Regulation of gluconeogenesis in type 2 diabetes mellitus: An investigation of the role of corticosteroids?

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Abstract

Type 2 diabetes mellitus (T2DM) is a complex disease involving various physiological factors, hormones and metabolites. Over expression of key gluconeogenic enzymes, such as PEPCK, cause features of T2DM including obesity and insulin resistance. Previous studies showed intravenous administration of corticotropin releasing factor (CRF) and sauvagine in rats cause marked hyperglycaemia, which is adrenal dependent. The 11-β hydroxylase inhibitor metyrapone augmented this hyperglycaemia raising important questions about the role of known and unknown corticosteroids in hepatic carbohydrate metabolism.

The goal of this project was to identify and characterise the gluconeogenic activity of adrenal corticosteroids secreted under basal and stimulated conditions. This was accomplished by establishing a rapid, sensitive and robust multi-well assay for measurement of PEPCK activity in the hepatocyte cell line H4-II-E-C3. A sensitive fluorescent method for assay of glucose production by these cells was also developed.

Extracts of media samples from adrenal glands incubated with sauvagine and metyrapone significantly increased hepatocyte glucose production (HGP) despite low corticosterone concentrations. HPLC characterisation of these extracts revealed increases in 11-deoxycorticosterone (DOC) and other unidentified peaks. However, none of these individual fractions significantly affected HGP in culture.

Commercially available corticosterone, which contains DOC as an impurity, had greater gluconeogenic effect compared to purified corticosterone alone. Based on these observations and discrepancies in the literature, the effect of DOC on hepatic carbohydrate metabolism was characterised. Surprisingly, DOC suppressed the activity of gluconeogenic enzyme PEPCK in fed rat hepatocytes and enhanced insulin

stimulated glycogen stores in cultured hepatocytes at higher glucose concentrations (25 mM) over a 24 hour period. In serum-starved, fasted hepatocytes these effects were not significant, suggesting the need for a detailed investigation of the signalling pathways and regulatory control of DOC on GK, GS, G6Pase and PEPCK.

Chapter 1: Introduction

Chapter 6: General discussion

List of Abbreviations

AA	Arachidonic acid
ACTH	Adrenocorticotropic hormone
ADP	Adenosine di phosphate
AF	Activating Function
AMPK	AMP- activated Protein Kinase
ANG II	Angiotensin II
ATP	Adenosine tri phosphate
AVP	Arginine Vasopressin
cAMP	cyclic Adenosine mono-phosphate
CEBP	CCAT Enhancer Binding Protein
CNS	Central Nervous System
CRF	Corticotropin Releasing Factor
CRF-BP	CRF binding protein
CRU	Cyclic AMP response units
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's modified eagle's medium
DOC	11-deoxycorticosterone
DOCA	11-deoxycorticosterone acetate
18-OH DOC	18-hydroxy 11-deoxycorticosterone
DTT	Dithiothreitol
EBSS	Earl's Balanced salt Solution
EGFR	Epidermal growth factor

EGTA	Ethylene glycol tetra-acetic acid
G6P	Glucose 6 phosphate
G6pase	Glucose-6-phosphatase
GLUT	Facilitative glucose transporters
GNG	Gluconeogenesis
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
GRU	Glucocorticoid response units
GSK3	Glycogen synthase kinase 3
GTP	Guanosine tri phosphate
GWA	Genome wide association
11-β HSD	11-β-hydroxysteroid dehydrogenase
HGP	hepatic glucose production
HNF	Hepatic Nuclear factor
HPA	Hypothalamic Pituitary-adrenal Axis
HPLC	High performance liquid chromatography
HRE	Hormone responsive element
HRP	Horse radish peroxidase
HSP	Heat-shock protein
IGFBP-1	Insulin like growth factor binding protein-1
IGT	Impaired glucose tolerence
IRU	Insulin response units
ITP	Inosine tri phosphate

LIP	Liver enriched Inhibitory protein
LLRM	Luciferase-luciferin reaction mix
MAPK	Mitogen Activated Protein Kinase
MR	Mineralocorticoid receptor
MS	Metabolic syndrome
NADP	Nicotinamide adenide dinucleotide phosphate
NFkB	Nuclear factor kappa B
OAA	Oxaloacetic acid
PBS	Phosphate buffered saline
PEP	Phosphoenol pyruvate
PC	Pyruvate Carboxylase
PCOS	Polycystic Ovary Syndrome
PGC-1α	PPAR-coactivator
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pyruvate Kinase
PKRB	Pyruvate kinase reaction buffer
PPAR	Peroxisome proliferator-activated receptor
R1LB	Reaction 1 lysis buffer
RARU	Retinoic acid response units
RB	Reaction Buffer
SGLT	Sodium glucose carriers
SPE	Solid phase extraction
T2DM	Type 2 diabetes mellitus

TAT	Tyrosine amino transferase
TRU	Thyroid hormone response units
TX-100	Triton X-100
UCN	Urocortins
WHO	World Health Organisation
ZR	Zona reticularis

Chapter 1
General Introduction

1.1 Overview

Type 2 diabetes mellitus (T2DM) is a complex and heterogeneous disease with a poorly understood aetiology. It is characterised by impaired beta cell function and varying degrees of insulin resistance leading to reduced tissue uptake of glucose and varying degrees of hyperglycaemia (Gerich, 1998). It is generally considered that there is a strong genetic predisposition, which becomes evident when exposed to a sedentary western lifestyle.

Since 2006 many studies have been published based on High-Throughput genome wide association (GWA) studies. These studies have uncovered new genetic loci linked to diabetes. Five large studies were conducted using a two-stage strategy consisting of a GWA screen in an initial cohort of unrelated cases and controls followed by replication of the most significant findings in additional sets (Florez *et al.*, 2007; Hanson *et al.*, 2007; Hayes *et al.*, 2007; Rampersaud *et al.*, 2007; Salonen *et al.*, 2007). From these studies at least fifteen loci emerged as being consistently associated with T2DM across multiple studies. Three of these (TCF7L2, KCNJ11, and PPARG) have already been linked with T2DM. The other 12 represent potential new T2DM genes (Table 1.1).

T2DM has increased rapidly over the past 30 years. According to the World Health Organisation (WHO), the numbers of cases worldwide are around 170 million and the number is expected to rise to 350 million by 2025 (Wild *et al.*, 2004). The predominant reasons for the global rise in T2DM are obesity, physical inactivity and the increasing number of elderly people. Of the existing diabetes worldwide, 95% suffer from T2DM.

Gene Symbol	*Cellular Function*	*OMIM Number*	*Odds Ratio*	*Frequency of Risk Allele*
NOTCH2	Regulator of cell differentiation	600275	1.13	0.11
THADA	Unknown	611800	1.15	0.90
ADAMTS9	Proteolytic enzyme regulating extracellular matrix	605421	1.09	0.76
PPARG	Transcription factor receptor for TZDs and prostaglandins	601487	1.17	0.85
IGF2BP2	IGF2 mRNA-binding protein 2	608289	1.14	0.29
CDKAL1	Presumed regulator of cyclin kinase	611259	1.0-1.20	0.31
JAZF1	Zinc-finger protein of unknown function	606246	1.10	0.50
SLC30A8	Zinc transporter 8	611145	1.18	0.65
CDKN2A CDKN2B	Cyclin-dependent kinase inhibitor 2A and 2B	600431	1.20	0.83
CDC123	Required for S phase entry of the cell cycle		1.11	0.18
CAMK1D	Mediator of chemokine signal transduction in granulocytes	607957		
IDE	Neutral metallopeptidase that can degrade many peptides	146680	1.13	0.53
HHEX	Homeobox transcription factor	604420		

KIF11	Kinesin related motor in microtubule & spindle function	148760		
TCF7L2	High mobility group transcription factor	602228	1.31-1.71	0.26
KCNJ11	Inwardly rectifying potassium channel	600937	1.14	0.47
TSPAN8	Cell surface glycoprotein	600769	1.09	0.27
LGR5	Orphan G protein receptor	606667		
FTO	58kD protein with nuclear localization signal	610966	1.27	0.38

Table 1.1: Genetic loci in "common variety" in T2DM (Doria *et al.*, 2008).

WHO estimates that there are also 1.6 billion people who are overweight and around 400 million are obese worldwide. Obesity is a major concern as there is clear evidence of an association with the risk of developing T2DM (Gruber, 2006). Sedentary lifestyle, lack of exercise, consumption of foods rich in saturated fat and sugar lead to an increase in obesity (Swinburn *et al.*, 2004), resulting in insulin resistance and subsequent T2DM (Caterson *et al.*, 2002). Although there is a strong association between excess body fat and T2DM, not all obese and overweight subjects develop diabetes. The relationship to the risk of T2DM is closer in subjects with abdominal obesity (Bordier *et al.*, 2009). This is also consistent with increased visceral fat being linked to insulin resistance and dyslipidaemia; two characteristics of the metabolic syndrome and T2DM. The majority of patients with T2DM suffer from hypertension, as well as microvascular and macrovascular diseases which result in a

high incidence of cardiovascular disease, blindness, amputation and renal failure (2008).

The aetiology of type 2 diabetes primarily involves peripheral insulin resistance and impaired insulin secretion, but several other defects contribute to the development and progression of type 2 diabetes. These include increased hepatic glucose production, a lack of glucagon suppression, impaired incretin signalling, and increased expression and activity of renal glucose transport proteins (Tahrani *et al.*, 2009). Together, all of these factors contribute to hyperglycaemia; in turn, the resulting glucotoxicity worsens all of these defects. Increased hepatic glucose production due to dysregulation of gluconeogenesis and glycogen metabolism and decreased glucose uptake can cause hyperglycaemia (Clore *et al.*, 1992; Krssak *et al.*, 2004). In T2DM this hyperglycaemia is not controlled because of defects in insulin secretion and action. It is now well recognised that in addition to the defects in secretion of insulin and tissue sensitivity to the actions of insulin, there are a number of other hormones that potentially play a role in development and progression of T2DM.

As the molecular defects causing diabetes are still largely unknown the factors triggering insulin resistance or deficiency as a primary defect still need to be resolved. Steroids derived from the adrenal gland are well known glucose modulators with glucocorticoids closely linked with hyperglycaemia and T2DM (McMahon *et al.*, 1988). However research of various clinical conditions exhibiting insulin resistance or hyperglycaemia raise interesting questions about adrenal abnormalities that may underline T2DM.

1.2 Pathophysiology of type 2 diabetes mellitus

1.2.1 Abnormalities in insulin secretion

T2DM involves impairments in both insulin secretion and insulin sensitivity (DeFronzo *et al.*, 1989). Insulin sensitivity is determined by the ability of insulin to promote glucose uptake and utilization. Normally insulin displays rapid variations in blood concentrations with early secretory peaks every 5 – 10 min and larger oscillations every 60 – 120 min. However in patients with overt T2DM these secretory peaks are found to be reduced or absent (O'Rahilly *et al.*, 1988; Polonsky *et al.*, 1988). In experimental models normal insulin secretion in response to intravenous glucose is characterised by an early peak after 3 – 5 min followed by a slower and more progressive phase that lasts as long as the glucose infusion. In T2DM this first phase is abolished, and the late phase is reduced and delayed (Cerasi *et al.*, 1967; Fujita *et al.*, 1975). This early phase insulin secretion suppresses hepatic glucose production during the transition stage from fasting to the fed state (Luzi *et al.*, 1989).

Specific immunoassays have shown that insulin deficiency in T2DM is often masked by unprocessed circulating pro-insulin. These prohormones may account for more than 40% of circulating insulin immunoreactivity in T2DM compared to 5% in non-diabetics (Temple *et al.*, 1990). Insulin secretory capacity is decreased by 50% at the time of diagnosis with a further decrease of 15% six years later (UKPDSG, 1995). This progressive reduction has been attributed to glucotoxicity (Rossetti *et al.*, 1990), lipotoxicity (Unger, 1995), advanced glycation of the insulin promoter gene (Matsuoka *et al.*, 1997), and excess reactive oxygen species causing β-cell apoptosis (Butler *et al.*, 2003; Sakuraba *et al.*, 2002). Overall a 20 – 40% reduction in β-cell

mass is present in T2DM in contrast with β-cell hyperplasia observed in insulin resistant states associated with non diabetic obesity (Biarnes *et al.*, 2002).

TCF7L2 is a gene that was found to predict high susceptibility to T2DM; accounting for 20% of cases (Grant *et al.*, 2006). This gene was found to be associated with alterations in insulin secretion with severely impaired insulin secretion in carriers of the susceptibility variants (Saxena *et al.*, 2006). Low birth weight has also been associated with decreased β-cell mass and an increased chance of developing impaired glucose tolerance (IGT) and T2DM during adulthood (Hales *et al.*, 1991). There is a hyperbolic relationship between insulin secretion and sensitivity (Weyer *et al.*, 1999). β-cells in non-diabetics adapt their secretion rate according to the level of insulin sensitivity. If this compensation is impaired it results in hyperglycaemia.

1.2.2 Insulin resistance and endogenous glucose production

In addition to the deficiency of insulin some of the earliest studies showed variations in the responses of diabetic patients to insulin, suggesting the role of insulin insensitivity as the biochemical defect. This was supported by radioimmunoassay data that showed that subjects with adult (onset) T2DM had more than average circulating levels of insulin (Yalow *et al.*, 1960). Although a high proportion of this circulating insulin was later reported to be pro-hormones (Temple *et al.*, 1990).

Insulin resistance can be explained as an increased hepatic glucose production and decreased glucose uptake by skeletal muscle. A primary defect in insulin signalling pathways and dysfunction in adipose tissue further contributes to insulin resistance. Individuals who develop T2DM proceed through a phase of impaired glucose tolerance IGT (Tuomilehto *et al.*, 1992) as a result of defects in insulin secretion and action. As the tissues become more and more resistant to insulin, pancreatic β cells

secrete more insulin to compensate for this resistance. However, progressive β cell failure leads to an inability to compensate for insulin resistance which results in post-prandial hyperglycaemia, and eventually the fasting hyperglycaemia of the diabetic state. The risk of progressing from IGT to T2DM is known to vary depending on the population, ethnicity, obesity and other cardiovascular risk factors (Alberti, 1996). As long as pancreatic β cells compensate for the insulin resistance the disease never progresses from IGT to T2DM. A number of trials have shown a greater than 50% reduction in progression of IGT to T2DM by lifestyle measures which include moderate exercise and diet (Tuomilehto *et al*., 2001).

The transition from a pre-diabetic state to T2DM is characterised by three major changes

a) A reduction in insulin secretion by pancreatic β cells.

b) Overproduction of glucose by the liver.

c) Insulin resistance in the skeletal muscle

The manifestation of insulin resistance in T2DM is generally attributed to decreased glucose transport and metabolism in fat cells and skeletal muscle. Inability to suppress hepatic glucose output is also a major factor (DeFronzo, 2004). So, insulin resistance in the liver contributes to hepatic glucose production causing hyperglycaemia. Although the general assumption is that insulin resistance results in increased endogenous glucose production, there is also a possibility of a change in the hepatic metabolic flux that can lead to insulin insensitivity. Some studies showed that increases in the gluconeogenic regulator fructose 2,6-bisphosphate can overcome insulin resistance in T2DM (Wu *et al.*, 2006).

1.2.3 Insulin sensitivity and glucose transport

Obesity is characterised by insulin resistance. Eighty percent of all subjects with T2DM are obese. Also, obese individuals with T2DM are more insulin resistant than those without diabetes (DeFronzo *et al.*, 1996). In T2DM hepatic glucose production is uninhibited and peripheral glucose uptake is reduced in response to insulin. This indicates that both liver and peripheral tissues are involved in insulin resistance. Skeletal muscle is the major tissue responsible for insulin-mediated glucose uptake. It was quickly established that insulin resistance in skeletal muscle and liver did not result from a defect in the insulin receptor number but from a defect in the insulin signalling pathway (Garvey *et al.*, 1993). Furthermore, it was clearly demonstrated that insulin resistance in skeletal muscle is due to defects in insulin-dependent glucose transport (Bell *et al*., 1993). Due to the hydrophilic nature of glucose it cannot penetrate the lipid bi-layer of cell membranes and needs specific transporter proteins to facilitate its diffusion. This is mediated by two distinct families of hexose transport proteins: facilitative glucose carriers [glucose transporters (GLUTs)] and sodium glucose carriers (SGLTs) (Bell *et al*., 1993). Within the facilitative glucose transporters, class I transporters (GLUT 1, GLUT 3, and GLUT 4) have a high affinity for glucose and the level of these proteins on the cell surface greatly influence the rates of glucose uptake into cells. Of the three Class I GLUTS, GLUT 4 mediates insulin-stimulated glucose uptake by skeletal muscle, heart, white and brown adipose tissues by a mechanism involving translocation between cellular compartments. Insulin increases the cell surface expression of this protein by increasing the rate of externalisation and decreasing the rate of internalisation (Jhun *et al.*, 1992). The fundamental role of GLUT4 was demonstrated in animal models where muscle specific over expression significantly improved insulin action and reduced blood

glucose levels (Leturque *et al.*, 1996; Rencurel *et al.*, 1996). Several other studies have also shown that insulin increases glucose uptake in cells by stimulating the translocation of glucose transporters (GLUT4) to the cell surface from intracellular sites. Up to 75% of insulin dependent glucose disposal occurs in the skeletal muscle, whereas adipose tissue accounts for only a small fraction of this (Klip *et al.*, 1990). However, mice with an insulin receptor knockout in muscle had normal glucose tolerance (Bruning *et al.*, 1998), and those with knockout of insulin sensitive glucose transporter in fat developed impaired glucose tolerance (Abel *et al.*, 2001). This highlights the important role of adipose tissue in regulating glucose metabolism. It is also interesting to note that exercise causes an increase in AMPK in muscle, which in turn increases GLUT4 translocation in response to insulin (Goodyear *et al.*, 1998).

As the molecular defects causing diabetes are still largely unknown the factors triggering insulin resistance or deficiency as a primary defect still need to be resolved. Skeletal muscle, adipose tissue and the liver are all involved in the development of the disease state but, the most important site of insulin resistance is still the subject of debate (DeFronzo, 2004). Apart from insulin resistance and sensitivity, endogenous glucose production and dysregulation of gluconeogenesis are considered to be the key areas of inadequate understanding in T2DM, with excess hepatic glucose production being a major contributor to both fasting and postprandial hyperglycaemia in T2DM. Promising biological targets being investigated include those leading to insulin sensitization (11β-HSD-1 inhibitors, and antagonists of glucocorticoid receptor), reducing hepatic glucose output (antagonist of glucagon receptor, inhibitors of glycogen phosphorylase and fructose-1,6-biphosphatase), and finally increasing urinary elimination of excess glucose (SGLT inhibitors).

1.2.4 Adrenocortical dysregulation.

Adrenal glucocorticoids are increasingly implicated in the pathogenesis of metabolic syndrome (MS). Increased adrenal corticosteroid production represents an important pathogenic pathway in MS, T2DM, Cushing's syndrome and other disease states such as Polycystic ovary syndrome (PCOS), which exhibit insulin resistance and hyperglycaemia (Vassiliadi *et al.*, 2009). Alterations in carbohydrate metabolism occur in adrenalectomised animals. Administration of adrenal extracts raise blood sugar levels and liver muscle glycogen in both adrenalectomised and normal animals (Long *et al*., 1940). The most interesting finding linking the adrenal gland with the diabetic state is that adrenalectomy in obese Zucker rats prevents hyperinsulinaemia (Freedman *et al*., 1986). Similarly, in diabetic (ob/ob) mice, adrenalectomy reduces plasma insulin and glucose levels (Makimura *et al*., 2000). Animal models of lipoatropic diabetes have elevated glucocorticoids. Adrenalectomy in these models decreased the blood glucose, serum insulin, and glycated hemoglobin levels (Haluzik *et al.*, 2002). These mice also showed improved insulin sensitivity in both muscle and liver after adrenalectomy.

Although it is generally assumed that adrenalectomy simply eliminates the metabolic actions of corticosterone, such studies are not specifically aimed at investigating the effects of specific glucocorticoids and thus do not eliminate a role for other adrenal steroids and metabolites. The defects observed in adrenalectomised animals were not due to impaired cAMP production or subsequent signalling pathways. Freedman in his observations quoted that the "*nature of the defect following adrenalectomy is elusive and not easily reproducible*". This was based on the observations that adrenalectomy of obese Zucker rats improved insulin resistance (Freedman *et al*.,

1986). The hypothalamic pituitary-adrenal axis (HPA) plays an important role in the tight regulation of the circulating levels of glucocorticoids. However, glucocorticoid levels are locally enhanced in the liver and adipose tissue by the enzyme 11β-HSD1 (Tomlinson *et al.*, 2004). Transgenic mice with over expression of 11β-HSD1 either in adipose tissue or the liver exhibited components of MS (Masuzaki *et al.*, 2001).

It is now well accepted that glucocorticoids generally mobilise substrates for gluconeogenesis by stimulating the release of amino acids from skeletal muscle, fatty acids and glycerol from adipose tissue, and have a direct effect on gluconeogenic enzymes like phosphoenolpyruvate carboxykinase (PEPCK) (Gunn *et al.*, 1975). They also stimulate glycogen synthesis by activating glycogen synthase and inactivating the glycogen-mobilizing enzyme glycogen phosphorylase.

There has been an association between enlarged adrenal glands and T2DM in obese patients (Godoy-Matos *et al.*, 2006). Adrenal gland weight is also higher in rats fed a high-fat diet (Pitts *et al.*, 1977). From these observations it is important to look in more detail the role of adrenal corticosteroids in the collective effect on glucose homeostasis both *in vivo* and *in vitro*.

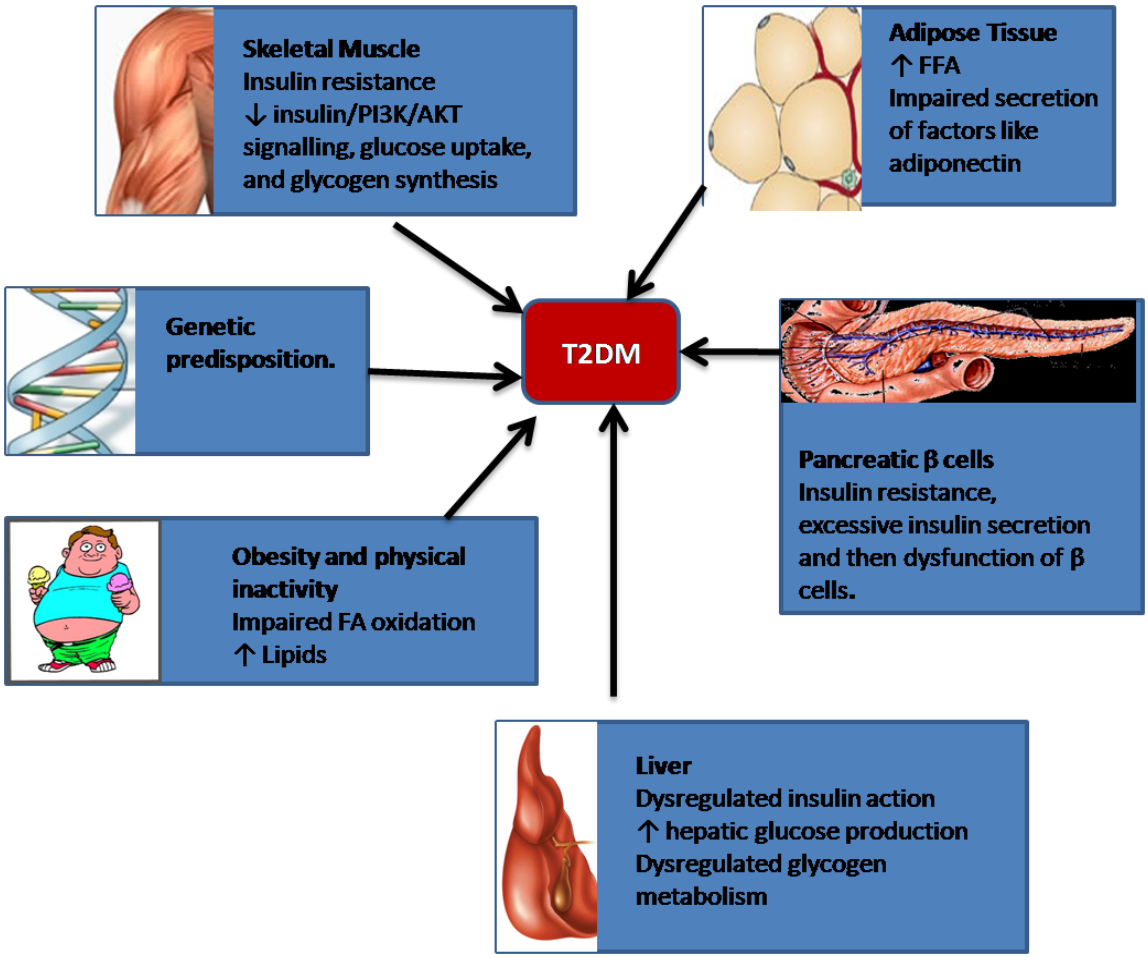


Figure 1.1 Overview of the pathogenesis of T2DM.

Current theories on T2DM include insulin resistance of muscle, dysfunction of adipocytes, and dysfunction of pancreatic β cells, obesity, excess hepatic glucose output, and genetic predisposition.

1.3 Adrenal corticosteroids

The adrenal cortex synthesises and secretes steroids while the medulla produces catecholamines and neuropeptides (Delarue *et al.*, 2001). The zona glomerulosa is specialized in the production of aldosterone whereas the zona fasciculata and zona reticularis synthesise glucocorticoids (Stewart *et al.*, 1972). In humans and other primates the zona reticularis and to a lesser extent the zona fasciculata produce C19 androgens including large amounts of precursor's dehydroepiandrosterone (DHEA) and DHEA-sulphate (Rainey *et al.*, 2004). Glucocorticoids and C19 steroids secretion are mainly controlled by adrenocorticotropic hormone (ACTH), whereas aldosterone secretion is mainly regulated by angiotensin II (ANG II). However, in physiological conditions, all adrenal steroids may also be regulated by a complex interaction of several systemic or paracrine factors (Ehrhart-Bornstein *et al.*, 1998).

1.3.1 Corticosteroid receptors

Mineralocorticoids (aldosterone, deoxycorticosterone) and glucocorticoids (corticosterone, cortisol) bind to corticosteroid receptors which belong to the large superfamily of 48 nuclear receptors proteins that are involved in eukaryotic gene expression. The corticosteroid receptors are mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Detailed biochemical studies of these receptor proteins, which were partially purified, revealed that their domain structure (N terminal, central DNA binding domain and the C-terminal) is very similar within the family of receptor proteins.

The progesterone and androgen receptors share many structural and functional features with MR and GR.

1.3.2 Structural domains of corticosteroid receptors

There are three main domains that are described as part of these receptors: 1) N-terminal region, 2) the central DNA binding domain, and 3) C-terminal region.

N-terminal region

This region differs the most among the receptor types in size and amino acid sequence. It contains the ligand independent activation function 1 (AF1) region. It is considered that this region acts as a docking platform for the recruitment of chromatin remodelling proteins (Warnmark *et al.*, 2003).

DNA binding domain

The central DNA binding domain is the most conserved between the receptor types. This region confers the specificity for the recognition of the hormone response element in gene promoter regions. It is also considered that the hinge region between the DNA binding domain and the C-terminal region contains nuclear localization signal motifs (Tyagi *et al.*, 1998).

C-terminal region

This region serves as the ligand binding domain of the receptor and serves many essential functions. It contains the specific binding pocket for the steroid hormone. Crystal structure studies have shown that the ligand binding induces a conformational change in the α-helix of the C-terminal region. This leads to a protein surface that allows interactions with transcriptionally active proteins. This surface is called the ligand-dependent activation function 2 (AF2).

1.3.3 Variants of corticosteroid receptors

The genes coding for GR and MR have similar DNA sequences composed of 9 exons. For both GR and MR, several different transcripts that differ in the 5´-untranslated region give rise to the same identical receptor proteins (Patel *et al.*, 1989; Turner *et al.*, 2005).

The human GR receptor has 3 splice variants that result in two distinct mRNAs and proteins namely GRα and GRβ. In GRβ, the 50 C-terminal amino acids are lacking. This makes it transcriptionally inactive, which has led to suggestions that it acts as a dominant negative transcription factor (Yudt *et al.*, 2003). Both the MR and GR receptors have different translation variants which differ in their transcriptional capacities and are expressed in a tissue-dependent manner (Yudt *et al.*, 2002). From these studies it suggests that MR or GR receptors are a set of closely related and possibly functionally different proteins that may differ in hormone sensitivity and intrinsic activity.

1.3.4 Expression of the corticosteroid receptors

Of the two receptors the GR is present in almost all tissues. It is highly expressed in the immune system, bone, lungs, liver, adipose tissue and the brain. These expression levels reflect the main clinical use (anti-inflammatory) and side effects (hepatic glucose output) of synthetic glucocorticoids. MR expression was initially shown by immune-histochemistry in kidney (Krozowski *et al.*, 1989; Lombes *et al.*, 1990). MR expression was also detected in the colon (Lombes *et al.*, 1984), lung (Krozowski *et al.*, 1981), sweat glands (Kenouch *et al.*, 1994), liver (Duval *et al.*, 1974) and in the inner ear (Teixeira *et al.*, 2006). In the brain both the MR and GR are co-localised in the hippocampus.

1.3.5 Selectivity of the corticosteroid receptors

The MR is capable of binding mineralocorticoids (aldosterone, 11-deoxycorticosterone), glucocorticoids (corticosterone, cortisol) and progesterone with high affinity. Aldosterone is considered to be the main primary ligand for MR *in vivo* in most tissues. In some tissues cortisol may be the primary ligand for MR.

1.3.6 Mechanism of action of corticosteroid receptors

1.3.6.1 General mechanism of action of Glucocorticoids

In general corticosteroids enter the cells through passive diffusion across the cell membrane and bind to the steroid receptors in the cytoplasm. The corticosteroid receptors are located predominantly in the cytoplasm as multiprotein complexes including heat-shock proteins (HSP), immunophillins and other kinases (Gustafsson *et al.*, 1987; Pratt *et al.*, 2006). This combination of ligand and the receptor causes steric changes in the receptor, which leads to the dissociation of the HSPs and other factors. This dissociation of the GR results in its translocation from cytoplasm to the nucleus within minutes of glucocorticoids binding to its receptor (Buttgereit *et al.*, 2009). This dissociation uncovers nuclear localization motifs of the receptor. These motifs are recognized by the transport machinery of the cells and members of the importin family proteins direct the ligand activated receptor to gated channels of the nuclear membrane. Within the nucleus, the GR interacts with the DNA (with or without transcription factors) to regulate gene expression (Hager *et al.*, 2000).

Normally, ligand activated steroid receptors are considered to modulate the initiation rate of transcription (Sabbah *et al.*, 1998). However, it is also proposed that many rapid random transcriptionally unproductive complexes are formed in conjunction

with the association of the appropriate factors at specific DNA sites. These initial random interactions have been proposed to be essential in scanning the whole genome before the sequential and ordered recruitment of the co-regulator proteins at high affinity binding sites (Fletcher *et al.*, 2002).

Although glucocorticoids have pleiotrophic effects, the target genes are mostly cell specific (So *et al.*, 2007). The co-regulator protein recruitment is a major determinant to this effect. These proteins mediate the transduction of the signal from the DNA bound steroid receptors to the transcription machinery. Co-regulator proteins are enzymatically active and they reorganize the chromatin environment after recruitment by the ligand activated receptor. They exist in multi component complexes supplying the promoters with the variety of enzymatic activities and forming specific blocking platforms for the recruitment of other co-regulator proteins. Activation of the glucocorticoid receptor can result in either the stimulation or repression of the target gene (Figure 1.2). Stimulation typically involves the direct binding of GR to specific DNA sites called the glucocorticoid responsive elements (GREs) and subsequent recruitment of co-regulator proteins, most of the transactivation through DNA binding requires dimerisation of the GR.

Repression is brought about by direct interference of the activated GR with other transcriptional factors such as activated protein 1 (AP-1) and nuclear factor kappa B (NFκB). Transcription of the genes can also be inhibited by the direct interaction between activated GR and negative GREs such as alpha fetoprotein and prolactin genes (Sakai, 1985). It is also been suggested that glucocorticoids suppresses transcription of IL-1 and IL-2 via negative GREs. In general it takes hours or days before changes can occur on cellular tissue or organism level. However, some of the

immunosuppressive, anti-inflammatory and anti-allergic effects of glucocorticoids occur too fast to be explained by the classical genomic mechanisms of action (Buttgereit *et al.*, 2002; Falkenstein *et al.*, 2000).

1.3.6.2 Mechanism of action of non-genomic effects of Glucocorticoids:

At least three different mechanisms have been proposed to explain the rapid non-genomic effects of glucocorticoids (Figure 1.3).

Non specific interaction of glucocorticoids with cell membranes:

It was shown that at high concentration of glucocorticoids interchelate into the cell membranes. This changes the physiochemical properties of the membrane and also affects the activities of the membrane associated proteins (Buttgereit *et al.*, 2002). This results in reduced calcium and sodium cycling across cell membranes of immune cells. This contributes to the rapid immunosupression and a reduction in the inflammatory processes (Buttgerit and Scheffold, 2002). In addition to these effects glucocorticoids are suggested to increase mitochondrial proton leaks resulting in impaired ATP production. As ATP is important for housekeeping activities of the immune cells it affects the functions such as cytokine synthesis, phagocytosis etc.

Non-genomic effects caused by the cytosolic ligand activated glucocorticoid receptors:

The unligated cGR is located in the cytoplasm as a multi protein complex consisting of HSPs and several kinases including the MAP kinases (MAPKs). Due to the glucocorticoids ligand binding the cGR is dissociated from these kinases. Certain signalling molecules like the Src are released from the GR multi protein complex. These Srcs are responsible for certain rapid effects observed (Croxtall *et al.*, 2000).

Arachidonic acid (AA) is essential for metabolic and inflammatory reactions in producing prostaglandins. AA is released from the cell membrane associated phospholipids and is controlled by different mediators such as MAPK and lipocortin-1. This release of AA is inhibited by glucocorticoids in a cGR dependent mechanism (Croxtall *et al.*, 2000). These studies show that cGR is not only important in the genomic effects but also involved in the non-genomic effects.

Non-genomic effects caused by the membrane bound glucocorticoid receptors:

The existence of the membrane bound glucocorticoid receptor (mGR) was shown initially in the amphibian neuronal membranes and lymphatic cells (Gametchu *et al.*, 1999). These mGRs were later identified in human peripheral blood mononuclear cells using immunofluorescent staining (Buttgereit *et al.*, 2004). Using this sensitive method it was shown that over expression of cGR did not increase the mGR expression on the cell surface. Hence, it is considered that the mGR is not just a cGR that has been transported to the cell surface. It is suggested that, it could be a splice variant of the GR. In patients with rheumatoid arthritis an increased number of mGR positive monocytes and lymphocytes correlate with higher disease activity scores (Buttgerait et al., 2005). Many other correlations in inflammatory diseases suggest that mGR may play a role in pathogenesis of chronic inflammatory disease.

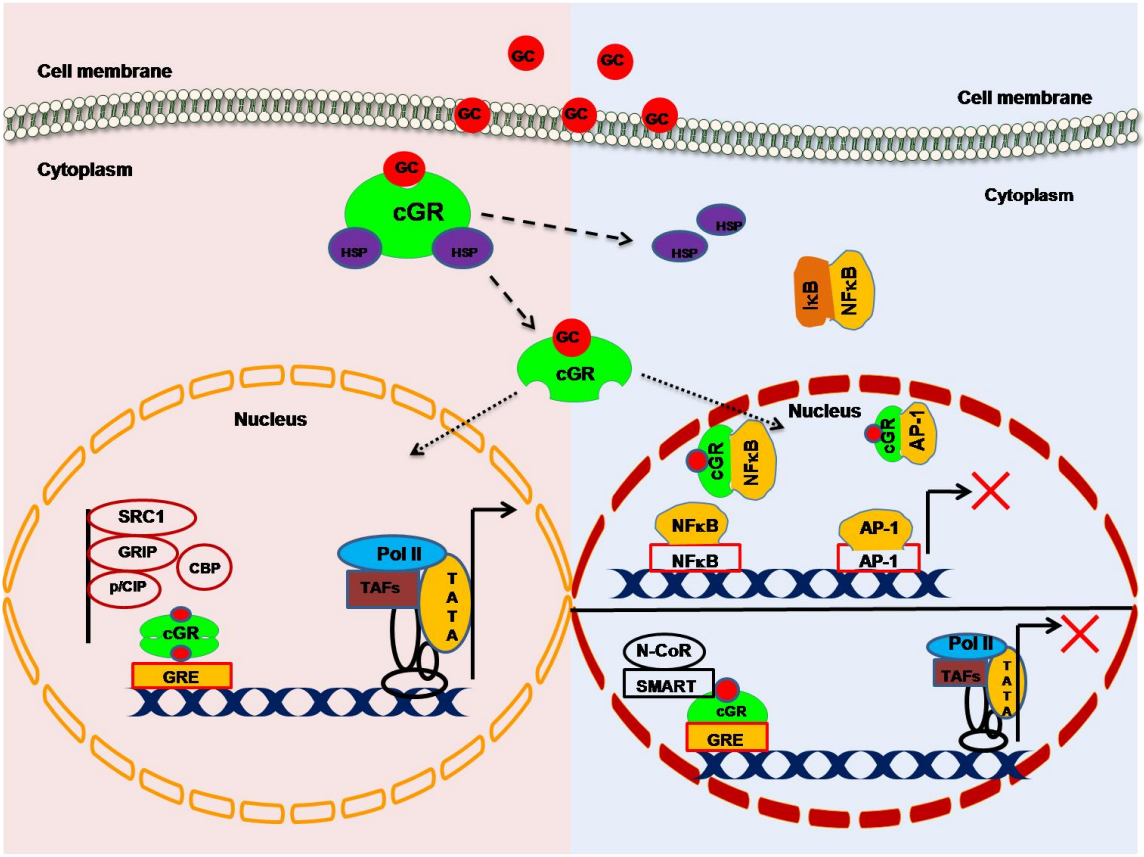


Figure 1.2 General genomic mechanism of action of Glucocorticoids

The glucocorticoids (GC) diffuse into the cell and bing to cytoplasmic glucocorticoid receptor (cGR). This induces conformational changes causing disassociation of the chaperone proteins. Subsequently the receptor translocates into the nucleus and either induces or represses gene expression.

1.3.7 Mineralocorticoid Receptor:

Mineralocorticoid Receptor (MR) like the GR is located in cytoplasm and was cloned and characterized in many species. It is now regarded as a crucial transcription factor involved in many important physiological processes and pathological disorders (O'Malley, 2007). The ligand binding domain (C-terminal region) of MR contains a ligand dependent activation factor 2 (AF-2) consisting of helices H3, H4, H5 and H12. Once the ligand is bound to the MR a rearrangement of the ligand binding

domain (LBD) occurs forming a hydrophobic cleft on the surface. These changes are essential for the activation of MR transcriptional activity. Like the GR, the MR interacts with a variety of proteins such as HSPs, Immunophillins, etc., in its non-liganded state. Once the mineralocorticoid binds to the MR these proteins are dissociated and the MR undergoes nuclear translocation. Inside the nucleus this acts as transcription factor by binding to specific Hormone Responsive Elements (HRE) in target genes. These HREs are located up to 10Kb upstream or downstream of the transcription start site (So *et al.*, 2007). When the MR binds to the HREs it recruits chromatin remodelling complexes to release the nucleosome structure and also brings in the transcriptional machinery to activate transcription. In mineralocorticoid sensitive epithelial tissue (kidney and colon) MR regulates salt balance and water homeostasis by stimulating the expression of ionic transporters (ENaC, $Na^{+,}$ K^{+}-ATPase pump).

1.3.6.1 Non genomic effects of Mineralocorticoids:

Non genomic effects of mineralocorticoids, especially aldosterone have been described on various target organs like smooth muscle, endothelial cells (Wehling, 1994), skeletal muscles, lymphocytes, cardiac myocytes (Sato *et al.*, 1997), colonic epithelial cells (Doolan *et al.*, 1996) and kidney cells (Gekle *et al.*, 1997). A specific membrane MR (mMR) has been proposed based on several studies. High affinity binding sites for aldosterone have also been described and the most commonly reported second messenger response is an increase in intracellular calcium concentration (Gekle *et al.*, 1996). It was also demonstrated that epidermal growth factor receptor (EGFR) is used by aldosterone to stimulate MAPK signalling cascade (Gekle *et al.*, 2002). This was also shown in M1 kidney cell line. These studies suggest that a rise in intracellular calcium and MAPK phosphorylation may be

general mediators of the rapid aldosterone effects. It is also suggested that there could be an interaction integrating both genomic and non-genomic aspects of mineralocorticoids (Losel *et al.*, 2003). Firstly, steroid hormones elicit rapid activation of protein kinases such as PKAs and MAPKs through non-genomic mechanisms and these kinases in turn phosphorylated transcription factors and co-factors activating early genomic responses.

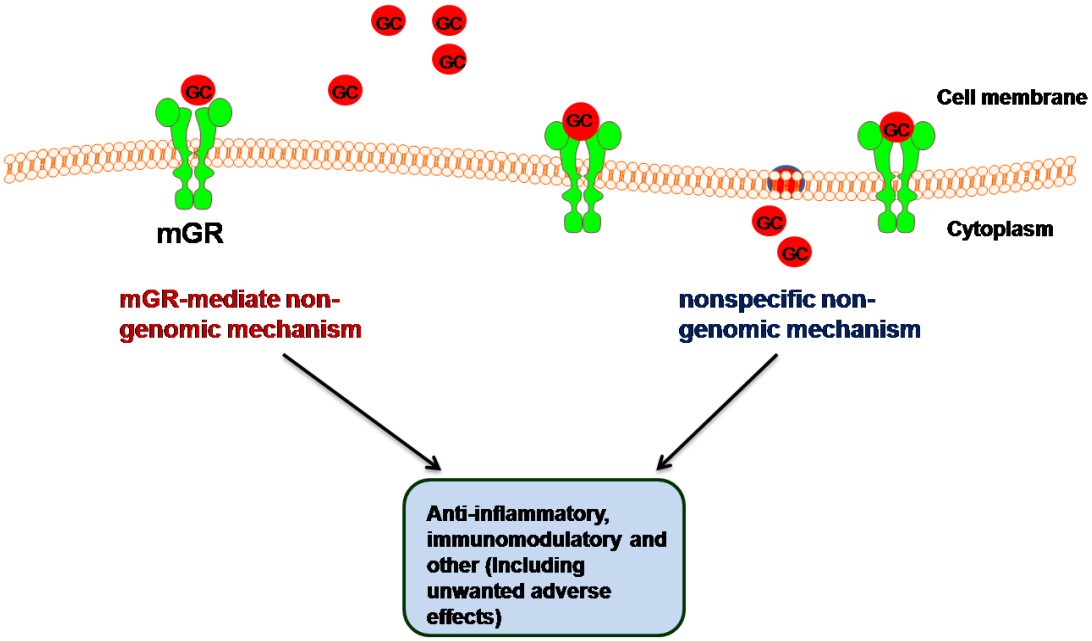


Figure 1.3 Non-genomic mechanism of action of corticosteroids

1.4 The diverse role of glucocorticoids

Glucocorticoids (cortisol/corticosterone) exert widespread actions in the body which are essential for the maintenance of homeostasis and enable the organism to prepare, respond and cope with physical and emotional stress (Sapolsky *et al.*, 2000). They promote the breakdown of carbohydrate and protein, and exert complex effects on lipid deposition and breakdown. They are also important regulators of immune and

inflammatory processes and are required for numerous processes associated with host defence system. These properties underlie many of the stress-protective actions of the steroids as they quench the pathophysiological responses to tissue injury and inflammation and, thereby, prevent them proceeding to a point where they threaten the survival of the host (Munck *et al.*, 1992). This is also why they are used as anti-inflammatory and immunosuppressive drugs. Glucocorticoids also have other actions. They raise blood pressure and have aldosterone-like effects in some cases. These actions were suggested to be due to their ability to alter the sensitivity of tissues to catecholamines. Glucocorticoids also have an effect on bone growth. In the central nervous system (CNS), glucocorticoids affect the growth and development of neurons and glial cells. During development they help in the organization of the brain and during adulthood they contribute to neuronal plasticity. They are also associated with complex changes in mood and behaviour. They affect food intake, body temperature, pain perception and neuroendocrine function (Brown, 2009).

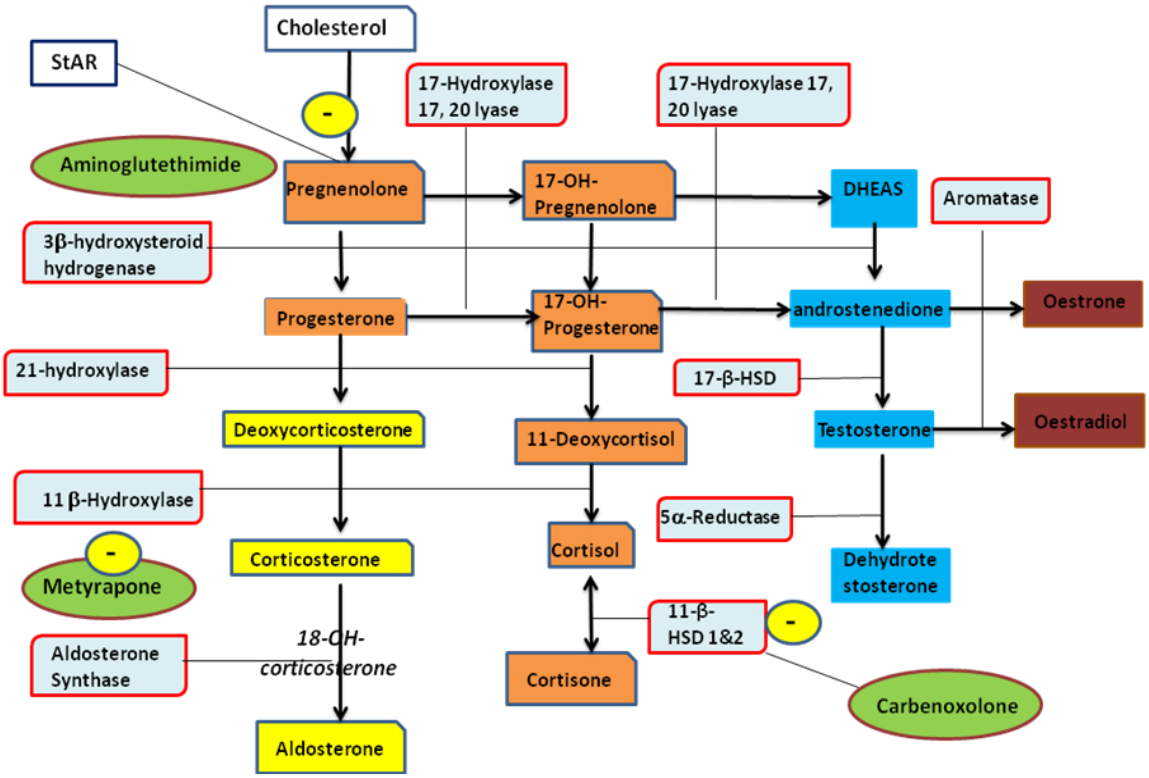


Figure 1.4 Principal pathways for the biosynthesis of adrenocorticosteroids.

