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I am submitting herewith a dissertation written by Pedro Del Corral entitled "Metabolic Effects of Cortisol During Exercise in Humans." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Education.

Edward T. Howley
Edward T. Howley, Major Professor

We have read this dissertation
and recommend its acceptance

David R. Barrett, Jr.
Dr. L. J.
Charm A. Mestral

Accepted for the Council

Curmink
Associate Vice Chancellor and
Dean of The Graduate School

Metabolic Effects of Cortisol During Exercise in Humans

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Degree

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ABSTRACT

This study examined the physiological effects of an acute reduction in cortisol (C) during exercise in humans. The effects of normal C (NC) were compared to metyrapone-induced low C (LC) on plasma substrate availability and utilization during 2 h of exercise @ ~60% $\dot{V}O_{2\max}$ in 5 men and 4 women. The C responses were compared to pre-exercise (PRE) levels and to a rest day (CON). At rest, C was attenuated by ~70% during LC compared to NC, whereas resting plasma glucose (GLU), lactate (LA), glycerol (GLY), hydroxybutyrate (HYD), alanine (AL), branched chain amino acids (BCAA), insulin (I), glucagon (GLG), growth hormone (GH), epinephrine (EPI) and norepinephrine (NOR) were similar under LC and NC ($p > 0.05$). During exercise under NC, plasma C increased compared to PRE whereas it remained unchanged during LC. During NC, plasma C was elevated at 90 min (compared to CON) and at 120 min (compared to CON and PRE). The exercise response of GLU and LA were similar during NC and LC. Plasma GLY, HYD, BCAA and AL were higher ($p < 0.05$) under NC. Plasma I declined ($p < 0.05$) to a greater extent but GH, EPI and NOR tended to be higher ($0.05 < P \leq 0.10$) during LC. Plasma GLG increased over time ($P < 0.05$) with no difference between treatments. The respiratory exchange ratio did not differ between conditions during exercise. We conclude that during exercise: 1) C normally plays a demonstrable role by accelerating lipolysis, ketogenesis and proteolysis; 2) under LC glucoregulatory hormone adjustments maintain glucose homeostasis during exercise; and 3) despite the changes in substrate availability LC does not alter whole-body substrate utilization; and 4) under acute hypocortisolemia does not alter exercise tolerance.

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CHAPTER I

Introduction

Humans use carbohydrates, lipids and proteins to produce energy during exercise. The type of substrate and the rate at which it is utilized depends to a large extent on the intensity and duration of the exercise bout, as well as on the diet and training status of the individual. Blood glucose is a key substrate during prolonged exercise not only because of its contribution to energy production by the working muscle, but also because of the reliance of the brain on this substrate. The liver maintains the blood glucose level by two processes. The first process is glycogenolysis, the release of glucose from glycogen stores, which represents the first line of action to maintain glycemia. Gluconeogenesis is the second process for glucose production. As liver glycogen stores decrease, glucose production from lactate, glycerol, alanine and other amino acids increases (Coggan, 1991; Wasserman and Cherrington, 1991, Wasserman, 1995). During dynamic cycle exercise of moderate intensity the demand for plasma glucose uptake by working muscle increases ~ 10-fold (Wahren and Felig, 1971) and is met by a 2-to-3 fold increase in hepatic glucose production (Coggan et al., 1992; Coggan et al., 1995a). Plasma glucose concentrations are normally maintained within narrow limits despite the body's normal fluctuations in demand and supply.

The onset of dynamic exercise is associated with an immediate increase in skeletal muscle uptake of non-esterified fatty acids (NEFA) that precedes the onset of lipolysis, leading to a temporary decline in the plasma NEFA concentration (Friedberg et al., 1960). The rates of NEFA uptake and oxidation are dependent on the rate of lipolysis in adipose tissue and the oxidative capacity of muscle. Under metabolic

conditions associated with high rates of NEFA oxidation such as prolonged fasting, diabetes and exercise, the liver produces considerable quantities of acetoacetate and β -hydroxybutyrate which may be utilized by extrahepatic tissues. Amino acids can also participate in energy production via entering as intermediates of the tricarboxylic acid cycle and be oxidized by the working muscle, or they may enter gluconeogenesis.

The rates of hepatic glucose production, lipolysis and proteolysis are normally regulated by the nervous and endocrine systems (i.e., insulin and the counterregulatory hormones). During intense exercise ($>85\%$ $\text{VO}_{2\text{max}}$) hepatic glucose production increases 6-to 8-fold, matching or slightly exceeding glucose uptake. This accelerated hepatic glucose production is observed in the presence of little or no change in plasma glucagon and plasma insulin concentrations. On the other hand, plasma catecholamines are 7-to 10-fold elevated suggesting that during intense exercise the major regulators of hepatic glucose production are the catecholamines. (Coggan et al., 1995b; Kjær et al., 1986 Marliss et al., 1991; Sigal et al., 1996),

At moderate exercise intensities and longer durations (i.e., 60% $\text{VO}_{2\text{max}}$, 60 min) the importance of the catecholamines on maintaining hepatic glucose production is questionable. Marker et al. (1991) blocked the alpha and beta receptors while allowing or preventing changes in insulin and glucagon with the islet-clamp technique. The results of this study were compared to a control study in which subjects underwent islet-clamp alone. During exercise (i.e., 60% $\text{VO}_{2\text{max}}$, 60 min) under adrenergic blockade alone, plasma glucose fell 12% compared to a saline infusion study. When insulin and glucagon were held constant, without alpha and beta adrenergic blockade plasma glucose declined $\sim 35\%$. However, when insulin and glucagon were held constant and adrenergic blockade was present, plasma glucose declined progressively to $\sim 55\%$ of baseline and overt hypoglycemia developed. The investigators concluded that during exercise of moderate intensity and duration a drop in insulin and an increase in glucagon

are most important to maintain hepatic glucose production. Furthermore, sympathochromaffin activation plays a minor role when the insulin and glucagon responses are sufficient but, catecholamines may become critical in the prevention of hypoglycemia during exercise when changes in insulin and glucagon do not take place. Additionally, these investigators reported that during exercise serum NEFA and β -hydroxybutyrate responses were blunted during adrenergic blockade, but were eliminated when insulin and glucagon were held constant and catecholamine actions were blocked. This suggests that the drop in insulin and the increase in the catecholamines are critical to promote lipid catabolism

The progressive hypoglycemia developed during exercise when insulin and glucagon do not change and the catecholamines actions are blocked, suggests a critical role for these hormones. However, this does not prove that other hormones or neurotransmitters are not involved in the prevention of hypoglycemia during exercise. It is now well known that cortisol and growth hormone play important roles in the defense against prolonged hypoglycemia at rest (Boyle and Cryer, 1991, De Feo et al., 1989a; De Feo et al., 1989b). Thus, it is plausible to suggest that during prolonged exercise (>60 min) the roles of cortisol and growth hormone may become apparent. Additionally, there is paucity of data on hormonal regulation of glucose, lipids and amino acids during prolonged exercise.

When humans are challenged by stressful stimuli, such as hypoglycemia, surgery, sepsis, or exercise the hypothalamic pituitary adrenal (HPA) axis is activated, and results in cortisol secretion by the adrenal cortex. Additionally, cortisol controls its own release by means of negative feedback on the hypothalamus and the adenohypophysis. In this manner it can modulate the release of corticotropin-releasing-hormone (CRH) and adrenocorticotropin (ACTH). Approximately 90 to 95% of the circulating cortisol binds to corticotropin-binding-globulin (CBG, also known as

transcortin), albumin and the erythrocytes, whereas the remaining 5-10% is free. The free fraction of cortisol is the biologically active form. In response to glucocorticoid binding, steroid receptors are thought to interact with the specific target DNA to modulate the transcription of certain genes (Burnstein, 1989). Although the aforementioned mechanism is thought to be the chief mechanism of action of glucocorticoids, observations of non-genomic steroid effects are being published at an increasing rate [see (Wehling, 1996) for review].

Cortisol is the chief glucocorticoid produced by humans and it affects carbohydrate, lipid and protein metabolism. Some of the metabolic functions of cortisol related to fuel homeostasis include: (a) a modest increase in hepatic glucose production; (b) proteolysis, allowing amino acid oxidation and/or amino acid entrance into gluconeogenesis; (c) lipolysis, allowing oxidation of NEFA, glycerol's entrance into gluconeogenesis, and ketogenesis; (d) a reduction in insulin dependent peripheral glucose uptake, thus assuring blood glucose supply to the brain; and (e) an increasing the rate of synthesis of gluconeogenic enzymes in the liver (De Feo et al., 1989a; Divertie et al., 1991; Friedman, 1994; Garrel et al., 1995; Rizza et al., 1982; Simmons et al., 1984)

The elevation of plasma cortisol in the morning has been found to be of physiological significance by virtue of its involvement in maintaining higher fasting plasma glucose, increasing gluconeogenesis, proteolysis and ketogenesis (Dinneen et al., 1993; Dinneen et al., 1995). Furthermore, studies using patients with cortisol and growth hormone deficiencies receiving basal hormone replacement have indicated that despite elevated catecholamines, the rise of cortisol and GH are involved in the prevention of prolonged insulin induced hypoglycemia. These findings suggest that the elevation of these hormones may play a critical role in the defense against prolonged hypoglycemia (Boyle and Cryer, 1991).

In humans, the HPA axis is activated during intense (Elias et al. 1991) or prolonged exercise (Tabata et al., 1991) and as a consequence, cortisol levels increase. Davies and Few (1973) were the first to establish a quantitative relationship between exercise intensity (percent of $\text{VO}_{2\text{max}}$) and adrenocortical activation during exercise. Furthermore, the increases in the concentration of serum cortisol during exercise have been attributed to increases in cortisol secretion (Few, 1974).

Studies on the mechanism of activation of the HPA axis have suggested that a decrease in the blood glucose concentration to ~ 3.3 mmol/L triggers the HPA axis to secrete cortisol during low intensity prolonged exercise (Tabata et al., 1991). However, others (Deuster et al., 1992) have reported increases in cortisol levels during prolonged exercise under normoglycemia (4.5 to 5.5 mmol/L). The exercise-induced activation of the HPA axis may be sensitive to a marker of metabolic disturbance that would be detected locally, namely muscle chemoreceptors (Few et al., 1980). In keeping with this suggestion, Kjær et al. (1989) have demonstrated that during exercise afferent neuronal feedback from the working muscle stimulates the secretion of ACTH. This group later proved that there was a direct relationship between stimulation of group III and IV afferent fibers and plasma ACTH (Vissing et al., 1994).

Recently, Czerwinski and Hickson (1990) have shown that following the administration of labeled glucocorticoid in adrenalectomized (ADX) rats, glucocorticoid receptor activation steadily increases to a similar extent during rest and exercise. Receptor activation was $\sim 17\%$, 35% , 40% , 65% and 70% at rest, 5 min, 30 min, 60 min and 90 min, respectively, suggesting that glucocorticoids may exert effects within the time frame of an exercise bout. Numerous studies that examined the metabolic effects of glucocorticoid excess or deficit in animals models indicate that during exercise glucocorticoids : (a) are likely to exert effects within the time frame of a prolonged exercise bout and suggests that the rise of plasma cortisol during exercise may have of metabolic significance, especially in

trained animals; (b) increase substrate availability by stimulating gluconeogenesis, lipolysis and glycogenesis; (c) play a pivotal role in alanine availability and metabolism; (d) modestly increase endurance time to exhaustion, suggesting an ergogenic effect; (e) operate via gene transcription-protein synthesis; and (f) appear to mediate catabolic actions by binding the glucocorticoid type II receptor (Czerwinski and Hickson, 1990; Devenport et al., 1993; Friedman, 1994; Gorostiaga et al., 1988; Sellers et al., 1988; Shaw et al., 1976; Toode et al., 1993; Viru and Smirnova, 1985; Viru et al., 1994).

In regards to humans, several studies have examined adrenocortical insufficient patients and found that these patients: (a) readily remove exogenous cortisol during exercise; (b) have a reduced work capacity due to cardiovascular instability and skeletal muscle weakness, which may be improved by appropriate cortisol replacement; and (c) during heavy exercise under cortisol deficiency may experience cardiovascular collapse and death (Hill et al., 1951; MacBryde and De la Balze, 1944; Mier et al., 1988; Parsons et al., 1984; Zelinsky et al., 1987). Although these studies provide useful information in regards to work capacity, and cardiovascular and muscle function, they failed to examine endocrine profiles, and substrate availability and utilization during exercise.

Others (Barwich et al., 1980; Barwich et al., 1981; Hoelzer et al., 1986; Järhult and Holst, 1979; Johnson et al., 1971) have reported that hypopituitary and adrenalectomized subjects showed proper or even exaggerated metabolic responses during moderate to heavy exercise when glucocorticoid and mineralocorticoid replacement was adequate. Unfortunately, these studies did not provide information on substrates or hormonal responses when the subjects were off cortisol replacement.

To date, there is a paucity of data on the effects of high and/or low cortisol on blood glucose and other substrates during exercise in humans. Our understanding of the effects of cortisol during exercise are based mostly on studies performed on animals [see Viru (1985); Terjung (1978) and Tharp (1975) for a review]. In brief, these studies

used ADX as the experimental model and revealed consistent decreases in endurance time and/or substrate availability. Furthermore, very little is known concerning the physiological significance of the transient increase of cortisol levels during exercise. This lack of information is probably because many of cortisol's effects are delayed when compared to the more fast-acting hormones. However, this does not negate its potential significance during exercise.

The objective of this study is to evaluate the physiological effect of different cortisol levels on: (1) the concentration of plasma substrates; (2) whole-body substrate utilization; (3) the endocrine profile; and (4) selected cardiovascular responses during prolonged exercise. To address this question, we administered metyrapone (i.e., a pharmacological agent that suppresses cortisol production) to induce a pharmacological adrenalectomy and compared that treatment to a control condition in which the subjects received a placebo (allowing the morning cortisol peak).

CHAPTER II

Review Of The Literature

The rates at which carbohydrates, lipids and proteins are used to produce energy during exercise depends on the intensity and duration of the exercise bout, as well as on the diet and training status of the individual. Plasma glucose concentrations are normally maintained within narrow limits despite the body's normal fluctuations in demand and supply. This task is normally regulated by the neuroendocrine system (i.e., counterregulatory hormones). During exercise, increments in glucose utilization are normally matched precisely by increments in hepatic glucose production, allowing plasma glucose levels to remain unchanged. During intense exercise, the major regulators of hepatic glucose production are the catecholamines (Sigal et al., 1996). At lower exercise intensities and longer durations (e.g., 60 min @60% $\text{VO}_{2\text{max}}$) the primacy of insulin and glucagon in preventing hypoglycemia has been established (Hirsch et al., 1991).

Cortisol is the chief glucocorticoid produced by humans and its acute metabolic effects at rest include lipolysis, proteolysis and under certain conditions glucose production. There is a paucity of data on the effects of cortisol on blood glucose and other substrates during exercise in humans. Furthermore, very little is known concerning the physiological significance of the transient increase in cortisol during exercise. This lack of information is probably because most of its effects are delayed when compared to the more fast-acting hormones. However, this does not negate its potential significance during exercise. This chapter will cover the following topics: 1)

substrate metabolism during exercise; 2) the effects of the endocrine system on substrate metabolism at rest and during exercise; 3) the physiology of cortisol and its acute and chronic effects on fuels; 4) the effects of exercise on cortisol levels; and 5) the metabolic effects of cortisol during exercise

1. Substrate Metabolism During Exercise

The Respiratory Exchange Ratio

The respiratory quotient (RQ) is defined as the ratio of CO₂ produced to O₂ consumed. The ratio provides an estimate of carbohydrate, lipid and protein oxidation. Typically, following an overnight fast the RQ is 0.82-0.85. The respiratory exchange ratio (RER) measured at the mouth, represents alveolar gas exchange and is believed to represent whole-body RQ (Ferranini, 1988).

Similarities between RER and the muscle RQ have been previously examined (Jasson et al. 1982). In this study the authors investigated the effect of 25 min of cycle exercise at 65% VO_{2max} on the RER and RQ under high carbohydrate and high lipid diets. The results of the study indicated that although the RER tended to be slightly lower (0.02) than the muscle RQ, the former was able to track the latter under both dietary conditions. The potential benefits of using gas exchange measurements to study metabolism were realized long ago, and nowadays the RER is a standard procedure in exercise physiology.

Carbohydrates

Muscle glycogen

Muscle glycogen is the most abundant form of carbohydrate storage in the human body, with about 400 gm in a 70 kg man (Wahren, 1983). The hydrated polysaccharide is in close proximity to the myocyte contractile unit and may be readily hydrolyzed at the onset of muscle contraction. The resulting glucosyl units are phosphorylated, enter the glycolytic pathway and are either oxidized to lactic acid or processed through the tricarboxylic acid cycle depending on the metabolic state of the myocyte. The intensity and duration of muscle contraction will determine the degree of muscle glycogenolysis. A recent study by Romijn et al. (1993) reported the effects of exercise intensity and duration on the relative contributions of energy substrates on 5 trained subjects during 2 h of exercise at 25 and 65% $\text{VO}_{2\text{max}}$ and 30 min at 85% $\text{VO}_{2\text{max}}$. At 15 min of exercise at 65% $\text{VO}_{2\text{max}}$ muscle glycogenolysis contributed ~40% of the energy expenditure and by the end of the 2 h it declined to nearly 20% of energy expenditure. When the subjects exercised at 85% $\text{VO}_{2\text{max}}$ muscle glycogenolysis contributed over 60% of the energy expenditure. In contrast, at 25% $\text{VO}_{2\text{max}}$ muscle glycogenolysis was negligible throughout the 2 h period.

Muscle glycogen provides most of the carbohydrate to perform endurance events, and the longer the exercise duration the greater the muscle glycogen depletion (Coggan and Coyle, 1991). In fact, it has been documented in both animal and human studies that fatigue in activities requiring approximately 60-85% $\text{VO}_{2\text{max}}$ is closely associated with muscle glycogen depletion (see [Coggan and Coyle, 1991; Conlee, 1987] for a review). In light of muscle glycogen depletion during prolonged or intense exercise, it follows that other forms of carbohydrate must become available to sustain carbohydrate oxidation. The other form of carbohydrate available to the working muscle is blood glucose.

Blood glucose

Blood glucose is a key substrate during prolonged exercise. This is not only true because of its contribution to energy production by the working muscle, but also because of the dependence of the brain on this substrate. If the homeostasis of this substrate is seriously disturbed, cerebral function may be compromised (Gerich, 1993). Fortunately, in healthy individuals the liver releases glucose at approximately the same rate as glucose is taken up by the working muscles and other tissues, and hypoglycemia (< 3.0 mmol/L) seldom develops (Ahlborg and Felig, 1982).

