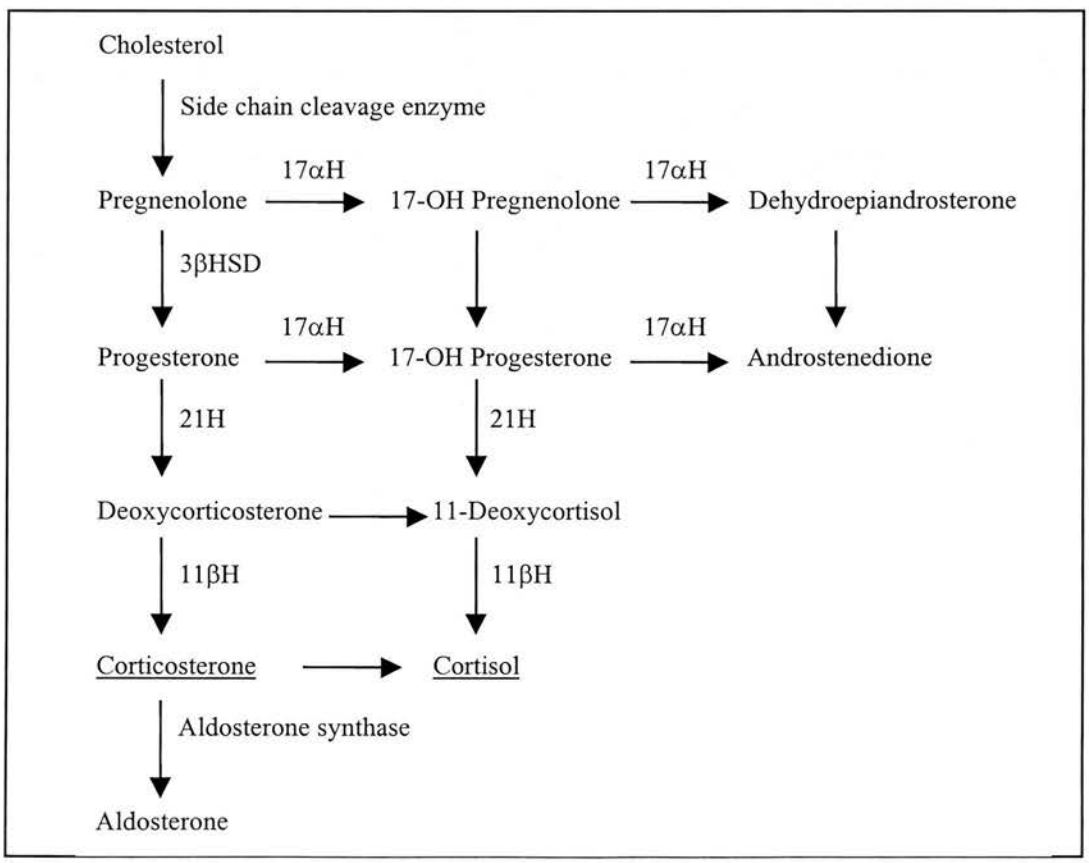


Figure 1-4 Summary of the steroid synthetic pathway of the adrenal cortex.

Cortisol and corticosterone are synthesised from cholesterol in the endoplasmic reticulum and mitochondria of cells in the zona reticularis and zona fasciculata of the adrenal cortex. Aldosterone production occurs exclusively in the zona glomerulosa.

Abbreviations used: 17αH: cytochrome p450-linked 17α-hydroxylase, 21H: 21-hydroxylase, 11βH: 11β-hydroxylase, 3βHSD: 3β-Hydroxysteroid dehydrogenase.



Once secreted, glucocorticoids are mostly transported bound either to albumin, or with high affinity to transcortin / corticosteroid-binding globulin (CBG), synthesised by the liver (Rosner 1991). The unbound glucocorticoid (8% of cortisol in humans) is the form that can diffuse through cellular membranes to achieve an effect. Secreted molecules become bound to receptor or degraded, with a half-life of 90 minutes, within the liver or elsewhere. This metabolism is undertaken by a variety of enzymes, including 5α- and 5β-reductases and 11β-Hydroxysteroid dehydrogenase (11β-HSD) type II (Finken *et al.* 1999), which is discussed further below. Conjugation of products in the liver by glucuronidation or sulphatation precedes excretion through the bile or kidney. This occurs in a 1:3 ratio in humans, while biliary excretion predominates in rodents.

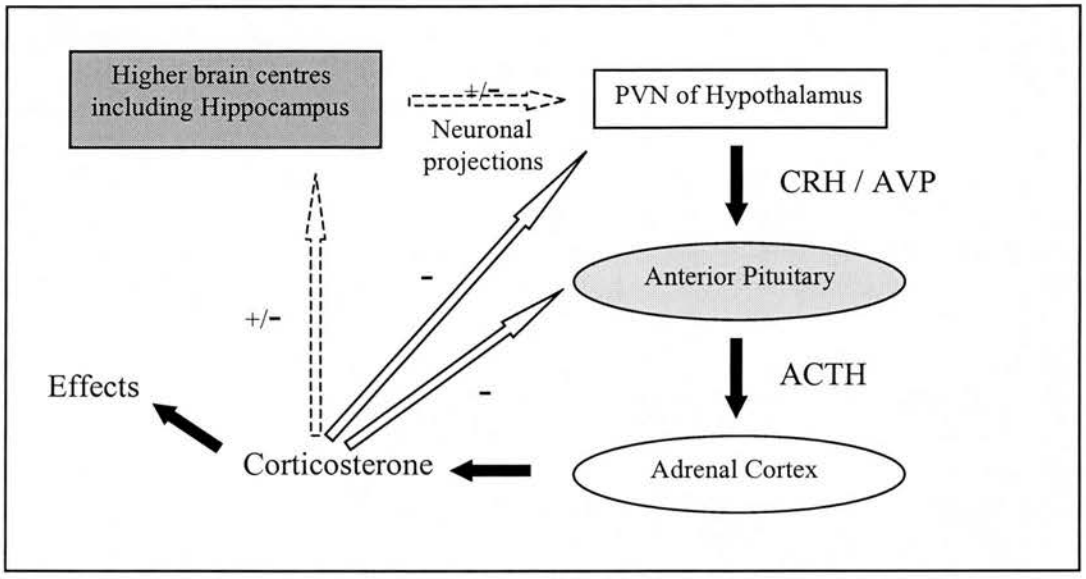
1.4.2 The Hypothalamic-Pituitary-Adrenal (HPA) axis

Glucocorticoids are secreted by the adrenal cortices in response to activation of adenyl cyclase to produce cAMP by adrenocorticotrophic hormone (ACTH), a polypeptide secreted by the anterior pituitary. It is derived by cleavage of the preprohormone pro-opiomelanocortin (POMC), which also yields melanocyte-stimulating hormone, beta-lipotrophin and beta-endorphin. ACTH is released under stimulation from corticotrophin releasing hormone (CRH) and / or arginine vasopressin (AVP), arriving through the hypophyseal portal system from the hypothalamus. These components comprise the HPA axis (Guyton & Hall 1996), and are pictured in **Figure 1-5**. CRH and AVP are also polypeptides, synthesised within the cell bodies of specific neurones in the paraventricular nucleus (PVN), and subject to regulation through numerous neuronal connections from other brain regions, mainly the limbic system and lower brain stem. Through these connections, stress induces CRH secretion, and hence glucocorticoid production. Particular attention has been paid to inputs from the hippocampus, which down-regulate CRH production (Sapolsky *et al.* 1985). Glucocorticoids have a negative feedback effect upon their own secretion through binding to receptors in the hippocampus, hypothalamus, and the pituitary.

Figure 1-5 The Hypothalamic-Pituitary-Adrenal Axis of the Rat.

Corticosterone is secreted by the adrenal cortex in response to the secretion of ACTH by the anterior pituitary, secretion of which is in turn triggered by CRH and AVP release by the paraventricular nucleus of the hypothalamus. Corticosterone has a negative feedback effect upon its own secretion at the hypothalamus and pituitary, and also influences the activity of neuronal projections to the hypothalamus from higher brain centres.

Key: + up-regulation, - down-regulation



In the absence of other stimuli, CRH, ACTH, and glucocorticoid secretion oscillate through the day, but conform to a circadian rhythm overall. In man and other diurnal animals, peak secretion occurs in the early morning, while the nadir is usually around midnight, while in rodents, the cycle is reversed, reflecting their nocturnal activity (Dallman *et al.* 1993).

1.4.3 Physiological Effects

Glucocorticoids have wide-ranging effects on metabolism and immunity, which typify the changes seen in the body during periods of "stress". These effects are exerted primarily through altered transcription rate of target genes. A list of metabolic genes regulated by glucocorticoids is displayed in **Table 1-1**.

Table 1-1 Relevant metabolic targets for glucocorticoid action in skeletal muscle and white adipose tissue

Effects of glucocorticoids on transcription from target genes and the activity of their products in skeletal muscle (M) and white adipose tissue (A) are shown, as detailed in the literature. ↑ signifies a resulting increase, and ↓ a decrease in transcription or activity.

Metabolic target	Effect of glucocorticoids	Tissue	References
Glucocorticoid Receptor	↓ mRNA / binding	M	(Korn *et al.* 1998; McKay *et al.* 1997)
11 β-HSD type I	↑ mRNA / activity	A	(Bujalska *et al.* 1999)
GLUT-4	No effect on expression	A M	(Haber & Weinstein 1992; Dimitriadis *et al.* 1997)
	↓ recruitment to plasma membrane		(Weinstein *et al.* 1998; Dimitriadis *et al.* 1997)
Glycogen synthase	↑ activity	M	(Koerts-de Lang *et al.* 1999)
	↓ sensitivity to insulin-induced activation		(Dimitriadis *et al.* 1997; Coderre *et al.* 1991)
Fructose 2,6-bisphosphatase	↑ mRNA / activity	M	(Marker *et al.* 1989)
PEPCK	↓ mRNA / activity	A	(Franckhauser-Vogel *et al.* 1997; Glorian *et al.* 1998)
Hormone-sensitive lipase	↓ activity	A	(Samra *et al.* 1998)
Lipoprotein lipase	↓ or ↑ mRNA / activity	A	(Fried *et al.* 1993; Ottosson *et al.* 1994; Ong *et al.* 1992)
UCPs	↓ mRNA	A M	(Scarpace *et al.* 2000; Zakrzewska *et al.* 1999)
PPARγ	↑ mRNA (synergism with insulin)	A	(Vidal-Puig *et al.* 1997b)
Leptin	↑ mRNA	A	(De Vos *et al.* 1995)
Resistin	↑ mRNA	A	(Haugen *et al.* 2001)

The effects of glucocorticoids on metabolism are broadly antagonistic to those of insulin. Moreover, they inhibit insulin signalling in target tissues (de Pirro *et al.* 1980; Kahn *et al.* 1978), and reduce insulin secretion (Novelli *et al.* 1999; Delaunay

et al. 1997). Whereas they do not affect overall levels of GLUT-4 (Haber & Weinstein 1992), insulin-stimulated translocation of GLUT-4-containing vesicles to the plasma membrane of both muscle and fat is inhibited (Weinstein *et al.* 1998; Dimitriadis *et al.* 1997; Carter-Su & Okamoto 1987), hence peripheral glucose uptake is reduced. Increased plasma glucocorticoid and NEFAs cause increased hepatic VLDL secretion (Brindley 1995) and fatty acid oxidation in muscle and fat (Bitar 2001; Guillaume-Gentil *et al.* 1993), which may also inhibit glucose uptake through the Randle cycle (Randle *et al.* 1963). Furthermore, glycogen synthesis and glucose oxidation are reduced (Dimitriadis *et al.* 1997). Glucose utilisation is reduced in the liver by glucocorticoids (Rooney *et al.* 1993), while hepatic glucose output is increased as a result of induction of PEPCK, and therefore gluconeogenesis (Hanson & Reshef 1997). The net result of all these changes is increased blood glucose.

Glucocorticoids antagonise the effects of insulin by mobilising fatty acids and promoting β-oxidation, hence their secretion results in an increase in plasma triglycerides. They also promote the mobilisation of amino acids from muscle and reduce amino acid transport into non-hepatic cells and protein synthesis throughout much of the body. Glucocorticoid excess hence results in muscle wasting, occurring preferentially in fast twitch fibres (Polla *et al.* 1994). Effects are also exerted upon other hormones directly: glucocorticoids induce leptin expression, for example (De Vos *et al.* 1995).

In addition to their metabolic effects, glucocorticoids also prevent the development of inflammation and speed its resolution, through effects on lysosomes, capillary endothelia, white blood cells, and cytokine secretion.

1.4.4 Pre-receptor Modulation

The effects of glucocorticoids are mediated in a tissue-specific manner by expression of the glucocorticoid receptor, but also by expression of the two isoforms of the enzyme 11β-HSD. 11 β-HSD type I is NADPH-dependent and is a predominant

reductase, converting cortisone to cortisol in man, and 11- dehydrocorticosterone to corticosterone in rodents. Conversely, 11 β-HSD type II is NAD-dependent, and converts cortisol to cortisone in man, and corticosterone to 11- dehydrocorticosterone in the rodents. I.e. the type II enzyme is responsible for the de-activation, and the type I enzyme the re-activation of glucocorticoids (Seckl & Chapman 1997).

11β-HSD type I is widely expressed in tissues which also express the glucocorticoid receptor, and is especially abundant in liver and adipose tissue, where it locally amplifies the effect of glucocorticoids (Chapman *et al.* 1997). Its expression is under the regulation of glucocorticoids (Jamieson *et al.* 1999) and other hormones (Moore *et al.* 1999; Napolitano *et al.* 1998), and its absence results in attenuated glucocorticoid-inducible responses (Kotelevtsev *et al.* 1997).

11β-HSD type II is expressed in tissues also expressing the mineralocorticoid receptor (MR), including the kidney, and also in the placenta (Edwards *et al.* 1996). This enzyme prevents exposure of MR to active glucocorticoids, which act as a ligand for MR, and circulate in higher concentrations than aldosterone. Absence, mutation, or pharmacological inhibition of this isoform results in hypertension due to excessive salt retention (Stewart *et al.* 1994; Kotelevtsev *et al.* 1999). Protection of the foetal HPA axis from the higher concentrations of glucocorticoids in the maternal circulation is similarly engendered by the type two isoform in its placental location (Benediktsson *et al.* 1997). The enzyme is expressed at the materno-foetal junction, until towards the end of pregnancy in the rat (Waddell *et al.* 1998). The implications of this will be discussed further in the section on programming.

1.4.5 The Glucocorticoid Receptor

The receptor for glucocorticoids is a member of the steroid-thyroid-retinoid receptor super-family, and it is typically expressed in its unbound form in the cytoplasm of cells, although membrane receptor has been reported in the CNS (Orchinik *et al.* 1991). The members of the super-family have a common functional domain structure. This includes a variable N-terminal domain, often important for trans-

activation of transcription; a well conserved DNA-binding domain, crucial for recognition of specific DNA sequences and protein: protein interactions; and at the C-terminal end, a ligand-binding domain, important for hormone binding, protein: protein interactions, and additional trans-activation activity (Kumar & Thompson 1999). Members of this family are active only as dimers, and GR homodimerises to yield a 94 kDa protein (Chalepakis *et al.* 1990; Tsai *et al.* 1988). The GR gene consists of 9 exons, the untranslated first containing the promoter region, and the remaining structural exons 2-9 (Encio & Detera-Wadleigh 1991).

In addition to the widely-expressed GRα, a GRβ has been identified, derived from an alternate splice transcript, and differing in the addition of 15 amino acids at the carboxyl terminus, which has been reported to exert a dominant negative effect upon GRα trans-activation in man (Brogan *et al.* 1999; Oakley *et al.* 1999), perhaps modulating tissue GR sensitivity (Bamberger *et al.* 1995). However, the importance of this receptor isoform is still controversial (Carlstedt-Duke 1999), especially in view of its absence in rodents (Otto *et al.* 1997). Recently, a 91kDa alternately translated form of the receptor has also been identified in human cell lines, leading to subdivision of human GRα into types A and B. The latter form is reported to be twice as efficient at trans-activation, and probably arises from a leaky ribosomal scanning mechanism (Yudt & Cidlowski 2001).

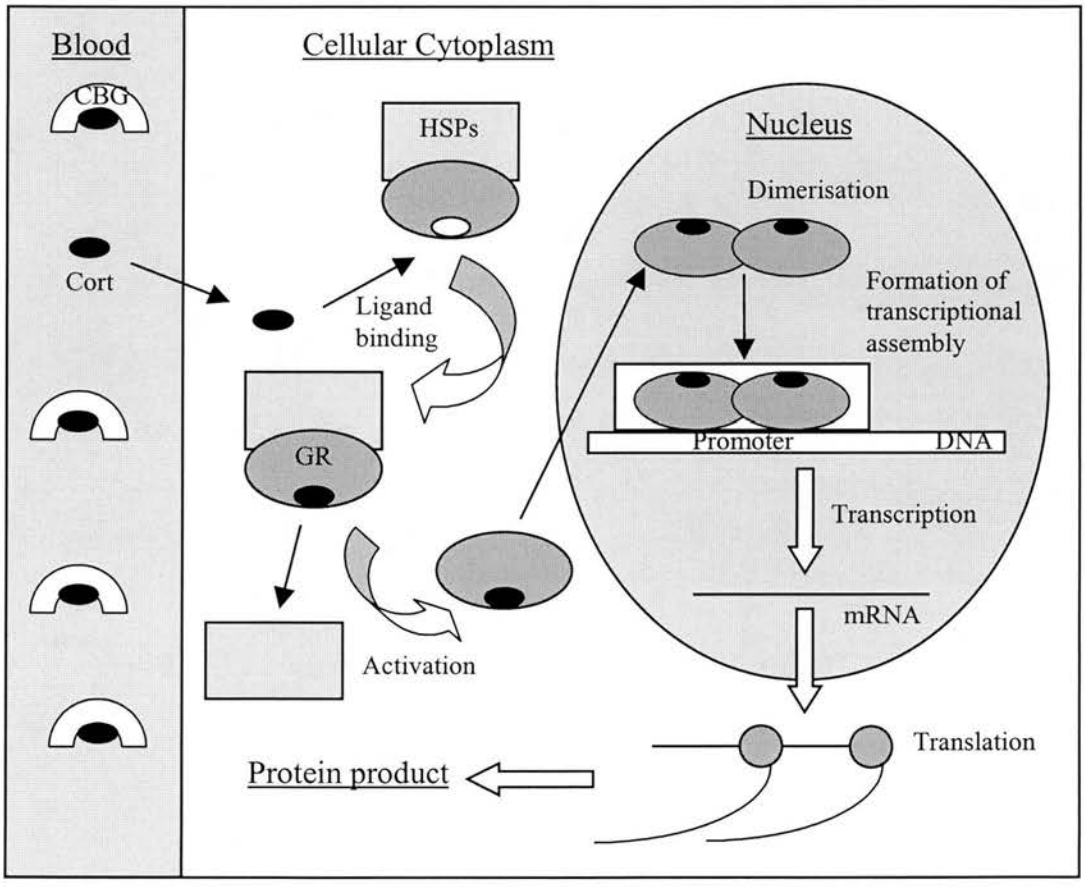
1.4.6 Transcriptional activation by GR

As glucocorticoids are fat-soluble, they enter cells by simple diffusion and activate cytoplasmic receptor via the ligand-binding domain. This process involves dissociation from the heat-shock proteins (HSPs) that form a complex with unbound receptor (Gustafsson *et al.* 1989; Pratt *et al.* 1993), and translocation of the hormone-ligand complex across the nuclear membrane (Hache *et al.* 1999). Once its nuclear effect has been achieved, receptor is recycled to the cytoplasm, where it re-associates with HSPs (Yang & DeFranco 1996). A summary of the events involved in the activity of GR is depicted in **Figure 1-6**. Recent work implies that the phosphorylation status of the receptor is key in its degree of activation (Orti *et al.*

1992), and also in determining its half life (Webster *et al.* 1997), according to the degree of activation of a proteasomal degradation mechanism (Wallace & Cidlowski 2001).

Figure 1-6 **Model of Glucocorticoid Receptor activity** (after Litwack &Schmidt, in "Textbook of Biochemistry", Ed. Devlin, 1997)

Free hormone dissociates from the circulating transport protein (CBG), diffuses into the cytosol, and binds to inactive GR. This activates the receptor by causing dissociation of the heat-shock protein complex (HSP), and the receptor dimerises and is translocated into the nucleus. Here, the activated receptor binds to glucocorticoid-response elements in the promoter region of target genes, and recruits other elements of the transcriptional apparatus of the cell. Transcribed mRNA is translated into protein products by ribosomes in the cytosol.



Liganded receptor classically exerts an effect through attachment of the DNA-binding domain to specific sequences of DNA in the promoter regions of target genes, known as glucocorticoid response elements (GREs) (Payvar *et al.* 1983; Scheidereit *et al.* 1983; Becker *et al.* 1986). It can also achieve an effect by protein: protein interactions with other transcription factors, which bind DNA (Gottlicher *et*

al. 1998; Reik *et al.* 1994). Complexes are also believed to be formed with various co-activators or co-repressors, which modulate the induced transcriptional activity of target gene promoters (Jenkins *et al.* 2001; Sheppard *et al.* 1998). Binding of GR and other transcription factors initiates chromatin remodelling and modulates the assembly of the transcriptional machinery, such that target gene transcription in activated or repressed (de Lange *et al.* 1997). Appropriate response elements have now been identified in multiple genes involved in carbohydrate and lipid metabolism (Lange *et al.* 1989; Hanson & Reshef 1997).

1.4.7 Regulation of GR expression

In addition to its role in regulating transcription from other genes, the expression of GR itself is regulated, because its abundance determines tissue sensitivity to glucocorticoids (Schmidt & Meyer 1994). Control of expression is exerted through binding of transcription factors to response elements within its promoter region, and also through post-translational mechanisms (Dong *et al.* 1988), including enhanced proteasomal degradation rate (Wallace & Cidlowski 2001). The most frequently studied mechanism is the induction or repression of GR transcription or activity by glucocorticoids. This can be achieved through binding of liganded receptor to GREs in the promoter region of the GR gene (Okret *et al.* 1986; Burnstein *et al.* 1991), and permits feedback control on glucocorticoid-regulated events at multiple levels.

A further mechanism of GR regulation could be through usage of alternate promoter regions of the gene (McCormick *et al.* 2000; Gearing *et al.* 1993). Multiple transcription initiation sites have now been identified within exon 1 of rats (McCormick *et al.* 2000), mice (Strahle *et al.* 1992) and man (Breslin *et al.* 2001) that yield transcripts with different 5' untranslated regions, permitting transcriptional control through different specific response elements on each promoter region. These numerous facets of control of GR permit very specific expression of the receptor according to developmental stage (Kalinyak *et al.* 1989; Cole *et al.* 1995), age (Tohgi *et al.* 1995; Djordjevic-Markovic *et al.* 1999), tissue (Kalinyak *et al.* 1987;

McCormick *et al.* 1998), sex (Liu *et al.* 2001; DuBois & Almon 1984), and hormonal and metabolic milieu (Burnstein *et al.* 1991; Okret *et al.* 1986).

1.5 The Metabolic Syndrome

1.5.1 Definition and epidemiological significance

Having discussed normal carbohydrate and lipid metabolism, and its control, especially by glucocorticoids, I will now discuss relevant associated pathophysiology.

The "metabolic syndrome" or "syndrome X" is a cluster of abnormalities that are risk factors for the development of ischaemic cardiovascular disease. These include insulin resistance and compensatory hyperinsulinaemia, glucose intolerance, increased plasma triglycerides, decreased high-density lipoprotein cholesterol, smaller denser low-density lipoprotein particles, and hypertension (Reaven 1995). Obesity is an additional risk factor that usually co-exists (Bjorntorp & Rosmond 1999), the effects of which are often very difficult to disentangle from those of insulin resistance (Steppan *et al.* 2001). In fact, there exists a "chicken and egg" question between these two syndromes. Aberrant lipid deposition in muscle as a result of obesity is important in determining local insulin resistance (Simoneau *et al.* 1999; Kelley *et al.* 1999), but muscle insulin resistance can lead to increased adipose deposition (Kim *et al.* 2000b).

Occurrence of this syndrome is traditionally associated with lifestyle factors such as poor diet, lack of exercise, and consumption of alcohol and cigarettes (Barker *et al.* 1989). The significance of this group of disease entities is emphasised by examining the World Health Organisation report of 1998, which rated cardiovascular disease as the number one cause of mortality in the developed world for the preceding year, at 46% (World Health Organisation 1998).

1.5.2 Insulin Resistance, Diabetes Mellitus and Cardiovascular Risk

Diabetes mellitus is a series of diseases characterised by lack of control over glycaemia. Type I diabetes refers to the early onset disease caused by an inability of the pancreatic β-cells to secrete sufficient insulin. Type II or non insulin-dependent diabetes (NIDDM), however, has a typical onset in middle-age, and is related to poor glucose tolerance. Resistance to insulin-stimulated glucose uptake is present in the majority of human patients with impaired glucose tolerance or type two diabetes, and in approximately 25% of non-obese individuals with normal oral glucose tolerance. In these conditions, deterioration of glucose tolerance can only be prevented if the β-cell is able to increase its insulin secretory response and maintain a state of chronic hyperinsulinemia (Reaven 1988). There is still debate on the relative contribution of pancreatic insufficiency (Kulkarni *et al.* 1999; Novelli *et al.* 1999) and resistance of tissues to insulin in the development of NIDDM, but it seems most likely that insulin resistance comes first (Bjorntorp *et al.* 1999; Reaven 1995).

The insulin resistance syndrome reflects as a reduced capacity for many tissues to respond to insulin. Gluconeogenesis proceeds at an increased rate in the liver (Boden *et al.* 2001), but reduced glucose uptake and glycogen synthesis in liver, adipose tissue, and especially skeletal muscle are quantitatively more significant (Abel *et al.* 2001; Bonadonna *et al.* 1996; Cline *et al.* 1999; Kelley *et al.* 1996). As one would also expect, there is also reduced fatty acid synthesis in adipocytes (Richardson & Czech 1978), and impaired uptake of fatty acids by adipose tissue (Maheux *et al.* 1997; Reynisdottir *et al.* 1997), explaining the elevated plasma triglycerides and LDL observed.

The link between insulin resistance and hypertension, and subsequent cardiovascular disease is as yet not fully explained. However, insulin has been shown to be responsible for peripheral vasodilatation, which may partially determine glucose uptake (Baron 1994), and it is also implicated in salt sensitivity (Sechi 1999), and linked with the sympatho-vagal balance of cardiac innervation (Flanagan *et al.*

1999). Since salt retention, tachycardia and peripheral vasoconstriction all contribute to hypertension, cardiovascular or renal insulin resistance could be implicated. These links are being further explored in models such as the spontaneously hypertensive rat, in which glucose uptake is impaired (Reaven 1991; Collison *et al.* 2000).

1.5.3 Mediators of insulin resistance

There have been many factors implicated in the mechanism of development of insulin resistance, reflected in a vast literature. This thesis is concerned with the importance of glucocorticoids in insulin resistance, which will be discussed further below, but I will review here a selection of other proposed mechanisms.

Impairment in insulin-signalling mechanisms have been identified, which would reduce the effect of circulating insulin on metabolism. The IP_3 cascade (Cusi *et al.* 2000) and early post-receptor phosphorylation steps (Paez-Espinosa *et al.* 1999) have both been implicated. There has been much attention paid to involvement of aberrant lipid metabolism, since the observation that accelerated release of fatty acids impaired glucose uptake (Randle *et al.* 1963). It has further been shown that elevated fatty acids are associated with reduced glucose oxidation and glycogen synthesis elsewhere (Ebeling & Koivisto 1994; Boden *et al.* 1994). Redistribution of lipid from adipose tissue to muscle (Dobbins *et al.* 2001; Turcotte *et al.* 2001) and liver (Mori *et al.* 1997; Kim *et al.* 2001a) occurs, and is associated with insulin resistance in these tissues (Pan *et al.* 1997; Kim *et al.* 2001a). Adverse lipoprotein profile (high LDL, low HDL) is a key part of the syndrome which may be implicated in this re-distribution, and apolipoprotein abnormalities have been shown to cause insulin resistance (Castellani *et al.* 2001).

Altered secretion of hormones or cytokines from adipose tissue has been implicated in the development of insulin resistance, and in the linkage of insulin resistance and obesity. Tumour necrosis factor α is over-secreted from adipose tissue and muscle of obese, insulin resistant individuals (Hotamisligil *et al.* 1993; Saghizadeh *et al.* 1996), and has a negative effect on insulin signalling (Kroder *et al.* 1996). Subnormal leptin

secretory responses (Liu *et al.* 1999) and the associated over-expression of melanin-concentrating hormone (Ludwig *et al.* 2001) are also associated with both related syndromes. Secretion of adiponectin has been shown to correlate inversely with fat mass, and reduced secretion is associated with obesity, insulin resistance, and endothelial inflammation (Ouchi *et al.* 1999; Yamauchi *et al.* 2001). Finally, the recently described product, resistin, secreted in larger amounts in obese rodents, has been claimed to reduce peripheral glucose uptake through reduction in insulin sensitivity (Steppan *et al.* 2001). Reduced levels of the hormone also have been noted in several models of obesity (Masuzaki *et al.* 2001; Way *et al.* 2001a), so its expression may additionally be secondarily influenced by insulin resistance.

1.5.4 Importance of glucocorticoids in insulin resistance and obesity

As has been discussed earlier, the effects of glucocorticoids upon lipid and glucose metabolism are broadly antagonistic to those of insulin. The potential importance of glucocorticoids in these two related syndromes can be readily appreciated, therefore, by considering the features of the syndrome associated with chronic over-secretion of glucocorticoids: Cushing's Syndrome. This condition is associated with increased truncal adiposity, which may be a result of specific up-regulation of GR (Bronnegard *et al.* 1990), 11β-HSD type I, and LPL in this fat depot (Bujalska *et al.* 1997; Rebuffe-Scrive *et al.* 1988). Several features of the metabolic syndrome are also seen in Cushing's: patients have impaired glucose uptake, hyperlipidaemia, and hypertension (Friedman *et al.* 1996) associated with peripheral insulin resistance (Heaney *et al.* 1997).

Evidence for the importance of glucocorticoids in insulin resistance comes both from evidence of increased glucocorticoids in tissues, and increased glucocorticoid-mediated metabolism. Epidemiological associations have been identified between increased HPA axis activity and the presence of cardiovascular risk factors (Reynolds *et al.* 2001), and obesity especially (Rask *et al.* 2001; Pasquali *et al.* 1993). This may be mediated through increased circulating NEFAs, as the feeding of a high fat diet causes HPA hyperactivity (Tannenbaum *et al.* 1997). At the receptor

level, improved insulin sensitivity results from lower GR in muscle (Vestergaard *et al.* 2001), while receptor polymorphisms have been associated with both insulin resistance and obesity (Weaver *et al.* 1992; Rosmond *et al.* 2000). Finally, up-regulation of adipose 11β-HSD I is present in obese rats and humans, which would increase local glucocorticoid concentrations (Rask *et al.* 2001; Livingstone *et al.* 2000a). Hyperglycaemia would be predicted as a result of the magnification of the metabolic effects of glucocorticoids on carbohydrate metabolism in tissues described above.

1.6 Foetal Origins of Adult Disease

1.6.1 Determinants of foetal development

Growth is an ongoing process between fertilisation and adulthood, consisting of hypertrophy, hyperplasia, and differentiation of cells. It is, however, not a uniformly continuous process, as different cells and tissues are occupied in each or these processes, or are quiescent, during different periods of normal development. *In utero*, growth is determined by the genetic composition of the foetus, maternal physiology, and the placenta, which is responsible for selective exchange of materials between the two, and is an endocrine organ. Placental function is crucial to permit the supply of the foetus with sufficient metabolic substrates and oxygen, and for the removal of waste. Periods of more rapid growth of placenta and foetus do not necessarily coincide, hence potential demands on the placenta also vary throughout gestation (Robinson *et al.* 1995). Placental secretion of luteinising hormone, progesterone, and oestrogen is important in maintaining pregnancy, but other endocrine products of the placenta have also been identified, including CRH (Goland *et al.* 1993).

After the glands have formed, foetal endocrine control of development becomes very significant. Whereas growth hormone assumes more importance after birth, thyroid hormones, glucocorticoids and insulin all have crucial effects prenatally. Insulin

promotes tissue accretion by stimulating cell proliferation, and has anabolic effects on foetal metabolism (Fowden 1989). Thyroid hormones affect differentiation and proliferation of different tissues at different times during gestation, and hence have an asymmetric effect on growth (Fowden 1995). In contrast, glucocorticoids are mainly concerned with differentiation, especially in preparing the foetus for birth (Liggins 1994). For example, they promote the production of surfactant, which is required for lung inflation (Liley *et al.* 1989), and induce PEPCK transcription, allowing perinatal gluconeogenesis (Girard 1986). In most species, glucocorticoid levels rise towards the end of pregnancy, and are involved in the initiation of parturition (Karalis *et al.* 1996). Hormones and glucose concentration affect the production of IGFs, through which many of their effects are probably achieved. *In utero*, IGF-II predominates, while IGF-I increases after birth (Sara & Hall 1990), but both stimulate growth, and it seems that foetal IGF-I may be more significant in modulating cell proliferation in response to the specific endocrine and hormonal conditions prevailing *in utero* (Fowden 1995; Oliver *et al.* 1999).