Cholesterol, the precursor to all steroid biosynthetic pathways, is converted to a variety of steroid molecules in a series of reactions catalyzed by several cytochrome P450 (cyp 450) enzymes.

1.4.1 Glucocorticoids and the hypothalamo-pituitary axis.

All glucocorticoids are synthesised from cholesterol in the adrenal cortex and are released into the systemic circulation in a distinct circadian pattern. Serum glucocorticoid concentrations vary 3–5 fold over a 24 h period. In humans, plasma cortisol/corticosteroid concentrations are maximal around dawn and decline thereafter to reach a low phase early in the sleep phase. Glucocorticoids are also released in response to physical and emotional trauma. This ‘stress response' is superimposed over the existing circadian tone and varies in magnitude according to the nature, intensity and duration of the stimulus and the individual's previous experience (Buckingham *et al.*, 1996).

The circadian and stress-induced secretion of glucocorticoids is governed by the hypothalamo-pituitary axis (HPA). The hypothalamus receives monitors and integrates information from many sources, and acts as a sensor of changes in the external and internal environment. Using this information, the hypothalamus responds to adverse physical or emotional changes by activating the pathway for glucocorticoid synthesis. The first step is the release of two hypothalamic neurohormones, corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), from parvocellular neurones that project from the paraventricular nucleus to the median eminence. CRF and AVP travel from the hypothalamus via the hypophyseal–portal blood vessels to the anterior pituitary gland where they act synergistically via specific receptors [type 1 corticotropin-releasing factor receptor (CRF-R1) and type 1b vasopressin receptor, respectively] to trigger the release of adrenocorticotrophic hormone (corticotrophin, ACTH) from the corticotrophs into the systemic circulation. ACTH in turn acts on the adrenal cortex via type 2 melanocortin receptors to initiate the synthesis of cortisol/corticosterone, which is released immediately into the systemic circulation by diffusion. The sensitivity of the hypothalamo-pituitary-adrenocortical (HPA) axis to incoming stimuli is modulated by a negative feedback (servo) system through which the sequential release of CRF/AVP and ACTH from the hypothalamus and anterior pituitary gland are suppressed by the glucocorticoids themselves. The magnitude of the HPA response to stress thus depends upon the pre-existing glucocorticoid tone (Buckingham *et al.*, 1996).

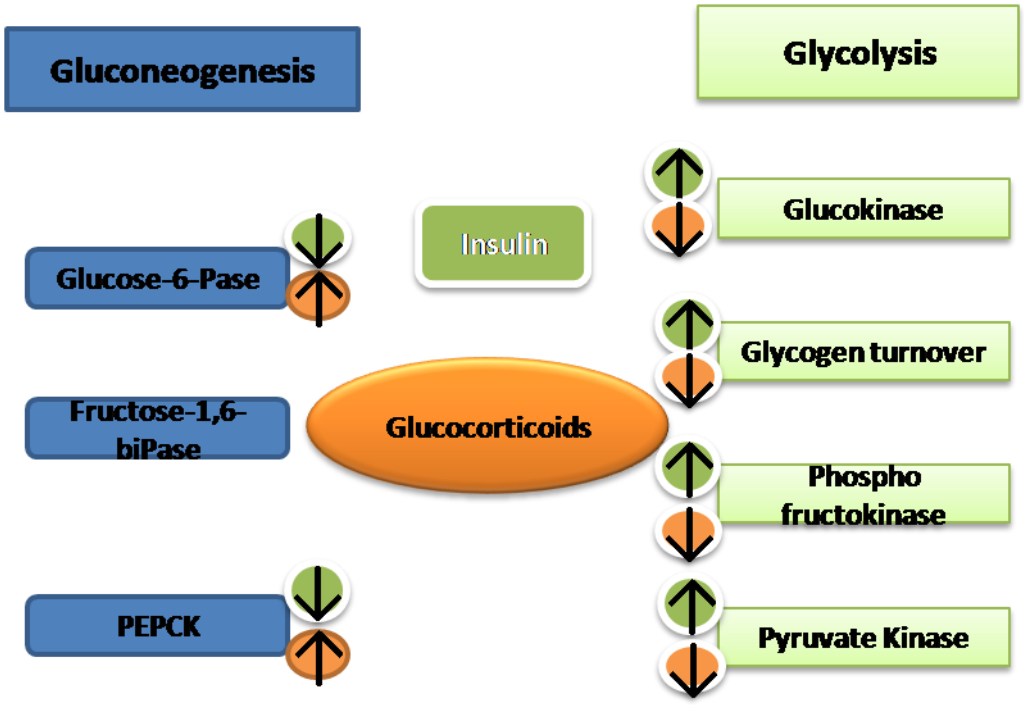


Figure 1.5 Inverse relationships between the effects of insulin (green arrows) and glucocorticoids (orange arrows) on key enzymes regulating hepatic glucose metabolism.

1.4.2 Glucocorticoids and regulation of hepatic glucose metabolism

The liver is a key organ in the control of mammalian glucose homeostasis. Glucocorticoids control many aspects of the hepatic energy metabolism. During fasting, free fatty acids or amino acid precursors are channelled into the gluconeogenic pathway that comprises various biochemical steps converting pyruvate to glucose. The main regulatory enzymes are pyruvate carboxylase (PC), which converts pyruvate into oxaloacetate, phosphoenolpyruvate carboxykinase (PEPCK) promoting decarboxylation of oxaloacetate to phosphoenolpyruvate, and finally glucose-6-phosphatase (G6Pase) hydrolysing glucose-6-phosphate into free glucose (Figure 1.4). PC catalyses the ATP dependent carboxylation of pyruvate to oxaloacetate, which may be utilised in the synthesis of glucose, fat, some amino acids

or their derivatives and several neurotransmitters. T2DM and hyperthyroidism increase the level of expression of pyruvate carboxylase in the long term, while its activity in the short term is controlled by the intra-mitochondrial concentrations of acetyl-CoA and pyruvate (Wallace *et al.*, 1998).

PEPCK expression is controlled by a variety of physiological stimuli including dietary carbohydrates, hormones and cellular intermediates (Hanson *et al.*, 1994). Over expression of PEPCK in the liver during diabetes is a major factor in the elevated hepatic glucose production that is characteristic of T2DM. The first convincing evidence for the role of PEPCK in T2DM came from studies performed on transgenic mice carrying a rat PEPCK mini-gene. This showed that over expression of PEPCK led to fasting hyperglycaemia, decreased glycogen storage, and altered glucose tolerance test: all features of T2DM (Valera *et al.*, 1994). Additional research showed hypoglycaemia in fasted animals treated with 3-mercaptopicolinic acid, which is a PEPCK inhibitor (DiTullio *et al.*, 1974). PEPCK is adaptively regulated at the transcriptional level in a manner that correlates with gluconeogenic flux (Cimbala *et al.*, 1982; Granner *et al.*, 1983), with most evidence indicating the importance of PEPCK in the pathophysiology of T2DM. PC and pyruvate kinase (PK) were suggested to play a greater role in gluconeogenesis than PEPCK (Groen *et al.*, 1986).

The pathways involved in hepatic energy metabolism are regulated by multiple mechanisms. Since PEPCK catalyses a reaction at the intersection of several fundamental pathways, the absence or over expression of this might have unpredictable effects on the accumulation of specific metabolites in disease states. PEPCK activity is reduced in the fed state whilst its activity is increased in the fasted animals (Granner *et al.*, 1990). PEPCK mRNA is not significantly expressed in livers

of fed rats, but its expression increases dramatically in fasted rats (Yoon *et al.*, 2001). Peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1 (PGC-1α) plays a key role in regulating lipid and carbohydrate metabolism. PGC1-α knockout mice showed diminished hepatic gluconeogenesis and lowered glucose production, highlighting the importance of this co-activator (Burgess *et al.*, 2006; Chevalier *et al.*, 2006).

Uncontrolled gluconeogenesis contributes significantly to hyperglycaemia in T2DM patients (Consoli, 1992). This is largely due to the induction of gluconeogenic gene expression by glucocorticoids (Jones *et al.*, 1993). Mice carrying a liver specific mutation of the glucocorticoid receptor (GR) gene displayed fasting hypoglycaemia due to reduced expression of gluconeogenic genes (Opherk *et al.*, 2004). These studies suggest a role of glucocorticoids in the induction of gluconeogenesis under insulin resistant diabetic conditions. Indeed liver GR antagonism with A-348441 (a liver specific analogue of RU486) and antisense oligonucleotides is sufficient to cause substantial improvement of hyperglycaemia and dyslipidaemia in animal models of T2DM (Jacobson *et al.*, 2005; Liang *et al.*, 2005). Also GR has been found to be over expressed in hepatocytes of diabetic rodent models (Liu *et al.*, 2005).

The PEPCK gene expression is increased not only in diabetes but also in hyperthyroidism and various nutritional states (Hanson *et al.*, 1997). The transcription of PEPCK is altered by hormones. PEPCK catalyses the conversion of oxaloacetate (OAA) to phosphoenol pyruvate (PEP). Its activity is only controlled at the transcriptional level as there are no known allosteric modifiers (Hanson *et al.*, 1994). During fasting, hormones like glucagon and glucocorticoids enhance the transcription of PEPCK. Insulin dominantly represses hormone induced expression. The transcription of PEPCK is also enhanced by retinoic acid and thyroid hormone.

1.5 PEPCK promoter response units

There are five major hormone response units in the PEPCK promoter region.

1) Glucocorticoid response units (GRU)
2) Retinoic acid response units (RARU)
3) Cyclic AMP response units (CRU)
4) Thyroid hormone response units (TRU)
5) Insulin response units (IRU)

The following figure shows the arrangement of the various response units in the promoter region (Figure 1.6).

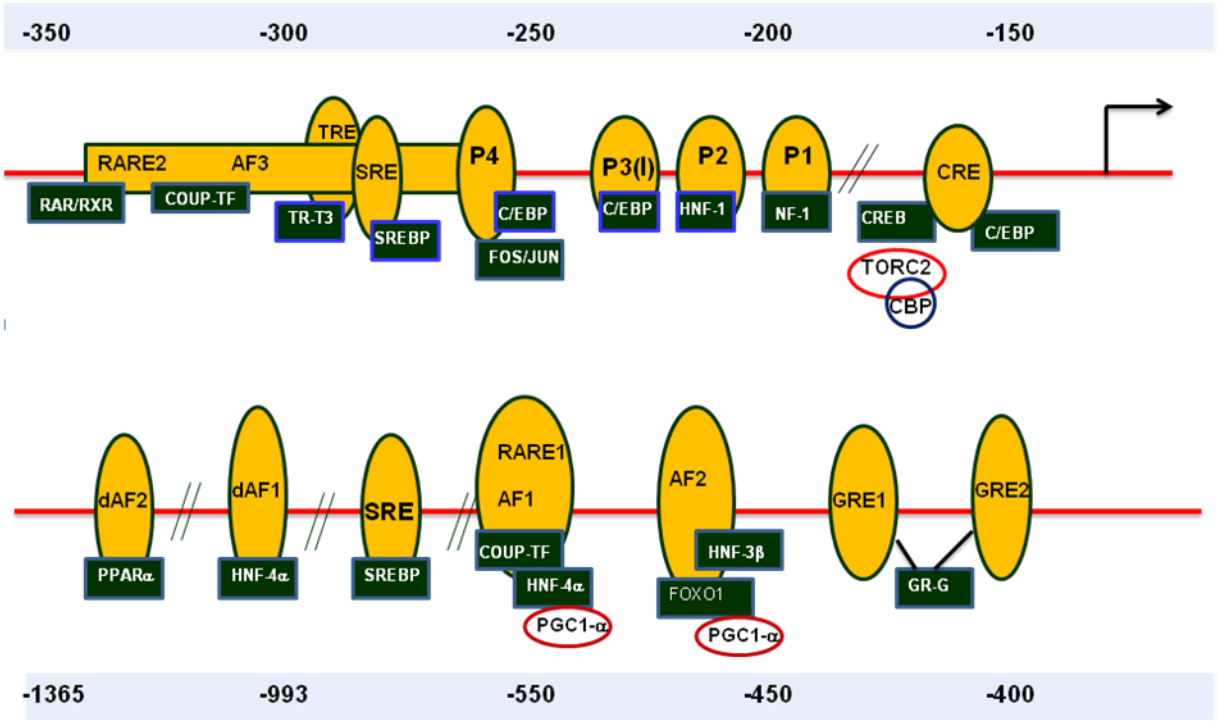


Figure 1.6 PEPCK gene promoter: response elements and the binding proteins. *dAF2, distant accessory factor element 2; dAF1, distant accessory factor element 1; SRE, sterol response element; RARE1, retinoic acid response element 1; AF1, accessory factor element 1; AF2, accessory factor element 2; GRE1, glucocorticoid response element 1; GRE2, glucocorticoid response element 2; RARE2, retinoic acid response element 2; TRE, thyroid response element; AF3, accessory factor element 3; CRE, cyclic AMP response element; PPARα, peroxisome proliferator activated receptor α; SREBP, sterol response element-binding protein; HNF-4α, hepatic nuclear factor-4α; HNF-3β, hepatic nuclear factor 3β; COUP-TF, chicken ovalbumin upstream transcription factor; GR–G, glucocorticoid receptor–glucocorticoid; RAR–RXR, retinoic acid receptor–retinoid receptor; TR–T3, thyroid receptor–thyroid hormone; C/EBP, CAAT enhancer-binding protein; HNF-1, hepatic nuclear factor 1; NF-1, nuclear factor 1; CREB, cyclic AMP response element binding protein; CEBPβ, CAAT enhancer-binding protein β; PGC-1α, peroxisome proliferator-activated receptor – γ coactivator-1α; TORC2, transducer of regulated CREB activity 2; CBP, CREB-binding protein*

1.5.1 Glucocorticoid response units (GRU)

The GRU of the PEPCK consists of two non-consensus glucocorticoid response elements (GRE) GRE1 and GRE2; three accessory factor (AF) elements AF1, AF2 and AF3 and cyclic AMP response elements (CRE). A series 5' deletion mutants of the promoter before the transcription start site defined the regulatory elements responsible for the response to glucocorticoids (Imai *et al.*, 1990). There had initially been a discrepancy between the boundary of functional GRU domain and the actual GREs. These studies led to the discovery of accessory factor elements. The cGR poorly binds to the GRE1 and GRE2 compared to a consensus GRE. It was shown that AF1 and AF3 create a high binding environment for cGR to bind to GRE1 and GRE2 (Stafford *et al.*, 2001). When AF1 and AF2 positions in the promoter regions are swapped the response to glucocorticoid is reduced (Wang *et al.*, 1999) this indicates that the exact positions of the accessory factor elements are necessary for full response. Also, any combination of two mutations of the AF1, AF2 and AF3 completely abolishes the glucocorticoid response (Scott *et al.*, 1996). So, it can be said that the PEPCK GR1 and GR2 are inactive in the absence of at least two accessory elements.

Hepatic nuclear factor 4 (HNF4) or chicken ovalbumin upstream promoter transcription factor (COUP-TF) was shown to serve as accessory factors for the induction of PEPCK by glucocorticoids via the AF1site (Hall *et al.*, 1995). Members of the hepatic nuclear factor 3 (HNF3) and CCAT enhancer binding protein (CEBP) bind to the AF2 element (O'Brien *et al.*, 1994). It appears that CEBP functions as an accessory factor for the GREs through the CRE (Yamada *et al.*, 1999). Distal accessory factors dAF1 and dAF2 were shown to be also important in the

glucocorticoid regulation of PEPCK (Cassuto *et al.*, 2005). For the complete response of the PEPCK gene to glucocorticoid response requires AF1, AF2, AF3, CRE and the two GREs (GR1 and GR2) along with the steroid receptor complexes. Of the two GREs GR1 is quantitatively more important than GR2 (Scott *et al.*, 1998).

It is interesting to note that the PEPCK gene transcription is positively regulated by glucocorticoid in the liver and negatively regulated in adipose tissue (Hanson *et al.*, 1994). Liver enriched transcription factors HNF3 and HNF4 serve as AF1 and AF2 respectively. These factors are not present in adipose tissue; this possibly explains differential regulation in different tissues. So, the whole of GRU allows for a graded response and it is also suggested that GRU is part of a much more complex assembly called metabolic controlled domain.

1.5.2 Retinoic Acid response unit (RARU):

In addition to their role in GRU, AF1 and AF3 elements also function as retinoic acid response element RARE1 and RARE2 by binding to a heterodimeric complex consisting of retinoic acid receptor (RAR) and 9-cis-retinoic acid receptor (RXR). So the RAR binding to the RARE1 enhances the PEPCK gene expression (Hall et al., 1992). RARE2 supports the function of the RAR and RXR heterodimer. The DNA binding elements of GRU and RARU are overlapping and suggest the existence of functional co-operativity. The combination of dexamethasone and retinoic acid has a synergistic effect on PEPCK expression in both primary hepatocytes and H4IIE cells (Wang *et al.*, 2004a).

1.5.4 Cyclic AMP response units (CRU)

The CRU is composed of one CRE, three CAAT enhancer binding proteins (CEBP) and one AP-1 site. The CRE is present in number of other promoter region genes whose transcription is induced by cAMP (Short *et al.*, 1986). The transcription factor binding to this CRE was cloned and identified as CRE binding protein (CREB) (Montminy *et al.*, 1987). The CRE with CREB and a co-activator CREB binding protein (CBP) is not enough to explain the liver specific response of PEPCK gene to cAMP. A distal region in the promoter spanning from -320 to -230 showed a distinctive liver specific binding pattern. Liver enriched transcription factor called the CEBP bound specifically to three sites in this distal region along with binding to the CRE (Park *et al.*, 1990). An AP-1 binding site was also identified later in the same region. A mutation in any of these cis-elements (CRE, CEBP and AP-1) significantly reduces the degree of cAMP response (Liu *et al.*, 1991). Unlike the glucocorticoid response unit rearrangement of these elements did not have any effect (Roesler *et al.*, 1994). It is interesting to note that CEBPβ when bound to CRE acts as an accessory factor in the GRU of the PEPCK promoter (Yamada *et al.*, 1999).

1.5.5 Thyroid hormone response units (TRU):

In the presence of thyroid hormone, the thyroid hormone receptor (TR) binds to its response element as a hetero dimer with RXR. P3(I) is another site which binds to CEBPα. It was shown that CEBPβ along with TR mediates thyroid hormone induction of PEPCK transcription. The P3(I) site is involved in the induction of PEPCK by both T3 and cAMP. Interestingly, CBP which is involved in cAMP induction also enhances PEPCK transcription by thyroid hormone. It is shown to

interact with steroid receptor coactivator (SRC-1) which is recruited by the ligand activated TR.

1.5.6 Insulin response units (IRU):

Many gene regulatory proteins mediating the actions of insulin have been identified but the actual insulin response units (IRU) have not yet been clearly established. The AF-2 element in the GRU mediates the inhibition of insulin using a heterologous thymidine kinase promoter (O'Brian et al., 1990). Insulin triggers the dissociation of the transcription initiation complex formed on the promoter in response to glucocorticoids (Hall *et al.*, 2007). CEBPα dependent transcription is enhanced by FOXO1. This transcription is inhibited by insulin although the exact mechanism is not known (Sekine *et al.*, 2007). However, mutation of IRE (AF-2) did not cause any alterations in insulin response in H4IIE cells (Yeagley *et al.*, 2001). Insulin seems to affect PEPCK transcription indirectly by affecting transcription factors and co-activators. Insulin increases the levels of liver enriched inhibitory protein (LIP) which natural inhibitor of CEBPβ. Insulin signalling phosphorylates CBP which affects the interaction of CBP with CREB.

1.6 Hypercortisolism and adrenal dysregulation in metabolic syndrome and T2DM

Although it appears that hypercortisolism is the common link between all of these disease states, closer examination reveals a more complex picture and shows our poor understanding of the mechanisms that are involved. Only mild hypercortisolism is observed in MS and T2DM patients compared to control subjects (Pasquali *et al.*, 2006; Walker, 2006). It has been suggested by a number of authors that inhibiting the

synthesis or actions of cortisol could provide a novel therapeutic approach for MS (Walker, 2006). A number of studies have reported circulating cortisol concentrations to be higher in patients with MS compared with healthy subjects. This difference is more evident in patients with MS and hypertension or IGT (Reynolds *et al.*, 2001). Furthermore, weight loss normalizes cortisol levels and improves insulin resistance (Reinehr *et al.*, 2004). Despite the fact that cortisol levels are within the normal range, there is evidence of increased activity of cortisol in the periphery and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Sen *et al.*, 2008; Walker, 2006).

Cortisol clearance seems to be inversely correlated with insulin sensitivity, and this correlation is independent of body fat (Holt *et al.*, 2007). It is also well documented that glucocorticoids promote the differentiation and proliferation of human adipocytes and that their receptors are more abundant in visceral adipose tissue (Rebuffe-Scrive *et al.*, 1992). They also redistribute adiposity from peripheral to central depots, increase the size and number of fat cells, and activate lipolysis and the release of free fatty acids into the circulation, which further increases insulin resistance.

Increased cortisol levels are also associated with insulin resistance (Phillips *et al.*, 1998; Reinehr *et al.*, 2004; Ward *et al.*, 2003). Higher cortisol concentrations were related to a reduced insulin secretion, a finding consistent with *in vivo* and *in vitro* data showing that glucocorticoids regulate insulin secretion (Delaunay *et al.*, 1997). However, higher insulin levels may also be compensatory to counter insulin resistance and raised blood glucose concentrations. Furthermore, a study conducted in obese children with or without insulin resistance showed that body weight reduction decreased both cortisol levels and insulin resistance in the insulin-resistant group. This was not shown in children without insulin resistance (Reinehr *et al.*, 2004).

1.7 Dysregulation of adrenal glucocorticoids and disease.

In certain conditions, when there are sustained and pronounced elevations in circulating glucocorticoids, the normal glucocorticoid effects become exaggerated and result in a lot of unwanted and undesirable physiological conditions. This increase in adrenal glucocorticoids is either due to hyper-secretion of endogenous steroids (Cushing's syndrome) or prolonged administration of exogenous steroids (treatment conditions). Increased circulating glucocorticoids result in a significant redistribution of fat giving rise to centripetal obesity, protein wasting, muscle weakness, hyperglycaemia and insulin-resistant diabetes mellitus (‘steroid diabetes'), hypertension, raised cholesterol, altered serum lipids, salt and water retention. Other adverse effects of glucocorticoids include immunodeficiency, poor wound healing, impaired growth and development, osteoporosis, menstrual irregularities, infertility, depression and, sometimes, impaired cognitive function. Conversely, insufficient glucocorticoid secretion, which may arise from Addison's disease (an autoimmune disorder causing degeneration of the adrenal cortex), the adrenogenital syndrome (an inherited disorder of glucocorticoid synthesis) or pituitary disease, is characterised by a vulnerability to stress, white blood cell excess, lymphoid tissue hypertrophy, hypotension, mood disturbances, weakness/lethargy, weight loss and hypoglycaemia.

Slight changes in glucocorticoid levels and prolonged changes contribute to the pathogenesis of a number of common diseases, including hypertension, cardiovascular disease, insulin resistance, obesity and T2DM, depression, autoimmune inflammatory disease and reproductive dysfunction (De Kloet *et al.*, 1998; Gold *et al.*, 2002; Seckl, 2004).

1.8 11β-hydroxysteroid dehydrogenase

Probably the most important factor regulating the access of endogenous glucocorticoids to their receptors (GR or MR) is local metabolism of the steroids within the target cells by 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes. 11β-HSDs catalyse the interconversion of cortisol and its inactive metabolite cortisone in humans (or corticosterone and 11-dehydrocorticosterone in rodents) (Seckl *et al.*, 2001). Its existence was first recognised in 1953 (Amelung *et al.*, 1953). In the late 1980's studies on a case of 11β-HSD deficiency revealed that in the normal state intra renal 11β-HSD inactivates cortisol and, thus, allows preferential binding of aldosterone to mineralocorticoid receptor (MR) (Stewart *et al.*, 1988). On the other hand, other tissues in which MRs are abundant (e.g. brain, heart) show little if any 11β-HSD activity, suggesting that cortisol (or corticosterone) is the primary MR ligand at these sites (Edwards *et al.*, 1996). These studies led to more research on the effects of interconversion of active and inactive glucocorticoids.

Initially 11β-HSD was purified from rat liver and complementary cDNA was prepared that hybridised with a product expressed in the kidney (Agarwal *et al.*, 1989; Stewart *et al.*, 1991). A second enzyme 11β-HSD2 was purified and cloned from human placenta (Albiston *et al.*, 1994; Brown *et al.*, 1993). This enzyme is highly expressed in aldosterone sensitive tissues like kidney, colon and sweat glands. It converts active cortisol into cortisone, thus preventing nonspecific binding to mineralocorticoid receptors (Whorwood *et al.*, 1992)

11β-HSD1 differs from 11β-HSD2 in many regards. It is expressed mainly in the liver, lung adipose tissue (particularly omental) and brain, but is also found in other tissues (Stewart *et al.*, 1999). It is subject to regulation by a variety of factors

including glucocorticoids, stress, sex steroids, cytokines and perioxisome proliferator-activator receptor ligands (Hermanowski-Vosatka *et al.*, 2000; Jamieson *et al.*, 1999). 11β-HSD1 is a low-affinity NADP(H)-dependent enzyme, which *in vitro* shows bidirectional activity (i.e. both dehydrogenase and reductase). However, *in vivo* it appears to function solely as a reductase, relying on hexose-6-phosphate dehydrogenase (with which it is co-localised in the endoplasmic reticulum) to generate NADP(H) (Draper *et al.*, 2003). 11β-HSD1 thus serves to regenerate biologically active cortisol/corticosterone from inert cortisone/11-dehydrocorticosterone..

As 11β-HSD1 is found mainly in tissues in which the high-affinity MR is sparse but the low-affinity GR is abundant, it has been argued that principal role of 11β-HSD1 is to amplify the local concentration of active glucocorticoids in those tissues in which the steroids have a key regulatory role, for example, the liver (Seckl *et al.*, 2001). The support for this hypothesis emerged from a number of studies, including phenotypic analysis of 11β-HSD1-null mice (Kotelevtsev *et al.*, 1997). These mice have raised corticosterone levels (due to impaired glucocorticoid feedback) but are resistant to the hyperglycaemia normally induced by stress or overfeeding. In addition, they show raised high-density lipoprotein cholesterol, with reduced low-density lipoprotein cholesterol and blood triglycerides (Harris *et al.*, 2001). The metabolic responses appear to be driven by key changes, which, by preventing amplification of the local corticosterone concentration, reduce gluconeogenesis and β-oxidation of lipids in the liver and possibly also attenuate glucocorticoid-dependent functions in visceral adipose tissue.

Interestingly, when placed on a high fat diet, 11β-HSD1-null mice gain less weight than their wild-type counterparts and tend to deposit fat in subcutaneous rather than the visceral sites associated with metabolic disease (Kershaw *et al.*, 2005). Furthermore, fat-specific 11β-HSD1 transgenic mice, which have enzyme activity increased to a similar extent to that seen in obese humans, develop visceral obesity with insulin and leptin resistance, dyslipidaemia and hypertension (Masuzaki *et al.*, 2001). In adipocytes, both antidiabetic PPARγ agonists and LXRα agonists significantly reduce 11β-HSD1 mRNA and enzyme activity, suggesting that suppression of 11β-HSD1 in adipose tissue may be one of the mechanisms by which these drugs exert beneficial metabolic effects (Masuzaki *et al.*, 2003).

1.9 Polycystic ovary syndrome

Polycystic Ovary Syndrome (PCOS) is defined by: (i) clinical and/or biochemical hyperandrogenism, (ii) chronic anovulation, and (iii) exclusion of related disorders. PCOS displays a strong association with metabolic syndrome (T2DM, obesity, and cardiovascular diseases) (Baillargeon, 2005). It is already known that hyperandrogenism is the main clinical feature of PCOS (Azziz *et al.*, 2006). This feature correlates positively with hyperinsulinaemia and it is agreed that adrenal androgens contribute to PCOS (Dunaif, 1997).

In PCOS, it is well known that altered cortisol clearance is associated with enhanced steroid 5α-reductase activity and that insulin is likely to increase the 5α-reduction of steroids (Stewart *et al.*, 1990; Tsilchorozidou *et al.*, 2003). Earlier these 5α metabolites were presumed to be inactive, but recent evidence suggests that these 5α metabolites and not the 5β metabolites bind and activate the glucocorticoid receptor

(GC) (McInnes *et al.*, 2004). GR is preferentially expressed in the zona reticularis of the adrenal cortex and this suggests a possible role of glucocorticoid metabolites in regulating adrenal steroid biosynthesis (Paust *et al.*, 2006). Metformin is an anti-diabetic drug that increases glucose utilization in insulin-sensitive tissues. As PCOS and T2DM share some altered parameters-such as abnormal glucose: insulin ratio, altered lipid metabolism and insulin-resistance syndrome, the use of metformin has become increasingly accepted and widespread in the treatment of PCOS. These observations suggest that local steroid metabolism might be dysregulated or defective, giving rise to novel steroids that have an effect on blood glucose regulation.

1.10 Hyperglycaemia induced by corticotropin releasing factor family of peptides

Intravenous administration of corticotropin releasing factor (CRF) or sauvagine cause a pronounced and long-lasting hyperglycaemia (Brown *et al*., 1982). Both sauvagine and CRF are equipotent stimulators of ACTH release through CRF-R1 receptor (Brown *et al*., 1982). So this hyperglycaemia could be a glucocorticoid response due to pituitary activation. However, administered in equal amounts sauvagine is more potent in causing hyperglycaemia than CRF. Therefore, it is most likely that this effect is due to a CRF-R2 receptor dependent mechanism, which implies that the anterior pituitary secretion of ACTH cannot account for this response.

1.11 Corticotropin releasing factor family of peptides

1.11.1 CRF

CRF is a 41-amino acid peptide abundantly expressed in neurons of the paraventricular hypothalamic nucleus that project to the median eminence to stimulate the secretion of adrenocorticotropic hormone (ACTH). CRF is the major controller of the basal and stress-induced activation of the HPA axis. It was observed that the only remarkable phenotype of the CRF knockout mouse is the pituitary–adrenal hyporeactivity (Muglia *et al.*, 2000).

The broad distribution of CRF neurons conforms to the many expected functions of the peptide. When injected centrally, CRF evokes autonomic responses and cause a widespread arousal (Koob *et al.*, 1990) and anxiety-like behaviours (Heinrichs *et al.*, 1999). It activates and inhibits respectively, the sympathetic and parasympathetic branches of the autonomic nervous system. It stimulates cardiorespiratory functions (Fisher *et al.*, 1982), as well as brown adipose tissue thermogenesis (LeFeuvre *et al.*, 1987), and inhibits digestive activity (Tache *et al.*, 1990). CRF also blunts the activity of the reproductive system (Rivest *et al.*, 1995) and induces anorexia (Heinrichs *et al.*, 1999). The view that CRF orchestrates the multiple facets of the whole animal response to stress has dominated the research area on CRF since the discovery of the peptide. However, apart from the brain, CRF like immunoreactivity was thought to be widely expressed in peripheral tissues, but now it seems more likely that this was due to cross reactivity with other structurally homologous peptides.

1.11.2 Urocortins

Urocortins are peptides that are homologous to CRF and the fish peptide urotensin I. These peptides bind to CRF receptors and show a particularly high specificity for CRF-R2 (Lewis *et al.*, 2001). Urocortin is a 40-amino acid peptide expressed in the mammalian brain as well as in peripheral tissues. It has 63% sequence homology with urotensin I and 45% with CRF (Vaughan *et al.*, 1995). Urocortin II and urocortin III are also included in the urocortin subfamily of peptides. Murine urocortin II is a 38-amino-acid peptide that selectively binds to the CRF-R2 with no appreciable affinity for the CRF-R1 (Reyes *et al.*, 2001). There are still uncertainties surrounding the exact chemical nature of the human ortholog of murine urocortin II (Hsu *et al.*, 2001). Urocortin III is a putative 38 amino acid peptide that is also referred to as stresscopin in humans (Hsu *et al.*, 2001; Lewis *et al.*, 2001). It is similar to urocortin II and selectively binds to the CRF-R2 receptor.

Of the three urocortins, urocortins II and III are not likely to be physiologically involved in either the basal or stress-induced pituitary activation as they show little activity on the CRF-R1 receptor, which mediates the hypophysiotropic effect within the CRF system (Smith *et al.*, 1998). Whereas, a central infusion of urocortin, which binds to the CRF-R1 with a high affinity, activates the HPA axis (Vaughan *et al.*, 1995) and elicits anxiety-like behaviours (Moreau *et al.*, 1997).

Because of their selectivity for the CRF-R2 urocortins II and III have been described as ‘stress-coping’ peptides (Hsu *et al.*, 2001). Both peptides are capable of reducing anxiety, blood pressure and arousal.

1.11.3 Sauvagine

Sauvagine is a 40-amino acid peptide isolated from the frog skin (Montecucchi *et al.*, 1980). When administered intravenously into rodents sauvagine causes an intense, long-lasting hypotension accompanied by tachycardia (Erspamer *et al.*, 1980). The degree of hypotension was not modified by either atropine or propranolol, indicating the fall in blood pressure was due to vasodilator activation. Tachycardia, on the contrary, was partially inhibited by propranolol indicating that it was due to baroreceptor mediated reflex activation of sympathetic function rather than a direct chronotropic action. In these studies there was a reduction in urine volume accompanied by a decrease in GFR and an increase in tubular Na^+ reabsorbtion.

Sauvagine shares significant structural homology with CRF and elicits a number of similar biological responses. CRF and sauvagine have similar potencies in stimulating pituitary ACTH secretion. However, sauvagine is 5-10 times more potent than CRF administered within the brain to increase plasma levels of catecholamines and glucose and to elevate mean arterial pressure. Sauvagine is likewise more potent than CRF outside of the brain to increase superior mesenteric artery flow and plasma glucose concentrations and to decrease mean arterial pressure (Brown *et al.*, 1982).

Regulation of energy homeostasis is a vital function of the central nervous system (CNS) requiring adaptive responses to maintain and support life after stress perturbations. The mechanisms whereby these processes occur are, however, only partially understood. A major determinate of these responses is corticotropin-releasing factor (CRF). The two CRF receptor subtypes, CRF-R1 and CRF-R2, have been hypothesized to play distinct roles in the alterations necessary for homeostatic maintenance. The function of CRFR2, in particular, has remained elusive despite its

presence in both the CNS and periphery. Gene deletion and pharmacological approaches have been used to elucidate the crucial role CRF-R2 plays in the regulation of regional tissue thermogenesis and adaptive physiology.

1.12 Corticotrophin releasing factor receptors

The CRF-R1 (Perrin *et al.*, 1993) and the CRF-R2 (Lovenberg *et al.*, 1995) together with the non-mammalian CRF-R3 receptor (Arai *et al.*, 2001) are G-protein-coupled receptor types that mediate the effects of the CRF family of peptides. In addition to binding to two receptors, CRF and related peptides also bind to CRF-binding protein (Behan *et al.*, 1989; Orth *et al.*, 1987; Potter *et al.*, 1992).

1.12.1 CRF-R1 receptor

The CRF-R1 binds CRF with high affinity (particularly ovine CRF), urocortin I with low affinity, and does not bind to urocortin III. CRF-R1 mRNA is broadly distributed in the brain with high densities of expression observed in cortical, hypothalamic, limbic and cerebellar regions (Potter *et al.*, 1994; Wong *et al.*, 1994). It is also markedly expressed in the pituitary and in some peripheral tissues (Dieterich *et al.*, 1997). Within the PVN, CRF-R1 mRNA is not detected under basal conditions but can be acutely induced by stressful stimuli. Glucocorticoids have been reported to suppress the expression of the CRF-R1 in anterior pituitary cell cultures (Pozzoli *et al.*, 1996). The pituitary and PVN are among the few regions for which modulatory effects on the CRF-R1 gene have been reported. These modulatory effects are consistent with the critical role played by CRF-R1 in mediating the hypophysiotropic action of CRF (Smith *et al.*, 1998).

Animal investigations tend to demonstrate that the CRF-R1 receptor activity is increased in obesity. Following stressful stimuli, obese rodents showed an enhanced activity of the HPA axis (Guillaume-Gentil *et al.*, 1990), which is under the control of the CRF-R1. In addition, food-deprived obese Zucker rats exhibit a neurogenic-stress-like response, which appears to be mediated by CRF receptors and was associated with the development of obesity (Timofeeva *et al.*, 2001; Timofeeva *et al.*, 1997). This is consistent with the hypothesis that development of obesity may be linked to a dysregulation of the HPA axis. Adrenalectomy reverses or attenuates the obese phenotype in genetically obese (*ob*/*ob*) mice (Feldkircher *et al.*, 1996; Yukimura *et al.*, 1978) and (*fa*/*fa*) Zucker rats (Marchington *et al.*, 1983). CRF-R1 antagonists have relatively mild effects on energy balance, but CRF-R1 blockade was shown to improve plasma variables related to metabolic syndrome in obese Zucker rats (Doyon *et al.*, 2007).

Pharmacological inhibition of CRF receptors in now considered a potential therapeutic target to treat depression (Grammatopoulos *et al.*, 2002). Interestingly depression is also linked with raised blood glucose and insulin resistance.

1.12.2 CRF-R2 receptor

The CRF-R2 receptors show high affinity towards urocortins and lower affinity towards CRF (Reyes *et al.*, 2001). The CRF-R2 has two splicing variants in rodents and three variants in humans. The three anatomically distinct splice variants are known as the $CRF_{2\alpha}$ receptor, the CRF-$R_{2\beta}$ receptor and the CRF-$R_{2\gamma}$ receptor (Perrin *et al.*, 1999). The CRF-$R_{2\alpha}$ receptor is mainly expressed in the hypothalamus and other areas of the brain. The $CRF_{2\beta}$ receptor is not expressed in the brain parenchyma and is principally expressed in the peripheral tissues including the adrenal glands

(Dermitzaki *et al.*, 2007). The CRF-$R_{2\gamma}$ receptor has solely been found in human brain (Kostich *et al.*, 1998). The CRF-R2 has greater affinity for the urocortins, urotensin I and sauvagine than for CRF itself (Dieterich *et al.*, 1997). There is evidence that the CRF-R2 mediates 'stress-coping' responses such as anxiolysis, 'dearousal' and hypotension (Hashimoto *et al.*, 2001). CRF-R2-ablated mice tend to exhibit an increased HPA activity following restraint stress (Coste *et al.*, 2000). In the periphery, the CRF-R2 mediates the inhibitory effect of CRF receptor agonists on gastric emptying.

1.12.3 Other CRF receptors and CRF binding protein

In mammals, the existence of CRF receptors other than CRF-R1 and CRF-R2 has yet to be described. However, the observation that CRF can produce effects in the absence of CRF-R1 or CRF-R2 suggests the presence of additional CRF receptors (Van Pett *et al.*, 2000). In addition to binding to two receptors, CRF and its related peptides also bind to CRF-binding protein (CRF-BP) (Jahn *et al.*, 2001). CRF-BP is a 32 KDa soluble glycoprotein. It has been detected in anthropoid primates and rodents but not in evolutionarily earlier species suggesting that a complex regulatory mechanism involving CRF-BP developed later in the evolution. In the circulation and interstitial spaces CRF-BP binds to either CRF or UCNs with high affinity. In primates the CRF-BP gene is expressed in several tissues including liver, placenta and brain. By binding to CRF and UCNs CRF-BP reduces their bioavailability and consequently their activity (Tsatsanis *et al.*, 2007).

1.13 CRF peptides and adrenal function.

Accumulating evidence suggests that CRF or its related peptides also affect the adrenals directly, through actions within the adrenal gland where they form paracrine regulatory loops (Tsatsanis *et al.*, 2007). However, very little is known about the physiological importance of intra-adrenal CRF and related peptides, or their receptors in regulating adrenal function. CRF and its related peptides, the urocortins [UCNs: UCN1, UCN2 and UCN3], their receptors CRF-R1 and CRF-R2 as well as CRF-BP are all expressed in adrenal cortical, medullary chromaffin and resident immune cells (Chatzaki *et al.*, 2002). The intra-adrenal CRF-based regulatory system is likely complex and depends on the balance between the local concentration of CRF ligands and the availability of their receptors.

Complex CRF based circuits exist within the human and rodent adrenal glands. These complex circuits may control adrenal cortical and medullary function, or the level of their interaction. An early study showed exogenous CRF enhanced adrenal steroidogenesis in hypophysectomised calves, indicating that CRF could have a direct effect independent of ACTH (Jones *et al.*, 1992). This is supported by a report that CRF stimulated cortisol production from isolated human fetal adrenocortical cells (Sirianni *et al.*, 2005). In addition to a direct effect, CRF enhances the adrenal response to ACTH; when a sub-maximal dose of CRF with a sub-effective dose of ACTH caused a marked increase in the release of corticosterone levels in rat adrenals *in vitro* (Andreis *et al.*, 1991).

Consistent with the pivotal role of the hypothalamus and pituitary in regulating the HPA axis, adult CRF (-/-) mice have low basal corticosterone and impaired glucocorticoid responses to stress. Histological examination showed marked atrophy

of the zona fasciculata/reticularis region (Muglia *et al.*, 1995). CRF-R1 (-/-) mice confirms that it is the main receptor for mediating the effects of CRF in the pituitary. These studies also confirmed that both CRF and CRF-R1 are required for normal adrenal development function and stress response (Smith *et al.*, 1998; Timpl *et al.*, 1998). While the zona fasciculata is markedly decreased in size in CRF-R1 (-/-) mice, the zona reticularis appeared relatively normal (Smith *et al.*, 1998).

CRF-R2 (-/-) mice has normal adrenal function and structure along with normal corticosterone secretion. However, they show a more rapid increase in glucocorticoid levels in response to stress and a quick decline compared to wild type mice. This suggests that these receptors mediate an inhibitory action on adrenal function (Bale *et al.*, 2000). However, it has yet to be established whether this effect involves CRF-R2 receptors locally in the adrenal, or the identity of the endogenous peptide involved in mediating this response.

1.14 Adrenal dysfunction in T2DM: unanswered questions?

1.14.1 Does the zona reticularis of the adrenal gland play a role in T2DM?

The key role of the adrenal gland in carbohydrate metabolism was recognised through observations of altered blood glucose in adrenalectomised animals. Administration of adrenal extracts raised blood sugar levels and glycogen in liver and muscle in both adrenalectomised and normal animals (Long *et al.*, 1940). Adrenalectomy in obese Zucker rats prevented hyperinsulinaemia (Freedman *et al.*, 1986). Similarly adrenalectomy in diabetic (ob/ob) mice reduced plasma insulin and glucose levels (Makimura *et al.*, 2000).

Intravenous injections of CRF or sauvagine are known to cause hyperglycaemia (Brown *et al.*, 1982). This hyperglycaemia was thought to be a glucocorticoid response due to pituitary activation. However, while sauvagine and CRF are equipotent stimulators of ACTH release through CRF-R1 receptor, sauvagine is more potent in causing hyperglycaemia than CRF (Brown *et al.*, 1982). Therefore, it is most likely that this effect is due to a CRF-R2 receptor dependent mechanism. Previous studies showed that this was an adrenal dependent response (Kaminski 2004), but not mimicked by dexamethasone or corticosterone (Barker and Corder, 1996; Kaminski, 2004). Interestingly, while surgical removal of the adrenal gland blocked the response, pharmacological adrenalectomy with metyrapone augments the hyperglycaemia produced by sauvagine, and causes hyperglycaemia when administered alone in rodents (Kaminski, 2004; Rotllant *et al*., 2002). These findings seriously question the relative contribution of known corticosteroids such as corticosterone to adrenal induced hyperglycaemia as well as to the precise mechanisms regulating carbohydrate metabolism. This raises the possibility of secondary pathways in steroidogenesis. If this hypothesis is correct, then what adrenal cells are responsible for the synthesis of these corticosteroids?

In this respect the location of CRF-R2 receptors in the adrenal may provide some insight as CRF-R2 receptors have been localised to the zona reticularis of the adrenal cortex (Fukuda *et al*., 2005). The zona reticularis (ZR) of the adrenal cortex could be the major site of production of a novel zona reticularis gluconeogenic factor (ZRGF). Moreover, there are marked differences in the zonation of certain enzymes in the adrenal gland. Pertinent to observations with metyrapone, there is less 11-β hydroxylase activity in the zona reticularis (Hyatt *et al*., 1983). Indeed, novel steroids may have been overlooked in previous analyses because marked synthesis, as a

compensatory mechanism may occur only during hypoglycaemia or fasting or in disease states such as T2DM when normal regulatory mechanisms are lost. Interestingly, in early reports of glucose regulation by synthetic steroids adrenal extracts showed a greater activity in adrenalectomised rats.

1.14.2 Is there a role for a novel adrenal corticosteroid in regulating hepatic glucose output?

Metyrapone is an inhibitor of 11-β hydroxylase and its administration inhibits the final conversion of deoxycorticosterone to corticosterone. However, aminoglutethimide does not cause a similar hyperglycaemic effect to metyrapone (Kaminski 2004). Aminoglutethimide inhibits 20α-hydroxylation preventing the conversion of cholesterol to pregnenalone. Unlike metyrapone it also inhibits both 21 and 18 hydroxylation blocking both aldosterone and corticosterone synthesis (Dollery, 1999). These differences in action point to a defective steroid metabolism resulting in the production of a novel gluconeogenic corticosteroid.

Aims of this thesis

The main aim of this thesis is to test the hypothesis that the adrenal gland produces a novel gluconeogenic steroid, which may play a role in physiological or pathophysiological regulation of glucose metabolism.

CHAPTER 2
Development of a multi-well format, high-throughput-bioluminescent methodology for measurement of PEPCK activity.

2.1 Introduction.

PEPCK (EC 4.1.1.32) is a gluconeogenic enzyme that catalyses the conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP) (reaction 1, Figure 2.1). PEPCK contains 13 cysteine residues all in the reduced state (Nordlie *et al.*, 2010). The purified enzyme needs thiol compounds like DTT or β-mercaptoethanol to maintain maximal activity (Ballard *et al.*, 1969). PEPCK has a dual cation requirement: millimolar concentrations of Mg^{2+} are associated with the nuclear substrate, and micromolar concentrations of a second cation (Mn^{2+} or Fe^{2+}) bind to a specific enzyme site as an activator (Snoke *et al.*, 1971).