The liver maintains blood glucose levels by two processes. The first process is glycogenolysis, the release of glucose from glycogen storage, which represents the first line of action to maintain glycemia. Gluconeogenesis is the second process for maintaining blood glucose. As liver glycogen stores decline, glucose production from lactate, glycerol, alanine and other amino acids increases (Ahlborg et al., 1974; Ahlborg and Felig, 1982; Coggan, 1991; Wasserman and Cherrington, 1991; Wasserman, 1995). During dynamic cycle exercise of moderate intensity the demand for plasma glucose uptake by working muscle increases 10-fold (Wahren and Felig, 1971) and is met by a 2-to 3-fold increase in hepatic glucose production (Coggan et al., 1992; Coggan et al., 1995a). Furthermore, the demand for plasma glucose increases disproportionately above the "ventilatory threshold" (Cooper et al., 1989) and "lactate threshold" (Coggan et al., 1992). In addition, as length of exercise increases, so does the reliance on plasma glucose and hence hepatic glucose production (Ahlborg and Felig, 1982; Coggan et al., 1995; Wahren and Felig, 1971). For instance, glucose uptake by the legs during cycle exercise at $\sim 60\%$ $\text{VO}_{2\text{max}}$ increases nearly 40% between 40 and 90 minutes of exercise (Ahlborg and Felig, 1982). The increase in plasma glucose utilization over time may partially compensate for decreases in the rate of muscle glycogenolysis (Coggan and Coyle, 1991; Romijn et al., 1993). Thus, plasma glucose

is an important energy source, supplying between 25 to 50% of the total oxidative energy and between 25-100 % of the total carbohydrate oxidized (Coggan, 1991). The variation in these values depends on the training and nutritional status of the individual, and the intensity and duration of the exercise bout (see [Coggan, 1991] for a review).

Early in the course of submaximal exercise, the liver produces glucose primarily by glycogenolysis. However, as liver glycogen declines, glucose requirements must be met by gluconeogenesis (Coggan, 1991; Wasserman and Cherrington 1991, Wasserman, 1995). In fact, it has been shown that 1 h of heavy exercise reduces liver glycogen by 50% (Hultman and Nilsson, 1971). Following an overnight fast and 40-240 min of exercise gluconeogenesis may supply 25 to 60 % of the hepatic glucose production (Ahlborg et al., 1974; Ahlborg and Felig, 1982; Coggan et al., 1995a; Stanley et al., 1988; Wasserman and Cherrington 1991). These estimates are not different from those found in overnight fasted subjects (Magnusson et al., 1992; Petersen et al., 1996; Tayek and Katz, 1996). Gerich, (1993) estimates that in subjects fasted 8-16 h gluconeogenesis accounts for ~ 50% of hepatic glucose production.

Lactate

Excluding blood glucose, lactate is probably the single most studied carbohydrate metabolite in exercise physiology. This compound is produced by myocytes and other cells (i.e., erythrocytes, adipocytes) of the human body under resting conditions as well as accelerated metabolic conditions such as sepsis and exercise. Lactate production is affected by PO_2 and the redox state of the cell (Katz and Shalin, 1988). With increasing exercise intensities lactate production increases and can be oxidized by nearby myocytes or can diffuse to the circulation and be extracted by heart and liver. If extracted by the liver, lactate will undergo gluconeogenesis. In fact, lactate is a major gluconeogenic substrate during exercise in humans (Ahlborg and Felig,

1982; Stanley et al., 1988). Ahlborg and Felig (1982) demonstrated that during prolonged cycle exercise there is a 2-to 3-fold increase in lactate fractional extraction by the splanchnic bed and that the increased lactate delivery to the splanchnic bed is not only the result of leg lactate output, but also that of less active muscles (e.g., forearm). Later Stanley et al. (1988) examined the importance of lactate gluconeogenesis during 40 min of cycle exercise at 45% $\text{VO}_{2\text{max}}$. By using radioactive isotopes the authors demonstrated that ~ 25% of lactate removed appeared as glucose, whereas the remaining 75% underwent direct oxidation, primarily in exercising muscle. Additionally, it is now known that improved lactate removal during progressive exercise after training is due mainly to an increased lactate oxidation rather than an accelerated lactate gluconeogenesis (MacRae et al., 1995)

Although muscle glycogen, blood glucose and lactic acid contribute a large fraction of the carbon flux during moderate to heavy exercise, lipids also contribute to the substrate oxidation pool. In fact, as the limited carbohydrate store is depleted, non-esterified free fatty acid (NEFA) from peripheral adipocytes and intramuscular triacylglycerol depots, coupled with ketone bodies and glycerol, become fundamental fuel sources in the latter stages of a prolonged (> 60 min and < 60% $\text{VO}_{2\text{max}}$) exercise bout. Furthermore, in endurance-trained subjects lipids provide most of the energy expenditure during light to moderate exercise (Romijn et al., 1993)

Lipids

NEFA, intramuscular triacylglycerides and glycerol

Visceral and subcutaneous adipose tissue supply NEFA to the working muscles. At the onset of adipose tissue lipolysis, NEFA and glycerol are released into the blood stream. The NEFA bind albumin allowing for transport to muscle, liver, heart and other organs to be taken up and oxidized for the production of energy. Glycerol, a water soluble compound, may be extracted by the liver to enter gluconeogenesis or it may be used to form triacylglycerol depending on hormonal signals and the metabolic state of the hepatocyte (Wasserman and Cherrington, 1991). Typically, lipids are the major fuel during low intensity exercise (i.e., 25% $\text{VO}_{2\text{max}}$) and they become increasingly important as exercise duration increases (Romijn et al., 1993). Following an overnight fast, lipids supply ~ 50-75% of the resting caloric needs in healthy subjects. However, the onset of dynamic exercise is associated with an immediate increase in skeletal muscle uptake of NEFA that exceeds the onset of lipolysis, leading to a temporary decline in plasma NEFA concentrations (Friedberg et al., 1960). The rates of NEFA uptake and oxidation are dependent on the rate of lipolysis in adipose tissue, which in turn varies depending on whether the adipocyte store is subcutaneous, intraabdominal or intramuscular (Martin, 1996). Additionally, it has been demonstrated that whole-body lipolytic rates are greater in endurance trained athletes than in sedentary controls during high intensity exercise at the same relative intensity (Klein et al., 1996), whereas glycerol kinetics were the same when trained and untrained subjects exercised at a low intensity (Klein et al., 1994).

The other source of NEFA is the intramuscular triacylglycerol store which appears to be more abundant in type IIa > type I, > type IIb myocytes (Martin, 1996). The individual contribution of peripheral and intramuscular NEFA to energy metabolism

was not clearly understood until recently. Romijn et al. (1993) examined the effects of exercise intensity and duration on intramuscular triacylglyceride and peripheral adipocyte NEFA mobilization and utilization. The investigators used stable isotopes in 5 trained cyclists during exercise at 25, 65 and 85% $\text{VO}_{2\text{max}}$ for 2 h, 2 h, and 30 min respectively. The findings suggest that the peripheral lipolytic response after 30 min of exercise was not significantly different at the three intensities. At 25% $\text{VO}_{2\text{max}}$ the RER of 0.75 reflected primary reliance upon lipid oxidation. At 65% $\text{VO}_{2\text{max}}$, the RER was 0.83 and peripheral lipolysis and intramuscular triacylglycerols contributed equally to lipid oxidation. Similarly, at 85% $\text{VO}_{2\text{max}}$ intramuscular triacylglycerols and peripheral lipolysis contributed to a similar extent to lipid oxidation. Interestingly, total lipid oxidation did not differ between exercise at 25 and 85% $\text{VO}_{2\text{max}}$, whereas at 65% $\text{VO}_{2\text{max}}$, lipid oxidation was significantly higher than at 25 and 85% $\text{VO}_{2\text{max}}$.

Other forms of lipids such as plasma lipoproteins and ketone bodies contribute to energy metabolism during exercise. For instance plasma lipoprotein (VLDL and chylomicrons) may supply up to ~ 10% of the energy derived from free fatty acid oxidation (Martin, 1996).

Ketone bodies

Under metabolic conditions associated with high rates of NEFA oxidation such as prolonged fasting, diabetes and exercise, the liver produces considerable quantities of acetoacetate and β -hydroxybutyrate (Gorki, 1990; Wasserman and Cherrington, 1991). Acetoacetate continuously undergoes spontaneous decarboxylation to yield acetone. These three compounds (acetoacetate, β -hydroxybutyrate and acetone) are collectively known as ketone bodies. With the exception of acetone which is lost through the lungs, ketone bodies may be utilized by extrahepatic tissues during prolonged fasting and exercise. Isotopic estimates with labeled acetoacetate and β -hydroxybutyrate have

demonstrated that, during 2 h of cycle exercise at 50% $\text{VO}_{2\text{max}}$, the rate of appearance and the metabolic clearance rate of ketone body increases (Ferry et al., 1983; Ferry et al., 1986). These findings suggest that, despite an increase in ketone body uptake, ketone body concentration increases during exercise. These findings were confirmed and further extended by direct measurement of the splanchnic output and leg uptake of ketone bodies at rest and during 60 min of cycle exercise at 45% $\text{VO}_{2\text{max}}$ (Wharen et al., 1984). The authors demonstrated that the increase in ketogenesis was associated with the rise in splanchnic inflow and fractional extraction of NEFA as well as an increased intrahepatic interconversion of NEFA to ketone bodies. Although leg uptake of ketone bodies rose 2-fold during exercise, calculated ketone body oxidation accounted for only 3% of energy expenditure (Ferry et al., 1983; Ahlborg et al., 1984). However, this substrate is an important fuel during the recovery period after prolonged exercise (Ferry et al., 1983; Ferry et al., 1986). Finally, fuels other than carbohydrate and lipids may contribute to the total substrate pool, namely amino acids.

Amino Acids

Proteins are involved in numerous functions in the body, and when broken-down by proteases, plasma free amino acids increase. Amino acids are probably the most flexible carbon skeletons; they can be converted to glucose, tricarboxylic acid intermediates, NEFA and ketone bodies (Stryer, 1988). Amino acids can participate in energy production as intermediates of the tricarboxylic acid cycle or they may enter gluconeogenesis. From the amino acid pool, alanine, glutamine, and branched chain amino acids (BCAA) appear to be the key contributors to energy metabolism (Felig and Wahren, 1971; Gerich, 1993; Hood and Terjung, 1990; Perriello et al., 1996). The amino acid pool is dependent on the rates of protein synthesis and degradation, both of

which are influenced by exercise. Of the 20 alpha-amino acids only the BCAA (leucine, isoleucine and valine), alanine, glutamate and aspartate are oxidized at a significant rate by skeletal muscle (Hood and Terjung, 1990).

Alanine

During exercise plasma alanine and other free amino acids increase in concentration (Carraro et al., 1994; Coggan et al., 1995; Einsphar and Tharp, 1989; Felig and Wahren, 1971) and contribute up to 10% of energy needed during prolonged exercise (Hood and Terjung, 1990). It has been shown that amino acids are released from the working muscles during exercise (Felig and Wharen, 1971). This group showed that exercise of light, moderate and high intensity induces a net release of alanine. Recently, a group of investigators provided evidence that although the major fraction of alanine flux is derived from *de novo* synthesis from pyruvate during light exercise ($\sim 40\%$ $\text{VO}_{2\text{max}}$), alanine provided by proteolysis is also accelerated (Carraro et al., 1994). This increase may be important in the maintenance of blood glucose levels via the glucose-alanine cycle. Additionally, alanine is thought to play a pivotal role in amino acid nitrogen transfer to the liver (Felig and Wahren, 1971; Hood and Terjung, 1990).

Branched chain amino acids

BCAA may be released from the splanchnic bed (Felig and Wahren, 1971) and be subsequently taken up by active muscle during exercise (Ahlborg et al., 1974). William et al. (1996) examined leucine kinetics during exercise in a dog model by using arteriovenous differences in amino acid concentrations and tracer techniques. The authors reported that most ($\sim 70\%$) of leucine's whole-body rate of appearance came from gut proteolysis as opposed to muscle proteolysis. The authors concluded that

proteins mobilized from the gut provide amino acids that may be utilized to reduce muscle proteolysis during exercise. During exercise the rate of leucine oxidation appears to be directly related to the whole body metabolic rate and has been reported to increase 3-to 5-fold during prolonged exercise of moderate intensity (Hood and Terjung, 1990). Although the contribution of leucine oxidation to energy metabolism may account for ~1%, when total BCAA is taken into account, it may provide 3% of energy expenditure. Additionally, BCAA appear to play a key role in nitrogen transfer to alanine, glutamine and aspartate which may be instrumental for both proper nitrogen metabolism, and the maintenance of a high gluconeogenic rate (Hood and Terjung, 1990).

2. Effect Of The Endocrine System On Substrate Metabolism At Rest

Plasma glucose concentrations are normally maintained within narrow limits despite the body's normal fluctuations in demand and supply. This task is normally regulated by the neuroendocrine system (i.e., counterregulatory hormones). However, under severe insulin-induced hypoglycemia (i.e., < 1.8 mmol/L), hepatic autoregulatory mechanisms serve as a final defense mechanism to prevent further hypoglycemia and central nervous system damage (Bolli et al., 1985). At rest, the role of the counterregulatory hormones insulin, glucagon, epinephrine, triiodothyronine, cortisol and growth hormone has been demonstrated [see (Cryer, 1993; Gerich, 1993) for review]. Recently, it has been postulated by Cryer (1993) that there is a hierarchy among the glucose regulatory factors. The first line of action against decrements in blood glucose is a drop in insulin secretion. Among other regulatory factors, glucagon plays a key role, and epinephrine, which is not normally critical, becomes critical when glucagon is deficient. Cortisol and growth hormone are involved in the recovery from

prolonged insulin-induced hypoglycemia. Cortisol and growth hormone not only control glucose production and utilization, but lipolysis and ketogenesis. On the other hand, acute proteolysis appears to be primarily under the control of cortisol (see [De Feo 1996] for a review). However, although we understand how blood glucose is regulated at rest, less is known about the role of the counterregulatory hormones during exercise particularly about the physiological effects of cortisol.

3. Effect Of The Endocrine System On Substrate Metabolism During Exercise

High Intensity

During exercise, increments in glucose utilization are normally matched precisely by increments in hepatic glucose production, allowing plasma glucose levels to remain unchanged. However, during intense exercise ($>85\% \text{ VO}_{2\text{max}}$) there is a mismatch between the glucose rate of appearance (R_a) and rate of disappearance (R_d) leading to increases in plasma glucose. During such exercise there is only a modest, if any, increase in plasma glucagon whereas plasma insulin remains unchanged or declines slightly (Coggan et al., 1995; Kjær et al., 1986; Marliss et al., 1991; Sigal et al., 1996). Although these small changes may be of physiological significance (Wasserman and Cherrington, 1991; Wasserman, 1995) it may not account for the much larger rise in glucose R_a (5 to 8 fold). In contrast, the plasma concentration of epinephrine and norepinephrine increase 10-to 15-fold (Coggan et al., 1995; Kjær et al., 1986; Marliss et al., 1991; Sigal et al., 1996). Furthermore, Marliss et al. (1991) reported significant

correlations between plasma norepinephrine ($r=0.82$) and Ra, and epinephrine ($r=0.71$) and Ra, suggesting a major glucoregulatory role for the catecholamines.

In a recent study Sigal et al. (1996) examined the relative roles of catecholamines versus glucagon and insulin responses in stimulating glucose Ra. Eleven subjects exercised at 87% VO_{2max} for ~ 13 min during an islet cell clamp in which a somatostatin analog (Octreotide), insulin, glucagon and growth hormone were infused at baseline levels to maintain euglycemia. A group of 8 served as control subjects and exercised without islet cell clamp. Glucose Ra increased 6-fold in the islet cell clamp and control group, and plasma catecholamines increased >10-fold with no differences between groups. This study suggested that during intense exercise, the major regulators of hepatic glucose production are the catecholamines, because insulin, glucagon and growth hormone did not change in either group.

Moderate Intensity

At moderate exercise intensities (e.g., 60% VO_{2max}) and longer durations (e.g., > 60 min) the primacy of the catecholamines in maintaining hepatic glucose production is questionable (Marker et al., 1991). Marker et al. (1991) blocked the alpha and beta receptors while allowing (i.e., saline) or preventing (with the islet cell clamp technique) changes in insulin and glucagon. The results of this study were compared to a control study in which subjects underwent an islet clamp alone. During exercise (60 min @ 60% VO_{2max}) under adrenergic blockade alone plasma glucose fell (12%) compared to a saline infusion study in which plasma glucose fell 6%. When insulin and glucagon were held constant without alpha and beta adrenergic blockade, plasma glucose declined ~ 35%. However, when insulin and glucagon were held constant and adrenergic blockade was present plasma glucose declined progressively to ~55% of

baseline and overt hypoglycemia developed. The investigators concluded that sympathochromaffin activation plays a minor role when insulin and glucagon are allowed to respond normally. However, catecholamines may become critical to the prevention of hypoglycemia during exercise when changes in insulin and glucagon do not take place.

Similarly, bilaterally ADX humans (glucocorticoid and mineralcorticoid replaced) who exercised at 60% $\text{VO}_{2\text{max}}$ for 60 min showed an increase in glucose Ra not different from control subjects (Hoelzer et al., 1986). Additionally, others have reported stable blood glucose responses during more vigorous exercise intensities in this patient population (Barwich et al., 1981; Järhult et al., 1979). Furthermore, intact hepatic innervation is not necessary during exercise (40 and 75% $\text{VO}_{2\text{max}}$) to sustain hepatic glucose production in Celiac ganglion blocked (i.e., blocking norepinephrine release to the liver) subjects (Kjær et al., 1993) and liver transplant recipients (Kjær et al., 1995). Taken together, these findings indicate that neither norepinephrine nor epinephrine are essential for hepatic glucose production during exercise of moderate intensity. Thus, during moderate exercise other regulatory factors must modulate hepatic glucose production.

During moderate exercise, the role of NEFA and ketone bodies and other hormones becomes important. The control of lipolysis and ketogenesis is complex and affected by numerous factors such as nutritional status, exercise intensity, and duration, plasma lactic acid, blood flow to the adipose tissue, and hormones (Martin, 1996). In the complex hormonal milieu the catecholamines and insulin stand as key regulators of lipolysis (Martin, 1996). Any hormone or neurotransmitter that regulates NEFA mobilization, hepatic NEFA extraction, or intrahepatic lipid oxidation may play a role in the regulation of ketone bodies during exercise (Wasserman and Cherrington, 1991).

The study by Marker and associates (1991) showed that serum NEFA and β -hydroxybutyrate responses were blunted during adrenergic blockade and were prevented when insulin and glucagon were held constant and catecholamine actions were blocked. Chalmers et al. (1979) examined the role of somatostatin on the hormonal and substrate response to exercise. Six healthy males cycled for 30 min at 70% $\text{VO}_{2\text{max}}$ under somatostatin and under normal conditions. The results of that study showed that insulin, glucagon and growth hormone were suppressed at rest as well as during exercise with somatostatin, whereas NEFA, glycerol and ketone bodies increased similarly under both exercise conditions. Other investigators examined the effects of light exercise on plasma substrates under constant high insulin and glucagon concentrations versus low insulin and glucagon concentrations. Subjects cycled at 40% $\text{VO}_{2\text{max}}$ for 60 min under the 2 experimental conditions and a control trial (saline infusion). The different insulin infusions had pronounced effects on resting NEFA concentrations. The high insulin group had depressed NEFA levels in the basal state and throughout exercise as compared to the low insulin and control group, whereas the NEFA concentration during low insulin and control trials were not different (Wolfe et al., 1986). These findings, coupled to those of Marker and associates (1991), suggest that insulin and the catecholamines play a pivotal role in the hormonal control of lipolysis and ketogenesis during exercise.