1.6.2 Epidemiological associations between birth weight and adult disease

Barker's original observations were based upon the re-examination of adults for whom rigorous birth records had been retained in hospitals in Preston and Hertfordshire, UK. Strong inverse and continuous correlations between birth weight and the incidence of hypertension, insulin resistance and cardiovascular disease in middle age were identified (Barker *et al.* 1989; Barker *et al.* 1990; Hales *et al.* 1991). The basic association between low birth weight and adult disease has been made now in numerous studies in populations on four continents (Fall *et al.* 1995b; Curhan *et al.* 1996; Hofman *et al.* 1997). In most studies, findings were independent of adult lifestyle factors, such as alcohol intake, and adult body mass (Gale *et al.* 2001; Curhan *et al.* 1996; Barker *et al.* 1990).

Birth weight is used as a surrogate for growth of the foetus *in utero* in these studies, but this measurement is crude at best. Separate associations have also been made, however, between thinness at birth (measured as ponderal index) and insulin

resistance and cardiovascular disease in later life (Phillips *et al.* 1994; Barker *et al.* 1993). This may implicate reduced resources during gestation for the liver and pancreas specifically in the development of insulin resistance. Placental weight has also been considered as a risk factor for later disease, since the placenta controls the access of nutrients to the foetus. One study found a clear beneficial effect of a large placenta (Thame *et al.* 2000), but the combination of a large placenta and a small foetus has been found to be most detrimental (Barker *et al.* 1990; Barker *et al.* 1993).

Some workers have further attempted to control for confounders, including genetic composition, by examining homozygotic twins. Although birth weight has not correlated with later disease in all studies of this sort, many have recapitulated the original associations in identical twins (Dwyer *et al.* 1999; Poulter *et al.* 1999; Levine *et al.* 1994; Poulsen *et al.* 1997), implying that the effect of small variations in the maternal environment to which the twins are exposed, can outweigh that of genetic factors.

Much work has also addressed the timing of development of differences between high and low birth weight cohorts, and the effect of catch-up growth in modifying associations between birth data and later disease. It seems that individuals born small, who go on during childhood to reach equivalent size to their peers, are at still greater risk of cardiovascular disease (Mortaz *et al.* 2001; Ong *et al.* 2000; Fall *et al.* 1995b). Although risk factors for cardiovascular disease have been looked for most frequently, altered foetal growth has additionally been associated with changes in immunity (Beasley *et al.* 1999; McDade *et al.* 2001a; McDade *et al.* 2001b), osteoporosis (Dennison *et al.* 2001), intelligence (Haddow *et al.* 1999) and renal impairment (Hinchliffe *et al.* 1992).

1.6.3 Potential mechanisms for the foetal origin of adult disease

During periods of rapid growth or differentiation, tissues are more susceptible to the effects of external stimuli, i.e. specific "windows" exist for the environment to have an effect on development (Widdowson & McCance 1975). Research in the 1970s showed that very discrete interventions, such as a single dose of androgen, could have permanent effects on physiology, through altered development, if they occurred during one of these "windows" (Arai & Gorski 1968; Gustafsson & Stenberg 1974b). This phenomenon was termed "programming".

Investigation into the mechanisms of the programming of adult disease by *in utero* growth retardation is lagging some way behind the accumulation of epidemiological associations. However, a number of changes in maternal physiology have now been demonstrated to be associated with, or to cause, IUGR and subsequent deranged metabolism and disease. The earliest hypothesis, and the one that still receives the majority of attention, is that maternal malnutrition is responsible (Godfrey *et al.* 1994; Roseboom *et al.* 2001b). This is discussed further in the next section. Alternative hypotheses implicate secreted factors in the maternal circulation. Administration of cytokines to rats during pregnancy causes reduced insulin sensitivity, increased adipose mass, and increased stress responsiveness in the offspring (Dahlgren *et al.* 2001). The HPA axis has also been programmed by postnatal endotoxin treatment (Shanks *et al.* 1995), and handling of pups (Liu *et al.* 2001; O'Donnell *et al.* 1994), with effects on stress-responsiveness.

In view of the significance of endocrine control of foetal metabolism and development, perturbations in hormone levels *in utero* could potentially have long term effects. And, indeed, this is the case, as altered androgen (Goldman *et al.* 1976; Gustafsson & Stenberg 1974a), oestrogen (Vom Saal *et al.* 1997), and thyroid hormone status (Castello *et al.* 1994; Haddow *et al.* 1999) have been associated with permanent effects on reproduction and brain function respectively. Prenatal over-exposure to glucocorticoids has marked effects (Seckl *et al.* 2000; Edwards *et al.* 1993), and is discussed further below.

A contrasting alternative hypothesis has been put forward by Hattersley, the "Foetal insulin hypothesis" (Hattersley & Tooke 1999). This proposes that genetically determined insulin resistance results in impaired insulin-mediated growth in the foetus, as well as insulin resistance in adult life. It is quite likely that this mechanism operates in tandem with environmental effects.

1.6.4 Programming by nutrition

Many investigators propose that maternal malnutrition during pregnancy is responsible for low birth weight and subsequent cardiovascular disease in their offspring, however there are few prospective studies in human populations yet published that support this contention. An exhaustive survey of the literature produced three papers that correlated combinations of poor maternal weight gain, low skin-fold thickness, and low maternal haemoglobin during pregnancy, with offspring hypertension (Clark *et al.* 1998; Adair *et al.* 2001; Godfrey *et al.* 1994). A further paper inversely correlates, retrospectively, protein / carbohydrate intake ratio and blood pressure (Roseboom *et al.* 2001b). Other surveys have examined populations affected by historical famines, but have not produced consistent associations (Stanner *et al.* 1997; Stein *et al.* 1995). Finally, the one genuine prospective study examining maternal food intake showed no association between birth or placental weight, and any macro-nutrient (Mathews *et al.* 1999).

The evidence for this hypothesis, therefore, is mostly restricted to the results of animal studies, which have been conducted mainly in the rat or the sheep, and typically involve either restriction of food availability (Woodall *et al.* 1996) or protein intake (Hales 1997) in the former species, and timed restriction of availability in the latter (Gallaher *et al.* 1998; Whorwood *et al.* 2001). The phenotype of each model shows a degree of variation, but in general, tends to re-capitulate that of human *in utero* growth retardation. One commonly used model involves the feeding of a 6-9% protein diet (versus an 18-20% protein diet) to pregnant mothers, which reduces the amino acid supply to the offspring; amino acid supply has been shown to be subnormal in growth-retarded babies (Cetin *et al.* 1988).

In the low protein rat model, offspring have reduced birth weight, undergo catch-up growth (Vehaskari *et al.* 2001; Ozanne 1999) and develop insulin resistance (Ozanne & Hales 1999; Holness *et al.* 2000) and hypertension (Langley-Evans *et al.* 1996c; Vehaskari *et al.* 2001; Kwong *et al.* 2000) in adulthood. Defects in the kidney have been reported at birth in two models (Vehaskari *et al.* 2001; Merlet-Benichou *et al.* 1994), and the reduced availability model (30-50% normal intake of food) shows delayed puberty (Engelbregt *et al.* 2000), and hyperphagia and obesity in adulthood (Vickers *et al.* 2000; Jones *et al.* 1984). Clearly under-nutrition, when severe, and in various guises, can have a marked effect on development and long-term health. Whether there is a genuine variation in offspring health as a direct result of the more subtle variation in the nutrition of First World mothers, is still a matter for debate.

1.7 Prenatal Programming by Glucocorticoids

1.7.1 Rationale and Proposed Mechanism

An alternative hypothesis has been proposed to explain the programming effect, based upon the over-exposure of the foetus to glucocorticoids (Edwards *et al.* 1993). This hypothesis is reasonable, given that these hormones are important in the development of various organ systems before birth (Liggins 1994; Fowden 1995), and high dose administration *in utero* has teratogenic effects (Mosier *et al.* 1982; LaBorde *et al.* 1992). Additionally, foetal cortisol is known to be elevated in naturally-occurring IUGR (Goland *et al.* 1993), while exogenous glucocorticoids have been shown to reduce birth weight in humans and animals (Reinisch *et al.* 1978; Mosier *et al.* 1982). Furthermore, glucocorticoids are directly associated in adulthood with both hypertension and insulin resistance (Walker *et al.* 1998), as described above.

As discussed earlier, placental 11β-HSD type II is responsible for maintaining the lower foetal level of circulating glucocorticoid, by inactivating endogenous hormone

(Benediktsson *et al.* 1997). However, administration of dexamethasone, which is a poor substrate for the enzyme, to pregnant rats, results in over-exposure of the foetus to glucocorticoid, and resulting offspring have low birth weight and develop subsequent hypertension (Benediktsson *et al.* 1997). If the enzyme is inhibited by liquorice derivatives, such as carbenoxolone, the effects are similar (Lindsay *et al.* 1996a; Lindsay *et al.* 1996b). A summary diagram of this proposed mechanism can be seen in **Figure 1-7**.

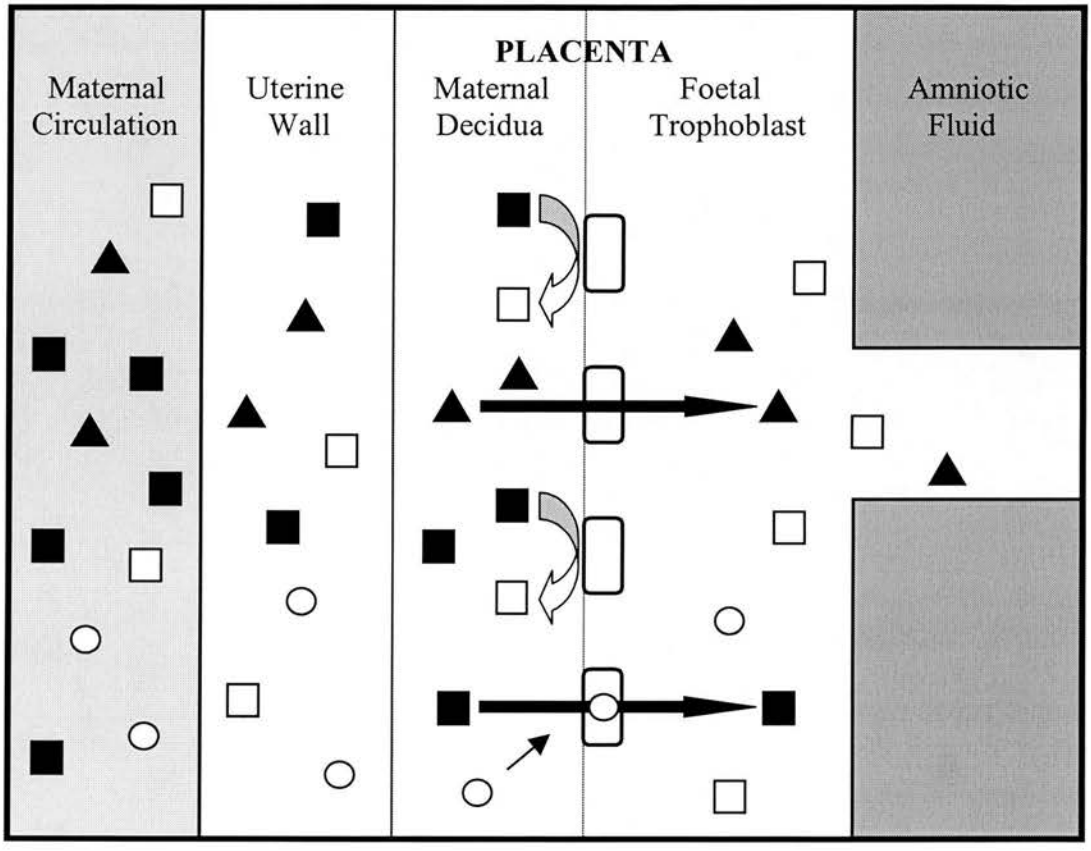
Activity of placental 11β-HSD type II has been found to correlate positively with term weight (Benediktsson *et al.* 1997; Stewart *et al.* 1995), while feeding of a restricted diet causes a reduction in placental enzyme activity (Bertram *et al.* 2001; Lesage *et al.* 2001; Langley-Evans *et al.* 1996b), an observation which may provide the mechanistic link between dietary and endocrine models. The basic similarity between the models is further underlined through the observations that maternal weight gain is interrupted as a result of prenatal dex (Nyirenda *et al.* 2001), while administration of metyrapone to protein malnourished mothers prevents the programming of offspring hypertension (Gardner *et al.* 1997). Glucocorticoids have also been shown to down-regulate placental glucose transporters, which could restrict nutrient supply to the foetus (Hahn *et al.* 1999).

The window for the programming effect has been elucidated to some degree in the rat model. Glucocorticoid over-exposure throughout pregnancy (Benediktsson *et al.* 1993), or during the third week alone (Levitt *et al.* 1996), creates low birth weight offspring, which later become hypertensive, but treatment during the third week alone is required to cause insulin resistance in adulthood (Nyirenda *et al.* 1998) (Lindsay, unpublished observations). Hence, the most striking effects are specifically exerted during the period that is the rough equivalent of the human third trimester.

Figure 1-7 The placental barrier, and the proposed mechanism of programming by *in utero* over-exposure to glucocorticoids

Corticosterone concentration is maintained at a lower level in the foetus than in the maternal circulation by the enzyme 11β-Hydroxysteroid dehydrogenase type II, which inactivates the majority of maternal glucocorticoid diffusing across the placenta. However, over-exposure of the foetus to glucocorticoid occurs if dexamethasone is administered to the mother, which is a poor substrate for the enzyme, or if carbenoxolone is administered during pregnancy, which inhibits the enzyme.

Legend: rectangles: 11β-HSD type II molecules; black squares: corticosterone molecules; white squares: 11-dehydrocorticosterone molecules (inactive); black triangles: dexamethasone molecules; circles: carbenoxolone molecules.



1.7.2 Adult Phenotype of Glucocorticoid Over-exposure Model

A number of groups have now examined the effect of excess glucocorticoid on long-term health and metabolism, considering a number of systems. As mentioned, dex-treated rats are born with an 11-16% reduction in their body mass, versus controls (Nyirenda *et al.* 1998; Welberg *et al.* 2001; Levitt *et al.* 1996; Sugden *et al.* 2001), and develop relative hypertension (Benediktsson *et al.* 1993; Levitt *et al.* 1996; Sugden *et al.* 2001) from 2-3 months of age. Administration of dexamethasone or

carbenoxolone *in utero* also results in glucose intolerance and insulin resistance from around six months (Nyirenda *et al.* 1998; Saegusa *et al.* 1999), i.e. some major components of the metabolic syndrome are present. Adverse effects of the treatment have also been noted in the kidney (Celsi *et al.* 1998), heart (Langdown *et al.* 2001), lung (Price *et al.* 1992), immune system (Bakker *et al.* 2000), and brain (Carlos *et al.* 1992; Welberg *et al.* 2000; Welberg *et al.* 2001), the latter affecting behaviour.

Effects of prenatal glucocorticoid have also been noted in guinea pigs (Liu *et al.* 2001) and sheep. Repeated injection with dex during early pregnancy programmes hypertension (Dodic *et al.* 1998) and reduced lipolytic capacity (Gatford *et al.* 2000), but glucose intolerance is only induced by treatment spanning mid to later gestation (Moss *et al.* 2001). Additionally, one study showed that pre-term treatment with glucocorticoids results in relative hypertension in adolescent humans (Doyle *et al.* 2000). Hence, not only are differing long term effects achieved through different windows of susceptibility, there is also inter-species variation in the timing of these.

1.7.3 Programming of Insulin-Glucose Metabolism

Much work is currently addressing potential underlying molecular mechanisms for the programming of insulin resistance. It has been shown that *in utero* dex and perinatal dietary manipulations reduce glucose-induced insulin secretion long term (Holness & Sugden 2001b; Aalinkeel *et al.* 2001; Petrik *et al.* 1999), probably due to impaired pancreatic development resulting in a programmed reduction in β-cell mass (Berney *et al.* 1997; Garofano *et al.* 1999; Petrik *et al.* 1999). Insulin target tissues are also affected by prenatal manipulations, however. Permanent up-regulation of PEPCK has been reported in both models (Desai *et al.* 1997b; Nyirenda *et al.* 1998a), while glucokinase is down-regulated in the low protein model (Desai *et al.* 1997b). The net result of reduced glycolytic flux and increased gluconeogenesis is likely to be increased hepatic glucose output.

Insulin-stimulated glucose clearance is impaired in dex offspring (Holness & Sugden 2001b), but molecular changes explaining this have not yet been documented in

muscle and fat. In young offspring of protein-malnourished dams, glucose uptake into muscle (Ozanne *et al.* 1996) and adipocytes (Ozanne *et al.* 1999) is actually improved, and this is associated with increased membrane insulin receptor and GLUT-4 in muscle (Ozanne *et al.* 1996). Increased presence of insulin-signalling components in fat further explains these observations (Ozanne *et al.* 1997), but belies a reduced effect of insulin on lipolysis (Ozanne *et al.* 1999; Holness *et al.* 1998). Low protein rats have reduced blood triglycerides, as a result of this, and probably reduced synthetic activity (Ozanne *et al.* 1998a), and resist ketosis (Ozanne *et al.* 1998b).

Reduced food intake during pregnancy has also been shown to programme hyperphagia and obesity, a phenotype which is exacerbated by subsequent feeding of a hypercaloric diet (Vickers *et al.* 2000). The co-existent paradoxical hyperleptinaemia is hypothesised to dysregulate the adipo-insular axis, leading to adipogenic diabetes (Vickers *et al.* 2001). Additionally, a low protein diet *in utero* exacerbates the loss of insulin sensitivity induced by high fat feeding in later life (Holness & Sugden 1999). The added effects of obesity upon those of *in utero* malnutrition have also been recognised in humans (Stanner *et al.* 1997), but are difficult to separate from the increased prevalence of obesity in low birth weight groups (Curhan *et al.* 1996; Ong *et al.* 2000).

1.7.4 Programming of Glucocorticoid Action

In addition to the programming effects of *in utero* glucocorticoids, the HPA axis and GR are both targets for programming. Epidemiological data show that low birth weight babies develop hyper-activity of the HPA axis later in life (Reynolds *et al.* 2001; Clark *et al.* 1996) associated with elevated adrenal androgens (Clark *et al.* 1996; Dahlgren *et al.* 1998), and that elevated cortisol is associated with glucose intolerance and hypertension (Phillips *et al.* 1998). Hyperactivity of the HPA axis was proposed as a factor linking low birth weight with later insulin resistance as a result of this. Elevated corticosterone has been reported in the adult offspring of dex-treated models (Levitt *et al.* 1996; Welberg *et al.* 2000), although reduced adrenal

size has been recorded in pups more aggressively dex-treated or malnourished *in utero* (Hristic *et al.* 1997; Lesage *et al.* 2001). Reduced GR in the hippocampus has been found several times in different prenatal programming models (Lesage *et al.* 2001; Langley-Evans *et al.* 1996a; Levitt *et al.* 1996; Welberg *et al.* 2000; Welberg *et al.* 2001), while GR up-regulation is programmed by postnatal handling of pups (Meaney *et al.* 1989; O'Donnell *et al.* 1994). Since, as previously discussed, the hippocampus has an important feedback input into the HPA axis, these findings are probably responsible for HPA hyper- and hypo-activity respectively in these animal models.

Altered GR expression and / or binding capacity has additionally been observed in other tissues. Increased GR is found in the liver of the dexamethasone and low protein models (Nyirenda *et al.* 1998; Bertram *et al.* 2001), localised in the former case to the periportal zones. It is likely that this leads to the elevation of PEPCK in these models. A preliminary report also suggests GR is increased in the pancreas of the dexamethasone model (Sugden & Bulmer 2001), and this change has also been reported in the kidney, whole brain, and lung of low protein rats (Bertram *et al.* 2001) and nutrient-restricted sheep (Whorwood *et al.* 2001). Increased GR implies increased tissue sensitivity to glucocorticoids, and this is exacerbated in the kidney and adrenal of low protein rats and sheep by reduced activity of 11β-HSD type II (Whorwood *et al.* 2001; Bertram *et al.* 2001).

The level of GR expression is not known as yet in tissues mediating peripheral disposal of glucose. Furthermore, it has not been investigated whether changes in GR expression are important in determining the adult phenotype of dex-programmed offspring, or whether they are merely a part of it. Dysregulation of GR could be secondary to insulin resistance, in view of the associations observed between the two (Vestergaard *et al.* 2001), or secondary to hypercorticosteronaemia, since tissue-specific effects of plasma glucocorticoid concentration on GR expression have been observed previously (Kalinyak *et al.* 1987). The resolution of this conundrum could be a significant step forward in the understanding of the molecular basis of the programming of insulin resistance by prenatal glucocorticoids.

1.7.5 Stress and programming

Maternal stress during late gestation has also been shown to exert long term programming effects on rat offspring (Meaney *et al.* 1996), particularly on stress-responsiveness and glucocorticoid sensitivity of brain regions (McCormick *et al.* 1995; Koehl *et al.* 1999; Takahashi *et al.* 1992). These findings are similar to those in the offspring of rats given dex in the last week of pregnancy (Welberg *et al.* 2001). Glucocorticoids are responsible for the mobilisation, production and distribution of energy substrates during stress, and hence it is not surprising that stressors cause hyper-activation of the maternal HPA axis (Williams *et al.* 1999). An increase in exposure of the foetuses to endogenous glucocorticoids is the likely consequence of this, which may explain the similarity between offspring phenotypes generated by prenatal dex and by stress. Programming effects are not restricted to prenatal manipulations, however, as neonatal handling (Meaney *et al.* 1989; O'Donnell *et al.* 1994) and stress (Meaney *et al.* 1996) have also been shown to exert long-term effects on stress susceptibility and brain GR expression. However, the long term metabolic consequences of perinatal stress have not been investigated.

1.8 Aims

Against this background, my thesis addresses the key questions listed below:

1) Is skeletal muscle and white adipose tissue metabolism programmed by prenatal over-exposure to glucocorticoids?

2) Is GR expression altered in these tissues?

3) Is programming of GR expression a primary determinant of the phenotype of the model, or is its level of expression set secondary to circulating glucocorticoid excess or insulin resistance?

4) What is the effect of perinatal stress upon the phenotype of *in utero* dex-treated and control rats?

2 Chapter Two – Materials and Methods

2.1 Materials

Materials were obtained from the following sources:

2.1.1 General Laboratory Chemicals

Bactotryptone Yeast extract Agar	*Becton Dickinson, Cowley, Oxon, UK.*
Liquid Nitrogen	*British Oxygen Company Gases, Guildford, Surrey, UK.*
Ethanol	*Hayman Ltd, Witham, Essex, UK.*
Other Organic Solvents Acids Formamide Formaldehyde (38%)	*BDH-Merck, Poole, Dorset, UK.*
Malate Dehydrogenase	*Roche Diagnostics Ltd, Lewes, East Sussex, UK.*
Solid chemicals	*Sigma-Aldritch Company Ltd, Poole, Dorset, UK.*

2.1.2 Molecular Biologicals

Restriction enzymes	
Nucleotides	
Transcription buffer	
DTT	
BSA solution	
RNA polymerases	
HB101 and JM109 cells	
p-GEM 3 and p-Gem-T Easy Vector cloning kit	
Taq DNA polymerase and buffer	
Wizard Plus Miniprep DNA purification System	*Promega Ltd, Southampton, Hants, UK.*
Agarose	*BioWhittaker Molecular Applications, Wokingham, Surrey, UK.*
100bp DNA ladder	
1kb DNA ladder	
First strand buffer	
Superscript enzyme	
Low Melting Point Agarose	
TRIzol^{R}	*Invitrogen Life Technologies / Gibco BRL, Paisley, UK.*
Qiagen PCR purification kit	*Qiagen Ltd, Crawley, West Sussex, UK.*
Oligonucleotides	*TAGN, Newcastle-Upon-Tyne, UK.*
ThermoSequenase Radiolabelled Terminator Cycle Sequencing Kit	*Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK.*

Dialysis tubing	*Phillip Harris Scientific, Ashby-de-la-Zouch, Leicestershire, UK.*
p-TRI-B-Actin-125-Rat plasmid containing cDNA insert	*Ambion Inc, Austin, Texas, USA.*
PVL167: pGEM-T Easy vector, Containing 186bp GR exon 2. Derived by 5'-RACE PCR from rat Thymus RNA	*Courtesy of Val Lyons and Dr. Karen Chapman, Endocrinology Unit.*
Kodak Biomax MR film	
Tris-saturated phenol	
Phenol / Chloroform / Iso-amyl alcohol (25:24:1 ratio)	*Sigma*
Electrophoresis Power Pac 300 Model 583 Gel Dryer	*Bio-Rad Laboratories, Ltd., Hemel Hempstead, Hertfordshire, UK.*

2.1.3 Antibodies

Anti-Corticosterone antibody	*Raised in rabbits by Dr. Chris Kenyon, then of Glasgow University.*
GR (M-20) anti-GR polyclonal antibody	*Santa Cruz Biotechnology Inc, (Autogen Bioclear UK Ltd, Calne, Wiltshire, UK).*
RaIRGT anti-GLUT 4 polyclonal Ab	*Biogenesis Ltd, Poole, Dorset, UK.*

Anti-rabbit horseradish peroxidase-linked Secondary Ab	*Amersham Pharmacia Biotech.*

2.1.4 Radioisotopes

[α-^{32}P]-GTP, 111 TBq/mmol	
[1,2,4,6,7-^{3}H] Dexamethasone, 3.37 TBq/mmol	
[1,2,6,7-^{3}H] Corticosterone, 2.85 TBq/mmol	
[α-^{33}P] dideoxyNTPs, 55.5TBq/mmol	*Amersham Pharmacia Biotech*
2-[^{3}H(G)]-Deoxy-D-Glucose, 222GBq/mmol	*NEN Life Science Products Inc, Boston, Massachusetts, USA.*

2.1.5 Animals

Wistar rats were supplied by *Charles River UK Ltd, Margate, Kent, UK*, or *Harlan Orlac, Bicester, Oxon, UK.* Females were purchased for mating at 200-250g, along with males of an equivalent age.

Feed (Standard rat chow) was supplied *by Special Diet Services, Witham, Essex, UK*, and contained 61.9% carbohydrate, 18.8% protein, 3.4% oil and 0.6% salt.

2.1.6 Drug substances

Corticosterone-21-acetate	
Dexamethasone	
Metformin (1,1 Dimethylbiguanide)	*Sigma*

Rosiglitazone (BRL 49653C)	*GlaxoSmithKline, Welwyn Garden City, Hertfordshire, UK.*

2.1.7 Materials for Protein Work

Glass microfibre filters	
Meltilex melt-on scintillator sheets	*Wallac Oy, Turku, Finland / Perkin-Elmer Life Sciences, Cambridge, UK*
Protease Inhibitors	
Tween 20 (Polyoxyethylenesorbitan Monolaurate)	
Colour Protein Markers	*Sigma*
ECL Western Blotting Detection Reagents	
Hybond ECL nitrocellulose membrane	*Amersham Pharmacia Biotech*
Non-Fat Dry Milk Blocker	
Mini-Protean 2 vertical electrophoresis System	
Bradford Protein assay reagent	*Bio-Rad Laboratories, Ltd*

2.1.8 Materials for Cell Glucose Uptake Assay

Type 1 Collagenase	*Worthington Biochemical Corporation, Lakewood, New Jersey, USA.*
Adenosine	
Bovine Serum Albumin Fraction 5	
2-Deoxy-D-Glucose	
1% Triton-X-100	
Cytochalasin B	*Sigma*

Human Actrapid Insulin 600μM 100iu/ml	*Novo Nordisk, Crawley, West Sussex, UK.*
Dow Corning 200/100cS fluid	*BDH-Merck.*

2.1.9 Materials for Ribonuclease Protection Assay (RPA)

Nick sephadex G-50 DNA column	*Amersham Pharmacia Biotech*
Picofluor-40 scintillation fluid	*Canberra-Packard, Pangbourne, Berkshire, UK.*
Aquasafe 300 Plus scintillation fluid	*Zinsser Analytic, Maidenhead, Berkshire, UK.*

Hybspeed RPA kit
(Containing 5mg/ml Torulla Yeast RNA, 5M Ammonium acetate, Hybspeed hybridisation buffer, Hybspeed RNase digestion buffer, Hybspeed inactivation / precipitation mix, Gel loading buffer 2, RNase A/T_1 solution).

Century RNA size markers	*Ambion Inc.*
RNase A RNase T_1	*Roche Diagnostics Ltd*
Kodak D19 developer Amfix high speed fixer	*H.A. West, Edinburgh, UK.*

2.1.10 Materials for plasma assays

Anti-rabbit scintillation proximity assay Reagent	*Amersham Pharmacia Biotech*

Mouse Leptin ELISA Kit
(Containing Antibody-coated microplates, 2.56ng lyophilised leptin standard, sample diluent, guinea pig anti-mouse leptin serum, anti-guinea pig IgG enzyme conjugate stock solution, enzyme conjugate diluent, enzyme substrate solution, enzyme reaction stopping solution, 20x washing buffer stock solution.)

Rat Insulin ELISA Kit
(Containing Antibody-coated microplates, 2ng lyophilised rat insulin standard, lyophilised sample diluents 1 and 2, guinea pig anti-insulin lyophilised serum, anti-guinea pig lyophilised antibody enzyme conjugate, o-phenylenediamine tablet, enzyme substrate diluent, phosphate-buffered saline powder, enzyme reaction stopping solution, 20% Tween-20.) *Crystal Chem Inc, Chicago, Illinois, USA.*

Infinity Glucose Reagent
(Containing 2.1 mM ATP, 2.5 mM NAD, >1500U/l yeast hexokinase, >3200 U/l Glucose-6-phosphate dehydrogenase.)
β-Hydroxybutyrate Assay kit
(Containing β-HBA reagent (4.6 mM NAD, oxamic acid and buffer, pH 7.6), 50 units/ml β-Hydroxybutyrate dehydrogenase, pH 7.6, 50 mg/dl D-β- Hydroxybutyrate Calibrator.)
10, 25 and 75 mg/dl D-β-
Hydroxybutyrate Calibrators *Sigma Diagnostics*

2.1.11 Equipment

Ultra-Turrax T8 auto-homogeniser	*Ika Labortechnik, Staufen, Germany.*
Dri-block DB Series	*Techne, Cambridge, Cambridgeshire, UK.*
GeneQuant RNA/DNA Calculator	*Amersham Pharmacia Biotech.*

Hybaid Thermal Reactor (RTs and RNA denaturation)	*Hybaid Ltd.*
1450 Microbeta Plus Liquid Scintillation Counter (used for standard cpm counting)	*Wallac Oy.*
Minaxi β Tricarb 4000 Liquid Scintillation Counter	*Canberra Packard, Pangbourne, Berkshire, UK.*
Eppendorf Mastercycler gradient (PCRs)	
Eppendorf Centrifuge 5415C (used for <2ml volume Eppendorf tubes)	*Eppendorf AG, Hamburg, Germany.*
Labofuge 400R Centrifuge (used for 15 and 50ml volume Falcon and Corning tubes)	*Heraeus, Brentwood, Essex, UK.*
Optima™ TLX Ultracentrifuge (rpm > 14000) Beckman J2-MC Centrifuge (used for maxi-preps, preparation of competent cells and cytosol preps.)	*Beckman Instruments, High Wycombe, Buckinghamshire, UK.*
Gallenkamp Orbital Incubator	*Sanyo Gallenkamp plc, Loughborough, Leicestershire, UK.*
Jouan EB115 Incubator (for Petri dishes)	*Jouan Ltd, Ilkeston, Derbyshire, UK.*

UV-160A UV-Visible recording Spectrophotometer	*Shimadzu Europa, Milton Keynes, Buckinghamshire, UK.*
EL 312e Bio-Kinetics Microplate Reader	*Bio-Tek Instruments Inc, Winooski, Vermont, USA.*
Combi cell harvester Vacuum pump	*Skatron Instruments, Dolasketta, Lier, Norway.*
Tomtec harvester 96 Mach III M Autotrap 24	*Tomtec, Orange, Connecticut, USA.*
Appligene Transilluminator "The Imager"	*Q-biogene, Harefield, Middlesex, UK.*
Northern light B95 Precision Illuminator	*Imaging Research Inc, St. Catharine's, Ontario, Canada.*
Dage MTI CCD72S imaging camera	*Dage Inc, Michigan City, Indiana, USA.*
Phosphorimager FLA-2000	*Raytest Scientific Ltd.*
Phosphorimager screens	*Fuji Photo Film Company Ltd, Tokyo, Japan.*
Whirlimixer	*FSA Laboratory Supplies, Fisons plc, UK.*
Orbital Shaker	*Bellco Biotech, Vineland, New Jersey, USA.*

Water Bath	*Grant Instruments, Cambridge, Cambridgeshire, UK.*

2.1.12 Software

Multicalc Advanced v2.0	*Wallac Oy*
GeneRunner v.3.05	*Hastings Software Inc, USA. (www.generunner.com)*
Radlig	*BioSoft, Cambridge, UK.*
MCID-M4 Image Analysis V.3.0 Rev 1.5	*Imaging Research Inc.*
Fujifilm Fluorescent Image Analyser FLA-2000 V.1.0 Aida 2.0 Auto Image Data Analyser	*Raytest Scientific Ltd, Sheffield, UK.*
SigmaStat for Windows v.2.0 SigmaPlot for Windows v.4.0	*SPSS Science, Chicago, Illinois, USA.*
Statistica v.5.0	*Statsoft, Tulsa, Oklahoma, USA.*

2.1.13 Standard solutions

Ultra-pure water	dH_2O, UV-treated and autoclaved.
DEPC-treated water	0.5ml DEPC was added to 500ml deionised water, agitated and left to stand for 1-24 hours prior to autoclaving.
DEPC-treated sodium chloride	5M sodium chloride solution, treated with DEPC as above.