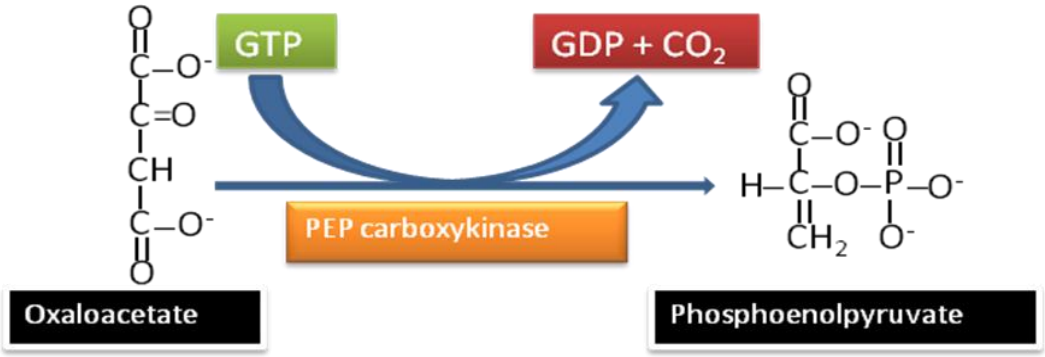
Extremely sensitive methods are required to determine PEPCK activity in various tissues. Four different methods have been employed to measure PEPCK enzyme activity: (1) determination of PEPCK activity by coupled fluorimetric method (Burch *et al.*, 1978); (2) determination of PEPCK activity using an assay in which the coupling enzyme, malate dehydrogenase, which reduces OAA to malate concomitantly with the oxidation of NADH to NAD^+ (Petrescu *et al.*, 1979); (3) determination of γ-32 P incorporation in phosphoenolpyruvate from γ-32 P GTP, as a phosphate donor (Vandewalle *et al.*, 1981); and (4) bioluminescent assay for the determination of PEPCK activity in tissue samples (Wimmer, 1988).

Methods (1) and (3) are complicated and time consuming. Method (2) is the preferred choice by many groups and is in use. But this method is not suitable for the rapid analysis of multiple samples required for this project. Method (4) as described by Wimmer is not practical for use in cell culture studies and involves performing the measurements under oil, which is technically demanding and not easily reproducible. However, the principle of Wimmer's bioluminescent method has the potential for

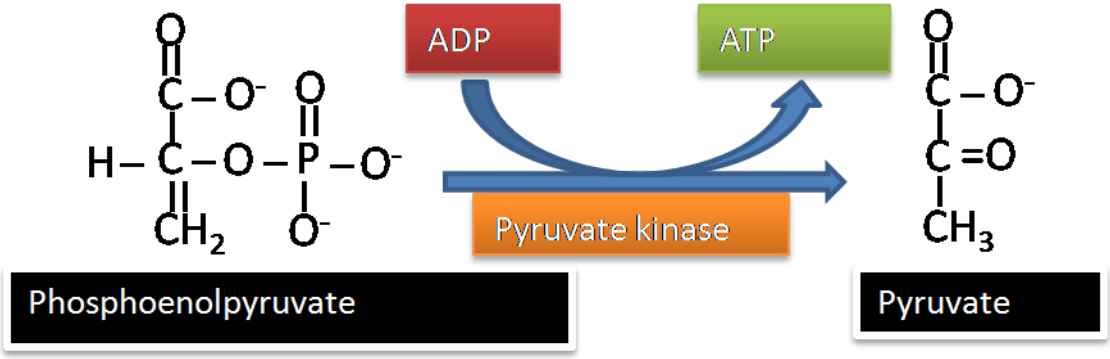
highly sensitive measurements with low amounts of sample. Therefore, it was used as the basis for developing an optimised multi-well format method for measurements of PEPCK activity in cultured cells and tissue samples to meet the needs of this project.

This method is based on the measurement of ATP generated in three reaction steps. The first reaction is the formation of phosphoenolpyruvate (PEP) from oxaloacetate and GTP in the PEPCK reaction (reaction 1, Figure 2.1). The PEP generated in this reaction is used in the second reaction to generate ATP in the presence of PK and ADP (reaction 2, Figure 2.1). ATP generated in reaction 2 is used in the next step in which the amount of ATP is quantified by the luminescence produced in the presence of luciferin and beetle luciferase (reaction 3, Figure 2.1). The luminescence signal produced is proportional to the amount of PEPCK in reaction 1.

Reaction1



Reaction2



Reaction 3

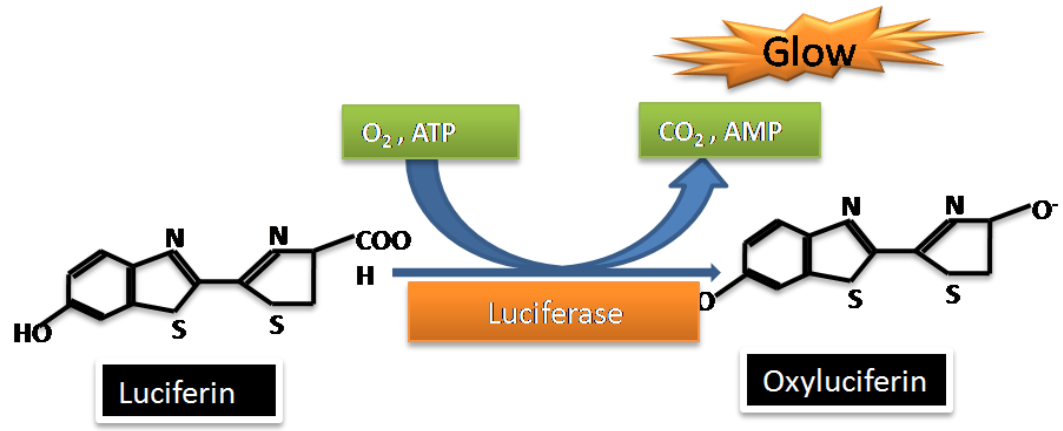


Figure 2.1 Schematic representations of the three coupled reactions in the PEPCK bioluminescent assay.

2.1.1 Need for a high-throughput PEPCK assay for screening of a novel diabetogenic compound.

T2DM is characterised by post-prandial and fasting hyperglycaemia. This hyperglycaemia at least in part results from increased hepatic glucose production through increased gluconeogenesis (Mitrakou *et al.*, 1992; Perriello *et al.*, 1997) and in particular increased activity and transcription of PEPCK (Veneziale *et al.*, 1983).

The main goal of this PhD project was to screen plasma, tissue and cell culture medium samples for potential novel gluconeogenic adrenal steroids. Separating and purifying a gluconeogenic component from a complex mixture like plasma or incubation medium requires the use of purification techniques such as solid phase extraction (SPE) and high performance liquid chromatography (HPLC). This requires testing intermittent pools and fractions in a bioassay on cultured hepatocytes and using a rapid sensitive method to measure PEPCK activity in multiple samples.

In order to achieve this, the PEPCK activity assay should be cost effective, time saving and should be suitable for use in a multi-well format so that HPLC fractions collected using different separation techniques can be tested for an effect on the PEPCK enzyme activity.

2.1.2 Overview of the method development.

This PEPCK method relies on the successful coupling of the initial two reactions involving PEPCK and PK (Figure 2.1). The resultant ATP is measured using the luciferin/luciferase reaction.

The success of this assay is in the detection of the final signal, which involves the sensitive measurement of ATP using bioluminescence (reaction 3). Therefore this

reaction was optimised by determining the luciferase enzyme reaction time course. The initial velocity region of the luciferase reaction was determined and in all subsequent experiments ATP measurements were conducted in this linear range.

The second reaction involving PK generates ATP from the dephosphorylation of PEP. This reaction was optimised by coupling with luciferin/luciferase reaction (reaction 3) to determine the ATP generated. Validation experiments were performed to determine the optimal dilution factor of the ATP generated in PEP/PK (reaction 2) so that it was in the ideal signal range of reaction 3.

The initial PEPCK reaction (reaction 1) generates PEP, the substrate for reaction 2. This reaction was fully optimised to be coupled with reaction 2. As this method is for determination of PEPCK activity in cultured hepatocytes, cell lysis conditions were optimised to achieve reproducible protein levels. PEPCK has a very sensitive dual cation requirement with Mg^{2+} and Mn^{2+}, with Mn^{2+} also being inhibitory at higher concentrations.

Experiments were performed to establish assay parameters, reagent stability and suitability, linearity of enzyme activity, signal window and data analysis. Rat hepatocytes (H4-II-E-C3 cell line) were initially used to validate the assay using known stimulators and inhibitors of PEPCK activity. Reproducibility, coefficient of variance and limit of detection were established. This was further extended to tissue samples collected from *in vivo* animal models.

2.2 Materials and Methods

2.2.1 Materials

Reagents:

All dilutions were prepared in H_2O (uHQ Elga) unless mentioned. Phosphoenolpyruvate (PEP) was purchased from Fluka. Luciferin, luciferase, and pyruvate kinase (PK) were purchased from Promega. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), oxaloacetate (OAA), inosine triphosphate (ITP), Triton X-100 (TX-100), Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), 8-Br-cAMP, insulin, and EGTA were purchased from Sigma-Aldrich. H4-II-E-C3 cell line was obtained from European collection of animal cell cultures (ECACC; ECACC no. 85061112). Unless mentioned, all other reagents and chemicals used were purchased from Sigma.

Equipment:

Luminescence was measured using a set programme on the Wallac TRILUX 1450 Microbeta counter using 96-well black Costar plates. Luminescence measured was plotted as relative luminescence units (RLU).

Data Analysis:

All data are expressed as mean ± SEM. Data were analysed using Graphpad Prism and Statview software. Statistical differences were evaluated by one-way (or) two-way analysis of variance (ANOVA), followed by a Bonferroni's post-hoc test where appropriate. Statistical differences were accepted as $P < 0.05$.

2.3 Determination of ATP

2.3.1 Reaction 3 Chemistry

Luciferase (EC 1.13.12.7) catalyzes the oxidative decarboxylation of luciferin (LH_2) in the presence of ATP, O_2, and Mg^{2+}, producing yellow-green light (λ_{max} = 560 nm). Like all mono-substrate enzyme reactions the initial rate varies hyperbolically with ATP concentrations for a fixed concentration of luciferase.

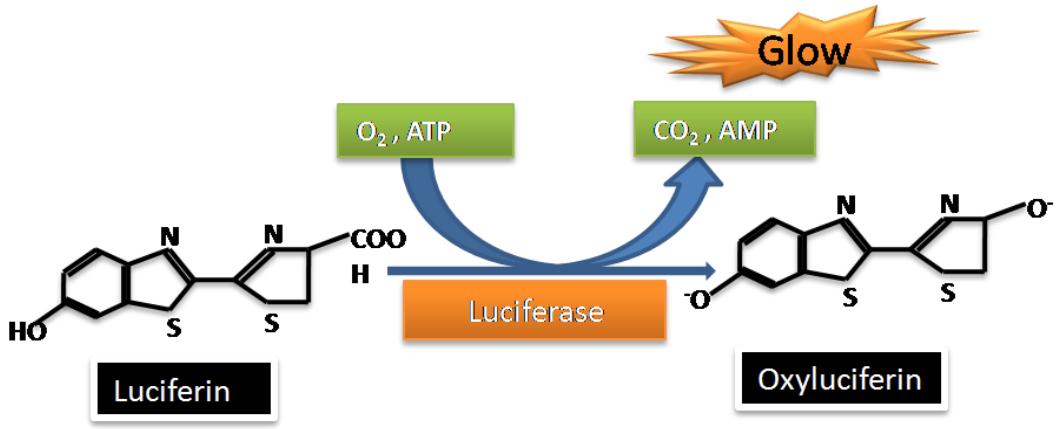


Figure 2.2 Oxidative decarboxylation of luciferin (LH_2) by luciferase in the presence of ATP, O_2, and Mg^{2+}, producing yellow-green light (λ_{max} = 560 nm).

2.3.2 Luciferase-Luciferin reaction mix

Luciferase-luciferin reaction mix (LLRM) reaction buffer (RB) was prepared and stored as a 20x stock containing 500 mM tris-HCl buffer (pH 7.8), 100 mM $MgSO_4$, 2 mM EDTA, and 2 mM sodium azide. LLRM (10 ml) was prepared by adding 8.9 ml H_2O, 0.5 ml 20x RB, 0.1 ml 0.1 M DTT, 0.5 ml 10 mM D-luciferin and luciferase (2.5 µl).

2.3.3 Luciferase reaction progress curve

The reaction was initiated by adding working LLRM (100 µl) to 10 µl of ATP standard dilutions (0.01 – 1 mM). Luminescence was measured at 10 min. The reaction rate (slope V_o) for each concentration was calculated by $\Delta Y/\Delta X$ and plotted against the concentration in a Michaelis-Menten plot. V_{max} of ATP was 513 ± 37 and the K_m =33 ± 8 µM ATP (Figure 2.5A).

2.3.4 Establishing the linear range of measurable ATP using the initial velocity conditions.

Dilutions of ATP (1 – 10 µM) (10 µl) were incubated with working LLRM (100 µl). The reaction was protected from light with an aluminum foil and incubated for 10 min at room temperature before measuring luminescence (Figure 2.5.B). The luminescence measured is plotted against ATP (µM). The assay produced linear regression of r^2=0.99 up to 10 µM.

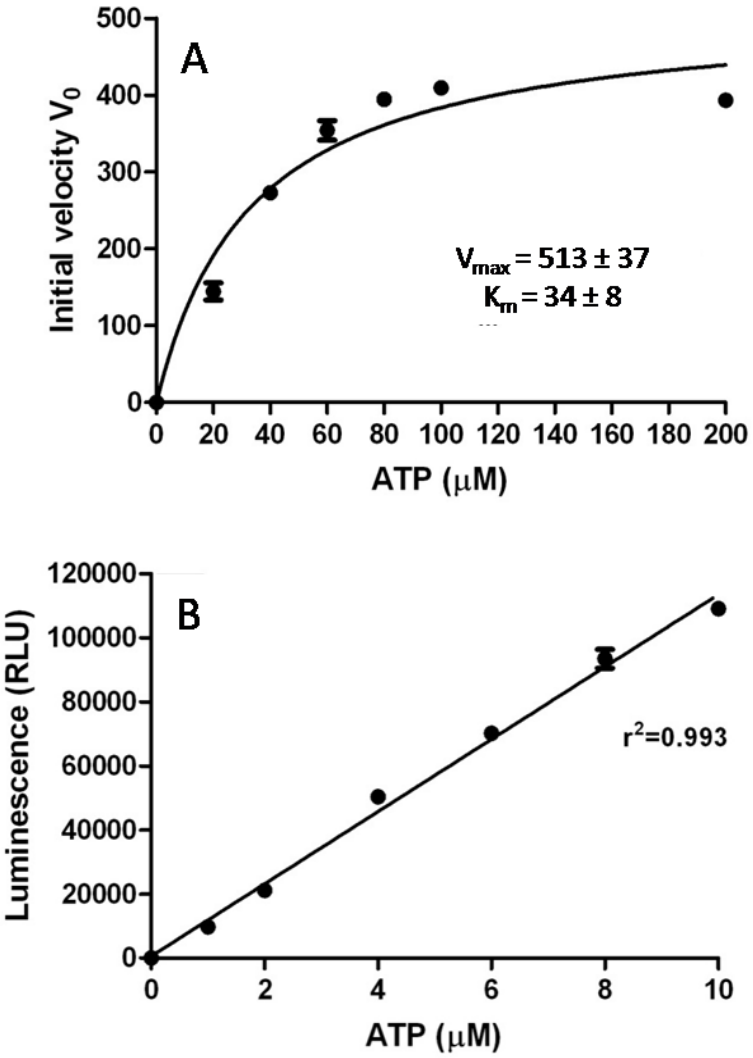


Figure 2.3 Initial velocity conditions of Luciferase/luciferin reaction with changes in ATP.

Working LLRM (100 µl) was incubated with 10 µl ATP (0 – 200 µM). Slope V_o for each concentration is calculated by Δluminescence/Δtime (sec). (A) Michaelis-Menten plot of V_o vs ATP (µM) at saturated Luciferin (0.5 mM). (B) ATP standard in the linear range (0 – 10 µM). Linear regression was calculated using Graphpad Prism.

2.3.5 Optimisation of reaction 3 incubation time.

LLRM (100 µl) was added to ATP (range 1 – 62 pmol/10 µl) in a 96-well plate. Luminescence was measured after 5, 10 and 20 min (Figure 2.4). ATP standard curves had a regression of r^2 =0.99, 0.98 and 0.99 respectively for 5, 10 and 20 min. 10 min reaction time was chosen for practical reasons when handling large sample numbers.

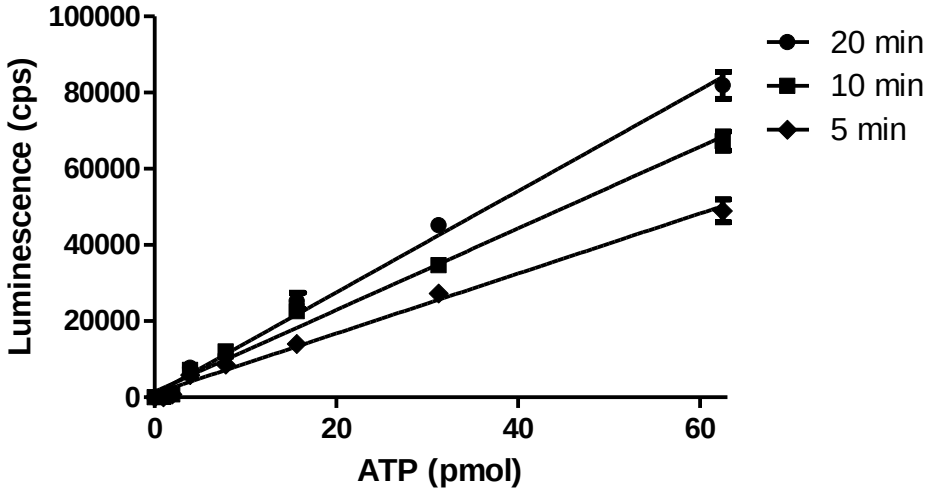


Figure 2.4 Reaction 3 incubation time.

LLRM (100 µl) were incubated with 10 µl of ATP dilutions (1 – 62 pmol). The linear regression was calculated using Graphpad Prism. Results are mean from 3 separate experiments.

2.4 Formation of ATP using Pyruvate kinase.

2.4.1 Reaction 2 Chemistry

Pyruvate kinase (EC 2.7.1.40) catalyzes the dephosphorylation of PEP in the presence of ADP, K^+, and Mg^{2+}producing pyruvate, and ATP. This reaction is irreversible under intracellular conditions.

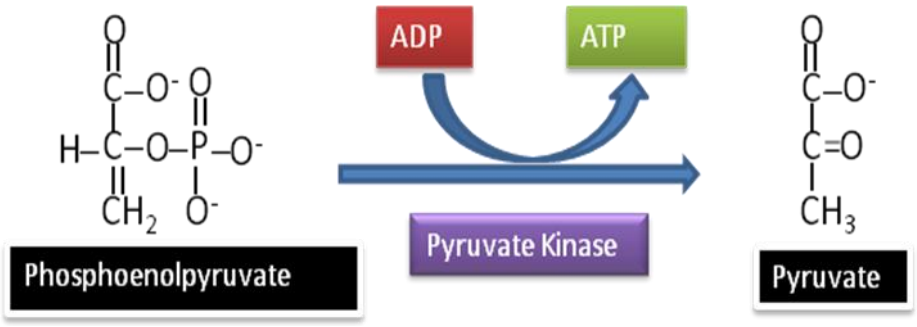


Figure 2.5 Dephosphorylation of PEP by pyruvate kinase in the presence of ADP, K^+, and Mg^{2+}producing pyruvate, and ATP

2.4.2 Pyruvate Kinase reaction buffer.

A stock solution of phosphoenolpyruvate (PEP) (100 mM) was prepared in H_2O. PK was diluted (0.5 U/µl) in enzyme dilution buffer containing 50 mM sodium phosphate buffer (pH 7.5) and 40% glycerol. Pyruvate kinase reaction buffer (PKRB) was prepared from stock solutions to attain the final working concentrations of 5 mM $MgCl_2$, 50 mM potassium phosphate buffer, 2 mM ADP and 2.5 mM KCl.

2.4.3 ATP formation in reaction 2

PEP dilutions (2.5 – 25 µM) (10 µl) were incubated with PKRB (40 µl). The reaction was incubated on a shaker at room temperature for 40 min. The reaction was then stopped and 10 µl of this incubated reaction was transferred to a 96-well plate. ATP dilutions (0.01 – 10 µM) were used to determine a control response in order to compare the coupling of the two reactions. LLRM (100 µl) was added to each well, and the sealed plate was incubated in the dark and luminescence was measured after 10 min (Figure 2.6). ATP standard curve was linear with little or no background signal in the control wells, r^2= 0.99. PEP standard curve on the other hand had a high background noise and resulted in a poor standard curve with r^2=0.86. Further experiments were carried out to find the interference in PKRB that resulted in very high background luminescence.

2.4.4 Interference in reaction 2

In order to find the interference observed in reaction 2, three conditions were tested: (i) 10 µl H_2O + 40 µl PKRB (5 mM $MgCl_2$, 50 mM potassium phosphate buffer, 2 mM ADP and 2.5 mM KCl). (ii) 10 µl of 5 µM ATP dilution + 40 µl H_2O, (iii) 10 µl 5 µM PEP + 40 µl PKRB. All three conditions were incubated at room temperature for 40 min. Samples (10 µl) of the incubation mix were incubated with LLRM (100 µl) and luminescence was measured after 10 min (Figure 2.7). PKRB, ATP, and PEP +PKRB gave 96752±6176, 76270±2302, and 160359±10236 RFU (cps) respectively. The results confirmed that PKRB contained a source of ATP, which was responsible for generating the very high background signal in reaction 3. In order to further investigate the main cause of the high background signal, each of the components were individually assayed in reaction 3

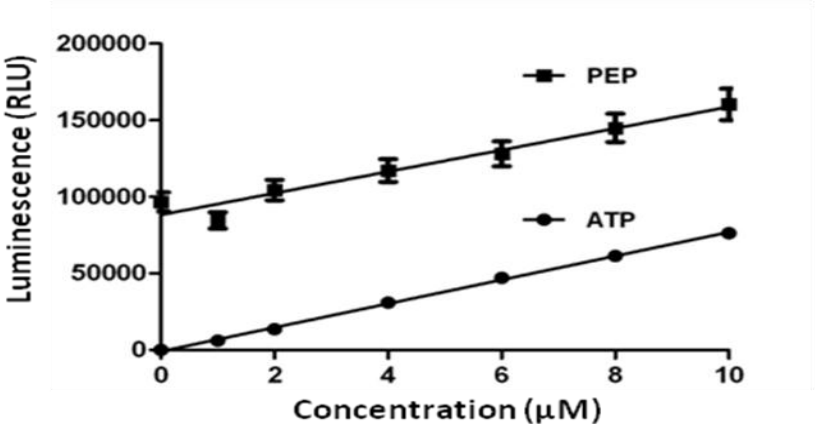


Figure 2.6 ATP formation from PEP using Pyruvate Kinase.

Reaction 2 sample (10 µl) containing an effective PEP concentration (1 – 10 µM) or ATP (1 – 10 µM) were incubated with LLRM (100 µl) and luminescence measured after 10 min. n=6. Linear regression r^2 was 0.99 and 0.89 for ATP and PEP respectively.

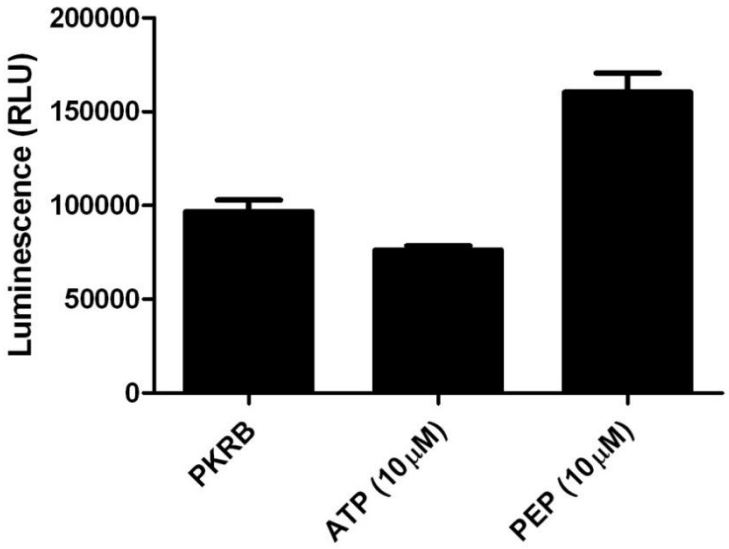


Figure 2.7 Interference in reaction 2

PKRB (5 mM $MgCl_2$, 50 mM potassium phosphate buffer, 2 mM ADP and 2.5 mM KCl) 40 µl was added to 10 µl H_2O, 10 µM ATP, and 10 µM PEP. The reaction was incubated for 40 min at room temperature and 10 µl of the reactions were assayed for luminescence in reaction 3. n=8.

2.4.5 Interference in PKRB

In order to identify the interfering component within PKRB (5 mM $MgCl_2$, 50 mM potassium phosphate buffer, 2 mM ADP and 2.5 mM KCl), 10 µl of all the 5 components of the buffer were incubated with LLRM (100 µl) for 10 min. ADP (10 µl) alone produced a signal of 75243±476 (Figure 2.8). This indicated that ADP purchased from Sigma (catalogue no A5285) was not pure as it contained ATP. Further experiments were carried out to purify this ADP and to remove the interfering ATP.

2.4.6 Purification of ADP using ion-exchange chromatography

Strong anion exchange resin was purchased from Sigma Aldrich (DOWEX 1x8 200) and packed in a mini-column (1 cm bed volume). The resin bed was washed with 10 ml of 2 M KCl and equilibrated with 40 ml of 10 mM KH_2PO_4. For purification, 2 ml of ADP (50 mg/ml) was prepared in 10 mM KH_2PO_4 and loaded onto the column. The eluant was collected into 5 ml tubes and the bound adenosine nucleotides were eluted by serial elution with (KH_2PO_4) with increasing salt concentrations as follows: 2 ml x 1 (0.1 M KH_2PO_4), 2 ml x 2 (0.3 M KH_2PO_4), 2 ml x 3 (0.4 M KH_2PO_4), 2 ml x 5 (0.75 M KH_2PO_4), 2 ml x 5 (1 M KH_2PO_4). Adenine content of each fraction (1:1000 dilution) was estimated by measuring absorbance at A_{260}. Fractions containing ATP were identified by incubating 10 µl of the eluted fraction with LLRM (100 µl) for 10 min to measure the luminescence signal. A_{260} and luminescence signal were plotted to identify the fractions containing ADP alone with no interfering ATP luminescence with LLRM (Figure 2.9).

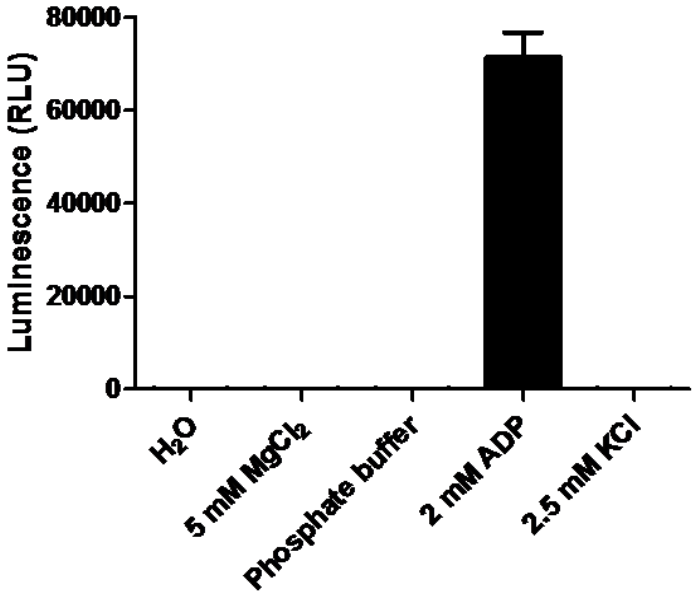


Figure 2.8 Interference within PKRB.

Samples (10 µl) of H_2O, 5 mM $MgCl_2$, 50 mM phosphate buffer, 2 mM ADP, and 2.5 mM KCl were incubated with LLRM (100 µl) and luminescence measured after 10 min. n=4.

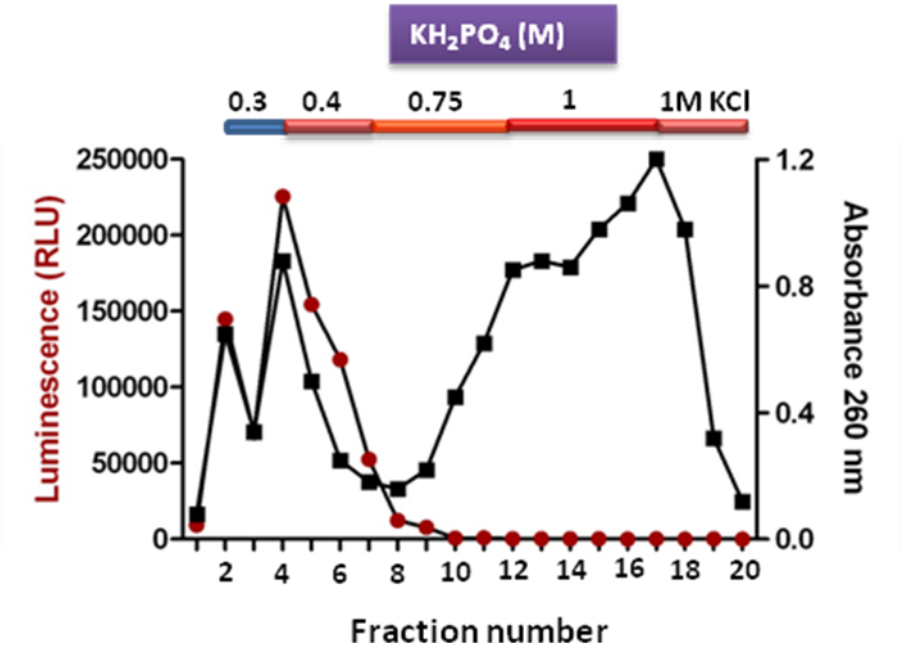


Figure 2.9 Purification of ADP for use in PKRB.

Representative chromatogram of fractions collected from Dowex (strong-anion-exchange resin) by elution with KH_2PO_4. Fractions assayed for luminescence in reaction 3 and plotted against absorbance (A 260 nm) to identify the fractions for use in PKRB.

2.4.7 Optimisation of reaction 2 incubation time using purified ADP.

PEP (250 pmol/10 µl) was incubated with PKRB prepared using the purified ADP (5 mM $MgCl_2$, 50 mM potassium phosphate buffer, 2 mM purified ADP and 2.5 mM KCl). The reaction was stopped at 5 min time intervals by addition of 10 µl of termination buffer (1 M NaOH) up to 50 min. The terminated reaction was neutralised again by addition of 1 M HCl (10 µl). Neutralised reaction (10 µl) was incubated with LLRM (100 µl). Luminescence was measured after 10 min and plotted against time to determine the optimal incubation time (Figure 2.10). Luminescence signal reached a plateau at 20 min but increased slightly to 40 min. The reaction 2 incubation time was standardised at 40 min to allow conversion of all the PEP formed in reaction 1 to pyruvate and ATP.

2.4.8 Verification of the coupling of reaction 2 with reaction 3 using purified ADP.

PEP dilutions and ATP dilutions (1 – 10 µM) were prepared. PKRB with purified pooled ADP (40 µl) were incubated with PEP dilutions (10 µl) for 40 min at room temperature on a shaker. Reaction 2 (10 µl) and ATP dilutions (10 µl) were incubated for 10 min with LLRM (100 µl). Luminescence measured at 10 min was plotted against concentration to compare the stoicheometric conversion of PEP into ATP (Figure 2.11).

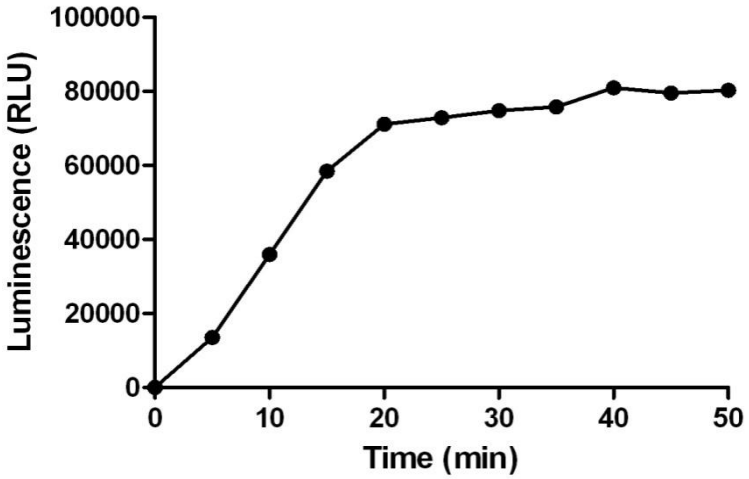


Figure 2.10 Reaction 2 incubation time.

PEP samples (25 µM) (10 µl) were incubated with PKRB (5 mM $mgCl_2$, 50 mM potassium phosphate buffer, 2 mM purified ADP and 2.5 mM KCl). ATP formed in 10 µl of the reaction was measured using LLRM in reaction 3 and the luminescence plotted against reaction 2 incubation times.

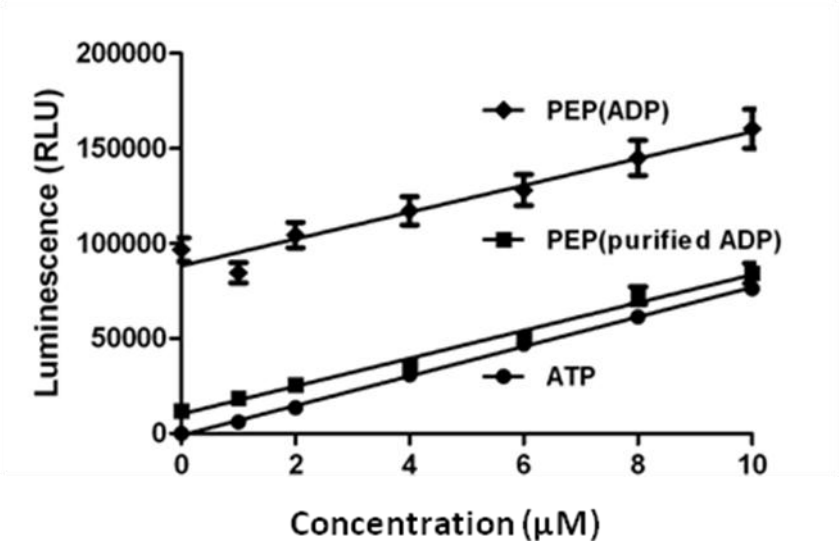


Figure 2.11 Coupling of reaction 2 with reaction 3

PEP (2.5 – 25 µM) was incubated with 40 µl PKRB for 40 min. 10 µl of this reaction was incubated with LLRM (100 µl) for 10 min. The bioluminescence was measured and compared with that obtained with ATP (1 – 10 µM).n=6.

2.5 Generation of phosphoenolpyruvate (PEP) with intracellular PEPCK.

2.5.1 Reaction 1 chemistry.

PEPCK catalyses the conversion of oxaloacetate to phosphoenolpyruvate and has a dual cation requirement: millimolar concentrations of Mg^{2+} and micromolar concentrations of a second cation (Mn^{2+} or Fe^{2+}).

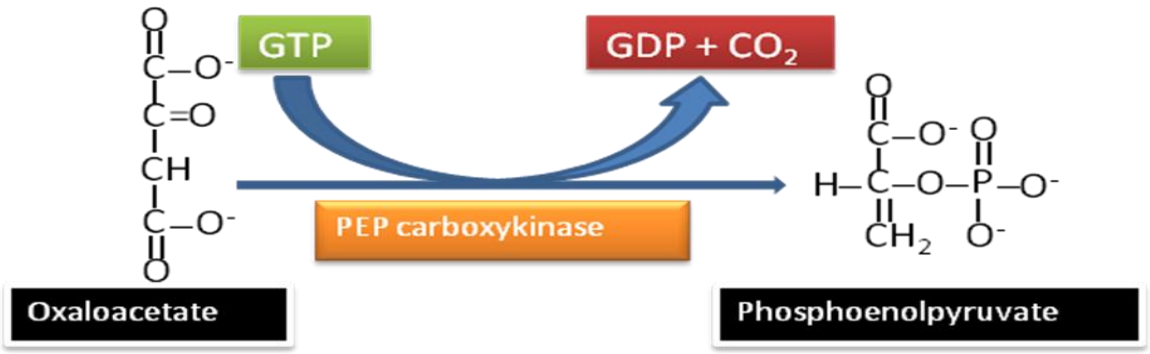


Figure 2.12 Decarboxylation of oxaloacetate to phosphoenolpyruvate by PEPCK in the presence of divalent cations Mg^{2+} and Mn^{2+}.

2.5.2 Reaction 1 Lysis buffer.

Reaction 1 lysis buffer (R1LB) was prepared fresh before each experiment. It contained 50 mM potassium phosphate buffer, pH 7.5, 10 mM $MgSO_4$, 0.1 mM $MnSO_4$, 0.1 mM EGTA, 1 mM mercaptoethanol, 3 mM inosine triphosphate (ITP), and 2.5 mM oxaloacetate.

2.5.3 Choice of non-ionic detergents in reaction 1 lysis buffer

Detergents break the lipid barrier surrounding cells by solubilising proteins and disrupting lipid-lipid, protein-protein and protein-lipid interactions. Detergents like lipids, self associate and bind to hydrophobic surfaces. They are comprised of a polar hydrophilic head group and a non-polar hydrophobic tail and are categorized by the nature of the head group as either ionic (cationic or anionic), non-ionic or zwitterionic. Their behaviour depends on the properties of the head group and tail.

As there is no standard protocol available for selecting a detergent to use for membrane lysis. The ideal detergent depends on the intended application. In general, non-ionic and zwitterionic detergents are milder and less denaturing than ionic

detergents and are used to solubilise membrane proteins where it is critical to maintain protein function and/or retain native protein: protein interactions for enzyme assays or immunoassays. CHAPS, a zwitterionic detergent, and the Triton-X series of non-ionic detergents are commonly used for these purposes.

H4-II-E-C3cells were seeded in multiwell plates; 6 well (9.5 cm^2 growth area; 1.2 x 10^6 cells) or 24 well (2 cm^2 growth area; 2.5 x 10^5 cells) and grown to 100% confluence in DMEM with 10% FCS. Cells were washed twice in ice cold PBS and stored frozen at -80^oC for subsequent use. R1LB with varying TX-100 concentrations (0 – 1%) was added to the cells on thawing. The cells were incubated for 10 min at room temperature and the contents of each well were collected for total protein measurement (Figure 2.12 A). R1LB containing 0, 0.01, 0.1, 0.5 and 1% TX-100 produced 80±15, 90±12, 138±8.5, 175±13.6, 180±15.4 total protein (µg/2x10^5cells). R1LB (0.5% TX-100) was used to compare an equimolar PEP and ATP standard (Figure 2.13 B). TX-100 (0.5%) did not interfere in reaction 2 & 3 resulting in PEP standard r^2= 0.996, and ATP r^2=0.993. For the rest of the experiments R1LB with TX-100 (0.5%) was used to lyse cells during reaction 1.

2.5.4 PEPCK progression curve and Km of oxaloacetate in reaction 1.

R1LB without oxaloacetate (OAA) (50 mM potassium phosphate buffer, pH 7.5, 10 mM $MgSO_4$, 0.1 mM $MnSO_4$, 0.1 mM EGTA, 1 mM mercaptoethanol, 3 mM ITP) was incubated with 50 µl OAA dilutions (0.5 – 20 mM) and 50 µl of H4-II-E-C3 total protein (150 µg/ml). For each concentration, the reaction was stopped at 5, 10, 15 and 20 min with 1 M NaOH (20 µl). 10 µl of each reaction time point were transferred into 96 well plate and reaction 2 and reaction 3 were carried out as described in section 2.4.8. Luminescence was plotted against time for each OAA concentration and

the slope V_o for each concentration was calculated by $\Delta Y/\Delta X$. V_o of each concentration was plotted against the corresponding OAA concentration in a Michaelis-Menten plot. V_{max} of PEPCK at the given reaction conditions was 11717 ± 650 and the K_m for OAA was 5 ± 1 mM (Figure 2.14).

2.5.5 Optimisation of Mg2+ and Mn2+ in reaction 1.

As mentioned earlier, PEPCK has a dual cation requirement and the concentration of Mg^{2+} and Mn^{2+} affect the reaction rate and the sensitive determination of enzyme activity. Low concentrations of Mn^{2+} are essential for PEPCK activity, but are inhibitory at higher concentrations for both PEPCK and PK. Therefore it was necessary to optimise the Mn^{2+} concentrations in R1LB to ensure maximum activity in reaction 1, and a negligible effect in reaction 2.

R1LB (50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EGTA, 1 mM mercaptoethanol, 3 mM ITP, and 2.5 mM OAA) with varying Mn^{2+} (0.01 – 10 µM) were prepared with and without 10 mM Mg^{2+}. Each of these reaction 1 buffer conditions (150 µl) was incubated with 50 µl of H4-II-E-C3 total protein (150 µg/ml). Reaction 1 was incubated for 20 min and reaction 2 and 3 were carried out as described in section 2.4.8. Luminescence was plotted against Mn^{2+} concentrations (Figure 2.15).

2.5.6 Reaction 1 incubation time

R1LB (50 mM potassium phosphate buffer, pH 7.5, 0.1 mM EGTA, 1 mM mercaptoethanol, 3 mM ITP, and 2.5 mM OAA, 10 mM Mg^{2+}, and 0.5 mM Mn^{2+}) (150 µl) was added to 50 µl of H4-II-E-C3 total protein (150 µg/ml) in Eppendorf tubes. The reaction was stopped at 5, 10, 15, 20, 25 and 30 min with 1 M NaOH (20

µl). Reaction 2 and 3 were performed as described in section 2.4.8. Luminescence was plotted against time (Figure 2.16). Reaction 1 incubation time was optimised at 10 min to maximise the differences between treatments in PEPCK activity.

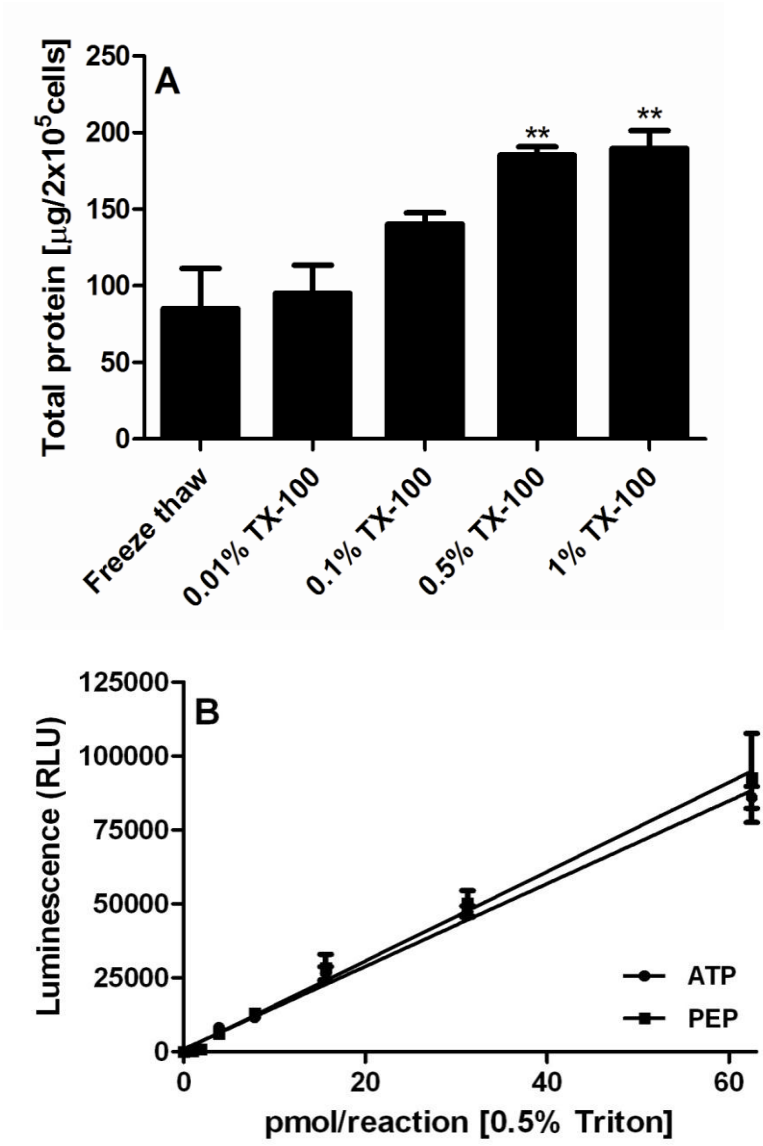


Figure 2.13 Optimising TX-100 in R1LB.

*(A) 150 µl of R1LB with TX-100 (0.01 – 1%) was added to H4-II-E-C3cells grown to confluence in a 24 well plate. After 10 min, the cell lysate was assayed for total protein using Bio-Rad detergent compatible assay. n=4 (B) PEP (2.5 – 25 µM) and ATP (0 – 10 µM) dilutions were assayed in reaction 2 and 3 in the presence of 0.5% TX-100 to test for any interference in the assay. n=4. ** = P < 0.01 vs Freeze thaw protein*

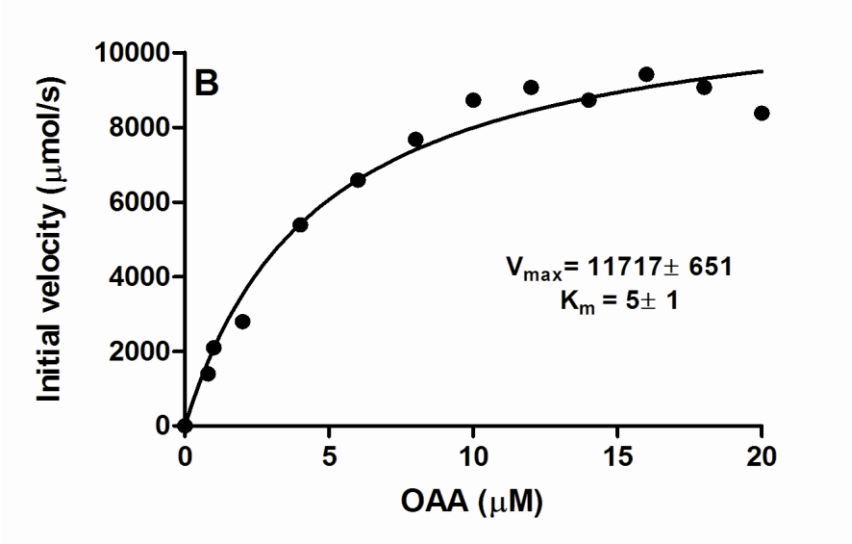


Figure 2.14. PEPCK time course and K_m of oxaloacetate in reaction 1

R1LB (150 µl) without oxaloacetate was incubated with 50 µl OAA dilutions (0.8 – 20 mM) and 50 µl of H4-II-E-C3 total protein (150 µg/ml). For each concentration, the reaction was stopped at 5, 10, 15 and 20 min and the corresponding slope V_o plotted against the OAA concentration in a Michaelis-Menten plot.