In the study by Chalmers et al. (1979), somatostatin induced a significant drop in blood glucose when compared to the control condition. Whereas insulin levels differed only slightly, glucagon levels differed by a large margin. This suggests that the drop in insulin is not the sole factor accelerating glucose production and indirectly provides some evidence for the importance of the increase in glucagon. On the other hand, Wolfe et al. (1984) reported that when changes in insulin and glucagon were prevented, hepatic glucose production did not keep up with glucose utilization and

hence, plasma glucose fell ~ 45% from baseline. Björkman and associates (1981) reported that somatostatin induced a drop in hepatic glucose production and the plasma glucose concentration when compared to a control condition. However, by the 30th min of exercise hepatic glucose production and the plasma glucose concentration were normal. The authors suggested that during exercise the rise in plasma glucagon is not essential to maintain hepatic glucose production.

Hirsch and associates (1991) went a step further and examined the individual effects of insulin and glucagon on substrate availability during exercise. A group of men were studied during 60 min at 60% $\text{VO}_{2\text{max}}$ under 4 different somatostatin, insulin, and glucagon infusion regimens to: 1. hold insulin and glucagon constant; 2. decrease insulin; 3. increase glucagon; 4. decrease insulin and increase glucagon. Growth hormone was replaced at basal levels in all trials and an additional control trial (saline infusion) was performed. Under the first three experimental conditions blood glucose declined similarly, ~ 40% compared to 6% in controls. However, when insulin was decreased and glucagon was increased, blood glucose declined only 18%, not significantly different from the control study, suggesting that both insulin and glucagon are involved in the prevention of hypoglycemia during exercise of moderate intensity and duration.

Taken together, these studies (Chalmers et al. 1979; Hirsh et al. 1991; Wolfe et al. 1986) demonstrate the critical roles of insulin and glucagon in preventing hypoglycemia during exercise. Wasserman and Cherrington (1991) suggested that insulin and glucagon operate by signaling increments in glucose production.

The progressive hypoglycemia developed during exercise when insulin and glucagon do not change and the catecholamines actions are blocked, suggests an important role for these hormones. However, it does not prove that other hormones or neurotransmitters are not involved in the prevention of hypoglycemia during exercise.

Additionally, there is a paucity of data on hormonal regulation of glucose, lipids, and amino acids during prolonged exercise (>60 min). In fact, the studies that have examined the roles of insulin and glucagon via somatostatin have consistently failed to replace the growth hormone rise during exercise, potentially confounding their results. On the other hand, no studies have clamped cortisol at different concentrations during exercise. It is now well known that at rest cortisol and growth hormone play important roles in the defense against prolonged insulin-induced hypoglycemia (Boyle and Cryer, 1991, De Feo et al. 1989a; De Feo et al. 1989b). Thus, it is plausible to suggest that during prolonged exercise the roles of cortisol and growth hormone may become more important.

There is a paucity of data on the effects of cortisol on blood glucose and other substrates during exercise. Furthermore, very little is known concerning the physiological significance of the transient increase of cortisol levels during exercise. This lack of information is probably because most of its effects are delayed when compared to the more fast acting hormones. However, this does not negate its potential significance during exercise. Current understanding of the effects of cortisol during exercise are based mainly on animal studies [see (Terjung, 1978; Tharp, 1978; Viru, 1985) for a review]. The evaluation of these findings is often difficult and may lead to erroneous conclusions because of the potential differences in exercise metabolism between humans and different animal species (Coggan et al. 1995).

4. Cortisol

Hypothalamic Pituitary Adrenal Axis

Table 1 lists some of the historical landmarks in our understanding of the adrenal gland and its relationships to energy metabolism and exercise. When humans are challenged by stressful stimuli such as starvation, insulin-induced hypoglycemia, surgery, sepsis or infection, the hypothalamus receives input via the limbic system (hippocampus, septum and amigdala), higher brain centers, and/or direct blood borne chemical stimulation (e.g., hypoglycemia). The arcuate, supraoptic and paraventricular nuclei of the hypothalamus will synthesize and release corticotropin releasing hormone (CRH) and vasopressin which in turn stimulates the adenohypophysis to synthesize proopiomelanocortin. This peptide will in turn be cleaved and glycosylated to yield ACTH (Jacobson and Sapolski, 1991). Other factors such as oxytocin, angiotensin II and interleukin 6 have been found to stimulate ACTH secretion (Abou-Samra et al., 1988; Plotzki et al., 1985). As this short-lived peptide reaches the adrenal cortex it will stimulate the synthesis and release of cortisol into the blood stream where it exists free in plasma, and bound to transcortin, albumin and erythrocytes (Al Ansary et al., 1982; James and Few, 1985; Kehlet and Binder, 1976; Hiramatsu and Nisula, 1988; Vining et al., 1983). Interestingly, there is evidence that extrahypothalamic immunoreactive-CRH may be involved in pituitary ACTH secretion (Sasaki et al., 1987). Moreover, Azar et al. (1992) has postulated an ACTH-independent effect of CRH on cortisol secretion. The authors speculated that CRH may have a direct effect on the human adrenal gland, or it may release ACTH-like factors that stimulate the human adrenal cortex to secrete cortisol.

Table 1. Some Landmarks On Adrenal Cortical Chronology

YEAR	EVENT	RESEARCHERS
1563	Adrenals described	Eustacheo
1855	Adrenal insufficiency in man	Addisons
1856	ADX fatal in dogs	Brown-Sequard
1910	Hypoglycemia in Addison's	Porges
1927	First active adrenal extract	Hartman et al
1931	Decrease work capacity in ADX animals	Briton & Silvette
1942	Decrease work capacity in ADX humans	MacBryde & De La Balze
1945	Partial synthesis of cortisone	Sarret et al
1955	Cortisol t 1/2 was determined	Peterson et al
1955	Cortisol response during exercise-humans	Stahealin et al
1955	Addisons patients and exercise	Kägi
1973	Cortisol threshold during exercise	Davies and Few
1983	Cortisol deficiency fatal during exercise	Parson's et al
1991	HPA axis during prolong exercise	Tabata et al.
1991	HPA axis during intense exercise	Elias et al

* Modified from Tepperman (1988)

Synthesis And Secretion

Cortisol is also known as compound F, and 11- β , 17 α , 21-trihydroxypregn-4-ene-3,20-dione. This 21-carbon steroid is the primary and most potent glucocorticoid synthesized by humans (Figure 1). Its synthesis takes place in the zona fasciculata and reticularis of the adrenal cortex. Cholesterol is the precursor for adrenal steroid hormone biosynthesis and the bulk of adrenal cholesterol is derived from the uptake of plasma cholesterol. Cholesterol esters and triacylglycerols are packed into lipoproteins that contain small amounts of apoproteins. The apoproteins bind to the lipoprotein receptors on the adrenal target cells and are believed to stimulate endocytosis and the transport of the lipoprotein into the cells, an event enhanced by the action of ACTH. In the basal state, there is a balance between the cholesterol delivered to the adrenal cells and the amount of steroid hormone secretion (James and Few, 1985).

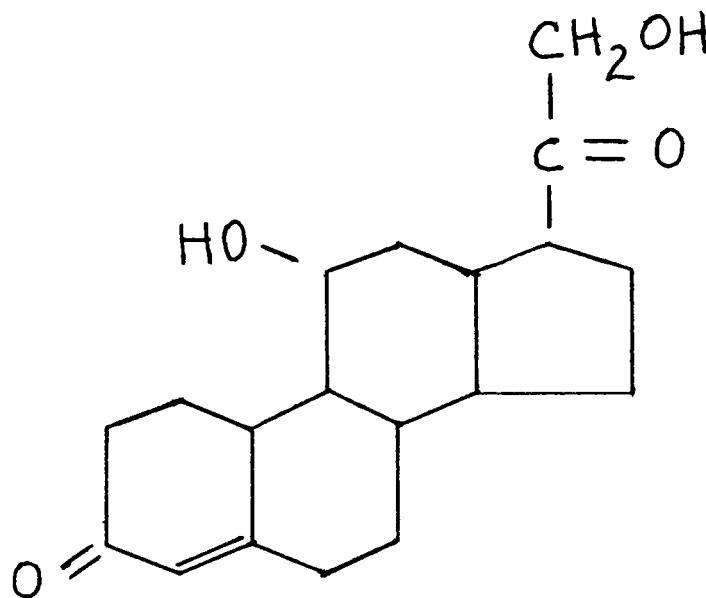


Figure 1. Structural formula of cortisol.

The first step in the biosynthetic pathway of cortisol occurs in the mitochondria and involves the cleavage of the terminal six carbons of the 27 carbons of cholesterol. This results in the formation of pregnenolone in a step mediated by a cytochrome P₄₅₀ side chain cleavage enzyme. Pregnenolone is then released from the mitochondria into the smooth endoplasmic reticulum where a double bond is shifted from the fifth to the fourth position and the hydroxyl group at the C₃ position is oxidized into a keto group. The remaining steps of cortisol synthesis consist of an additional hydroxylation, at position 11 by various NADPH-dependent hydroxylases. A common pathway is as follows: pregnenolone ----> 17- α hydroxypregnenolone -> 17- α hydroxyprogesterone -> 11-deoxycortisol -> cortisol (James and Few, 1985; Pincus, 1955; Simpson and Waterman, 1988). See figure 2 for an schematic representation of adrenal steroidogenesis.

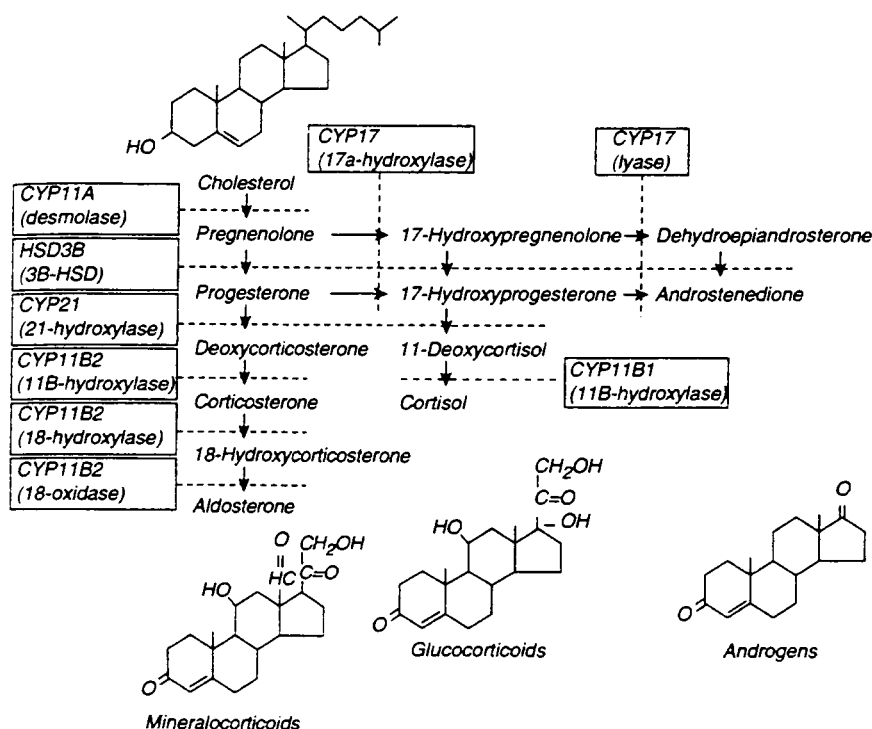


Figure 2. Steroid synthesis pathways. Adapted from Griffin, J. E. and S. R. Ojeda (1996)

Control Of Synthesis & Secretion

When corticotropin releasing hormone (CRH) is released from the hypothalamus it induces ACTH release from the adenohypophysis, which in turn triggers the adrenal cortex to synthesize and release cortisol. Adrenocorticotropin binds to the plasma membrane of the zona fasciculata cells and activates adenylate cyclase. This increases intracellular cAMP, causing a rapid increase in the intracellular levels of cholesterol in the vicinity of cytochrome P₄₅₀_{sc} in the inner mitochondrial membrane. This is followed by the activation of one or more phosphoproteins protein kinases leading to the rate-limiting step of cholesterol to pregnenolone conversion and initiates steroidogenesis.

In humans, cortisol production rate is 12 to 15 mg·m⁻²·day⁻¹ (Kenny et al. 1966). However, recent findings (Esteban and Yergey, 1990) have questioned the validity of the early studies and have suggested a lower production rate of nearly half the reference value. There is a diurnal pattern of cortisol release in the blood. The regularity of this pattern appears to be a function of the sleep/wake cycle (Krieger et al., 1971; Van Coevorden et al., 1991; Weibel et al., 1996) as well as the eating and exercise habits (Branderberger et al., 1982; Kern et al., 1995) of the individual. Additionally, other forms of stress such as prolonged fasting may induce significant increases in the cortisol production rate; these are mediated by an increase glucocorticoid secretory burst mass, rather than changes in secretory burst frequency (Bergendahl et al., 1996). In the last decade new findings have shown that the resting adrenal cortex is under paracrine (by the adrenal medulla) control [see (Nussdorfer, 1996) for a review]. The adrenal medulla contains and releases a number of regulatory molecules that may exert stimulatory or inhibitory effects and act directly or indirectly on the cortex. Stimulatory molecules include epinephrine and norepinephrine, serotonin, CRH-ACTH, arginine-vasopressin, oxytocin, enkephalins, neuropeptide Y, pancreatic polypeptide, vasoactive-intestinal peptide, pituitary adenylate

cyclase activating polypeptide, and galanin. Inhibitory molecules include dopamine, thyrotropin-releasing hormone, dynorphin, β -endorphin, and natriuretic peptides (Nussdorfer, 1996). The author suggests that under basal conditions, the stimulatory and inhibitory paracrine effects exerted by the adrenal medulla on the cortex are in balance and cancel each other. Whether this is true under stress conditions such as exercise remains to be established.

Typically, serum cortisol levels are highest at 0800 h and lowest at around 2300 h (Krieger et al., 1971; Riad-Fahmy et al., 1982; Van Coevorden et al., 1991; Weitsman et al., 1971). Additionally, cortisol exerts its own control of release by means of negative feedback on the hypothalamus and the adenohypophysis. In this manner it can modulate the release of CRH and ACTH. Typical plasma cortisol values for a resting healthy human at 0800 h are 135-552 nmol/L and at 2300 h 28-280 nmol/L. However, normal plasma cortisol levels may vary according to the measuring technique used. The levels cited above are used as a reference for radioimmunoassay (RIA) and protein binding radioassay of cortisol.

Pituitary-adrenal function is fully functional by the seventh day of birth (Ng et al., 1997). When stimulated with human-CRH the infants produce a pituitary adrenal response comparable to older children and adults. This suggests that pituitary-adrenal function is developed at an early stage of life (Ng et al., 1997). However, there may be qualitative differences in adrenocortical function. For instance, infants may produce considerably higher quantities of cortisone than cortisol (Fujitaka et al., 1997). This underscores the need for simultaneous measurements of cortisone and cortisol concentrations. Knutsson et al. (1997) examined the relationship between serum cortisol and age, sex, weight, height and body composition and pubertal stage. A total of 235 children (162 boys and 73 girls) ranging from 2.2 to 18.5 yr were investigated. No evidence was found for differences in temporal placement or level of the circadian cortisol rhythm in relation to age, growth,

gender or body composition. The authors reported a broad range of cortisol levels with individual mean diurnal levels ranging from 100 - 510 nmol/L (Knutsson et al., 1997). It has been demonstrated that this variability in cortisol's circadian rhythmicity is, at least in part, predetermine genetically (Linkowski et al., 1993; Meikle et al., 1988)

Importantly, a recent study has demonstrated the existence of marked gender and age effects on basal cortisol secretion and diurnal rhythmicity (Van Cauter et al., 1996). Van Cauter and associates (1996) examined 177 temporal profiles of plasma cortisol in 90 men and 87 women by using frequent blood sampling. The authors found that in young adulthood overall plasma cortisol levels are lower in women than in men because the female response to the circadian signal is slower and of lesser magnitude, and the return to quiescence is more rapid. Additionally, the authors indicated that during aging there appeared to be a progressive decline in the endogenous inhibition of nocturnal cortisol secretion in both men and women. Finally, the researchers also demonstrated an increase responsiveness of corticotropic activity in older women compared to men in the same age range.

Glucocorticoid Transport

Cortisol is secreted unbound to protein. However, due to its hydrophobic characteristics 90-99% of circulating cortisol binds to protein that will transport it to the target tissue. For instance, corticosteroid-binding globulin (CBG, same as transcortin) transports ~ 80% of cortisol with high affinity and intermediate capacity, whereas albumin (Khelet and Binder, 1976) and the erythrocyte (Hiramatsu and Nisula, 1988) transports ~ 10 to 15% with a low affinity and a very high capacity. The remaining 1-10% is free. The binding sites of transcortin become saturated at ~550 nmol/L and any further increase in cortisol secretion results in an increase in plasma free cortisol (Al

Ansary et al., 1982; James and Few, 1985; Robin et al., 1977; Riad Fhamy et al., 1982). For instance, in Cushing's disease plasma free cortisol represents 25%, whereas in Addison's disease it is ~ 6% of the total cortisol pool (Robin et al., 1977). The free fraction of cortisol is the biological active form and its level in the blood depends on the total cortisol concentration.

Cortisol's half life ($t_{1/2}$) of 60 to 90 minutes was first determined more than 40 years ago (Peterson et al., 1955) and was later confirmed by many others (Bright, 1995b; Bruno et al., 1971; Few, 1974; Levin et al., 1978; Veldhuis et al., 1989; Weitzman et al., 1971). The variability in cortisol $t_{1/2}$ is in part explained by the ability of transcortin to modulate the cortisol concentration to variable production rates. In fact, 40% of the variance in cortisol concentration can be attributed to the concentration of transcortin (Bright and Darmaun, 1995a). In a companion manuscript, Bright (1995b) reported that significant decreases in cortisol R_d , prolonged $t_{1/2}$, increased pool size and smaller volumes of distribution are all associated with increases in the transcortin concentration. Additionally, recent evidence demonstrated transcortin-specific cell membrane receptors in extravascular sites (Hryb et al., 1986; Maitra et al., 1993). This suggests that transcortin may play a role not only as a plasma protein carrier, but may facilitate the intracellular transport of cortisol. For instance, Khun (1988) has postulated that CBG may augment the free fraction of steroid by binding, internalization, and carrying the bound steroid into the cell. Internalized CBG may also function to protect the steroid from metabolism within the cell, and aid in the delivery of steroid to the nuclear receptors (Khun, 1988). Additionally, the author reported that when the pH of the reaction mixture was dropped from 7 to 6, CBG binding sites were increased 3-fold. This observation is of interest because during heavy exercise intramuscular pH can drop under 7, raising the question as to whether heavy exercise increases the

internalization of bound-CBG. This could enhance the intracellular cortisol concentration and perhaps augment cortisol's biologic effects.