Borate buffer	8.25g Boric acid and 2.7g sodium hydroxide were dissolved in 1l dH_2O and set to pH 7.4 with hydrochloric acid.
Deionised Formamide	150ml formamide was mixed with 15g mixed bed ion-exchange resin for >1 hour, filtered twice and stored away from light.
20 x GTB (glycerol tolerant buffer)	216g tris, 72g taurine and 4g EDTA dissolved in 1l dH_2O.
10 x MOPS buffer	42g MOPS, 16.6ml 3M sodium acetate and 20ml 0.5M EDTA were dissolved in 1l deionised water, adjusted to pH 7, and treated with DEPC as above.
10 x TBE buffer	104.9g Tris, 55.7g boric acid and 4.7g sodium EDTA dissolved in 1l deionised water and autoclaved.
Molybdate buffer	10mM tris, 2mM DTT, 1.5mM EDTA, 0.1M sodium molybdate, 10% glycerol, set to pH 7.2.
DNA loading buffer	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol in water.
Alternative loading buffer	0.35% (w/v) orange G, 40% glycerol in water.
Ambion gel loading buffer 2	95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 18mM EDTA, 0.025% SDS.
RNase A/T_1 mixture	25mg (1250 Kunitz units) RNase A powder dissolved in 2.3ml 10mM Tris 15mM sodium chloride pH 7.5. 0.2ml RNase T_1 (20000 units) in 3.2M

	ammonium sulphate pH 6 added. Mixed, aliquoted and frozen at minus 80°C.
20 x TBS buffer	24.2g tris and 80g sodium chloride dissolved in 500ml dH_2O, pH 7.6.
2 x Laemmli buffer	4% w/v SDS, 20% glycerol, 2mM DTT, 125mM tris, 10% β-mercaptoethanol in water, pH 6.8, with bromophenol blue added to effect.
Western Blot Running Buffer	3.03g tris, 14.4g glycine and 1g SDS made up to 1litre with dH_2O.
Western Blot Transfer Buffer	3.03g tris, 14.4g glycine and 200ml methanol made up to 1litre with dH_2O and chilled to 4°C.
LB broth	10g bactotryptone, 5g bacto yeast extract, 5g sodium chloride in 1l deionised water, autoclaved immediately.
LB agar	LB broth plus 15g agar per litre added prior to autoclaving.
GTE	50mM glucose, 25mM tris, 10mM EDTA in deionised water, adjusted to pH8 with hydrochloric acid. (used fresh)
5M Potassium acetate	245.6g potassium acetate was dissolved in 300ml water, and the volume made to 500ml with 57.5ml glacial acetic acid and 142.5ml water.
Alkaline SDS solution	0.2M sodium hydroxide, 1% w/v SDS.
TE buffer	10mM tris, 1mM EDTA, adjusted to pH8 with conc. hydrochloric acid. Autoclaved before use.

Caesium chloride / TE buffer	100g CsCl dissolved in 100ml TE buffer.
Anthrone Reagent	38ml of freshly opened concentrated sulphuric acid added to 15ml dH_2O on ice. 150mg of anthrone mixed in. (70% H_2SO_4 4mM anthrone).

2.2 Methods

2.2.1 Maintenance of Animals

All animal procedures were carried out under the terms of the Animals (Scientific Procedures) Act 1986 and Project Licence number 60/2466. Keith Chalmers ,Vince Ranaldi and Donald Hay were responsible for the maintenance of and prenatal injections given to the rats.

Animals were under the primary care of the animal technicians of the Biomedical Research Facility, or the Medical Faculty Animal Area throughout the experiments. All rats were maintained under controlled lighting (0700h – 1900h daily) and temperature (22°C) conditions, with *ad lib* access to food and water. Adult and post-weaning rats were kept four to a cage or two to a cage when their size required, and cleaned out weekly.

2.2.2 Production and care of offspring

Bought-in rats were allowed to acclimatise to their environment in home cages in the animal unit for a period of at least a week prior to mating. Single virgin females were placed in breeding cages with a male to permit mating, and recorded as such when an expelled vaginal plug was noticed. This was designated day zero of pregnancy. Males were used to impregnate an average of three females each. Females were

housed singly during pregnancy, and littered down on day 22. Litters were weighed individually at birth and culled to eight, retaining as many males as possible. Pups were weaned at three weeks of age and males retained for experiments in the company of litter-mates. Offspring were weighed at days 25, 40, 60, 80, 100 etc. In all cases, experimental cohorts comprised male offspring selected randomly from as many litters as possible (usually a maximum of two offspring per litter were used per experiment). Males alone were used to complement the results from previous studies using this model (Nyirenda *et al* 1998; Nyirenda *et al* 2001; Welberg *et al* 2001), and to avoid the confounding influence of varying progesterone levels on glucocorticoid-mediated metabolism (Xu *et al* 1990; Hirota *et al* 1985).

2.2.3 Prenatal administration of dexamethasone

Pregnant mothers were injected subcutaneously with a solution of 100μg per kg dexamethasone in 0.9% saline containing 4% ethanol (**Dex** mothers) or with an equivalent volume of vehicle (1ml per kg) (**Saline** mothers) each morning between days 15 and 21 of pregnancy inclusive.

2.2.4 Killing and harvesting of tissues

After mating or weaning was completed, bought-in rats were killed by cervical dislocation. Subject offspring were killed similarly or by decapitation at between six and nine months of age, between 9am and 12pm, without prior withdrawal of food. Unless indicated otherwise, relevant tissues were removed by dissection, weighed where appropriate, and quickly frozen on dry ice or in liquid nitrogen. Trunk blood was collected into a plastic tube containing 1ml 100mM EDTA through an EDTA-coated funnel, and removed to ice. As soon as possible thereafter, the tubes were centrifuged at 2000g for 7 minutes, and the supernatant plasma removed to Eppendorf tubes. Plasma and tissues were stored thereafter at minus 80°C.

2.2.5 Oral Glucose Tolerance Test

Rats were starved for 17 hours prior to oral glucose tolerance testing. Commencing between 9 and 9:30am, rats were weighed, and then the distal 5mm of their tails removed with a scalpel. Each tail was then massaged distally and the basal (time=0) blood sample collected into a 1.5ml Eppendorf tube pre-coated with 5000iu/ml heparin. The rats were then given 2g/kg glucose solution (0.2g/ml, 10ml/kg) by gavage and replaced in their cages. After 20 or 30, and 120 minutes, a further blood sample was collected by "milking" the tail, as before. In each instance, approximately 300-400µl of whole blood was collected, and centrifuged as above for collection of plasma, which was frozen at minus 80°C.

2.2.6 Measurement of Plasma [Insulin] and [Leptin] by ELISA

The concentration of insulin in plasma samples obtained during oral glucose tolerance testing was measured using Crystal Chem^{R} ELISA kits, according to the manufacturer's instructions (see Materials). The kits measure [insulin] in the range 156 to 10000 pg/ml; intra- and inter-assay CVs are reported as 3.5 and 6.3%. Leptin concentration was measured in stored trunk plasma using the Crystal Chem^{R} mouse leptin ELISA kit. The manufacturer reports that the anti-mouse leptin antibody supplied shows 90% cross-reactivity with rat leptin. The assay is designed for [leptin] between 200 and 12800 pg/ml, and intra- and inter-assay CVs of 5.4 and 6.9% respectively are quoted.

2.2.7 Measurement of Plasma [Glucose] by Hexokinase Assay

This assay was undertaken on starved plasma samples collected during oral glucose tolerance testing, using the Sigma Infinity^{TM} glucose reagent. Hexokinase catalyses the phosphorylation of glucose, and the glucose-6-phosphate produced is oxidised to 6-phosphogluconate, with the generation of NADH. The amount of NADH formed is proportional to the glucose present, and was measured by the increase in UV absorbance of the reaction mixture.

1ml of dH_2O was added to 10µl of each plasma sample and the non-specific absorbance at 340nm measured on the spectrophotometer, zeroed with water. The UV absorbances of duplicate or triplicate 10µl plasma aliquots or glucose standards in dH_2O (0, 2, 4, 6, 8, 10, 15 and 20mM) were then measured in a single assay after the addition of 1ml reagent and 5-60 minutes' incubation at RTP. Standard curves of Glucose / mM versus UV absorbance units were constructed and sample glucose concentrations calculated after the deduction of non-specific absorbance.

2.2.8 Measurement of Plasma [β-Hydroxybutyrate]

β-Hydroxybutyrate was measured in trunk plasma of rats. The assay relies on the equimolar generation of NADH during the oxidation of β-hydroxybutyrate to acetoacetate by β-hydroxybutyrate dehydrogenase, with detection as for the glucose assay.

Sigma calibrators were diluted with water to obtain standards of 0, 2, 4, 6, 8, 10, 15, 25, and 35 mg/dl. UV absorbances of samples and standards in duplicate were measured in a single assay on the spectrophotometer at 340nm, zeroed with dH_2O. To 17µl of water, standard or sample, 1.02ml of β-HBA reagent was added, and the mixture was warmed in a hot-block to 37°C. 17µl of β-Hydroxybutyrate dehydrogenase was mixed in and the UV absorbance of the incubate measured swiftly after 10-15 minutes at 37°C. Where possible, sample blanks in dH_2O were measured and deducted from measured absorbances. Standard curves of [β-Hydroxybutyrate] / mg/dl versus absorbance units were used to calculate sample concentrations.

2.2.9 Measurement of Plasma lipid parameters

These measurements were made by Dr. Philip Wenham, Department of Medical Biochemistry, Western General Hospital, Edinburgh.

Automated assays based upon a colourimetric end-point were used, utilising kits obtained from Wako Pure Chemical Industries Ltd, Osaka, Japan (Non-Esterified Fatty Acids) or Roche Diagnostics Ltd (Total and High Density Lipoprotein Cholesterol, Triglycerides).

2.2.10 Measurement of Plasma [Corticosterone] by Radioimmunoassay

Plasma cort concentration was measured in aliquots of thawed plasma by radioimmunoassay. The assay was developed by Dr. Chris Kenyon, who found intra and inter-assay variations of 9.4 and 9.2% respectively, and cross-reactivities for progesterone, deoxycorticosterone and cortisol of 7.7, 6.5 and 5.3% respectively, compared to cort (100%) (MacPhee *et al.* 1989).
25µl of plasma was mixed with 225µl of borate buffer containing 0.5% BSA, 1% methanol and 0.1% ethylene glycol, and heated to 75°C for 30 minutes to destroy corticosterone-binding globulin.

The solvent was evaporated from an aliquot of ^{3}H cort in a glass vial, and the steroid was suspended in borate buffer to give between 8000 and 13000 cpm per 50µl solution on the β-counter. 20µl aliquots of diluted plasma or cort standard (320, 160, 80, 40, 20, 10, 5, 2.5, 1,25, 0.625 and 0nM cort in ethanol) were incubated in duplicate with 25µl rabbit anti-cort Ab (final dilution 1:40000) and 25µl diluted ^{3}H cort for one hour in 96 well plates.

50µl anti-rabbit scintillation proximity assay reagent diluted with borate buffer was then added to each well, the plates sealed, shaken, and left at RTP for 24 hours to equilibrate. Plates were counted on the β-counter and [cort] in each sample calculated from a graph of cpm versus [cort] generated using the Multicalc programme.

2.2.11 Systolic Blood Pressure Measurement

Systolic BP was measured in rats using a tail cuff plethysmography method, first outlined by (Evans *et al.* 1994), and utilising an identical set of apparatus. This provides a quick, non-invasive measurement of BP, based upon the detection of a pulse in the tail of a rat, while the pressure in a more proximal occluding cuff is varied. Its accuracy is limited by movement artefacts and the effect of stress in raising the BP of the subjects. Other workers have published comparisons of plethysmography with arterial cannulation, however, which demonstrate a close correlation between the values obtained (Yamakoshi *et al.* 1979; Evans *et al.* 1994).

Rats were pre-warmed for 15 minutes in an incubator to 35°C, restrained in a drape, and an appropriately sized cuff was attached to each tail, as far proximal as possible. Four cycles of inflation and deflation of the cuff were performed, and the pulse wave generated was followed on the computer screen. The pressure of the cuff at the moment of the restoration of the pulse wave, on each deflation, is equivalent to the systolic BP. This was automatically recorded, and the mean value was calculated for each rat, excluding those cycles in which movement occurred. Each rat underwent this procedure on three separate occasions. The results of all sets of measurements were averaged for each rat, as no effect of familiarisation with the technique was observed over the course of these repetitions.

2.2.12 Extraction of total RNA from tissue

2.2.12.1 Muscle:

RNA was extracted with TRIzol^{R}, using a method derived from that of (Chomczynski & Sacchi 1987). Portions of muscle were ground in a mortar and pestle in liquid nitrogen and the powder emptied into 15ml Falcon tubes on dry ice. 10-15 volumes of TRIzol^{R} were added and the mixture stood at room temperature for five minutes to permit dissociation of nucleoprotein complexes. It then was vigorously agitated and vortexed ± auto-homogenised with the Ultra-Turrax, involving an average of three 10 second bursts at the three-quarter speed setting. The

tubes were centrifuged at 3000g for ten minutes at 4°C to remove insoluble material and the supernatant aliquoted into 1.5ml Eppendorf tubes. One fifth volume of chloroform was added and the tubes were vigorously shaken for fifteen seconds. After centrifugation at 4°C for 15 minutes at 12000g, the upper aqueous phase, was removed by pipette to fresh tubes. One half volume of propan-2-ol was added and the agitated mixture stood at room temperature for a further 10 minutes to precipitate the RNA. Centrifugation at 12000g for 20 minutes at 4°C pelleted the RNA, and the supernatant was discarded. The pellet was washed by vortexing in ≥ one volume of 75% ethanol, followed by centrifugation at 7500g for five minutes at 4°C. The wash was removed by drawn-out pasteur pipette and the pellet dried, before resuspending in DEPC-treated water. Resuspended RNA from the same tissue portion was recombined in a single tube.

2.2.12.2 Adipose tissue:

As for muscle, except that tissue was first auto-homogenised in TRIzol^{R} in place of grinding and then centrifuged at 600g for 10 minutes at 4°C. The upper lipid layer was discarded, and the subnatant aliquoted into Eppendorf tubes for RNA extraction.

2.2.12.3 Liver:

As for muscle, except that tissue was first auto-homogenised in TRIzol^{R} filled Eppendorfs in place of grinding.

2.2.13 Quantitation and agarose gel electrophoresis of extracted RNA

1-2µl of resuspended RNA from each tissue sample was diluted in 100µl total DEPC-water and its UV absorbance at 260 and 280nm measured on the GeneQuant. The RNA concentration calculated was recorded and the original [RNA] in the sample calculated. The ratio of Absorbance (260nm) / Absorbance (280nm) was also recorded, which is indicative of the purity of the RNA. Values as near 2.0 as possible are desirable.

The intactness of extracted RNA was assessed by agarose gel electrophoresis:

2.2.13.1 Method 1:

A denaturing gel was made using 0.3g agarose (1%), mixed with 2ml formalin and 3ml 10 x MOPS, made up to 30ml with DEPC-water. This mixture was heated in a microwave oven to melt the agarose and poured into a plastic mould, with a comb placed to create loading wells upon its cooling back to solid form. 1-2µg of the RNA solution was mixed with 2.5µl formaldehyde, 2.5µl 10x MOPS and 10µl deionised formamide and denatured at 65°C for 15 minutes or 95°C for five minutes. 2µl of a mixture of 1 part ethidium bromide / 20 parts loading buffer type 3 was added and the mixture loaded on to the gel. The gel was run at <70mA for approximately 30 minutes in 1x MOPS prepared in baked glassware, and the results viewed on the trans-illuminator at 240nm. The presence of intact 28S, 18S and 5S Ribosomal RNA bands indicated that the preparation was undegraded.

2.2.13.2 Method 2:

A 30ml 1% agarose gel was made containing 3ml 10 x TBE and 0.5µl ethidium bromide using RNase-free materials. 0.5-1.0µg of RNA was loaded in 2.5µl loading buffer per sample, and the electrophoresis performed in 0.5 x TBE. All other details were as above.

2.2.14 Preparation of cDNA templates by PCR

cDNA templates were prepared by PCR for various genes of interest in fat and muscle, to be used subsequently for synthesis of riboprobes for use in RPA. Forward and reverse oligonucleotide primers were designed to target rat mRNA sequences of interest of between 200 and 500bp, based upon published sequences, usually available through the BLAST database (National Centre for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA):

UCP-3	forward	TTGGC CTCTA CGACT CTG		
	reverse	GACAC CTTTC CCTGA ACC	anneal:	57°C
LPL*		ACTGC CACTT CAACC ACAG		
		CCCAA TACTT CGACC AGG		53°C
PPARγ		AGATT TGAAA GAAGC TGTGA ACC		
		TGTGA ACGGG ATGTC GTCTT CATAG		53°C
Leptin*		CCAAA ACCCT CATCA AGACC		
		GTCCA ACTGT TGAAG AATGT CCC		53°C
Resistin		TGTGC CCTGC TGCTG AGCTC TC		
		GCTAG TGACG GTTGT GCCTT C		58°C

* Primers kindly provided by Dr. Nik Morton.

Total RNA was used to prepare cDNA populations, and oligonucleotides were used to amplify the sequences of choice by PCR. 2-5μg of RNA was diluted to 60μl volume with ultra-pure water and denatured by heating at 65°C in a PCR block for 10 minutes, then transferred to ice. On occasion, RNA solutions were digested with DNase prior to the denaturation to remove genomic contamination. RNA was mixed with 1μl 10 x DNase buffer, 1μl RNase-free DNase I, and made up to 10μl total with ultra-pure water, and left for 10 minutes at RTP; the digestion was terminated with 1μl of 25mM EDTA. Reverse transcription of the RNA was undertaken using RT mixes containing multiples of 4μl first strand buffer, 1μl of 10mM mixed dNTPs, 2μl 0.1M DTT, 1μl random priming hexamers and 2μl superscript II enzyme. For the positive RT reaction, multiples of 10μl of RT mix were then added to 10μl of denatured RNA and placed at 42°C for 90 minutes in a PCR block. A negative RT reaction was usually set up in parallel, in which water was added to the mixture in place of enzyme, to reveal any genomic contamination of the RNA.

In subsequent PCR reactions, multiples of 2μl cDNA (+RT product), 5μl 10 x PCR buffer, 0.5μl 10mM mixed dNTPs, 2μl each of 10pg/ml forward and reverse primers corresponding to the gene of interest, 0.5μl Taq DNA polymerase, and 38μl ultra-

pure dH_2O were mixed in 0.5ml Eppendorf tubes. For each reaction utilising a +RT product, a parallel reaction was usually set up containing a –RT product in its place. Tubes were placed in thermal cycler blocks under a heated lid and subjected to 37 cycles of PCR amplification, consisting of 1minute at each of 95°C (denaturation), the specific annealing temperature for the primers used, and 72°C (polymerisation). This was followed by a final extension period of 10 minutes at 72°C, and the tubes were then held at 4°C until removed.

2.2.15 Checking and purification of DNA

The length and purity of the DNA was determined by agarose gel electrophoresis of 1µl of the solution in loading buffer against 100bp DNA ladder (PCR products) or 1kb DNA ladder and 1µl of uncut plasmid (linearised plasmids). 1% gels were made, containing 0.5µl of ethidium bromide, which were run at 130V for one hour in 0.5 x TBE. Single bands were desired, and their lengths were estimated by comparison with the appropriate ladder, and compared with that desired. DNA solutions showing a single band of the appropriate length were either gel-purified or phenol / chloroform-purified. The latter tended to be used for cleaning up linearised plasmids, while the former method was preferred for PCR products, as it eliminated the possibility of contamination with nucleotides or primer-dimers.

Gel purification involved similarly electrophoresing the entire volume on low melting point gels, from which specific bands were cut under brief illumination at 365nm. DNA was extracted from excised gel pieces using phenol / chloroform separation, sometimes followed by spin-column separation.

In the former case, gel blocks were melted in a hot-block at 68°C for 10 minutes, then one volume of Tris-saturated phenol was added to each, and the mixture was vortexed thoroughly, before centrifugation at 12000g for 15 minutes. The upper aqueous layer was removed from each tube and this was vortexed with one volume of phenol / chloroform / iso-amyl alcohol in fresh tubes. After five minutes'

centrifugation at 12000g, the upper phase was again removed and the extraction repeated. One tenth volume of DEPC-treated 5M sodium chloride solution and 2.5 volumes of absolute alcohol were added to the final upper phases, and the mixtures were vortexed and incubated at minus 80°C for 20 minutes to precipitate the DNA. This was pelleted by 10 minutes' centrifugation, and the pellets were washed in 75% ethanol, air-dried and resuspended in ultra-pure water. Where DNA solutions were purified by phenol / chloroform extraction alone, only a one-stage phase separation was employed, prior to DNA precipitation.

Where gel-separated DNA species were additionally column-purified, a silica gel membrane-based spin preparation kit was used, according to the manufacturer's instructions. This ensured the removal of any traces of extraneous organic compounds remaining.1µl aliquots of the putative purified products were electrophoresed as above to verify that the DNA was still present, and of the expected length. The [DNA] present was determined in each case by dilution and spectrophotometric assay on the GeneQuant.

PCR products were of the following lengths, corresponding to that predicted from sequence information: 260bp (UCP-3), 469bp (LPL), 262bp (resistin) (Kim *et al.* 2001b), 394bp (leptin) and 239bp (PPARγ).

2.2.16 Ligation of cDNA into vector

Purified PCR products were ligated into pGem-T Easy plasmid utilising –A/-T overhangs generated by the Taq polymerase. The volume of purified PCR product containing an amount of DNA equal to three times the molar quantity of vector per microlitre in the kit was calculated in each case. This volume was mixed with 5µl of 2 x Ligase buffer, 1µl linear vector and 1µl of T4 DNA Ligase and made up to 10µl with ultra-pure water. Positive and negative control ligations were set up, containing 2µl of control DNA insert in place of PCR product, or no DNA respectively. The mixtures were incubated at 4°C overnight.

2.2.17 Cloning of plasmid DNA

Ligated plasmids containing the DNA fragment of interest were used to transform competent *Escherichia coli* cells, in which large quantities of DNA could be produced during microbial replication. All procedures were carried out using sterile solutions, equipment and technique.

Competent cells (capable of readily taking up plasmids) were either bought-in (JM109 – in vector kit) or made using cultures of HB101 cells. In the latter case, a single colony was inoculated into 5ml of L-Broth and grown overnight at 37°C in a shaking incubator. The following morning, 1ml of this culture was pipetted into 50ml of L-Broth in a conical flask, and this volume was incubated for a further 90 minutes, at which time the bacteria would be in their log phase of growth. The broth suspension was then centrifuged at 3500g for 5 minutes at 4°C, and the resulting cell pellet was resuspended in 20 ml of ice-cold 0.1M calcium chloride solution. After at least 10 minutes' incubation on ice, the suspension was re-centrifuged as above, and the cells resuspended in 2ml 0.1M $CaCl_2$. The cells were ready for use after a further 2 – 24 hours on ice.

Petri dishes were prepared, containing LB-agar, which had been melted, cooled, and 1ml per litre of 100mg / ml ampicillin added (to a final concentration of 100µg / ml). Where colour-selection of colonies was desired, 200µl of 40mM IPTG and 20µl of 50 mg/ml X-Gal were spread across each plate just before use, and given 30 minutes to dry in.

Competent cells were transformed as follows:

2.2.17.1 Method 1 (JM109s – ligation products):

2µl of each ligation reaction was pipetted into a 1.5ml Eppendorf on ice, to which 50µl of freshly thawed JM109s were added. After gentle mixing, the tubes were left on ice for 20-30 minutes, then heat-shocked at 42°C for 50 seconds, and returned to

ice for another 2-3 minutes. 950µl of L-broth was added to each tube of transformed cells, and they were incubated at 37°C for 90 minutes. The cells were then pelleted by centrifugation at 1000g for 10 minutes, resuspended in 200µl of L-broth, and 100µl of this suspension distributed across each agar plate. Each culture was incubated overnight (16-20h) at 37°C, and colonies inspected the following day, at which stage the petri dishes were transferred for storage at 4°C. Colonies should have only developed from bacteria which had taken up religated plasmids (and contained an ampicillin-resistance cassette). If blue / white screening was undertaken, white colonies were further utilised, as these bacteria should have taken up an insert-containing plasmid, which could no longer express β-galactosidase activity.

2.2.17.2 Method 2 (HB101s – high concentration vectors):

The procedure was generally as above, except that 200µl of competent cells were transformed per 2µl of DNA, and typically, transformed cells were not initially grown up in L-broth, but the whole volume was plated out directly on to LB-AMP plates.

The presence of plasmids containing the insert of interest within a colony was checked sometimes by a PCR technique, using primers for the T7 and SP6 RNA polymerase sites flanking the insertion site.

Primers:	(annealing temperature 54°C)	(provided by Dr. David Brown)
T7 site	TGTAATACGACTCACTATAG	
SP6 site	GATTTAGGTGACACTATAG	

A small amount of each colony picked was taken on a pipette tip into 20µl of ultra-pure water, boiled at 99°C for 10 minutes to destroy the cells, and centrifuged at 12000g for five minutes to pellet the disrupted cells. 2µl of each supernatant, containing the plasmid DNA, was mixed with 2µl of 10 x PCR buffer, 1µl of 5pg/ml T7 primer, 1µl of 5pg/ml SP6 primer, 0.4µl of 10mM dNTPs, 0.2µl of Taq DNA polymerase and 13.4µl of ultra-pure water. The tubes were cycled as for the original

cDNA synthesis, and aliquots of each mixture were examined by agarose gel electrophoresis as also described above. Single bands of lengths corresponding to the size of the insert ligated in, plus the portion of the vector (180bp) between the polymerase sites were looked for.

2.2.18 Plasmid preparations

2.2.18.1 Maxipreps

This method was used when large quantities of DNA were required for use in multiple RPAs.

Single colonies identified as containing vector with the insert of interest were inoculated into 2ml of LB and grown until appreciable turbidity was apparent. This culture was then transferred into a conical flask containing 500ml LB, which was incubated for 14-16 hours in the orbital incubator. Each culture was centrifuged in two 250ml bottles at 3800g for five minutes at 4°C, and the cell pellet was resuspended in 12ml of GTE by pipetting. 24ml of fresh 0.2M NaOH / 1% SDS was added, the suspension shaken vigorously, and left on ice for at least 10 minutes. 16ml of cold pH 4.8 5M potassium acetate was then added, and the procedure repeated. The resulting lysate was then centrifuged for at 3800g for 10 minutes at 4°C and the supernatant filtered through Whatman number 4 filter paper into a fresh bottle, to which 32ml of isopropanol was added. Thirty minutes later, this was spun at 11000g for three minutes, and the resulting pellet was resuspended in 2.2ml TE by pipette. 2.95g of caesium chloride was dissolved in this suspension, and 100µl of ethidium bromide was also added. Ultracentrifuge tubes were filled with this mixture, balanced with 1% w/v caesium chloride in TE, sealed, and centrifuged for 17 hours at 200000g. This yielded a red band across the middle of each tube, in which ethidium-associated DNA was to be found. This layer was removed to a fresh tube with a syringe and needle, and the centrifugation process repeated to re-band the separated DNA, usually by spinning for four hours at 410000g. Each new band was syringed into a test tube, and an equal volume of isopropanol added. Having mixed this in, the upper layer was discarded and the process was repeated until all colour

due to the presence of ethidium had vanished. The resulting product was dialysed overnight at 4°C in three changes of fresh TE (2ml 0.5M EDTA and 10ml pH 8 1M Tris in one litre of water) to remove salts, and the [DNA] quantified spectrophotometrically.

2.2.18.2 Minipreps

Small-scale preparations of DNA were undertaken using the Wizard Plus^{R} kit to identify cells transformed by specific plasmids. Single colonies identified as containing vector with the insert of interest were inoculated into 2ml of LB and grown until appreciable turbidity was apparent (between five and 15 hours). The manufacturer's protocol was followed thereafter. DNA was purified from cells, protein and RNA by lysis, RNase digestion and passage through mini-columns. The products were eluted in ultra-pure water and the [DNA] quantified spectrophotometrically.

2.2.19 Restriction enzyme digestion of DNA

Restriction enzyme digestion was used for the linearisation of insert-containing vector in advance of riboprobe synthesis, the excision of inserts to verify their size (LPL, leptin, resistin and PPARγ), and to determine the orientation of inserts by asymmetric digestion.

When preparing templates for use in RNase Protection Assay, clones were selected from which antisense riboprobes could be transcribed using SP6 RNA polymerase, as this enzyme was found to be more reliable in producing a single RNA species of the predicted length. In the case of LPL, this orientation of DNA insert could only be achieved by excising the DNA insert from pGEM-T Easy using *Sph I* and *Sac I*, and re-ligating into pGEM-3.

In general terms, 1-2µl of enzyme was mixed with one tenth the total volume of the supplied buffer (or the optimum buffer for two enzymes used simultaneously) and 1-

5µg of DNA, ±BSA, and made up to between 20 and 60µl total volume with DEPC-treated or ultra-pure water. This was incubated for 90 to 180 minutes at 37°C (or the optimum temperature for the enzyme in use), and an aliquot electrophoresed on agarose gels to verify linearisation or to examine the length of products with reference to DNA ladder.

2.2.20 Sequencing of cDNA by dideoxy method

Chain termination sequencing involved the PCR synthesis of DNA strands from the cDNA template of interest, starting from a single primer-annealing site at the vector T7 or SP6 RNA polymerase sites. 3' elongation occurs in the presence of dNTPs and is terminated by the random incorporation of a dideoxyNTP, which is radiolabelled and present in low concentration. Four reactions, each containing a different ^{33}P-labelled ddNTP, allow the production of DNA chains of all lengths between two nucleotides and the total number of nucleotides in the sequence. After electrophoresis, the nucleotide sequence of the cDNA can then be read from bands produced by autoradiography. This procedure was carried out to verify the identity of the UCP-3 clone, with assistance from Val Lyons.

Termination mixes were prepared on ice. For each cDNA of interest, 2µl of the DNA solution was mixed with 2µl of 1pmol/µl T7 or SP6 primer (see above for sequence), 2µl of 10 x ThermoSequenase buffer, 2µl of ThermoSequenase DNA Polymerase and 12µl of water. 4.5µl aliquots of this were pipetted into four 0.5ml Eppendorfs and mixed with 2.5µl of one of the nucleotide mixtures. These were prepared from 2µl of mixed dGTP, dATP, dTTP and dCTP, and 0.5µl of one of the ^{33}P-labelled ddNTPs each. 35 thermal cycles of 95°C for 60 seconds, 55°C for 60 seconds, and 72°C for 90 seconds were allowed, then 4µl of stop solution / loading buffer was added to each tube. 3.5µl of each reaction was loaded on to a sequencing gel after denaturing at 70°C for 10 minutes.

The 6% sequencing gel was made by dissolving 42g of urea in 4ml 20 x GTB, 15ml 40% acrylamide and 35 ml dH_2O. After re-warming, the solution was made up to 100ml with dH_2O, filtered, 600µl of ammonium persulphate and 40µl TEMED added, and the gel poured. Electrophoresis occurred at <50mA for two hours, and the gel was exposed against Kodak Biomax MR film until readable. The order of occurrence of bands in each of the nucleotide lanes was compared with the published sequence for the gene of interest to verify its identity and orientation within the vector.