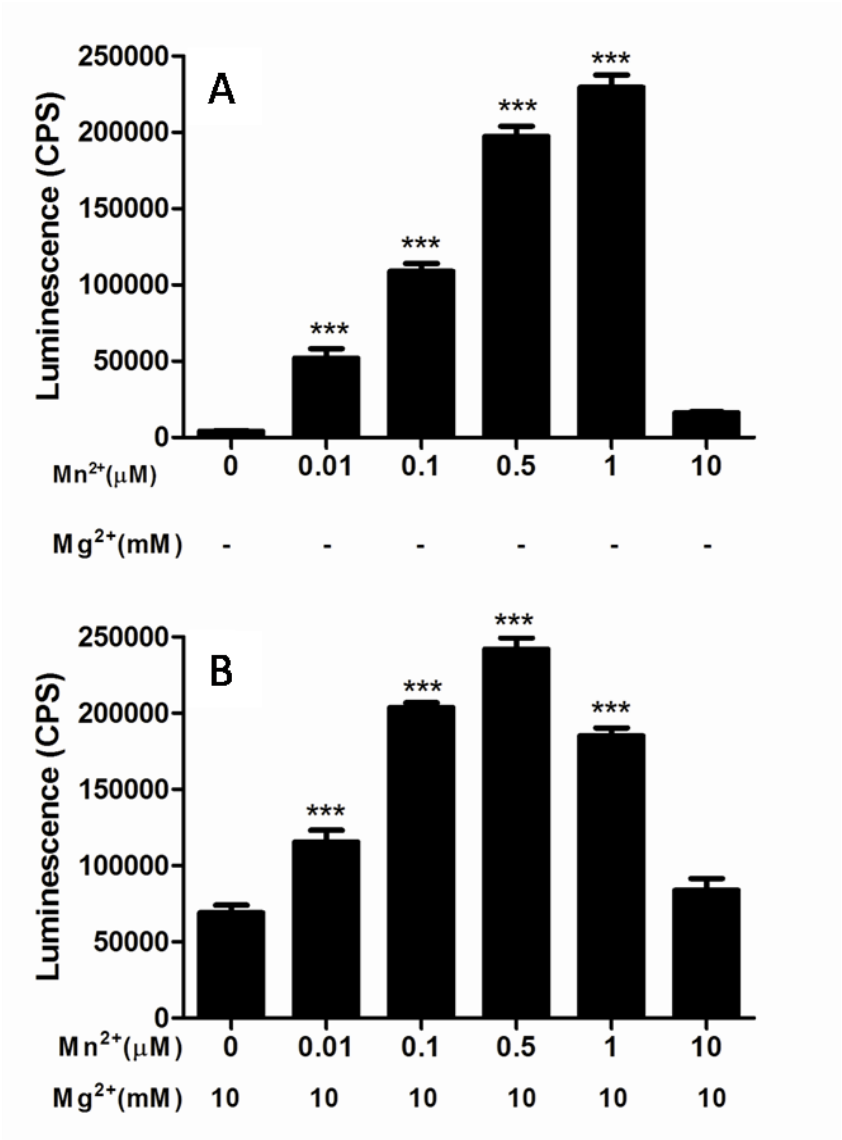


Figure 2.15 Optimisation of the Mg^{2+} and Mn^{2+} concentration in R1LB.

*R1LB was prepared with varying concentrations of Mn^{2+} (0 – 10 µM) with and without Mg^{2+} (10 mM). R1LB (150 µl) was incubated with 7.5 µg total protein. Reaction 1 was stopped after 10 min and 10 µl assayed in reaction 2. 10 µl of 1:100 dilution of reaction 2 was assayed in reaction 3 and the luminescence measured. n=3. Statistical differences determined by one-way ANOVA. *** = P<0.001 vs control.*

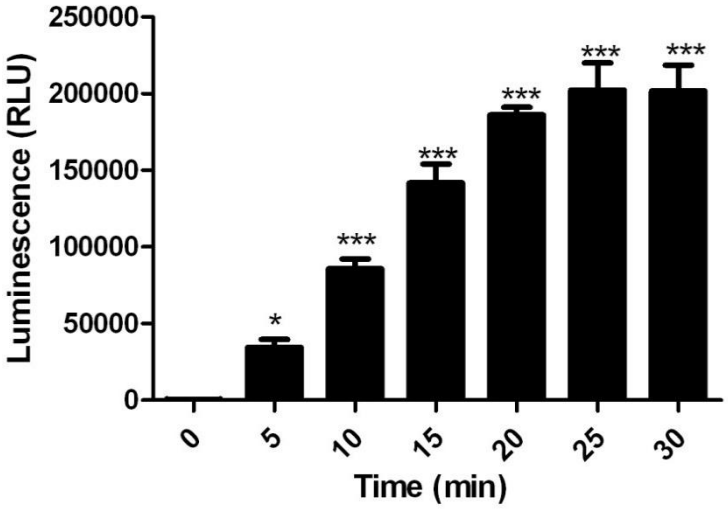


Figure 2.16 Optimisation of Reaction 1 Incubation time.

H4-II-E-C3 total cell protein (7.5 µg) was incubated with 150 µl optimised R1LB. The reaction was stopped with 1 M NaOH (20 µl) at 5 min time intervals up to 30 min. Neutralised reaction 1 was assayed in reaction 2 and 3 as described in section 2.4.8. Luminescence is plotted against time. n=6. Statistical differences determined by one-way ANOVA. = P< 0.05; ***= P<0.001 vs 0 min.*

2.6 Optimised multi-well format PEPCK activity method.

For all further PEPCK activity measurements, cells were frozen at -80^{o}C after treatment. On the day of the assay, the plates were thawed at room temperature. R1LB (150 µl/well) (50 mM phosphate buffer, pH 7.5, 0.5 µM $MnSO_4$, 10 mM $MgSO_4$, 0.1 mM EGTA, 1 mM mercaptoethanol, 3 mM ITP, 2.5 mM OAA and 0.5% TX-100) was added to each well and then incubated on an orbital mixer at room temperature for 10 min. The reaction was terminated with the addition of 20 µl 1 M NaOH and the plates were heated at 75^{o}C in a water bath for 10 min before being neutralised with 20 µl 1 M HCl. Neutralised reaction 1 (10 µl) was incubated with 40 µl PKRB (50 mM HEPES buffer, 2 mM purified ADP and 2.5 mM KCl, and PK (0.5 U/µl)) for 40 min at room temperature. Reaction 2 was diluted (1 in 50 or 1 in 100) in H_2O. Diluted reaction 2 (10 µl) was incubated with LLRM (100 µl) for 10 min before measuring the luminescence signal generated. Control samples of reaction 1 are taken through reaction 2 and 3 without the addition of PKRB. The ATP signal generated from these samples in reaction 3 is subtracted from the corresponding values generated with PKRB for the same sample.

2.7 Experimental evaluation of PEPCK activity method

2.7.1 The effect of 8-Br-cAMP on PEPCK activity.

H4-II-E-C3 cells were grown to confluence in 10% FCS containing DMEM. Cells were washed with PBS and incubated for 6 h with 8-Br-cAMP (75 – 600 µM) in EBSS with 10 mM lactate and 2 mM pyruvate. Medium was aspirated and cells were frozen at -80°C. PEPCK activity assay was performed on these cells as described in

section 2.6. 8-Br-cAMP (75 – 600 µM) caused a concentration dependent increase in PEPCK activity with 1.2 ± 0.1, 1.6 ± 0.1, 1.9 ± 0.1, and 2.2 ± 0.1 fold increase from untreated cells with 75, 150, 300 and 600 µM cAMP respectively. Insulin (10 ng/ml) suppressed the cAMP induced PEPCK activity in all the treatments to below the control values (Figure 2.19).

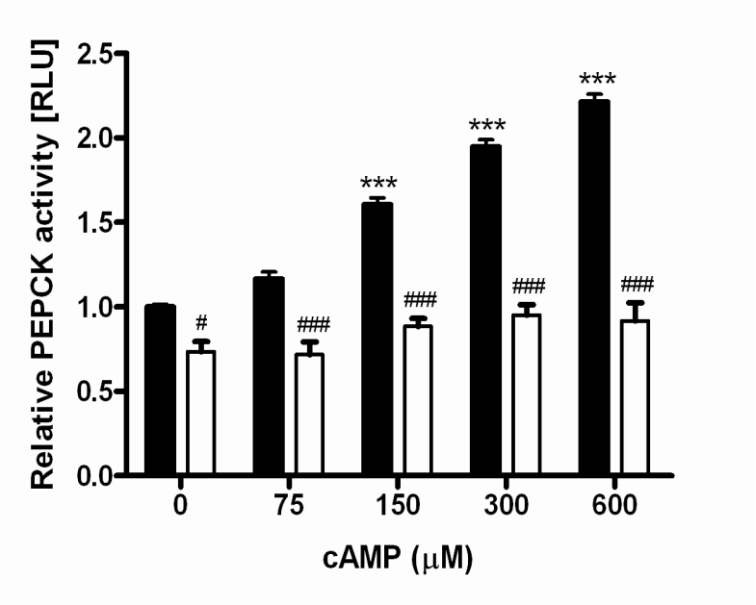


Figure 2.19 Effect of cAMP and Insulin on PEPCK activity in H4-II-E-C3cells.

*H4-II-E-C3 cells were treated with 8-Br-cAMP (75 – 600 µM) for 6h with and without 100 ng/ml insulin. The cell lysate were assayed for PEPCK activity using the optimised method described in section 2.6. Relative PEPCK activity is plotted against concentration. n=6. Statistical analysis was performed using a two-way ANOVA with a Bonferroni post hoc test. *** = P <0.001 vs 0 cAMP treatments; #=P < 0.05; ## = P < 0.01 and ### = P < 0.001 vs the corresponding cAMP treatments.*

2.8 Discussion

PEPCK plays an important role in the conversion of pyruvate to glucose and acts as the first bypass reaction of gluconeogenesis. It is often referred to as the rate-limiting enzyme in gluconeogenesis. The PEPCK assay was optimised to achieve a reproducible method capable of measuring multiple samples in a competitive manner for gluconeogenic activity.

Extremely sensitive methods are required to determine PEPCK activity in various tissues. The principle of Wimmer's bioluminescent method was used to develop and optimise this multi-well format that can be used on cultured cells and mg quantities of tissue to suit the needs of this project.

Reaction 1 buffer was optimised to contain all the substrates and cofactors required by PEPCK along with a mild detergent TX-100 to facilitate cell lysis and permit reproducible application in multi-well format. Possible interferences were addressed. As the initial PEPCK reaction is the rate limiting of all the three reactions the incubation time was optimised such that the reaction is terminated during the initial velocity conditions such that the signal measured in reaction 3 is a measure of the PEPCK enzyme concentration in the lysed cells.

Reaction 1 is terminated with changes in pH and heating at 70^{o}C for 10 min. This denatures all enzymes that could interfere in the subsequent reactions. PEP formed in reaction 1 is carried into reaction 2 in the presence of optimal conditions for PK. Non PEPCK generation of ATP as a contributor to the measurement can be excluded by taking control samples of reaction 1 through reaction 2 and 3 without the addition of PKRB. The ATP generated from these control samples is subtracted from the

corresponding values generated with the samples from the same wells incubated with PKRB containing PK. The change in pH and the heating at 70°C denatures all the protein in the samples preventing any other way of generating ATP apart from that generated due to PK. Purification of ADP was required to reduce the background signal. The ATP generated in reaction 2 is measured in reaction 3. The relative luminescence measured is a measure of the PEPCK activity. The ability to measure functional differences in PEPCK activity was confirmed using a cell permeable cAMP analogue to stimulate PEPCK gene expression. One of the key limitations of this assay method is that the reactions have to be carefully optimised for all reactions to be coupled in order to yield a quantitative measure. The control samples to subtract the background ATP are very important.

The specificity of the methodology for measurement of PEPCK may be further tested by the addition of mercaptopicolinic acid as a PEPCK inhibitor (Wimmer, 1988) or the use of cells without PEPCK (siRNA). Other negative controls that can be considered as part of the assay are to lyse and heat treat the control samples before conducting the PEPCK measurements. Results were comparable with previous reports describing cAMP regulation of PEPCK and suppression with insulin. Insulin only marginally down regulates PEPCK activity at baseline. This may be due to its ability to suppress the mRNA and expression, but the PEPCK protein already present in the cell before treatment could be responsible for the activity noticed in these experiments. The evaluation of PEPCK responses with the optimised reactions conditions confirmed the suitability of this methodology for the studies to be undertaken in this project.

CHAPTER 3

Definition of experimental conditions for studying regulation of gluconeogenesis by adrenal corticosteroids.

3.1 Introduction

The liver is the major organ for glucose production *in vivo*. Experimental studies using isolated perfused livers or liver slices are difficult to perform and not always reproducible. Nevertheless hepatic glucose release has been measured in a liver slice culture system. This method may be helpful for characterization of direct insulin action and resistance in a complex tissue as the liver. It has been applied for the analysis of drug effects on hepatic glucose metabolism (Buettner *et al.*, 2005). This method of culturing liver slices is not suitable for the current project as we need a robust system to test a lot of samples under highly comparable conditions.

Hepatocyte cultures allow the detailed investigation of the effect of various compounds on hepatic metabolism and secretion. They form an important experimental tool for pharmacological, toxicological and metabolic studies. Traditionally isolated hepatocytes are cultured as a monolayer on collagen coated plates. Primary cultures of adult rat liver hepatocytes have been extensively used in studying hepatocyte cell function. The short-term controls of glycogen synthase and glycogen phosphorylase by major regulators, such as insulin, glucose, catecholamine, and glucagon, were compared in adult rat hepatocytes in primary culture (Nakamura *et al.*, 1984). However hepatocytes cultured under such conditions tend to de-differentiate rapidly and their liver specific functions such as gluconeogenesis are lost during the culture process (Kang *et al.*, 2002; Sidhu *et al.*, 2004; Wang *et al.*, 2004b).

Several laboratories have explored new experimental conditions in order to prevent hepatocyte dedifferentiation. There are now accumulating data suggesting that culturing cells in a 3D matrix offers an environment that mimics native tissue architecture in order to establish hepatocyte integrity (Cho *et al.*, 2008; Yamamoto *et*

al., 2006). Cultured hepatocytes in a 3D collagen matrix have demonstrated advantage over monolayer cells in terms of liver-specific protein expression, glucose metabolism, and cytochrome (CYP450) activities (Richert *et al.*, 2002). However, for the current study these complex culture systems are time consuming and restrict the potential for measurement of rapid changes in protein expression or glucose production.

The human hepatic carcinoma cell line HepG2 is widely used in studying liver metabolism in culture. These cells respond to insulin by altering the synthesis and secretion of insulin like growth factor binding protein-1 (IGFBP-1) in a manner that mimics many of the changes in plasma IGFBP-1 levels observed *in vivo*. Popular rat hepatoma cell lines, such as H4-II-E-C3 from Reuber hepatoma, and H35 and HTC from Morris hepatoma 7288C are routinely used in studies of hepatocyte function. Glucose uptake and insulin signalling pathways have been studied using H4-II-E-C3 cell lines (Curran *et al.*, 2010; Dickens *et al.*, 1998). These cell lines have also been used to study the effect of various compounds on PEPCK gene expression (Juan *et al.*, 2010). H4-II-E-C3 cells have also been used to determine total glucose production in culture conditions (de Raemy-Schenk *et al.*, 2006). These studies have evaluated the effects of gluconeogenic stimulators, and suppression by insulin and metformin.

Adrenal corticosteroids have long been known to regulate glucose homeostasis. Administration of adrenal cortex extracts to fasted adrenalectomised rats leads to increased blood glucose levels and liver glycogen deposition (Long *et al*., 1940). This increase in glucose levels was demonstrated to be due to stimulation of glucose production and not an inhibition of glucose utilization (Welt *et al.*, 1952). Actions of glucocorticoids *in vitro* on liver to stimulate gluconeogenesis have also been

demonstrated. Cortisol and corticosterone stimulated glucose production in liver tissue slices from fasted, adrenalectomised animals (Haynes, 1962; Uete *et al.*, 1963).

It was proposed that glucocorticoids play a permissive role in the hormonal regulation of gluconeogenesis. Livers from adrenalectomized rats were shown to be less responsive to glucagon and epinephrine, yet became fully responsive within 60 min of dexamethasone addition to the liver perfusions (Friedmann *et al.*, 1967). Dexamethasone alone caused no significant stimulation in these studies.

A physiologically relevant increase in serum cortisol levels alone does not significantly increase the rate of glucose production. Although infusion of cortisol alone had no effect on glucose production, the addition of cortisol markedly accentuated hyperglycaemia produced by glucagon and (or) epinephrine primarily by sustaining the increases in glucose production produced by these hormones. These data indicate that changes in glucose metabolism in circumstances in which several counter regulatory hormones are elevated (e.g., "stress hyperglycaemia") are a consequence of synergistic interactions among these hormones (Eigler *et al.*, 1979). This synergistic effect with glucagon was also shown *in vitro* in isolated hepatocytes using dexamethasone (Allan *et al.*, 1984).

The adrenal glands have been studied since the 1870s in relation to blood glucose. In the 1900’s many studies reported that subcutaneous or intravenous administration of adrenal extracts caused glycosuria (Blum, 1901). Production of corticosteroids by adrenal glands *in vitro* has been extensively used in assay methods for corticotrophin (ACTH) (de Wied *et al.*, 1969; Saffran *et al.*, 1952; Vinson, 1966).

Different approaches have been preferred to investigate in vitro steroidogenesis using adrenal glands. Adrenal slices and homogenates have been found to produce steroids,

incorporate radioactive precursors and respond to ACTH (Cooper *et al.*, 1958; Vinson, 1966). Adrenal slices were used to study aldosterone and corticosterone production *in vitro* (Kaplan, 1965; Laplante *et al.*, 1966). Adrenal glands were perfused to study the response of ACTH stimulation on cortisol release (Madill *et al.*, 1973). Guinea pig adrenal segments were used to study the effects of ACTH effect on adrenal glands (Chayen *et al.*, 1976).

Pathways of corticosteroid biosynthesis from ^{3}H-pregnenolone and ^{14}C-progesterone have been studied *in vitro* using duck (Whitehouse *et al.*, 1967), sheep (Vinson, 1967) and human (Whitehouse *et al.*, 1968) adrenal tissue. Many groups have studied *in vitro* steroid metabolism using adrenal cells obtained by trypsin or collagenase digestion. However, it was observed that *in vitro* studies using adrenal quarters produced reliable results (Hofmann *et al.*, 1954).

Previous studies have shown that sauvagine and metyrapone infusions cause long lasting hyperglycaemia in rats. This was an adrenal dependent response that could not be reproduced by corticosterone alone (Kaminski, 2004). The hyperglycaemic effect of metyrapone strongly suggested that perturbation of corticosterone synthesis led to the production of an adrenal steroid with even greater gluconeogenic activity. Despite more than 100 years of research on adrenal regulation of glucose homeostasis, there is a lack of studies describing comparative data for the gluconeogenic activity of different adrenal steroids. Therefore, this chapter describing optimisation of the conditions to study the relative gluconeogenic activity of adrenal steroids on cultured hepatocytes. In addition, solid phase extraction methods were optimised to recover the steroids from adrenal incubations, and initial bioassays of extracts from adrenal incubations with sauvagine and metyrapone are included.

3.2 Aims of this chapter.

1. Optimisation of cell culture conditions for studying gluconeogenesis *in vitro.*

2. Characterisation of H4-II-E-C3 cell responses to standard stimuli (8-Br cAMP, glucagon, insulin and corticosterone)

2. Extraction of steroids from isolated adrenal gland incubations with sauvagine and metyrapone, and evaluation of their biological activity on cultured hepatocytes.

3.3 Methods

3.3.1 Cell Culture

The rat liver hepatoma cell line, H4-II-E-C3 (European collection of animal cell cultures; ECACC no. 85061112) was used for all experiments. The cell line was originally derived from Ruber H-35 hepatoma, which was induced in an AXC rat by the carcinogen N-2 fluorenyldiacetamide (Weinstein, 1972). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 5 mM glucose, non-essential amino acids and 10% (v/v) foetal calf serum (FCS), and incubated at 37°C in a humidified CO_2 incubator (92% air, 8% CO_2). Cells were passaged using 0.05% trypsin in phosphate buffered saline (PBS) containing 0.02% EDTA. Cells were seeded in multi-well plates: 6 well (9.5 cm^2 growth area; 1.2 x 10^6 cells) or 24 well (2 cm^2 growth area; 2.5 x 10^5 cells) and grown to 100% confluence. Treatments were carried out in either glucose-free Earl's balanced salt solution (EBSS; 0.264 g/L $CaCl_2$, 0.4 g/L KCl, 0.2 g/L $MgSO_4.7H_2O$, 6.8 g/L NaCl, 2.2 g/L $NaHCO_3$, 0.158 g/L $NaH_2PO_4.2H_2O$) or DMEM containing 5 mM glucose.

3.3.2 Glucose assay

Glucose was assayed using a protocol adapted from the method described by Hugget and Nixon (Bergmeyer, 1963). Media samples (50 µl) were added to reaction buffer (100 µl) containing 0.12 M sodium phosphate (pH 7), 9 U/ml glucose oxidase, 6 U/ml horseradish peroxidase (HRP), 1 mM Amplex red® and incubated in the dark for 10 min. The assay is based on two sequential enzymatic reactions. Initially, glucose oxidase catalyses the conversion of glucose to gluconic acid and hydrogen peroxide (H_2O_2) and secondly, H_2O_2 is reduced by HRP and in turn reacts with Amplex Red®

to form resorfin which is highly fluorescent. Amplex red reacts with H_2O_2 in a 1:1 stoichiometry. Thus, the presence of glucose is indicated by the measure of fluorescence. Measurements were made in a 96 well plate using a Cytofluor II fluorescent plate reader (Ex: 560; Em: 590 nm) (Perspective Biosystems, US). Data were analysed by linear regression using Graph Pad prism software.

3.3.3 PEPCK Activity assay

PEPCK activity was measured using the bioluminescent method described in section 2.10. At the end of each cell experiment media samples were collected for glucose measurement and the cells were frozen at -80^{o}C for subsequent PEPCK activity assay.

3.3.4 Adrenal incubations in vitro.

Male Wistar rats (250 – 300 g; Charles River, UK) were used in all experiments. Rats were killed using an approved method and their adrenal glands were removed. The peri-capsular fat was carefully removed and each adrenal gland was cut into 4 quarters in a Petri dish. These adrenal sections were washed twice in 2 ml PBS and then incubated in phenol red free DMEM (5 mM glucose) with treatment compounds (sauvagine 3 µM, metyrapone 30 µM, or ACTH 1 nM) for 6 hours at 37°C in a CO_2 incubator. The incubation media were collected and stored at -20°C for extraction and bioassay.

3.3.5 Corticosterone measurements.

Corticosterone concentrations in the adrenal incubation media were measured by using an EIA corticosterone kit (Assay Designs, Inc USA). The kit uses a polyclonal antibody to corticosterone to bind in a competitive manner. All the reagents were allowed to warm to room temperature before performing the experiment. Standards

were prepared in glass tubes. Assay buffer was prepared by diluting 10 ml of the buffer provided with 90 ml of deionised water. Wash buffer was prepared by diluting 5 ml of the supplied concentrate with 95 ml of deionised water. Standards and samples (100 µl) were dispensed into the appropriate wells in the strips provided. Conjugate (50 µl) was added to each well except the total activity well and the blank well, and antibody (50 µl) was added to each well except the blank, TA and NSB wells. The plate was incubated on a plate shaker for 2h. The contents of the plate were emptied and the wells washed three times with 400 µl of wash solution. Conjugate (5 µl) was added to the TA well, and pNpp substrate (200 µl) was added to every well. The plate was then incubated for 1 h at room temperature. Stop solution (50 µl) was added to each well and the plate was read immediately at 405 nm.

The % crossreactivity of the assay to deoxycorticosterone (28.6%), progesterone (1.7%), aldosterone (0.18%) and cortisol (0.046%).

3.3.7 Extraction of steroids using Sep-pak C18 cartridges.

Media samples were acidified with 10% 1 M HCl and extracted on Sep-pak® 1 cc (100 mg) C_{18} cartridges (Waters). The cartridges were initially equilibrated with methanol (2 x 1 ml) followed by a wash step with water (1 x 1 ml). The cartridges were further acidified with 50 mM HCl (1 x 1 ml) before loading the acidified samples. After loading samples the columns were washed with 50 mM HCl (1 x 1 ml) and water (1 x 1 ml). Bound steroids were eluted with 80% methanol (2 x 0.5 ml) into glass tubes and dried with a stream of N_2 at 40°C on a heated block. The dried extracts were reconstituted in 200 µl 50% ethanol and stored for further analysis on cells and for HPLC analysis.

3.3.8 Extraction of steroids using Strata-X Cartridges.

The media samples were acidified with 10% 1 M HCl and extracted on Strata-X cartridges (Phenomenex). The Strata-X matrix is described by the manufacturer as a mixed anion-exchange hydrophobic interaction resin. The cartridges were initially equilibrated with methanol (2 x 1 ml) followed by a wash step with water (1 x 1 ml). The cartridges were further acidified with 50 mM HCl (1 x 1 ml) before loading the acidified samples. The columns were washed with 50 mM HCl (1 x 1 ml) and water (1 x 1 ml). The steroid conjugates were eluted following the manufacturer's recommended elution conditions with 5% ammonia in methanol (2 x 0.5 ml) into glass tubes and dried under N_2 gas at 40°C on a heated block. The dried extracts were reconstituted in 200 µl 50% ethanol and stored for further analysis on cells and for HPLC analysis.

3.4 Results

3.4.1 Optimisation of cell culture conditions for studying gluconeogenesis.

3.4.1.1 Effect of Lactate/Pyruvate ratio on hepatocyte glucose production

H4-II-E-C3 cells were treated with increasing concentrations of lactate (2 – 20 mM) and 2 mM pyruvate in EBSS. Control cells were treated with EBSS alone with no substrates. The cells were incubated for 6 h in a CO_2 incubator at 37^{o}C. Significant increases in glucose production were observed with 5, 10 and 20 mM lactate forming 4.6 ± 0.6, 8.4 ± 0.7, and 10.4 ± 1.2 (nmol/2x10^5 cells) respectively (Figure 3.1).

3.4.1.2 Time course of glucose production in fed and fasted H4-II-E-C3 cells.

H4-II-E-C3 cells were grown to confluence in DMEM (5 mM glucose) supplemented with 10 % FCS. Cells were then maintained for 18 h in serum-free DMEM (5 mM glucose).

For the fed cell experiments, cells were treated without any prior fasting for 6 h in EBSS with and without gluconeogenic substrates (20 mM lactate/2 mM pyruvate). Glucose production in cells without substrates increased with time up to 180 min producing 9.0 ± 1.2 (nmol/2x10^5 cells) and subsequently decreased to 3.2 ± 1.3 (nmol/2x10^5 cells) at 360 min. Glucose production in cells incubated with substrates reached a maximum at 240 min (15.9 ± 4 nmol/2x10^5 cells) and decreased by 30 – 40% at 360 min (10 ± 2 nmol/2x10^5 cells) (Figure 3.2 A).

For the fasted cell experiments, cells were starved in DMEM (0 mM glucose) for 4 h and then incubated for 6 h in EBSS with and without gluconeogenic substrates (20 mM lactate/2 mM pyruvate). Glucose production in cells without substrates did not

increase in the 360 min. With the substrates the glucose production increased significantly with time up to 240 min when steady state glucose levels were reached (13 ± 2 nmol/2x10^5 cells) (Figure 3.2 B).

3.4.1.3 cAMP mediated glucose production in H4-II-E-C3 cells.

Fasted H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing 8-Br-cAMP (75 – 600 µM) with and without insulin (10 ng/ml). Media samples were collected for glucose measurements and the cells were frozen at -80^{o}C for PEPCK activity measurements.

Glucose production increased significantly with 300 µM and 600 µM cAMP to 25 ± 4 and 35 ± 6 glucose (nmol/2x10^5 cells) respectively. Insulin (10 ng/ml) suppressed this glucose production to below basal levels (Figure 3.3 A). The corresponding PEPCK activities in cells treated with 300 µM and 600 µM cAMP were increased 1.8 ± 0.1 and 2.2 ± 0.5 fold basal values (Figure 3.3 B).

3.4.1.4 Effect of glucagon on hepatocytes

Fasted H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing glucagon (1 – 1000 nM) with and without insulin (10 ng/ml). Media samples were collected for glucose measurements and the cells were frozen at -80^{o}C for PEPCK activity measurements.

Glucose production increased significantly with 100 nM and 1000 nM glucagon to 18 ± 3 and 24 ± 3 glucose (nmol/2x10^5 cells) respectively. Insulin (10 ng/ml) suppressed this glucose production to basal levels (Figure 3.4 A). The corresponding PEPCK activities in cells treated with 100 nM and 1000 nM glucagon were increased to 1.4 ±

0.2 and 1.7 ± 0.5 fold basal values. The PEPCK activity was suppressed in cells treated with insulin (10 ng/ml) (Figure 3.4 B).

3.4.2 Effect of adrenal corticosteroids on gluconeogenesis.

3.4.2.1 Effect of corticosterone on hepatic glucose production and PEPCK activity.

Fasted H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing corticosterone (0 – 10,000 nM) with and without insulin (10 ng/ml). Media samples were collected for glucose measurements and the cells were frozen at -80^{o}C for PEPCK activity measurements.

Glucose production increased significantly with 1000 nM and 10,000 nM corticosterone to 23 ± 3 and 30 ± 6 glucose (nmol/2x10^{5} cells) respectively. Insulin (10 ng/ml) suppressed this glucose production to basal levels of 9 ± 1 and 11 ± 3 glucose (nmol/2x10^{5} cells) respectively (Figure 3.5 A). The corresponding PEPCK activities in cells treated with 1000 nM and 10000 nM corticosterone were increased to 1.9 ± 0.1 and 2.2 ± 0.2 fold basal values. These increases in PEPCK activity were blocked in cells treated with insulin (10 ng/ml) (Figure 3.5 B).

3.4.2.2 Effect of DHEA, pregnenolone, allopregnenolone and their sulphated conjugates on hepatic glucose production.

Fasted H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing corticosterone, DHEA, PREG, ALPREG, DHEA-S, PREG-S, and ALPREG-S (1 µM). Untreated cells produced 12 ± 1 glucose (nmol/2x10^{5} cells).

In the presence of corticosterone (1 µM) glucose production was significantly increased to 22 ± 3 glucose (nmol/2x10^{5} cells). The same concentrations of DHEA,

PREG, ALPREG, DHEA-S, PREG-S and ALPREG-S did not cause any significant increase in glucose production. A trend for decreased glucose production was observed in the DHEA and PREG incubations (Figure 3.6).

3.4.2.3 SPE extraction of corticosterone.

DMEM was spiked with corticosterone to obtain a final concentration of 1 µM. Extraction with Sep-pak® 1cc (100 mg) C_{18} cartridges resulted in recovery of 84 ± 3% of corticosterone.

3.4.2.4 Adrenocorticotropic hormone induced corticosterone production in vitro.

Adrenal gland sections were incubated in DMEM (5 mM glucose) with and without adrenocorticotropic hormone (ACTH; 1 nM). Incubation media was collected at 1 h time intervals for 6 h and corticosterone was measured by enzyme immunoassay. The Corticosterone values for untreated adrenal sections were 7.2 ± 3, 28 ± 6, 72 ± 9, 74 ± 6 (ng/100 mg tissue), and for ACTH stimulated adrenals 6 ± 1, 171 ± 23, 256 ± 16, and 281 ± 23 (ng/100 mg tissue) at 0, 2, 4 and 6 h respectively (Figure 3.8).

3.4.2.5 Adrenal Incubations with sauvagine and metyrapone.

Adrenal sections were incubated for 6 h with DMEM (5 mM glucose) under basal (B) conditions or with sauvagine (S, 3µM), metyrapone (M, 30 µM), sauvagine and metyrapone (S, 3 µM; M, 30 µM). There were no significant increases in corticosterone production compared to basal secretions. The corticosterone measurements of the incubation medium were 93 ± 10, 105 ± 9, 43 ± 5, and 53 ± 6 (ng/100 mg) for B, S, M and SM respectively (Figure 3.9)

3.4.2.6 Comparison of the effect of SPE extracts of adrenal incubations on total glucose production and PEPCK activity.

SPE extracts were prepared using Sep-pak C18 cartridges and Strata-X AW columns from adrenal incubations collected after 6 h basal (B) conditions or with sauvagine (S, 3μM), metyrapone (M, 30μM), sauvagine and metyrapone (S, 3 μM; M, 30 μM). The extracts were reconstituted in EBSS (20 mM lactate/2 mm pyruvate) and incubated with fasted H4-II-E-C3 cells for 6 h. Corticosterone (C, 1 μM) was also used to compare the glucose production. Glucose production was 11 ± 3, 20 ± 3, 29 ± 4, 62 ± 9, 66 ± 12 and 48 ± 5 (nmol/2x10^5 cells) for Sep-pak C_{18} extracts from U, C, B, S, M, and SM incubations respectively. The corresponding glucose levels were 11 ± 3.3, 14.2 ± 2.7, 17 ± 3.4 and 17.6 ± 4 (nmol/2x10^5 cells) for extracts prepared with Strata-X AW columns for B, S, M and SM incubations respectively (Figure 3.11A). Relative PEPCK activity measurements showed that corticosterone and the Sep-pak extracts of B, S, M and SM incubations produced significant increases in PEPCK activity, which were 1.7 ± 0.2, 1.8 ± 0.1, 2.1 ± 0.4, 2 ± 0.3, and 2.3 ± 0.4 times the levels of untreated cells respectively. PEPCK activity did not increase significantly in the cells incubated with extracts obtained from Strata-X AW columns (Figure 3.11B).

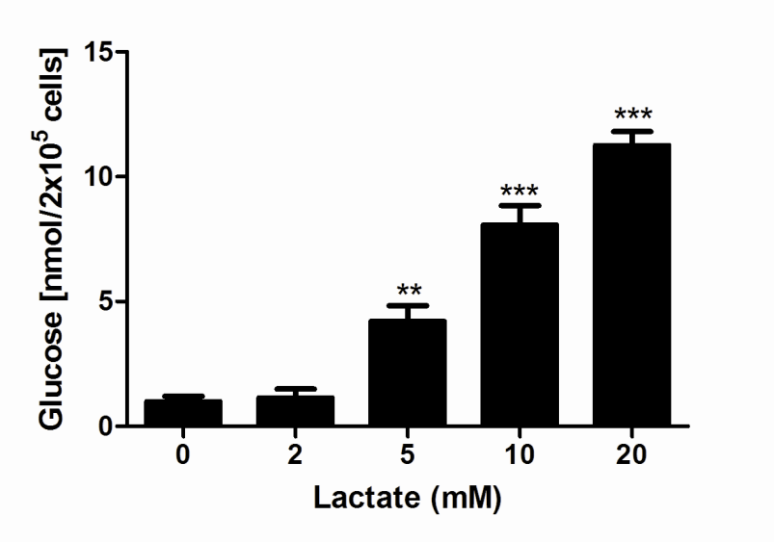


Figure 3.1 Concentration dependent increases in glucose output with lactate.

*H4-II-E-C3 cells were incubated for 6 h with lactate (2 – 20) mM in addition to pyruvate (2 mM). Results are mean ± SEM. n= 8. Statistical differences were determined by ANOVA (** = p<0.01, *** = p<0.001, vs pyruvate alone)*

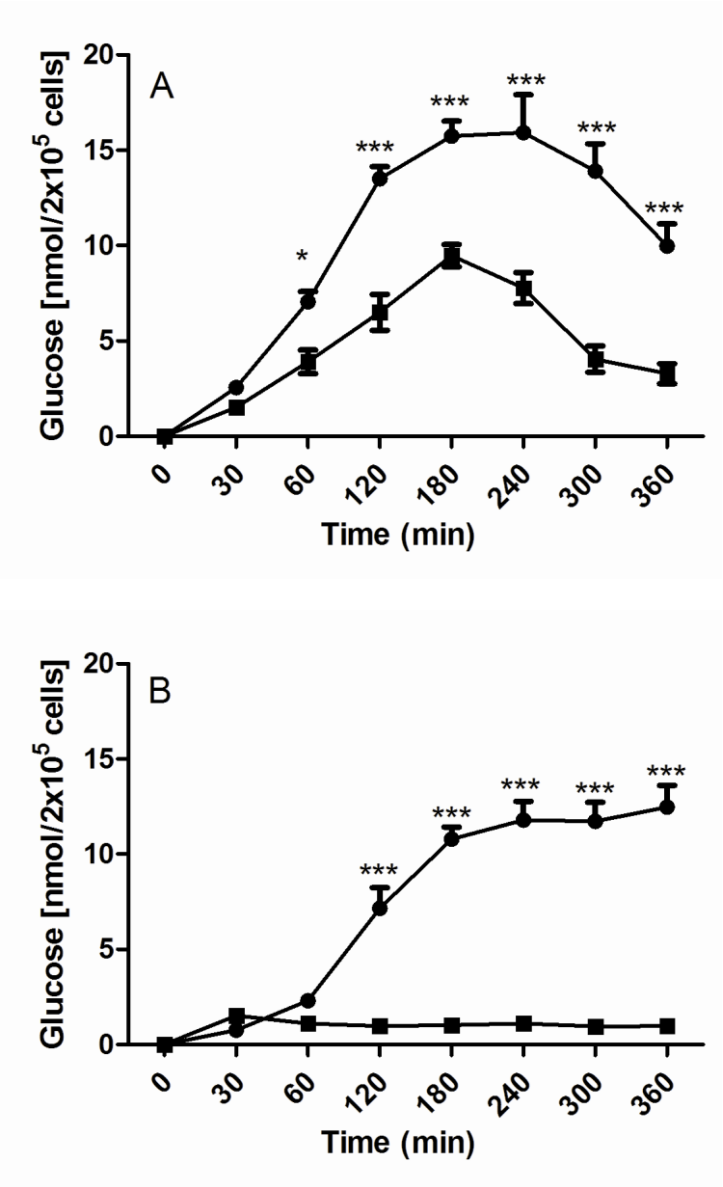


Figure 3.2 Time course of glucose production by fed and fasted cells.

(A) Fed cells; (B) fasted cells, incubated with (dark circles) and without (dark squares) substrates (20 mM lactate/2 mM pyruvate) in EBSS for 6h with glucose measured at the indicated time points. Results are mean ± SEM. n= 8. Statistical differences were determined by ANOVA (= p<0.05, *** = p<0.001, vs the corresponding time points without substrates.)*

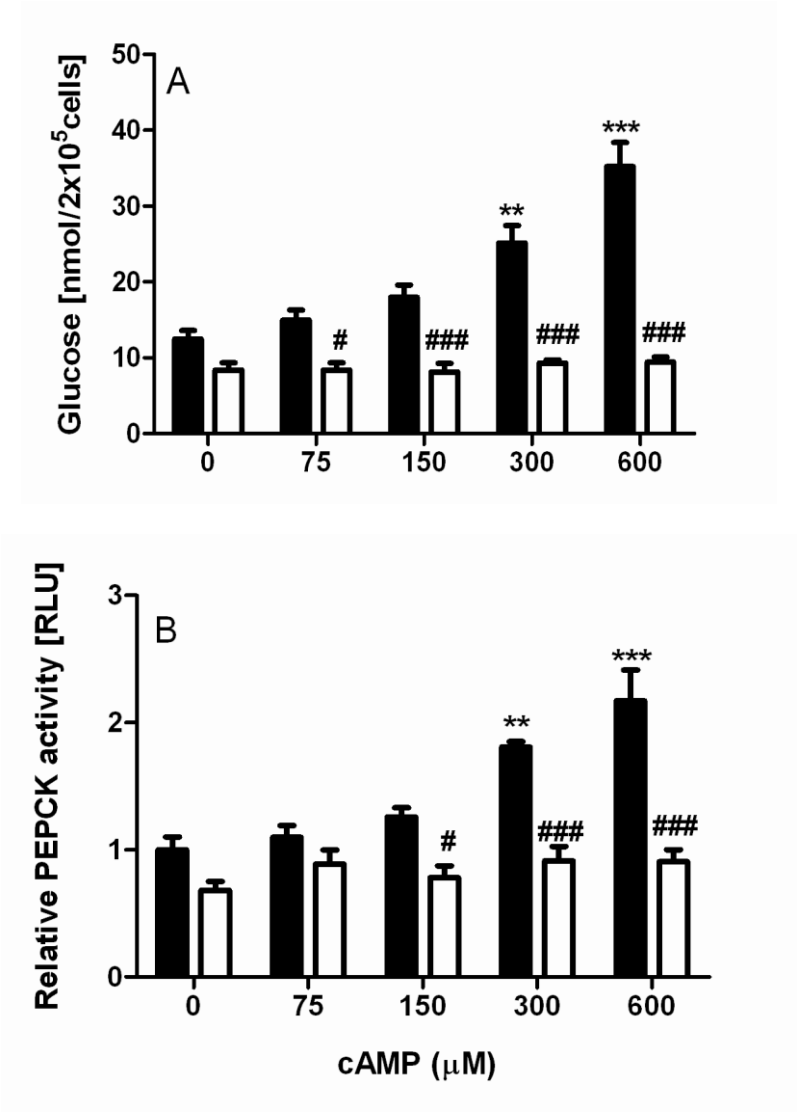


Figure 3.3 cAMP stimulated hepatic glucose production in vitro.

*Fasted H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing 8-Br-cAMP without (dark bars) and with (white bars) insulin (10 ng/ml). (A) Glucose measurements at 6 h. (B) PEPCK activity measurements in cells at 6 h. n=8. Statistical differences were determined by two-way ANOVA followed by Bonferroni post test (**=P<0.01; ***=P<0.001 vs control; # = P<0.05; ###=P<0.001 vs the corresponding cAMP treatment without insulin)*

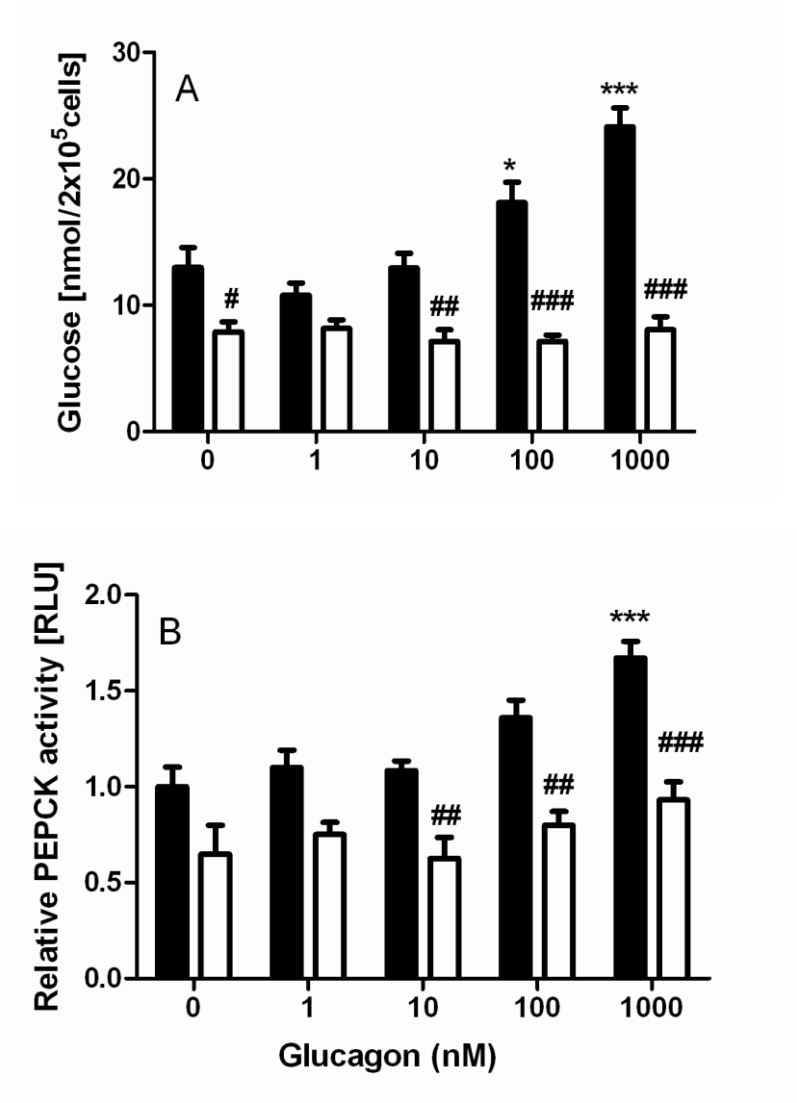


Figure 3.4 Effect of glucagon on hepatic glucose production.