Glucocorticoid Receptor

Glucocorticoid receptors are localized in virtually every nucleated cell of the body (Ballard et al., 1974; Baxter, 1971). Glucocorticoid receptor density depends on the cell type, state of differentiation, phase of the cell cycle and the endocrine status of the cell (Burnstein et al., 1989; Ballard et al., 1974). As cortisol reaches the target tissues it will diffuse through the plasma membrane and binds to the glucocorticoid cytoplasmic type I (Arriza et al., 1987) or type II receptor (Hollenberg et al., 1985; Miesfeld et al., 1984). Both glucocorticoid receptors are ligand-dependent transcription factors that can be recycled and reutilized (Orti et al., 1992). Glucocorticoid action within cells is initiated by the binding of the steroid to a cytoplasmic receptor. After this interaction the steroid-receptor complex, undergoes activation by phosphorylation before it is translocated to the nucleus and binds with DNA (Carson-Jurica et al., 1990). In response to glucocorticoid binding, steroid receptors are thought to interact with the specific target DNA to modulate the transcription of certain genes (Burnstein et al., 1989) mediated via kinases and phosphatases (Orti et al., 1992). Figure 3 shows the general scheme of glucocorticoid transport, binding and mechanism of action in the hepatocyte.

The chief mechanism of action of glucocorticoids is via induction of protein synthesis. However, this does not explain some of the glucocorticoid rapid-acting effects. The ability of this hormone to act on the cell membrane has been known for some time (Baxter, 1971; Trueba et al., 1987) and it helps, at least in part, to explain some of its rapid-acting effects. Some of these *in vitro* effects have been found in as little as 1-2 min (Durant et al., 1983), 9 min (Sistare and Haynes, 1985) less than 30 min (Toode et al.,

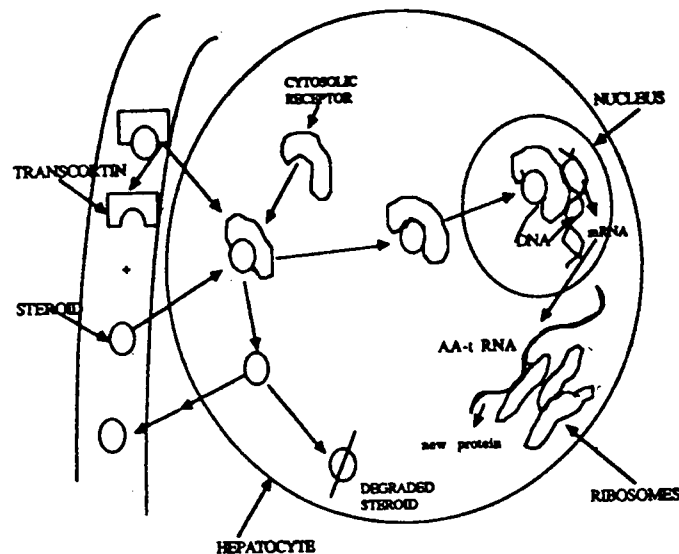


Figure 3. Mechanism of action of glucocorticoid in liver (from Berdanier, 1989).

1993), or 60 min *in vivo* (Perkoff et al., 1965). For instance, one of the mechanisms proposed to explain the fast acting effects include the promotion of Ca^{++} -dependent binding of calmodulin (Sze and Iqbal, 1994) which stabilizes the calmodulin-membrane complex from dissociation. Observations of non-genomic steroid effects are presently being published at an increasing rate [see (Wheling, 1996) for review] and their thrust covers effects of almost all steroids on nearly all kinds of tissues and species. In addition to glucocorticoids, numerous rapid effects have been reported for aldosterone, progesterone, and neurosteroids.

The glucocorticoid type I (also called mineralcorticoid receptor) receptor is a high affinity low-capacity receptor that binds both mineralcorticoids (aldosterone) and cortisol with similar affinities. The steroid affinity is most likely dependent on the enzyme 11- β -hydroxysteroid dehydrogenase (Tempel and Leibowitz, 1994) which metabolizes

glucocorticoids to compounds of low affinity for the type I receptor. This receptor responds to low circulating levels of glucocorticoids and has been associated with anabolism (Tempel and Leibowitz, 1994). Cortisol is the main glucocorticoid type II receptor ligand. Surprisingly, this receptor has a lower affinity for cortisol than the type I receptor. However, at higher concentrations cortisol readily binds to the type II receptor. This receptor mediates the cortisol negative feedback control mechanisms leading to reduction in steroid synthesis, and catabolism. In the rat, the type I and II receptors are similar in structure, with a similar 50% homology in the ligand binding domains of the receptors, and 94% homology in DNA binding domains, but only 15% homology in the regulatory domains [see (Tempel and Leibowitz, 1994) for a review]. Although, glucocorticoid receptors are localized in virtually every nucleated cell of the body glucocorticoid receptor density is most pronounced in liver, and in certain hypothalamic nuclei, limbic structure, and brain stem cell groups. The type I mineralocorticoid receptor and its m-RNA exist in classic mineralocorticoid target tissues, such as the kidney, salivary glands, gut, and the brain (Tempel and Leibowitz, 1994).

Metyrapone

Chart et al. (1958) and Jenkins et al. (1958) were the first to described the inhibitory effects of 2 methyl-1,2 bis - (3 pyridyl)-1-propanone, also known as SU4885 and later, metyrapone. Figure 4 depicts the chemical structure of metyrapone. Following the administration of SU4885 in doses of 10-300 mg·kg⁻¹ either intravenously, orally or intragastrically to dogs, investigators reported a fall in adrenal vein corticoids ranging from 25 to nearly 100% below pre- treatment control values (Chart et al., 1958; Jenkins et al., 1958). This effect lasted for 1 to 4 h. Later that year Liddle et al. (1958) reported similar findings in humans undergoing adrenal or renal surgery procedures. Liddle and coworkers

(1958) postulated that SU4885 might act as a specific inhibitor of adrenal cortex 11- β hydroxylation. The mechanism of action of metyrapone was elucidated by in vitro studies of isolated adrenocortical mitochondria. Metyrapone interferes with the mitochondrial steps of steroidogenesis at the level of cytochrome P₄₅₀ operating with an NADPH-P₄₅₀ reductase complex. Specifically, metyrapone interferes with the cholesterol side-chain cleavage, 11-hydroxylation, and 18-hydroxylation of adrenacortical substrates (Caballeira et al., 1976); See figure 2. Metyrapone is a pharmacological agent that reversibly inhibits the biosynthesis of cortisol in the adrenal cortex by blocking the 11- β -hydroxylation of 11-deoxycortisol to cortisol, leading to elevated levels of the former and depressed levels of the latter. In addition to this effect, there is an elevation of ACTH. Other studies using labeled cortisol have demonstrated that metyrapone exerts extra-adrenal effects such as increases in plasma cortisol Rd (e.g., decreases $t_{1/2}$) by ~ 50% (Bruno et al., 1968; Blichert-Toft et al., 1972).

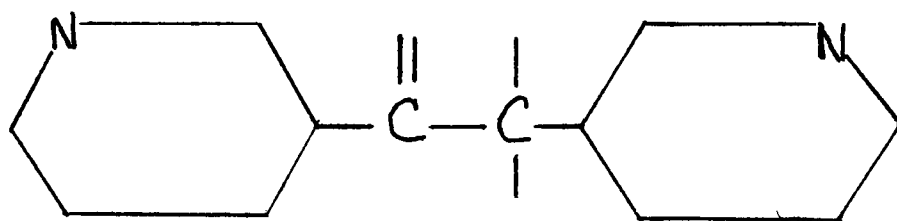


Figure 4. Structural fomula of metyrapone.

Blechert-Toft et al. (1972) suggested that metyrapone might be acting on peripheral 11 β -hydroxysteroid dehydrogenase; this was recently confirmed *in vivo* by Raven et al. (1995) who demonstrated that metyrapone inhibits the back reduction of cortisone to cortisol in peripheral tissue.

Metyrapone is typically used to diagnose latent ACTH deficiency, to examine adrenocortical reserve, and as a supplementary treatment of Cushing's disease. Additionally, this agent has been used in the diagnosis (Del Corral et al., 1966) and prognosis (Del Corral et al., 1967) of amenorrheic and neurosurgical patients, respectively. When this drug is administered for prolonged periods of time (i.e., Cushing's treatment) or at high dosages, gastrointestinal disorders (nausea and vomiting), dizziness, sedation, hypotension, mild facial flushing and adrenal insufficiency symptoms may occur (Verhelst et al., 1992). Jubiz et al. (1974) reported no side effects after administering the single dose metyrapone test on 346 patients. Recently, Fiad and colleagues (1994) administered 576 tests with no serious side effects. Seven patients reported one or more of the following: feeling faint, nausea, vomiting and dizziness. However, side effects were most prevalent in subjects with hypothalamic-pituitary-adrenal dysfunction. The authors concluded that unless there is objective evidence of adrenal insufficiency (i.e., postural hypotension, nausea and vomiting), the Overnight Single Dose Metyrapone Test (OSDMT) is suitable for out-patient testing.

The OSDMT was first described by Jubiz and associates (1970). In that communication they recommended a single dose of $\sim 30 \text{ mg}\cdot\text{kg}^{-1}$, administered at midnight with a glass of milk and a snack, not only to decrease gastrointestinal distress but also to delay the drug's absorption. Blood was drawn at 0800 h and analyzed for cortisol, 11-deoxycortisol (Sparks, 1971) and ACTH. However, Staub and associates (1979) have indicated that the maximal ACTH response is obtained at 0730 h, whereas

the cortisol response remains unchanged from 0730 to 0800 h. It is generally accepted that the decrease in plasma cortisol stimulates ACTH secretion by decreased negative feedback. However, Holmes and associates (1993) administered metyrapone and despite frequent blood sampling found that the large increase in plasma ACTH was not accompanied by a detectable rise in peripheral plasma levels of CRH or arginine-vasopressin. The authors suggested that a decrease in cortisol feedback increases the "gain" of AVP and CRH on pituitary ACTH release, or that factors other than AVP or CRH may modulate the metyrapone-induced ACTH release.

The recommended metyrapone dose has consistently proven to provide an appropriate adrenocortical blockade (>50%) (Atiea et al., 1989; Dicktein et al., 1986; Jubiz et al., 1970; Kaspersen et al., 1992; Schöneschöfer et al., 1977; Schöneschöfer et al., 1980; Sparks 1971; Staub et al. 1979; Steiner et al. 1994). There are, however, other forms of administering metyrapone including the Morning Single Dose Metyrapone Test (Thornton et al., 1994), 250 mg every 2 h (Buss et al., 1961), 500 mg every 4 h, 750 mg every 4h, and 1500 mg every 6 h (see Streeten et al., 1984 for a review). When using these clinical tests it is necessary to ensure that the antibody used to measure cortisol has a low cross reactivity with 11-deoxycortisol which is elevated 10 to 100 fold (Kinsley et al., 1996; Sparks, 1971), otherwise the test would reveal falsely elevated cortisol levels (Kinsley et al., 1996). Additionally, estrogen therapy causes an acceleration of the hepatic conjugation of metyrapone, less 11 β -hydroxylase inhibition and a subnormal ACTH rise. These findings suggest that in estrogen-treated females, the OSDMT should be interpreted with caution (Meikle et al., 1970; Vermeulen et al., 1967). Importantly, previous studies have demonstrated that the OSDMT has no effects on blood glucose and the glucoregulatory hormones (Atiea et al., 1990; Bright et al., 1980; Kinsley et al., 1996). Similarly, metyrapone has been found to have no intrinsic effects on ketone bodies and NEFA (Samra et al., 1996; Schade et al., 1978).

Furthermore, the elevated 11-deoxycortisol is unlikely to have any effects on carbohydrate metabolism (Fajans, 1951).

5. Metabolic Effects Of Cortisol

Introduction

Cortisol has numerous physiologic effects on the immune (Jefferies, 1991), cardiovascular (Whithworth et al., 1995; Fallo et al., 1994), and digestive systems (Baker, 1955) to name a few (see [Baxter, 1971] for a review). However, this review will emphasize the effects of cortisol in energy metabolism. As it is evident from their name, glucocorticoids have important effects on intermediary metabolism including carbohydrate, lipid and protein metabolism. In humans, cortisol is the chief glucocorticoid secreted by the adrenal cortex. Some of the metabolic functions of cortisol related to fuel homeostasis include: (a) proteolysis, allowing the entrance of free amino acids into gluconeogenesis; (b) lipolysis; (c) reducing insulin dependent peripheral glucose uptake thus assuring blood glucose supply to the brain; and (d) increasing the rate of synthesis of a number of gluconeogenic enzymes in the liver. This section will first review the effects of acute hypercortisolemia on carbohydrate, lipid and protein metabolism, and be followed by the effects of chronic hypercortisolemia. The review will focus on human data; animal studies will be kept to a minimum.

Hypercortisolism

There are four possible sources of excess cortisol. The most obvious and most common is prolonged administration of glucocorticoids for therapeutic reasons. The

other 3 sources of hypercortisolism can be categorized as endogenous Cushing's disease: a) pituitary hypersecretion of ACTH; b) ectopic production of ACTH and CRH by non-endocrine neoplasm, and c) hypersecretion of cortisol by adrenal adenoma, carcinoma, or nodular hyperplasia (Engelhardt and Weber, 1994; Robins et al., 1994; Liddle, 1972). Figure 5 shows a hyperplastic adrenal gland due to a pituitary hypersecretion of ACTH (β -cell adenoma), whereas figure 6 shows an adrenal nodular hyperplasia. Definitive surgical treatment is the therapy of choice in all forms of Cushing's syndrome; however, the outcome of surgical treatment largely depends on the etiology of the excess cortisol. A pituitary hypersecretion of ACTH can be corrected by transphenoidal pituitary microsurgery in ~ 50 to 80% of all patients, whereas adrenal-dependent hypercortisolism can be cured by unilateral or bilateral adrenalectomy. However, about one third of the hypercortisolinemic patients require temporary or permanent pharmacologic treatment (i.e., adrenolytic drugs and steroid receptor antagonists). Some of these agents include: trilostane, aminoglutethimide, ketoconazole, etomidate and metyrapone (Engelhardt and Weber, 1994).



Figure 6. Adrenal nodular hyperplasia. Right and left adrenal gland weights were 13.5 and 14.0 gm, respectively. A normal adrenal gland weighs 4 gm. Courtesy of Dr. John Neff, Department of Pathology, University of Tennessee Medical Center, Knoxville, TN



Figure 5. Pituitary-dependent hyperplastic adrenal gland due to a pituitary hypersecretion of ACTH (β -cell adenoma). Right and left adrenals weight 12.5 gm and 13 gm, respectively. A normal adrenal gland weighs 4 gm. Courtesy of Dr. John Neff, Department of Pathology, University of Tennessee Medical Center, Knoxville, TN

Acute Hypercortisolemia

The effects of acute hypercortisolemia may be of interest because of its potential metabolic effects on energy metabolism during stressful conditions such as an acute bout of prolonged exercise. Shamooin et al. (1980) examined the role of physiological hypercortisolemia (~ 1100 nmol/L) in the regulation of fuel metabolism during a 5-h cortisol infusion and compared it to a saline-infusion trial. Thirteen healthy subjects were studied at rest after an overnight fast; glucose turnover was calculated, and blood samples were analyzed for glucose, BCAA, ketone bodies and NEFA. Blood glucose increased ~ 0.85 mmol/L despite no change in hepatic glucose production. A decrease in glucose uptake and clearance accounted for the cortisol-induced hyperglycemia, suggesting insulin resistance. Similarly, during cortisol infusion there was a 40%, 25% and a 3-fold increase in BCAA, phenylalanine and ketone bodies, respectively. NEFA tended to increase but did not reach statistical significance. These changes took place in the absence of significant changes in insulin and glucagon concentrations. Some of the effects were evident within the first hour (e.g., glucose uptake), whereas others took 2-3 h (BCAA and ketone bodies). The study did not clamp insulin and glucagon, allowing small changes which could have affected hepatic glucose production.

Clerc et al. (1986) examined the effects of cortisol on hepatic glucose production. Nine subjects underwent an euglycemic-hyperinsulinemic clamp with placebo or 80 mg of cortisol. The researchers reported a decrease in hepatic glucose production during the placebo compared to the cortisol trial. The authors suggested that during hypercortisolemia exogenous insulin failed to suppress hepatic glucose production, probably via cortisol-induced hepatic insulin resistance.

Lecavalier and colleagues (1990) examined the effect of cortisol on glucagon-induced hepatic glucose output and lactate gluconeogenesis using an insulin clamp in a group of healthy subjects. There were 4 experimental conditions: (1) normoglucagonemia-normocortisolinaemia; (2) isolated hyperglucagonemia; (3) isolated hypercortisolinaemia (~900 nmol/L); and (4) combined hyperglucagonemia-hypercortisolinaemia. Following an overnight fast the clamp was administered and subjects were studied from 0800 to 1300 h. Hyperglucagonemia increased hepatic glucose output and lactate gluconeogenesis whereas hypercortisolinaemia had no effect on hepatic glucose output but increased lactate gluconeogenesis. Hyperglucagonemia-hypercortisolinaemia further increased lactate gluconeogenesis in an additive fashion without increasing the overall hepatic glucose output. Importantly, the authors indicated that the failure to demonstrate an increase in hepatic glucose output during hypercortisolinaemia was probably due to the small number of subjects examined (hyperglucagonemia n=8; hypercortisolinaemia n=5). The authors concluded that within a 3-h period hypercortisolinaemia increases hepatic glucose output by augmenting gluconeogenesis rather than glycogenolysis.

Divertie and associates (1991) examined the effect of physiological hypercortisolinaemia (~ 1000 nmol/L) on lipolysis in humans. Following an overnight fast and 3 h of isotopic equilibrium (¹⁴C palmitate), 7 healthy individuals underwent a pancreatic clamp (insulin and glucagon were replaced at basal levels) on two occasions with saline or hydrocortisone for a 6 h period. Cortisol concentration increased 3-fold and palmitate Ra increased by 60% when compared to saline infusions. The increase became significant within a 2-h period and continued to increase until the end of the hydrocortisone infusion. This study demonstrated that when pancreatic hormones are not allowed to change, cortisol by itself may stimulate lipolysis.