2.2.21 Ribonuclease Protection Assay

This method was derived from that of (McCormick *et al.* 2000). RPAs were used to determine the relative abundance of mRNA transcribed from a gene of interest between tissues of treated and control rats. Expression was compared to that of a so-called "housekeeping" gene, β-actin, a cytoskeletal protein which is commonly used for this purpose. ^{32}P-labelled cRNA probes for β-actin and the gene of interest are synthesised using linearised cDNA templates. These are hybridised in excess with complementary sequences contained within total RNA extracted from tissue, and the unprotected single-stranded RNA remaining is digested by ribonucleases. Radiolabelled species are visualised then by electrophoresis and autoradiography. The relative intensity of test versus β-actin bands can then be quantified, as the intensity of each will be directly proportional to the amount of complementary RNA in the mixture.

Riboprobes and RNA markers were synthesised *in vitro* transcription. 1µl of linearised template, 2µl of 100mM DTT, 4µl of a 1:1:1:1 mixture of 10mM rATP, rUTP, rCTP and DEPC-treated water, 4µl of 5x transcription buffer, 0.5µl of 40u/µl RNase Inhibitor, 0.5µl of 10mg/ml BSA, 5µl of ^{32}P-GTP, 2.5µl of cold GTP, and 1µl of 20u/µl RNA polymerase were mixed. This mixture was incubated for 90-120 minutes at 37°C (T7 polymerase) or 40°C (SP6 polymerase) to permit polymerisation. The remaining DNA was removed by digesting with 1µl of 1u/µl

RNase-free DNase for 15-20 minutes at 37°C. Labelled riboprobes and markers were purified from unincorporated nucleotides by passage through sephadex G-50 DNA columns, with elution in DEPC-treated water. 1ml of liquid scintillant was added to 1µl aliquots of each eluted fraction, vortexed, and counted on the β-counter. 1-200000 cpm of each fraction was electrophoresed in type III loading buffer on a denaturing 4% polyacrylamide gel for 15 minutes at 25mA to verify that single labelled RNA species of the appropriate length were present. Gels consisted of 1ml 40% acrylamide, 1ml 10 x TBE, 3.6g urea, 100µl ammonium persulphate, and 10µl TEMED made up to 10ml with dH_2O. Probes were either used immediately or frozen at minus 20°C overnight.

The first phase of the RPA was the precipitation of each RNA sample and its re-dissolution in hybridisation buffer. To a volume of solution containing the desired quantity of RNA, made up to 50µg with yeast t-RNA if required, an appropriate number of cpm of both the test and β-actin riboprobes were added. Positive controls consisted of 50µg of yeast t-RNA to which only one probe was added, and negative controls contained 50µg of yeast t-RNA and both probes. If the total volume was below 20µl, DEPC-water was used to make it up to this total. One tenth volume of ammonium acetate and 2.5 volumes of ethanol were then added, the mixture was vortexed, and incubated at minus 20°C for 20 minutes. The precipitated RNA was pelleted by centrifugation at 12000g for 15 minutes and air-dried.

Pellets were re-suspended in 20µl of hybridisation buffer by heating to 95°C for 15 minutes, with vortexing every five. Tubes were swiftly transferred to a second hot-block at 68°C for 10 minutes' hybridisation, before being allowed to cool. 100µl of digestion buffer was added to the positive control tubes, and 100µl of a 1:10 dilution of RNase A/T_1 mixture in digestion buffer to the samples and negative control tubes. Incubation for two 15 minute spells at 37°C with an intervening vortex permitted digestion of non-hybridised RNA in sample tubes and therefore all RNA in the negative control tube.

After this time was up, the reaction was stopped, and the remaining intact RNA was re-precipitated by the addition of 150µl of precipitation / inactivation buffer to each tube, and incubation at minus 20°C for 15 minutes. Hybridised RNA was pelleted by centrifugation as before, and pellets re-dissolved in 8µl each of loading buffer by heating to 95°C and vortexing. The denatured RNA solutions were loaded on to 5% polyacrylamide gels alongside controls and markers. 100-150000 cpm of markers were heat-denatured prior to parallel loading, and positive controls were diluted in loading buffer to a number of cpm similar to the sample lanes.

The gel mixture consisted of 5ml 40% acrylamide, 5ml 10 x TBE, 21g urea, 100µl fresh ammonium persulphate and 40µl of TEMED, made up to 50ml with water. Electrophoresis took place at 30mA for 50-60 minutes, and gels were subsequently dried for c. 75 minutes, before placing them against phosphorimager screens and Kodak X-AR5 film at minus 80°C sequentially to develop images.

No bands appeared in the negative control lanes, while bands corresponding to the full-length probe (from vector RNA polymerase site to utilised restriction site) could be seen in the positive control lanes. Sample RNA lanes contained bands of lengths equal to the specific cDNA insert only. Band intensity was determined from phosphorimager-generated images using Fluorescent image analyser and Auto-image data analyser software, and mean values of test band divided by β-actin band intensity were calculated for each treatment group, and the results compared. For quantitation between gels and sets of probes, comparison between band intensities produced by bulk standard RNA preparations on each gel were made.

2.2.22 Protein concentration by the Bradford method

The concentration of total protein in a solution was determined using the method of Bradford (Bradford 1976). The assay is based upon the binding of Coomassie brilliant blue G-250 to basic and aromatic amino acid residues, and consequent colour change in proportion to the amount of protein of ≥ 3000kDa present.

Samples were diluted with buffer to give solutions in the linear range of 0.05 – 0.5mg protein/ml, and BSA standards were prepared in the same buffer to 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. To duplicate 10µl aliquots of samples and standards in a 96 well plate, 200µl of a 1:4 dilution of Bio-Rad Protein Assay reagent were added. After agitation and five minutes' incubation at RTP, the absorbances in each well were measured in a microplate reader at 570nm. The [protein] in each sample was calculated from the mean absorbance, the dilution factor, and a graph of [protein standard] versus mean absorbance at 570nm.

2.2.23 Competitive receptor-binding assay

The quantity of functional receptor protein in a tissue can be measured using an assay in which various concentrations of "cold", unlabelled ligand are incubated with a defined concentration of tracer ligand, labelled with tritium. Bound and unbound ligand are separated, and the amount of label retained with the receptor in each incubate is measured by β-emission. From this information, the amount of receptor present per unit mass of tissue can be determined. The method described by (Panarelli *et al.* 1995) was utilised for the quantitation of GR binding in tissue.

Portions of liver were harvested from freshly killed rats and placed immediately into ice-cold Molybdate buffer, which stabilises GR-hsp complexes. On return to the laboratory, tissue was rinsed of blood and the buffer replaced, and it was subsequently minced with scissors. Auto-homogenisation in approximately three volumes of buffer followed, with care taken not to warm the buffer above 4°C. Homogenates were centrifuged at 20000g for 20 minutes at 4°C to generate crude cytosol preparations. Further centrifugation of supernatants at 105000g for 60 minutes at 4°C yielded refined supernatants. The [protein] within was measured as described above, and supernatants were diluted subsequently to 4mg/ml and used immediately, or frozen at minus 80°C.

96 well microtitre plates were prepared containing duplicate mixtures of 100µl cytosol, 50µl ^{3}H dexamethasone (diluted in Molybdate buffer to 6nM) and 50µl of "cold" dexamethasone dilutions (0, 0.632nM, 2nM, 6.32nM, 20nM, 63.2nM, 200nM, 632nM, 2µM, 6.32µM, 20µM and 200µM). These were incubated overnight at between zero and 4°C.

The following day, the contents of each well were vacuum-aspirated through glass microfibre filters on a cell harvester. This separates steroids bound to receptor from unbound steroid, as the latter is free to pass through the filter, while the former is retained. The filters were dried, and scintillator sheets melted on to their surfaces. The β-emission from each incubate was assessed by five minutes' counting per corresponding area of the filters, indicative of the amount of radioactive steroid bound to GR in each.

Plots of mean cpm versus [Dex standard] were constructed to verify that a typical sigmoid dissociation curve was described by the data. K_d and B_{max} for the tissue were calculated from Scatchard analysis of the data, using the Radlig package: on a plot of Ratio [Bound receptor] / [Free Receptor] versus [Bound receptor], the intercept on the abscissa is equal to B_{max}, and K_d is equal to the negative reciprocal of the gradient.

2.2.24 Western Blotting

The quantity of a specific protein present in a tissue was determined by western blotting. Total protein from lysed cells or a subcellular fraction of homogenate was prepared as detailed below. The protein content of each protein preparation was measured as described above, and diluted 1:1 with 2 x Laemmli buffer.

For determination of GR protein content, muscle lysates were prepared. 1-200mg of each muscle was minced in ice-cold 1.05ml LSM buffer, containing 20mM HEPES, 10mM KCl, 20mM sodium molybdate, 1mM EDTA, 1mM EGTA, 0.1mM sodium

orthovanadate, pH 7.9, 0.2% Nonidet P-40, and 10% glycerol (Eickelberg *et al.* 1999), to which 2μg/ml each of pepstatin A, leupeptin, aprotinin, soybean trypsin inhibitor (STI), and antipain, had been added immediately before use, to inhibit intracellular proteases. 400μM PMSF then was added, and the mixture auto-homogenised using the Ultra-Turrax^{R}. Homogenates were centrifuged at 400g for 15 minutes, to remove insoluble matter, and the supernatant frozen at minus 20°C over-night.

Low density microsomes (LDM) (consisting of membranes and vesicles) were prepared for measurement of total GLUT-4 in muscle, using a method derived from those of (Kim *et al.* 2000a) and (Castello *et al.* 1994). Less than 24 hours before electrophoresis, 3-400μg of muscle was minced and auto-homogenised in ice-cold buffer B (30mM HEPES, 600mM KCl, pH 7.4), containing 5μg/ml pepstatin A, 4μg/ml leupeptin, 3μg/ml aprotinin, 2μg/ml aprotinin and STI, and 400μM PMSF, prepared as above for the LSM buffer. Homogenates were centrifuged at 11,400g for 10 minutes, and the supernatants at 356,000g for 60 minutes. The resulting LDM pellets were resuspended in 100-250μl of ice-cold buffer C (20mM Tris, 255mM Sucrose, 1mM EDTA, pH 7.4).

1mm thick SDS-PAGE gels were prepared in the vertical electrophoresis system as follows. An 8% resolving gel containing 5.3ml dH_2O, 2.0ml 40% acrylamide, 2.5ml 1.5M tris.HCl pH 8.8, 0.1ml 10% SDS, 75μl 10% ammonium persulphate and 10μl TEMED was poured between the glass plates to two-thirds of their height. Its surface was covered with water-saturated butanol while the gel set, which was then washed out and a 4% stacking gel poured on top, and a comb added. This consisted of 7.565ml dH_2O, 1.0ml 40% acrylamide, 1.25ml 1M tris.HCl pH 6.8, 0.1ml 10% SDS, 75μl 10% ammonium persulphate and 10μl TEMED.

A pre-determined quantity of protein in Laemmli buffer was denatured for four minutes by heating to 95°C and immediately loaded into each well. Samples were electrophoresed in Running buffer at 40mA versus molecular weight colour markers

until the dye front reached the base of the resolving gel. Protein species are separated according to their mass, with smaller species migrating further over the same period. The resolving gel was subsequently removed from the assembly and pre-soaked in cold Transfer buffer, along with ECL blotting membrane, for 20 minutes. Proteins were transferred to the membrane by electroblotting in cold Transfer buffer at 250mA for 2.5-3 hours. Complete transfer was verified by the loss of marker dyes from the gel, or occasional staining of the gel with coomassie blue, which revealed any remaining protein.

Membranes were transferred to trays containing 5% milk powder and 0.1% Tween-20 in 2.5 x TBS and left overnight at 4°C on an orbital shaker, to minimise the availability of non-specific antibody-binding sites. The following day, dilutions of primary antibody in the same solution were applied to membranes for two hours, followed by three five-minute washes on the orbital shaker. Dilutions of the appropriate secondary antibody (directed against rabbit or mouse IgG) were then applied to the membranes for one hour. Three further five-minute washes in 0.1% Tween-20 in 2.5 x TBS and a final wash in 2.5 x TBS followed to remove any unbound antibody.

Antibody complex bound to the protein of interest was visualised using the ECL chemoluminescence method. Secondary antibodies are attached to a horseradish-peroxidase molecule, which catalyses the oxidation of luminol in the presence of hydrogen peroxide. The light produced exposes chemoluminescence-sensitive film in areas corresponding to the specific protein bands on the membrane. 1:1 mixtures of ECL reagents 1 and 2 were made and applied to membranes for one minute, and then ECL film placed against the wrapped membranes. The film was left in place for a period sufficient to give a suitable exposure, after which it was developed and fixed. Based upon the intensity of the visible bands, further exposures were made until clear specific bands were visible. Film images were captured on the MCID software via an imaging camera and illuminator, and band intensity on the resulting files analysed using AIDA.

A second antibody application could be made to membranes using the same protocol after existing bound antibody had been stripped. Blots were incubated for one hour on the orbital shaker with 0.2M glycine 0.1% SDS 0.1% Tween-20 pH 2, washed three times in 0.1% Tween-20 2.5 x TBS, and blocked as above for one hour at RTP or overnight at 4°C.

2.2.25 PEPCK assay

PEPCK catalyses the decarboxylation and phosphorylation of oxaloacetate to form phosphoenolpyruvate during gluconeogenesis, as well as the reverse reaction (incorporation of carbon dioxide and dephosphorylation, with dGDP as phosphate acceptor). The assay measures PEPCK activity by coupling oxaloacetate formation with its reduction by excess malate dehydrogenase to yield malate, with the equimolar consumption of NADH. The rate of decrease of [NADH], measured spectrophotometrically, is proportional to the activity of PEPCK (Petrescu *et al.* 1979).

Portions of liver from freshly killed rats were taken into ice-cold 250mM Sucrose / 5mM HEPES buffer pH 7.4 and minced with scissors. They were homogenised with the Ultra-Turrax in two 2ml Eppendorfs per sample, and centrifuged at 8000g for 10 minutes at 4°C. The crude cytosolic supernatants from each pair of Eppendorfs were then combined and re-centrifuged at 105000g for 60 minutes. The supernatants (refined cytosol) were then frozen at minus 70°C, apart from an aliquot of each which was used for determination of protein concentration by the Bradford method. Sample mixtures were pre-incubated in duplicate at 30°C for ≥ three minutes. Each 1ml reaction mixture contained 50mM HEPES pH 6.5, 50mM sodium bicarbonate, 1mM manganese (II) chloride, 0.25mM NADH, 1mM phosphoenolpyruvate, 1.5 i.u. malate dehydrogenase and 50-150µl cytosol preparation. The reactions were initiated in a spectrophotometer, set to "Kinetic" mode, using 0.15mM dGDP, and the drop in absorbance at 340nm followed for four to seven minutes. Reaction mixtures lacking bicarbonate were introduced periodically as negative controls. PEPCK activity was

calculated from the rate of decrease in absorbance during the linear phase of the curve, and the molar absorptivity of NADH.

2.2.26 Assay of 2-deoxy-D-glucose uptake by adipocytes

The rate of glucose uptake by adipocytes can be estimated by assaying the uptake of 2-deoxy-D-glucose, which is a close structural analogue of glucose. This compound is transported into cells by glucose transporters with equal efficiency to glucose and is phosphorylated by hexokinase, but it is not further metabolised. Since the activity of hexokinase is not rate-limiting, the rate of accumulation of 2-deoxy-D-glucose in cells is equivalent to the rate of glucose uptake by glucose transporters (Jenkins *et al.* 1986).

Cytochalasin B inhibits glucose transporter activity, and hence assays in its presence show the uptake of glucose into cells by simple diffusion. 2-deoxy-D-glucose uptake was measured in the presence of insulin (GLUT-4 mediated) and without insulin (basal uptake, predominantly GLUT-1 mediated), and the non-specific uptake, measured in the presence of cytochalasin B, deducted in each case (Gibbs *et al.* 1988).

Buffers to be used were freshly prepared each day, and warmed to 37°C in a water bath. 1 litre of KRH buffer was prepared, containing 118mM NaCl, 5mM $NaHCO_3$, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 25mM HEPES, 2.5mM $CaCl_2$ and 0.2μM adenosine, pH 7.4. 500ml of KRH/BSA buffer was made by dissolving 1% Fraction V BSA in KRH and re-setting to pH 7.4. 100ml of KRH/BSA/glucose was made by dissolving 3.0mM glucose in KRH/BSA and re-setting to pH 7.4. 15-20ml of KRH/BSA/glucose was warmed to 37°C in a 50ml Falcon tube and placed in a thermos flask in water of between 37 and 40°C for transport to the Animal Unit. Both epididymal fat pads were dissected out from a freshly killed rat, weighed, and placed into the warm buffer for immediate return to the laboratory.

A suitable piece of fat was weighed and quickly placed in 4ml / g fat of KRH/BSA/glucose in a Falcon tube at 37°C. To this, 2mg / ml collagenase was added and the fat was minced with scissors. The tube was then shaken in the water bath for 30 minutes and the resulting digest filtered through a tea strainer into another tube in the water bath. The collected adipocytes were allowed to float to the top of the liquid, and the buffer below was removed with a syringe and needle, prior to the addition of 20ml KRH/BSA. This flotation and washing step was repeated three times over a 20 minute period. The cells were then stored at 40% cytocrit until use (within 20 minutes).

Solutions for the glucose transport assay were prepared during the collagenase digestion. The sugar cocktail solution contained 400μM cold 2-deoxy-D-glucose and 16.7μM 2-[^{3}H(G)]-Deoxy-D-Glucose (3.7MBq / ml). The 10mM stock of cytochalasin B was diluted 1 in 10 with KRH for use, and the insulin was prepared in KRH to concentrations of 1nM and 25nM. These concentrations were chosen based upon preliminary experiments indicating that 10pM insulin would induce an approximately half-maximal, and 250pM insulin a maximal glucose uptake.
Six 50ml tubes were set up in the shaking water bath for each assay, containing 900μl KRH/BSA (glucose-free buffer) and 100μl of cells. 10μl of insulin dilution (0, 1 or 25nM) were added to two tubes each and incubated for 15 minutes. One minute prior to the end of this period, 10μl cytochalasin B was added to one tube containing each of the insulin concentrations. Afterwards, 25μl of sugar cocktail was added to each tube, and the mixture incubated for a further three minutes. Three 200μl aliquots of incubate were removed from each tube at the end of this period and added to 0.5ml Eppendorfs containing 200μl Dow Corning oil each, to terminate the reaction. Eppendorfs were centrifuged at 12000g for 30 seconds to collect the cells in pellets at the top of the oil. The adipocytes were transferred by Pasteur pipette to scintillation vials, and c. 1ml of 1% Triton-X-100 used to remove the remaining cells and solubilise them for at least 30 minutes. After vortexing in a large excess of scintillation fluid, dpm per vial were measured in the β-counter. Basal and insulin-

stimulated 2-deoxy-D-glucose transport rates into 100µl of adipocytes from the chosen rat were then calculated.

2.2.27 Assay of glycogen content by the Anthrone method

The method used was derived from that of (Van Handel 1965), with tissue preparation adapted from (Azpiazu *et al.* 2000). Portions of each muscle were powdered under liquid nitrogen and 12-22µg scraped into Eppendorfs, which were weighed before and after the addition. The powder was homogenised in 400µl 30% potassium hydroxide by syringe and needle, having been weighed again after each step to allow for variable pipetting and losses in the syringe. After heating in a hot-block at 100°C for 30 minutes to solubilise the tissue, 1.05ml absolute alcohol was added to each tube to precipitate the glycogen. After pelleting the glycogen by centrifugation at 6000g for 10 minutes, the pellets were washed with 1ml 70% ethanol. The tubes were re-centrifuged for five minutes and then the washing and centrifugation were repeated twice. The final pellets were dried and resuspended in 150-200µl of water by vortexing and heating to 70°C for 20 minutes. These were stored at minus 20°C overnight.

In duplicate or triplicate, 50µl of glycogen solution or glucose standard (0, 5, 10, 15, 20, 25, 30, 35 or 40µg glucose in dH_2O) was pipetted into pyrex tubes and placed into a 90°C water bath. 950µl of Anthrone reagent was added to each tube and marbles placed on top to minimise evaporation. After 20 minutes' incubation, the tubes were agitated and the absorbance of each incubate was measured at 620nm in a spectrophotometer blanked with dH_2O. The quantity of glucose and therefore glycogen per sample aliquot was estimated using a standard curve of [glucose] versus absorbance at 620nm. The glycogen content per gram wet weight of muscle was then calculated.

2.2.28 Statistical Methods

All data are quoted as the mean ± standard error of the mean. Results were taken to be significant with $p \leq 0.05$. Statistical analyses were undertaken using either SigmaStat or Statistica programmes.

The difference between the mean values of measurements made on two comparable treatment groups was tested by Student's t-test, and between multiple groups by One-Way ANOVA. Where multiple separate treatment types were involved, Two or Three-Way ANOVA was performed, with *post-hoc* testing using Tukey's Honest Significant Difference test, or Student's t-test. If measures were repeated, this was also taken into account. Analyses were made using equivalent non-parametric tests if data were not normally distributed, according to the statistics package used.

Linear regression analysis was used to examine the relationship between two continuous variables.

3 Chapter Three – Programming of Skeletal Muscle and Adipose Tissue Metabolism by Glucocorticoids

3.1 Introduction

Low birth weight and thinness at birth has been associated with increased incidence of hypertriglyceridaemia, hyperglycaemia, hyperinsulinaemia, and type two diabetes in later life (Hales *et al.* 1991; Phillips *et al.* 1994; Curhan *et al.* 1996; Fall *et al.* 1995a; Phillips *et al.* 1998), implying a pathophysiological link between *in utero* growth retardation and the development of insulin resistance. Hyperglycaemia and hyperinsulinaemia have been induced in the adult offspring of rats that were prenatally malnourished (Ozanne & Hales 1999; Holness & Sugden 1999), or over-exposed to glucocorticoids *in utero* (Nyirenda *et al.* 1998; Lindsay *et al.* 1996b), providing models with which to dissect this phenomenon.

Insulin resistance causes hyperglycaemia through the failure of inhibition of hepatic glucose output by insulin (Boden *et al.* 2001) and the attenuation of glucose uptake by GLUT-4 in skeletal muscle and adipose tissue (Bonadonna *et al.* 1996; Kelley *et al.* 1996). Similarly, fatty acid uptake by adipose tissue is attenuated, due to reduced LPL activity (Maheux *et al.* 1997; Reynisdottir *et al.* 1997), resulting in increased plasma triglycerides and LDL, at the expense of atheroprotective HDL (Daae *et al.* 1993). Multiple gene expression changes in muscle and fat have been associated with insulin resistance. For example, fat mass signalling is interrupted by insulin resistance, as the secretory response of leptin to insulin is subnormal (Liu *et al.* 1999; Hardie *et al.* 1996), muscular UCP-3 has been found to be differentially expressed in states of insulin resistance (Kageyama *et al.* 1998; Krook *et al.* 1998), while PPARγ, which is a transcriptional regulator of multiple genes in multiple insulin sensitive tissues (Way *et al.* 2001b), including UCPs (Sears *et al.* 1996), resistin (Way *et al.*

2001a) and leptin (Hollenberg *et al.* 1997), is itself up-regulated in muscle of obese subjects (Way *et al.* 2001a).

In the adult offspring of rats that were protein-restricted or given dex during pregnancy, permanently elevated expression and activity of hepatic PEPCK has been identified (Nyirenda *et al.* 1998; Desai *et al.* 1997). This implies increased gluconeogenesis in the liver, and therefore, increased hepatic glucose output. Insulin-stimulated glucose transport and insulin signalling mechanisms have been shown to be programmed in muscle (Ozanne *et al.* 1996) and fat (Ozanne *et al.* 1999) by a low protein diet, and these animals are also resistant to ketosis, in a similar manner to some type two diabetic patients (Ozanne *et al.* 1998b). Evidence also exists for dysregulation of the adipoinsular axis in malnutrition models (Vickers *et al.* 2001; Holness & Sugden 2001a). However, the effect of prenatal glucocorticoid over-exposure on glucose uptake and metabolism in muscle and adipose have not been investigated to date.

Changes in glucocorticoid activity in these tissues may mediate the observed metabolic changes in programmed offspring at a molecular level, through altered level of GR expression (Schmidt & Meyer 1994). Supporting evidence for this contention comes from findings that improved insulin sensitivity is associated with lower GR expression in muscle (Vestergaard *et al.* 2001), and the association of receptor polymorphisms with both insulin resistance and obesity (Weaver *et al.* 1992; Rosmond *et al.* 2000). Accordingly, differences in GR expression have been noted in both models of prenatal programming. In the dex rat, GR is elevated in periportal zones of the liver, which may mediate the increased PEPCK (Nyirenda *et al.* 1998), while GR is down-regulated in the hippocampus of adult dex offspring (Welberg *et al.* 2001). Moreover, widespread permanent GR up-regulation has been reported in the offspring of undernourished sheep and rats: in kidney, liver, lung and brain (Bertram *et al.* 2001; Whorwood *et al.* 2001), but the expression of muscle and fat GR in programmed offspring has not been reported.

Both white adipose tissue and skeletal muscle can be subdivided on a functional and anatomical basis. Muscle fibres are the functional units of skeletal muscle, and are subdivided firstly into type I and II fibres, which occur in different proportions in muscles with differing primary functions. For example, the rat soleus is composed of 87% slow twitch, type I, predominantly oxidative muscle fibres, while the EDL contains 98% fast twitch, type II, mainly glycolytic fibres (Armstrong & Phelps 1984). Muscle fibres types differ with respect to absolute levels of GR expression (Claus *et al.* 1996; DuBois & Almon 1984), the effects of glucocorticoids on them (Polla *et al.* 1994), and their metabolic function (Rivero *et al.* 1998). Intra-abdominal or visceral fat is less responsive to the anti-lipolytic effects of insulin than subcutaneous fat, and lipid accumulation in this depot is specifically associated with other features of the metabolic syndrome (Bjorntorp 1991). Visceral fat metabolism is particularly sensitive to glucocorticoids, since both GR (Pedersen *et al.* 1994; Montague & O'Rahilly 2000) and 11β-HSD type I (Bujalska *et al.* 1997) are highly expressed here. Additionally, the increased quantity of fatty acids released into the portal circulation from this depot in obesity may be responsible for induction of insulin resistance elsewhere (Benthem *et al.* 2000; Rebrin *et al.* 1996).

In this chapter, I present data to show that adult muscle and fat metabolism is programmed by *in utero* over-exposure to glucocorticoids. I have examined the expression of key metabolic genes in these tissues, and assayed various associated biochemical parameters. I have also further characterised the expression of GR in insulin target tissues of the model, as alterations in local glucocorticoid sensitivity may be important in the development of insulin resistance.

3.2 Methods

3.2.1 Animals

Female rats and their litters were maintained, bred, and administered with dexamethasone or vehicle during their third week of pregnancy as described in Chapter 2. Male offspring were killed at 6-8 months of age, and tissues and trunk plasma frozen as described in Chapter 2.

Tissues were obtained from animals used in other experiments (Welberg *et al.* 2001; Nyirenda *et al.* 1998), in which *in utero* dexamethasone administration had been shown to reduce birth weight and cause subsequent glucose intolerance, hyperinsulinaemia, hypercorticosteronaemia, and elevated hepatic PEPCK activity in adulthood.

3.2.2 Muscle Glycogen Assay

The glycogen content of portions of quadriceps muscle was assayed using the Anthrone method, as described in Chapter 2.

3.2.3 Measurement of plasma leptin, β-Hydroxybutyrate and lipids

These measurements were undertaken using thawed trunk plasma as described in Chapter 2. Measurements of plasma TAG, NEFAs, cholesterol, and HDL-cholesterol were made by Dr. Philip Wenham, Department of Medical Biochemistry, Western General Hospital, Edinburgh.

3.2.4 Cloning of DNA sequences

DNA templates were prepared to target each gene of interest, purchased (p-TRI-B-Actin-125-Rat plasmid) or obtained courtesy of Val Lyons and Dr. Karen Chapman (PVL167: pGEM-T Easy vector, containing 186bp GR exon 2, derived by 5'-RACE PCR from rat thymus RNA). Oligonucleotide primers were designed to amplify sequences from cDNA populations derived from rat quadriceps muscle (UCP-3) and retroperitoneal fat (all others). PPARγ primers were designed such both mRNA splice variants would be identifiable as separate bands on a RPA gel.

These methods are all described in detail in Chapter 2. A summary of the restriction enzymes used for insert excision, for determination of insert orientation, and for plasmid linearisation, and the predicted lengths of the DNA fragments, is shown below, in **Table 3.1**.

Table 3-1 Summary of DNA templates used in RNase Protection Assays

For each DNA template used in RNAse protection assays, the method of insert length and orientation determination within the vector are listed. Restriction enzymes used for this, and for linearisation of templates are detailed, along with the insert sizes produced, and the consequent length of protected RNA fragments on RNAse protection gels.

Key: Full: full length riboprobe; Prot: protected fragment on RPA gel; PCR: determined by PCR; SEQ: determined by sequencing; N/A: not applicable; *: position of PPARγ2 and total PPARγ protected bands, respectively.

DNA sequence	Insert length determination		Insert orientation determination		Linearisation		
	Enzyme	**Length**	**Enzyme**	**Length**	**Enzyme**	**Full**	**Prot**
GR	N/A	N/A	N/A	N/A	*Nco I*	383	186
β-actin	N/A	N/A	N/A	N/A	*BamH I*	218	108
UCP-3	PCR	440	SEQ	N/A	*Nco I*	236	163
Leptin	*Not I*	437	*BstX I*	110	*Nco I*	511	394
LPL	*Not I*	505	*Nco I*	449	*Apa I*	361	315
PPARγ	*Not I*	282	*Nco I*	101	*Sph I*	347	171* 239
Resistin	*Not I*	305	*Apa I*	149	*Sac II*	360	262

3.2.5 RNase Protection Assay

The relative quantity of specific mRNAs was assessed in insulin target tissues using RNase Protection Assay (RPA). Total RNA was extracted from frozen tissues using the TriZol^{R} method, quantified, and verified as intact as described in Chapter 2. Complementary ^{32}P-GTP-labelled riboprobes and Century^{R} markers were synthesised by *in vitro* transcription, using linearised DNA insert-containing plasmids, broadly as described in Chapter 2. The concentration of "cold" rGTP in the transcription mixture was varied according to the desired specific activity of the riboprobes. RPAs were undertaken as described, using appropriate quantities of total RNA , test and actin riboprobes. A summary of probe specific activities and RPA reaction constituents in each of the assays performed is shown below in **Table 3-2**. Typical riboprobe and polyacrylamide RPA gels are displayed as **Figure 3-1**.

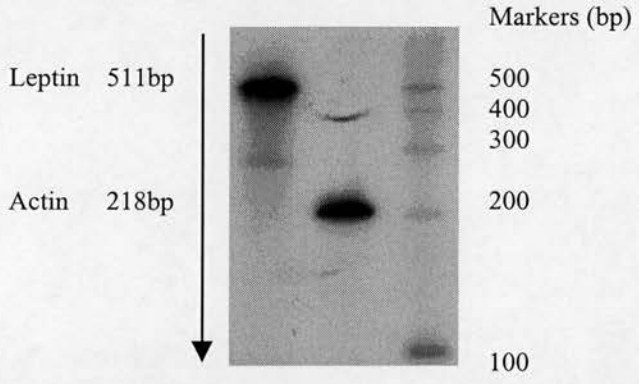
Table 3-2 Summary of riboprobe properties and reaction mixtures used in RNAse protection assays

For each target gene in each tissue examined, the final concentrations of unlabelled GTP used in the synthesis of test and actin probes (volumes and components as detailed on page 89) are shown. These were chosen based upon preliminary experiments to determine the optimum specific activity of each for quantitation of the relative abundance of the test probe. The quantity of labelled probe per microlitre was assessed by scintillation counting, and excess cpm of each probe, as determined in preliminary experiments, along with a sufficient quantity of total RNA to produce a signal, was mixed in each reaction tube.