*Fasted H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing glucagon (1 – 1000 nM) without (dark bars) and with (white bars) insulin (10 ng/ml). (A) Glucose measurements at 6 h. (B) PEPCK activity measurements in cells at 6 h. n=8. Statistical differences were determined by two-way ANOVA followed by Bonferroni post test (**=P<0.01; ***=P<0.001 vs control;# = P < 0.05, ##= P<0.01, ###=P<0.001 vs the corresponding cAMP treatment without insulin)*

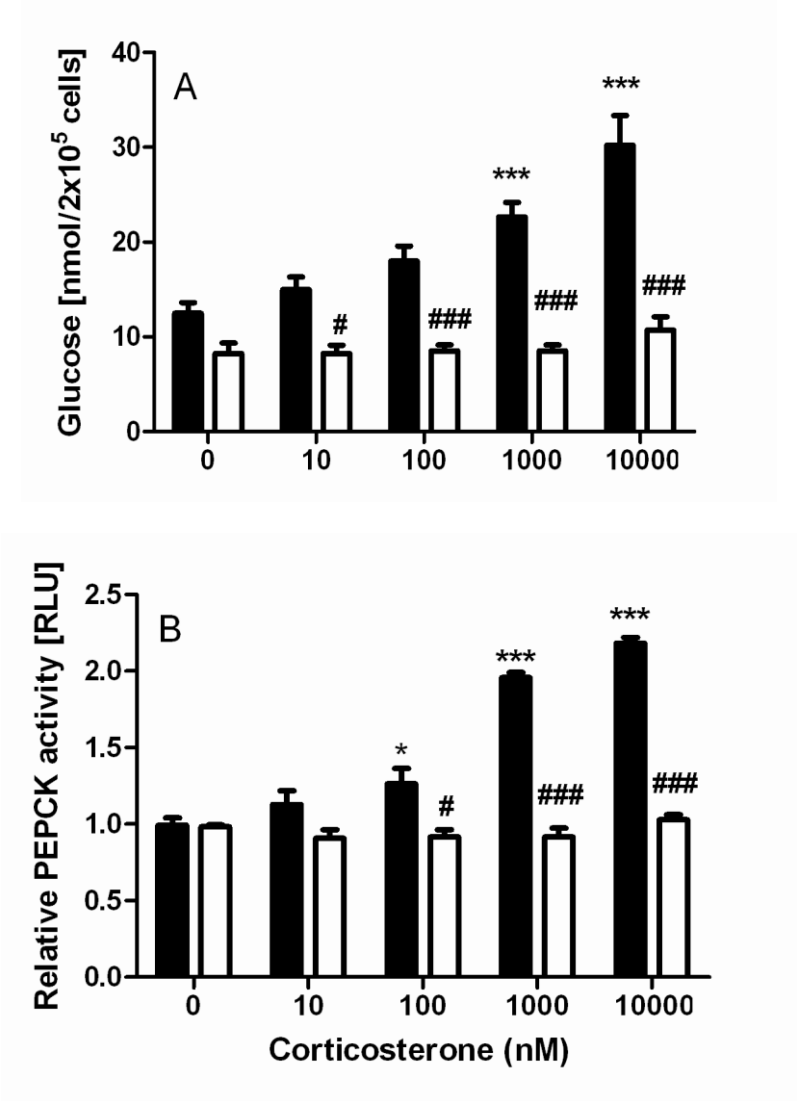


Figure 3.5 Effect of corticosterone on glucose production and PEPCK activity in H4-II-E-C3 cells

Fasted H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing corticosterone (10 – 10,000 nM) without (dark bars) and with (white bars) insulin (10 ng/ml). (A) Glucose measurements at 6 h. (B) PEPCK activity measurements in cells at 6 h. n=8. Statistical differences were determined by two-way ANOVA followed by Bonferroni post test (=P<0.05; ***=P<0.001 vs control; # = P < 0.05, ###=P<0.001 vs the corresponding cAMP treatment without insulin)*

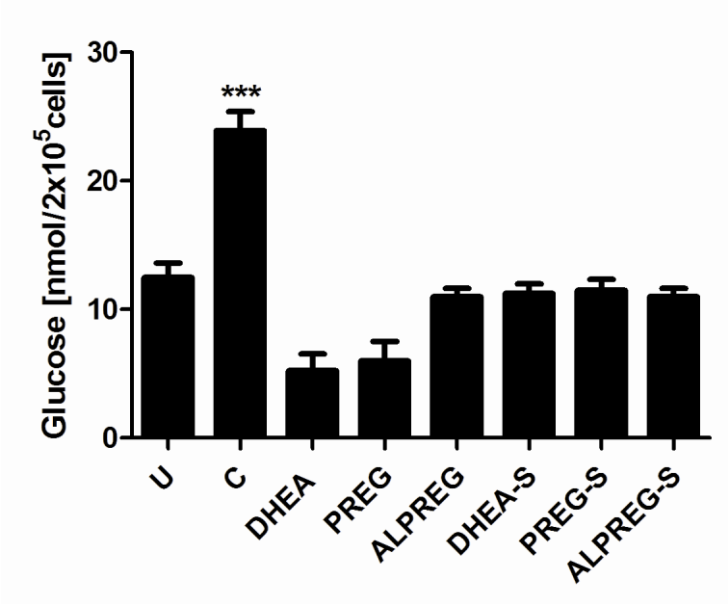


Figure 3.6 Effect of some common adrenal steroids on H4-II-E-C3 glucose production.

*Starved H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing corticosterone (C, 1 µM), dehydroepiandrosterone (DHEA 1 µM), pregnenolone (PREG 1 µM), Allo-pregnenolone (ALPREG 1 µM), DHEA sulphate (DHEA-S 1 µM), PREG sulphate (PREG-S 1 µM), and ALPREG sulphate (ALPREG-S 1 µM). n=6. Statistical differences were determined by one-way ANOVA (***=P<0.001 vs untreated cells).*

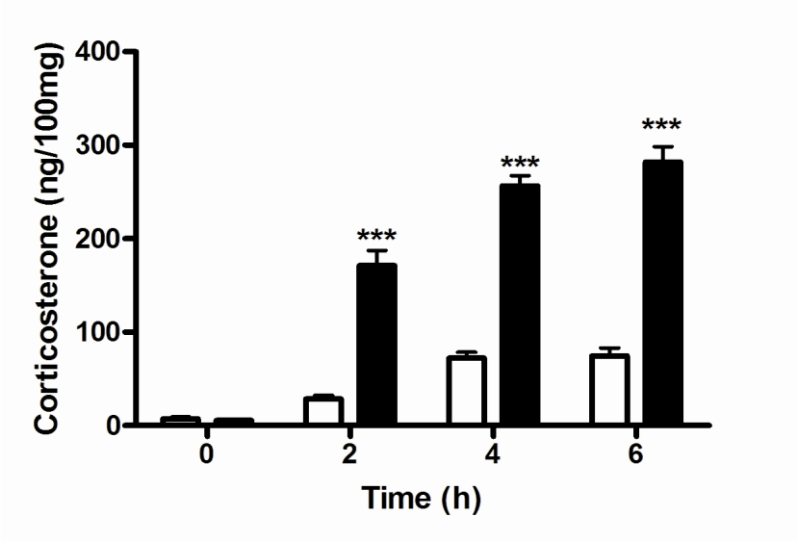


Figure 3.7 ACTH induced corticosterone production by adrenal sections.

*Adrenal sections were incubated in DMEM for 6h. Medium was collected and assayed for corticosterone levels. The clear bars show corticosterone (ng/100mg tissue) production by untreated adrenal gland sections and the dark bars show corticosterone production from adrenal sections stimulated with ACTH (10 nM). Statistical differences were determined by two-way ANOVA followed by Bonferroni post test. (***=P<0.001 compared to the corresponding incubation time without ACTH).*

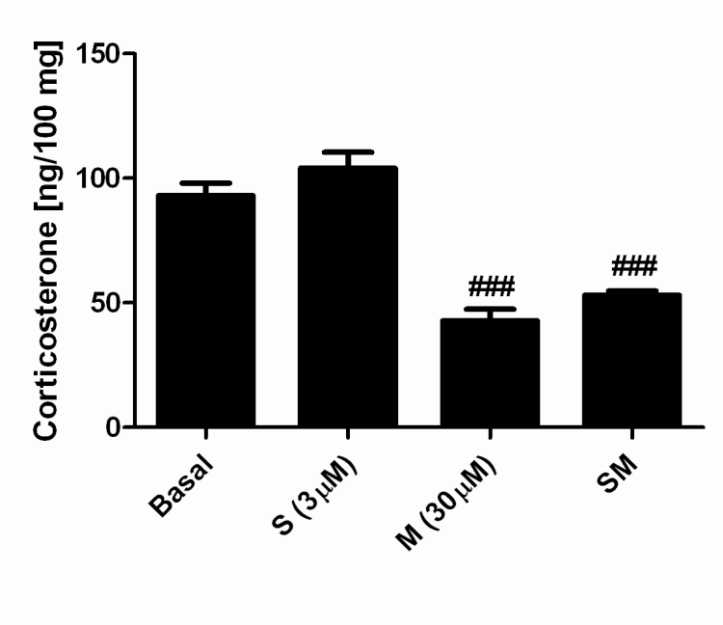


Figure 3.8 Secretion of corticosterone from adrenal sections incubated with sauvagine and metyrapone.

Adrenal sections were incubated for 6 h in DMEM (5 mM glucose) under basal conditions (B), sauvagine (S, 3µM), metyrapone (M, 30 µM), or sauvagine and metyrapone (S, 3 µM; M, 30 µM). n=6. Statistical analysis was performed using one-way ANOVA (###=P<0.001 vs B).

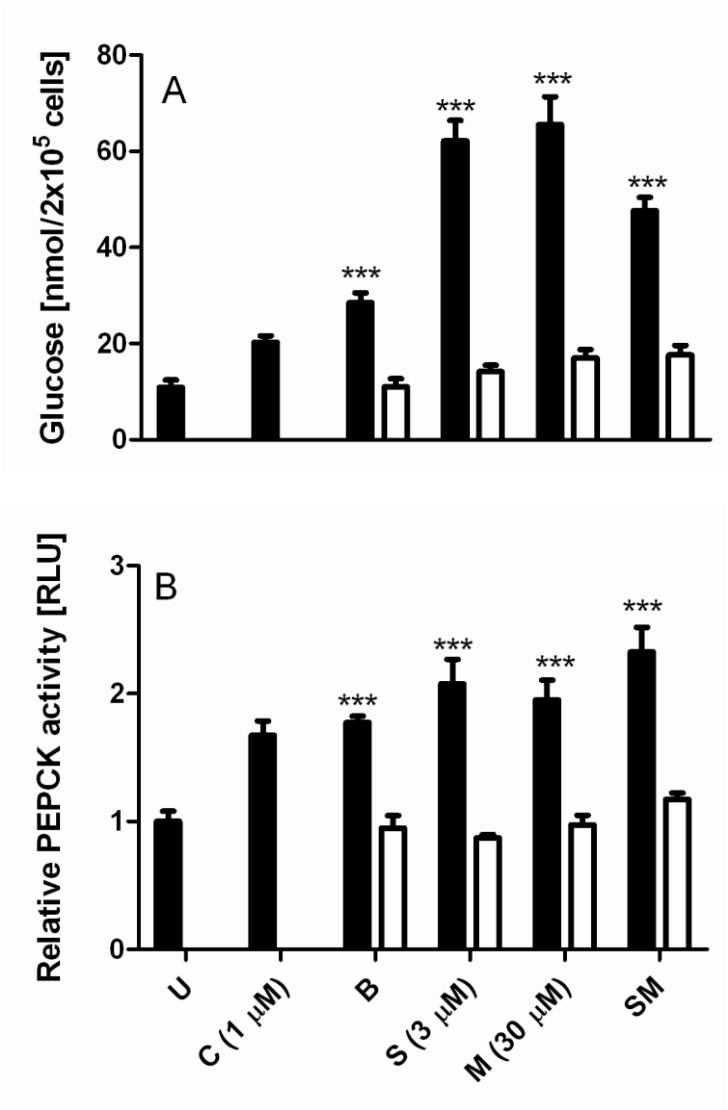


Figure 3.9 Comparison of biological activity of SPE extracts of adrenal incubations.

*SPE extracts from Sep-pak C_{18} cartridges (dark bars) and Strata-X AW columns (white bars) were reconstituted in EBSS (20 mM lactate/2 mM pyruvate) and incubated on fasted H4-II-E-C3 cells for 6 h. U= control EBSS. C=corticosterone (1 μM), B, S, M and SM correspond to adrenal incubation conditions described in figure 3.9. (A) Total glucose production was measured in media samples. (B) PEPCK activity is measured in cell lysate. n=6. Statistical differences were determined by two-way ANOVA followed by Bonferroni post test. (***=P<0.001 vs U).*

3.5 Discussion

Optimisation of conditions for studying hepatic glucose production used lactate and pyruvate as gluconeogenic substrates. In this study the ratio of lactate/pyruvate were optimised to maximize total glucose production in cell culture using H4-II-E-C3 cells. A lactate/pyruvate ratio of (10:1) in EBSS produced the maximal glucose in 6 h and was standardised as the substrate concentrations for this study. This ratio has previously been shown to be optional in hepatocytes isolated from starved rats (Rigoulet *et al.*, 1987).

Total glucose production is a result of both glycogenolysis and gluconeogenesis. Experiments were performed to observe the difference in glucose production during fed and fasted sates. Gluconeogenic genes are up-regulated during fasted states and should result in increased glucose production in the presence of gluconeogenic substrates (Owen *et al.*, 1969). Results in this chapter show that in the fasted states H4-II-E-C3 cells produce more glucose at any given time for the same substrate concentrations. These glucose release assays include both glucose uptake and release simultaneously. The net glucose release into the media in the presence of gluconeogenic substrates such as lactate and pyruvate and during starving is mostly attributed to increased gluconeogenic genes (Rigoulet *et al.*, 1987).

All further experiments with treatment conditions were carried out on starved cells. Well known gluconeogenic stimulators (Liu *et al.*, 2008) were initially tested under these experimental conditions. 8 Br-cAMP, glucagon and corticosterone produced a concentration dependent increase in glucose production and PEPCK activity which was suppressed to below control/untreated levels in the presence of insulin (10 ng/ml).

In order to study the role of adrenal corticosteroids in hepatic glucose production, *in vitro* adrenal incubations were performed. Adrenal glands were removed from male Wistar rats and the peri-capsular fat was quickly removed and the gland was chopped into four quarters, which were washed in PBS to minimise background levels of steroids due to any stress response occurring during animal handling. In order to confirm adequate responses in the adrenal incubations, initial experiments were performed to measure corticosterone production in the presence of ACTH ($1x10^{-9}$ M). The adrenal glands produced 4 fold more corticosterone with ACTH stimulation compared to un-stimulated adrenal glands. The corticosterone levels produced by the adrenal glands under basal conditions were comparable with published results (White *et al.*, 1992).

Adrenal incubation media collected from adrenal glands incubated with sauvagine and metyrapone were tested for biological activity. SPE of the steroids present in the adrenal incubation media was performed using two different columns. Sep-pak C_{18} columns were used to extract all steroids. This extraction was optimised using DMEM spiked with corticosterone. The optimised method provided >80% recovery of corticosterone.

Strata-X columns contain a weak anion exchange resin and are used to extract steroid conjugates from the adrenal incubation media. Initial experiments with spiked DMEM provided less than 10% recovery of corticosterone. This extraction was essential to determine the nature of the biological activity observed in hepatocyte incubations with adrenal media extracts. Sep-pak extracts of sauvagine and metyrapone caused a significant increase in total glucose production and relative PEPCK activity compared to untreated, corticosterone (1μM) and basal extracts. The corresponding Strata-X

extracts did not cause any significant change in glucose production or in PEPCK activity.

The corticosterone levels present in the adrenal incubations varied according to the different treatments. Basal and sauvagine incubations had maximum values that approximated to 100 nM corticosterone. Metyrapone incubations had significantly low corticosterone levels of around 40 nM. Despite these low levels of corticosterone present in the metyrapone incubations, the Sep-pak extracts increased glucose production to 2 fold the level of extracts from basal adrenal incubations, and to approximately 3.5 times the level of glucose production of cells incubated with corticosterone (1 µM). Even the extracts from adrenal incubations under basal conditions, with corticosterone levels below 100 nM produced 1.5 times the glucose output compared to that of cells incubated with corticosterone (1 µM). These results raise an important question as to the exact role of corticosterone in hepatic glucose production and the importance of the other steroid components in the extracts that are responsible for this gluconeogenic effect. Even under extreme stress conditions the circulating blood corticosterone levels never increase over (0.5 µg /mL) (Dugovic *et al.*, 1999). This implies that other endogenous factors are likely critical for a stress-induced hyperglycaemic response.

The results in this chapter conclude that adrenal incubations contain gluconeogenic steroids in addition to corticosterone, which are unlikely to be conjugated. Whether these steroids act in synergy with corticosterone or have an effect on their own is yet to be determined.

CHAPTER 4
Characterisation of gluconeogenic adrenal steroids

4.1 Introduction

The preliminary studies to evaluate the conditions for bioassay of secreted gluconeogenic steroids showed that despite low levels of corticosterone in extracts prepared from adrenal incubations with sauvagine and metyrapone, they had significant glucose producing activity. This chapter describes the investigations by high performance liquid chromatography used to characterise the steroids present in the conditioned media samples.

The historically important, initial experiments of extraction and characterisation of steroids from adrenal extracts involved complex chemical methods. Only six of the twenty-eight crystalline compounds that were isolated could prolong the life of adrenalectomised animals (Reichstein *et al*., 1938). Paper–partition chromatography was one of the earliest methods used to isolate and characterise the adrenal steroids (Zafforoni *et al*., 1951).

Gas chromatography coupled to mass spectrometry (GC–MS) is now considered the gold standard for quantification of steroids. It is highly specific and has been applied to quantify a large number of steroids (Ichimura *et al.*, 1986; Shackleton, 1993). However, this technique requires complicated and time-consuming sample preparation procedures which are not suitable for the needs of this project.

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is a better alternative to quantify steroids. Methods have been described for the determination of cortisol and cortisone (Taylor *et al.*, 2002), 17-hydroxyprogesterone (Etter *et al.*, 2006), 11-desoxycortisol (Guo *et al.*, 2006) and corticosterone (Ghulam *et al.*, 1999). Derivatisation is often necessary to achieve the required sensitivity to quantify endogenous levels of steroids.

Although all these methods are suitable to simultaneously quantify and study the steroid profiles under different conditions, a simple HPLC method was used in this study. Fractions were collected and dried and reconstituted in incubation media to test the activity on cells.

AIMS of this chapter:

1. Define a HPLC separation method to identify specific gluconeogenic steroids that are secreted during adrenal incubations with sauvagine and metyrapone.
2. Characterise the activity of potential gluconeogenic steroids.

4.2 Experimental

4.2.1 Chemicals and reagents

Corticosterone, 11-deoxycorticosterone (DOC) and 11-deoxycorticosterone acetate (DOCA) were obtained from SIGMA (Dorset, UK). 18-OH-DOC was purchased from Steraloids, UK.

4.2.2 Chromatography

HPLC was performed with a MERCK HITACHI L-6200 dual piston pump. Corticosteroids were detected at 240 nm using a Shimadzu SPD-6A UV detector. Chromatographic data was collected and processed by PRIME software. Chromatography was undertaken using a reverse phase Hichrom H5ODS H3724 column (4.6 x 150 mm).

A number of HPLC conditions were evaluated for their potential to separate the steroid components in extracts for identification of gluconeogenic activity. The flow rate for all these gradient conditions was 1.5 ml/min. A blank run was always conducted between steroid standards and test samples. For test samples, fractions (1 min duration) were collected automatically on a fraction collector throughout each HPLC run.

4.2.2.1 Conditions set 1: The column was equilibrated with a solvent mixture consisting of 7.7% methanol, 7.7 mm H_3PO_4 and 30% CH_3CN and eluted with a linear gradient over 20 min to 6.6% methanol, 6.6 mM H_3PO_4 and 40% CH_3CN. The column was further eluted with a gradient over 40 min to 0% methanol, 0 mM H_3PO_4 and 100% CH_3CN. The elution conditions were maintained at 100% CH_3CN for 5 min and the

column was then re-equilibrated to 7.7% methanol, 7.7 mM H_3PO_4 and 30% CH_3CN for 5 min.

4.2.2.2 Conditions set 2: The column was equilibrated with a solvent mixture consisting of 7.7% methanol, 7.7 mm HCl and 30% CH_3CN and eluted with a linear gradient over 20 min to 6.6% methanol, 6.6 mM HCl and 40% CH_3CN. The column was further eluted with a gradient over 40 min to 0% methanol, 0 mM HCl and 100% CH_3CN. The elution conditions were maintained at 100% CH_3CN for 5 min and the column the re-equilibrated to 7.7% methanol, 7.7 mM HCl and 30% CH_3CN for 5 min. This method was adapted from condition set 1 to overcome incompatibility for bioassay purposes of fractions containing H_3PO_4.

4.2.2.3 Conditions set 3: The column was equilibrated with a solvent mixture consisting of 8.5% methanol, 8.5 mM HCl and 15% CH_3CN and eluted with a linear gradient over 25 min to 6.0% methanol, 6.0 mM HCl and 40% CH_3CN. The column was further eluted with a gradient over 40 min to 0% methanol, 0 mM HCl, 90% CH_3CN and 0.01% tri-fluoro acetic acid (TFA). The elution conditions were maintained at 90% CH_3CN and 0.01% TFA for 5 min and the column was then re-equilibrated to 8.5% methanol, 8.5 mM HCl and 15% CH_3CN for 5 min.

4.2.2.4 Conditions set 4: The column was equilibrated with a solvent mixture consisting of 8.5% methanol, 8.5 mM HCl and 15% CH_3CN and eluted with a linear gradient over 30 min to 7.0% methanol, 7.0 mM HCl and 30% CH_3CN. The column was further eluted with a gradient over 40 min to 0% methanol, 0 mM HCl, 90% CH_3CN and 0.01% TFA. The elution conditions were maintained at 90% $CH3_3CN$ and 0.01% TFA for 5 min and the column then re-equilibrated to 8.5% methanol, 8.5 mM HCl and 15% CH_3CN for 5 min.

4.2.2.5 HPLC condition set 5

The column was equilibrated with a solvent mixture consisting of 8.0% H_20, 8.0 mM HCl and 20% CH_3CN and eluted with a linear gradient over 25 min to 5.8% methanol, 5.8 mM HCl and 42% CH_3CN. The column was further eluted with a gradient over 40 min to 0% methanol, 0 mM HCl, 90% CH_3CN and 0.01% TFA. The elution conditions were maintained at 90% $CH3_3CN$ and 0.01% TFA for 5 min and the column then re-equilibrated to 8.0% H_20, 8.0 mM HCl and 20% CH_3CN for 5 min.

4.2.2.6 HPLC condition set 6

The column was equilibrated with a solvent mixture consisting of 8.5% H_20 , 8.5 mM HCl and 15% CH_3CN and eluted with a linear gradient over 30 min to 7.0% H_20, 7.0 mM HCl and 30% CH_3CN. The column was further eluted with a gradient over 40 min to 0% H_20, 0 mM HCl, 90% CH_3CN and 0.01% TFA. The elution conditions were maintained at 90% $CH3_3CN$ and 0.01% TFA for 5 min and the column then re-equilibrated to 8.5% H_20, 8.5 mM HCl and 15% CH_3CN for 5 min.

4.2.3 Method for DOCA hydrolysis.

DOCA solution was incubated with 1M $NaHCO_3$ solution overnight in a 37°C water bath. The incubated solution was centrifuged and the steroids in the supernatant were extracted using Sep-pak C18 cartridges as described in section 3.3.7.

4.2.4 Bioassay of HPLC fractions

HPLC fractions were dried down with a stream of N_2 at 40°C, reconstituted in EBSS and incubated with H4-II-E-C3 cells in 24 well plates for 6 h. Media was collected and assayed for total glucose as described in section 3.3.2.

4.2.5 In vivo studies of the effect of sauvagine and metyrapone on blood glucose.

Male Wistar rats (220 – 300 g; Charles River, UK) were used. In the days prior to the experiment, rats were given standard chow and water *ad libitum* and subjected to a 12 h dark/light cycle. On the day of the experiment the rats were anaesthetised by i.p injection of thiopentone sodium (Intraval sodium; 100 mg/kg). Rats were tracheotomised and catheters were inserted in the carotid artery and jugular vein using polyethylene catheters of tubing size PP50 and PP25 respectively (Portex, UK). Experiments were conducted after a 30 min stabilisation period. Sauvagine treated rats were initially given an i.p injection of saline (200 µl) 60 min prior to sauvagine infusion for 25 min with sauvagine at the rate of 20 pmol/kg/min. For control rats, an initial i.p injection of saline was given 60 min prior to saline infusion for 25 min. For sauvagine and metyrapone treatments, rats were given an i.p injection of metyrapone (200 mg/kg) 60 min prior to sauvagine infusion for 25 min at the rate of 20 pmol/kg/min.

Blood glucose measurements were made using Accu-chek Aviva (Roche Ltd) by placing a drop of blood collected by pricking the tail vein at 15 min time intervals. At the end of the experiment 3 ml of arterial blood was collected to prepare plasma. Liver sections were snap frozen in liquid nitrogen for PEPCK activity measurements. Adrenals removed and subjected to incubation in DMEM for 6 h as described in section 3.3.4.

4.3 Results and discussion

Each experiment results discussed in this section is a representative of one or two experiments for the given condition. Mean ± SEM was not derived for the peak areas, but a comparison to the respective control under the same chromatographic condition was made. The main goal of these experiments was to generate fractions from the adrenal incubations to test for gluconeogenic activity on hepatocytes.

4.3.1 Initial evaluation of the HPLC methodology and comparison of adrenal incubation extracts.

Gradient elution was performed using the HPLC condition set 1 described in section 4.2.2.1. Corticosterone eluted at 6.1 min under these conditions.

4.3.2 Bioassay of the HPLC fractions collected using condition set 1.

Five consecutive fractions (0.5 ml) were pooled and dried under a stream of N_2. The dried fractions were reconstituted in EBSS (20 mM lactate/2 mM pyruvate) and tested on H4-II-E-C3 cells as described in section 3.

The cells incubated with the reconstituted fractions did not survive the 6 h incubation time period. This was assumed to be due to the high phosphate concentrations arising from H_3PO_4 used in the elution buffer, even though fractions were neutralised with $NaHCO_3$ prior to cell incubation.

4.3.3 Comparison of HPLC results for media extracts from basal incubations with sauvagine and metyrapone.

As the reconstituted fractions from condition set 1 containing H_3PO_4 were toxic to the cells, it was replaced by 10 mM HCl as described in HPLC condition set 2 in section

4.2.2.2 as this would be readily neutralised after drying down. Fractions were collected at 1 min time intervals. The collected fractions (0.5 ml) were pooled in series of five, dried under a stream of N_2 gas and reconstituted in EBSS (20 mM lactate/2 mM pyruvate). These fractions were used for bioassay.

The HPLC method was first evaluated using corticosterone. A representative chromatogram for corticosterone is shown in Figure 4.1. The retention time (RT) for corticosterone was 5.9 min under the given conditions. Sep-pak extracts of the adrenal incubations collected from basal (B), sauvagine (S) and metyrapone (M) treatments were reconstituted in equilibration mix (1.5 ml) and injected into the HPLC system under the same conditions. The differences between the chromatograms of the adrenal extracts from the three conditions are shown in Figure 4.2.

A corticosterone peak was present on all three chromatograms eluting at 6.1 min (RT). The corticosterone peak for the sauvagine sample had a relatively similar peak area to that of the basal extract. The metyrapone extract showed a 41% reduction in peak area compared to basal corticosterone levels. Various peaks were consistently found in all three media extracts. Of interest were the peaks at RTs of 5.2, 6, 8.5, 11.5 and 24.6 min (Figure 4.2). The peak areas and the relative changes are shown in table 4.1. The peak at 5.2 min was unchanged in the sauvagine extract compared to basal extract, while there was a 31% decrease in the metyrapone extracts. The identity of this peak is unknown. Another observed change was in the peak at 8.5 min. This peak was markedly increased in the sauvagine extract while minimal in the basal and metyrapone extracts. The peak at 11.5 min, which was subsequently identified as DOC, increased in both the sauvagine and metyrapone extracts. The small peak at 24.6 min was relatively constant in all extracts.

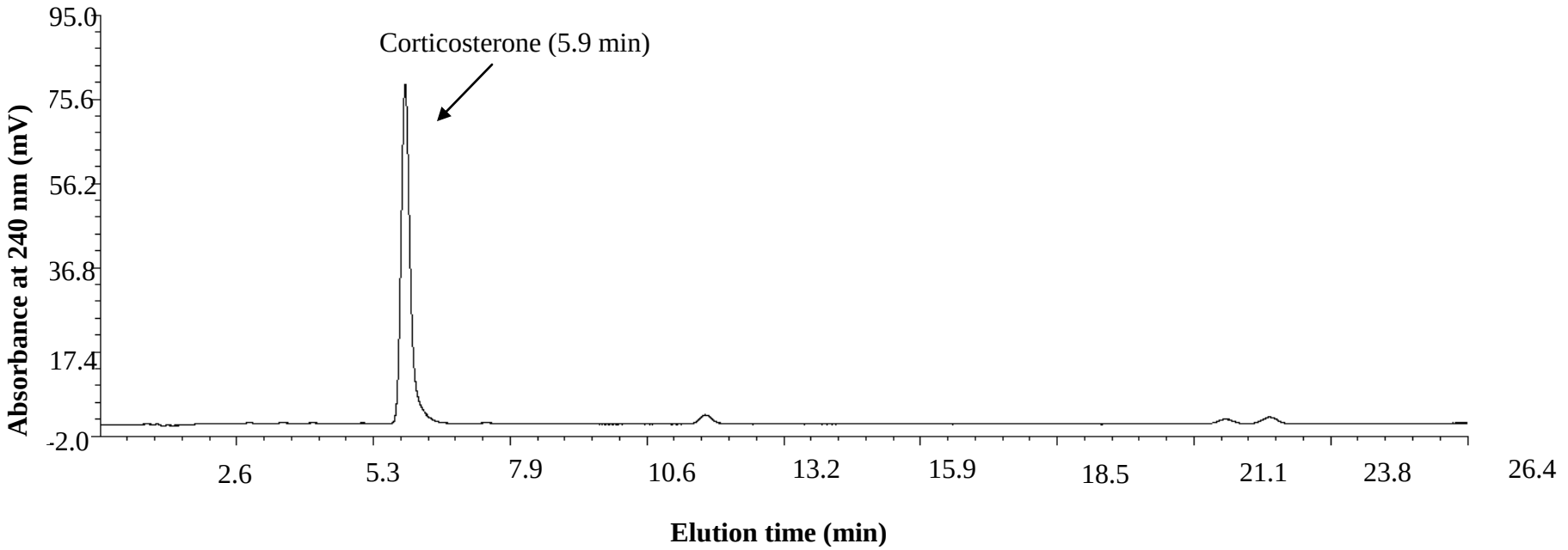


Figure 4.1 Representative chromatogram of corticosterone separation.

Column: Hypersil ODS (4.6 x 150 mm); Injection (0.6 nmol corticosterone); gradient: HPLC condition set 2 (section 4.2.2.2). Corticosterone RT is 5.9 min. Chromatogram integration and data analysis was done using PRIME software.

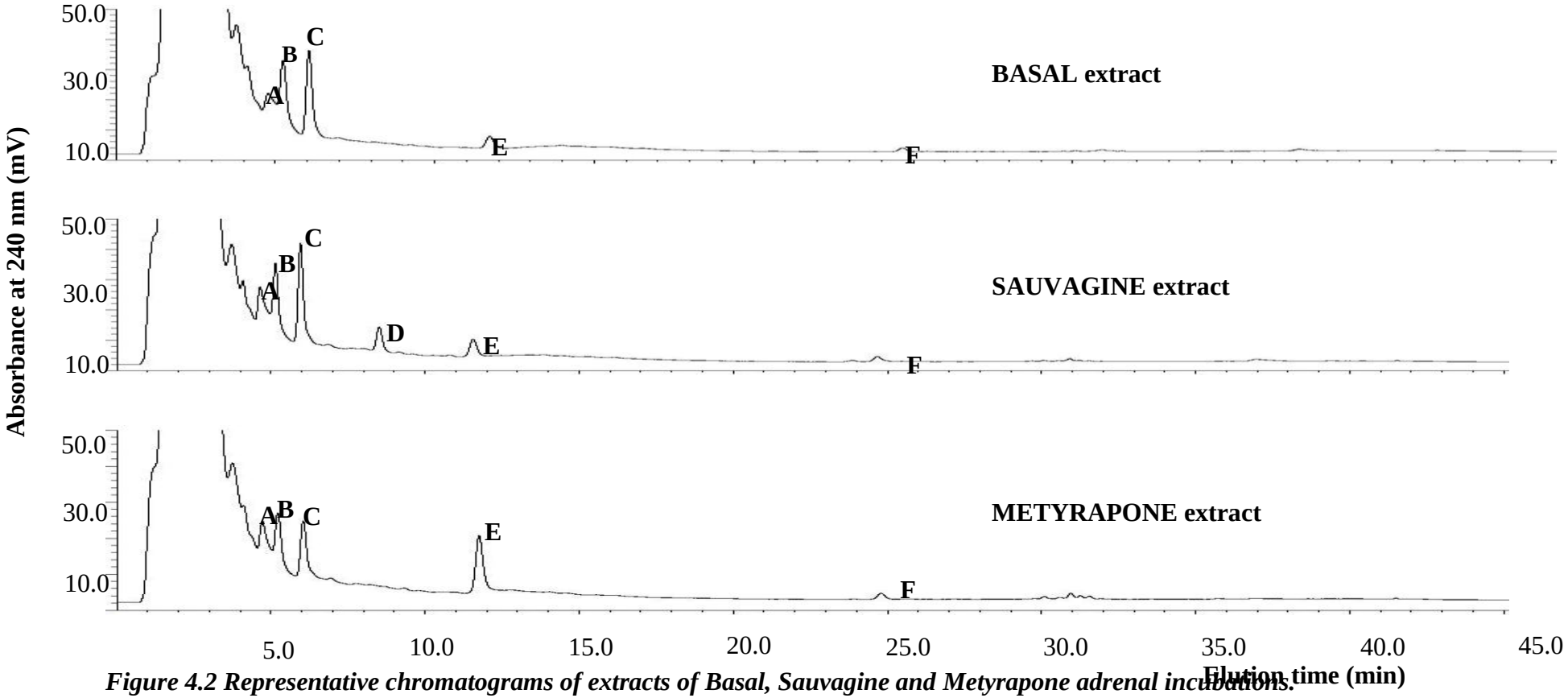


Figure 4.2 Representative chromatograms of extracts of Basal, Sauvagine and Metyrapone adrenal incubations.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Injection (1.5 ml of reconstituted adrenal extract in equilibration mix). Gradient setting: HPLC condition set 2 (section 4.2.2.2). Flow rate (1.5 ml/min). Peaks: (A) unknown 1 (4.7 min), (B) unknown 2 (5.2 min), (C) corticosterone (6.1 min), (D) unknown 3 (8.5 min), (E) unknown 4 (11.7 min), and (F) unknown 5 (24.6 min). Chromatogram integration and data analysis was done using PRIME software.

Table 4.1 Comparison of HPLC peak areas for extracts from adrenal incubations with basal, sauvagine and metyrapone.

Peak (RT)	Basal extract Peak Area	Sauvagine extract Peak Area	Metyrapone extract Peak Area
A (4.7 min)	82.4	156.5	120.3
B (5.2 min)	231.3	217.4	159.6
C (6.1 min)	470.0	505.0	277.1
D (8.5 min)	6.1	130.4	11.5
E (11.7 min)	87.6	117.7	337.08
F (24.6 min)	28.5	41.2	37.5

4.3.4 Bioassay of HPLC fractions.

HPLC fractions (0.5 ml) were pooled in series of 5 and dried under a stream of N_2 gas. The dried fractions were reconstituted in EBSS (1ml) containing 20 mM lactate and 2 mM pyruvate. Reconstituted fractions (300 µl) were then incubated on fasted H4-II-E-C3 cells in a 24 well plate for 6 h. Medium was collected and assayed for glucose (Figures 4.1, 4.2 and 4.3)

Untreated cells incubated with substrates produced 8 ± 1 glucose (nmol/2 x 10^5 cells). Glucose production with basal adrenal extract (containing 33 ng/ml ~ 0.1 µM corticosterone) and corticosterone (1 µM) was 31 ± 2 and 19 ± 1 nmol glucose/2 x 10^5 cells respectively. Direct incubation of the reconstituted fraction pools did not stimulate

any significant glucose production apart from the fraction pool (6 – 10) which produced 13 ± 1 nmol glucose/2 x 10^5 cells compared to control untreated cells (Figure 4.3). Glucose production with the fractions incubated along with corticosterone (1 µM) did not differ significantly from incubations with corticosterone alone (Figure 4.3). Sauvagine extracts (containing corticosterone ~ 0.1 µM) produced 58 ± 4 (nmol/2 x 10^5 cells) glucose (Figure 4.4). None of the collected fraction pools showed any significant glucose production compared to the untreated cells. To test whether gluconeogenic activity was facilitated by corticosterone, fractions were also incubated in its presence. However, fractions incubated with corticosterone (1 µM) did not show any significant increases in glucose production from the incubations with corticosterone alone (Figure 4.4). Although, there was a tendency towards increased glucose production with fraction pools 6 – 10 and 11 – 15 producing 22 ± 1 and 29 ± 9 glucose (nmol /2 x 10^5 cells) respectively. Similarly, metyrapone extracts (containing corticosterone ~ 0.04 µM) produced 73 ± 3 glucose (nmol /2 x 10^5 cells), but none of the fraction pools showed any significant glucose production compared to the untreated cells. Fractions incubated along with corticosterone did not show any significant increases in glucose production from incubations with corticosterone alone. Fraction pool (11 – 15) showed slight increases with 22 ± 4 glucose (nmol /2 x 10^5 cells). These values were not significantly greater compared to incubations with corticosterone alone which produced 21 ± 1 (nmol glucose/2 x 10^5 cells).

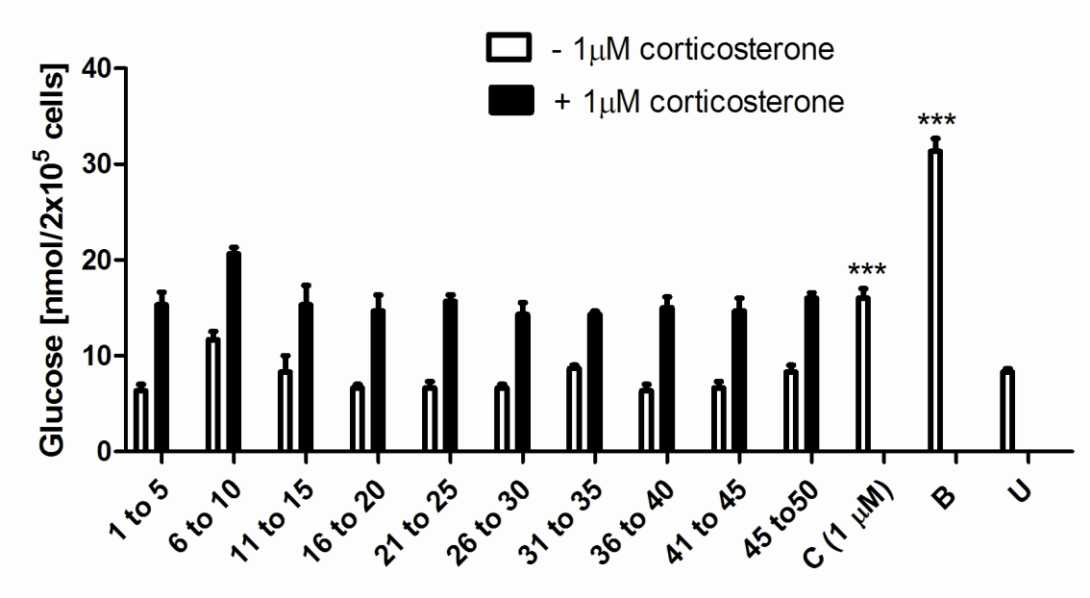


Figure 4.3 Effect of HPLC fractions obtained from adrenal medium from basal incubations on hepatocyte glucose production.

*Starved H4-II-E-C3 cells were incubated for 6 h with the pooled samples of HPLC fractions in EBSS (20 mM lactate/2 mM pyruvate) with (dark bars) and without (white bars) corticosterone (1 μM). Corticosterone alone (C), extract of basal adrenal media (B), untreated cells with EBSS and substrates (U). Glucose was measured in the conditioned media samples. Results are mean ± SEM (n=6). Statistical differences were determined by ANOVA (*** = P<0.001 vs U).*

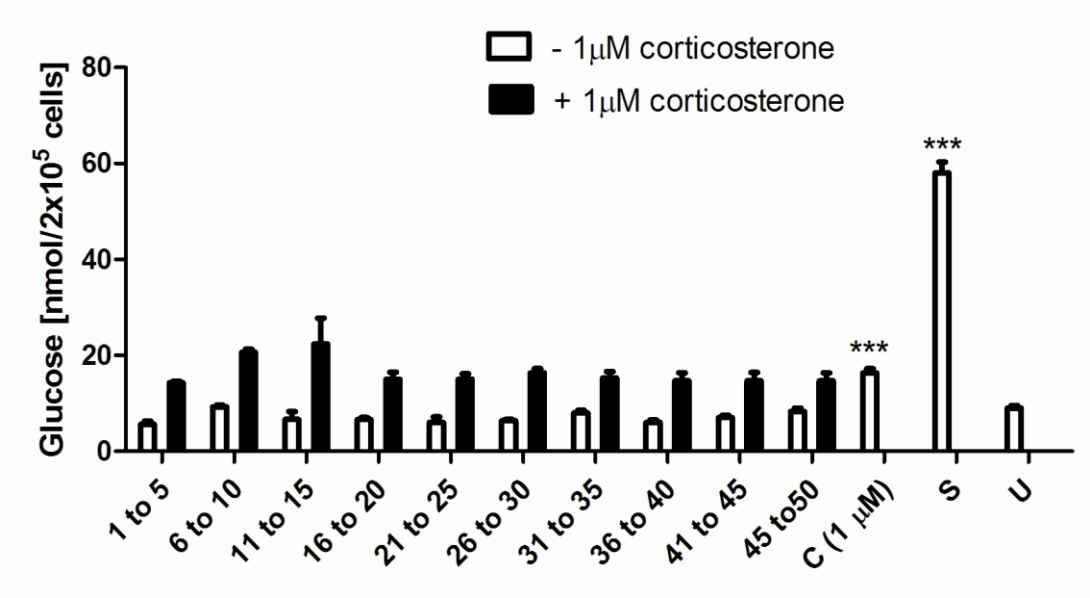


Figure 4.4 Effect of HPLC fractions obtained from adrenal medium from sauvagine incubations on hepatocyte glucose production.

*Starved H4-II-E-C3 cells were incubated for 6 h with the pooled samples of HPLC fractions in EBSS (20 mM lactate/2 mM pyruvate) with (dark bars) and without (white bars) corticosterone (1 μM). Corticosterone alone (C), extract of sauvagine adrenal media (S), untreated cells with EBSS and substrates (U). Glucose was measured in the conditioned media samples. Results are mean + SEM (n=6). Statistical differences were determined by ANOVA (*** = P<0.001 vs U).*

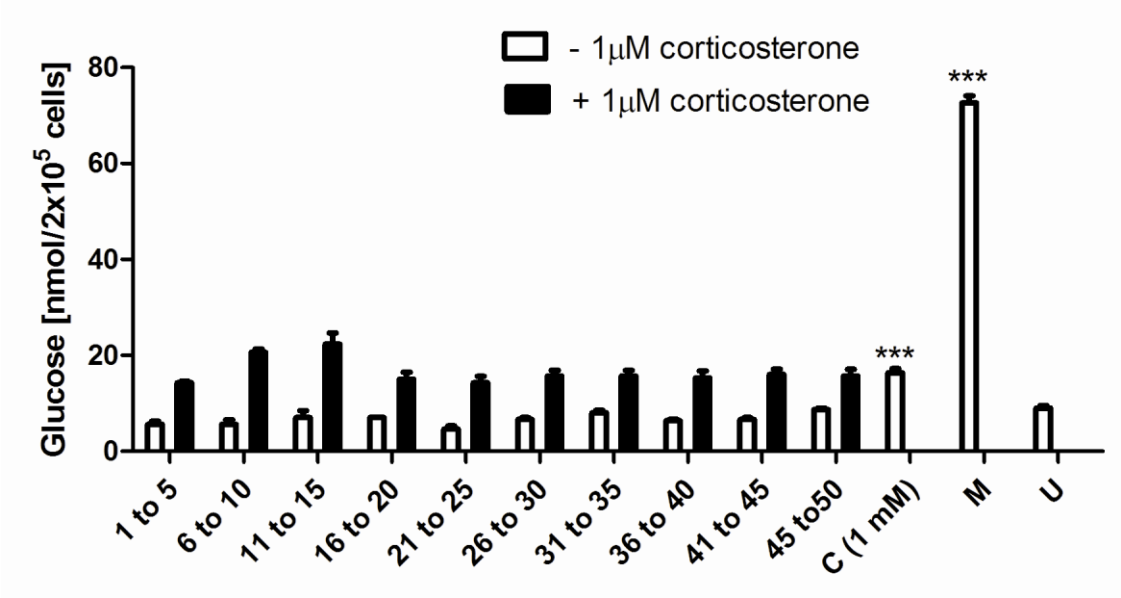


Figure 4.5 Effect of HPLC fractions obtained from adrenal medium from metyrapone incubations on hepatocyte glucose production.

*Starved H4-II-E-C3 cells were incubated for 6 h with the pooled samples of HPLC fractions in EBSS (20 mM lactate/2 mM pyruvate) with (dark bars) and without (white bars) corticosterone (1 μM). Corticosterone alone (C), extract of metyrapone adrenal media (M), untreated cells with EBSS and substrates (U). Glucose was measured in the conditioned media samples. Results are mean + SEM (n=6). Statistical differences were determined by ANOVA (*** = P<0.001 vs U).*

4.4 Impurities in the commercially available corticosterone and their effect on glucose production.

In the initial characterisation of the HPLC methodology it was observed that there were secondary peaks at 11.9 min similar to those observed in the adrenal extracts. In order to understand the importance of the contribution of the impurities found in the commercially available corticosterone on the hepatocyte glucose production a large scale purification of 25 mg of corticosterone (SIGMA; catalogue number C2505 Lot 109H0949) was carried out to obtain sufficient pure corticosterone for further studies of cell responses in the presence and absence of corticosterone (Figures 4.6 & 4.7).

For the large scale purification APEX hypersil C18 column (25 cm, 8 µ) was used with HPLC condition set 2 (section 4.2.2.2), with a flow rate of 2 ml/min. Absorbance was measured at 240 nm. Fractions were collected at 1 min time intervals.

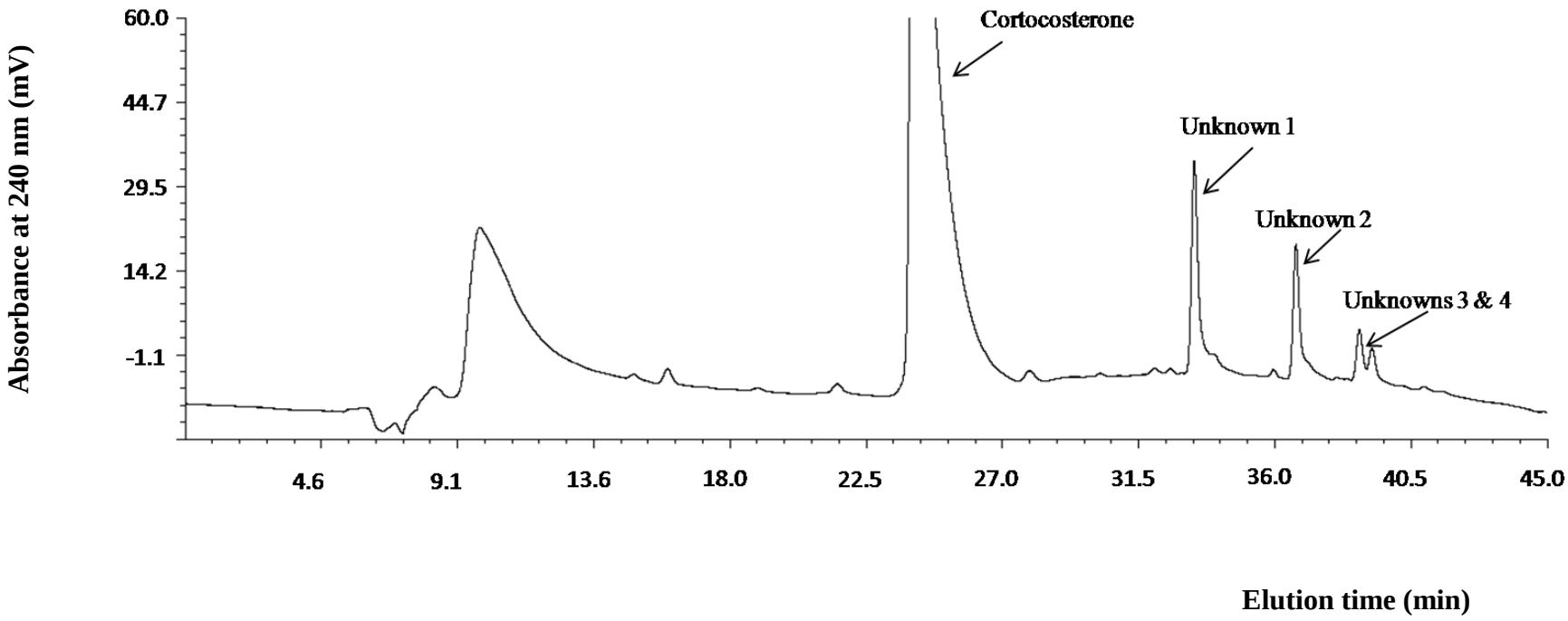


Figure 4.6 A typical chromatogram showing impurities in commercially available corticosterone.