Schade et al. (1978) explored the relationship between cortisol and plasma concentration of ketone bodies and NEFA in insulin dependent diabetic persons. Twenty-four h prior to sample collections, endogenous secretion of cortisol was blocked in 3 experiments with metyrapone. In 2 of these studies cortisol was replaced to bring cortisol to normal (~280 nmol/L) and high levels (~ 400 nmol/L), respectively. The suppression (~90%) of cortisol resulted in a 25% and 60% reduction in the plasma concentrations of ketone bodies and NEFA, respectively. When cortisol was replaced to achieve resting levels, plasma concentrations of ketone bodies and NEFA returned to near-control values. On the other hand, when cortisol was replaced to raise cortisol to levels seen during stress conditions there was hyperketonemia and elevated levels of NEFA. Taken together, the studies of Divertie et al. (1991) and Schade et al. (1978) suggest a direct and positive relationship between plasma cortisol levels and lipid catabolism.

To determine whether the physiologic increase in plasma cortisol increases proteolysis and *de novo* synthesis of alanine, Simmons and associates (1983) infused 7 subjects on 2 occasions for 8-h periods with cortisol (to ~1100 nmol/L) or saline (i.e., cortisol level ~ 380 nmol/L). Labeled alanine and leucine were used to trace changes in plasma amino acids. Plasma cortisol increased 4-fold with no change in plasma insulin, glucagon, or C-peptide during either infusion. The cortisol infusion induced an increase in plasma leucine concentration and leucine Ra when compared to the saline infusion. Similarly, alanine Ra increased during the cortisol infusion. The authors suggested that an increase in plasma cortisol within the physiologic range increases proteolysis within a 2 to 3 h period. Similarly, Karl et al. (1976) measured a 2.5-fold increase in muscle alanine release when an epitrochlearis preparation was incubated with glucocorticoids for 60 to 120 min compared to a control group.

To define the time course of physiological elevations of cortisol on amino acid metabolism, Darmaun et al.(1988) placed a group of healthy subjects on a standard diet before and during 64 h of cortisol infusion (to ~900 nmol/L). Labeled leucine, alanine, glutamate, and phenylalanine were infused before, at 12 h, and 60 h following cortisol infusion and the results were compared to a saline infusion study. Cortisol levels rose 3-fold compared to the saline study. The cortisol rise increased leucine and phenylalanine flux (15%), and glutamine (55%) and alanine (40%) concentrations. Additionally, the study suggested that the increase in alanine and glutamine flux were via *de novo* synthesis. The authors concluded that hypercortisolemia increases both protein breakdown and turnover of important non-essential amino acids.

Brillon et al. (1995) examined the effects of 3 cortisol infusions (to ~300, 940 and 1800 nmol/L) on amino acid metabolism, and the concentrations of plasma glucose, NEFA and the glucoregulatory hormones. Nine healthy subjects participated in the 4 parts of the study (3 cortisol infusions and a saline trial). The subjects ate a meal at 1800 h; at 2200 h the infusions started and continued until 1400 h the following day. In order to study the isolated effects of hypercortisolemia in the last 3.5 h, insulin and glucagon were clamped with somatostatin. Leucine, glutamine and phenylalanine amino acid tracers were infused during the last 7 hours of the study. Hypercortisolemia increased proteolysis, plasma glucose, NEFA, and insulin concentrations in a dose-response fashion. Interestingly, C-peptide levels were not increased which suggests that increasing cortisol reduced the insulin clearance rate and did not increase secretion. Institution of the clamp reduced plasma insulin but further increased proteolysis, plasma glucose and plasma NEFA, suggesting that insulin attenuates the catabolic effects of cortisol on protein and lipid metabolism. Hypercortisolemia increased the cycling of amino acids by increasing protein breakdown and *de novo* synthesis having a net energetic cost of 1-2% of the resting energy expenditure. This study gives no specific

indication on onset of the effects; 8 h elapsed from the start of the experiment to the first blood sample.

Although it is well known that glucocorticoids increase amino acid turnover, less is known about the molecular mechanisms involved. The rate limiting enzyme of branched chain amino acid catabolism, branched chain alpha keto acid dehydrogenase, is increased within 2 h following the acute administration of glucocorticoids. This enzyme is expected to be activated in vivo during stress-related hypercortisolemia (Block and Buse, 1990)

Chronic Hypercortisolemia

In light of the fact that glucocorticoid use in clinical medicine is typically for days or weeks, the metabolic effects of chronic elevation of cortisol have been studied extensively. Chronic cortisol elevations will be defined as >24 h. Chronic hypercortisolemia is known to affect amino acid metabolism. For instance, the decrease in leucine turnover in Cushing's disease patients (i.e., chronic hypercortisolemia) appears to be due primarily to a decrease in protein synthesis. This abnormality in protein metabolism is normalized upon corrective surgery (Reinartz et al., 1995). Chronic hypercortisolemia has also been associated with body weight gain that seems to be attributable to a positive energy balance induced by an increase in energy intake that is larger than the increase in energy expenditure (Tartarini et al., 1996).

Wajngot and associates (1990) explored the effects of a 48 h dexamethasone administration on hepatic glucose fluxes in a group of 8 healthy subjects. Dexamethasone increased glucose Ra measured with 2-³H glucose hepatic total glucose output, but not glucose Ra when measured with 6-³H glucose hepatic glucose production; and therefore glucose cycling increased. Glucose cycling involves the

phosphorylation of glucose to glucose-6-phosphate by glucokinase and its dephosphorylation by glucose-6-phosphatase. The authors suggested that dexamethasone increases hepatic total glucose output and glucose cycling by activating glucose-6-phosphatase directly, without affecting hepatic glucose production. This is consistent with previous findings that reported a stimulatory effect of the glucocorticoids on glucose cycling (Shaw et al. 1976). A different approach to studying the metabolic effects of a hormone is by examining its effects at high levels in patients with chronic hormone deficiency and/or via acute pharmacological intervention.

Goldstein et al. (1993) examined the effects of a 5-day elevation of plasma cortisol on hepatic fuel metabolism in the dog. The researchers used labeled glucose and alanine, and arteriovenous difference techniques to measure substrate fluxes and gluconeogenesis. The authors reported increases in the plasma concentrations of cortisol, plasma glucose, insulin and glycerol, and hepatic production of lactate (4-fold) and β -hydroxybutyrate (3-fold). Gluconeogenic conversion of alanine to glucose increased ~ 60%, but the gluconeogenic efficiency of the liver was unchanged. Furthermore, the glucose Ra was increased, whereas the net hepatic glucose output was not. The author suggested that glucose was being cycled to glycogen. In fact, cortisol infusions doubled liver glycogen storage. The authors concluded that a 5-day elevation of cortisol increased the substrate fluxed through the liver without altering the gluconeogenic efficiency within the liver. The failure of gluconeogenic efficiency to increase may have been due to the rise in plasma insulin.

In a similar study, Goldstein et al. (1994) examined the effects of 5 days of hypercortisolemia coupled with insulin deficiency and compared it to insulin deficiency alone in the dog. The authors reported that insulin deficiency plus hypercortisolemia increased glucose Ra (100%), hepatic lactate production, hepatic alanine uptake, and arterial alanine levels. Interestingly, the enhanced glucose Ra was

due to glycogenolysis and not to gluconeogenesis. The increase in glucose Ra during combined insulin deficiency plus hypercortisolemia was no surprise since other investigators have previously reported similar interactions between glucocorticoids and glucagon (Eigler et al., 1979; Issekutz and Borkow, 1973; Lecavalier et al., 1990), and glucocorticoids and epinephrine (Eigler et al., 1979; Issekutz and Allen, 1972). These studies concluded that glucocorticoids enhanced the glucose-producing properties of these hormones. Another mechanism by which glucocorticoids enhance glucose production, at least in the dog, is by increasing gluconeogenesis from glycerol (Shaw et al., 1976)

Malerbi and associates (1988) examined the effects of hypercortisolemia on hepatic glucose production in patients with cortisol deficiency, and compared them to healthy controls. The patients were studied during high-dose cortisone acetate treatment (3-fold higher than normal) for 2 weeks. The authors reported that following an overnight fast, hepatic glucose production was not altered by cortisol replacement when compared to controls, but there was a significant increase in plasma glucose and insulin levels in the high-dose cortisol treatment. It was suggested that 2 weeks of elevated glucocorticoids did not increase hepatic glucose production. However, the elevated insulin levels might have obscured the effects of cortisol on hepatic glucose production, whereas the elevated plasma glucose suggested insulin resistance.

Reinartz et al. (1995) examined glucose and amino acid concentrations and kinetics in 11 patients with pituitary-dependent Cushing's disease and compared them to 9 healthy controls. The patients were tested before, 1 week and 3 months after corrective surgery. Prior to surgery patients showed an increase in hepatic glucose production linked to hepatic insulin resistance and reduced leucine turnover. Both of these effects were normalized a week after surgery. However, the authors indicated that only 4 patients showed distinct increases in hepatic glucose production. It was

concluded that high cortisol (to ~ 1100 nmol/L) causes fasting hyperglycemia due to both increases in hepatic glucose production and decreased glucose utilization.

Other studies have reported a stimulatory effect of cortisol on glucose Ra despite elevated insulin levels (Rizza et al., 1982). Rizza et al. (1982) studied the effects of prolonged cortisol infusion (2.5-fold) on glucose production, utilization and insulin resistance on 6 healthy subjects infused with cortisol and compared to a 24-h saline infusion. The authors reported that hypercortisolemia increased postabsorptive plasma glucose (~ 25%), plasma insulin and glucose Ra and Rd. The results of this study indicate that the cortisol-induced insulin resistance in man is due to a decrease in both hepatic and extrahepatic sensitivity to insulin. Furthermore, McMahon et al. (1988) reviewed the *in vivo* and *in vitro* literature on the effects of glucocorticoids on glucose tolerance and concluded that a decrease in glucose utilization occurs despite counterregulatory changes in plasma glucose and insulin concentrations.

Clerc and colleagues (1986) demonstrated that acute cortisol excess resulted in unimpaired insulin-induced suppression of plasma NEFA and BCAA concentrations, whereas insulin's effect on endogenous insulin secretion and glucose kinetics was diminished. Others have reported that small increases in plasma cortisol may result in an abrupt inhibition of insulin secretion without changes in the glucose concentration. Furthermore, 4-6 h after a large cortisol elevation insulin resistance developed and lasted at least 16 h (Fowlle et al., 1989; Plat et al., 1996;).

Interestingly, Boden et al. (1996) recently suggested that circadian changes in insulin sensitivity are not related to blood levels of glucose and insulin or insulin clearance. They were caused by circadian changes in hepatic glucose production, which in turn were closely correlated to changes in plasma NEFA and cortisol. Furthermore, Guillaume-Gentil et al. (1993) showed that the glucocorticoid-induced muscle insulin resistance was prevented by etomoxir (i.e., antilipolytic drug), suggesting that the

resistance is, at least in part, mediated via excessive lipid oxidation. Others have postulated that perhaps glucocorticoids reduce skeletal muscle insulin sensitivity by altering the kinetics of GLUT4 subcellular trafficking in favor of GLUT4 partitioning to intracellular sites (Weinstein et al., 1995). For instance, glucocorticoids may inhibit the translocation of intracellular GLUT4 to the plasma membrane and/or by promoting the internalization of plasma membrane GLUT4 to the intracellular pool, decrease total GLUT4 protein (Weinstein et al. 1995).

Cortisol exerts profound effects on lipid metabolism, some of which appear to be inherent to this hormone, whereas others are mediated in the presence of insulin. In adipose tissue lipoprotein lipase (LPL) is an important regulating factor for the uptake of free fatty acids. Appeal and Fried (1992) examined the mechanisms by which insulin and glucocorticoids affected LPL synthesis and degradation. Subcutaneous and omental adipose tissue from lean to morbidly obese female and male subjects were cultured for 6 to 7 days. Four experimental conditions were used: 1) serum free medium; 2) insulin; 3) dexamethasone; and 4) insulin and dexamethasone. The results of the study indicated that insulin increased the heparin releasable and total LPL activity by 7-fold, 5-fold of which were mediated through increased LPL synthesis. Although dexamethasone had no significant effect by itself, it added to the insulin effect (1-fold), none of which was mediated by increased LPL synthesis. Pulse-chase studies revealed that in the presence of insulin, dexamethasone delayed newly synthesized LPL degradation from 69% to 29% during 90 min of incubation with dexamethasone (Appeal and Fried, 1992)

In humans, the pattern of lipid accumulation is affected by gender; women tend to accumulate more fat in the subcutaneous region, whereas men tends to accumulate more fat in the abdominal and visceral depot. Pedersen et al (1994) addressed this gender difference in relation to glucocorticoid receptor density and correlated this information to LPL activity. Eleven men and 8 women matched for age and body mass

index (kg/m^2) underwent intraabdominal surgery for non-malignant diseases. Biopsies were taken from subcutaneous abdominal depots and the intraabdominal depots (Omentum Majus). The results of the study revealed that glucocorticoid receptor binding in subcutaneous adipose tissue is similar between males and females. Similarly, glucocorticoid receptor binding in omentum was not different between the two genders. However, glucocorticoid receptor binding was 4-fold higher for omentum when compared to subcutaneous adipose tissue. This study also revealed that in subcutaneous adipose tissue LPL activity was higher in females than in males, whereas omentum LPL activity was similar in both genders. Recently, Samra et al. (1996) studied the effects of the morning rise in cortisol concentration on lipoprotein lipase. Ten healthy subjects were studied under placebo (i.e., allowing the morning rise in cortisol) and metyrapone (i.e., preventing the morning rise in cortisol). The authors reported that when the morning rise in plasma cortisol was prevented there was a significant reduction in the adipose tissue lipoprotein lipase rate of action.

In summary, 1) in the presence of insulin, dexamethasone increases LPL activity by delaying LPL degradation; 2) *in vivo*, cortisol may play a key role by inhibiting the degradation of LPL; 3) omental adipose tissue has 4-fold more glucocorticoid receptor binding than subcutaneous tissue; 4) hypocortisolemia is associated with a significant reduction in the adipose tissue lipoprotein lipase.

Hypocortisolemia

Adrenocortical hypofunction may be due to an anatomic or metabolic lesion of the cortex that impairs the output of cortical steroids, or it may be secondary to deficiency of ACTH. Primary acute adrenocortical insufficiency (adrenal crisis) is typically precipitated by any form of stress to which the glands are incapable of

responding. This condition stems from too rapid withdrawal of glucocorticoids from patients whose adrenals have been suppressed by long-term glucocorticoid administration, from failure to increase the level of administered glucocorticoids during stress in a bilateral ADX patient, or from acute pharmacological adrenalectomy (i.e., metyrapone, ketoconazole, etomidate or aminoglutethimide) coupled with stress. Additionally, massive destruction of the adrenals can occur in adrenal hemorrhage with concurrent bacteremia caused by meningococci, gonococci, pneumococci, or staphylococci. Primary chronic adrenocortical insufficiency (Addison's disease) is a rare condition caused by any chronic destructive lesion of the adrenal cortex. Although the etiology of this disease varies, more than 90% of all cases are attributable to autoimmune adrenalitis, tuberculosis or metastatic cancers. Furthermore, autoimmune adrenalitis is responsible for 60-70% of the cases. Secondly, adrenocortical insufficiency is characterized by any disorder of the hypothalamus and pituitary (i.e., metastatic cancer, infection, infarction or irradiation) that reduces the output of ACTH leading to a syndrome of hypoadrenalism similar to Addison's disease (Oelkers, 1996; Robbins et al., 1994; Liddle, 1972). Figure 7 depicts adrenal atrophy due to secondary adrenocortical insufficiency.

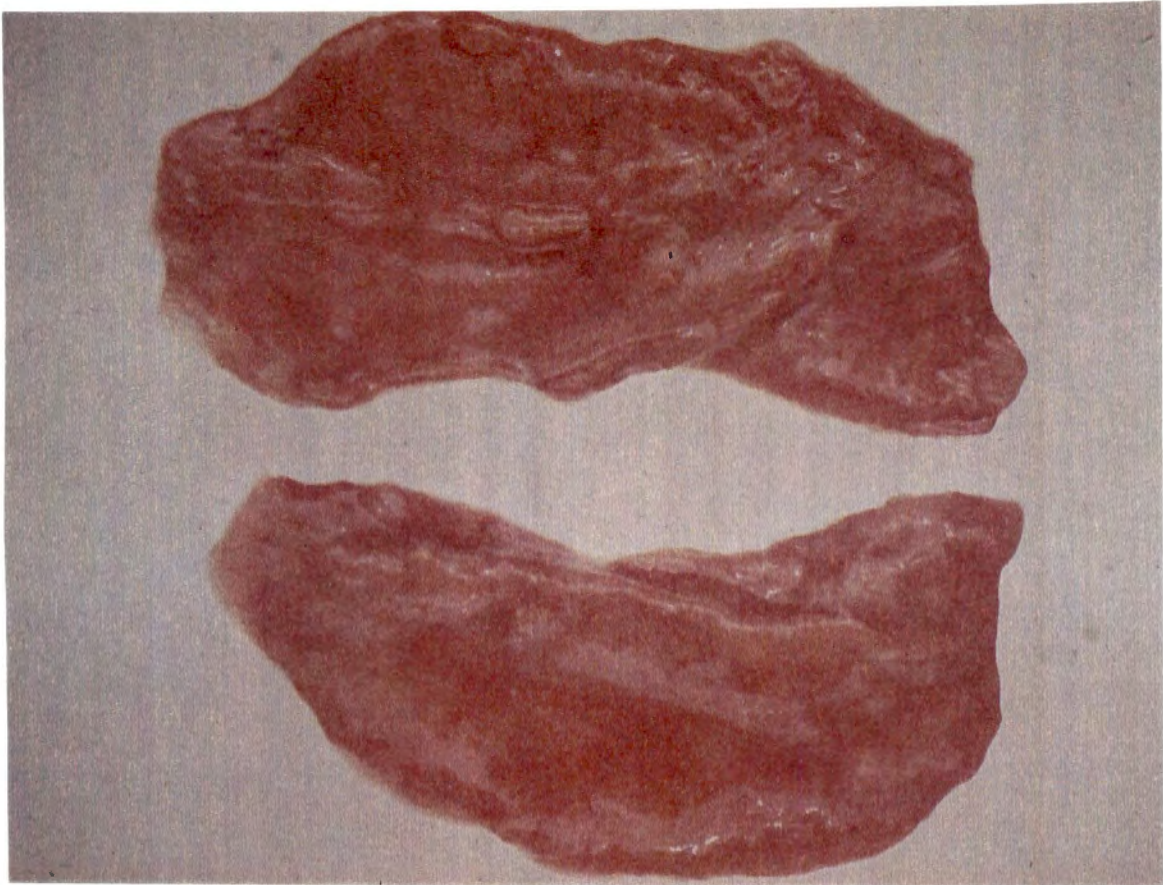


Figure 7. Adrenal atrophy due to secondary adrenocortical insufficiency (weight: 0.8 gm. A normal adrenal gland weighs 4 gm). Courtesy of Dr. John Neff, Department of Pathology, University of Tennessee Medical Center, Knoxville, TN

Chronic Hypocortisolism

Chronic adrenocortical insufficiency, also known as Addison's disease, was first described by Dr. Thomas Addison in 1855. This condition develops in patients with an immune disorder of the adrenal cortex (Addison's disease), untreated ADX, and hypopituitarism. Addison's disease is frequently characterized by cardiovascular instability (i.e., low blood pressure) and metabolic derangements (Frawley, 1955; Liddle, 1972; Oelkers, 1996; Veseley, 1982). Thorn et al. (1955) studied 158 patients with Addison's disease and found the following incidence of signs and symptoms: weakness and fatigability 100%; weight loss 100%; increased pigmentation 94%; anorexia nausea and vomiting 85%; diarrhea 21%; salt craving 19%; and muscle pain 16%. Some of the metabolic derangements may include: a) low plasma glucose when fasting or on low carbohydrate diet (D'albora and Martin, 1966; Lovinger et al., 1975); b) increased sensitivity to exogenous insulin; c) abnormally intense hypoglycemia following oral or intravenous administration of glucose; and d) high fasting respiratory quotient. Administration of hydrocortisone in physiological amounts reverses these effects (Fajans, 1961, McMahon et al., 1988; Takeda et al., 1987; Wajchenberg et al., 1964). Adrenocortical insufficiency is characterized by chronic low cortisol levels. Similar clinical presentation has been documented in isolated ACTH deficiency (Yamamoto et al., 1992). Hypoglycemia in adrenocortical insufficiency appears to be a consequence of inadequate gluconeogenesis (Fajans and Floyd, 1975). The weakness and hypoglycemia characteristic of Addison's disease are reversible by administration of cortisol (Frawley, 1955; Vesely, 1982 and Yamamoto et al., 1992).