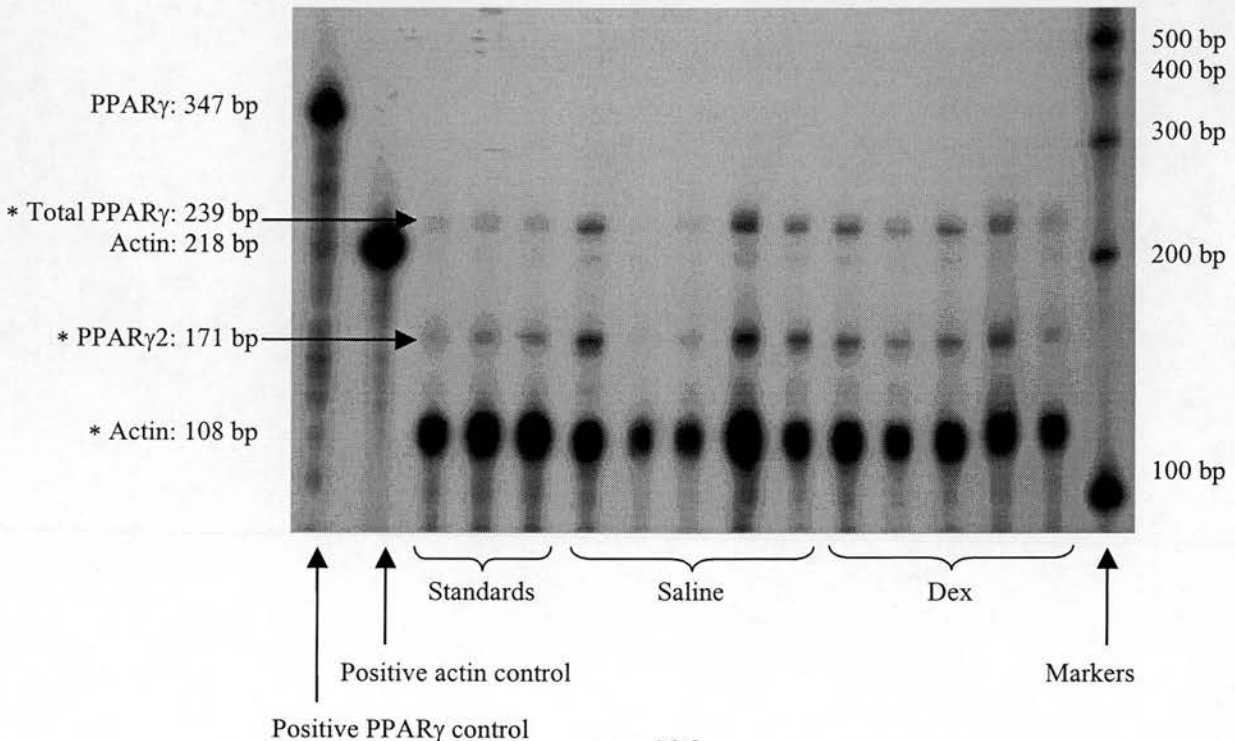
Probe	Tissue	[cold GTP] in Test probe / μM	Cpm Test probe in reaction	[cold GTP] in Actin probe / μM	Cpm Actin probe in reaction	Quantity of sample RNA in reaction / μg
GR	Liver, quadriceps	12.5	100,000	12.5	250,000	50
GR	EDL	12.5	100,000	12.5	250,000	30
GR	Soleus	6.25	100,000	12.5	250,000	30
GR	Retroperitoneal, subcutaneous fat	4.17	150,000	62.5	150,000	25
UCP-3	Quadriceps	12.5	120,000	12.5	150,000	30
Leptin	Retroperitoneal fat	25	100,000	12.5	150,000	5
LPL	RP Fat	62.5	100,000	12.5	150,000	7.5
PPARγ	RP Fat	4.17	75,000	25	175,000	15
Resistin	RP Fat	25	100,000	12.5	250,000	7.5

Figure 3-1 Typical examples of riboprobe and RPA polyacrylamide gels

a Leptin and actin riboprobes and century markers, synthesised from DNA templates as detailed on page 89 and in table 3-2. Aliquots of each probe and markers were electrophoresed on urea-PAGE gels and film autoradiographs obtained, such as that shown, with labelled species of the predicted lengths visible.



b Sample RPA gel for PPARγ mRNA quantitation in retroperitoneal fat, generated as described on page 90, and in table 3-2, visualised by autoradiography. Positive control, marker, 3 repeat standard and sample lanes (each from a different individual) are shown, with bands labelled according to length and identity. Key: *: protected RNA band; bp: base pairs of RNA; Standards: standard preparation of total RNA from adipose tissue; Saline: RPAs utilising RNA from control offspring; Dex: RPAs utilising RNA from dexamethasone-treated offspring

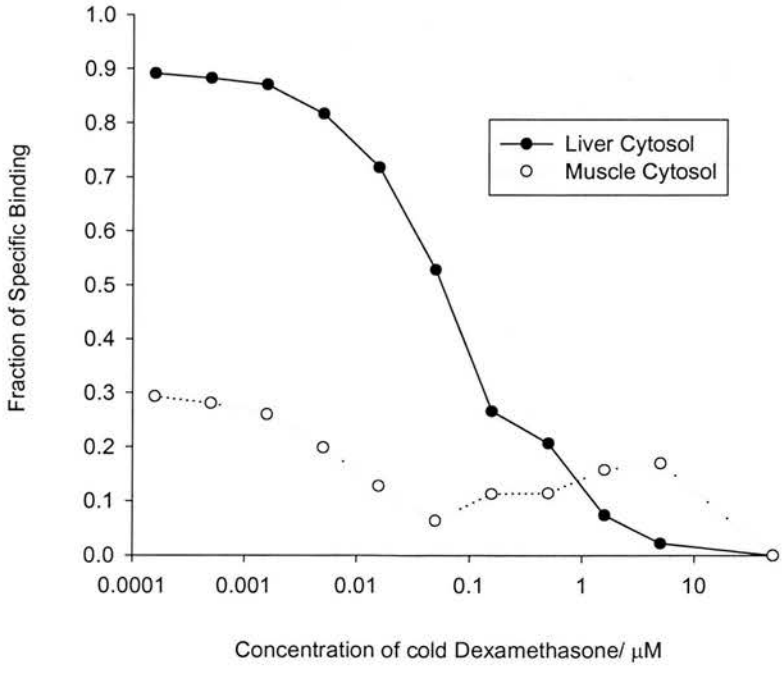


3.2.6 Western Immunoblotting

In order to determine whether differences in GR mRNA correlated with function, the development of a ligand-binding assay was attempted for skeletal muscle. A number of variations based upon the method of (Panarelli *et al.* 1995), and incorporating a scintillation proximity assay (Bosworth & Towers 1989) were attempted. Although the method could be successfully reproduced using liver cytosol, the amount of non-specific binding of dex by skeletal muscle was too great to permit quantification of GR binding. See **Figure 3-2**. Western blotting was utilised, therefore, as an alternative method of semi-quantifying GR protein in muscle homogenates, and was also used for assay of GLUT-4 protein. The biological relevance of results for GR protein was improved by standardising between blots using a bulk preparation of liver cytosol of known dex-binding capacity, which was stored at minus 80°C, and electrophoresed alongside muscle protein samples. The ligand-binding capacity of this standard preparation was determined as described in Chapter 2.

Figure 3-2 Specific binding of dexamethasone by liver and skeletal muscle cytosol of an adrenalectomised rat

Fraction of specific binding versus [Dexamethasone] in a preliminary ligand-binding experiment for GR in liver and quadriceps cytosol. Even in an acutely adrenalectomised rat, whereas liver GR binding follows a typical sigmoid dissociation curve, muscle GR binding is a minority of the total, making quantitation in this tissue unreliable.

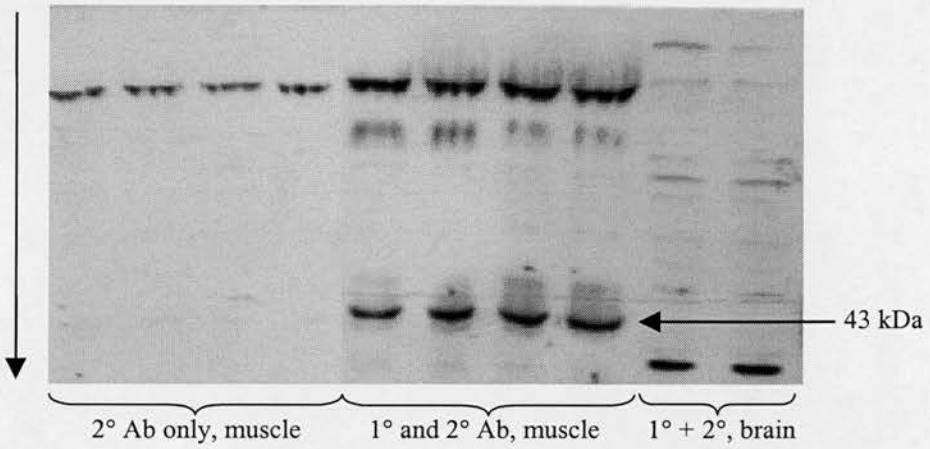


In the case of GR, measurement of protein content, SDS-PAGE and western blotting undertaken as described in chapter 2. On each gel, 5x 60μg muscle protein samples were electrophoresed alongside a lane containing colour protein markers, and lanes containing 4, 14, 26, and 40μg liver cytosol protein. Specific GR bands were identified according to the method detailed in Chapter 2. Blots were exposed to 1:500 dilutions of anti-GR Ab, followed by 1:5000 dilutions of anti-rabbit 2° Ab. After exposure to ECL^{R} reagents, blots were typically exposed to film for 1-2.5 minutes. Single bands at approximately 95kDa were typically produced using this method, as can be seen in the typical blot displayed as **Figure 3-5d**, later in this chapter. Plots of μg protein loaded versus GR band intensity for the liver cytosol were used to estimate the amount of GR protein present in each muscle homogenate. Three blots were generated for each muscle sample, and the mean B_{max} used to calculate overall mean binding capacity for the treatment group.

Total GLUT-4 content of quadriceps muscle was measured using a method derived from those of Kim (Kim *et al.* 2000a) and Castello (Castello *et al.* 1994). Quadriceps low density microsome (LDM) preparations were made as described in Chapter 2, including a bulk preparation of standard quadriceps LDM, which was electrophoresed alongside LDM samples to control for inter-gel variation in GLUT-4 protein band intensity. 25μg of LDM protein per sample were used in SDS-PAGE and western blotting, which were conducted as detailed in Chapter 2. 1° and 2° Abs were applied to blots at dilutions of 1:750 and 1:7500 respectively, and after ECL reagent application, blots were placed against film for 80s each. Triplicate blots were used to calculate the mean band intensity at 43kDa for quadriceps of each treatment group. See **Figure 3-3**.

Figure 3-3 Western blots for GLUT-4 in quadriceps muscle: specificity.

Although multiple bands appeared on each film using anti-GLUT-4 antibodies, bands corresponding to protein species of sizes other than 43kDa were also present either when 2° Ab was applied alone, or when both Abs were applied to brain lysates in preliminary experiments. Conversely, these lanes did not display the main 43kDa band, hence the 43kDa band could be regarded as the only specific GLUT-4 band. Gels containing muscle and brain protein lanes exposed to both antibodies or to secondary antibody alone are shown together below.



3.3 Results

3.3.1 Glucocorticoid Receptor in insulin target tissues

The effect of prenatal dex on GR expression was tissue-specific. In liver (**Figure 3-4 a**), it caused a 38% increase in GR mRNA, and in RP fat (**3-4 b**), a 50% increase. Conversely, in soleus (**3-4 d**), GR mRNA was reduced by prenatal dex (by 23%), while no difference in expression was seen in EDL muscle (**3-4 e**) or SC fat (**3-4 c**). In quadriceps muscle, GR mRNA and protein were not different between treatment groups (**Figure 3-5 a** and **3-5 b**). The trends displayed in **Figure 3-5** are similar for message and protein ($p = 0.12$ for protein, Student's t-test), and the results are weakly correlated ($r = 0.32$, $p = 0.17$)(**3-5 c**), implying that the abundance of GR protein follows that of mRNA. The Scatchard plot for the standard liver cytosol preparation used in western blotting is shown in **3-5 d** ($K_d = 3.5 \times 10^{-9}$M, $B_{max} = 6.3 \times 10^{-9}$M). An example of a western blot film is displayed in **3-5 e**.

Figure 3-4 GR mRNA expression in insulin target tissues of dexamethasone and saline-treated offspring.

GR mRNA measured by RNAse Protection in **A** liver, **B** retroperitoneal fat, **C** subcutaneous fat, **D** soleus muscle and **E** EDL muscle of 6 month old male rat offspring, and expressed as a ratio versus β-actin mRNA expression. n=8-10 per group. By Student's t-test, * $p<0.05$ versus saline control.

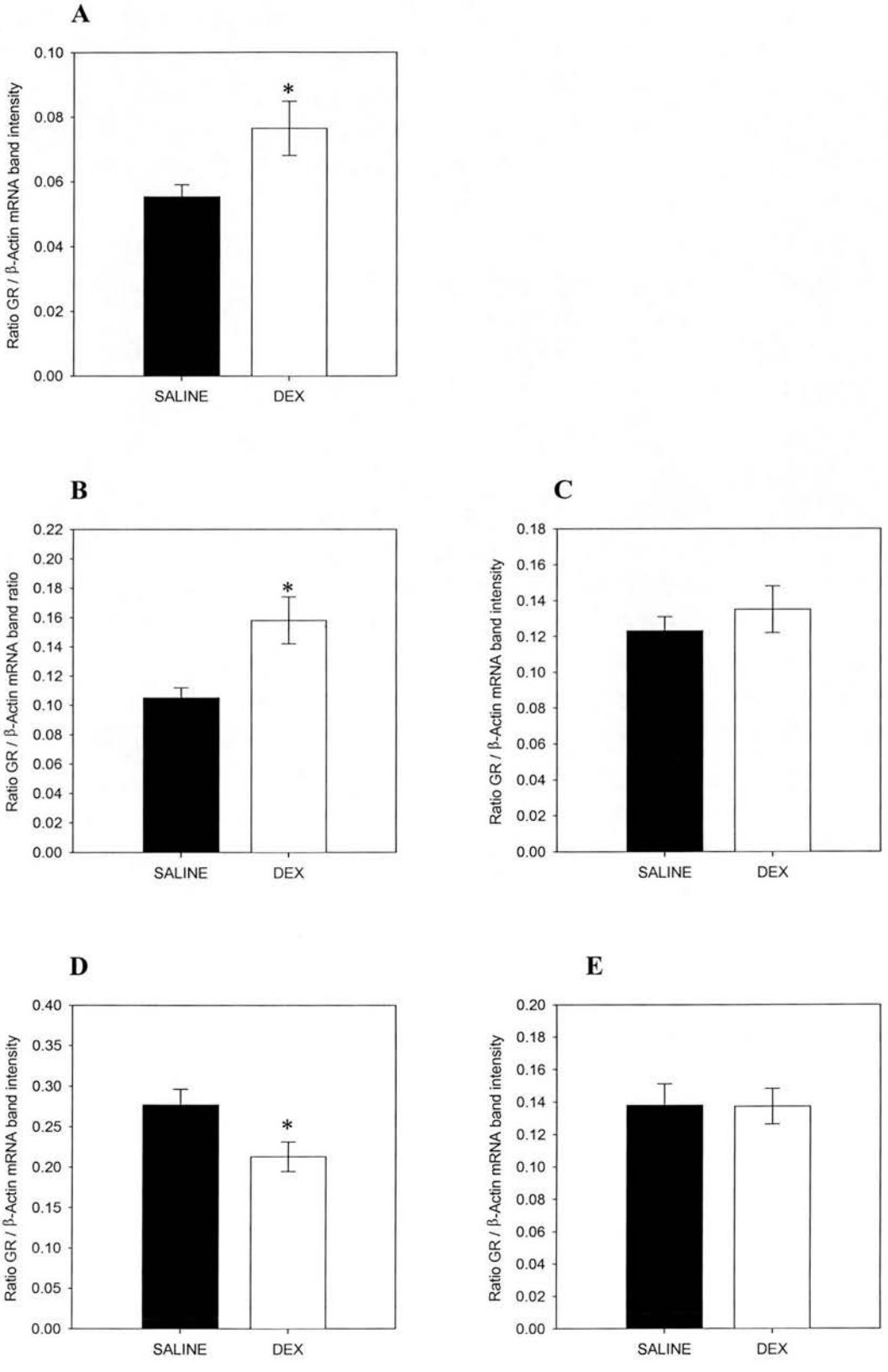
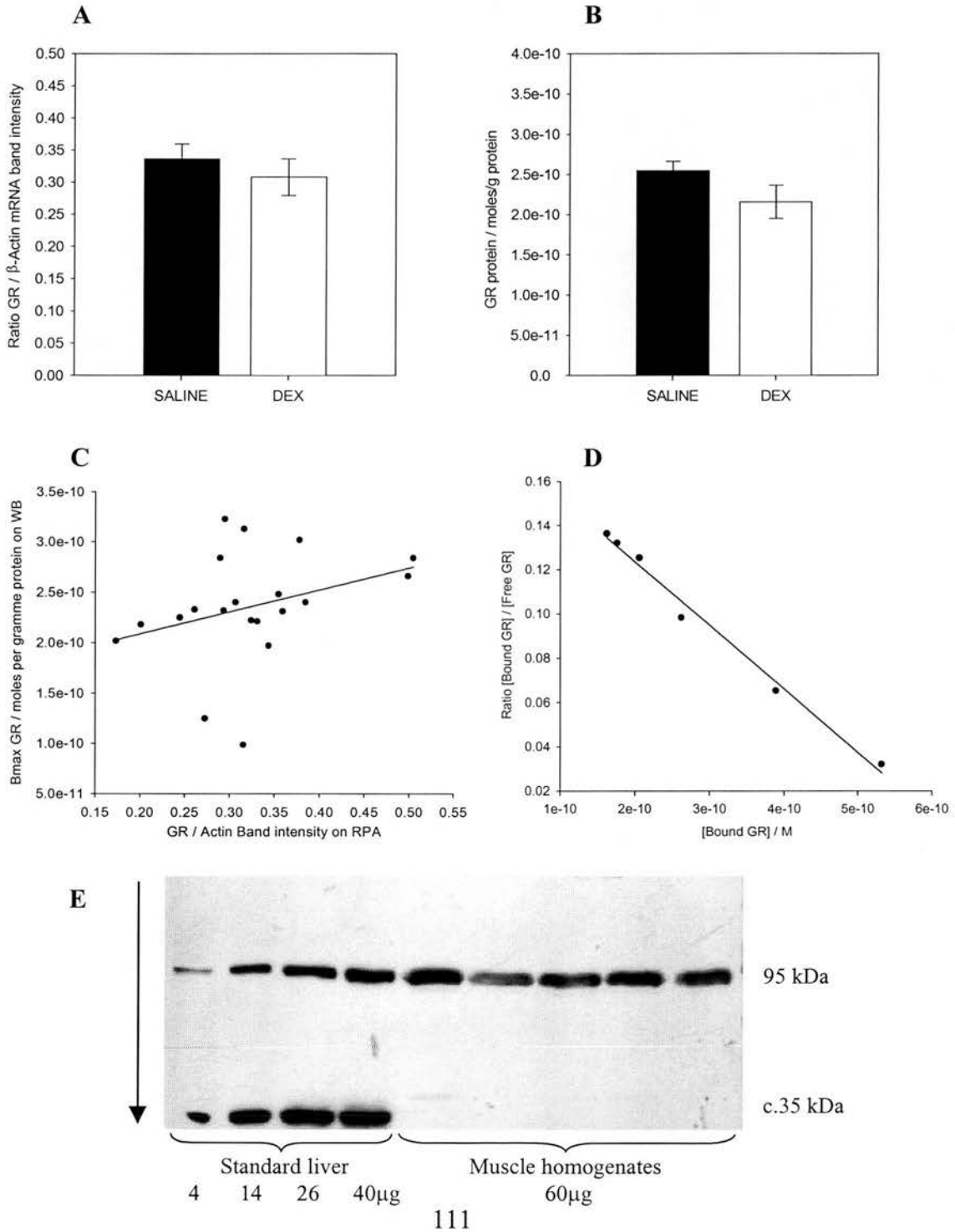


Figure 3-5 GR expression in Quadriceps muscle

A GR mRNA, quantified by RNAse protection analysis versus β-actin, and **B** GR protein, quantified by western blotting in triplicate, standardised using a bulk preparation of liver cytosol of known binding capacity, for 6 month old male offspring of dexamethasone and vehicle-treated rats. N=10 per group. **C** Scatter diagram showing estimated B_{max} for GR protein versus relative GR mRNA expression for all rats investigated. **D** Scatchard plot for bulk liver cytosol preparation used to quantify/ standardise between muscle GR western blots. From this, $K_d = 3.5 \times 10^{-9}$M (-1/gradient) and $B_{max} = 6.3 \times 10^{-9}$M (x intercept). **E** Typical western blot for GR protein in quadriceps. Total GR protein was detected as a single band at 95kDa in muscle lanes, with an additional non-specific band at c.35kDa in liver lanes, which was not used in quantitation. The quantity of total protein loaded into each lane is shown, with 4 different amounts of standard liver cytosol protein, and 5 lanes containing protein samples from vehicle (2, left) and dex-treated (3, right) quadriceps on each gel.



3.3.2 Biochemistry: Quadriceps glycogen and GLUT-4 content

The glycogen content of quadriceps muscle from adult dex offspring was 26% lower than that of control quadriceps (**Figure 3-6 a**). There was no significant difference in LDM GLUT-4 protein between groups, but there was a trend towards a lower level in muscle from dex rats ($p = 0.14$, Student's t-test) (**Figure 3-6 b**).

3.3.3 mRNA expression of muscle and adipose metabolic genes

There was no difference between groups in the relative abundance of mRNAs for UCP-3 in quadriceps (**Figure 3-7**), or for leptin or resistin in RP adipose tissue (**Figure 3-8 a** and **3-8 b**), as measured by RPA. There was, however, a 28% reduction in LPL mRNA in retroperitoneal fat of dex-treated rats (**3-8 c**). Whereas no significant difference was seen in total PPARγ expression (**Figure 3-9**), there was a trend towards increased PPARγ2 mRNA in dex-treated retroperitoneal fat ($p=0.09$, Student's t-test), and a small, but significant, shift from the PPARγ1 isoform to γ2.

3.3.4 Plasma parameters

In aliquots of freshly thawed plasma, there were no differences seen in the plasma levels of leptin, β-Hydroxybutyrate, triglycerides, non-esterified fatty acids, cholesterol, or HDL-cholesterol between treatment groups. See **Figure 3-10**.

Figure 3-6 **Quadriceps glycogen and GLUT-4 content**

Glycogen storage and GLUT-4 content of quadriceps muscle of 6 month old dex and saline-treated rats. **A** Glycogen content (μg per mg muscle), measured by the Anthrone method; n = 20 and 18 respectively. By Student's t-test, * $p = 0.01$ versus control. **B** GLUT-4 protein in low density microsome fraction of muscle, semi-quantified using western blotting; n = 10 per group.

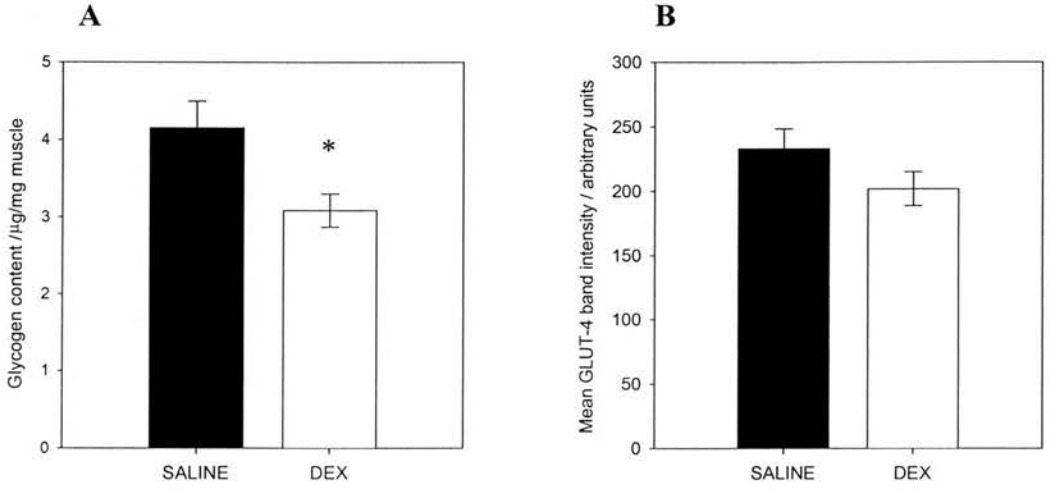


Figure 3-7 Quadriceps Uncoupling Protein-3 mRNA content

UCP-3 mRNA, measured using RNAse protection assay, normalised to β-actin, in 6 month old male offspring of dex and vehicle-treated rats. N=9-10 per group.

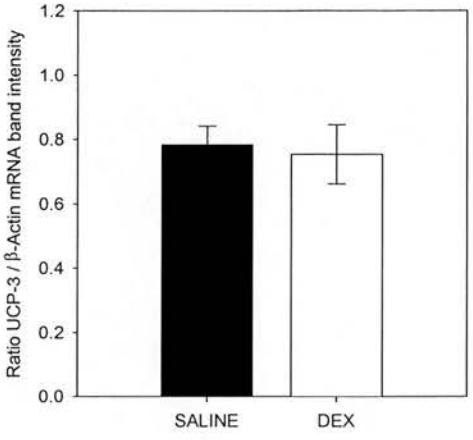


Figure 3-8 mRNA expression of key metabolic genes in retroperitoneal fat

mRNA levels quantified in retroperitoneal fat taken from 6 month old male offspring of dex and vehicle-treated rats using RNAse protection assays, normalised to β-actin. N=9-10 per group; by Student's t-test, * p=0.013 versus saline control. **A** Leptin **B** Resistin **C** Lipoprotein lipase.

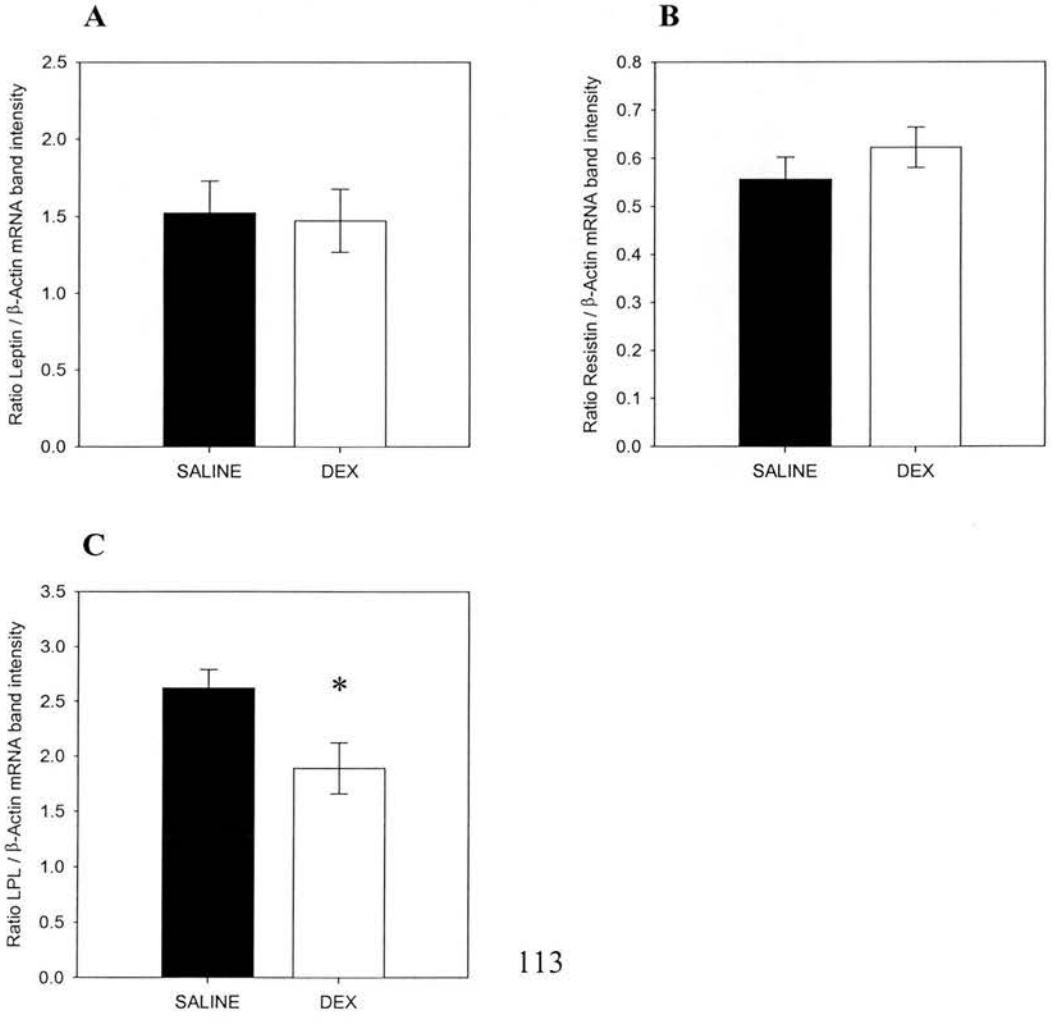


Figure 3-9 Expression of PPARγ isoforms in retroperitoneal fat

Level of expression of mRNA of PPARγ isoforms in retroperitoneal fat of 6 month old male dex and saline-treated offspring, measured by RNAse protection. **A** PPARγ1 (calculated from total PPARγ minus PPARγ2 band intensity), PPARγ2 and total PPARγ mRNA, normalised to β-actin mRNA expression. **B** Ratio of PPARγ1 to total PPARγ mRNA expression. N = 10 per group. By Student's t-test, * p=0.045 versus saline-treated control.

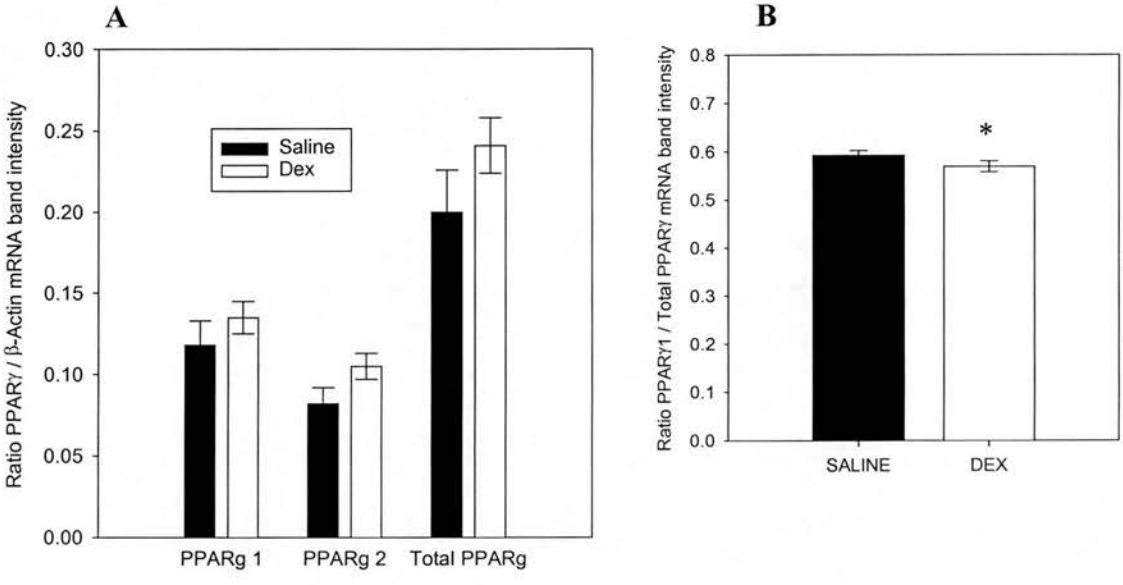
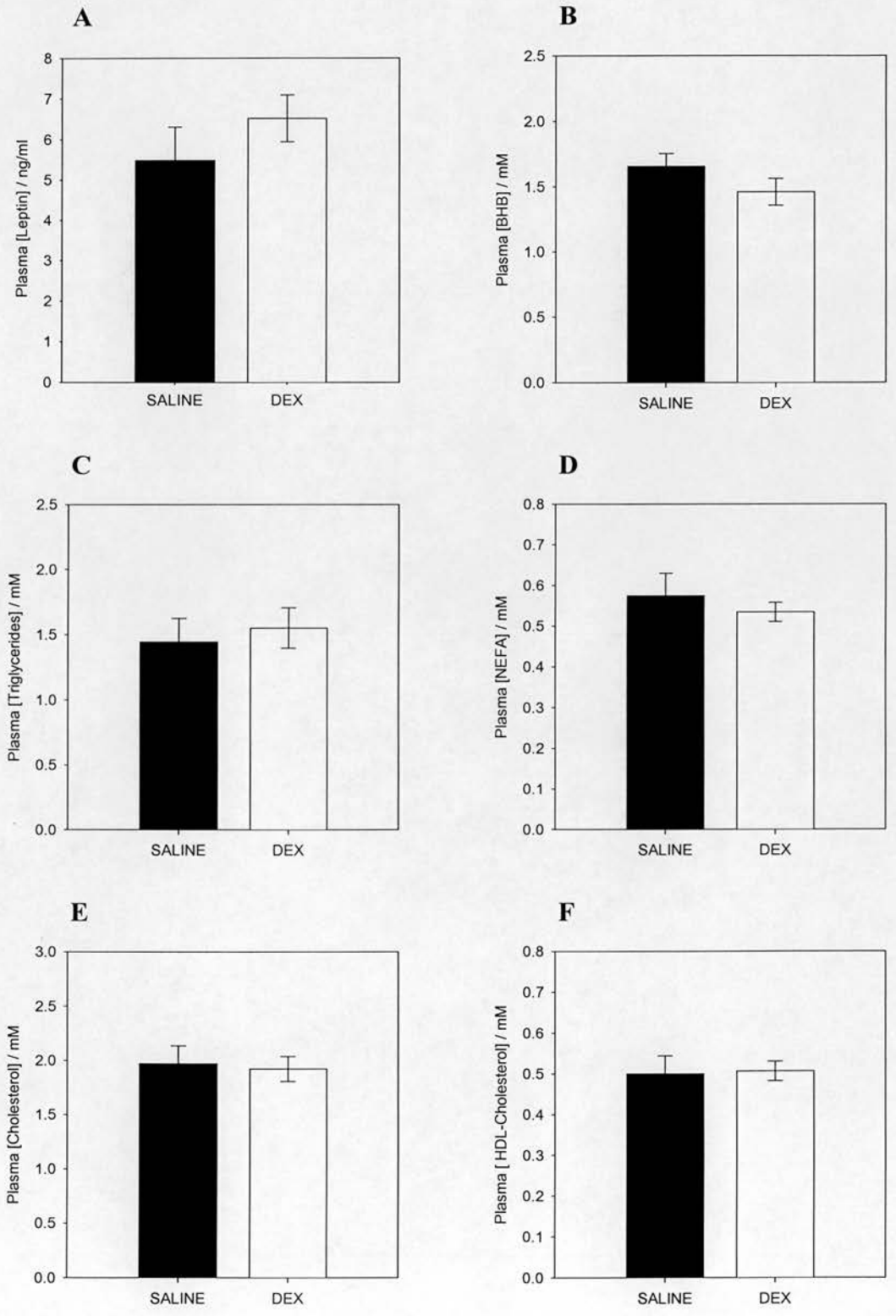


Figure 3-10 Plasma concentrations of substances related to lipid metabolism

Concentrations of substances related to lipid metabolism in trunk plasma of *ad lib* fed 6 month old male offspring of dex and vehicle-treated rats, measured using commercial kits. N=7 (saline group) and 12 (dex group). **A** Leptin **B** β-Hydroxybutyrate **C** Triglycerides **D** Non-esterified fatty acids **E** Total cholesterol **F** High density lipoprotein cholesterol.