Column: APEX prepsil ODS (10 x 250 mm); flow (2 ml/min); Gradient setting: HPLC condition set 2 (section 4.2.2.2). Peaks: Corticosterone (24.2 min), unknown 1 (33.3min), unknown 2 (36.7 min), unknown 3 (38.8 min). Chromatogram integration and data analysis was done using PRIME software.

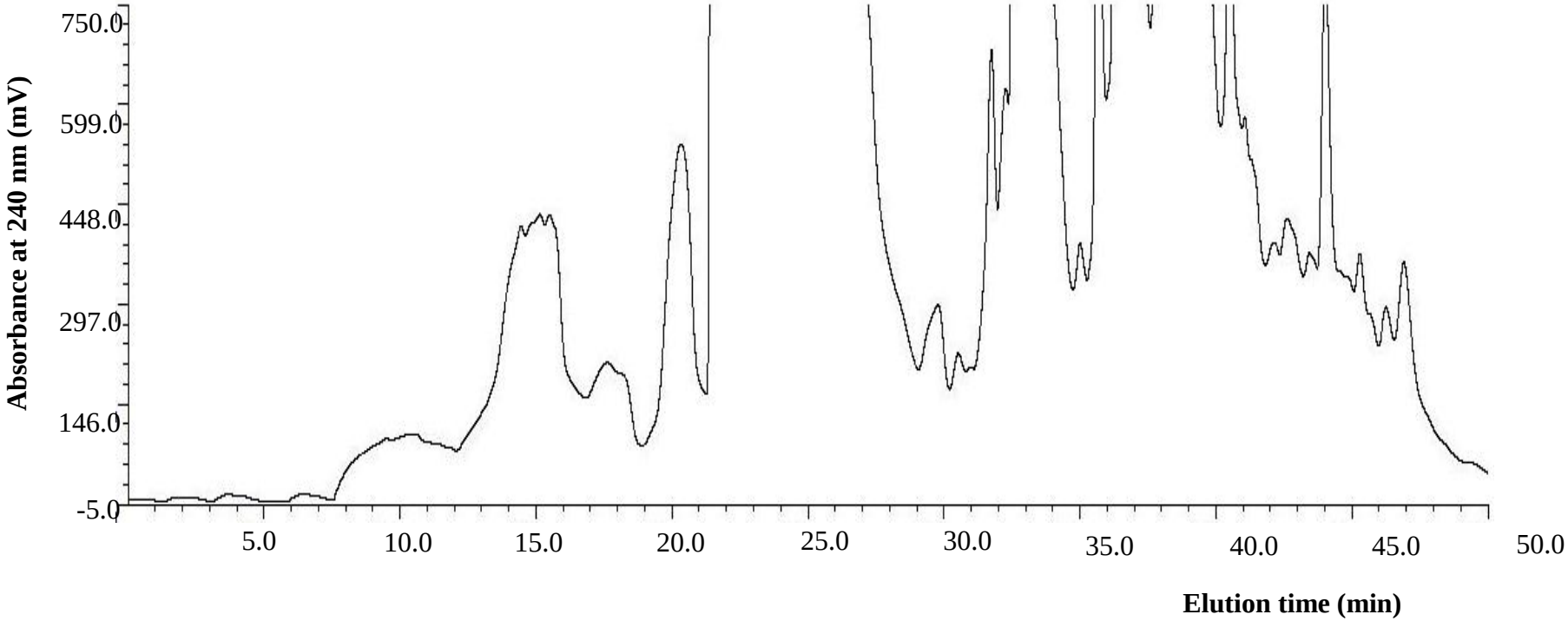


Figure 4.7 Representative chromatogram of large scale corticosterone purification.

Column: APEX prepsil ODS (10 x 250 mm); flow (2 ml/min); Gradient setting: HPLC condition set 2 (section 4.2.2.2). Fractions (1 min) were collected; Injection: Corticosterone (25 mg) in equilibration mix. Data integrated using PRIME software. Fraction 23 – 28 were collected to obtain pure corticosterone. UV detection at 240 nm.

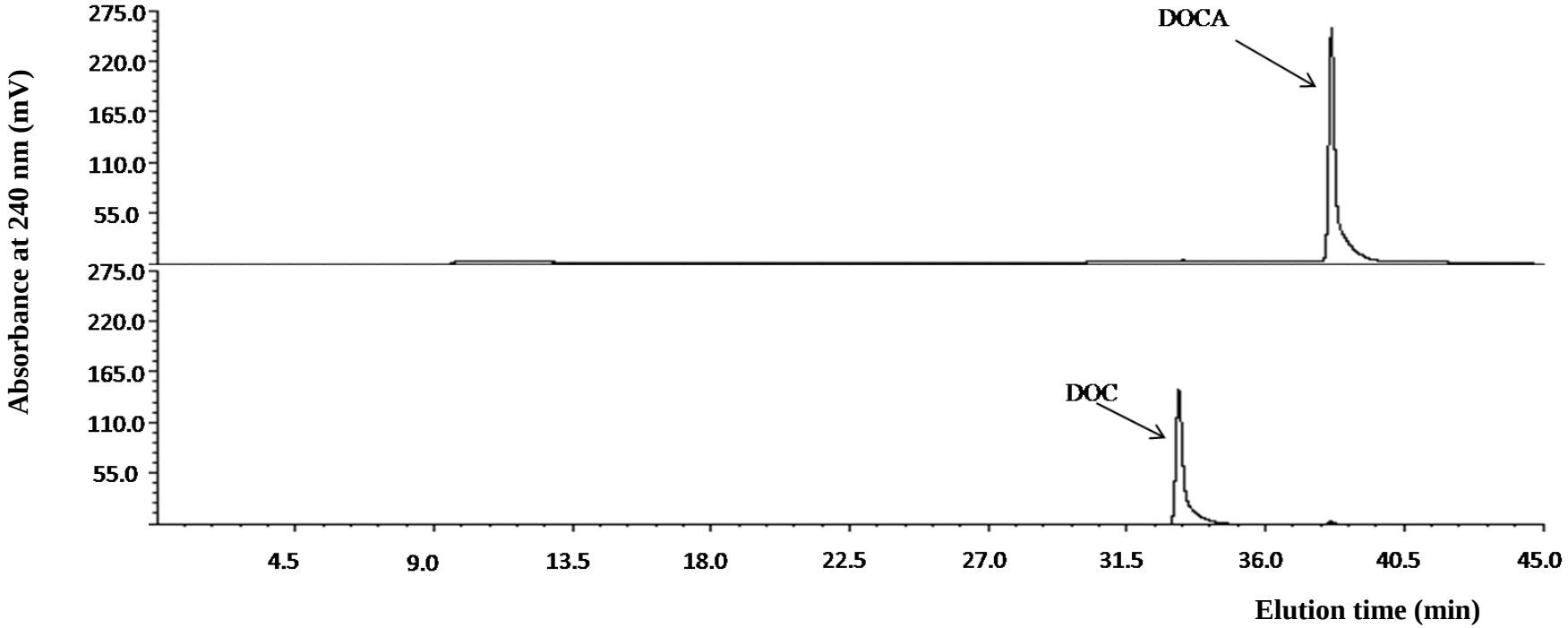


Figure 4.8 Representative chromatograms of DOCA and DOC.

Column: APEX prepsil ODS (10 x 250 mm); flow (2 ml/min); Gradient setting: HPLC condition set 2 (section 4.2.2.2). UV detection at 240 nm.

4.6 Identification of unknown peaks in commercially available corticosterone.

Deoxycorticosterone acetate (DOCA) was injected into the HPLC system under the same chromatographic conditions as used previously (section 4.2.2.2). DOCA eluted at 38.1 min (Figure 4.8) corresponding with one of the impurity peaks (unknown 3) eluted from commercially available corticosterone (Figure 4.6). Hydrolysis of DOCA produced DOC, which eluted at 33.1 min (Figure 4.8). This also corresponded to an impurity peak (unknown 1) observed in the corticosterone chromatogram (Figure 4.6).

4.7 Purification of media samples from adrenal incubations by higher resolution HPLC.

The HPLC method was changed to condition set 3 as described in section 4.2.2.3 to improve the resolution of the peaks before corticosterone in the chromatograms. This was the main part of the chromatogram showing some bioactivity, but it was unclear whether this was due to corticosterone or other undefined adrenal steroids. Under these new HPLC conditions DOC eluted at 19.8 min (Figure 4.9), 18-OH DOC eluted at 12.5 min (Figure 4.10), and corticosterone eluted at 13.6 min (Figures 4.11) (Table 4.2). Sep-pak extracts of the adrenal incubations under basal (B), sauvagine (S) and metyrapone (M) treatment conditions were reconstituted in equilibration mix (1.5 ml) and injected into the HPLC system under the same conditions. Other differences between the chromatograms of the adrenal extracts from the three conditions are shown in Figure 4.12.

Similar to previous observations DOC increased with sauvagine and metyrapone treatments. However, it was also observed that there was a double peak at 13.3 min and 13.6 min respectively that were not fully resolved (Figure 4.12) suggesting that a second peak (13.3 min) with similar elution characteristics to corticosterone was being resolved under these conditions.

4.8 Bioassay of fractions from higher resolution HPLC of adrenal media samples.

HPLC fractions (8 – 28) collected at 1 min time intervals were dried under a stream of N_2 gas and reconstituted in EBSS (1 ml) containing 20 mM lactate and 2 mM pyruvate. Reconstituted fractions (300 µl) were then incubated on fasted H4-II-E-C3 cells in a 24 well plate for 6 h. Media samples were collected and assayed for glucose (Figures 4.13, 4.14 and 4.15)

Untreated cells incubated with substrates produced 9 ± 1 glucose (nmol/2 x 10^5 cells). Glucose production with extract from basal incubation of adrenal sections and purified corticosterone (1 µM) was 31 ± 2 and 14 ± 1 glucose (nmol /2 x 10^5 cells) respectively. Direct incubation of the reconstituted fraction pools did not stimulate any significant glucose production in any of the fractions compared to control untreated cells (Figure 4.13). Glucose production with the fractions incubated together with purified corticosterone (1 µM) did not vary significantly from the incubations with corticosterone alone (Figure 4.14). Extracts of sauvagine stimulated adrenal media produced 57 ± 3 glucose (nmol/2 x 10^5 cells) (Figure 4.14). The collected fractions did not show any significant glucose production compared to the untreated cells. Fractions incubated with

purified corticosterone (1 μM) did not show any significant increases in glucose production compared to incubations with corticosterone alone (Figure 4.14). Extracts of media from metyrapone treated adrenals produced 69 ± 4 glucose (nmol /2 x 10^5 cells). But none of the fractions showed any significant glucose production compared to the untreated cells. Fractions incubated together with corticosterone did not show any significant increases in glucose production compared to incubations with corticosterone alone (Figure 4.15).

Standard compound	**Retention time (RT min)**
Corticosterone	13.6 min
18-OH 11-deoxycorticosterone	12.5 min
11-deoxycorticosterone	19.8 min

Table 4.2 Standard retention time with HPLC condition set 3

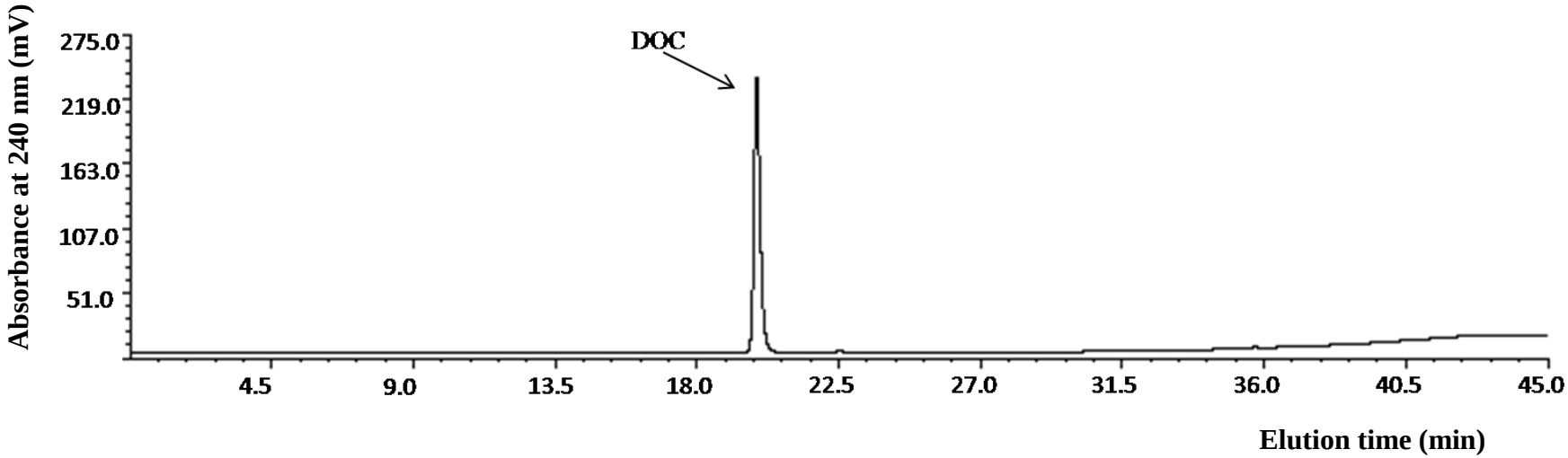


Figure 4.9 Representative chromatograms of DOC under HPLC condition set 3.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Gradient setting: HPLC condition set 3 (section 4.2.2.3). Flow rate (1.5 ml/min). DOCretention time 19.9 min. Chromatogram integration and data analysis was done using PRIME software.

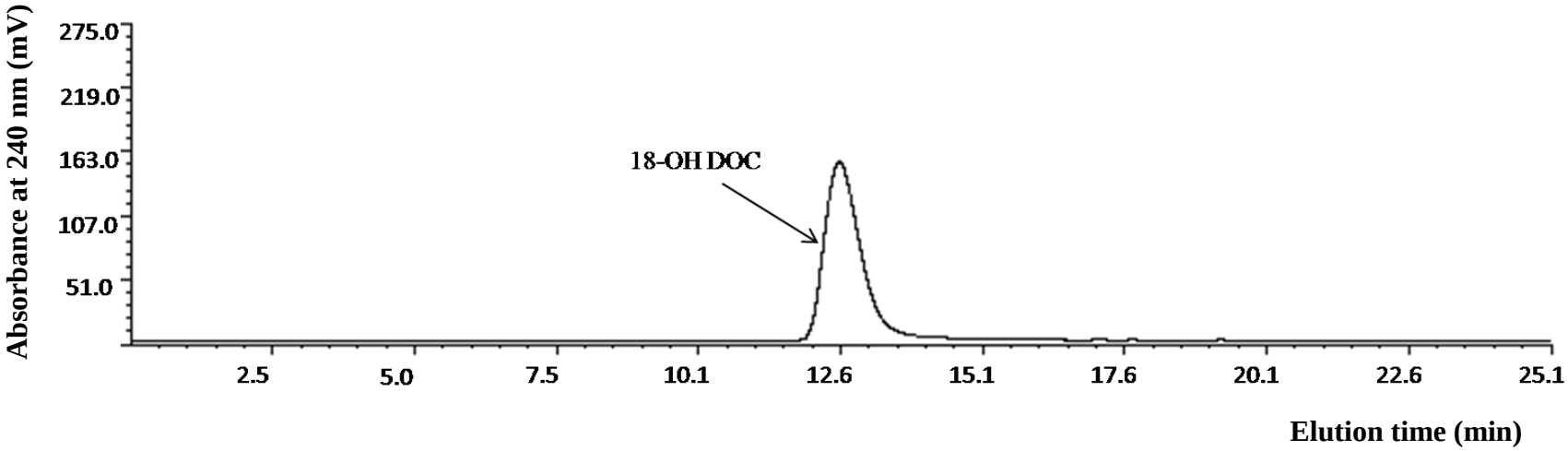


Figure 4.10 Representative chromatograms of 18-OH DOC under HPLC condition set 3.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Gradient setting: HPLC condition set 3 (section 4.2.2.3). Flow rate (1.5 ml/min). 18-OH DOC retention time 12.5 min. Chromatogram integration and data analysis was done using PRIME software.

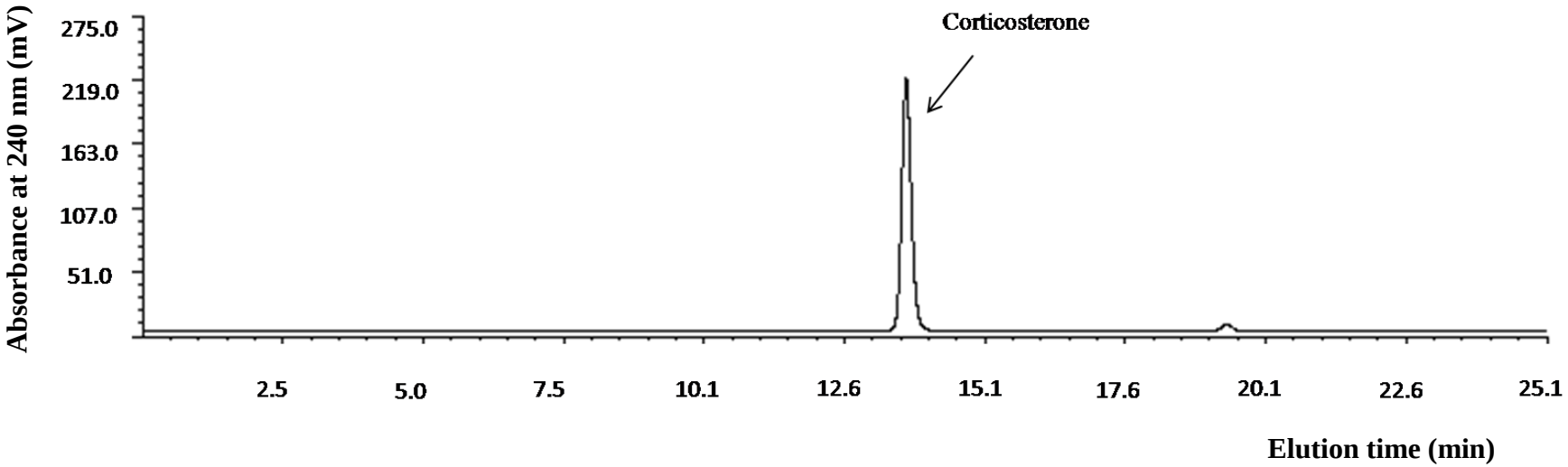


Figure 4.11 Representative chromatograms of corticosterone under HPLC condition set 3.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Gradient setting: HPLC condition set 3 (section 4.2.2.3). Flow rate (1.5 ml/min). Corticosterone retention time 13.6 min. Chromatogram integration and data analysis was done using PRIME software.

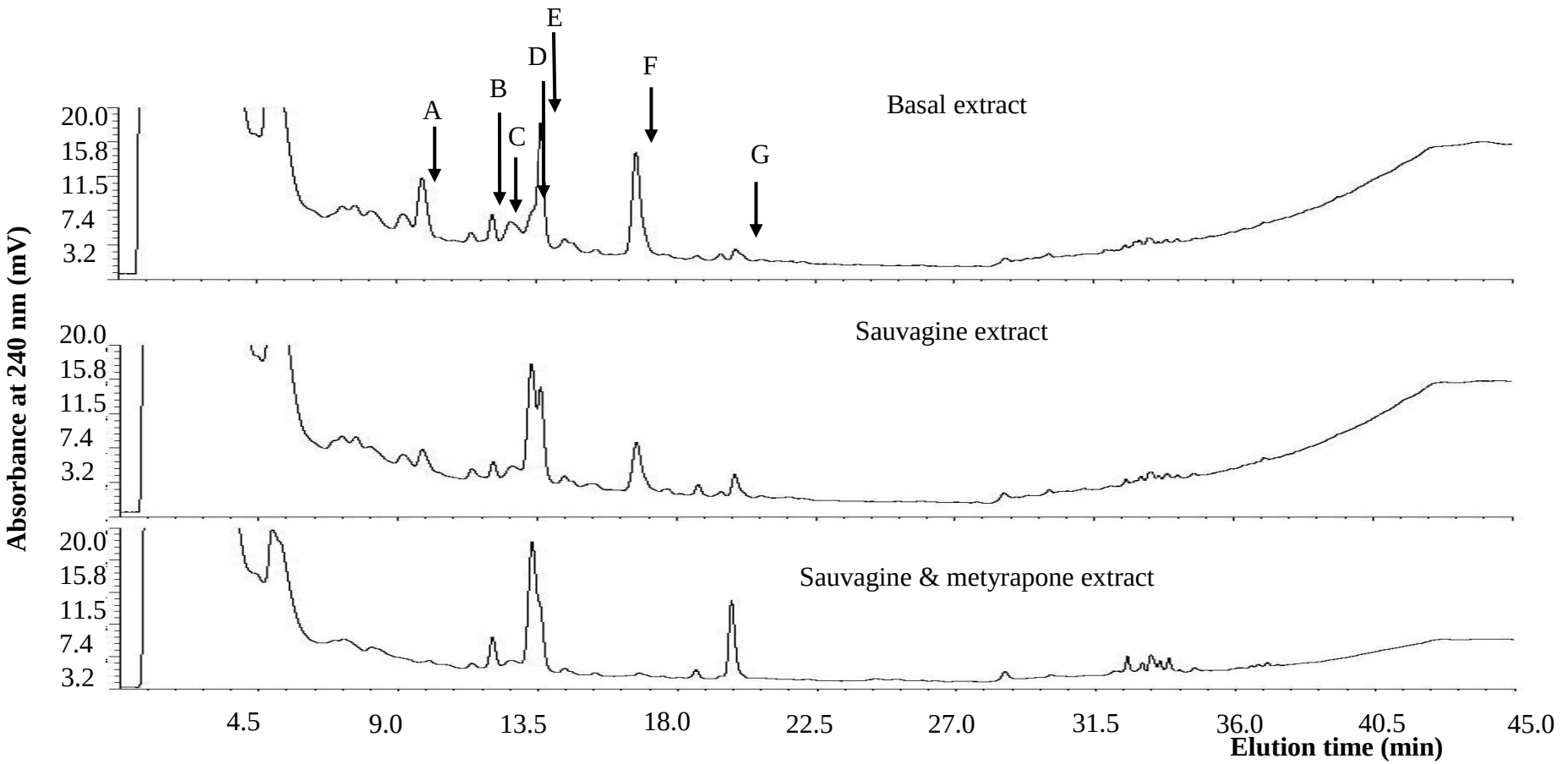


Figure 4.12 Representative chromatograms of extracts of adrenal from incubations under basal, sauvagine or sauvagine with metyrapone treatment conditions.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Injection (1.5 ml of reconstituted adrenal extract in equilibration mix). Gradient setting: HPLC condition set 3 (section 4.2.2.3). Flow rate (1.5 ml/min). Peaks: (A) unknown 1 (9.8 min), (B) unknown 2 (12.0 min), (C) 18-OH DOC (12.6 min), (D) unknown 3 (13.2 min), (E) Corticosterone (13.6 min), (F) unknown 4 (16.7 min) and (G) DOC (19.9 min). Chromatogram integration and data analysis was done using PRIME software.

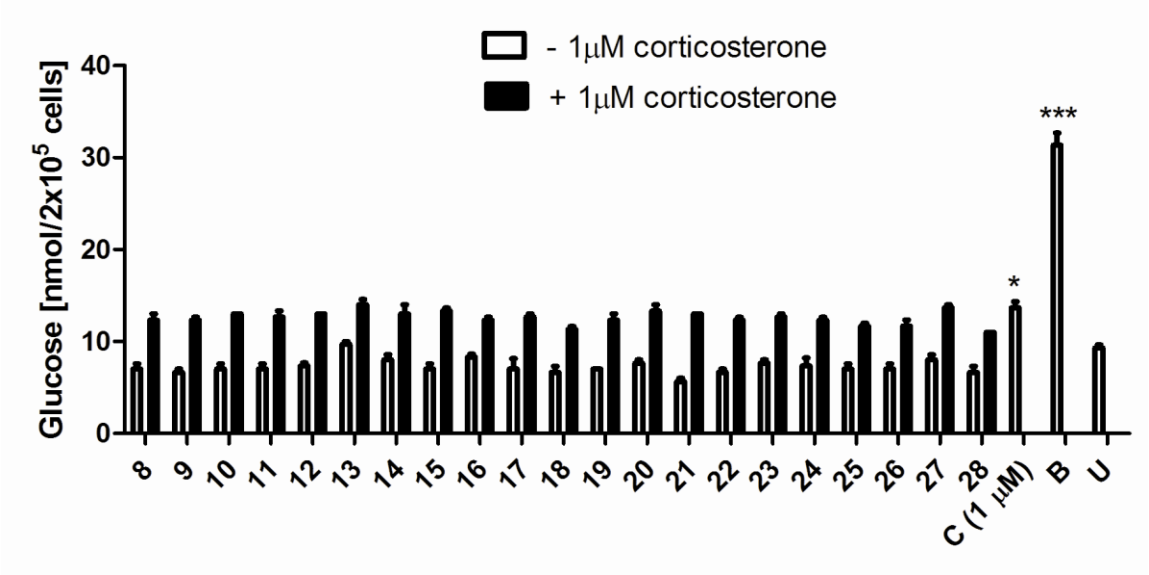


Figure 4.13 Effect of HPLC fractions obtained from adrenal medium from basal incubations on hepatocyte glucose production.

Starved H4-II-E-C3 cells were incubated for 6 h with the reconstituted samples of HPLC fractions in EBSS (20 mM lactate/2 mM pyruvate) with (dark bars) and without (white bars) corticosterone (1 μM). Corticosterone alone (C), extract of basal adrenal media (B), untreated cells with EBSS and substrates (U). Glucose was measured in the conditioned media samples. Results are mean of 3 experiments. Statistical differences were determined by ANOVA (= P < 0.05; *** = P <0.001 vs U).*

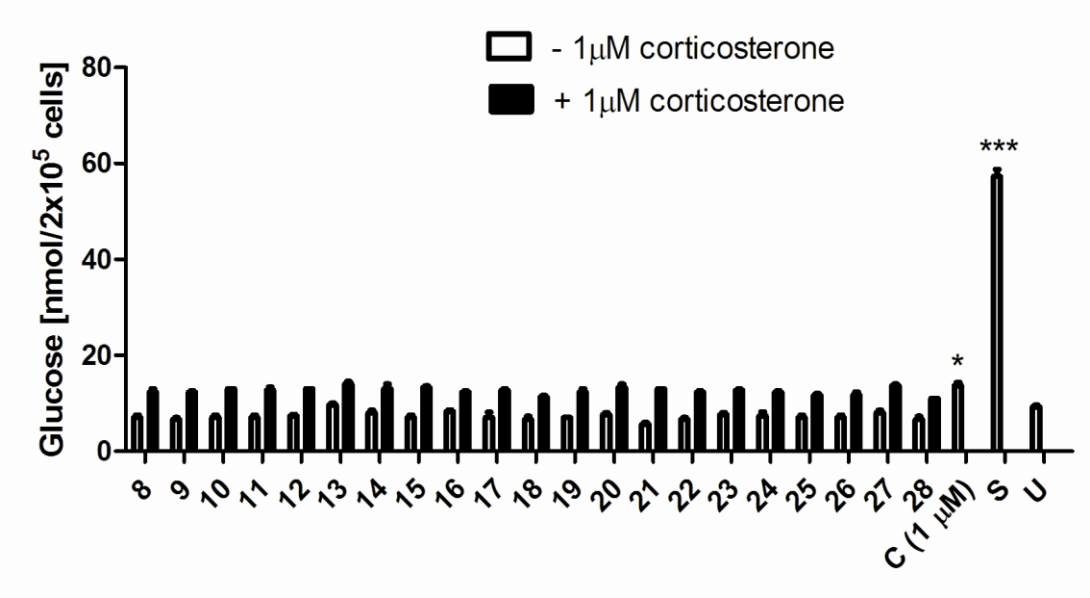


Figure 4.14 Effect of HPLC fractions obtained from adrenal media from sauvagine incubations on hepatocyte glucose production.

Starved H4-II-E-C3 cells were incubated for 6 h with the reconstituted samples of HPLC fractions in EBSS (20 mM lactate/2 mM pyruvate) with (dark bars) and without (white bars) corticosterone (1 μM). Corticosterone alone (C), extract of sauvagine adrenal media (S), untreated cells with EBSS and substrates (U). Glucose was measured in the conditioned media samples. Results are mean of 3 experiments. Statistical differences were determined by ANOVA (= P < 0.05; *** = P<0.001 vs U).*

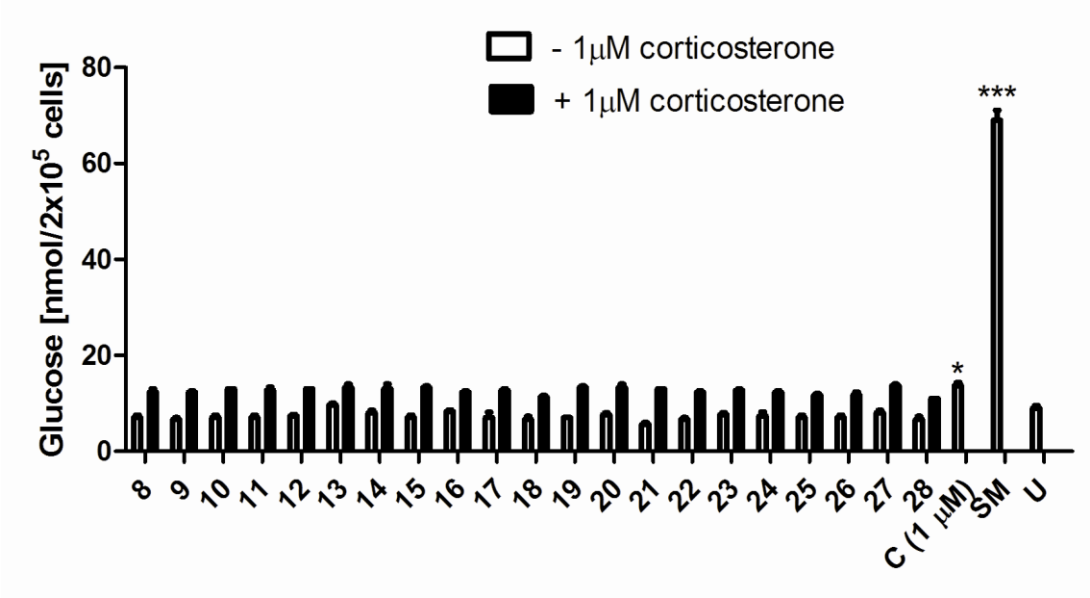


Figure 4.15 Effect of HPLC fractions obtained from adrenal media from incubation with sauvagine and metyrapone on hepatocyte glucose production.

Starved H4-II-E-C3 cells were incubated for 6 h with the reconstituted samples of HPLC fractions in EBSS (20 mM lactate/2 mM pyruvate) with (dark bars) and without (white bars) corticosterone (1 μM). Corticosterone alone (C), extract of sauvagine and metyrapone adrenal media (SM), untreated cells with EBSS and substrates (U). Glucose was measured in the conditioned media samples. Results are mean of 3 experiments. Statistical differences were determined by ANOVA (= P < 0.05; *** = P<0.001 vs U)*

4.10 Further improvements to the HPLC method to compare the adrenal extracts.

The HPLC method was changed to further improve the resolution of the peaks before corticosterone in the chromatograms. HPLC condition set 4 (section 4.2.2.4) was used in further HPLC separations.

Under these new HPLC conditions DOC eluted at 30.0 min (Figure 4.16), 18-OH DOC eluted at 17.6 min (Figure 4.17), and corticosterone eluted at 19.0 min (Figure 4.16) (Table 4.3). Sep-pak extracts of the adrenal incubations from basal (B), sauvagine (S) or metyrapone (M) treatment conditions were reconstituted in equilibration mix (1.5 ml) and injected into the HPLC system under the same conditions. The differences between the chromatograms of the adrenal extracts from these three conditions are shown in Figure 4.18.

Fractions were collected at 1 min time intervals. Similar to previous observations DOC increased with sauvagine and metyrapone treatments (Figure 4.18). But the double peak at the corticosterone retention time was not observed.

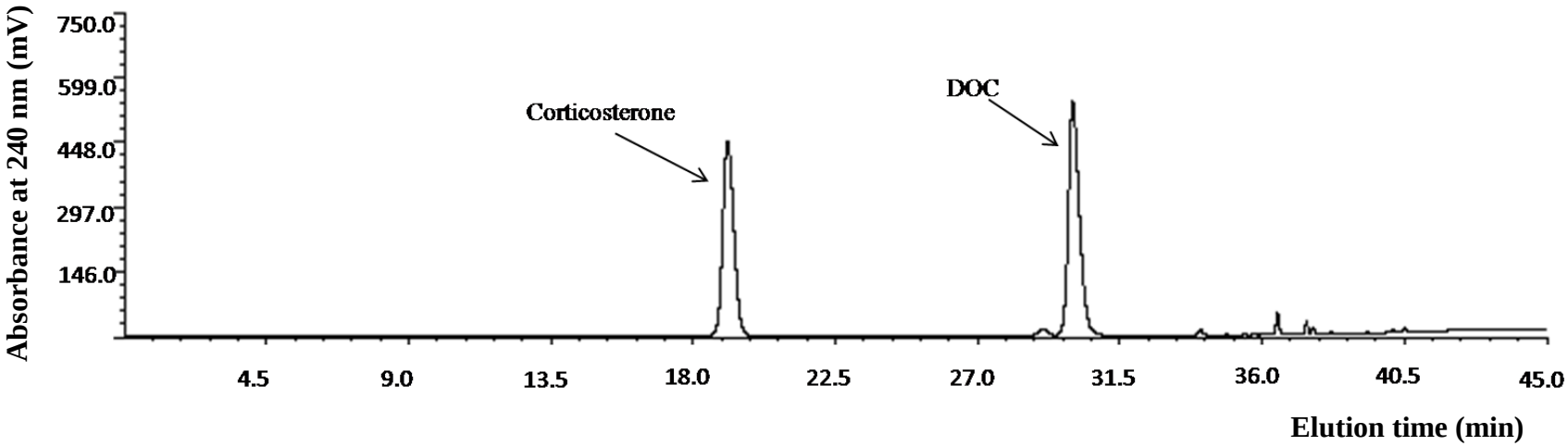


Figure 4.16 Representative chromatograms of corticosterone and DOC under HPLC condition set 4.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Gradient setting: HPLC condition set 4 (section 4.2.2.4). Flow rate (1.5 ml/min). Corticosterone retention time (19.0 min); DOC retention time 29.9 min. Chromatogram integration and data analysis was done using PRIME software.

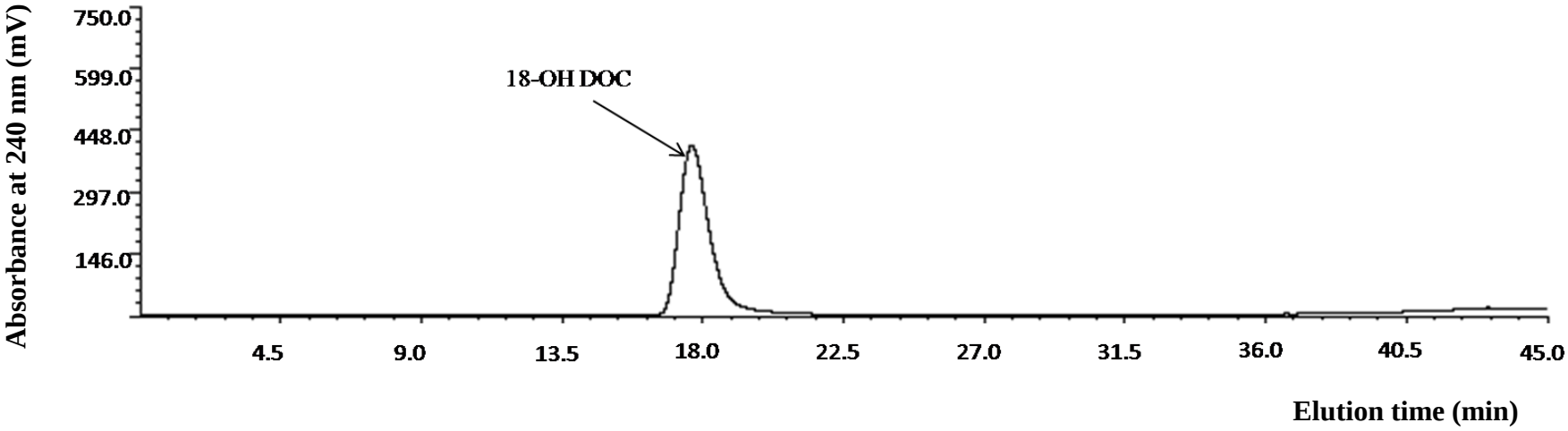


Figure 4.17 Representative chromatograms of 18-OH DOC under HPLC condition set 4.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Gradient setting: HPLC condition set 4 (section 4.2.2.4). Flow rate (1.5 ml/min). 18-OH DOC retention time 17.6 min. Chromatogram integration and data analysis was done using PRIME software.

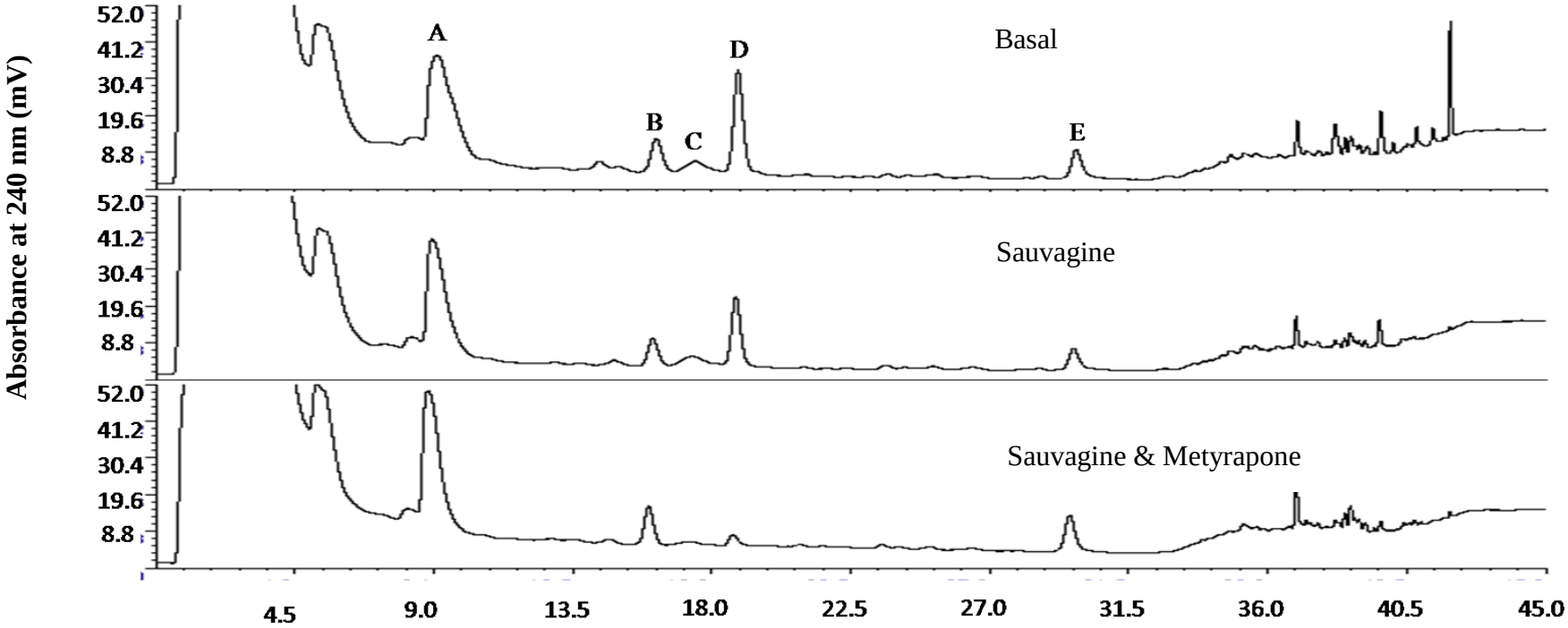


Figure 4.18 Representative chromatograms of extracts of adrenal incubations from basal, sauvagine, and sauvagine with metyrapone.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Injection (1.5 ml of reconstituted adrenal extract in equilibration mix). Gradient setting: HPLC condition set 4 (section 4.2.2.4). Flow rate (1.5 ml/min). Peaks: (A) unknown 1 (9.8 min), (B) unknown 2 (16.2 min), (C) 18-OH DOC (17.6 min), (D) Corticosterone (19.0 min), (E) DOC (29.9 min). Chromatogram integration and data analysis was done using PRIME software.

4.11 Further analysis of the incubation extracts with modified elution conditions replacing methanol with acetonitrile.

Because of the possibility of methanol affecting the elution of a few steroids more adrenal incubation extracts was analysed using HPLC condition set 5, which does not use methanol.

Under these new HPLC conditions DOC eluted at 19.3 min (Figure 4. 19), 18-OH DOC eluted at 10.7 min (Figure 4.19) and corticosterone eluted at 12.2 min (Figure 4.19). Sep-pak extracts of corticosterone and DOC spiked in DMEM showed 100% recovery (Figure 4.20). Adrenal incubations from basal (B), sauvagine (S) or metyrapone (M) treatment conditions were prepared under the same conditions and reconstituted in equilibration mix (1.5 ml) and injected into the HPLC system under these conditions. The differences between the chromatograms of the adrenal extracts from these three conditions are shown in Figure 4.21. It was interesting to note that 18-OH DOC now is in much higher concentrations in these samples when methanol was replaced with acetonitrile suggesting that methanol was not a good choice of eluent in the previous chromatographic conditions. It does seem to co elute with another steroid suggesting that the method still needs improvement. Corticosterone eluting at 12.3 min showed consistent changes as before with lower levels in the metyrapone treated samples. Increased DOC levels were found in sauvagine and sauvagine and metyrapone treated incubations consistent with previous observations (Figure 4.21).

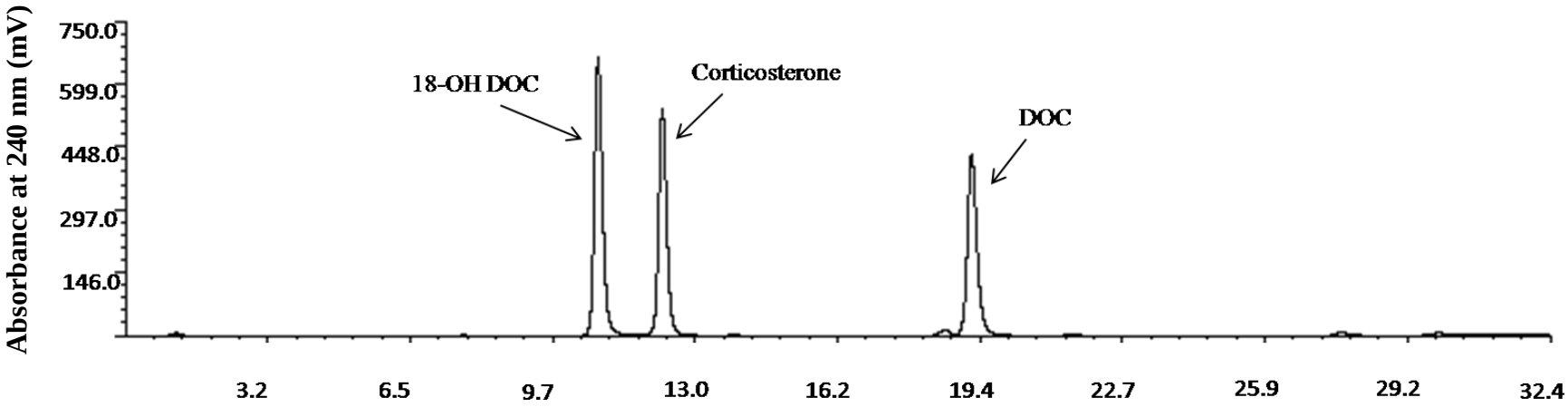


Figure 4.19 Representative chromatograms of 18-OH DOC, corticosterone and DOC under HPLC condition set 5.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Gradient setting: HPLC condition set 4 (section 4.2.2.4). Flow rate (1.5 ml/min). 18-OH DOC retention time 10.7 min. Corticosterone eluted at 12.3 min and DOC eluted at 19.3 min. Chromatogram integration and data analysis was done using PRIME software.

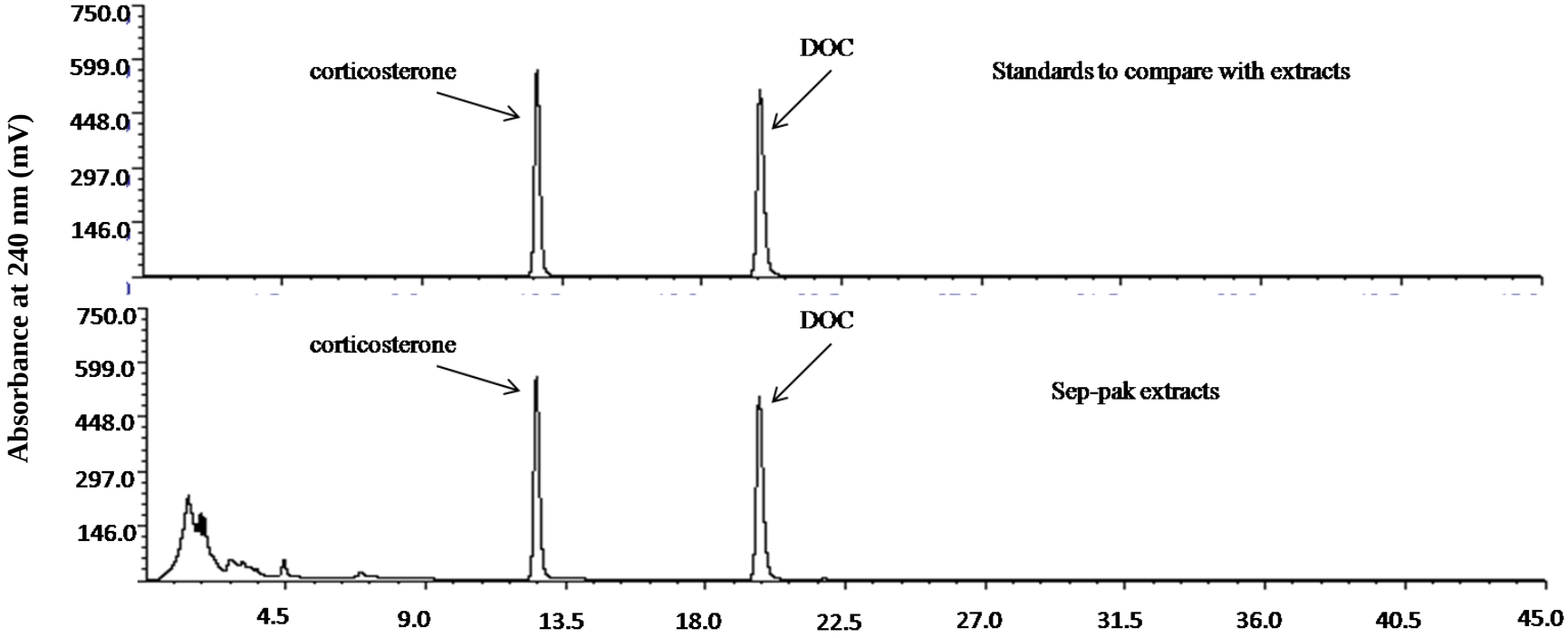


Figure 4.20 Representative chromatograms of corticosterone and DOC extracts from DMEM under HPLC condition set 5.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Gradient setting: HPLC condition set 4 (section 4.2.2.4). Flow rate (1.5 ml/min). Corticosterone eluted at 12.3 min and DOC eluted at 19.3 min. Chromatogram integration and data analysis was done using PRIME software.