Malerbi et al. (1988) examined the effects of no cortisol replacement on hepatic glucose production in patients with cortisol deficiency and compared them to healthy controls. Following an overnight fast, subjects were infused with labeled glucose for

120 min. Hepatic glucose production and plasma glucose did not differ when compared to controls. However, insulin levels were significantly lower in the untreated state. It was suggested that glucocorticoids may affect glucose production by altering insulin levels.

Boyle and Cryer (1991) studied a group of patients with cortisol and growth hormone deficiency with and without hormone replacement. The study used an insulin clamp to maintain blood glucose at 3 mmol/L. Glucocorticoid replacement was discontinued 48 h prior to their untreated trial, whereas under treatment, cortisol and GH were administered with an oral dose designed to mimic their circadian rhythms. Additionally, during the insulin clamp cortisol and GH were replaced intravenously at basal levels. Following an overnight fast, the subjects underwent a 2-h insulin infusion to reduce blood glucose to 3.6 mmol/L and then fixed the insulin infusion over an additional 10-h period. Despite identical insulin infusion rates and plasma insulin levels in the three groups, the final (12th h) plasma glucose concentration declined in both the basally treated (2.5 mmol/L) and untreated (2.4 mmol/L) state when compared to controls (2.9 mmol/L). Similarly, NEFA and hydroxybutyrate were markedly suppressed in patients (without replacement) compared to healthy controls. Plasma epinephrine and norepinephrine were significantly higher, and glucagon tended to be higher in patients. The authors indicated that despite elevated catecholamines, cortisol and GH are involved in the prevention of prolonged insulin-induced hypoglycemia. Additionally, the increase in cortisol and growth hormone was thought to play a critical role in the defense against prolonged hypoglycemia, not a permissive role.

Wajchenberg et al. (1964) studied carbohydrate tolerance in unreplaced adrenocortical patients and found that during insulin-induced hypoglycemia, the patients had higher insulin sensitivity and delayed recovery from hypoglycemia compared to control subjects. Similarly, an intravenous glucose tolerance test revealed an increase in

glucose disappearance rate. However, when the patients underwent a glucagon test (i.e., to evaluate liver function) no significant differences were found in plasma glucose between patients and controls. This study indicates that cortisol deficiency leads to an increased hypersensitivity to endogenous and exogenous insulin. Additionally, it suggests that chronic cortisol deficiency does not alter the effect of glucagon on the plasma glucose concentration.

Takeda et al. (1987) explored the mechanisms of insulin sensitivity in adrenocortical insufficient patients and compared them to controls and Cushing's patients. The patients were studied before and after cortisol replacement. Overnight fasted plasma glucose was low before treatment, and was increased but not normalized with cortisol replacement. The oral glucose tolerance test revealed an increase in insulin sensitivity before but not after cortisol treatment. When the researchers examined insulin binding to the red blood cell, specific insulin binding was high in all cases before treatment and was decreased to normal with cortisol replacement. The authors suggested that hypocortisolism increases insulin binding, chiefly through the mechanisms of increased insulin receptor concentration.

Although these studies (Boyle and Cryer, 1991; Malerby et al., 1988; Takeda et al., 1987; Wajchenberg et al., 1964) provide evidence of cortisol's role on fuel availability, some of the findings may be, at least in part, attributed to the population examined (i.e., hypopituitary and Addison's patients). Thus, the effects of acute hypocortisolism via pharmacological intervention in healthy individuals may provide additional insights.

Acute Hypocortisolism

In order to study the isolated effects of an impaired cortisol response on carbohydrate, lipid and protein metabolism, a series of studies have used metyrapone

and RU486. The former is a selective steroid synthesis inhibitor that blocks the last reaction prior to the synthesis of cortisol (Liddle et al., 1958), while the latter is an antiprogesterone with antiglucocorticoid activity that prevents cortisol from binding to its receptor (Ulman et al., 1990).

Kinsley and Simonson (1996) examined the role of cortisol and epinephrine in 11 healthy subjects during 3 h of insulin-induced hypoglycemia that lowered blood glucose from ~5 mmol/L to 2.2 mmol/L in steps of 0.55 mmol/L every 30 min. To examine the role of decreased cortisol and its availability on the epinephrine response to hypoglycemia a trial was performed following the OSDMT. The results were compared to a control trial. Before the insulin-clamp, there were no differences in plasma glucose and hormones between the control and metyrapone trial. During the insulin-clamp plasma glucose declined to similar levels under metyrapone compared to controls. Similarly, there were no differences in the plasma insulin, epinephrine, norepinephrine and GH levels under both experimental conditions. On the other hand, basal plasma cortisol did not differ between control and metyrapone treatment. In the former condition cortisol increased nearly 2-fold by the 3rd h, whereas on the latter it failed to increase. The authors concluded that although metyrapone abolished the cortisol response to hypoglycemia, the epinephrine response to hypoglycemia remained intact. During the metyrapone treatment the authors infused dexamethasone to prevent potential cardiovascular and other consequences of hypoglycemia during adrenal insufficiency. This, by itself, may have confounded their results. It is likely that the dexamethasone (a potent synthetic glucocorticoid) compensated for the cortisol deficiency the authors claimed. On the other hand, the elevated cortisol may be explained by the RIA kit used which, according to the manufacturer has a 33% cross-reactivity with 11-deoxycortisol which was elevated ~100-fold following metyrapone. Additionally, dexamethasone may have cross-reacted with cortisol, contributing to the elevated cortisol levels.

De Feo and colleagues (1989a) examined the role of cortisol during prolonged insulin- induced hypoglycemia in 8 subjects. The studies consisted of 4 parts and lasted 12 hours each. In study 1, insulin was infused for 12 h and glucose fluxes, substrates and counterregulatory hormones were monitored. In study 2, insulin, glucagon, growth hormone, and cortisol were clamped by the administration of somatostatin and metyrapone and replaced to simulate the blood glucose increase observed during hypoglycemia in study 1. Study 3 was similar to study 2 except that cortisol was not replaced and glucose was infused whenever needed to reproduce the plasma glucose concentration of study 2. Study 4 was identical to study 3 except that glucose was not replaced. Isolated cortisol deficiency (study 4) resulted in a 22% decrease in hepatic glucose production, and a 15% increase in peripheral glucose utilization. This resulted in greater hypoglycemia (2.05 vs. 2.89 mmol/L). In addition, cortisol deficiency was associated with a ~ 50% reduction in the plasma concentrations of NEFA, glycerol and β -hydroxybutyrate.

Dinneen and associates (1993) examined the effects of the nocturnal rise of cortisol on the disposition of a mixed meal when the morning rise of cortisol was prevented or mimicked by administering metyrapone and a basal or variable cortisol infusion. Following an overnight fast 8 subjects participated in the study. Blood glucose, NEFA, alanine concentrations and estimated gluconeogenesis (by the rate of labeled CO_2 incorporation into glucose) were measured. The variable cortisol infusion (i.e., morning peaked at ~550 nmol/L) was twice as high as the basal (i.e., morning peak ~240 nmol/L). Plasma glucose was significantly higher during variable infusion compared to basal infusion (5.27 Vs 4.88 mmol/L). Similarly, following meal ingestion, glucose's highest and lowest values were higher during variable than during basal infusion. Additionally, during the postprandial period glucose Ra, and palmitate Ra, and the plasma concentrations of alanine and β -hydroxybutyrate increased to higher

levels during variable than basal infusion. The increase in glucose Ra was the result of a greater incorporation of $^{14}\text{CO}_2$ into glucose, suggesting an accelerated gluconeogenesis. These findings suggested that the morning rise of plasma cortisol is of physiological significance and is involved in maintaining a higher fasting plasma glucose concentration, and increasing gluconeogenesis, proteolysis and ketogenesis.

In light of the fact that in the previous study the nocturnal rise of cortisol resulted in lower rates of insulin secretion and higher rates of glucagon secretion the authors could not distinguish between the direct effects of cortisol and those due to the resultant alterations in insulin and glucagon secretion. Thus, the authors repeated the study (Dinneen et al., 1995) in individuals with insulin-dependent-diabetes under a basal insulin infusion. Variable cortisol levels (~ 550 nmol/L) were twice as high as basal (~ 250 nmol/L), whereas fasting plasma glucose did not differ in either study. In contrast, following meal ingestion the glucose response was higher in the variable than in the basal infusion. By design, postprandial glucose concentration was the same during the basal and variable cortisol infusions in the "glucose-matched" portion of the study. Supplemental glucose was needed in the basal study to match the postprandial glucose of the variable cortisol study. Insulin, C-peptide, glucagon, and growth hormone did not differ in the basal and variable infusion. Postprandial rate of incorporation of CO_2 into glucose was higher during variable than basal cortisol infusion regardless of whether or not glucose concentrations were matched. Lactate and alanine did not differ either before or after meal ingestion in both studies, whereas β -hydroxybutyrate and palmitate were higher before and after meal ingestion in the variable than the basal cortisol study. Finally, carbohydrate oxidation was lower and lipid oxidation was higher during the variable than the basal cortisol infusion both before and during food ingestion. In summary, physiological increases in cortisol, independent

of insulin and/or glucagon, increases lipolysis, ketogenesis, gluconeogenesis and reduces carbohydrate oxidation.

Garrel and colleagues (1995) studied the effects of cortisol on metabolism by using RU486. The authors established the following 4 treatments: 1) saline and placebo; 2) saline and RU486; 3) cortisol and placebo; and 4) cortisol and RU486. Following an overnight fast, and during a 5-hour experimental period, they found that two hours after the administration of saline plus RU486, plasma glucose and hepatic glucose production showed decreases that were not seen in the saline and placebo controls. They did not observe an inhibitory effect on NEFA as a result of RU486 action, but they did find that RU486 reduced the rate of appearance, concentration and oxidation of leucine, all of which were higher in the presence of cortisol and the placebo. Because of the reduced hepatic glucose production and leucine Ra and oxidation in the presence of RU486, the authors suggested that cortisol is involved in the maintenance of blood glucose levels and proteolysis during a brief fast. Collectively, these studies (De Feo et al., 1989a; Dinneen et al., 1993; Dinneen et al., 1995; Garrel et al., 1995) indicate that under resting hypoglycemic and normoglycemic conditions, cortisol availability is critical to the maintenance of substrate levels.

The effects of acute hypocortisolemia have also been examined in diabetic patients. For instance, one study (Bright et al., 1980) looked at whether hypocortisolemia in insulin-dependent diabetic subjects would reduce the insulin requirements to maintain a euglycemic state. During hypocortisolemia, the authors reported that the total overnight insulin requirement to maintain euglycemia was lower, and no changes were noted in plasma growth hormone and glucagon concentrations between studies. Atiea et al. (1990) examined the effects of placebo and cortisol suppression by metyrapone on the "Dawn Phenomenon" (i.e., early morning rise in blood glucose) in non-insulin dependent diabetic subjects. The authors reported that

overnight hypocortisolemia reduced the morning rise of plasma glucose compared to control studies, and no significant changes were noted in insulin, C-peptide, glucagon, GH and catecholamines between the 2 studies.

Kitabachi et al. (1968) administered a oral glucose tolerance test to 12 healthy subjects before and after 24 h hypocortisolemia (metyrapone-induced). Their basal insulin and glucose were not different, but their peak insulin and glucose response to the oral glucose tolerance test were lower under treatment compare to the control study. This same group reported that in two mineralocorticoid-replaced Addison's patients the insulin response to the oral glucose tolerance test was attenuated compared to a different day under cortisol replacement. Interestingly, their basal overnight insulin levels were not different with or without cortisol replacement. The authors concluded that basal insulin is not significantly affected by lowering cortisol levels, however, hypocortisolemia reduces the amount of insulin needed to disposed of an oral glucose load. However, because peak glucose levels were lower it is no surprise that the insulin response was lower. Why was plasma glucose lower? Dinneen and associates (1993; 1995) reported comparable rates of carbohydrate absorption (as measured by D-xylose) under basal (240 nmol/L) and elevated (550 nmol/L) cortisol, but lower postprandial appearance of ingested glucose under the former than latter condition, indicating a mild inhibition of splanchnic glucose release due to a low cortisol concentration.

Physiological Significance Of Cortisol And Gluconeogenesis

The major function of gluconeogenesis is to prevent glucose levels from falling below normal. The rate of gluconeogenesis is therefore higher during prolonged fasting (Gerich, 1993) and exercise (Wasserman and Cherrington ,1991; Wasserman, 1995). Hormonal regulation of gluconeogenesis occurs via the major glucoregulatory

hormones. The acute regulation of hepatic glucose metabolism occurs through hormone-mediated changes in enzymatic activity, principally the phosphorylation or dephosphorylation of key enzymes and also by exerting long-term effects by changing the rate of enzyme synthesis (Jones et al., 1993; Pilkis, 1991). Glucocorticoids are well known for inducing the synthesis of a number of gluconeogenic enzymes [see (Lemaigre and Rouseau, 1994; Pilkis, 1991) for a review]. Glucocorticoids have been shown to induce glucose-6-phosphatase (Fowden et al., 1993; Weber et al., 1955), fructose 1,6 diphosphatase (Fowden et al., 1993; Mokrasch et al., 1956), pyruvate carboxylase (Fowden et al., 1993), and phospho-*enol*-pyruvate carboxykinase (Fowden et al., 1993; Shrago et al., 1963). In fact, it has been suggested that glucocorticoids are the most important factor in maintaining the expression of 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase (Pilkis, 1991). Moreover, glucocorticoids are the key regulator of phospho-*enol*-pyruvate carboxykinase, the rate limiting step of the gluconeogenic pathway (Pilkis, 1991). Glucocorticoid deficiency results in a blunted response by the liver to glucagon. The impairment does not appear to modify cAMP accumulation or cAMP-dependent protein kinase activation; the authors suggested that the impairment might be distal to the cAMP-dependent protein kinase activation (Exton et al., 1981).

In agreement with all these gluconeogenic effects *in vitro*, Lacavalier et al (1990) stimulated gluconeogenesis *in vivo* by hypercortisolemia by increasing lactate gluconeogenesis after a 4 h period. However, hyperglucagonemia-hypercortisolemia further increased lactate gluconeogenesis in an additive fashion without increasing the overall hepatic glucose output. Importantly, the authors indicated that the failure to demonstrate an increase in overall hepatic glucose output was probably due to the small number of subjects examined (hyperglucagonemia n=8; hypercortisolemia n=5). The authors concluded that within a 4-h period hypercortisolemia would increase hepatic

glucose output by augmenting gluconeogenesis rather than glycogenolysis. Similarly, other studies reviewed in earlier sections (Dinneen et al. 1993; Dinneen et al. 1995) have reported stimulatory effects of cortisol on the incorporation of $^{14}\text{CO}_2$ into glucose, suggesting accelerated gluconeogenesis in vivo.

6. Cardiovascular Effects Of Cortisol Deficiency

Patients with adrenocortical insufficiency have been described as hypotensive and extremely fragile because stimuli that can be tolerated by healthy subjects may lead to cardiovascular collapse (Clausen et al., 1992; D'albora and Martin, 1966; McGrady and Wright, 1989; Redman et al., 1987; Vesely, 1982). This has been associated with the inability of the cardiovascular system to respond to pressor agents (i.e., catecholamines), that is only restored by adrenal corticosteroids (Ross, 1961). Early studies in anesthetized rats exposed to acute surgery and to electrical stimulation demonstrated that the vascular smooth muscle became very insensitive to catecholamines unless pretreated with glucocorticoids (Fritz and Levine, 1951; Kalsner et al., 1969). This was confirmed in anesthetized dogs (Ramey et al., 1950) and the hypothesis was advanced that the cardiovascular collapse observed in individuals suffering from adrenal crisis was at least partially due to the cardiovascular system's insensitivity to pressor agents. Darlington et al. (1989) examined the changes in vascular responsiveness and the baroreflex after ADX in conscious, unrestrained and unstressed rats treated with a variety of replacement doses of corticosterone. The study demonstrated that physiological levels of corticosterone restored vascular responsiveness, whereas higher levels were necessary for the restoration of the baroreflex. Not long ago glucocorticoid receptors were found in neurons of the brain stem medulla and shown to modulate the baroreceptor signal and modulate sympathetic output (Harfstrand et al., 1986).

In untreated Addison's disease the renin-angiotensin system is altered (Brown et al., 1964), resulting in the production of large amounts of angiotensin I which is subsequently converted to angiotensin II by angiotensin converting enzyme. Angiotensin II is reported to be elevated in untreated Addison's disease, and is considered an important factor in maintaining blood pressure (Ogihara et al., 1979). Furthermore, this patient population presents elevated plasma renin activity and angiotensin converting enzyme levels which return to normal levels following the institution of mineralocorticoid and glucocorticoid replacement therapy (Falezza et al., 1985). However, the authors could not evaluate separately the effect of mineralocorticoid and glucocorticoid hormones, since all patients were treated with combined therapy

Fallo and associates (1994) evaluated the circadian blood pressure rhythmicity in a group of Addison's disease patients, steroid-replaced Addison's disease patients, and a group of normotensive subjects. These otherwise apparently healthy subjects underwent 24-h non-invasive blood pressure and heart rate monitoring. The authors reported that adrenocortical insufficiency is often characterized by a loss of circadian blood pressure rhythm, which is restored by corticosteroid replacement. This study suggested that at rest, cortisol plays a demonstrable role in blood pressure regulation. Interestingly, cortisol insufficiency is also associated with ECG abnormalities (Hartog and Joplin, 1968). These investigators reported that 81% of the patients with cortisol deficiency showed flattening or inversion of the T-waves in the standard and unipolar limb leads. Additionally, 33% showed prolonged Q-T intervals. However, the majority of the patients showed regression of the ECG changes after glucocorticoid replacement. Furthermore, the authors reported no electrolyte abnormalities, though they did not rule out an altered distribution of electrolytes within the intravascular and extravascular compartments (Hartog and Joplin, 1968).