3.4 Discussion

In line with the hypothesis, the results indicate that the effects of prenatal dex do indeed extend to skeletal muscle and white adipose tissue glucocorticoid sensitivity and metabolism. A programmed up-regulation of GR in retroperitoneal fat, similar to that previously observed in the liver, was accompanied by a reduction in LPL mRNA, and a small shift from PPARγ1 to PPARγ2 expression in this tissue. Despite this, no difference between groups was observed in plasma lipid composition, and the expression of leptin and resistin in RP fat of dex offspring was similar to controls. In contrast, there was a dex-programmed reduction in GR in soleus muscle, while GR mRNA was unchanged in two other muscles, in spite of elevated plasma corticosterone. Nevertheless, glycogen storage was reduced in the quadriceps, accompanied by a downward trend in total GLUT-4 protein, suggesting that glucose disposal into muscle may be attenuated.

Up-regulation of liver GR as a result of prenatal dex treatment is consistent with previously reported *in situ* hybridisation data (Nyirenda *et al.* 1998). This finding validates the RPA method used, in which quantification of GR was undertaken versus β-actin, to compensate for the variable gel loading seen using this technique. This finding is important, as the method assumes that the latter can be regarded as a "housekeeping" gene, a supposition which is hard to judge from RPA gels, due to inherent variation in gel loading. In practice, because of the extremely widespread transcriptional effects of insulin, a genuine "housekeeping" gene is hard to come by. Indeed, cytoskeletal actin is involved in the intracellular trafficking of GLUT-4 (Omata *et al.* 2000), and is probably transcriptionally regulated by insulin (Knight *et al.* 1995).

The hypothesis that increased GR expression in fat and muscle might mediate peripheral insulin resistance was partially confirmed, as GR programming was found to be specific to depot and fibre type, respectively. Whereas in EDL muscle, there

was no difference in GR between groups, GR mRNA was decreased by prenatal dex in soleus muscle, while an intermediate trend was apparent in quadriceps, consistent with its mixture of type I and II muscle fibres (Armstrong & Phelps 1984). A similar trend was identified in GR protein in quadriceps, with a weak correlation between individual protein and mRNA measurements. This is in line with expectation, in view of the predominantly transcriptional level of control of GR expression (Okret *et al.* 1986; Dong *et al.* 1988). The antibody used shows total GR content of the tissue, through direction against an epitope common to both human GRα and β at the amino terminus. The latter isoform does not bind ligand, and has been hypothesised to inhibit transactivation of glucocorticoid target genes by competition with active GRα for glucocorticoid response elements, thereby modulating tissue sensitivity to glucocorticoids (Bamberger *et al* 1995; Oakley *et al* 1999). However, GRβ is not present in the mouse (Otto *et al* 1997), and has not been reported in the rat, questioning its physiological significance, and implying that the assays used herein do not conceal a potential confounder of GR activity as part of their detection of "total" GR.

The reduced glucocorticoid sensitivity in soleus muscle, and therefore presumably in type I fibres, would be expected to ameliorate insulin resistance locally (Kusunoki *et al.* 1995; Dimitriadis *et al.* 1997), so this may be a protective adaptation of type I fibres against the prevalent hypercorticosteronaemia, as these fibres normally over-express GR relative to type II fibres (DuBois & Almon 1984). A lack of GR down-regulation in the face of hyper-corticosteronaemia, however, suggests that type II fibres may still be relatively susceptible to glucocorticoid-mediated effects.

Despite the lack of difference in quadriceps GR, the amount of glycogen stored by this muscle was reduced in dex offspring, implying either that glucose uptake is attenuated, or that the general level of muscular activity is increased in dex-programmed rats. The latter possibility is less likely, however, as empirical observation of rats in cages, and behavioural testing, for example using an "open field" (Welberg *et al.* 2000) do not support this contention. The downward trend in

GLUT-4 protein in the LDM fraction of quadriceps is also consistent with a reduction in glucose uptake by this muscle.

Glucose transport, and the phosphorylation and glycogen synthase-catalysed steps have all been suggested as rate-limiting in glycogen accumulation (Azpiazu *et al.* 2000; Cline *et al.* 1999), but glucose transport seems to be most significant (Cline *et al.* 1999). However, the primary control of glucose transport is at the level of intracellular trafficking of glucose transporters (Gibbs *et al.* 1988), so membrane fractionation, showing altered distribution of transporter molecules, might support the hypothesis more convincingly. In other models of insulin resistance, an altered ratio of plasma membrane GLUT-4: intracellular vesicular GLUT-4 has been recorded, as a result of defective translocation (Coderre *et al.* 1996; Weinstein *et al.* 1998). In support of this idea, young protein-restricted rats show increased glucose uptake, associated with unchanged total GLUT-4, but increased plasma membrane transporter. More may have been learned from assay of adipose GLUT-4, as hyperinsulinaemia has been shown to increase adipose, but not muscle GLUT-4 expression (Cusin *et al.* 1990), and adipose-selective reduction in GLUT-4 results in muscle and hepatic insulin resistance (Abel *et al.* 2001). The preservation of GR levels in this muscle suggests that the attenuated glucose uptake could occur as a result of the prevalent hypercorticosteronaemia, or could be programmed through an independent mechanism.

There is no evidence that mitochondrial uncoupling is programmed in quadriceps muscle, however, as no difference in UCP-3 expression was recorded, despite this being both glucocorticoid-regulated (Silva & Rabelo 1997), and altered in other models of insulin resistance (Kageyama *et al.* 1998; Krook *et al.* 1998). Down-regulation of UCP-2 and –3 has been noted in cardiac muscle of rats given dex continuously during their third week of pregnancy (Langdown *et al.* 2001b), however, implying that altered oxidative efficiency could be important in other tissues.

The pronounced increase in GR in retroperitoneal fat implies an increase in glucocorticoid sensitivity in this depot, in contrast to the lack of effect of prenatal treatment on subcutaneous fat. The combination of increased GR and plasma corticosterone would be expected to greatly amplify glucocorticoid-mediated effects in the former depot. Although measurement was also made of mesenteric GR, no difference in mRNA was found in this depot (data not shown). However, the freezing of this depot precluded assay of GR in adipocytes only, and the yield of RNA from this tissue suggested that there was a large degree of contamination with vascular or other GR, which probably masked the genuine result. For the purposes of interpretation, therefore, intra-abdominal retroperitoneal fat is used as a proxy for visceral adipose tissue.

A fuller consideration of local glucocorticoid sensitivity would incorporate measurement of 11β-HSD type I in these tissues, which re-constitutes active glucocorticoid (Chapman *et al.* 1997). This enzyme is expressed at high levels by visceral fat (Bujalska *et al.* 1997), and its activity is increased in this depot in a model of obesity and insulin resistance (Livingstone *et al.* 2000a). Moreover, over-expression of this enzyme in visceral fat of mice has recently been shown to cause an increase in [corticosterone] in the portal circulation, which may adversely affect hepatic insulin sensitivity (Masuzaki *et al.* 2001). Nevertheless, 11β-HSD type I was found not to be programmed by prenatal dex in the liver (Nyirenda *et al.* 1998).

The expression of several genes which are differentially regulated in insulin resistance was also examined in retroperitoneal fat, to determine whether there was a down-stream metabolic effect of this locally increased sensitivity to glucocorticoid action. Leptin (ob) mRNA and plasma leptin were unaltered by prenatal dex, probably implying a lack of obesity in adult offspring (Pelleymounter *et al.* 1995; Lonnqvist *et al.* 1995). However, leptin was increased in one year old rats given dex continuously during their third week of gestation (Sugden *et al.* 2001), and in others malnourished prepartum (Vickers *et al.* 2000), and it has been suggested that programmed rats have a sensitised leptin response to insulin (Holness & Sugden 2001a), so it is possible that there is an altered set point of leptin secretion relative to

fat mass in these rats. The lack of differential expression of resistin mRNA observed in RP fat is not surprising, however, if no difference in fat mass develops as a result of prenatal dex, as this adipose product is theorised to induce insulin resistance secondary to obesity (Steppan *et al.* 2001). However, increased expression might have been expected as a result of hypercorticosteronaemia, since dex has a direct effect upon adipocytes (Haugen *et al.* 2001).

Down-regulation of LPL mRNA in retroperitoneal fat is consistent with reduced import of fatty acids into this fat depot. LPL is down-regulated by glucocorticoids alone in isolated adipocytes (Ong *et al.* 1992), but up-regulated if insulin is also present (Ottosson *et al.* 1994; Fried *et al.* 1993), suggesting that this is not a direct effect of elevated plasma corticosterone. Furthermore, mutations at the LPL locus have been associated with insulin resistance (Zhang *et al.* 1997), and reduced activity is present in insulin resistant patients (Reynisdottir *et al.* 1997), so this change could be very significant in the development of the insulin resistant phenotype, through alteration of overall lipid status and distribution.

Since lipolysis of chylomicrons and VLDL by LPL liberates NEFAs, and this is a pre-requisite for the disposal of fatty acids to adipose tissue (Eckel 1989), it might be expected that measured plasma lipid parameters might also be different. However, plasma TAG, NEFAs, HDL-cholesterol and total cholesterol (and by extrapolation, LDL / VLDL) were unchanged by prenatal dex. However, these measurements were made on plasma from rats killed without a prior period of food withdrawal, so they bear repeating in fasted animals. Alternative explanations for this apparent dichotomy might be parallel reductions in the activity of HSL, which is rate-limiting in the re-mobilisation of fatty acids from adipose, or in lipoprotein synthesis. Finally, up-regulation of LPL activity in other tissues could be responsible, since up-regulation of LPL in muscle or liver results in increased triglyceride storage in these tissues, which is causatively associated with local insulin resistance (Kim *et al.* 2001a; Pan *et al.* 1997; Simoneau *et al.* 1999). Measurement of the LPL activity and triglyceride levels in muscle of dex-programmed rats would be interesting from this respect. Although the anti-lipolytic activity of insulin is impaired in low protein

offspring (Holness *et al.* 1998), no detailed molecular investigations have addressed these issues in other models of programming. Additionally, there was no difference in BHB, the principal ketone body in the plasma of dex rats, in contrast to *in utero* protein-malnourished rats (Ozanne *et al.* 1998b). This measurement also bears repeating when starved plasma is available.

Finally, a change in PPARγ expression may also mediate adipose insulin sensitivity in dex-treated offspring, and fatty acid uptake specifically. Although no overall change was observed in PPARγ mRNA in retroperitoneal fat, there was a trend towards up-regulation of the PPARγ2 isoform, and a small but significant shift from PPARγ1 to γ2. PPARγ has great importance in differentiation of and metabolic gene regulation in adipose tissue (Wahli *et al.* 1995; Way *et al.* 2001b). Thus, altered expression of PPARγ isoforms, alongside changes in GR, could be involved in mediating expression of metabolic genes in fat, and inducing local insulin resistance. PPARγ activation certainly affects LPL activity, although its effect is usually positive (Lapsys *et al.* 2000; Lefebvre *et al.* 1997). Interestingly, whereas PPARγ1 expression has not been found not to vary in obesity or insulin resistance in other studies (Kruszynska *et al.* 1998; Vidal-Puig *et al.* 1997b), the PPARγ2 isoform was increased in human obese subcutaneous fat, and γ2 / γ1 ratio correlated with body mass index (Vidal-Puig *et al.* 1997b). Furthermore, glucocorticoids predominantly increase the γ1 isoform (Vidal-Puig *et al.* 1997b), suggesting that this finding is not purely a result of hypercorticosteronaemia. The differential expression of PPARγ isoforms therefore may be of significance in the phenotype of the dex-programmed rat.

In summary, GR expression is programmed in the prenatally glucocorticoid over-exposed rat in a tissue-specific fashion. It is down-regulated in soleus muscle, but unchanged in two other muscles, possibly implying a protective mechanism against excessive effects of glucocorticoid in type I muscle fibres. However, visceral fat of dex-programmed rats has increased GR, and by analogy with liver (Nyirenda *et al.* 1998) may be hyper-sensitive to glucocorticoids. Elevated GR in liver and fat,

combined with elevated plasma corticosterone, is likely to result in a significant reduction in insulin sensitivity these tissues. Glycogen storage is reduced in muscle, suggesting attenuated glucose uptake, presumably programmed through a glucocorticoid-independent mechanism. GR up-regulation, alongside altered expression of PPARγ isoforms, is associated with reduced LPL expression in visceral fat, consistent with a programmed attenuation of fatty acid uptake by this tissue. All of these molecular changes could be involved in the development or phenotype of insulin resistance in rats over-exposed to glucocorticoids *in utero*.

The hypothesis that metabolic changes in liver and fat occur secondary to programmed increases in GR requires testing, perhaps through specific pharmacological inhibition of receptor activity in adulthood, or assessment of the sequence with which gene expression and metabolic changes develop. Furthermore, the mechanisms involved in the tissue-specific programming of GR require elucidation. The question of whether GR programming is important in determining insulin resistance in dex-programmed offspring, or whether it is merely a secondary feature of the adult syndrome is addressed in Chapter 4.

4 Chapter Four – Effect of corticosterone and insulin-sensitising drugs on dysregulation of GR in adulthood

4.1 Introduction

Tissue-specific differences in expression of glucocorticoid receptors seem to be important in determining key alterations in the physiology of adult dex-treated offspring. In the hippocampus, which is key in modulating feedback sensitivity of the HPA axis (Sapolsky *et al.* 1985), down-regulation of GR is apparent in dex-treated offspring (Levitt *et al.* 1996; Welberg *et al.* 2001), while in liver, GR is up-regulated (Nyirenda *et al.* 1998), specifically in the peri-portal zones in which gluconeogenesis is under positive regulation by glucocorticoids (Friedman *et al.* 1993). Increased hepatic glucose output is the likely result .Plasma glucose levels are also determined by peripheral disposal to skeletal muscle and adipose tissue, and in Chapter 3, I showed that GR is also selectively increased in visceral adipose tissue, but not in either subcutaneous adipose tissue or in skeletal muscle; indeed in the soleus muscle GR mRNA levels are reduced. Although GR in visceral adipose is important for glucose uptake and metabolic homeostasis, which may be pertinent to the insulin-resistant hyperglycaemic phenotype (Carter-Su & Okamoto 1987), the mechanism(s) underlying these tissue-selective changes in GR density in key metabolic tissues remain unclear.

GR expression determines tissue sensitivity to glucocorticoids (Schmidt & Meyer 1994), and its activity is regulated through control over transcription, through binding of transcription factors, including GR itself, and post-translationally. Previous *in vivo* studies have shown that short term increases in glucocorticoid levels down-regulate GR mRNA in liver (DuBois *et al.* 1995; Kalinyak *et al.* 1987) and skeletal muscle (McKay *et al.* 1997), while adrenalectomy up-regulates GR mRNA in muscle (DuBois *et al* 1984), but not in liver (Kalinyak *et al.* 1987), but there is

very little published information documenting GR auto-regulation in adipose tissue, in muscles of differing fibre type composition, or the effects of chronically altered glucocorticoid levels on GR. A key question then, is whether the observed changes in GR are important in determining the adult phenotype of dex-programmed rats, or whether altered GR expression is secondary to their hypercorticosteronaemia.

Glucocorticoids are known to attenuate insulin-stimulated glucose uptake and usage in muscle and fat (Dimitriadis *et al.* 1997; Sakoda *et al.* 2000), and excess glucocorticoid activity has been associated with the development of insulin resistance and syndrome X (Brindley 1995; Reynolds *et al.* 2001). It is also possible that hyperinsulinaemia could also affect GR expression, however, since insulin sensitivity has been inversely correlated with GR expression in diabetes (Vestergaard *et al.* 2001), hyperinsulinaemia has been associated with a GR polymorphism in obese women (Weaver *et al.* 1992), and cAMP levels are known to affect GR mRNA stability (Dong *et al.* 1989). A second key question, then, is whether changes in GR expression in dex offspring are secondary to their insulin resistant phenotype.

Thiazolidinediones, such as rosiglitazone, are pharmacological ligands for the peroxisome proliferator-activated receptor γ (PPARγ) (Guerre-Millo *et al.* 2000), which lower blood glucose, and have been shown to attenuate glucocorticoid-induced changes in insulin sensitivity (Anil & Marita 2000; Okumura *et al.* 1998). Metformin has been used for decades in the management of non insulin-dependent diabetes, and has insulin-sensitising, anti-hypertriglyceridaemic, and anti-hyperglycaemic effects (Wiernsperger & Bailey 1999). These are mediated particularly through improved cellular glucose uptake (Anil & Marita 2000; Thomas *et al.* 1998), as well as increased glycogen storage and suppression of hepatic gluconeogenesis and fatty acid oxidation (Wiernsperger & Bailey 1999). However, a molecular mechanism for these effects has yet to be identified.

In this chapter I examine whether the observed tissue-specific changes in GR expression in adult offspring of dams administered dex prenatally are permanent, or can be attenuated by manipulation of the HPA axis or insulin sensitivity. To these

ends, I chronically altered the concentration of circulating corticosterone (cort) in adult offspring, and in a separate experiment, administered insulin-sensitising drugs (metformin or rosiglitazone), and examined the resulting level of GR expression in primary insulin target tissues.

4.2 Methods

4.2.1 Animals

Rats were maintained and offspring generated as detailed in Chapter 2. Killing and harvesting of tissues from rats used in the drug treatment experiment was performed by Dr. Moffat Nyirenda.

4.2.2 Manipulation of plasma [corticosterone]

Only dex-treated offspring produced during the course of the investigations detailed in Chapter 5 were used in this experiment, as the evidence suggested that the phenotype of saline-treated animals was specifically compromised.

Dex offspring underwent bilateral surgical adrenalectomy (ADX), adrenalectomy plus corticosterone pellet implantation (CORT), or sham surgery (SHAM group), at 8 months. Adrenalectomy or sham surgery was performed through paralumbar incisions under halothane anaesthesia, and 5 corticosterone pellets were subcutaneously implanted through the same incisions, where appropriate. Adrenalectomised rats were maintained subsequently on 0.9% saline. After 3 weeks, animals were killed by decapitation between 9:00 and 11:00am. Organs were weighed, and trunk plasma and tissues frozen at -80°C.

Corticosterone 21-acetate pellets consisting of a 2:1 mixture of corticosterone 21-acetate with elastomer (Silastic^{R} medical grade, Dow Corning Corp, Midland, Michigan, USA) were prepared as previously described (Nyirenda *et al.* 1998). In preliminary experiments, pellets were incubated in 0.9% saline, and the quantity of hormone released was estimated by measurement of the spectrophotometric

absorption of the solution at 240nm. 5 pellets were calculated to release 0.6-0.7mg corticosterone over 24 hours.

4.2.3 Administration of insulin-sensitising drugs

Offspring of dex and saline-treated dams were given insulin-sensitising drugs dissolved in water, or water alone, by gavage from 5 months of age, by Dr. Dawn Livingstone. Each received 1mg/kg rosiglitazone (ROS group), 43mg/kg metformin (MET group), or 1ml/kg water (VEH group) daily, at 9:00am (Livingstone *et al.* 2000b). During this period, animals were weighed on alternate days, and food intake per cage was measured daily. Animals were killed after 3 weeks' treatment, as described above.

4.2.4 Plasma [Corticosterone]

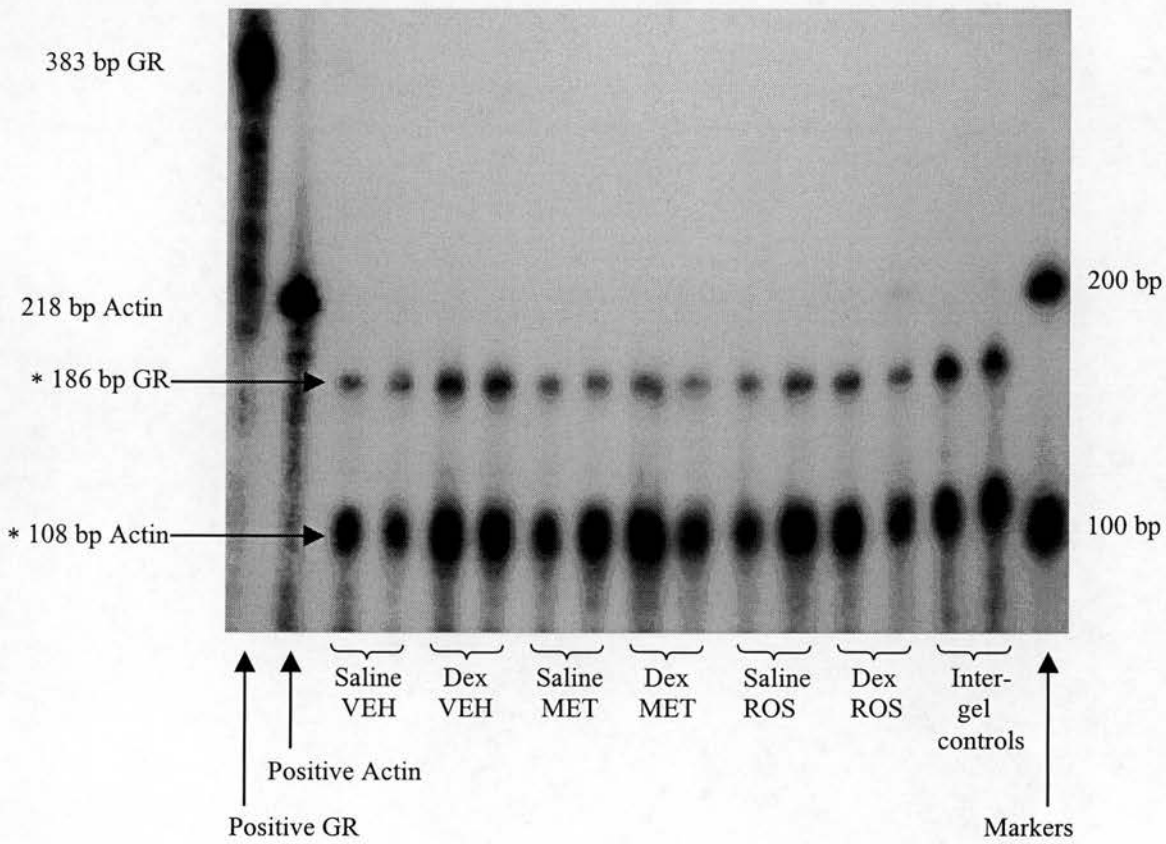
[Corticosterone] in trunk plasma of nine month old offspring was measured using the method outlined in Chapter 2.

4.2.5 RNase Protection assay

RNA was extracted from tissues as described in Chapter 2. GR and β-actin templates were linearised and used to synthesise riboprobes as described in Chapter 3. The concentration of "cold" rGTP in the transcription mixture was varied according to the desired specific activity of the riboprobes. RPAs were undertaken as described, using appropriate quantities of total RNA , GR and actin riboprobes. A summary of probe specific activities and RPA reaction constituents in each of the assays performed is shown in **Table 3-2**. A typical polyacrylamide RPA gel is displayed as **Figure 4-1**.

Figure 4-1 Sample RNase Protection gel for glucocorticoid receptor mRNA quantitation

Typical gel yielded during an assay of GR mRNA in quadriceps muscle of 6 month old male dex- and saline-treated offspring, subsequently given one of two insulin-sensitising drugs or vehicle as adults for three weeks, normalised using β-actin mRNA expression. Positive control lanes, containing undigested full length probes, century marker lane, repeated inter-gel control lanes, and sample lanes (n=2 per sub-group per gel) are labelled. Key: * protected band- showing size of digested riboprobe-mRNA hybrid; Saline: prenatal vehicle treatment; Dex: prenatal dexamethasone treatment; VEH: adult vehicle treatment; MET: adult metformin treatment; ROS: adult rosiglitazone treatment; bp: base pairs of RNA.



4.3 Results

4.3.1 Birth data

Analysis of the birth cohort used in the Cort experiment showed that prenatal dex treatment reduced birth weight by an average of 15.2% (6.04±0.05g versus 5.12±0.04g; n=83 and 162; $p<0.001$ by Student's t-test). There was no effect of dex on either litter size (9.2±0.7 versus 10.1±1.0; n=16 and 9; $p=0.43$ by Student's t-test) or sex distribution (47 male, 36 female versus 72 male, 90 female; $\chi^2=3.46$).

4.3.2 Effect of modifying plasma [corticosterone] in adulthood

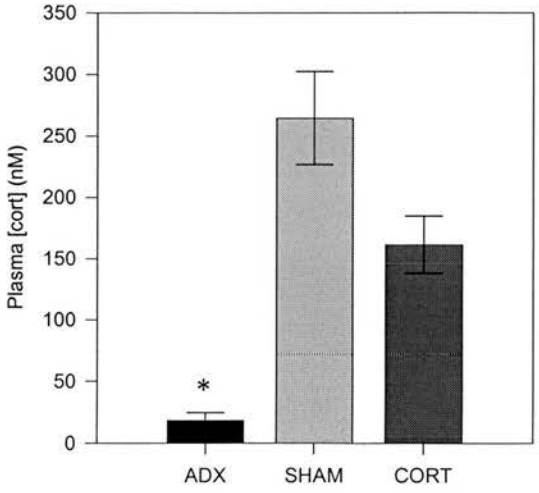
To determine whether chronic alterations in glucocorticoid levels may explain the tissue-specific differences in GR gene expression seen in dex offspring, adults were adrenalectomised ± cort replacement.

4.3.2.1 Plasma [corticosterone]

Trunk plasma [cort] was undetectable (<50nM) in ADX rats, and similar in SHAM and CORT rats (see **Figure 4-2**).

Figure 4-2 Plasma corticosterone concentration 3 weeks after surgery

Plasma [corticosterone], measured by radioimmunoassay of morning trunk plasma taken from 8-9 month old male dex-treated offspring, which had been adrenalectomised, sham-operated, or adrenalectomised and implanted with subcutaneous corticosterone pellets 3 weeks previously. N=6-7 per group. By 1-Way ANOVA, $p<0.001$ for effect of surgery. *Post-hoc*: * $p<0.05$ versus SHAM, by Tukey's Honest Significant Difference test.

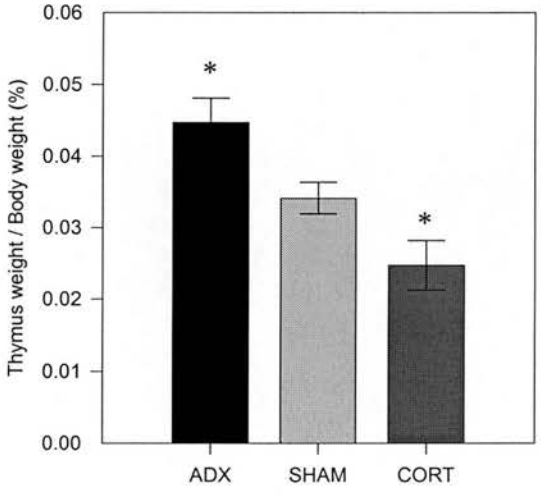


4.3.2.2 Body and Organ weight

There was no difference in the body weight between groups at the end of the experiment (ADX 470±14g, SHAM 513±35g, CORT 492±31g; n=7 or 8 per group; p=0.29 by 1-Way ANOVA). Thymus mass decreased as the chronic plasma [cort] increased (expressed as a fraction of body mass: n=7 per group) (see **Figure 4-3**).

Figure 4-3 Thymus weight after 3 weeks' manipulation of plasma corticosterone

Thymus weight as a percentage of body weight at death, 3 weeks after surgery (adrenalectomy, sham surgery or adrenalectomy / corticosterone pellet implantation). N = 7 per group, effect of surgery by ANOVA, $p<0.001$, *post hoc* * $p<0.05$ versus SHAM group using Tukey's HSD test.

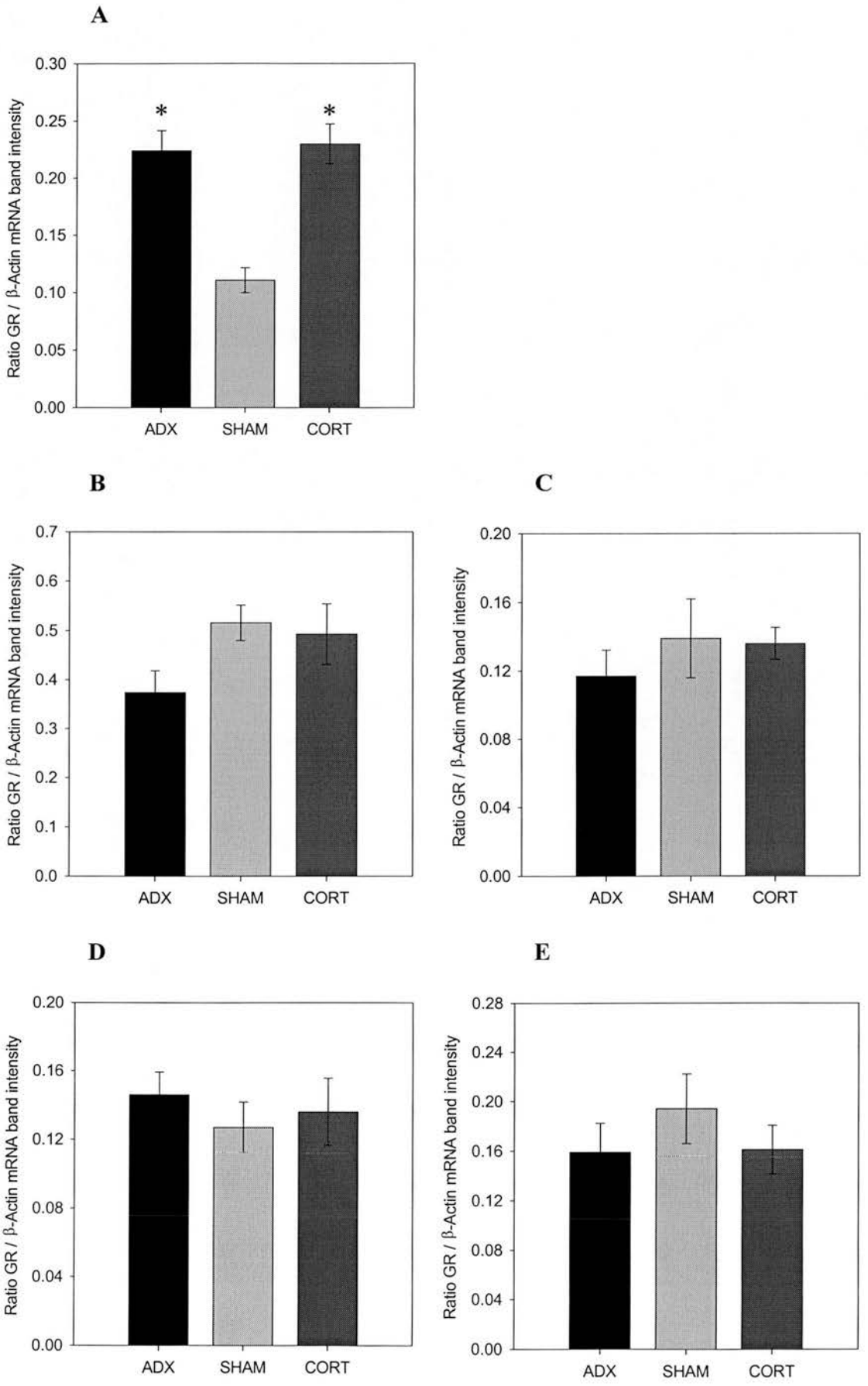


4.3.2.3 GR mRNA in tissues

In the liver, both ADX and CORT rats showed an approximate two-fold increase in GR mRNA expression relative to SHAM controls in rats prenatally over-exposed to glucocorticoids (see **Figure 4-4 a**). In contrast, these manipulations had no effect on GR mRNA in skeletal muscle (either EDL or soleus, **4-4 b** and 4-**4 c**) or adipose tissue (either subcutaneous or retroperitoneal, **4-4 d** and **4-4 e**).