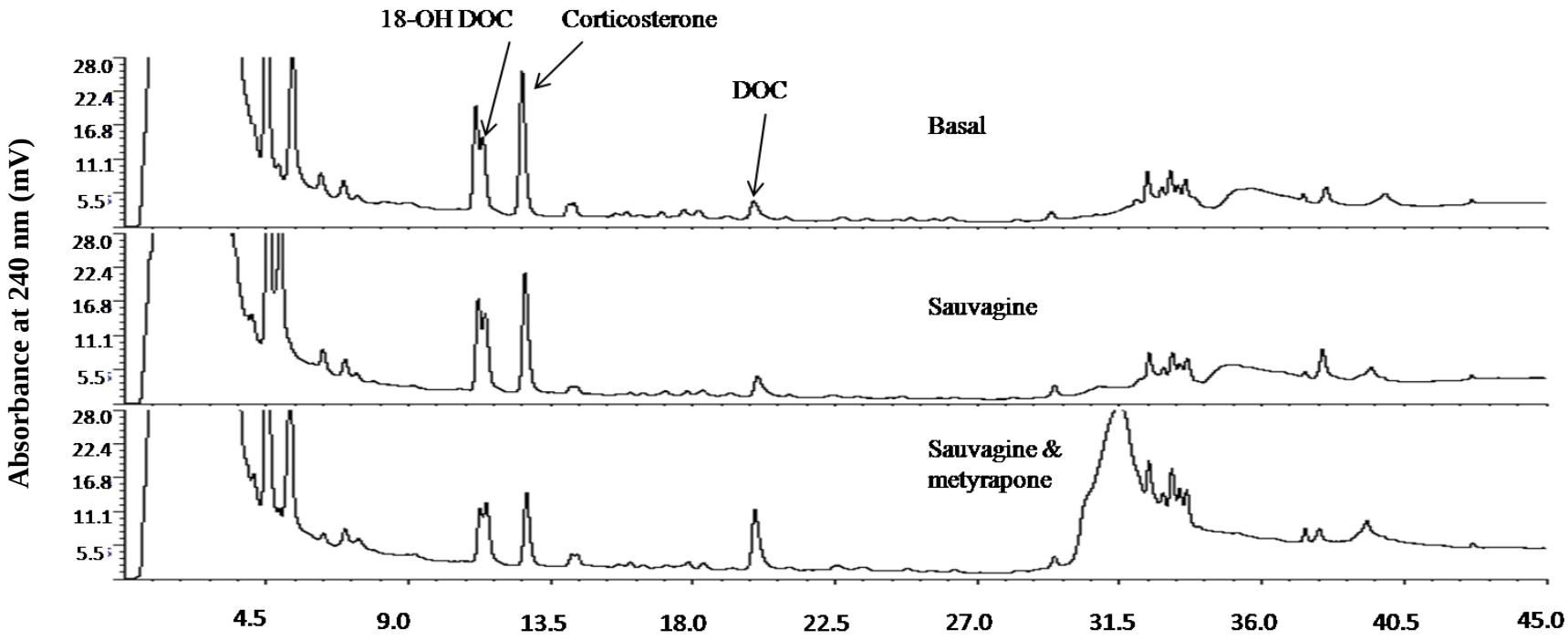


Figure 4.21 Representative chromatograms of extracts of adrenal incubations from basal, sauvagine, and sauvagine with metyrapone.

Column: Hichrom ODS Hypersil (4.6 x 150 mm); Injection (1.5 ml of reconstituted adrenal extract in equilibration mix). Gradient setting: HPLC condition set 5 (section 4.2.2.5). Flow rate (1.5 ml/min). Peaks: 18-OH DOC (10.7 min), Corticosterone (12.3 min) and DOC (19.3min). Chromatogram integration and data analysis was done using PRIME software.

4.12 Comparison of the effects of purified and unpurified corticosterone on hepatic glucose production.

Purified corticosterone (section 4.5), and commercially available corticosterone with impurities (SIGMA; catalogue number C2505) were compared on their ability to stimulate glucose production in H4-II-E-C3 cells. Control cells incubated with EBSS (20 mM lactate/2 mM pyruvate) produced 9 ± 3 glucose (nmol/2x10^5 cells). Cells incubated with the unpurified corticosterone (1 µM) produced 20 ± 2 glucose (nmol/2x10^5 cells). Insulin (10 ng/ml) suppressed this glucose production to below control levels. However, cells incubated with purified corticosterone (1 µM) produced only 12 ± 3 glucose (nmol/2x10^5 cells) (Figure 4.22).

4.13 Sauvagine and metyrapone infusions cause hyperglycaemia in the anaesthetised rat.

Control rats had a blood glucose level of 5 ± 0.2 mM before saline infusion and the levels did not change significantly after. Sauvagine infusions (40 pmol/kg/min) caused an increase in the blood glucose concentrations from 5.5 ± 0.5 to 9.2 ± 0.3 at 90 min. Rats pre-treated with metyrapone (100 mg/kg) for 60 min prior to sauvagine infusion showed a marked increase in blood glucose concentrations. Initial blood glucose levels were 6 ± 0.5 mM which steadily rose to 9 ± 0.5 mM in the first 1 hr and to 10.6 ± 0.6 mM by 90 min (Figure 4.23). Livers collected from sauvagine treated rats had a 0.2 ± 0.16 fold increase in PEPCK activity compared to control rat livers (Figure 4.24). Pre-treatment

with metyrapone for 60 min prior to sauvagine infusions increased the PEPCK activity by 0.4 ± 0.15 fold compared to control livers (Figure 4.24).

4.13 Plasma extracts from sauvagine and metyrapone treatments.

Plasma extracts were prepared from arterial blood collected from control rats (C), sauvagine-infused rats (S) and sauvagine-infused rats pre-treated with metyrapone (SM). Reconstituted extracts in EBSS were incubated on cells to test the effect on total glucose production. S and SM extracts produced 43 ± 2 and 68 ± 3 glucose (nmol/2 x 10^5 cells) respectively. C extracts produced 22 ± 2 glucose (nmol/2 x 10^5 cells). This glucose production was suppressed by insulin (10 ng/ml) (Figure 4.25).

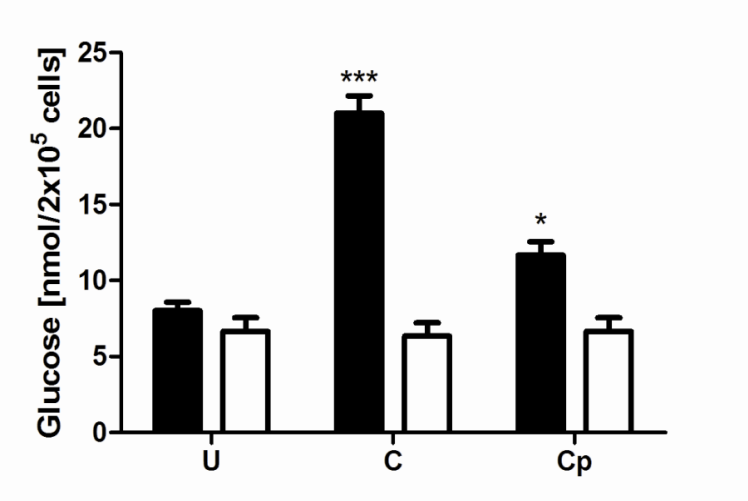


Figure 4.22 Comparison of purified and unpurified corticosterone on hepatocyte glucose production.

Starved H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) containing substrate alone (U), unpurified corticosterone (C, 1 µM), purified corticosterone (Cp, 1 µM) with (white bars) and without (dark bars) insulin (10 ng/ml). Results are mean ± SEM, (n=6). Statistical differences were assessed by ANOVA (= P < 0.05; *** = P < 0.001 vs control U).*

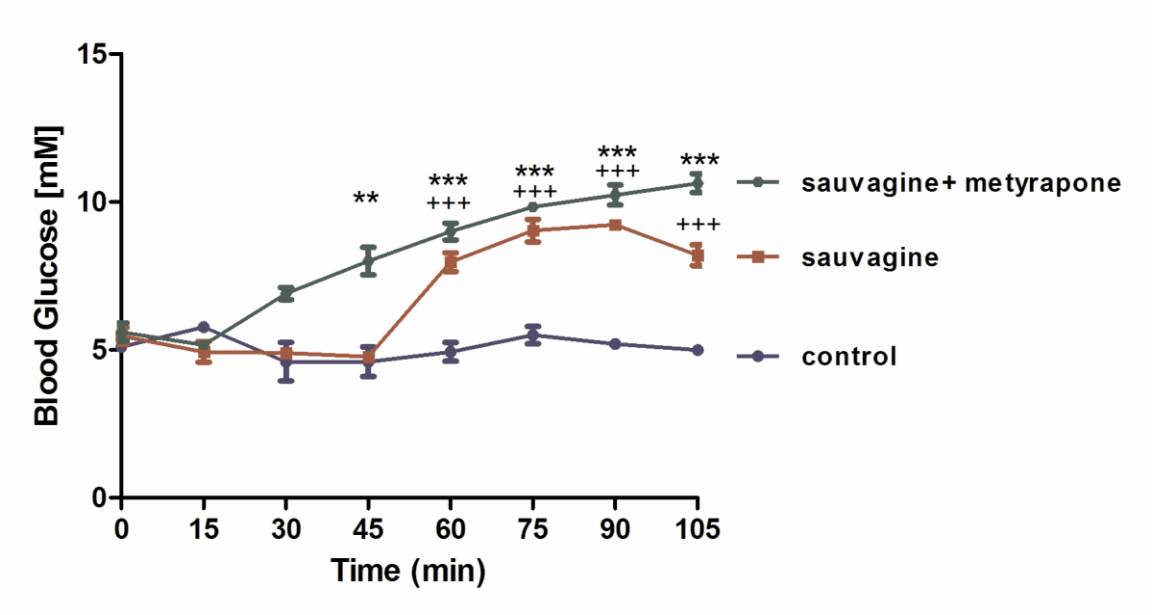


Figure 4.23 Blood glucose concentrations for rats under basal conditions or after treatment with sauvagine alone or with metyrapone.

*Tail vein blood was collected every 15 min from anaesthetized Wistar rats that were treated with sauvagine (40 pmol/kg/min) with or without metyrapone (100 mg/kg). Glucose was measured using Accu-chek® Aviva (Roche Ltd). Results are mean values from four animals for each time point. Statistical differences were determined by ANOVA (** = P <0.01, *** = P < 0.001, sauvagine + metyrapone vs control; +++ = P < 0.001, sauvagine vs control).*

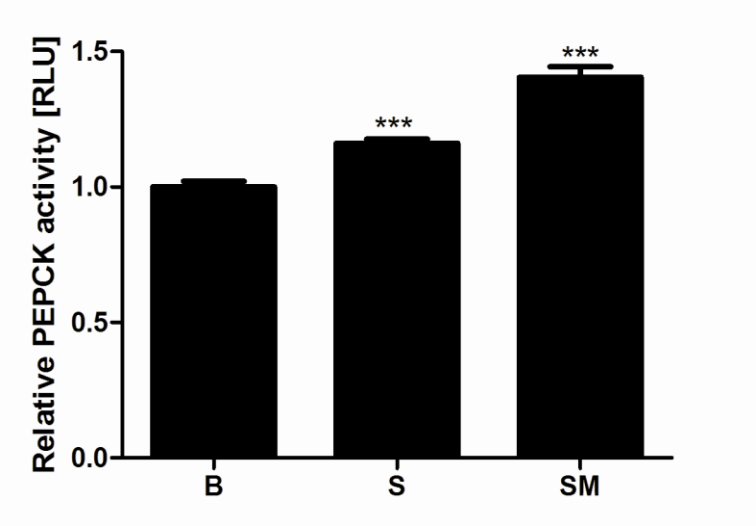


Figure 4.24 Relative levels of liver PEPCK activity in rats treated with sauvagine alone or with metyrapone.

*Liver sections (≈ 3 x 200 mg) were collected from each animal at the end of the 100 min treatment with sauvagine (40 pmol/kg/min) with or without metyrapone (100 mg/kg). PEPCK activity was measured in extracted liver protein. Results are means of 8 liver samples for each condition collected from 4 animals (2 sections per animal). Statistical analyses were performed using ANOVA (***=P<0.001 vs B). Control (C); sauvagine (S); sauvagine with metyrapone pre-treatment (SM).*

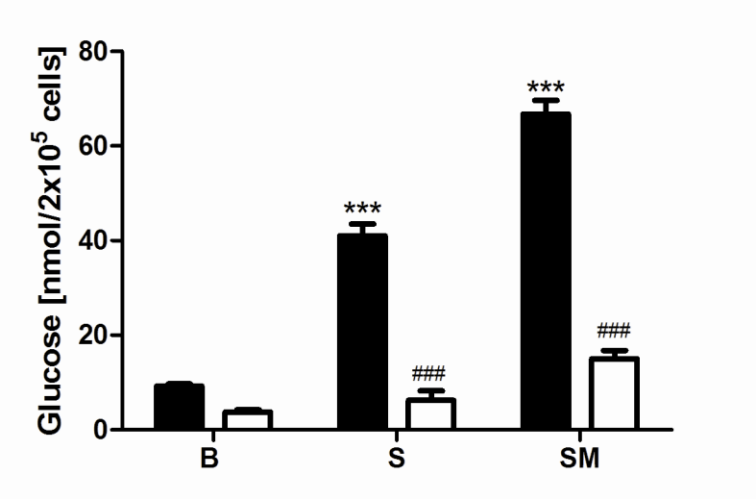


Figure 4.25 Effect of plasma extracts on hepatocyte glucose production.

*Plasma was prepared from arterial blood collected after 90 min from rats treated with sauvagine (40 pmol/kg/min) with and without metyrapone (200 mg/kg). Reconstituted extracts in EBSS (20 mM lactate/2 mM pyruvate) were incubated on fasted H4-II-E-C3 cells for 6 h. Glucose was measured in the medium. Dark bars represent treatment without and white bars indicate response in the presence of insulin (10 ng/ml). n=8. Statistical analysis was performed using a two-way ANOVA followed by a Bonferroni post test (*** = P < 0.001 vs B; ### = P < 0.001 vs the respective treatments without insulin).*

4.14 Discussion

In the previous chapter it was established that extracts prepared from incubations of adrenal sections with sauvagine alone or with metyrapone caused an increase in total glucose production from cultured rat hepatocytes. In order to further characterise the gluconeogenic steroids within these extracts HPLC separation methods were defined.

Initially, HPLC condition (set 1) included H_3PO_4. Under these conditions corticosterone eluted at 6 min and the chromatograms of the extracts from adrenal incubations with treatments showed good separation. However, H4-II-E-C3 cells died with the reconstituted fractions. It was likely this was a cytotoxic effect due to high phosphate concentrations. Therefore H_3PO_4 was replaced by HCl in HPLC condition set 2. Under the new HPLC conditions corticosterone eluted at 6 min and the collected fractions were not toxic on the hepatocytes. Comparison of chromatograms of extracts from adrenal incubations with sauvagine or metyrapone with that of extracts from adrenal incubations under basal conditions showed relative changes in 6 peaks with retention times 4.7, 5.2, 6.0, 8.5, 11.7 and 24.6 min. Peaks areas at retention times 4.7, 11.7 and 24.6 increased with sauvagine and metyrapone treatments. The peak area at 6 min decreased in the metyrapone treatment compared to the basal and sauvagine treatments in accordance with the corticosterone measurements in the extracts. However, none of the collected fractions had any significant effect on hepatocyte glucose production either alone or in combination with corticosterone.

It was interesting to note that the peak eluting at 11.5 min in the chromatograms of adrenal incubation extracts (Figure 4.2), was also present as an impurity in the commercially available corticosterone (Figure 4.6). To identify the impurities in

corticosterone and the peaks obtained from adrenal incubation extracts, DOCA was analysed with HPLC condition set 2. It was found to elute at 24.6 min. When DOCA was hydrolysed to DOC a second peak eluted at 11.7 min which correlates with the peak that increases in the chromatograms of the extracts of adrenals with sauvagine and metyrapone. The increase in DOC with metyrapone is consistent with its inhibitory action on 11-beta-hydroxylase. The sauvagine-stimulated increase in DOC was not expected.

The HPLC conditions were further altered with HPLC condition set 3 to increase resolution of the earlier peaks. Under these conditions corticosterone, DOC and 18-OH DOC eluted at 13.6, 19.8 and 12.5 min respectively. Chromatograms of extracts from adrenal incubations with sauvagine and metyrapone showed changes in at least 20 different peaks compared to that from extracts of adrenal incubation under basal conditions. As previously observed DOC peak areas increased with the treatment conditions compared to basal incubations. Of particular interest was the double peak that was unresolved at 13.2 and 13.6 min respectively. The relative peak areas of these two peaks changed with the treatment conditions, with the peak at 13.2 min increasing in the extracts from adrenal incubations with sauvagine and metyrapone. The peak at 13.6 min which corresponds to the corticosterone decreased in these two extracts compared to extracts prepared from adrenal incubations under basal conditions. The reconstituted fractions did not have any significant effect on hepatocyte glucose production alone or in combination with corticosterone. However, it was consistently observed that the total extract had a significant effect on hepatocyte glucose production compared to untreated cells and to the cells incubated with corticosterone (1 µM). HPLC condition set 4 was used to further resolve the double peak at 13 min. Under these new HPLC conditions corticosterone, DOC, 18-

OH DOC eluted at 19.0, 30.0, and 17.6 min respectively. As previously observed peak areas of DOC increased in the extracts obtained from adrenal incubations with sauvagine and metyrapone. Changes were observed in a peak eluting at 16.2 min that was not observed before in earlier comparisons (Figure 4.12). But no improvement in resolution of corticosterone was observed.

More studies of these fractions under different elution conditions will provide more insights into weather each of these peaks represent a single steroid. The lack of responses to corticosterone indicates that other factors in the adrenal extracts are important. The real test would be to combine a pooled sample of all the fractions collected and test for biological activity if different from the starting material. If there a novel steroid, it might have unknown or poor stability under the conditions used as demonstrated by the modification of 18-OH DOC by methanol in the eluting buffer. More careful evaluation of the appropriate extraction and HPLC conditions would be necessary to ensure that a pool of all HPLC fractions had the same activity as the starting material. Under such a set up, we can further investigate individual fractions for biological activity.

In vivo studies confirmed earlier observations (Kaminski 2004) that sauvagine infusions caused hyperglycaemia in the anaesthetised rats. Rats treated with metyrapone prior to sauvagine infusions had a more profound hyperglycaemic response. Plasma extracts from rats treated with sauvagine alone or with metyrapone had a more than 2 fold greater effect on stimulating glucose production in H4-II-E-C3 cells. Liver samples collected from these treatments showed an increase in PEPCK activity in the animals treated with sauvagine and metyrapone compared to control rat livers. A group of animals treated with metyrapone alone would have been a useful addition to the experiment. It would have been desirable to have measured plasma

corticosterone and insulin levels to obtain a clear insight into the hyperglycaemia observed in the sauvagine and metyrapone treated rats.

The results in this chapter show that there are significant differences in the steroids secreted by the adrenal glands incubated with sauvagine alone or with metyrapone compared to adrenals incubated under basal conditions. DOC is increased in the treatment conditions compared to basal incubations. There are several unknown peaks that change and are not consistent in the relative increases or decreases. This could be due to the differences in the stress levels of the rats or due to differences in handling of samples such that adrenal corticosteroids were modified by the extraction process. Sauvagine infusions in rats pre-treated with metyrapone have increased blood glucose levels due to increased hepatic glucose output, which is confirmed with the increases in PEPCK activities in the livers.

Extracts obtained from adrenal incubations with sauvagine and metyrapone are very potent in stimulating hepatic glucose production when used as a whole extract even with very low corticosterone levels (~ 0.04 μM). But the individual fractions did not show any significant effect on the hepatocyte glucose production. This could be due to loss of sample or the need for more than one or two combinations of steroid to have a synergistic effect. However, plasma extracts may be confounded by the presence of other factors that are co-extracted on Sep-pak but not recovered under the HPLC conditions being used.

CHAPTER 5
Investigation of the role of 11-deoxycorticosterone in hepatic glucose metabolism.

5.1 Introduction

It is well established that hepatic glucose regulation is tightly regulated by insulin, glucocorticoids, glucagon and epinephrine (Pilkis *et al.*, 1992). Glucocorticoids normally stimulate hepatic gluconeogenesis and antagonise the action of insulin (Ruzzin *et al.*, 2005). In the previous chapter commercial synthetic corticosterone was found to produce a greater increase in glucose than pure corticosterone in fasted hepatocytes. The major contaminants of the commercial synthetic product had HPLC elution characteristics of DOC, suggesting that these contaminants contributed to the biological activity of the unpurified commercial product. Studies of stress related adrenal corticosteroid secretion often show a concomitant increase in DOC and corticosterone (Gallant *et al.*, 1979). This suggests that DOC may have a physiological role in modulating or enhancing the actions of corticosterone.

For more than fifty years the two most commonly investigated adrenal corticosteroids in experimental studies have been corticosterone and DOC, mainly as the acetyl ester DOCA. Corticosterone is considered as a glucocorticoid, whereas DOC is considered as a weak mineralocorticoid. The extent to which these definitions are true remains an area for discussion and experimentation. Recent studies have now shown that DOC is a potent glucocorticoid (Brookes *et al.*, 2011). The glucocorticoid potential of a steroid largely depends on what is perceived to be the "glucocorticoid potential". Is it the ability to (1) increase liver gluconeogenesis, (2) increase liver glycogen deposition, (3) act as an anti-inflammatory, or (4) all the above? Recent detailed appraisals of the actions of corticosteroids have concluded that experimental data comparing DOC with corticosterone fails to distinguish between their actions (Vinson, 2009; Vinson, 2011).

Insulin normally increases hepatic glycogen deposition by increasing glycogen synthase (GS) activity and decreasing glycogen phosphorylase (GP) activity. Conversely, resistin induces insulin resistance in hepatocytes by disturbing glycogen metabolism (Yang *et al.*, 2009). Glycogen formation is determined by GS activity. GS activity is determined by both its covalent phosphorylation and the intracellular presence of glucose-6-phosphate (G6P). Resistin does not affect GS covalent phosphorylation, but has an effect on the G6P levels. It has also been observed that resistin affects glycogen metabolism in two different ways depending on the presence or absence of insulin. In the presence of insulin, resistin decreases the glucose uptake without changing hexokinase1 (HK) activity. In the absence of insulin resistin reduces HK activity (Niederwanger *et al.*, 2007).

Similarly, the effects of DOC in the presence and absence of insulin need to be investigated as it has been observed to improve insulin sensitivity in animal models (Dai *et al.*, 1992). It was found that, in non-diabetic rats, DOCA-induced hypertension was associated with normal glucose levels and glucose tolerance but with significantly lower levels of plasma insulin. DOCA-treated diabetic animals showed significantly lower levels of plasma glucose, but their plasma insulin concentrations were not significantly different from those of the DOCA vehicle treated diabetic rats. This suggests that DOCA may have either a direct or indirect action on the assimilation, production, or utilisation of glucose.

Aims of this chapter

- To characterise the effects of DOC on hepatic glucose production.
- To evaluate whether RU-486 (glucocorticoid antagonist) or eplerenone (mineralocorticoid antagonist) alter the response to DOC.

5.2 Methods

The methods used in this chapter are described in sections of early chapters – cell culture (3.3.1), glucose assay (3.3.2), PEPCK activity assay (2.10). H4-II-E-C3 cells were grown to confluence in DMEM (5 mM glucose) supplemented with 10% FCS. Cells were then maintained for 18 h in serum-free DMEM (5 mM glucose).

For fed cell experiments cells were treated for 6 h in EBSS (20 mM lactate/2 mM pyruvate). For fasted cells experiments cells were further incubated in DMEM (0 mM glucose) for 4 h and then incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate).

5.2.1 Glycogen measurements

For glycogen synthesis experiments, H4-II-E-C3 cells were incubated for 24 h in DMEM (5 - 25 mM glucose) with and without insulin in the presence of treatment compounds. The medium was aspirated and the cells were washed with PBS before being analysed for glycogen.

The plates were frozen at -80°C until the time of the assay. On the day of the assay the cells were scraped off the plate in ice cold PBS (300 µl), and the well contents were transferred into Eppendorf tubes. The cell contents were sonicated for 10 seconds and the sonicated cell suspension was heated at 75°C for 10 min to inactivate any enzyme activity that might interfere in the assay. The heat-treated samples were centrifuged to remove the cell debris. The supernatant was collected in fresh tubes and stored for glycogen assay.

Glycogen standards were prepared (2 – 10 µg/ml). Standards and samples (50 µl) were transferred into a 96 well plate. 100 µl of hydrolysis buffer containing 2U glucoamylase was added into each well. For glucose control of each sample, 100 µl of

the buffer without the hydrolysing enzyme was added. The plate was incubated at room temperature for 60 min for the hydrolysis to take place. The hydrolysed samples were heated at 75°C for 10 min to inactivate the enzyme, and then subjected to glucose assay (3.3.2) to measure the amount of glucose in each well. The glucose concentrations of the unhydrolysed samples from each well were subtracted from the hydrolysed concentrations to give the corrected value for the amount of glycogen in each well containing 2 x 10^5 cells.

5.3 Results

5.3.1 DOC causes a concentration dependent decrease in hepatic glucose production and PEPCK activity in the fed state.

Incubation of DOC (1 µM) with H4-II-E-C3 cells in the fed state decreased glucose production of control cells from 9.5 ± 0.6 to 7.7 ± 0.5 (nmol/2 x 10^5 cells). Corticosterone significantly increased glucose production to 18.5 ± 0.7 (nmol/2 x 10^5 cells), and this increase was significantly reduced in a concentration dependent manner to 16.6 ± 0.6, 13.8 ± 0.9, and 10.5 ± 0.7 when DOC concentrations of 0.1, 0.5 and 1 µM respectively were co-incubated with corticosterone (Figure 5.1 A). These changes in glucose production correlated with corresponding decrease in PEPCK activity in these cells. DOC (1 µM) on its own decreased relative PEPCK activity by 0.2 ± 0.06 fold compared to control untreated cells. It also significantly reduced corticosterone induced PEPCK activity in a concentration dependent manner from 1.8 ± 0.2 to 1.5 ± 0.09, 1.4 ± 0.04 and 1.2 ± 0.08 of the control values with DOC concentrations of 0.1, 0.5 and 1 µM respectively (Figure 5.1 B). Glycogen

concentrations under the same treatment conditions did not show any significant changes between the treatments (Figure 5.2).

5.3.2 The effect of RU-486, eplerenone and insulin on DOC mediated suppression of corticosterone induced hepatic glucose production and PEPCK activity in the fed state.

Insulin (10 ng/ml) suppressed total glucose production due to DOC (1µM), C (1 µM) and DOC + C (1 µM) from 8.5 ± 0.6, 18.7 ± 0.9 and 9.5 ± 0.6 to 6.5 ± 0.6, 7.5 ± 0.6 and 5.7 ± 0.8 glucose (nmol/2 x 10^5 cells) respectively (Figure 5.3 A). The corresponding PEPCK activity was also suppressed from 1 ± 0.04 to 0.8 ± 0.06 for untreated cells, 1.9 ± 0.1 to 0.7 ± 0.04 for corticosterone (1 µM) treated cells, 0.8 ± 0.08 to 0.62 ± 0.04 for DOC (1 µM) treated cells and 1.4 ± 0.09 to 0.47 ± 0.05 for corticosterone and DOC treated cells (Figure 5.3 B)

RU-486 (10 µM) suppressed total glucose production due to DOC (1µM), C (1 µM) and DOC + C (1 µM) from 8.5 ± 0.6, 18.7 ± 0.9 and 9.5 ± 0.6 to 7.8 ± 0.9, 7.7 ± 0.8 and 8 ± 0.5 glucose (nmol/2x10^5 cells) respectively (Figure 5.3 A). The corresponding PEPCK activity was not significantly changed for untreated cells and DOC (1µM) treatments. However, with RU-486 treatment PEPCK activity was suppressed from 1.9 ± 0.1 to 0.93 ± 0.06 for corticosterone (1 µM) treated cells, and 1.4 ± 0.09 to 0.92 ± 0.06 for corticosterone and DOC treated cells (Figure 5.3 B).

Eplerenone (1 µM) did not have any significant effects on either the total glucose production or the relative PEPCK activity observed with the different treatments (Figure 5.3 A & B).

5.3.3 DOC causes a concentration dependent increase in hepatic glucose production but does not affect the relative PEPCK activity in fasted H4-II-E-C3 cells.

Incubation of H4-II-E-C3 cells in the fasted state with DOC (1 µM) alone did not affect glucose production when compared to control cells (12.3 ± 0.8 versus 11.5 ± 0.6 nmol/2 x 10^5 cells). However, it significantly increased corticosterone induced glucose production in a concentration dependent manner from 20.5 ± 0.6 to 24 ± 0.7, 33 ± 1.6, and 39 ± 0.9 with DOC concentrations of 0.1, 0.5 and 1 µM respectively (Figure 5.4 A). These changes in glucose production occurred without a parallel increase in the corresponding PEPCK activity in these cells. DOC (1 µM) on its own did not cause any significant change in the relative PEPCK activity compared to control untreated cells. In addition, DOC treatment did not reduce the corticosterone induced increase in PEPCK activity at any of the test concentrations (Figure 5.4 B). Glycogen concentrations did not show any significant changes compared to the control untreated wells with either corticosterone, DOC, or the combination (Figure 5.5)

5.3.4 The effect of RU-486, eplerenone and insulin on DOC modulation of corticosterone induced hepatic glucose production and PEPCK activity in the fasted state.

Insulin (10 ng/ml) suppressed total glucose production due to DOC (1µM), C (1 µM) and DOC + C (1 µM) from 11 ± 1, 18.8 ± 0.9 and 36.5 ± 1 to 8 ± 0.4, 9.7 ± 0.5 and 8.2 ± 0.5 glucose (nmol/2 x 10^5 cells) respectively (Figure 5.6 A). The corresponding PEPCK activity was also suppressed from 1 ± 0.04 to 0.75 ± 0.06 for untreated cells, 0.9 ± 0.04 to 0.6 ± 0.02 for DOC (1 µM) treated cells, 1.9 ± 0.08 to 0.7 ± 0.04 for

corticosterone (1 µM) treated cells and 1.7 ± 0.1 to 0.75 ± 0.06 for corticosterone and DOC treated cells (Figure 5.6 B)

RU-486 (10 µM) suppressed total glucose production due to DOC (1µM), C (1 µM) and DOC + C (1 µM) from 11 ± 1, 18.8 ± 0.9 and 36.5 ± 1 to 8.2 ± 0.7, 8.5 ± 0.6 and 10.7 ± 0.5 glucose (nmol/2 x 10^5 cells) respectively (Figure 5.6 A). The corresponding PEPCK activity was also suppressed from 1 ± 0.04 to 0.85 ± 0.06 for untreated cells, 1.9 ± 0.1 to 0.75 ± 0.06 for corticosterone (1 µM) treated cells, 0.9 ± 0.04 to 0.8 ± 0.05 for DOC (1 µM) treated cells and 1.7 ± 0.1 to 0.97 ± 0.04 for corticosterone and DOC treated cells (Figure 5.6 B)

Eplerenone (1 µM) did not cause any significant effects on either the total glucose production or the relative PEPCK activity (Figure 5.6 A & B).

5.3.5 The effect of DOC on insulin stimulated glycogen formation in hepatocytes *in vitro*.

DOC had a varied effect on hepatocytes glycogen levels depending on the glucose concentrations. In the presence of 5 mM glucose concentrations untreated (U), corticosterone 1µM (C), and 11-deoxycorticosterone 1µM (D) treated cells had glycogen concentrations of 0.11 ± 0.03, 0.09 ± 0.01, and 0.22 ± 0.04 without insulin and 0.19 ± 0.03, 0.19 ± 0.04 and 0.15 ± 0.03 ug/2x10^5 cells with insulin (Figure 5.7 A). In the presence of 7 mM glucose the glycogen concentrations for U, C and D were 0.13 ± 0.03, 0.12 ± 0.03 and 0.12 ± 0.01 without insulin and 0.26 ± 0.02, 0.17 ± 0.03 and 0.24 ± 0.07 ug/2x10^5 cells with insulin (Figure 5.7 B). In the presence of 15 mM glucose the glycogen concentrations for U, C and D were 0.14 ± 0.03, 0.09 ± 0.03 and 0.12 ± 0.03 without insulin and 0.38 ± 0.04, 0.27 ± 0.01 and 0.42 ± 0.01 ug/2x10^5 cells with insulin (Figure 5.7 C). All the glycogen concentrations in the

presence of insulin at this glucose (15 mM) concentrations were significantly ($P < 0.001$) increased compared to the corresponding non-insulin treatments. When the cells were incubated with 25 mM glucose concentrations, the glycogen concentrations for U, C and D were 0.15 ± 0.04, 0.26 ± 0.04 and 0.29 ± 0.02 without insulin and 0.43 ± 0.06, 0.48 ± 0.01 and 0.63 ± 0.02 ug/2x10^5 cells with insulin (Figure 5.7 D). All the glycogen concentrations with insulin were significantly increased compared to the corresponding non-insulin treatments. The DOC treatments with insulin were significantly increased ($P < 0.05$) compared to insulin alone at 25 mM glucose concentrations (Figure 5.7 D).

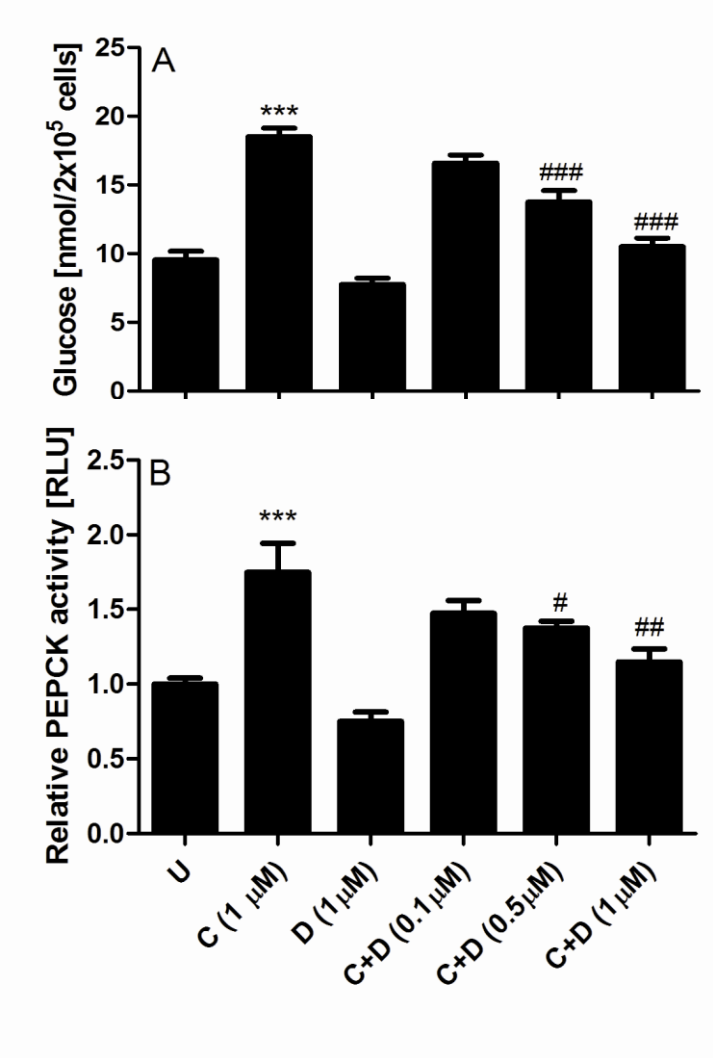


Figure 5.1 Effect of DOC on corticosterone induced glucose production and PEPCK activity in fed cells.

*H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) with deoxycorticosterone (D, 1 μM), corticosterone (C, 1 μM), and corticosterone (1 μM) with 0.1, 0.5 or 1 μM deoxycorticosterone (C + D). Results are mean ± S.E.M., n=6. *** = P < 0.001 vs U (untreated) conditioned media samples; # = P < 0.05 and ### = P < 0.001 vs C. One-way ANOVA with Bonferroni post hoc test was used to compare all columns.*

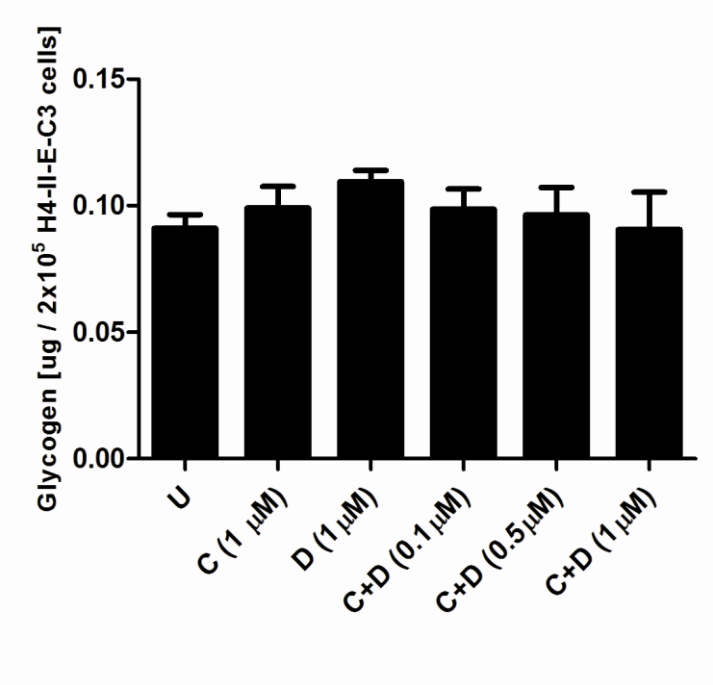


Figure 5.2 Effect of DOC and corticosterone on glycogen concentrations in fed H4-II-E-C3 cells.

H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) with deoxycorticosterone (D, 1 µM), corticosterone (C, 1 µM), and corticosterone (1 µM) with 0.1, 0.5 or 1 µM deoxycorticosterone (C + D). Results are mean ± S.E.M., n=6.

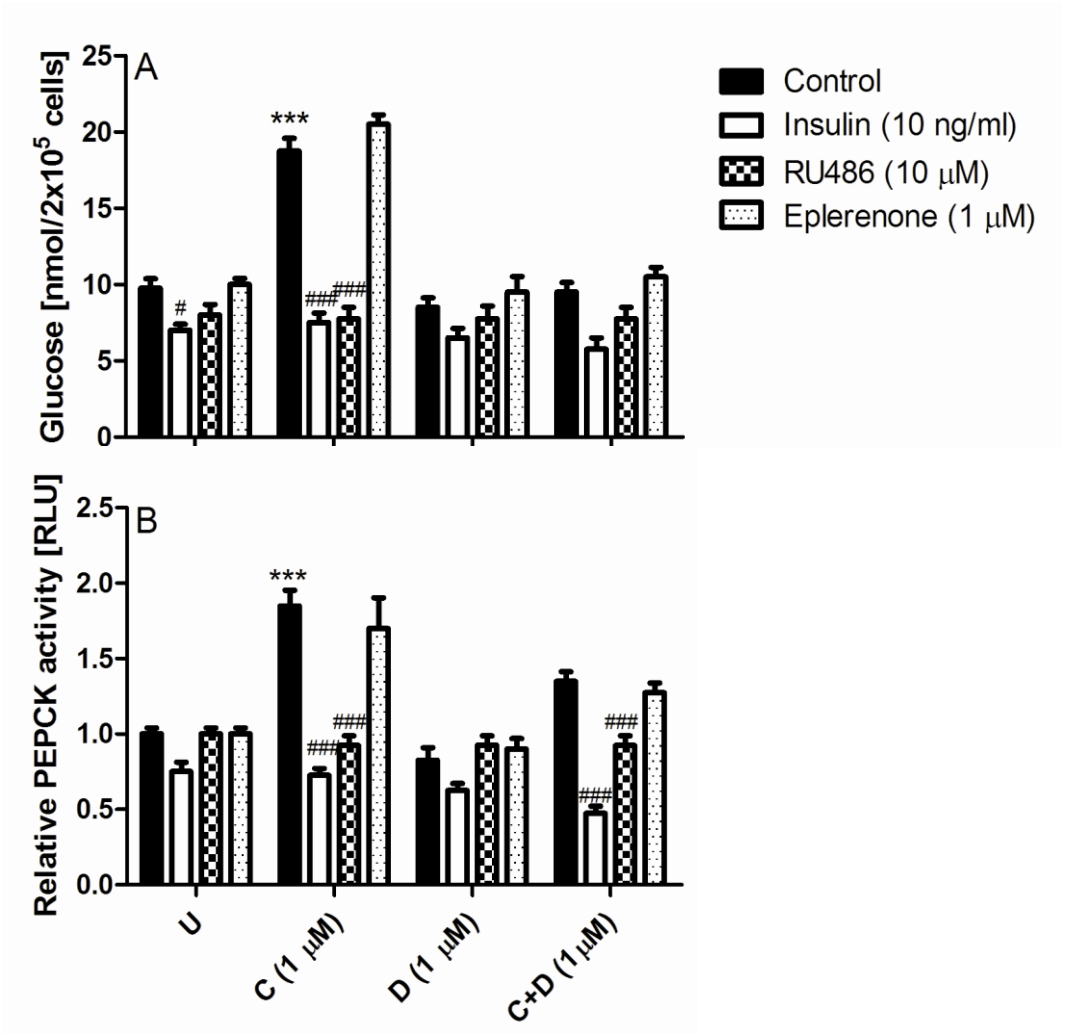


Figure 5.3 The effect of Insulin, RU486 and eplerenone on hepatic glucose production and PEPCK activity in the presence of DOC and corticosterone in fed H4-II-E-C3 cells.

*H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) with deoxycorticosterone (D, 1 µM), corticosterone (C, 1 µM), or corticosterone (1 µM) with deoxycorticosterone (C + D, both 1 µM) alone or combined with insulin (10 ng /ml), RU486 (10 µM), or eplerenone (1 µM). (A) glucose measurements in medium. (B) Relative PEPCK activity in the corresponding cells. Results are mean ± S.E.M., n=6. *** = P < 0.001 vs U. ### = P < 0.001 vs the corresponding control treatments under each group. Statistical differences were determined by two-way ANOVA with Bonferroni post hoc test.*

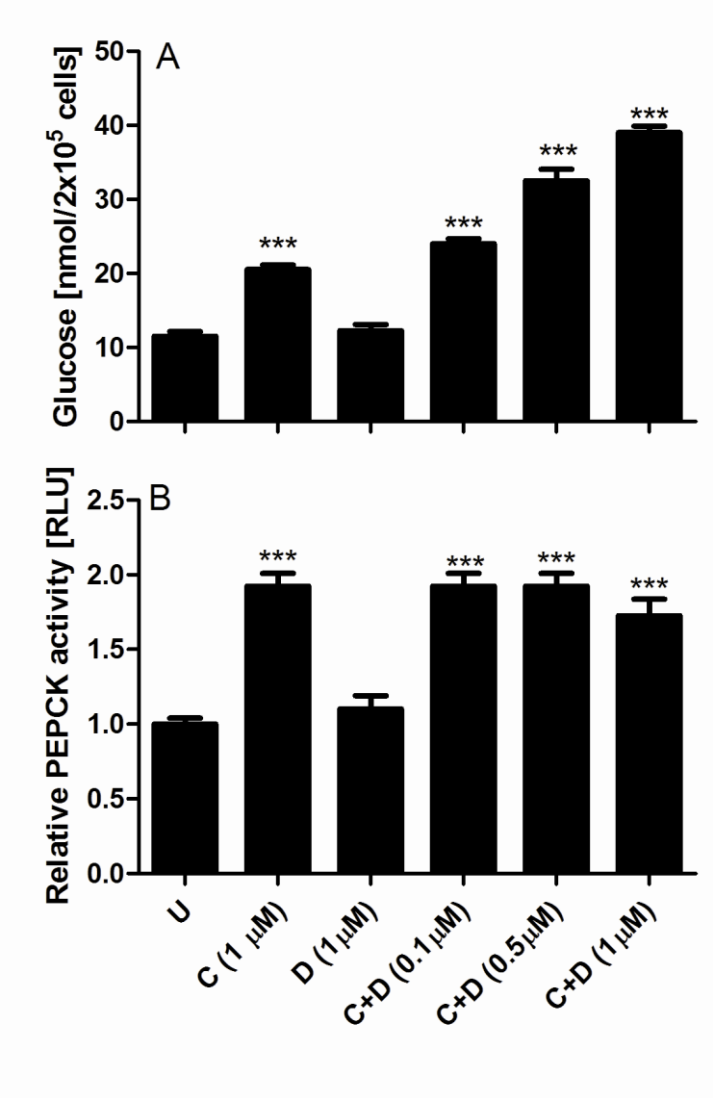


Figure 5.4 Effect of DOC on corticosterone induced glucose production and PEPCK activity in fasted cells.