7. The Effects Of Exercise On Cortisol

The Effects Of Intensity And Duration

In humans, the HPA is activated during intense (Elias et al., 1991) or prolonged exercise (Tabata et al., 1991) and as a consequence, cortisol levels increase. This was first established by Staehelin et al. (1955), who reported that cortisol levels in adults increase during the first 30 minutes of a two-h exercise bout (100 watts) on a cycle ergometer. Similarly, Sutton et al. (1969) reported that cortisol levels increased in both fit and unfit subjects performing 30 min of graded exercise to exhaustion. In contrast, Cornill et al. (1965), using the same exercise regimen as Staehelin et al. (1955), reported decreases in blood cortisol levels. In general, most of these studies were plagued with methodological problems with respect to controlling the intensity and/or duration of the exercise. For example, the studies performed by Staehelin et al. (1955) and Cornill et al. (1965) used absolute rather than relative work rates, which may have contributed to the discrepant results.

Davies and Few (1973) were the first to establish a quantitative relationship between exercise intensity, percent of $\text{VO}_{2\text{max}}$, and adrenocortical activation during exercise. They exercised subjects on a treadmill at various intensities including maximal work. At the lower intensities, cortisol levels fell. At higher intensities cortisol responses were more varied, but a marked increase in cortisol was observed when the exercise intensity exceeded approximately 60% of $\text{VO}_{2\text{max}}$. During submaximal steady state exercise performed for one hour or more at an intensity equal to or greater than 60% of $\text{VO}_{2\text{max}}$, blood cortisol levels have been reported to increase in many (Davies and Few, 1973; Deuster et al., 1992; Friedmann and Kindermann, 1989; Gabriel et al., 1992; Luger et al., 1987; Schwarz and Kindermann, 1989; Tabata et al., 1991), but not all studies (Barreca et

al., 1988). At higher intensities (70 to 91% of $\text{VO}_{2\text{max}}$) and shorter durations (15-to 40 min) increases in plasma and salivary cortisol and urinary free cortisol levels have been commonly reported (Bonen, 1976; Del Corral et al., 1994; O'Connor and Corrigan, 1987; Odink et al., 1986; Ruddel et al., 1986;). Similarly, increases in the concentration of cortisol have been found at exercise intensities equal to the anaerobic threshold as defined by the $4 \text{ mmol}\cdot\text{l}^{-1}$ lactate concentration (Kindermann et al., 1982; Schwarz and Kindermann, 1989). The cortisol response to submaximal exercise does not appear to be affected by age, since it has been reported that children (Del Corral et al., 1994) as well as the elderly demonstrate an (Silverman and Mazzeo, 1996) increase in plasma cortisol levels during 30 to 45 min of submaximal exercise.

At supramaximal intensities ranging from 100 to 156% of $\text{VO}_{2\text{max}}$ for one to four and a half minutes, serum cortisol levels are elevated (Buono et al., 1986; Kindermann et al., 1982; Kraemer et al., 1989). There is inconclusive evidence of adrenocortical activation during short term anaerobic exercise at intensities exceeding 175% $\text{VO}_{2\text{max}}$ and/or durations of less than a minute; some investigators have shown an increase in cortisol levels (Buono et al., 1986; Kuoppasalmi et al., 1980) while others have not (Kuoppasalmi et al., 1976; Schwarz and Kindermann, 1990).

Cortisol levels have also been reported to increase following graded exercise to exhaustion in blood (Buono and Yeager, 1991; Farrell et al., 1983; Hyypä et al., 1986; Mathur et al., 1986). Further, plasma cortisol levels have been reported to be elevated 10 to 30 minutes following graded exercise to maximal effort (Barreca et al., 1988; Cumming et al., 1986; Davis et al., 1981; Elias et al., 1991; Hyypä et al., 1986; Oleshanski et al., 1990; Rolandi et al., 1985; Rowbottom et al., 1995; Sutton et al., 1969). Few studies have reported no increase in cortisol levels following a graded exercise test (Jacobson et al., 1983; Odink et al., 1985). Importantly, all but one of these studies (Rowbottom et al., 1995) measured total cortisol concentration. Rowbottom et al. (1995) measured free and total

cortisol concentration and found that the increase in free cortisol was disproportionate, and of much greater relative magnitude, to the increase in total cortisol concentration. The authors suggested that the direct measurement of free cortisol should be considered in future work. Furthermore, the use of absolute cortisol values to assess its metabolic effects ignores any difference in glucocorticoid sensitivity which might exist between individuals. To date, there is a lack of any robust marker of glucocorticoid activity making it difficult to assess glucocorticoid sensitivity (Howlett, 1997)

In summary, it is evident that the activation of the HPA axis during exercise depends on both the intensity and duration of the exercise bout. Furthermore, any increase in the concentration of serum cortisol during exercise reflects a change in the rate of secretion and the rate of removal from the blood stream.

Cortisol Secretion And Removal

The mechanisms by which the changes in plasma cortisol during exercise take place are best understood by turnover studies in which ^3H -cortisol is administered. Asmal et al. (1974) examined arterial-venous plasma differences across the forearm and found no detectable differences in plasma cortisol. However, when labeled cortisol was infused intra-arterially into the forearm, there was evidence for retention of cortisol by the local tissue. Furthermore, it was demonstrated that cortisol retention was due to penetration of the tissues and not to prolonged transit time in the vascular bed. In a group of normal healthy male subjects $[1,2\text{-}^3\text{H}]$ cortisol was injected to determine the concentration and specific activity of plasma cortisol. Subjects exercised on a treadmill for one h at 65-80% of $\text{VO}_{2\text{max}}$. Plasma cortisol began to rise within 20 min and reached a peak value 10 to 30 min after exercise ceased. The author found a decrease in the specific activity of plasma cortisol

throughout exercise, suggesting that the rise in concentration was due to an increase in production and not due to a decrease in the rate of removal (Few, 1971).

To further study the secretion of cortisol, Few et al. (1974) administered ^3H -cortisol to twelve healthy individuals. Four individuals ran at the highest intensity they could sustain (moderate exercise, 73% of $\text{VO}_{2\text{max}}$) for one hour, four subjects walked on a treadmill for the same period of time at an intensity known not to increase cortisol secretion (light exercise, HR of 115-130 $\text{b}\cdot\text{min}^{-1}$), and four subjects served as resting controls. During light exercise, plasma cortisol levels fell, while during moderate exercise plasma cortisol levels increased. The half-lives for ^3H -cortisol were 74.5, 43.8, and 31.5 min for the resting, light, and moderate exercise, respectively. The authors concluded that during light exercise cortisol is removed more rapidly than at rest resulting in a decrease in the cortisol concentration. During moderate exercise even though the rate of removal of ^3H -cortisol is greater than during light exercise, the plasma cortisol levels increased. As there was a sharp fall in cortisol specific activity during exercise, this rise was attributed to an increase in cortisol secretion.

Cashmore et al. (1977) used multiple regression analysis to study the contribution of the rate of secretion and the rate of removal on cortisol levels in the blood. Following an oral administration of ^3H -cortisol, ten men exercised at workloads exceeding 65% of $\text{VO}_{2\text{max}}$ for one hour. The results of this study indicated that during exercise the rate of secretion of cortisol was a more important determinant of plasma cortisol than the rate of removal.

In order to study the effects of prolonged exercise on cortisol metabolism, Few and Thompson (1980) used a 100 kilometer race as the experimental condition. In this study [$4\text{-}^{14}\text{C}$] cortisol was employed to study cortisol metabolism by measuring urinary 17-OHCS and urinary free cortisol. Following the race, renal function was found to be normal as indicated by creatinine excretion, and decreases in the ^{14}C specific activity of cortisol

metabolites excreted by the runners suggested that they secreted cortisol two to four times the rates of the basal controls.

The influence of a 56 km endurance run on cortisol kinetics and production rate was evaluated in 6 horses administered ^3H -cortisol. Exercise resulted in an immediate 2-to-3 fold increase in plasma cortisol, 2.5-fold increase in clearance and 6-fold increase in production rate, suggesting that the plasma concentration may underestimate adrenal cortical activity during exercise (Lassourd et al., 1996). The authors suggested that this high rate of clearance could indicate a high rate of cortisol uptake by the working muscles. Interestingly, however, this does not appear to be the case in humans. Rennie and associates (1974) measured cortisol arterial-venous differences across the leg at rest and during 30 min of cycle exercise at 60% $\text{VO}_{2\text{max}}$ and found a 20% extraction under both conditions. It is possible that the liver and peripheral tissues may be clearing a large fraction of plasma cortisol, and that the intensity and duration of exercise, if anything, could have marginally stimulated cortisol secretion.

Although the rate of secretion and removal are two important factors in the concentration of cortisol, other factors can modulate the adrenocortical response to exercise. These include novelty and other psychological factors associated with exercise, blood glucose levels, muscle afferent activity, feeding, and pre-exercise cortisol levels.

Factors Affecting Cortisol Release During Exercise

Psychological

Psychological factors have been known to affect glucocorticoid output in humans prior to exercise. In a classic study, Hill et al. (1956) found that glucocorticoid levels increased significantly more on race days compared to practice days even though the work accomplished was comparable. Steadman and Sharkey (1969) studied the excretion of

urinary 17-ketogenic steroids which reflects the level of cortisol and cortisone elaborated by the adrenal gland. The subjects performed a modified Balke test (on Mondays and Wednesdays) that was terminated when the subject reached a HR equal to $180 \text{ beats} \cdot \text{min}^{-1}$; this was followed by graded exercise to exhaustion on Fridays. This was repeated for a period of four weeks. They found an initial increase in 17-ketogenic steroids excretion in the first two weeks, but in the third and fourth week 17-ketogenic steroids excretion levels had returned to control levels. This finding indicated that the early increase in 17-ketogenic steroid excretion was brought about by concerns about the severity of the task and the novelty of the treadmill protocol. It was concluded that exercise by itself did not increase adrenocortical activity, and that the emotional stress associated with exercise may be more important in this regard than the exercise itself.

In a study by Mason et al. (1973) it was demonstrated that the novelty of the exercise task could affect cortisol levels at rest. In addition, this group found that the anticipatory response of exercise to exhaustion was greater prior to a high-intensity (70% of $\text{VO}_{2\text{max}}$) compared to a lower-intensity (40% of $\text{VO}_{2\text{max}}$) trial. However, after seven weeks of training the anticipatory response was reduced. Raymond et al. (1972) found that elevated plasma cortisol levels during light exercise were present only in those subjects who showed tachycardia and apprehension, which indicates a high degree of emotional stimulation. Similar findings were obtained by Sutton and Casey (1975). Davis et al. (1981) examined the differences in serum cortisol levels between non-experienced laboratory subjects and experienced subjects to an identical short term graded exercise test to exhaustion. The results of this study demonstrated that the non-experienced subjects had higher cortisol levels before as well as following exercise compared to experienced subjects.

It is well known that other non-exercise related stressors such as heat, cold, altitude (Steadman & Sharkey, 1969) and intravenous catheterization (Mason et al., 1973) may

increase cortisol levels. It is also evident that resting cortisol levels may increase prior to competitive exercise and laboratory testing; and that the emotional component of the subjects can certainly affect the cortisol response to exercise. Therefore, appropriate familiarization with procedures and apparatus is strongly recommended.

Blood glucose

The effects of blood glucose on the adrenal cortex were reported as early as 1955 (Staehelin et al., 1955) when it was known that hypoglycemia induced an increase in plasma hydroxycorticosteroids. This would suggest that blood glucose is an important trigger for ACTH and cortisol release. More recently, Tabata et al. (1984) examined the interaction between blood glucose and the pituitary-adrenal axis during exercise. Ten subjects exercised for 3 h at 50% of $\text{VO}_{2\text{max}}$ and although they found no consistent time point for the increase in ACTH and cortisol levels among the subjects, they found a 3 mmol/L blood glucose threshold for the increase in serum cortisol. A few years later the same authors, Tabata et al. (1991), examined the effect of prolonged exercise on the HPA axis during a similar protocol. The authors showed that if blood glucose was maintained above 3.3 mmol/L during prolonged exercise by glucose infusions, the rise in CRH, ACTH and cortisol level was suppressed. These results demonstrate that decreases in the blood glucose concentration trigger the hypothalamic-pituitary-adrenal axis to become active during low intensity prolonged exercise.

Sotsky et al. (1989) examined the role of blood glucose and exercise on serum cortisol by using insulin clamps and glucose infusions. Ten subjects performed graded exercise at 45, and 53% of $\text{VO}_{2\text{max}}$ for 20 min, and at 65% $\text{VO}_{2\text{max}}$ for ten minutes under normoglycemic (4.8 mmol/L) and moderate hypoglycemic (3.3 mmol/L) conditions. During normoglycemia, cortisol levels remained stable at all intensities, while exercise at the highest intensity during hypoglycemia elicited a 150% increase in cortisol levels. In a

second experiment, plasma glucose was clamped in an identical manner as the hypoglycemic trial and subjects exercised at the same intensities and durations. After 25 min of exercise glucose was infused and normoglycemia was restored. However, it failed to suppress the increase in plasma cortisol levels.

Deuster et al. (1992) investigated the cortisol response to 2 h of treadmill running at 60-65% of $\text{VO}_{2\text{max}}$. The subjects performed 3 exercise trials. In each trial one of the following solutions was administered: a) 200 ml of a 7% carbohydrate and electrolyte solution, b) water, or c) a vitamin-enriched water solution. The different solutions were given orally immediately prior to exercise, and every thirty minutes into the run. Cortisol levels remained unchanged only in the trial in which the subjects ingested the carbohydrate and electrolyte solution. However, during the two trials in which the carbohydrate solution was not ingested blood glucose levels were above 5 mmol/L at all times and cortisol levels still increased.

The findings of Deuster et al. (1992) support the view that the activation of the HPA axis during exercise does not appear to be solely dependent upon blood glucose levels. In fact numerous investigators have reported an increase in cortisol levels during exercise under normoglycemia (Bloom et al., 1976; Kindermann et al., 1982; Farrell et al., 1983; Friedmann and Kindermann, 1989; Ruddel et al., 1986). Therefore, it is clear that other factors can stimulate the activation of the HPA axis during exercise. For instance, meals and time of day may modulate the HPA axis response to exercise.

Meals And Time Of Day

It is well known that cortisol levels vary throughout the day and that the circadian rhythm is affected by feeding (Brandenberger and Follenius, 1975). This same group of investigators later compared the effects of light and moderate (25 and 55% $\text{VO}_{2\text{max}}$) exercise at two different times of the day to see if exercise-induced and meal-induced

effects were additive. Moderate exercise during the postprandial cortisol peak (1300 h) resulted in a blunted cortisol response compared to the exercise-induced rise during the cortisol quiescent period (1000 h). Plasma cortisol responded similarly during light intensity exercise. The authors concluded that the exercise-induced and the meal-induced cortisol levels are not additive and demonstrated that high plasma levels of cortisol diminish the subsequent stress response (Brandenberger and Follenius, 1975). Food intake is another factor that may affect cortisol levels during exercise. A third study examined the diurnal changes in cortisol response to moderate exercise (90 min at 75% $\text{VO}_{2\text{max}}$) performed during quiescent periods (i.e., 1000, 1430, 1700 and 2130). Exercise performed during quiescent periods induced a similar increase in plasma cortisol levels when related to control days. However, when the same exercise was performed at 1300 or 2000 h coinciding with the cortisol peaks, an increase in cortisol concentration was not observed (Brandenberger et al., 1982). Taken together these studies indicate that the diurnal changes in the cortisol response to exercise are not only affected by circadian rhythm variation, but are related to meal peaks and more specifically to the proximity of food to the onset of exercise.

Studies by Brandenberger and Follenius's (1975;1982) suggested that small changes in cortisol secretion may only be identified by comparison with the same time interval on a control day. Their findings have been widely disregarded. Recently, however, the circadian rhythm of cortisol and its implications during exercise was revisited (Thuma et al., 1995). This group examined whether the neglect of the cortisol circadian rhythm would confound its response to exercise. Subjects exercised for 40 min at ~ 70% $\text{VO}_{2\text{max}}$ at 0800 and 2000 h and serum and salivary cortisol were measured before, during and after exercise as well as during a rest day at the same time intervals. Areas under the curve for serum and salivary cortisol calculated by reference to pre-exercise concentration underestimated salivary and serum cortisol by 93 and 84% in the morning and by 37 and

35% in the evening. Similarly, rises in serum and salivary cortisol were underestimated when calculated relative to the pre-exercise baseline method.

Mechanisms Of Activation Of The HPA Axis

There is little information available on the role of muscle afferent information and its possible link to the activation of the HPA axis during exercise. It has been shown that for a given workrate exercise with one leg is a more potent stimulus to increase plasma cortisol levels than the same exercise performed with two legs (Few et al., 1980). The authors suggested that the exercise-induced activation of the HPA axis may be sensitive to a marker of metabolic disturbance that would be detected locally, namely muscle chemoreceptors (Few et al., 1980).

In a study by Kjær et al. (1989), six subjects underwent epidural anesthesia at vertebrae L3-L4 to evaluate the importance of afferent neural feedback from working muscles. During the anesthesia, cutaneous sensory blockade was present below segments T11-T12. The subjects first exercised at 60% of VO_{2max} for 20 min, rested for 10 min, and then completed a graded exercise test to exhaustion. On a different day, the subjects repeated the sequence without anesthesia. The results of the study showed an increase in ACTH during maximal and submaximal exercise only in the trial without anesthesia. The authors concluded that during exercise afferent neuronal feedback from the working muscles stimulate the secretion of ACTH. This group later showed that there was a direct relationship between stimulation of group III and IV afferent fibers and plasma ACTH. This study used an *in situ* electrical frequency stimulation protocol in a cat preparation. The results indicated that as the frequency of stimulation increased the plasma concentration of ACTH rose, and provided evidence for a reflex control of pituitary secretion of ACTH (Vissing et al. 1994). Interestingly, Lassourd and associates (1996) have shown in the

horse that secretion rate was instantaneously increased and dramatically reduced at the onset and end of exercise, respectively. The authors suggested that during exercise the apparent on/off response supports the view of a neural rather than humoral activation of the HPA axis.

To determine whether corticotrophin releasing hormone (CRH) was the sole factor mediating ACTH release during exercise a group of subjects were given at separate times a 6-h infusion of ovine-CRH and saline. When the plasma concentration of CRH was elevated enough to saturate the adenohypophysis corticotroph-receptor, the subjects completed a high intensity intermittent run. Plasma ACTH and cortisol increased ~ 2-fold during the ovine-CRH infusion, with a further increase (~ 4-fold) stimulated by exercise. On the other hand, during saline infusion exercise induced a ~ 5-fold rise in ACTH. The authors suggested that some factor in addition to CRH causes ACTH release during exercise; vasopressin was mentioned as a possible candidate (Smoak et al., 1991).