Figure 4-4 Glucocorticoid Receptor mRNA expression in insulin target tissues of dex-programmed rats after 3 weeks' manipulation of plasma corticosterone level

GR mRNA expression in tissues of 8-9 month old male dex offspring, measured by RNAse protection analysis, normalised to β-actin mRNA, 3 weeks after surgery (adrenalectomy or sham surgery or adrenalectomy / corticosterone pellet implantation.. **A** Liver **B** EDL muscle **C** Soleus muscle; **D** Subcutaneous fat **E** Retroperitoneal fat. N=6-7 per group. By 1-way ANOVA, groups were different in liver only ($p<0.001$). *Post-hoc*: * $p<0.05$ versus SHAM.



4.3.3 Effect of treatment of adults with insulin-sensitising drugs

To examine the possible role of the documented insulin resistance in this model in producing the changes in GR expression, two classes of insulin-sensitising drugs were exploited.

4.3.3.1 Body Weight / Appetite

Examining all 24 rats utilised from each of the two prenatal treatment groups revealed that dex offspring were heavier at the start (Saline 296.5±3.3g, Dex 322.5±6.4g; p=0.003) and end of the experiment. The administration of insulin-sensitising drugs did not alter body weight gain (see **Table 4-1**). Dex offspring consumed more chow compared to controls, while treatment with both metformin and rosiglitazone resulted in a further increase, which was most pronounced in dex rats given rosiglitazone as adults.

Table 4-1 Effects of insulin sensitising drugs on food intake and body weight

Body weight was monitored during the 3 week period of treatment of 5-6 month old male dex and saline-treated offspring with either metformin (43 mg/kg), rosiglitazone (1mg/kg) or vehicle by gavage. Appetite was quantified on a daily basis by division of total food eaten per cage by number of rats in the cage. By 2-Way ANOVA, both prenatal ($p<0.001$) and adult treatment ($p<0.001$) had effects on appetite. Both metformin ($p<0.05$) and rosiglitazone ($p<0.05$) increased appetite, independent of prenatal treatment. *Post-hoc* * $p<0.05$ versus prenatal control by Tukey's HSD test. Drug treatment had no effect on body weight at the end of the experiment, but a difference due to prenatal treatment was maintained (2-Way ANOVA, $p<0.001$ overall).

Treatment group		Mean Food intake per cage (g/day)	Body weight at end of experiment (g)
Prenatal	Adult		
Saline	VEH	157 ± 4	318 ± 9
Saline	MET	172 ± 3	327 ± 6
Saline	ROS	168 ± 3	320 ± 7
Dex	VEH	162 ± 2	335 ± 13
Dex	MET	175 ± 2	350 ± 11
Dex	ROS	187 ± 2	371 ± 10 *

4.3.3.2 GR mRNA in tissues

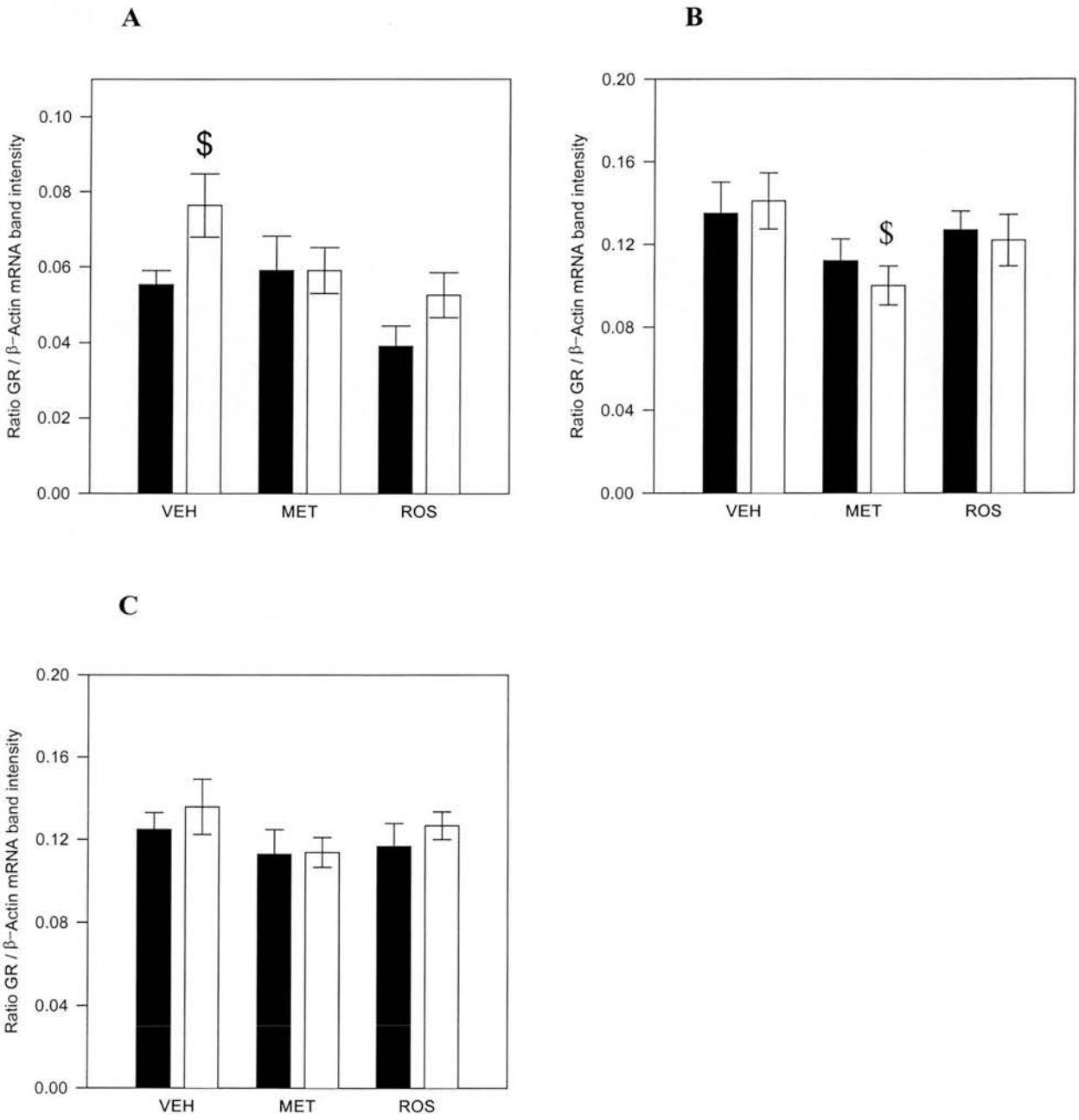
There were independent effects of both prenatal dex and treatment with insulin-sensitising drugs on GR expression in liver (see **Figure 4-5a**). Prenatal exposure to Dex during the third week of pregnancy resulted in a 38% increase in liver GR expression in adulthood. Rosiglitazone reduced GR mRNA expression in liver independently of prenatal treatment. In striking contrast, metformin reduced GR mRNA levels preferentially in liver of dex-programmed animals, and thus abolished the difference in liver GR levels between offspring exposed to dex and vehicle prenatally.

In quadriceps muscle, no significant effect of prenatal treatment was seen (**4-5b**). There was no effect of rosiglitazone administration on GR mRNA, but in contrast, metformin reduced GR mRNA levels in skeletal muscle. Once again, this was predominantly an effect in prenatally dex-programmed animals: there was a significant 29% reduction in GR mRNA in Dex-MET versus Dex-VEH rats, while the reduction in Saline-MET versus Saline-VEH rats was not significant.

In subcutaneous fat, no effect of either prenatal treatment or administration of insulin-sensitising drugs on adult GR mRNA was observed. (**4-5c**).

Figure 4-5 Glucocorticoid receptor mRNA expression in tissues of prenatally treated rats, given insulin sensitising drugs as adults

GR mRNA quantified by RNAse protection assay versus β-actin mRNA, in tissues removed from male 6 month old dex and saline-treated offspring, given metformin (43mg/kg), rosiglitazone (1mg/kg) or vehicle by gavage for 3 weeks previously. N = 7-8 per group. **A** Liver. By 2-Way ANOVA, effects of prenatal ($p=0.043$) and adult ($p=0.018$) treatment were significant. Rosiglitazone reduced GR compared to vehicle ($p<0.05$). *Post-hoc* \$ $p<0.04$ versus prenatal control, by Student's t-test. **B** Quadriceps muscle. By 2-Way ANOVA, there was an effect of adult treatment ($p=0.037$), with metformin reducing GR relative to vehicle ($p<0.05$). *Post-hoc* \$ $p<0.03$ versus Saline-VEHICLE by Student's t-test. **C** Subcutaneous fat. Key: Black bars = saline, white bars = dex-treated offspring. VEH = adult vehicle-treated, MET = adult metformin treated, ROS = adult rosiglitazone-treated.



4.4 Discussion

The results show that in adult rats prenatally exposed to dex, GR mRNA expression in skeletal muscle and adipose tissue is unaffected by chronic manipulation of plasma cort. In liver, GR mRNA appeared to be down-regulated in sham-operated animals, but was unaffected by chronic manipulation of plasma cort. In contrast, treatment of dex-treated offspring with metformin in adulthood normalised GR expression in liver and muscle. These effects were not seen with another insulin-sensitising drug, rosiglitazone, which reduces liver but not muscle GR irrespective of prenatal treatment. This suggests that hyperinsulinaemia is not of itself responsible for up-regulation of GR expression in programmed animals. Further investigation of the action of metformin on GR regulation may, however, provide key insights into its mechanism of action and the determinants of programming of adult GR expression by prenatal dex.

Consistent with prior observations, dex treatment of rats in week three of pregnancy resulted in a significant reduction in birth weight of the offspring, with no alteration in either litter size or sex distribution of pups (Levitt *et al.* 1996; Nyirenda *et al.* 1998). By five months of age, however, dex treated offspring were 9% heavier than controls, which differs from previous results, where offspring prenatally over-exposed to glucocorticoids were of the same weight (Lindsay *et al.* 1996b; Nyirenda *et al.* 1998), or lighter in adulthood (Welberg *et al.* 2000). During the three week period that food intake was monitored, dex offspring showed a marginal, but significant hyperphagia. This may be the cause of the weight gain, as hyperphagia was found to mediate obesity in the offspring of undernourished dams (Vickers *et al.* 2000). Further analysis of growth trajectory and body composition of programmed offspring would be interesting in the light of the association between low birth weight and later obesity in other animal models (Vickers *et al.* 2000; Jackson *et al.* 1996) and human populations (Ong *et al.* 2000).

Plasma [cort] was chronically manipulated by adrenalectomy ± supraphysiological cort replacement to determine whether dysregulation of GR mRNA in the tissues of

adult dex offspring is determined by hypercorticosteronaemia. Preliminary investigations, and data from a previous experiment (Nyirenda *et al.* 1998) suggested that implantation of five cort pellets subcutaneously would achieve hypercorticosteronaemia over the course of the experiment. The inverse relationship between expected three week [cort] and thymus mass confirmed that adrenalectomy and cort pellet implantation had their desired effects (Akana *et al.* 1985). Plasma [cort] was minimal in ADX animals, and highly elevated relative to the circadian mean in the CORT rats (Dallman *et al.* 1993; Welberg *et al.* 2001). [Cort] was similarly elevated at the time of sampling in SHAM rats, probably as a result of acute stress, which could not be reflected by the adrenalectomised CORT rats.

No effect of chronic cort manipulation was seen in either of two different skeletal muscles, the soleus (87% slow twitch, type I, predominantly oxidative muscle fibres) and EDL (98% fast twitch, type II, mainly glycolytic fibres) (Armstrong & Phelps 1984). Similarly, no effect of altering circulating glucocorticoid was found in either of the white adipose tissue depots tested. The similarity of GR mRNA levels in liver of the CORT and ADX groups suggests that there is, also no effect of chronic alterations in plasma cort on liver GR mRNA. However, lower GR mRNA was observed in SHAM operated animals. This may have been an effect of acute hyper-corticosteronaemia, resulting from stress immediately prior to killing, to which adrenalectomised animals would not respond. In support of this contention, liver GR mRNA down-regulation has been recorded previously within 30 minutes of administration of dex *in vivo* (DuBois *et al.* 1995). Furthermore, an effect of sham surgery *per se* has been previously identified on the expression in liver of 11β-Hydroxysteroid dehydrogenase type I, the enzyme responsible for re-activation of endogenous glucocorticoid (Jamieson *et al.* 1999).

Previous studies have not considered the effect of altered [cort] on GR mRNA in muscles of varying fibre type composition (DuBois *et al* 1984; McKay *et al* 1997) or adipose depots, or the effect of such chronic manipulations on liver (DuBois et al. 1995; Kalinyak et al. 1987). However, down-regulation of GR provoked by addition of dex to hepatoma cells reverses within a few days (Okret *et al.* 1986), while the

effect of adrenalectomy on hippocampal GR is abolished within 2 weeks (Holmes *et al.* 1995), supporting the implication of this study that GR auto-regulation is a relatively acute phenomenon. These findings indicate that chronic hypercorticosteronaemia is not responsible for tissue-specific dysregulation of GR mRNA levels in dex-programmed rats. Control of glucocorticoid sensitivity is also exerted post-translationally, through phosphorylation (Webster *et al.* 1997) and proteasomal degradation (Wallace & Cidlowski 2001), however, thus examination of GR binding or protein abundance would be required for completeness.

In a separate series of experiments, insulin-sensitising drugs were administered to adult dex-treated and control rats, to evaluate whether normalisation of hyperinsulinaemia would affect programmed GR expression. Metformin and rosiglitazone, two drugs used widely in the clinical treatment of type two diabetes, were given using the same protocol as has been successfully applied previously to reduce the glucose intolerance and insulin resistance of Zucker obese rats, as assessed by oral glucose tolerance tests (Livingstone *et al.* 2000b).

The appetite of rats on both of these treatments was stimulated, independent of any programmed effect, but this did not affect weight gain during the course of the experiment. This effect is in contrast to that seen in Zucker obese rats, where appetite was depressed by metformin in one study (Rouru *et al.* 1992), and unaffected in another (Livingstone *et al.* 2000b). Indeed, reduction of appetite and therefore obesity has been suggested to play a role in the clinical effect of metformin (Lee & Morley 1998). In this study and those of Livingstone (Livingstone *et al.* 2000b) and Wang (Wang *et al.* 1997), rosiglitazone caused an increase in appetite, but the increased weight gain of the obese Zuckers was not replicated during this study. These effects may be due to down-regulation of leptin expression by thiazolidinediones (Hollenberg *et al.* 1997).

Liver GR mRNA was up-regulated in adult rats given dex prenatally in this study, as previously demonstrated, using an *in situ* hybridisation technique (Nyirenda *et al.* 1998). The effects of insulin-sensitising drugs differed between tissues and between

classes of drug. In liver, whereas rosiglitazone reduced GR expression irrespective of prenatal treatment, metformin normalised the programmed over-expression of GR. In skeletal muscle, metformin but not rosiglitazone reduced GR mRNA, again having a greater effect in dex-treated offspring. In adipose tissue, however, neither rosiglitazone nor metformin affected GR mRNA expression, which perhaps reflects the use of SC rather than RP fat, in which GR up-regulation was recorded, in the assay. It would be instructive to examine the effect of these drugs on the up-regulation of GR in RP fat. Thus, the effect of metformin in reducing GR in both tissues is specific to programmed animals, whereas that of rosiglitazone in the liver is non-specific. As both drugs have similar effects to normalise hyperinsulinaemia (Livingstone *et al.* 2000b), yet they do not both normalise GR, we can conclude that GR gene expression in dex-programmed rats is not determined simply by hyperinsulinaemia. However, programming of GR gene expression in this model does appear susceptible to a specific, and novel, action of metformin. Moreover, the effect of rosiglitazone on liver GR mRNA was unexpected and may be important.

Thiazolidinediones, such as rosiglitazone have anti-hyperglycaemic effects (Anil & Marita 2000; Thomas *et al.* 1998), mediated through binding to PPARγ, predominantly in adipose tissue (Guerre-Millo *et al.* 2000). However, PPARγ activation also suppresses hepatic glucose output, despite low levels of expression in the liver (Kimura *et al.* 2000). It is possible that rosiglitazone has direct effects on the liver, since thiazolidinediones have been shown to modify GR-mediated effects in cell culture (Johnson *et al.* 1999). However, it is widely supposed that effects on hepatic metabolism are mediated by factors released from adipose tissue which are altered by thiazolidinediones, such as resistin (Steppan *et al.* 2001), free fatty acids (Rebrin *et al.* 1996) or glucocorticoids (Masuzaki *et al.* 2001). Which of these might influence GR expression in liver has not been tested, but this is an important question for further study.

Metformin has well-recorded physiological effects in reducing hyperglycaemia, through insulin-sensitising and non-insulin dependent means (Wiernsperger & Bailey 1999). The specific site of action has not been identified, but possibilities have been

suggested, such as potentiation of pyruvate kinase (McCarty 1999), vasodilatory effect in skeletal muscle (Katakam *et al.* 2000), and enhanced translocation of glucose transporter proteins (Rouru *et al.* 1995). This study shows effects of metformin in reducing GR expression in two insulin-sensitive tissues, most potently in insulin-resistant dex-programmed animals.

The mechanism of programming of GR mRNA remains obscure, but it has been proposed that its tissue-specificity is explained by differential regulation of alternate promoters influencing transcription at alternate exon 1 start sites (McCormick *et al.* 2000). The specific effect of metformin on GR mRNA in programmed animals offers a tool to dissect pathways which influence these tissue-specific promoters, which may respond directly or indirectly to metformin, and thereby unravel the molecular mechanism of programming. Equally importantly, down-regulation of GR may be a component of the poorly understood insulin-sensitising effect of metformin (Vestergaard *et al.* 2001). Moreover, this effect may recommend metformin as an appropriate therapeutic agent for amelioration of the metabolic syndrome in individuals of low birth weight. Further work is required to determine whether the effect of metformin on GR is direct or indirect, in order to assess its significance in the treatment of diabetes.

In summary, these studies provide evidence that neither hypercorticosteronaemia nor insulin resistance is sufficient to explain all the tissue-specific changes in GR gene expression observed in rats prenatally over-exposed to glucocorticoids, implying that GR expression is permanently programmed and may be a primary mechanism of insulin resistance. In addition, it has been shown that rosiglitazone lowers hepatic GR mRNA independently of programming, which may be important in the hepatic insulin sensitising effect of thiazolidinediones. Finally, metformin reduces GR expression specifically in insulin resistant liver and muscle, which may offer insights into the molecular determinants of programming of GR and the mechanism of action of this drug.

5 Chapter Five – Effect of perinatal stress upon programming by glucocorticoids

5.1 Introduction

The HPA axis is strongly activated by stressful stimuli, which come in many forms, including both psychological and physical varieties. Increased secretion of CRH and AVP is initiated through neuronal inputs from poorly identified higher brain centres, which also receive ascending inputs from other tissues. Acute stress results in the secretion of adrenaline additionally, which potentiates the effects of CRH and AVP in the pituitary. The corticosterone / cortisol secreted aids the individual in coping with stressors through adaptations including increased availability of metabolic substrates, anti-inflammatory and vascular effects. Chronic or repeated stress is detrimental to health, however, through persistence of these adaptive responses. Loud noise is a stressful stimulus for rodents and humans (Armario *et al.* 1984; Andren *et al.* 1982), and rodents possess a hearing range that incorporates both ultra- and subsonic frequencies. Acute or chronic, intermittent noise causes hyperactivation of the HPA axis (Armario *et al.* 1984; Vogel & Jensh 1988), and demonstrable physiological effects, such as increased mean arterial pressure (Andren *et al.* 1982).

Prenatal stress has been shown to programme subsequent physiology and behaviour in a number of rodent studies. Exposures to stressful stimuli during pregnancy results in HPA axis activation in both mother and foetuses before birth (Williams *et al.* 1999), that persists until weaning (Takahashi *et al.* 1992). There are long term implications, however, as anxious behaviour and increased stress responsiveness are manifested into adulthood (Takahashi *et al.* 1992; Vallee *et al.* 1997). These behavioural effects are associated with altered GR expression in brain regions (Koehl *et al.* 1999; McCormick *et al.* 1995) and alterations in the circadian rhythm of corticosterone secretion (Koehl *et al.* 1999), suggesting ongoing involvement of HPA-axis dysregulation. Further evidence that the *in utero* effects of stress are

induced through excessive foetal glucocorticoid comes from observations that the long term effects of prenatal stress are analagous to those induced by prenatal dex treatment. Specifically, prenatal dex treatment results in life-long hyperactivation of the HPA axis (Levitt *et al.* 1996) and altered GR expression in various brain regions, associated with increased anxiety-related behaviour (Welberg *et al.* 2001). Alteration in stress-susceptibility can also be induced by early post-natal manipulation, such as handling of pups, which reduces anxiety through altered HPA feedback sensitivity and hippocampal GR expression (Meaney *et al.* 1989; O'Donnell *et al.* 1994).

This chapter relates investigations into the phenotype of offspring of rats that were dex- or vehicle-treated during their third week of pregnancy, but which were subsequently discovered to have been exposed to ultrasonic and / or subsonic frequencies prenatally and / or postnatally, as a result of emission from strip lights and / or adjacent building work, respectively. This affected cohorts of rats housed in the Biological Research Facility at the Western General Hospital between late 1999 and early 2001. This unit was refitted, with the installation of a new lighting system in early 1999, and is situated next to the new Anne Ferguson building, on which building work was completed in summer 2000.

5.2 Methods

5.2.1 Animals

Female rats and their litters were maintained, bred, and administered with dexamethasone or vehicle during their third week of pregnancy as described in Chapter 2. Male offspring were weighed at days 25, 40, 60, 80, 100 etc. Some animals were killed at approximately two, six and nine months.

8-9 month old rats underwent triplicate measurements of systolic blood pressure on consecutive days by tail cuff plethysmography, as described in Chapter 2. After a further week, oral glucose tolerance tests (OGTT) were performed on each animal, as described, and after three weeks total, animals were killed by decapitation between

9:00 and 11:00am. Muscles were weighed, and trunk plasma and tissues frozen at -80°C.

5.2.2 Plasma [corticosterone]

[Corticosterone] in trunk plasma of nine month old offspring was measured using the method outlined in Chapter 2.

5.2.3 RNase Protection Assay for GR

The relative abundance of GR mRNA was assayed in the liver of nine month old rats, as described in Chapters 2 and 3.

5.2.4 Muscle Glycogen Assay

The glycogen content of portions of quadriceps muscle taken from nine month old rats was assayed using the Anthrone method, as described in Chapter 2.

5.2.5 PEPCK Activity Assay

Measurement of PEPCK activity was made in cytosol derived from homogenates of liver obtained from offspring killed at two months of age, according to the method described in Chapter 2. This assay was undertaken with the assistance of Dr. Moffat Nyirenda.

5.2.6 Measurement of plasma parameters

Measurement of plasma TAG, NEFAs, cholesterol, and HDL-cholesterol were made on aliquots of thawed trunk plasma from nine month old rats by Dr. Philip Wenham, Department of Clinical Biochemistry, Western General Hospital, Edinburgh, as described in Chapter 2. Measurement of plasma leptin was undertaken on the same material using the method described previously. Glucose, insulin and BHB were assayed in plasma samples collected during the OGTT, using the methods described in Chapter 2.

5.2.7 Deoxyglucose uptake by adipocytes

Preparations of isolated adipocytes were made from the pre-weighed epididymal fat pads of dex- and vehicle-treated offspring killed at six months. The basal and insulin-stimulated uptake of 2-[^{3}H(G)]-Deoxy-D-Glucose by aliquots of cells from each animal was measured, in order to compare the ability of adipocytes from rats in each treatment group to import glucose from the circulation. The methods are described in detail in Chapter 2.

5.2.8 Assessment of ultrasonic noise pollution

A "bat detector", set at 80kHz, was used to periodically sweep the animal unit for sources of ultrasound, after the atypical phenotypes of prenatally treated animals were characterised.

5.3 Results

5.3.1 Birth data

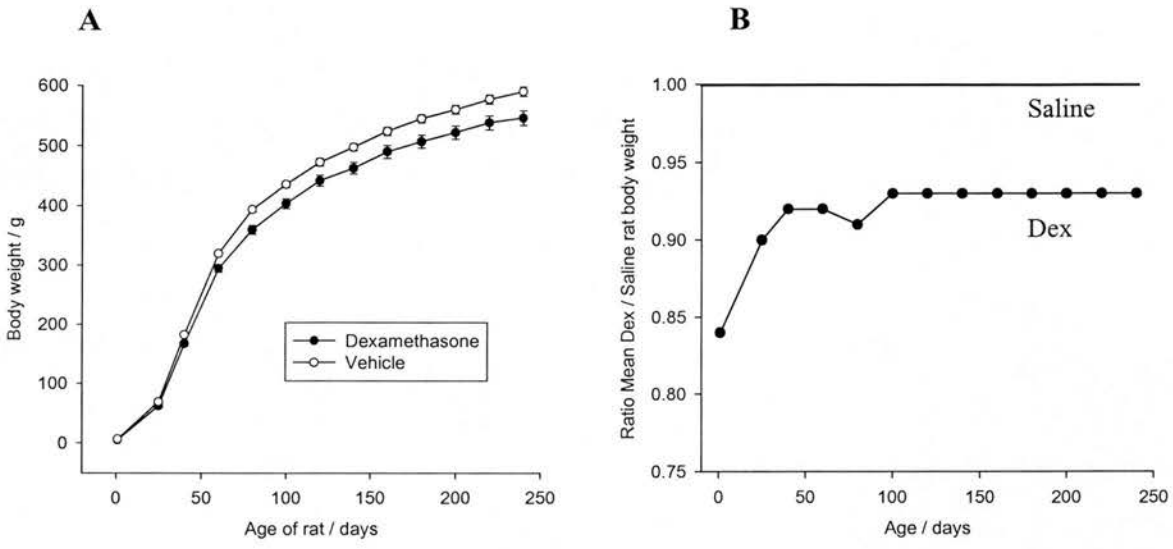
Birth weights for control and dex-treated rats subsequently killed at two months were 6.26 ± 0.07g and 5.21 ± 0.05g (n = 102 and 104 respectively, $p<0.001$ by Student's t-test), representing a mean 16.8% reduction in birth weight as a result of prenatal dex treatment. There was no difference in either litter size or sex ratio between dex and saline-treated rats (results not shown).

5.3.2 Growth trajectory

Figure 5-1 shows that rats in both treatment groups gained weight throughout the course of measurement. Catch-up growth by dex-treated rats occurred until approximately three months of age, when this ceased, and the mean weight of dex-treated offspring remained thereafter approximately 7% less than that controls. At 240 days of age, mean body masses were 589 ± 7.9g and 545 ± 12.0g for vehicle and dex-treated offspring (n = 25 and 21 respectively, p = 0.003 by Student's t-test).

Figure 5-1 **Growth of offspring to 8 months of age**

Dex and saline-treated offspring were weighed every 20 days from birth until euthanasia. **A** Growth trajectory of each cohort. **B** Catch-up growth of dex-treated rats: ratio of mean body weights of dex versus saline-treated offspring over the same period.

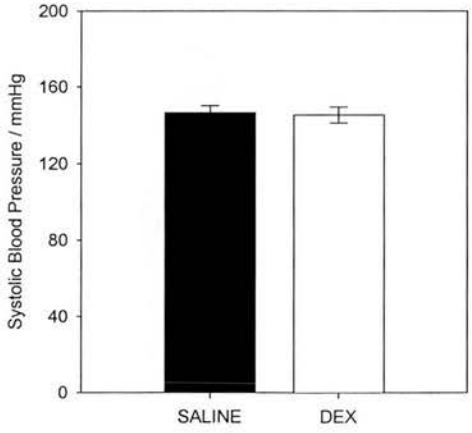


5.3.3 Blood pressure

There was no effect of prenatal treatment on blood pressure (see **Figure 5-2**).

Figure 5-2 **Systolic Blood Pressure of rats at 8 months of age**

Systolic blood pressure of male dex and vehicle-treated offspring at 8 months, measured in triplicate by tail cuff plethysmography. N = 8 per group.



5.3.4 Oral glucose tolerance test

No difference was observed between prenatal treatment groups in either plasma glucose (**Figure 5-3 a**) or insulin (**5-3 b**) during the OGTT.

5.3.5 Plasma [corticosterone]

There was a trend towards increased plasma corticosterone in dex-treated offspring (Saline 178 ± 29nM versus Dex 264 ± 38nM, n = 7-8 per group, p = 0.079 by Student's t-test).

5.3.6 Organ masses

At six months, the epididymal fat pad of dex-treated offspring was on average 20.9% lighter than that of controls, relative to total body weight (2.78 ± 0.10% versus 2.20 ± 0.10%; n= 6 / 7, p = 0.002 by Student's t-test). The weights of muscles taken from nine month old rats are shown below, in Table 1. There were no significant effects of prenatal treatment.

Figure 5-3 **Plasma glucose and insulin during an oral glucose tolerance test**

Plasma [glucose] and [insulin] quantified in blood taken from 9am from the tail-tip of rats starved overnight. N = 7-8 per group **A** Plasma Glucose before, and at 20 and 120 minutes after an oral glucose load, measured by hexokinase assay. **B** Plasma Insulin before, and 20 minutes after an oral glucose load, measured by ELISA.

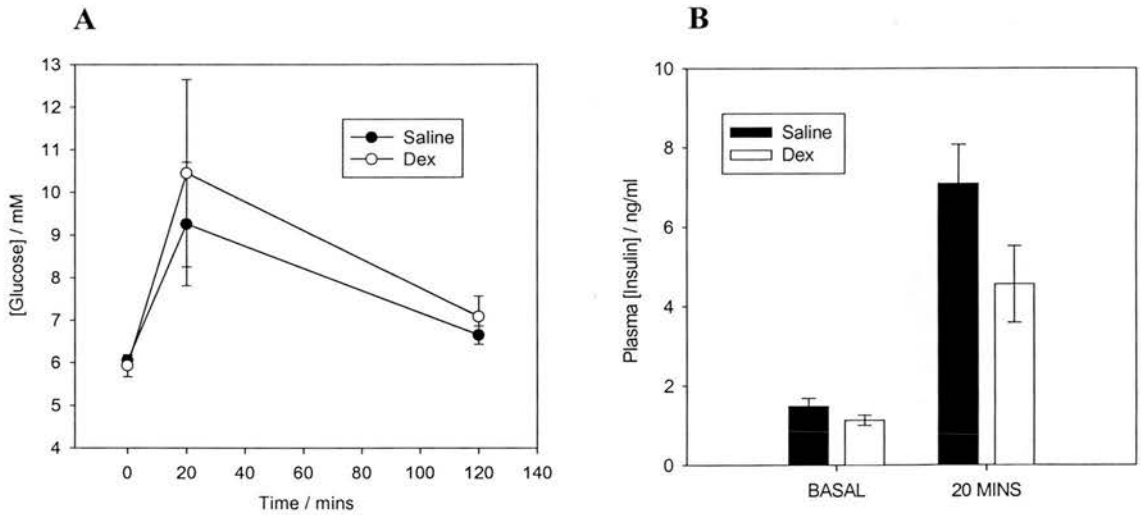


Table 5-1 Weight of muscles from 9 month old offspring

Weights of soleus, EDL and quadriceps muscles removed from male dex and saline-treated offspring, expressed as a percentage of body weight at death. n = 8 per group.