*H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) with deoxycorticosterone (D, 1 μM), corticosterone (C, 1 μM), and corticosterone (1 μM) with 0.1, 0.5 or 1 μM deoxycorticosterone (C + D). Results are mean ± S.E.M., n=6. *** = P < 0.001 vs U (untreated) conditioned media samples. One-way ANOVA with Bonferroni post hoc test was used to compare all columns.*

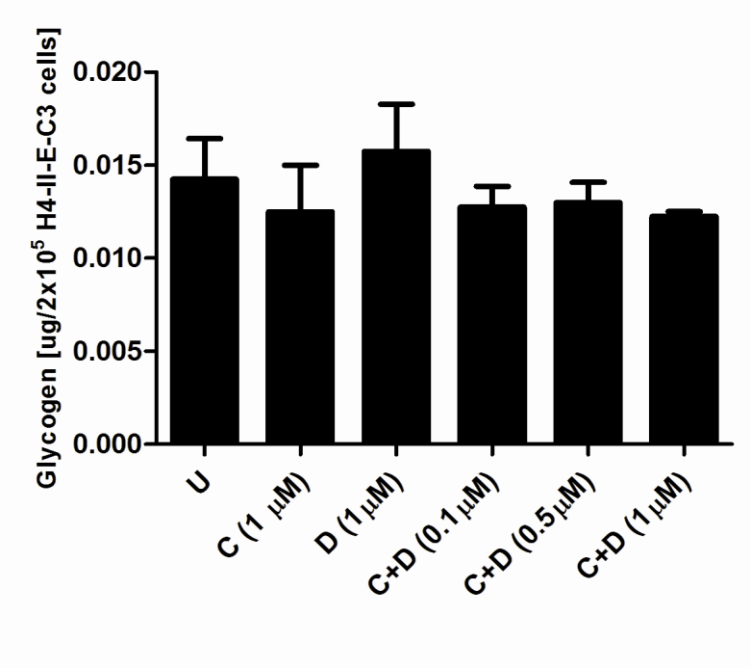


Figure 5.5 Effect of DOC and corticosterone on glycogen concentrations in fasted H4-II-E-C3 cells.

H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) with deoxycorticosterone (D, 1 µM), corticosterone (C, 1 µM), and corticosterone (1 µM) with 0.1, 0.5 or 1 µM deoxycorticosterone (C + D). Results are mean ± S.E.M., n=6.

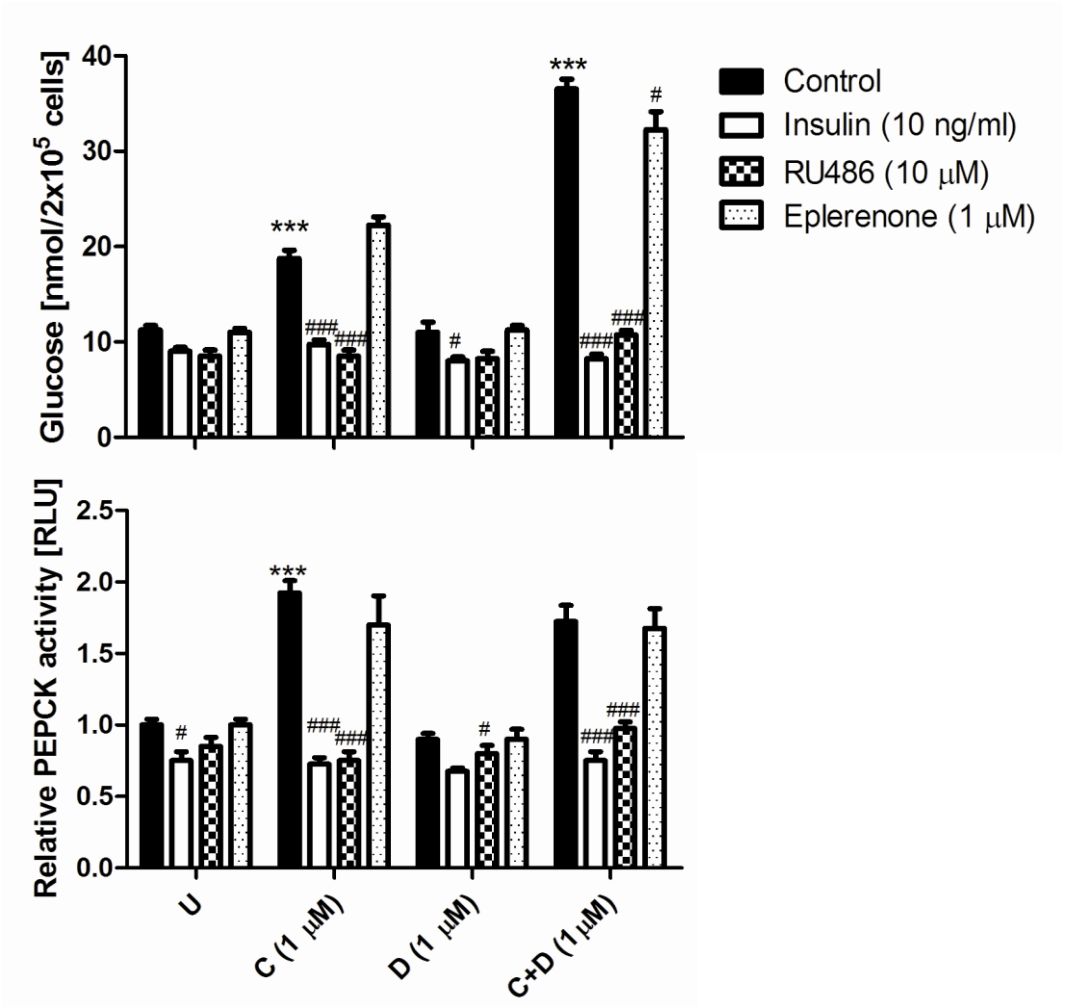


Figure 5.6 The effect of Insulin, RU486 and eplerenone on hepatic glucose production and PEPCK activity in the presence of DOC and corticosterone in fasted H4-II-E-C3 cells.

*H4-II-E-C3 cells were incubated for 6 h in EBSS (20 mM lactate/2 mM pyruvate) with deoxycorticosterone (D, 1 μM), corticosterone (C, 1 μM), and corticosterone with deoxycorticosterone (C + D, both 1 μM) with insulin (10 ng /ml), RU486 (10 μM), and eplerenone (1 μM).(A) glucose measurements in medium. (B) Relative PEPCK activity in the corresponding cells. Results are mean ± S.E.M., n=6. *** = P < 0.001 vs U. # = P < 0.05, ### = P < 0.001 vs the corresponding control treatments for each group. Statistical differences were determined by two-way ANOVA with Bonferroni post hoc test.*

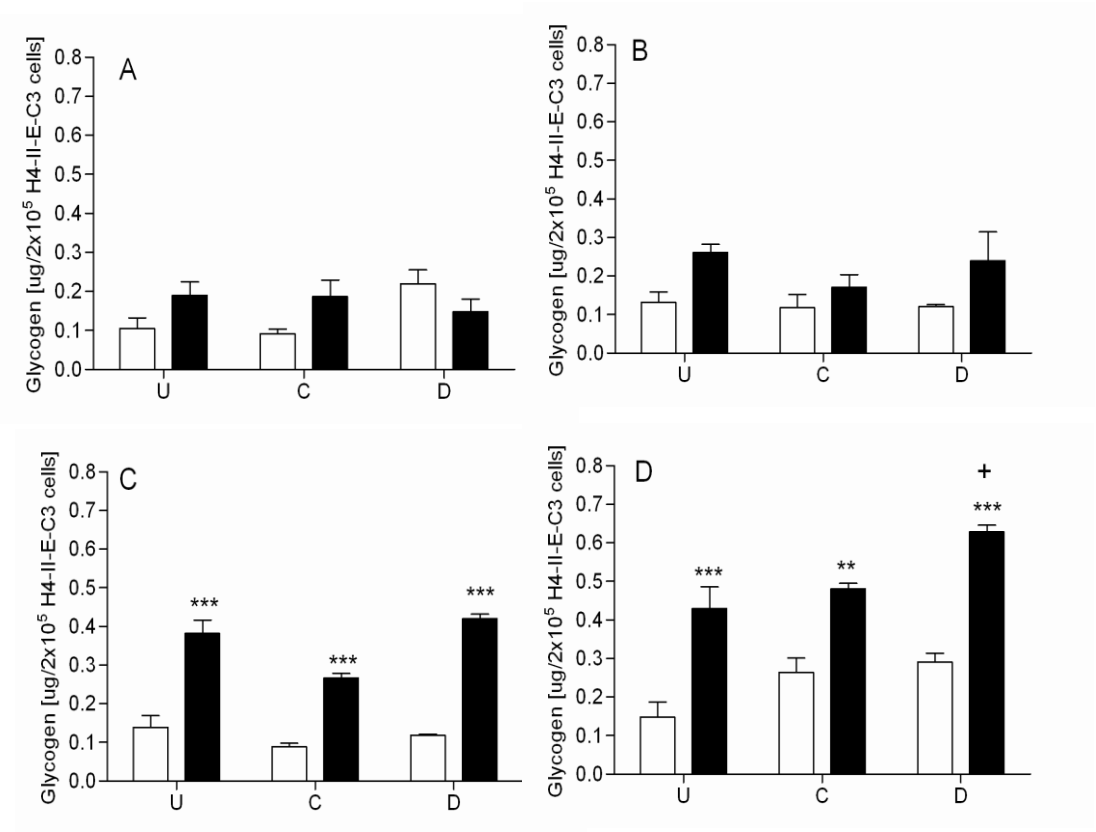


Figure 5.7 The effect of corticosterone, DOC and insulin on hepatic glycogen concentrations in H4-II-E-C3 cells under different glucose concentrations.

*H4-II-E-C3 cells were incubated for 24 h in DMEM containing (A) 5 mM (B) 7 mM (C) 15 mM and (D) 25 mM glucose with untreated (U), DOC (1 µM) (D), and Corticosterone 1 µM (c) both in the presence (black bars) and absence (Clear bars) of insulin (10 ng/ml). Results are mean ± S.E.M., n=6. *** = P < 0.001 vs the corresponding treatments without insulin. + = P < 0.05 vs Insulin alone. Statistical differences were determined by two-way ANOVA with Bonferroni post test.*

5.4 Discussion

In this chapter it was observed that DOC had effects on hepatocyte glucose production which varied according to the incubation conditions. DOC had a markedly different effect on hepatic glucose production under different energy states of the cells. In the fed state with glycogen reserves and surplus gluconeogenic substrates, DOC suppressed hepatic glucose production induced by corticosterone. It is interesting to observe that DOC suppresses PEPCK activity under these conditions. Further studies measuring mRNA and protein levels by western blot would be required to substantiate this finding. This would require specific PCR primers for measuring cytosolic and mitochondrial PEPCK, and selective antibodies for PEPCK1 and PEPCK2, to assess whether these changes were entirely due to altered cytosolic PEPCK1. However, intracellular glycogen concentrations under these experimental conditions were low and did not vary significantly between the different treatments.

In the fed state, RU486 blocked corticosterone-stimulated increases in total glucose production and PEPCK activity. Whereas eplerenone did not modify corticosterone responses under these conditions, which supports the established opinion that these gluconeogenic actions of corticosterone are mediated through GR rather than MR. DOC alone had no effect on glucose production or PEPCK activity in the fed state, but when co-incubated with corticosterone it suppressed the corticosterone induced increase in glucose production and attenuated the increase in PEPCK activity (Figure 5.3). The inhibitory effect of DOC was not reversed by the use of eplerenone, which tends to exclude an action mediated through MR, and suggests that suppression of corticosterone responses by DOC could be due to other metabolic changes in the cell. Whether this involves receptors other than GR or MR is difficult to determine with

currently available antagonists. RU486 completely blocks responses to corticosterone and DOC, however RU486 is not a specific GR antagonist, so it cannot be used to rule out the possibility of other receptors that might be involved in mediating the actions of DOC.

In the fasted state, with increased glycogenolysis and gluconeogenic enzymes, DOC, acted in synergy with corticosterone increasing total glucose production into the external medium. There is constant glucose uptake and release in these conditions and the source of this glucose cannot be determined from these experiments. The PEPCK suppression by DOC was not observed during the fasted state indicating a different regulation (probably due to the increased gluconeogenic substrates) in the fasted state. The glycogen concentrations in these cells were low compared to the fed state, but again there were not significant differences between the treatments. This is not surprising as it was shown that for maximal effects on glycogen production in the presence of insulin the hepatocytes have to be co incubated with a low concentration of a glucocorticoid such as dexamethasone (Lopez *et al.*, 1984). In order to distinguish the true effect of DOC on these cells future investigations should include comparisons of the responses to dexamethasone in the presence .

In the fasted state the cells are geared to increased gluconeogenesis, with other metabolites helping to overcome potential suppressors of glucose production. Under these conditions the combination of DOC with corticosterone increased glucose production compared to corticosterone alone. PEPCK activity with the combination was not different from corticosterone alone. Like the studies in the fed state, RU486 completely blocked the actions of corticosterone when used alone or combined with DOC. Eplerenone did not modify the corticosterone responses or the synergistic effects of DOC, indicating that MR are not involved in these responses. While the

results of these studies are in agreement with GR rather than MR mediating the actions of glucocortiocids, non-genomic actions of DOC through membrane receptors or intracellular actions that are still unknown could be contributing to the increased flux for gluconeogenesis and glycogen turnover in these cells.

The concentration of RU486 (10 μM) may be too high to notice more finite differences between these treatments. Further work with lower concentrations of RU486 or other specific GR antagonists, including confirmation of changes in the gluconeogenic genes using western blots and PCR, is needed to elucidate the mechanism of this interaction between DOC and corticosterone.

However, when hepatocytes were incubated with different glucose concentrations, with and without corticosterone and deoxycorticosterone, the differences in glycogen stores were measurable and significant only in the presence of insulin at higher glucose concentrations above 15 mM. The overall glycogen measures were low probably due to the lack of a fasting step and the inclusion of dexamethasone (10 nM) in all treatment conditions in the experiment. Literature suggests that fasting for 24 h prior to the treatments significantly enhances glycogen synthesis (Boyd *et al.*, 1981).

DOC is considered to have no effect on carbohydrate metabolism or on glycogen deposition in the livers of fasted adrenalectomised animals (Kendall, 1941; Ingle *et al*., 1945). However, others concluded that the difference in action on carbohydrate metabolism of these two corticosteroids is only in the time and rate at which they act, DOC acting more slowly (Montigel *et al*., 1943). It was also shown that DOC was capable of restoring the liver and muscle glycogen content to normal in adrenalectomised rats (Wang *et al*., 1949) confirming that in longer term experiments the differences between corticosterone and DOC are not marked. Large doses of

DOCA have been reported to have a glycogenic effect in adrenalectomised mice (Nissim, 1952). A later study to compare the different effects of corticosterone and DOC showed that in adrenalectomised rats both DOC and corticosterone restored the hexose phosphate levels to the levels of normal rats, with corticosterone being more potent (Conway *et al.*, 1953). The differences observed in these studies are likely due to the differences in the metabolic state of the animal models, which could influence the effect of DOC on carbohydrate metabolism.

There are no studies so far that describe the effect of DOC on glucose production using cultured hepatocytes. But there have been observations of an unknown mechanism or abnormalities in carbohydrate metabolism in animal studies with DOCA. A study investigating the metabolic changes in DOCA hypertensive rats showed that these animals had increased glycogen stores and hyperinsulinaemia but decreased blood glucose levels (Hernandez *et al.*, 2000). However, DOCA treated Wistar rats had significant increases in blood glucose levels compared to control rats (Campion *et al.*, 1998). The differences in these observations could be due to the strains of animals used and the fed and fasted states. So far, no explanation has been provided for these DOCA induced changes in blood glucose levels.

DOC has recently been shown to be both a potent mineralocorticoid and glucocorticoid (Brookes *et al.*, 2012). Previous studies have shown that DOC had no effect on a glucose tolerance test (Soffer *et al*; 1940), which was considered as indirect evidence of a lack of effect on insulin sensitivity (Vinson, 2011). In contrast, DOCA has also been shown to enhance insulin sensitivity (Cheng *et al.*, 1949). In this study it was observed that DOC significantly increased the glycogen stores when used in combination with insulin compared to insulin alone at higher glucose concentrations or when compared to insulin with corticosterone. This suggests that

DOC is an insulin sensitiser. It has yet to be determined whether DOC improves glucose disposal in hepatocytes or in skeletal muscle in the *in vivo* models. The finding that DOC suppresses PEPCK activity, suggests a role similar to that of insulin. DOC suppression of PEPCK activity was not found when cells were fasted. Under these circumstances based on the cells energy needs there is increased gluconeogenesis and DOC may not be a potent suppressor to override the regulatory mechanisms in the fasted state. The increased hepatic glucose output in the fasted state when DOC is used in combination with corticosterone probably reflects increased glycogen turnover.

There is increasing interest in the potential role of Ang II, mineralocorticoids (aldosterone and deoxycorticosterone) and the mineralocorticoid receptor (MR) in the pathogenesis of insulin resistance. High fat fed diabetic mice exhibiting phenotypic characteristics of the metabolic syndrome, including insulin resistance, hypertension, dyslipidemia, and fatty liver showed improved glucose and insulin tolerance tests when co-treated with spironolactone, a mineralocorticoid antagonist. Interestingly, PEPCK mRNA levels were suppressed by spironolactone suggesting a MR dependent signalling mechanism for regulation of gluconeogenic genes. These results indicate that inhibition of mineralocorticoid receptor might be a beneficial therapeutic approach for diet-induced phenotypes of metabolic syndrome and fatty liver (Wada *et al*., 2010). In addition, aldosterone causes hyperglycaemia in fasted mice (Yamashita *et al.*, 2004). Consistent with this, it is reported that in cultured mouse hepatocytes, aldosterone produced a concentration dependent increase in gluconeogenic enzymes such as G6Pase, PEPCK and F1,6Pase (Yamashita *et al.*, 2004). However, because this response was blocked by RU486, the authors concluded that the effect of aldosterone was mediated via a GR dependent mechanism.

Interestingly, the liver specific analogue of RU486 (a bile acid conjugate), A-348441 decreased hepatic glucose output in fasted dogs and fasted fa/fa rats. In freely feeding ob/ob mice chronic administration of very high doses of A-348441 reduced glucose levels without influencing the elevated insulin levels. This treatment had no effect on post-prandial glucose levels in ob/ob mice, suggesting that RU486 interferes with GR-dependent regulation of gluconeogenesis only in the fasted state (Jacobson *et al.*, 2005). However, RU486 shows a poor degree of receptor selectivity being both a progesterone receptor and a glucocorticoid receptor antagonist at nM concentrations (Attardi *et al.*, 2004). So, given this lack of receptor selectivity, RU486 could conceivably block the actions of corticosteroids mediated through other mechanisms not involving either GR or MR.

These observations along with the results in this chapter suggest the interplay between glucocorticoids, mineralocorticoids and 11-deoxycorticosteroids in the regulation of hepatic glucose metabolism merits further investigation. A greater degree of understanding of the receptors and signalling pathways participating in these responses could identify new targets for regulating gluconeogenesis in T2DM.

Chapter 6
General discussion

Regulating blood glucose concentrations within tightly controlled limits is very important for maintaining health. In this respect, the ability to increase or decrease gluconeogenesis is of fundamental importance. Although gluconeogenesis has been studied for around 80 years, many key regulatory mechanisms are only now being elucidated.

Previous experiments leading to the current project showed that intravenous administration of corticotropin releasing factor or CRF family peptides such as sauvagine cause a marked hyperglycaemia (Barker & Corder, 1996; Brown *et al*., 1982). This hyperglycaemia could be a glucocorticoid response due to ACTH release. However, CRF and sauvagine are equipotent stimulators of ACTH release through the pituitary CRF-R1 receptor. Yet sauvagine is at least two fold more potent in inducing the observed hyperglycaemia. Therefore it was considered more likely to be via a CRF-R2 receptor.

Earlier attempts to characterise this hyperglycaemic response showed that it was adrenal-dependent (Kaminski, 2004). However, consistent with other *in vivo* studies of glucocorticoids, acute administration of large pharmacological doses of dexamethasone or corticosterone could not mimic this response. Interestingly, although adrenalectomy blocked the response, metyrapone augmented the hyperglycaemia. Others have also reported metyrapone to cause hyperglycemia in rats (Rotllant *et al.,* 2002). These findings raise important questions about the role of known adrenal corticosteroids and indicate the existence of secondary pathways in adrenal steroidogenesis that result in corticosteroids with greater hyperglycaemic activity.

The goal of this project was to identify corticosteroids secreted by the adrenal gland responsible for the abnormal hyperglycaemia observed after treatment with sauvagine or metyrapone as these may play a role in T2DM and other diseases exhibiting hyperglycaemia or insulin resistance.

6.1 PEPCK activity measurements

PEPCK is reconsidered as a crucial rate-limiting enzyme in this process (Ballard *et al.*, 1969). One of the main aims of this project was to screen cell culture media samples for potential novel gluconeogenic corticosteroids. In order to achieve this, a rapid and sensitive method to measure the effect of extracted fractions (HPLC and SPE) on hepatocyte PEPCK activity was required.

Chapter 2 describes the optimisation of the PEPCK assay to achieve a reproducible method capable of measuring multiple samples in a comparative manner for gluconeogenic activity. The current methods available do not allow this flexibility and certainly cannot be used in a multi-well format. The cell lysis conditions were optimised to obtain reproducible protein quantities from each well without affecting PEPCK activity. The coupling of reaction 1 with reaction 2 required careful optimisation of the Mg^{2+} and Mn^{2+} concentrations as PEPCK required millimolar concentrations of Mg^{2+} and micromolar concentrations of Mn^{2+}. The final reaction required optimisation of the dilution of reaction 2 sufficiently, so that the ATP levels generated in the reaction 2 fall within the linear range of reaction 3. Agents known to regulate PEPCK expression such as 8-Br-cAMP, and insulin were used to validate the reproducibility of the method. The method has been successfully used in this project

and in studies of the effect of endotoxin and fructose on hepatic regulation of PEPCK (Caton *et al.*, 2009; Caton *et al.*, 2010; Caton *et al.*, 2011).

6.2 Experimental conditions for regulation of gluconeogenesis.

The ATP/ADP ratios, NAD/NADH ratios dictate which metabolic pathways are favoured. In the fasted state, there is increased expression of gluconeogenic enzymes including PEPCK (Vegiopoulos *et al.*, 2007). Glycogen stores in the liver cell also contribute to glucose production. In the fed state, the total glucose produced over 6 h is a combination of both gluconeogenesis from substrates, and also from glycogen reserves. These conditions were found to be unsuitable to study the differences between the gluconeogenic potential of the various steroids (Chapter 3). However, when cells are fasted for 4 h, more glucose is produced by the cells in the presence of lactate/pyruvate. The glucose produced by the cells is mainly due to gluconeogenesis and these conditions can differentiate the effects of the various steroids.

The levels of corticosterone in the metyrapone incubations were 40% lower compared to basal incubations. This is consistent with inhibition of 11-β hydroxylase. However, C18 Sep-pak extracts from adrenal incubations with sauvagine and metyrapone had a significantly greater effect on the total glucose productions despite lower corticosterone levels.

6.3 Characterisation of the glucocorticoid potential of adrenal steroids.

Chapter 4 showed that there are significant differences in the steroid extracts prepared from the incubation media from adrenal glands treated with sauvagine and

metyrapone compared to those incubated under basal conditions. Corticosterone levels measured by immunoassay in these extracts when diluted for incubation with hepatocytes were below 0.05 µM. Despite the low levels of corticosterone in extracts from adrenal incubations, these extracts had a stimulatory effect on total glucose production which was 3 times that produced by 1 µM corticosterone. This has not been previously observed under these cell culture conditions, but is consistent with observations that corticosterone on its own does not cause hyperglycaemia *in vivo* (Barker & Corder, 1996; Kaminski 2004).

However, when these extracts were subjected to HPLC none of the fractions collected showed any significant biological activity. It is possible that there were not sufficient quantities of the isolated corticosteroid to have an effect on its own. But the observation that impure corticosterone containing small quantities of DOC, is more potent as an inducer of glucose production compared to purified corticosterone raised questions about the glucocorticoid potential of physiologically secreted corticosteroids. In particular, the possibility that gluconeogenesis is regulated by a combination of corticosteroids with different potencies exerted via a number of distinct mechanisms such that the secreted combination in a given metabolic state might cause a maximal response that is insulin resistant.

It is therefore interesting to note that many of the *in vivo* studies of the effect of corticosterone or other steroids on carbohydrate metabolism have been done in fasted, adrenalectomised animals (McMahon *et al.*, 1988). The effects observed under these experimental conditions do not actually tell us the true story, as they do not consider the possibility of complex interactions of the various steroids and other hormones. The actions of corticosteroids are often complexly related to the functions of other

hormones. The permissive actions of corticosterone has been widely remarked and observed (Ingle, 1961; Vinson, 2009).

Basal levels of corticosterone/cortisol are necessary to allow the effects of other hormones that otherwise can go unnoticed. The results in this thesis show that extracts from adrenal incubations composed of a combination of steroids along with basal levels of corticosterone have a greater effect on hepatocyte glucose production compared to individual fractions or incubation with a high concentration of corticosterone alone.

A detailed study of the synergistic effects of the adrenal steroids without classifying them as mineralocorticoids or glucocorticoids does not appear to have ever been undertaken. It seems likely that the many steroid products that are released into the blood from the adrenal gland have an important role, rather than just representing an overflow of unwanted steroids.

6.4 The importance of glycogen regulation in HGP.

Immunosuppressive doses of prednisolone significantly lower liver glycogen content in normal rats (Riegel *et al.*, 1990), but increase liver glycogen in adrenalectomised and fasted rats (Lee *et al.*, 1983). Administration of 0.1 or 1 mg of prednisolone to fed mice caused a 5-fold activation of glycogen synthase in the liver after 3 h, without significant changes in the circulating levels of glucose or insulin, or in the hepatic concentration of cyclic AMP. But this was not observed in adrenalectomised rats. However, cortisol (10 mg) increased hepatic liver glycogen synthase in adrenalectomised fasted mice (Vanstapel *et al.*, 1982).

Although it has been known for some time that glucocorticoids increase endogenous glucose production, the exact mechanism is still not clear. Glucocorticoids regulate a large number of genes involved in hepatic carbohydrate metabolism (Vegiopoulos *et al.*, 2007). However, it has been suggested that the diabetogenic effects of glucocorticoids were more pronounced in the fasted states of healthy individuals (Wajngot *et al.*, 1992). Glucocorticoid induced hepatic glucose production is currently explained by at least five different mechanisms. First, glucocorticoids induce the expression of key regulatory enzymes like PEPCK and G6Pase (Jin *et al.*, 2004; Vander Kooi *et al.*, 2005). Second, long term exposure to glucocorticoids leads to break down of protein and fat, thus increasing alanine and glycerol as gluconeogenic substrates for the liver (Zimmerman *et al.*, 1989). Thirdly, glucocorticoids facilitate metabolite transport across the mitochondrial membrane enhancing gluconeogenesis in the liver (McMahon *et al.*, 1988). Fourth, glucocorticoids potentiate the effects of other glucoregulatory hormones like glucagon (Dirlewanger *et al.*, 2000). Fifth, glucocorticoid induced increases in hepatic glucose production are dependent on PPAR-α expression. The list of mechanisms is increasing with more and more research on glucocorticoids being performed with fresh perspectives.

It is now well established that 11-β HSD1 amplifies the glucocorticoid (cortisol/corticosterone) signal in the liver and adipose tissue. Selective inhibitors of 11-β HSD1 improved insulin resistance and glucose tolerance in rodent models (Stimson *et al.*, 2007). 11-β HSD1 knockout mice have reduced gluconeogenesis and glycogenolysis suggesting that local corticosterone levels play a very important role in hepatic glucose regulation (Kotelevtsev *et al.*, 1997).

Starvation and stress causes increases in corticosterone levels (Johnstone *et al.*, 2004). It is not entirely clear if these increases in corticosterone alone are responsible for the observed increases in glucose production as similar or even greater (10 μM) concentrations of purified corticosterone *in vitro* fail to achieve the same effect on hepatocytes. The sensitivity of different tissues or cell types to a given level of cortisol/corticosterone varies and the synergistic effect of 11-deoxycorticosteroids in fasted states could play an important role in this general observation in all laboratories.

It was shown that cortisol regeneration significantly increases glycogen turnover in both healthy and T2DM subjects (Andrews *et al.*, 2003; Walker *et al.*, 1995). There is already substantial evidence of increased liver glycogen in patients with T2DM (Krssak *et al.*, 2004). During fasting breakdown of glycogen reserves is expected with subsequent production of glucose by gluconeogenesis, as explained classically. However, some studies have surprised scientists with paradoxical results. In a study involving patients with T2DM, glycogen stores in the liver were assessed by measuring the increases in glucose responses to glucagon after a three-day fast (Clore *et al.*, 1992). Measurements were taken after one day and three days of fasting. Results were compared to those of lean and obese non-diabetic patients. In obese diabetic patients significantly greater glucose responses to glucagon were observed after a three day fast compared to lean and obese control subjects and also compared to the same subjects after a one day fast. This accumulation of glycogen during the fast in the patients with T2DM occurred despite basal rates of hepatic glucose output on the third day of the fast being greater than those of obese non-diabetic subjects (Clore *et al.*, 1992). It was later shown that hepatic glycogen content influences both

the absolute rate of endogenous glucose production and the percentage contribution of gluconeogenesis to total glucose production (Wise *et al.*, 1997).

Animal studies have also found similar surprising results relating to glycogen synthesis and storage. The rate of glycogen synthesis was higher in hepatocytes from fasted obese Zucker rats compared with hepatocytes from lean Zucker rats (McCune *et al.*, 1981). In the Zucker rats, hepatic glycogen content was higher after a six day fast than that found in animals after a two day fast (Triscari *et al.*, 1980). Glycogen synthesis in adrenalectomised animals seems to be different from normal. Liver sections from fasted normal rats showed limited dispersed glycogen synthase activity in both periportal and centrilobular regions. In contrast, activity for glycogen synthase in hepatocytes from fasted adrenalectomised rats appeared as large aggregates in random hepatocytes throughout the lobule (Michaels *et al.*, 1993).

The rate of glycogenolysis among T2DM patients is also controversial. It was reported to be reduced (Boden *et al.*, 2001), unchanged (Gastaldelli *et al.*, 2000), and increased (Basu *et al.*, 2004). These controversies can only be attributed to differences in methods for assessing glycogenolysis and gluconeogenesis. The reason for an increased and preserved glycogen stores in humans and rats during a prolonged fast is completely unexplained. Increased glycogen synthesis by GS activation has been proposed (van de Werve, 1990). Increased gluconeogenesis was put forward as an alternative explanation (Clore *et al.*, 1992), and other studies have indeed demonstrated active glycogen synthesis through gluconeogenic pathways (Jin *et al.*, 2007). The same group also showed that obese Zucker rats had higher fluxes of hepatic pyruvate cycling, PEPCK flux and TCA cycle compared to age-matched controls.

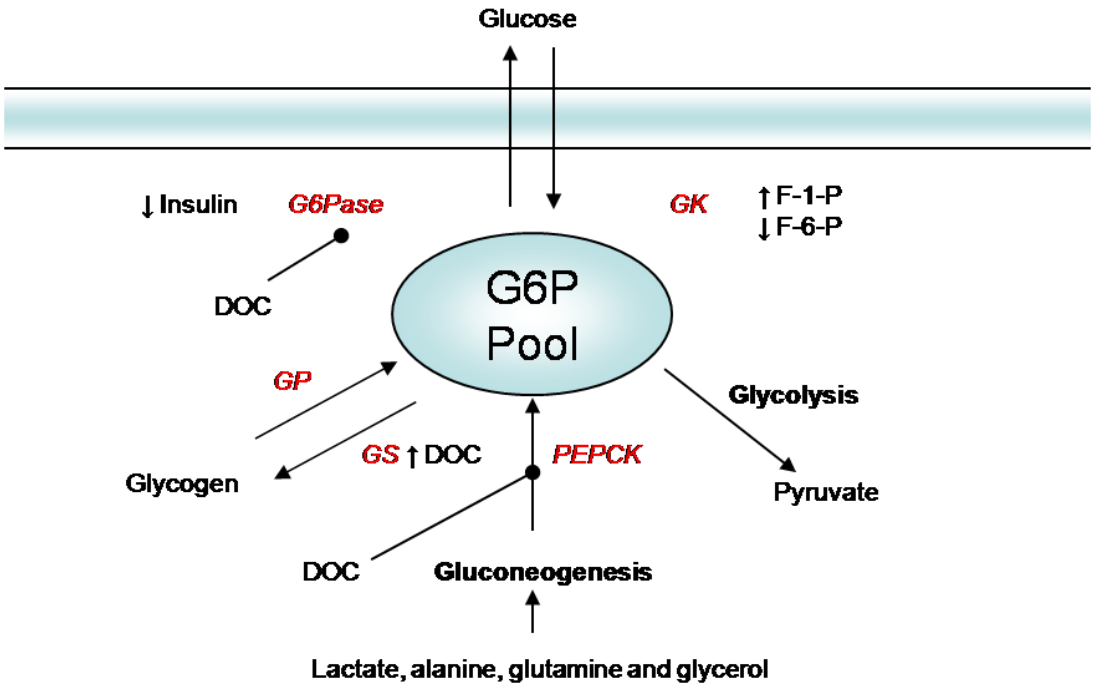


Figure 6.1 The metabolic control of G6P.

Schematic representation of the G6P formed from gluconeogenic precursors and glycogen. The fate of G6P is determined by many regulatory pathways and the energy state of the cell. The key enzymes involved are in red.

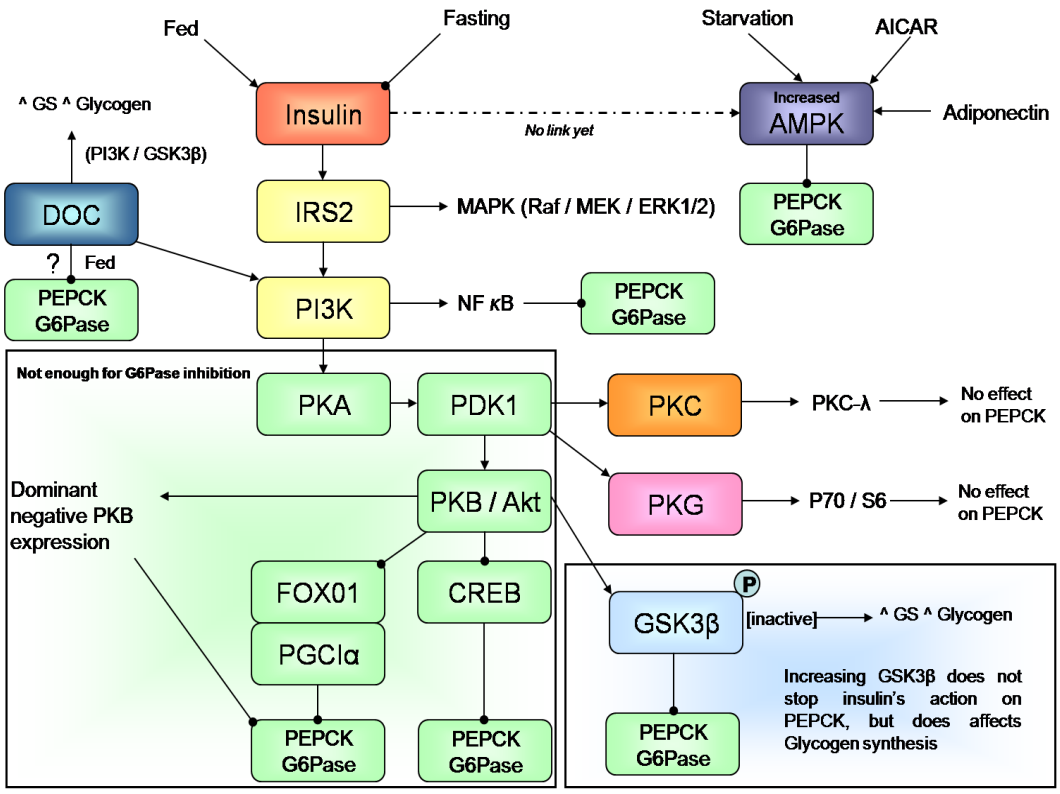


Figure 6.2 An overview of the insulin signalling pathways and various ways of increasing glycogen and suppressing gluconeogenesis.

6.5 A new role for DOC in hepatic carbohydrate regulation.

DOC is generally considered to be a weak mineralocorticoid. A recent review has clearly revealed the mislabelling of this steroid making it more of a universal steroid with both mineralocorticoid and glucocorticoid activities (Vinson, 2011).

The studies described in this thesis bring a new perspective to the role of DOC on hepatic glycogen stores. Two major findings in the studies performed so far are that DOC suppresses the activity of gluconeogenic enzyme PEPCK in fed rat hepatocytes and enhances insulin stimulated glycogen stores in cultured hepatocytes at higher glucose concentrations over a 24 h period. The finding that DOC suppresses PEPCK is novel and brings a new perspective to the role of DOC in the control of gluconeogenesis (Figure 6.2). DOC has recently been shown to have the potential to bind the glucocorticoid receptor and has significant glucocorticoid activity by inducing liver glycogen deposition in adrenalectomised rats and also inducing TAT activity in hepatocytes (Brooks *et al.*, 2011). Whether the binding of glucocorticoid receptor leads to increases in PEPCK or a gluconeogenic flux resulting in glycogen deposition is not clear. However, these two observations may not be related.

Glucocorticoids such as corticosterone and dexamethasone increase hepatic gluconeogenic flux and subsequently regulate glycogen synthesis by increasing the G6P pool. DOC might act differently to this mechanism in activating the GS via the GSK3 phosphorylation and in turn cause a mass pull of the G6P resulting in diverting the gluconeogenic pathway and glucose phosphorylation into glycogen synthesis. It was previously shown that DOC induces phosphorylation of glycogen synthase kinase 3 (GSK3) in the heart (Wyatt *et al.*, 2006). Such effects have yet to be investigated in the liver. GSK3 is a protein kinase, which phosphorylates GS, thus inactivating it.

GSK3 is elevated in diabetic and obese mice (Eldar-Finkelman *et al.*, 1999). Inhibition of GSK3 leads to increase in glycogen synthesis and increase in insulin sensitivity (Henriksen *et al.*, 2006). More recent studies using knockout mice have shown that GSK3α regulates glycogen metabolism in the liver and not in the muscle (MacAulay *et al.*, 2007).

Whether DOC phosphorylates GSK3 in liver is yet to be determined. If one can prove the signalling pathway of DOC in hepatocytes, it could activate GS in similar fashion to insulin. This would then fit with our observation that DOC is insulin sensitizing in the fed state both by increasing glycogen levels and also by decreasing PEPCK levels. This effect is not observed in the fasted state, which suggests other mechanisms are able to override this effect in the fasted state. This is not surprising as there is an absolute requirement for extra cellular glucose. These observations highlight the need for a detailed investigation of the signalling pathways and regulatory actions of DOC on GK, GS and G6Pase and PEPCK.

In the fasted H4-II-E-C3 cells, glycogen reserves have been depleted. Under these conditions the cells would be expected to have decreased cellular ATP levels, with metabolism geared towards glucose production (increased TCA cycle and PEPCK flux through mitochondrial enzymes). Treatment of cells under these metabolic conditions with corticosterone and DOC caused a significant increase in glucose release into the external media. This contrasted with observations from fed cells. It can be explained by an increase in the gluconeogenic flux both due to the cells energy state (ATP/ADP; NAD/NADH ratios) and increased GS activation due to the inactivation of GSK3 as a result of the phosphorylation induced by DOC. Under these conditions the increased GSK3 inactivation by DOC causes a pull of the

gluconeogenic flux through glycogen synthesis and eventual glycogenolysis and glucose release by the activation of G6Pase.

6.6 A case for the role of DOC in insulin mediated hepatic glycogen regulation

Insulin affects HGP directly by binding to the insulin receptor thus activating the insulin signalling pathways and suppressing gluconeogenic genes (Claus *et al.*, 1976; Fisher *et al.*, 2003), or indirectly by reducing glucagon secretion and decreasing lipolysis and proteolysis (Gupta *et al.*, 2002) (Figure 6.3). Glycogen synthesis and breakdown seems to be very complex and varies in different energy states and also in culture (Figure 6.2). Not all glycogen made by the hepatocytes is made directly from the extracellular glucose (Bergman *et al.*, 1982) and the presence of gluconeogenic substrates play a very important role in the indirect process of glyconeogenesis (Boyd *et al.*, 1981; Parniak *et al.*, 1985). The direct route is preferred in cells isolated from fed rats (Parniak *et al.*, 1985), whereas, the indirect route dominates in cells from fasted rats (Spence *et al.*, 1985). This raises a lot of questions about our interpretation of liver glycogen deposition and gluconeogenic ability of steroids in fasted ADX rats. It has to be noted that both these studies indicate that the carbon in glycogen assimilated via the indirect path way can be derived from the glucose which has been through the glycolysis pathway until the triose-phosphate level.

Despite reduced insulin receptor content in certain knockout mice, a normal Akt phosphorylation is seen in the liver suggesting other compensatory mechanisms for insulin signalling pathway (Okamoto *et al.*, 2005). DOC's effect on hepatocytes

(hypothetic diagram Figure 6.1) could play an important role in insulin signalling pathways and is subject to further confirmatory studies.

Fructose on the other hand is a potent stimulator of glycogen synthesis (Woods *et al.*, 1982) and enters the glycolytic/gluconeogenic pathways at the level of triose-phosphate and its metabolite fructose-1-phosphate inhibits phosphorylase and activates GK promoting both the indirect and direct glycogen synthetic pathways (Youn *et al.*, 1987). One wonders if glucocorticoids (such as dexamethasone) and other corticosteroids such as DOC enhance insulin's ability to restore hepatic glycogen stores similar to fructose by affecting the GK and phosphorylase a activities, especially when hepatic glycogenolysis, but not gluconeogenesis is sensitive to acute changes in insulin and glucose (Edgerton *et al.*, 2006).

Amino acids markedly stimulate glycogen accumulation in hepatocytes, but this effect was not reversed when PEPCK was inhibited by mercaptopicolinic acid in hepatocytes prepared from fed rats (Okajima *et al.*, 1979). Did these amino acids somehow increase the flux from glucose to glycogen? However, in fasted intact rats, the use of the same PEPCK inhibitor (mercaptopicolinic acid) virtually abolished hepatic glycogen accumulation (Sugden *et al.*, 1983). This shows the importance of gluconeogenic flux in the contribution to glycogen synthesis in the fasted state. As a homeostatic mechanism it seems logical for new glycogen molecules to be made after a fast before releasing glucose molecules from hepatocytes. This would work even better if there is simultaneous glycogenolysis and glyconeogenesis. This was in fact shown to happen both *in vivo* and *in vitro* (Barrett *et al.*, 1988; Shulman *et al.*, 1987). This would be an ideal scenario during fasting. This also means that glycogenolysis is a significant contributor to HGP.

6.7 The importance of the regulation of G6Pase in hepatic glucose regulation.

Free glucose outside the liver indirectly leads to the activation of GS by a prior inactivation of phosphorylase. Over expression of GK in primary cultures greatly enhances glycogen accumulation and improves glucose utilisation and storage in culture hepatocytes in diabetic rats (O'Doherty *et al.*, 1996; Seoane *et al.*, 1999). Another confirmatory study showed that the G6P arising from gluconeogenesis is as effective as G6P produced by GK in activating GS (Gomis *et al.*, 2003). This suggest that both gluconeogenesis and glucose uptake releases the G6P to the same pool. However, G6P from this pool can also be hydrolysed by G6Pase as shown in the studies where over expression of the gluconeogenic enzyme reduced glycogen deposition (Seoane *et al.*, 1997).

It is quite surprising that glucocorticoids increase hepatic glycogen levels in the fasted adrenalectomised rats also increase G6Pase expression. One can assume that the increased gluconeogenesis is diverted to glycogen synthesis before being released as glucose. It should be noted that the allosteric inhibition of GS by GP only works when the concentration of the G6P pool remains constant. An increase in this pool overrides the inhibition in the culture hepatocytes. GK has a high positive coefficient in glycogen deposition (Agius *et al.*, 1996) and on the other hand G6Pase catalyses the reverse reaction of GK and has a high negative coefficient of glycogen deposition. In other words, hepatic glycogen deposits from glucose are under the control of activated GS, which is in turn controlled by GK, GK reactive protein (GKRP) and G6Pase.

In (H6PDH) knockout mice models, glycogen storage is increased in the fed state reflecting increased flux of G6P through to glycogen (Lavery *et al.*, 2007). These

knockout animals have less G6Pase compared to wild type controls despite the increased concentrations of G6P (substrate for G6Pase). On the other hand PEPCK levels are relatively the same in both groups. It appears that the regulation of G6Pase in the fed and fasted states might be key in understanding hepatic carbohydrate metabolism in diseased states such as diabetes, as well as providing new insights into the metabolic effects of adrenal corticosteroids.

6.8 Future work to characterise the role of GR and MR in gluconeogenesis.

This project showed that corticosterone and DOC in combination resulted in a synergistic increase in glucose production. Future work needs to investigate in more detail the steroid ligands that are able to induce this synergistic interaction. This will enable an assessment as to whether it is a DOC-specific effect, define the relative importance of GR in this response, and confirm lack of importance of MR relative to other yet to be defined receptors. Initial experiments should define the minimum concentration of DOC required to potentiate the corticosterone-induced glucose responses of hepatocytes (H4-II-E-C3 and primary hepatocytes). This concentration of DOC should be evaluated in combination with dexamethasone to assess whether the response to a more selective GR ligand is also enhanced. Further studies should also be conducted to confirm that MR is not involved in this response by testing the combinations of aldosterone with dexamethasone, and aldosterone with corticosterone.

In addition studies are required to define the minimal concentration of RU486 required to block the actions of corticosterone and DOC, as this may enable greater

insights into whether additional receptors besides GR are involved in these responses. To complement these investigations gluconeogenic enzyme activities with protein levels should be determined with DOC and corticosterone combinations at different concentrations, with and without the minimum effective concentration of RU486 to block the corticosterone response. These experiments will give a greater insight into the exact mechanism of action of the increased glucose production observed in the fasted state due to DOC and corticosterone combinations.

Experiments on hepatocytes in the fed state need to further investigate the inhibitory effect of DOC to get a complete picture of its role in hepatic carbohydrate metabolism. More experiments are required to establish the minimum concentration of corticosterone that causes a maximum increase in glucose output. Under these conditions a range of DOC concentrations should be investigated to study the inhibitory effect of DOC. Measurement of mRNA levels for PEPCK, G6Pase and GS will help elucidate the mechanism underlying DOC inhibition of corticosterone-induced glucose production.

6.9 Conclusions

Studies in this thesis indicate that insulin induced glycogen synthesis is potentiated when incubated along with DOC. Further evaluation of this effect should be done in in fed and fasted states with the inclusion of lactate and fructose to observe significant glycogen synthesis. Other studies have reported similar effects after DOCA administration to diabetic animals, which caused an improvement in glucose levels and a decrease in insulin levels (Dai *et al.*, 1992). Future work should investigate whether DOC induces GSK3 phoshorylation and GS activation, as this would create a

metabolic torque at the G6P level. Such actions would support the hypothesis that DOC plays a crucial in glycogen uptake during re-feeding following a fast.

We now know that DOC has glucocorticoid potential (Brookes *et al.*, 2011) such that the detailed signalling and biochemical studies in both cultured hepatocytes and animal models warrant immediate attention. In addition, an investigation of DOC levels in patients with T2DM patients compared to controls is needed. Further studies investigating the effect of various corticosteroids on GK, phosphorylase a, G6Pase and PEPCK will give us a better understanding of the regulatory mechanisms in place during fasting and re-feeding.

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