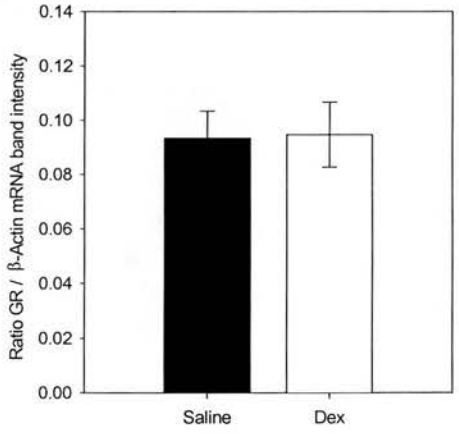
Muscle	Saline	Dex
Soleus	0.035 ± 0.002	0.036 ± 0.003
Quadriceps	0.760 ± 0.020	0.694 ± 0.025
EDL	0.040 ± 0.001	0.040 ± 0.001

5.3.7 Liver GR

As can be seen in **Figure 5-4**, there was no difference in GR expression between treatment groups at nine months of age.

Figure 5-4 Glucocorticoid receptor mRNA expression in liver

GR mRNA expression in liver of 8-9 month old male dex and vehicle-treated offspring, quantified by RNAse protection assay versus β-actin expression. N = 8 / 6 in saline / dex groups respectively.

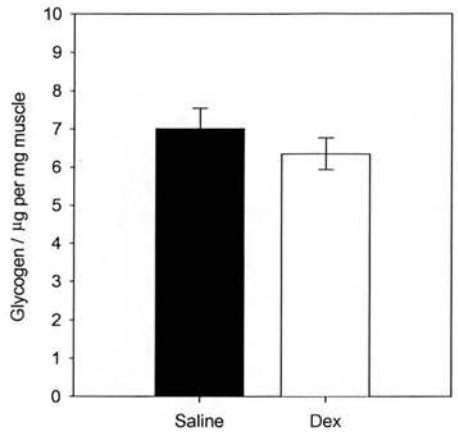


5.3.8 Quadriceps glycogen

Figure 5-5 shows that glycogen storage by the quadriceps muscle of dex-treated offspring was no different from controls.

Figure 5-5 Quadriceps Glycogen content

Micrograms glycogen per mg quadriceps muscle removed from 8-9 month old dex and saline-treated offspring, measured by the Anthrone method. N = 8 per group

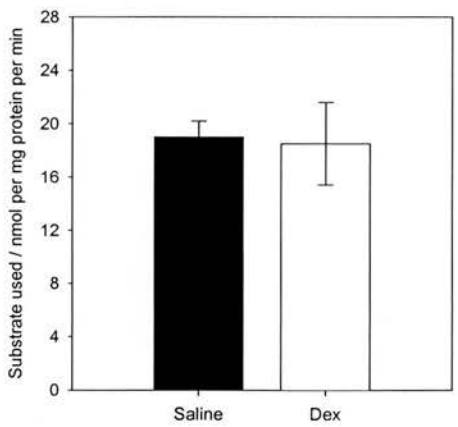


5.3.9 Liver PEPCK activity

By inspection of **Figure 5-6**, it is clear that at two months of age, no difference in PEPCK activity of offspring livers was apparent.

Figure 5-6 Hepatic Phosphoenolpyruvate carboxykinase activity

PEPCK activity measured in microsomal extracts from livers of 2 month old male dex and saline-treated offspring. N = 8 per group

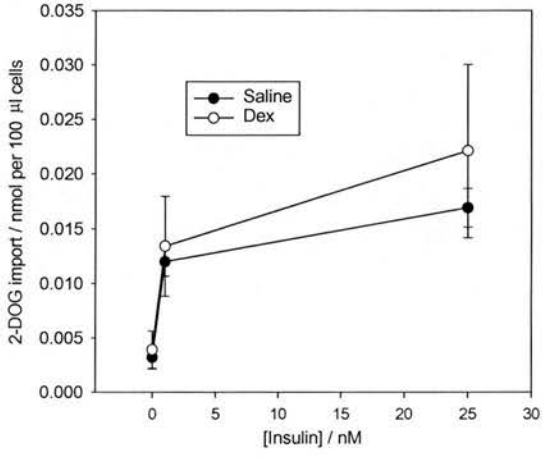


5.3.10 Deoxyglucose uptake by adipocytes

Figure 5-7 shows the deoxyglucose uptake by epididymal adipocytes from six month old offspring. Although uptake was different at each time point ($p < 0.05$ by 2-Way ANOVA), there was no significant difference in deoxyglucose import between treatment groups ($p=0.12$).

Figure 5-7 **2-Deoxyglucose uptake by epididymal adipocytes**

Net 2-DOG uptake by glucose transporters (total uptake minus that in the presence of cytochalasin B) measured in isolated epididymal adipocytes from 6 month old male dex and saline-treated offspring, at 3 different insulin concentrations. N = 6-7 per group.

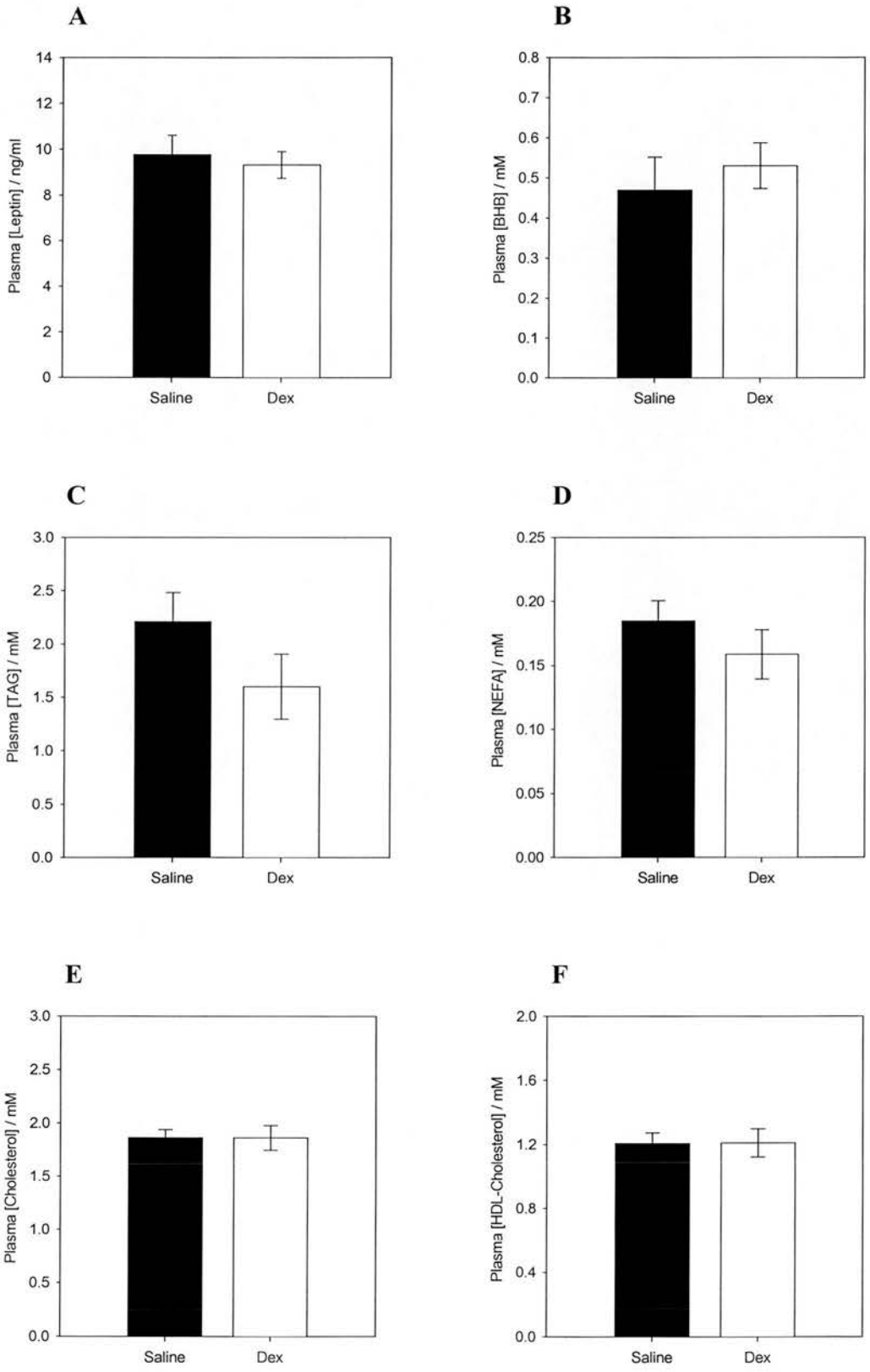


5.3.11 Plasma parameters

There were no differences between prenatal treatment groups in plasma leptin (**Figure 5-8 a**), BHB (**5-8b**), NEFA (**5-8d**), cholesterol (**5-8e**) and HDL-cholesterol (8f) as a result of prenatal dex, but there was a slight trend towards lower TAG in dex-treated offspring ($p = 0.16$ by Student's t-test) (**5-8c**).

Figure 5-8 Plasma concentrations of substances related to lipid metabolism

Concentrations of substances related to lipid metabolism in trunk plasma of *ad lib* fed or in tail-tip plasma of starved (β-Hydroxybutyrate only) 8-9 month old male offspring of dex and vehicle-treated rats, measured using commercial kits. N=7-8 per group. **A** Leptin **B** β-Hydroxybutyrate **C** Triglycerides **D** Non-esterified fatty acids **E** Total cholesterol **F** High density lipoprotein cholesterol.



5.3.12 Detection of noise pollution

Substantial emission of ultrasound at 80kHz was detected close to the fluorescent lighting fitted in the animal rooms of the Biological Research Facility, in close proximity to caged rats.

The frequent but irregular passage by the building of lorries heading to the adjacent building site was noted.

5.3.13 Comparison of offspring phenotypes

Many of the results generated from these cohorts of offspring contrasted with those reported in Chapter 3, and previously. A summary of the results demonstrating discrepancies between offspring cohorts generated for this study, and those before and afterwards, is shown in **Table 5-2**.

Offspring cohorts generated simultaneously in another animal unit showed the expected phenotypic differences. These are also being re-established in the Biological Research Facility, after the cessation of building work, and modification of the lighting system.

Table 5-2 Comparison between results confounded by perinatal stress, and those obtained using unaffected cohorts of rat offspring

Measurements of Systolic BP, Plasma glucose, insulin and leptin, hepatic PEPCK activity and liver GR expression in adult male offspring of dex and saline-treated mothers are compared before, during and after the period between late 1999 and early 2001, when ultrasound-emitting lights were installed, and when building work adjacent to the animal house was in progress. All statistical testing utilises Student's t-test.

Parameter	Before 1999	Late 1999 – Early 2001	Late 2001 onwards
Systolic Blood pressure	Saline 133±3 mmHg Dex 144±2 mmHg p<0.01 (Levitt *et al.* 1996)	Saline 146±4 mmHg Dex 145±4 mmHg p=0.84	Saline 132±3 mmHg Dex 147±3 mmHg p<0.01 (performed by David O'Regan)
Basal Plasma glucose	Saline 4.3+/-0.2mM Dex 5.3+/-0.3mM p=0.04 (Nyirenda *et al.* 1998)	Saline 6.1±0.1mM Dex 5.9±0.3mM p=0.67	
Peak glucose in OGTT	Saline 7.5+/-0.2 Dex 8.7+/-0.4 P=0.03 (Nyirenda *et al.* 1998)	Saline 9.3±0.3mM Dex 10.5±0.8mM p=0.23	Saline 9.3±0.2mM Dex 10.6±0.5mM P=0.03
Peak Insulin in OGTT	Saline 3.8±0.5 ng/ml Dex 6.1±0.5 ng/ml p=0.01 (Nyirenda *et al.* 1998)	Saline 7.1±1.0 ng/ml Dex 4.6±1.0 ng/ml p=0.09	
Plasma Leptin	Saline 5.5±0.8 ng/ml Dex 6.5±0.6 ng/ml p=0.31	Saline 9.8±0.8 ng/ml Dex 9.3±0.6 ng/ml p=0.66	
Liver GR mRNA	Increased by 31% by dex; p=0.05	No difference; p=0.94	
Hepatic PEPCK activity	Increased by 60% by dex; p<0.01 (Nyirenda *et al.* 1998)	No difference; p=0.87	Increased by 42% by dex; p=0.03 (David O'Regan)

5.4 Discussion

This chapter relates data accumulated during the course of investigations intended to further define the insulin resistant phenotype of the dex-programmed rat. What it shows, however, is that the phenotype can probably be confounded in certain cohorts of offspring by perinatal stress, induced by noise. During the period following the refurbishment of the Biological Research Facility, it was discovered that strip-lighting had been altered to a type that emitted ultrasonic noise locally, as demonstrated with a "bat detector". Furthermore, there was a period of months during which the animal unit was frequently and irregularly passed by lorries accessing an adjacent building site, which would have involved emission of infrasound. Either of these frequencies are audible to rodents, and may have caused stress, either pre- or early postnatally (Armario *et al.* 1984; Andren *et al.* 1982; Vogel & Jensh 1988), to both dex and saline-treated rat cohorts, either through the maternal HPA axis, or potentially through the placental CRH-ACTH axis (Challis *et al.* 1995).

Many previously described characteristics of the adult phenotype of rats over-exposed to glucocorticoids were not apparent in these cohorts of animals, despite comparable *in utero* growth retardation. However, **Table 5-2** suggests that this might be because vehicle-treated offspring have developed phenotypic characteristics similar to those of dex offspring, without any appreciable change in the phenotype of the latter. The lack of any further effect of stress upon the insulin resistant phenotype of dex-treated offspring implies that there may be a "ceiling" for the effects of perinatal glucocorticoids. In the complex milieu of endocrine and other influences upon development, it is possible that a factor exists which is capable of antagonising the effects of glucocorticoids, such that their long term effects can be limited.

Birth weights were of the same order of magnitude, and dex offspring showed a similar reduction in weight to that previously reported (Levitt *et al.* 1996; Nyirenda *et al.* 1998), implying that noise stress may have occurred only after birth, or only

have had an impact post-natally. Alternatively, it may be that perinatal stress did not influence the growth of the foetus, but did have an effect on later insulin sensitivity. The implication of this is that prenatal stress might programme later insulin resistance but not growth *in utero*, implying that separate mechanisms might exist for the development of each, rather than insulin resistance being the consequence of poor prenatal growth. Reports documenting altered adult insulin sensitivity as a result of *in utero* malnutrition, in the absence of an effect on birth weight, lend weight to this theory (Roseboom *et al.* 2001a). This is an important possibility that requires further dissection in models of prenatal programming.

Catch-up growth of dex-treated offspring occurred subsequently, but this was incomplete, since between three and eight months of age, dex offspring remained on average 7% lighter than controls. It is not possible to say with certainty whether this growth pattern differs from the norm, however, as previously reported dex-treated cohorts were lighter (Welberg *et al.* 2000), or there was no difference between groups (Nyirenda *et al.* 1998; Lindsay *et al.* 1996b), whereas in Chapter 4, dex programmed animals were heavier at six months of age. Perhaps group sizes did not permit statistical analysis to detect a difference in the case of the quoted studies, but, in any case, larger scale investigation of catch-up growth in this model is warranted. A variety of effects has also been seen in other programming models, including complete catch-up (Desai *et al.* 1995; Woodall *et al.* 1996) or lack of catch-up (Langley & Jackson 1994) a few months after prenatal malnutrition, and induction of obesity in adult rats as a result of severe malnutrition *in utero* (Vickers *et al.* 2001), or prenatal treatment with cytokines (Dahlgren *et al.* 2001). This is an important issue in the context of the association between catch-up growth and later obesity (Ong *et al.* 2000), and presumably therefore, other features of the metabolic syndrome, in later life.

At nine months, there was no difference in systolic blood pressure detectable using tail cuff plethysmography (Evans *et al.* 1994). This contrasts with previous reports that prenatal over-exposure to glucocorticoids results in adult rats with a relative hypertension of approximately 10mmHg (Levitt *et al.* 1996; Lindsay *et al.* 1996a).

The lack of difference seems to reflect an increase in systolic BP in vehicle-treated rats to the previously documented level of glucocorticoid over-exposed offspring (Lindsay *et al.* 1996a), an effect which is not additive to that of prenatal dex. This may reflect perinatal programming of increased blood pressure by noise stress. Although this phenomenon has not been identified *per se*, it would most likely be mediated through elevated glucocorticoids *in utero*, or in later life, both of which are features of the dex model (Levitt *et al.* 1996). Although the tail cuff technique has been widely used in investigations of this type, the stress of the procedure could affect the results generated, and it is unlikely to detect differences in BP of <10mmHg in treatment groups of this size. If smaller differences were present, they would be better detected using arterial cannulation or radiotelemetry methods of BP measurement. However, the technique was able to detect reduced BP as a result of adrenalectomy in contemporaneous measurements, consistent with previous reports (van den Berg *et al.* 1990), and the known effect of corticosterone upon BP (data not shown).

Similarly, no difference was observed as a result of prenatal dex on either plasma glucose or insulin measured at three time points during an OGTT, in contrast to the previously reported effect (Lindsay *et al.* 1996b; Nyirenda *et al.* 1998). However, glucose administration resulted in an increase in plasma glucose and insulin after 20 minutes, as expected (Nyirenda *et al.* 1998), suggesting that there was no problem with the procedure. Consistent with BP findings, the plasma [glucose] measured in vehicle-treated offspring was elevated relative to expected values, such that it was similar to that of dex offspring.

There was a trend for dex offspring to have higher plasma corticosterone than controls, consistent with previous findings (Welberg *et al.* 2001; Levitt *et al.* 1996). A significant difference may not have been generated because of stress at the time of euthanasia (both mean values are high), or because of the confounding effect of perinatal stress. There were no differences between cohorts in the weight of any of three muscles containing different proportions of muscle fibre types at euthanasia (Armstrong & Phelps 1984), relative to body weight. Storage of glycogen by

quadriceps, expression of GR in liver, and hepatic PEPCK activity in dex rats were no different to controls in these cohorts. These findings are in contrast to the reduced storage of glycogen, reported in Chapter 3, and the previously documented up-regulation of GR and PEPCK in liver of dex-treated offspring (Nyirenda *et al.* 1998). The implied lack of difference in both glucose uptake by muscle and hepatic gluconeogenesis are consistent with the similar insulin-glucose status of these offspring cohorts.

Despite the presence of stressful stimuli around birth, differences between groups were identified in fat. Epididymal fat pads were reduced in size by prenatal dex, but this was not associated with a difference in plasma leptin, although values were higher than those measured in treated and control offspring in Chapter 3. This result is in stark contrast to the increased fat mass associated with programming by both cytokines and prenatal malnutrition (Dahlgren *et al.* 2001; Vickers *et al.* 2001). Although the dominant effect of increased circulating glucocorticoid would be to induce plasma leptin (De Vos *et al.* 1995), this response is attenuated in diabetes (Liu *et al.* 1999), which probably explains the lack of effect of hypercorticosteronaemia in these, and standard dex-programmed offspring. There were no changes in plasma BHB, NEFA, cholesterol, or HDL-cholesterol associated with the reduced fat mass, but there were trends toward lower plasma TAG and increased deoxyglucose uptake by adipocytes. These differences are consistent with improved insulin sensitivity in dex rats under these circumstances, which is consistent with the increased glucose uptake in young adult offspring of protein-restricted dams (Ozanne *et al.* 1997), although these rats later become insulin resistant, with reduced glucose uptake (Holness & Sugden 1999). Increased lipolytic activity is also a feature of *in utero* protein-restricted offspring in later life (Ozanne *et al.* 1999; Holness *et al.* 1998), which would normally be associated with increased plasma TAG and NEFA. It seems in general that, with respect to adipose tissue, dex-treated rats are relatively resistant to the development of obesity and consequent adipose insulin resistance, when compared with perinatally stressed vehicle-treated animals. The explanation for this phenotype is likely to be obscure, and it could be that both cohorts of animals

are relatively more obese than unstressed control offspring, since plasma leptin in both groups is higher than previously measured.

In summary, it is clear that perinatal stress induced by environmental noise, is sufficient to confound dex-programming experiments. Insulin resistance and hypertension were induced in vehicle-treated animals such that a similar phenotypes were apparent in both treatment groups. No additional effect on insulin-glucose status or BP were noted in dex-treated offspring, suggesting that there may be a ceiling effect of perinatal stress / glucocorticoids. This would not be surprising, in view of the importance of glucocorticoids in mediating stress-induced changes in physiology. Despite these effects on metabolism, *in utero* growth differences were preserved, suggesting that effects on growth may be programmed separately. Given time, of course, repetition of many of these measurements would be instructive in further elucidating the phenotype of the dex-programmed rat. The re-establishment of programmed differences in metabolism should permit this.

6 Chapter Six – Discussion

6.1 Background and Aims

This thesis concerns a potential mechanism explaining the "Foetal origins of adult disease" hypothesis (Barker 1995), which was put forward following the the repeated identification of epidemiological links between low birth weight and increased incidence of disease in later life; most notably insulin resistance and non insulin-dependent diabetes mellitus (Hales *et al.* 1991). Edwards et al proposed that over-exposure of the foetus to glucocorticoids could explain both the reduced birth weight and later insulin resistance (Edwards *et al.* 1993). This hypothesis was supported by experiments in rat models in which dexamethasone was given daily during the third week of pregnancy (Nyirenda *et al.* 1998), or in which the placental barrier to glucocorticoids, 11β-HSD type II, was inhibited using liquorice derivatives (Lindsay *et al.* 1996b): treated offspring showed both reduced birth weight, and hyper-insulinaemia and hyperglycaemia in adulthood.

Further work demonstrated that dex-programmed offspring were hypercorticosteronaemic, resulting from an overactive HPA axis (Levitt *et al.* 1996), associated with reduced GR mRNA in hippocampus (Welberg *et al.* 2001). Up-regulation of PEPCK in the liver, the rate-limiting enzyme of gluconeogenesis, implied that increased hepatic glucose output contributed to the observed hyperglycaemia, and this was also associated with an increase in expression of GR in liver (Nyirenda *et al.* 1998).

The aims of the thesis were to determine firstly whether metabolism in skeletal muscle and white adipose tissue was also programmed by prenatal dex, especially with regard to glucose disposal. Secondly, glucocorticoid sensitivity, as indicated by GR expression, was assessed in these tissues; and thirdly, it was asked whether

dysregulation of GR was important in determining the insulin resistant phenotype, or whether it was secondary to hyperinsulinaemia or hyper-corticosteronaemia.

6.2 Programming of insulin resistance

The results presented in this thesis add further weight to the hypothesis that *in utero* over-exposure to glucocorticoids can programme insulin resistance in later life. Evidence is presented that glucose uptake into skeletal muscle of rats is attenuated by prenatal dex treatment, implying that increased hepatic glucose output is not the sole cause of hyperglycaemia in this model. This effect exists despite concurrent hyperinsulinaemia, so resistance to the action of insulin in skeletal muscle can be inferred. Furthermore, visceral fat of programmed rats has a reduced lipolytic capacity, suggesting that there is also attenuated insulin-stimulated fatty acid uptake into adipose tissue. This change may be mediated by increased glucocorticoid sensitivity, and a shift in the expression of PPARγ isoforms.

Analogous findings have been made in alternative models of programmed insulin resistance: a single prenatal glucocorticoid injection is sufficient to programme glucose metabolism in lambs (Moss *et al.* 2001), while various forms of prenatal malnutrition have also been demonstrated to induce insulin resistance in adult rats (Ozanne & Hales 1999; Vickers *et al.* 2000). These collective findings reinforce the foetal origins hypothesis, by providing a further line of evidence for the programming of insulin resistance in addition to that derived from epidemiological associations.

Furthermore, genes identified as being differentially expressed between prenatally manipulated and control animals become potential targets for investigation of the mechanisms linking poor growth *in utero* with later insulin resistance in human clinical studies. In the longer term, therapeutic interventions targeting genes underpinning this association could be used in those individuals deemed at risk because of low birth weight, to ameliorate or prevent the development of the insulin resistance syndrome.

The degree and significance for glycaemic control of variation in supply of endogenous glucocorticoids to the human foetus remains to be established. However, one study has shown that cortisol supply is increased to growth retarded foetuses (Goland *et al.* 1993). Administration of exogenous glucocorticoids to pregnant mothers is a practice which has long been pursued to prevent respiratory distress syndrome in cases of threatened premature delivery (Liggins & Howie 1972). It is now also becoming increasingly common to administer glucocorticoids for longer periods pre-partum to prevent the development of virilising congenital adrenal hyperplasia, due to foetal 21-hydroxylase deficiency (Mercado *et al.* 1995). It has been known for some time that large doses of glucocorticoids can result in low birth weight babies (Reinisch *et al.* 1978), and although rare, reports of maternal side-effects (Pang *et al.* 1992) and deleterious long term consequences for the offspring (Trautman *et al.* 1995; Doyle *et al.* 2000) are starting to appear. The direct relevance of examination of the long term effects of prenatal dex on insulin sensitivity in animal models, in order to assess the potential consequences of this practice, is clear. In the light of the further evidence presented in this thesis, however, caution is required in the use of these interventions.

Data presented in this thesis reinforces previous results suggesting that the third week of pregnancy is the crucial "developmental window" in the rat, during which insulin resistance can be programmed (Nyirenda *et al.* 1998). There is scope for this window to be further defined, however, as administration of dex during the last two days of pregnancy alone did not result in adult hyperglycaemia (Nyirenda *et al.* 2001). The window for programming by dex is species-specific, as insulin resistance is caused by a relatively earlier treatment in sheep (Moss *et al.* 2001), which may reflect differences in the relative timing of differentiation of pancreatic or insulin-sensitive tissues. Epidemiological studies have suggested that infant size and proportion, and the nature of subsequent metabolic derangement varies according to the trimester during which malnutrition occurred (Osmond & Barker 2000; Roseboom *et al.* 2001a). The precise nature of the systems susceptible to dex

treatment during particular windows will be defined by consideration of foetal gene expression and biochemistry throughout gestation.

6.3 Programming of glucocorticoid sensitivity

Results presented in this thesis have extended our knowledge of the tissue-specific nature of programming of GR by glucocorticoids. Programmed up-regulation of GR occurs in visceral but not subcutaneous fat, in addition to the liver. No change in expression was recorded in muscle of mixed fibre type, however, or in muscle composed predominantly of type II fibres. In contrast, down-regulation occurred in type I fibre-rich muscle, in common with the situation in hippocampus. It has been hypothesised that increased sensitivity to glucocorticoids may be responsible for resistance to the actions of insulin to inhibit gluconeogenesis in the liver (Nyirenda *et al.* 1998), and it indeed may mediate the observed reduction in lipolytic capacity in visceral fat.

The significance of increased GR in these tissues as a potential primary determinant of the programmed insulin resistance has been reinforced by the demonstration herein that neither hyperinsulinaemia nor hypercorticosteronaemia are sufficient to explain it. Furthermore, observations of specific effects of both metformin and rosiglitazone in reducing or attenuating GR over-expression suggest interesting avenues for further investigation of their respective modes of action.

The mechanism whereby GR expression can be determined through programming, auto-regulation, and drug administration in a tissue-specific fashion has yet to be established. However, a candidate mechanism that might permit such exquisite control over GR expression is the regulation of transcription from multiple alternate promoters. Tissue specific patterns of transcription have been identified already, which are modified by two different programming paradigms (McCormick *et al.* 2000). Metformin in particular could prove a useful tool to dissect the pathways influencing the expression of specific GR transcripts.

6.4 Effect of perinatal stress on programming

I have inferred from the data in Chapter 5 that the imposition of noise stress on dex-treated and control offspring perinatally altered the published rat phenotype (Benediktsson *et al.* 1993; Nyirenda *et al.* 1998). The programming effects of stress alone upon insulin-glucose metabolism have not been established, but since any effects are likely to be at least partially mediated through increased glucocorticoid secretion, the results of perinatal stress may be directly superimposed upon those of the intended prenatal treatments with dex or saline.

Dex-treated and control birth phenotypes normally observed in this paradigm were preserved, but the previously identified differences in adult phenotype were abolished in these experiments. Consideration of the absolute results for glucose tolerance and blood pressure suggested that the phenotype of the control offspring had been shifted towards that of the dex-treated animals, supporting the above contention, but also implying that there might be a "ceiling" for the programming effects of stress and glucocorticoids. The preservation of the birth phenotypes may imply that stress was only present post-natally, and "hard-wired" the metabolism of control offspring at this stage, as has been previously observed with respect to the HPA axis (Meaney *et al.* 1996).

Alternatively, and more intriguingly, it could be that perinatal stress exerts a programming effect on insulin-glucose metabolism but not on birth weight, implying a mechanistic separation between the two phenomena. Insulin resistance has been previously recorded as a result of prenatal manipulation in the absence of reduced birth weight (Roseboom *et al.* 2001a; Gatford *et al.* 2000), hence this is a possibility. Birth weight is at best an approximate surrogate for foetal growth, as it fails to reflect differences in body proportions and underlying differences in development of particular organs or tissues. Indeed, thinness at birth in particular has been associated with later insulin resistance (Phillips *et al.* 1994), presumably reflecting under-development of abdominal organs, most crucially the pancreas and / or liver. It is possible that the development of insulin-sensitive tissues is specifically affected by

perinatal stress, but not prenatal growth overall. Reduced fat mass in later life may reflect this.

6.5 Further work indicated

There is a great deal of scope for further characterisation of the insulin resistant phenotype of the dex model. Clearly, elements of the work presented in Chapter 5 bear repeating in the absence of the confounding effect of perinatal stress, in particular basic measurements such as muscle and fat depot size. Measurement of glucose uptake by adipocytes and muscle strips could confirm the attenuation of peripheral glucose uptake, but a particularly elegant method of elucidating the relative contributions of hepatic glucose output and peripheral glucose uptake to the hyperglycaemia would be to utilise hyperinsulinaemic-euglycaemic clamp methods, incorporating multiple radiolabelled tracers (Kraegen *et al.* 1990).

Further analysis of the role of GR in the model is also indicated. As insulin resistance does not arise in prenatally treated rats until well into adulthood, it would be interesting to establish at what age elevated GR expression develops, as this may chronologically precede the development of insulin resistance, if it is a causative influence. Treatment with metformin prior to the development of insulin resistance could help explore this possibility. Extension of the insulin-sensitising drug treatment experiment to examine effects on GR in visceral fat, and on the HPA axis could further strengthen the arguments made herein. The importance of these findings would also be emphasised if the expression of down-stream metabolic targets were also found to be altered. An obvious first gene to consider would be hepatic PEPCK.

There is clearly a need for corroboration of the novel effects of metformin and rosiglitazone on GR expression described, as these could be of great potential interest to clinicians involved in diabetes management. Examination of the effect of these drugs in other models of insulin resistance, in which elevated GR has been observed, would be worthwhile, in order to establish whether the effects are applicable to syndromes with different aetiology. The specific mechanism through which each

drug acts also requires elucidation, however. Tissue culture techniques might be suitable to determine whether direct or indirect effects are involved.

There are a number of further issues that have not been addressed in the glucocorticoid over-exposure model. No-one has yet examined whether glucose-sensing or insulin-secretory mechanisms are intact in the pancreas of these rats. This is important in establishing the relative importance of insulin secretion and resistance in this model. Epidemiological studies have also associated low birth weight with altered immune function (Beasley *et al.* 1999; McDade *et al.* 2001a; McDade *et al.* 2001b), osteoporosis (Dennison *et al.* 2001), and renal impairment (Hinchliffe *et al.* 1992) in later life. Whether glucocorticoid over-exposure *in utero* might be responsible for these abnormalities could also be addressed.

The potential significance of early life events is reinforced strikingly by recent work which has also identified effects on the health of the subsequent generation. Barker found a clear inverse correlation between maternal birth weight and offspring blood pressure, independent of offspring birth weight (Barker *et al.* 2000), and a study in rats (Martin *et al.* 2000) showed that maternal malnutrition *in utero* resulted in F_2 offspring with pronounced insulin resistance. Work investigating the potential for prenatal dex to influence glycaemic control in the subsequent generation is underway in the laboratory.

6.6 Summary

In summary, this thesis adds weight to the hypothesis that *in utero* over-exposure to glucocorticoids can programme insulin resistance in later life, and supports a role for both skeletal muscle and adipose tissue metabolism in mediating this. Moreover, it seems that programmed dysregulation of GR in insulin target tissues plays a significant role in determining the offspring phenotype. These findings may be valuable in determining targets for aetiological investigation and future therapeutic intervention in humans.

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