8. Cardiovascular Effects of Cortisol During Exercise

During the mid-1940's Schweitzer (1945) reported that ADX animals showed a decrease in mean arterial blood pressure to 60-70 mmHg which impaired *in situ* muscle contraction. A few years later Ramey and associates (1950) examined muscle strips to study the characteristic muscle fatigability of ADX animals and found that the muscle itself was not associated with fatigue. The authors suggested inadequate circulatory adjustments as the primary cause of fatigue. Additionally, this impairment was corrected by high doses of pressor agents but not by mineralocorticoid replacement (Goldstein et al., 1950). The authors suggested that it might be maladaptation of the neuro-circulatory system due to lack of C₁₁ oxysteroids (i.e., cortisol). The same laboratory (Fritz and Levine, 1951) later reported that the abnormal blood pressure response during exercise

in the ADX animal was due to attenuation of vasomotor tone. For instance, the animal's small vessels were unresponsive to known vasoconstrictive norepinephrine concentrations. However, at higher norepinephrine concentrations or when 11-oxysteroids were administered, the vasomotor tone was reestablished. The authors concluded that blood vessels constrict only if they are "primed" by 11-oxygenated steroids.

Other investigators have shown that adrenocortical hormones, particularly glucocorticoids, participate in the regulation of water and electrolyte transmembrane-shifts during exercise (Korge and Viru, 1971a; Korge and Viru, 1971b; Korge and Rooson, 1975). For instance, during heavy exercise corticosteroids increase the shift of water and Na⁺ from the intracellular to the extracellular space in cardiac (Korge and Viru, 1971a) and skeletal (Korge and Viru, 1971b) muscle of trained animals.

Adrenocortical-insufficient patients are characterized by generalized fatigue and hypotension. Typically, stimuli that can be tolerated by healthy subjects may lead to cardiovascular collapse. It is no surprise that for these reasons these patients have been reported to have a low physical work capacity (Grecu et al., 1972; MacBryde and De la Balze, 1944; Barwich et al., 1980; Mier et al., 1988; Zelinsky et al., 1987), a condition which can be improved to near-normal levels upon administration of glucocorticoid replacement therapy (MacBryde and De La Balze, 1941; Zerlinsky et al., 1987).

Adrenocortical-insufficient patients have a low work capacity due to muscle weakness. For instance, Mier et al (1988) reported a case of a patient who became breathless when walking up stairs. Pulmonary function tests revealed diaphragmatic weakness, and maximal voluntary contraction strength of the quadriceps was ~ 30% of normal values. When stimulated with ACTH, the patient's plasma cortisol remained consistently under 120 nmol/L. Ten months later, following cortisol replacement, pulmonary function tests were normal, maximal voluntary contraction strength of the

quadriceps had increased to ~ 75% of normal values and the patient was able to climb two flights of stairs. The authors concluded that Addison's disease may cause a myopathy which may affect respiratory muscles and limit the work capacity of the patient.

Zerlinky et al. (1987) examined 48 male and female Addison's disease patients and compared them to 20 healthy male and female controls. The subjects underwent a graded exercise test to maximal effort and a submaximal trial at the ventilatory threshold. During maximal exercise, 41/48 and 26/48 patients complained of muscle weakness and muscle soreness, respectively, which lasted 5-10 min post-exercise. Only ten patients could reach a $\text{VO}_{2\text{max}} \sim 25 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. When the patients did submaximal exercise at $\sim 55\% \text{ VO}_{2\text{max}}$, 27% showed a drop in systolic blood pressure of 10-15 mmHg by the tenth min of exercise. Glucocorticoid and mineralcorticoid treatment reduced muscle pain, prevented the drop in systolic blood pressure and improved but did not normalize work capacity to control levels. Hoelzer and associates (1986) exercised four glucocorticoid-mineralcorticoid replaced ADX subjects at $\sim 60\% \text{ VO}_{2\text{max}}$ for 60 min and reported that systolic blood pressure tended to decrease. In contrast, others have reported that these patients are able to increase blood pressure during 18 min of graded exercise to maximal effort, provided appropriate glucocorticoid and mineralocorticoid replacement therapy had occur.

The vital role of glucocorticoids during exercise was reported not long ago. Parsons and associates (1984) reported on June 1983 a case of a 45 year old runner who collapsed at 3.5 miles after the onset of a race; resuscitation attempts failed and he died 30 minutes later. In October 1980 the subject was diagnosed with sarcoidosis and treated with prednisolone (i.e., a synthetic glucocorticoid) which continued with reducing dosage until his death. In 1981 he started gradual recreational running. Prednisolone (2.5 mg) was accidentally omitted two days before the race (taken

normally the day before the race). An autopsy revealed the occlusion of the two major branches of the left coronary artery (80% and 50% focally) by atheroma without thrombosis, active sarcoidosis and adrenal cortex atrophy with lipid depletion. The authors concluded that three years of prednisolone therapy led to adrenocortical atrophy and that the stress of running and absence of sufficient prednisolone replacement precipitated acute adrenocortical insufficiency with hypotension. Myocardial ischemia and cardiac arrest followed as a result of reduced blood flow through the partially stenotic atheromatous coronary arteries. The authors emphasized the need to increase the glucocorticoid dose prior to a stressful situation when the adrenal cortex has been suppressed.

These studies (Hill et al., 1951; MacBryde and De la Balze, 1944; Mier et al., 1988; Parsons et al., 1984; Zelinsky et al., 1987) suggest: (1) that these patients readily remove exogenous cortisol during exercise; (2) their condition reduces work capacity which may be improved by appropriate cortisol replacement ; (3) the decrease in work capacity is characterized by cardiovascular instability and skeletal muscle weakness; and (4) that under rare circumstances and during exercise, cortisol deficiency may lead to cardiovascular collapse and death.

9. Metabolic Effects Of Cortisol During Exercise

Animal Model

It has been over 60 years since the effect of adrenal cortical hormones on exercise tolerance was first documented (Briton and Silvette, 1931). From the 1930's to the early 1950's many investigators examined the effect of ADX on exercise tolerance

(see [Tharp, 1975] for a review) and concluded that chronic adrenal cortical insufficiency decreased "work capacity".

With the onset of exercise, cortisol is removed from the circulation and, depending on the exercise intensity, cortisol release will be lower, match or exceed the rate of removal. As cortisol reaches its target tissues, it will bind to its receptor and initiate its effects on the cell. Little is known about the cortisol-receptor interaction during exercise. Recently however, Czerwinski and Hickson (1990) showed in ADX rats that glucocorticoid receptor activation as quantified by DNA-binding cellulose, steadily increased with an injection of labeled triamcinolone acetonide to a similar extent at rest and during exercise. Receptor activation was ~ 15, 35, 40, 65 and 70% at rest, 5, 30, 60 and 90 min, respectively suggesting that glucocorticoid receptor activation does not differ between resting and exercise conditions.

Sellers et al. (1988) investigated the effect of the increase in glucocorticoids on endurance in the rat. Rats were either sham operated or ADX and given subcutaneous aldosterone and corticosterone replacement. Five minutes before a run to exhaustion one group of ADX rats was given corn oil and the other was given corticosterone subcutaneously, but both groups were given epinephrine via a jugular catheter. The endurance time of the ADX rats injected with corn oil was reduced by 35% compared to sham operated, whereas the steroid replaced rats endurance time was reduced by 17%. Inhibition of the rise of glucocorticoids had no effect on liver AMP and glycogen, plasma insulin, glucose, lactate, glycerol, β -hydroxybutyrate, plasma norepinephrine, and muscle glycogen. However, plasma NEFA was significantly depressed at exhaustion. This study suggests that glucocorticoids exert their effects within the time frame of a prolonged exercise bout and play a role in increasing endurance in rats.

Similarly, others have examined the effect of glucocorticoids on substrate availability in Sprague-Dawley rats. The experimental animals were injected with

cortisol acetate 21-h prior to a treadmill run and a control group was injected with the vehicle. Immediately prior to exercise, the cortisol-treated rats had ~ 40% higher plasma NEFA and liver glycogen concentrations, and the muscle glycogen concentration was also elevated. Endurance time to exhaustion was increased 20%, O₂ consumption increased 11% and muscle glycogen depletion was more pronounced compared to vehicle-treated rats. The authors concluded that elevation of NEFA, liver and muscle glycogen by a single injection of cortisol can improve endurance but not nearly to the extent expected from the metabolic changes (Gorostiaga et al., 1988).

Toode et al. (1993) compared the actions of glucocorticoids and insulin on epinephrine's lipolytic action in adipocytes from trained and untrained subjects. The study used trained and untrained humans and rats. A group of rats remained as controls whereas the other group was swim trained 5 days a week for 45 min per day during the first week, with subsequent 15 min increments a week for a total of 6 weeks. Suprailiac white adipose tissue was obtained and incubated for thirty min under six conditions: a) no hormonal treatment, b) epinephrine, moderate concentration, c) epinephrine, high concentration, d) high epinephrine and dexamethasone, e) moderate epinephrine and insulin, f) moderate epinephrine, insulin and dexamethasone. The glycerol release into the incubation medium was used as an indicator of lipolysis in isolated adipocytes. The basal rate of lipolysis was found to be lower in adipocytes from trained than untrained. No significant difference were found between the stimulatory effects of the two different doses of epinephrine on glycerol release. However, the administration of dexamethasone enhanced the epinephrine effect significantly in trained but not in untrained humans and rats. Interestingly, the enhancing effect of the glucocorticoid on lipolysis took place in ~ 30 min. However, others have shown that glucocorticoids do not appear crucial to the rise of NEFA during exercise since blockade of glucocorticoids

by administering metyrapone for 3 days in normal male albino rats forced to swim for 15 to 60 min had no effects on the rise of NEFA (Lefebvre et al., 1972).

Viru et al. (1994) evaluated the interaction of exercise-induced increases in alanine and glucocorticoids on ADX and normal rats. In normal rats a 3-h swim induced increases in alanine levels in blood, quadriceps and liver, whereas ADX blunted these effects. Similarly the increase in muscle alanine aminotransferase activity was prevented by ADX. However, when glucocorticoids were replaced the exercise induced increases in alanine levels, and alanine aminotransferase activity were restored. These results support the view that glucocorticoids promote alanine supply and utilization in the liver during exercise.

The metabolic implications of glucocorticoid administration during exercise are of interest because they enhance our understanding of cortisol on energy metabolism. Shaw et al. (1976) examined glucose and glycerol metabolism at rest and during a 3-h treadmill run on alloxan-diabetic dogs treated with methylprednisolone (i.e., a synthetic glucocorticoid) or placebo. They found that a higher fraction of glycerol turnover was converted to glucose in methylprednisolone-treated dogs than in untreated diabetic dogs and controls. Additionally, methylprednisolone increased hepatic glucose output modestly but increased glucose cycling by 5- and 8-fold when compared to healthy and diabetic dogs.

Recently, Friedman et al. (1994) examined the role of glucocorticoids on phosphoenolpyruvate carboxykinase (PEPCK), the major regulatory point in the flux of the gluconeogenic pathway. The synthesis of this key enzyme increases linearly with swimming for up to 2 h. Transgenic mice carrying a gene consisting of the PEPCK promoter linked to a reporter gene for bovine-GH exhibited nearly a 5-fold increase in hepatic bovine GH mRNA in response to exercise. However, transgenic mice with a deletion in the PEPCK glucocorticoid regulatory unit showed no change. This

observation is supported by the finding that an exercise-induced increase in PEPCK mRNA is markedly attenuated in ADX rats, and dexamethasone corrects the impairment. Additionally, endurance capacity was reduced. These results suggest that during exercise, glucocorticoids act as transcriptional activators. A possible role for glucocorticoids in the molecular mechanisms for activation of hepatic genes involved in energy metabolism during exercise is suggested, particularly PEPCK.

To test the hypothesis that glucocorticoids operate via induction of protein synthesis Viru and Smirnova (1985) examined the effect of glucocorticoids and actinomycin D (a protein synthesis inhibitor) on the working capacity of intact and the ADX rats. In intact rats the blockade of RNA synthesis by actinomycin D during the 3 days prior to the swimming test substantially decreased time to exhaustion whereas the 3 day cortisol treatment had no effect. ADX significantly decreased time to exhaustion by ~ 55% and cortisol attenuated the decline to ~ 35%. However, this improvement was abolished by actinomycin D; in fact, the work capacity was slightly lower than ADX rats on saline. Similarly, work capacity was decreased by ~ 33% in the sham-operated rat receiving actinomycin D. The results of this study show that blockade of protein synthesis 3-days prior to testing decreases work capacity, and that improvement of work capacity in ADX rats by glucocorticoids involves protein synthesis.

Devenport et al. (1993) examined intact and ADX rats treated with dexamethasone (type II receptor agonist) or aldosterone (type I receptor agonist), or corticosterone (mixed agonist), or vehicle 24, and 2-h prior to a 1-h run. The endurance of intact animals was unaffected by the treatments. ADX advanced the onset of fatigue and aldosterone exacerbated this effect. Corticosterone improved endurance in the ADX rat and dexamethasone was even more potent in this effect. This study suggests that endurance in the rat is directly related to glucocorticoid type II receptor activity.

Taken together, these studies (Czerwinski and Hickson, 1990; Friedman, 1994; Gorostiaga et al., 1988; Sellers et al., 1988; Shaw et al., 1976; Toode et al., 1993; Viru and Smirnova, 1985; Viru et al., 1994) indicate that during exercise glucocorticoids: (1) are likely to exert effects within the time frame of a prolonged exercise bout and suggests that the rise of plasma cortisol during exercise may be of metabolic significance, especially in the trained animal; (2) increase substrate availability, by stimulating gluconeogenesis, lipolysis and glycogenesis; (3) play a pivotal role in alanine availability and metabolism; (4) modestly increase endurance time to exhaustion, suggesting an ergogenic effect; (5) operate via gene transcription- protein synthesis; and (6) appear to mediate endurance via glucocorticoid type II but not type I receptor activity.

Human Model

Adrenocortical-insufficient patients are characterized by generalized fatigue and hypotension, which can be improved to normal levels upon administration of glucocorticoid replacement therapy (MacBryde and De La Balze, 1944; Zerlinsky et al., 1987). Additionally, low plasma glucose on fasting or on low carbohydrate diet, increased sensitivity to exogenous insulin, abnormally intense hypoglycemia following oral or intravenous administration of glucose, and a high fasting respiratory quotient have all been reported in this patient population (Boyle and Cryer, 1991; Fajans, 1961; Vesely, 1982; Takeda et al., 1987; Wajchenberg et al., 1964). With the onset of exercise, these patients remove cortisol from the circulation at a fast rate (Kägi, 1955). Similarly, Hill and associates (1956) reported that increased physical activity in patients with Addison's disease on a constant dose of cortisol was associated with a small decrease with 17-hydroxycorticosteroid excretion. Exercise tolerance and

normoglycemia are restored in Addison's patients administered pork adrenal-cortex extract one hour prior to an exercise bout (MacBryde and De La Balze, 1944). The main merit of this study is the fact that they reported an increase in work capacity and normalization of blood glucose levels when glucocorticoids were replaced in patients previously lacking cortisol.

In general, it appears that glucocorticoid-replaced patients (i.e., hypopituitary, ADX for treatment of Nelson's tumor, Cushing's or bilateral pheochromocytoma) may have a normal (Hoelzer et al., 1986; Järhult and Holst, 1979) or exaggerated (Barwich et al., 1980; Barwich et al., 1981; Johnson et al., 1971) substrate and hormonal response to exercise. For instance, Hoelzer et al. (1986) exercised four glucocorticoid-mineralcorticoid replaced ADX subjects at $\sim 60\%$ $\text{VO}_{2\text{max}}$ for 60 minutes and compared them to a control group. Glucose Ra, plasma NEFA, glycerol, β -hydroxybutyrate, lactate, alanine, insulin, glucagon, GH, epinephrine, norepinephrine, and cortisol were measured. The subjects were able to increase glucose production and maintain a substrate and hormonal response no different from controls.

In summary, hypopituitary and ADX subjects appear to show proper or even exaggerated metabolic responses to moderate and heavy exercise provided that glucocorticoid and mineralcorticoid replacement is adequate. However, these studies (Barwich et al., 1980; Barwich et al., 1981; Hoelzer et al., 1986; Järhult and Holst, 1979; Johnson et al., 1971) failed to provide information on substrates or counterregulatory hormones responses when the subjects were off cortisol replacement. Little is known about the acute effects of cortisol on substrate availability and utilization during exercise. A few studies have examined the effects of exogenous glucocorticoid (Viru and Smirnova, 1982) and ACTH (Soetens et al., 1995) on performance and blood substrates.

Viru and Smirnova (1982) examined the relationship between physical work capacity and glucocorticoid level during short term exercise in 18 trained and untrained men. Cortisol levels were decreased by dexamethasone (3 day treatment) or increased by ACTH injection 30 minutes prior to a VO_{2max} and PWC_{170} test. The results of this study showed that ACTH increased plasma cortisol by ~65%, dexamethasone modestly suppressed cortisol by ~ 13 - 50% and no apparent effects were noted on either VO_{2max} or PWC_{170} . The authors concluded that physical working capacity during a short graded exercise test does not depend on an increase in the glucocorticoid response. Unfortunately, the design of the study had a major flaw. Dexamethasone does not provide an adequate model to induce cortisol deficiency because, although it reduces cortisol secretion, dexamethasone will induce the effects of cortisol. Thus, the effects of cortisol deficiency on work capacity can not be assessed under this scheme.

Recently Soetens and associates (1995) examined the effect of ACTH or placebo on 16 professional cyclists who cycled at 60% VO_{2max} for 60 min with subsequent 10 watt increments every minute until exhaustion. Subjects received ACTH injections 45 min prior to exercise. Plasma cortisol and NEFA concentrations increased 2-and 3-to 5-fold, respectively during ACTH compared to placebo treatment. Similarly, blood glucose was significantly higher during ACTH compared to placebo treatment. The subject's rating of fatigue was lower throughout the submaximal exercise under ACTH treatment compared to placebo. The enhanced lipolysis, improved glucose homeostasis, and decreased rates of fatigue suggest an ergogenic effect.

10. Summary

It has been over 40 years since the effects of exercise on glucocorticoids levels were first described. Since then, we have learned that the increase in cortisol is

dependent on the intensity and duration of the exercise bout. Furthermore, it is well known that cortisol levels may increase by 2 independent mechanisms. One is glucose independent and associated with intense or prolonged efforts. This increase in cortisol is thought to be induced via afferent feedback from the working muscles to the central nervous system. The second mechanism is associated with a drop in plasma glucose levels that is typically seen during prolonged submaximal exercise, suggesting that the HPA axis is sensitive to alterations in glucose homeostasis. Furthermore, any increase in the concentration of serum cortisol during exercise reflects a change in the rate of secretion and the rate of removal from the blood stream.

Although the rate of secretion and removal are two important factors in the concentration of cortisol, other factors also modulate the adrenocortical response to exercise. These include novelty and other psychological factors associated with exercise, blood glucose levels, or other factors including muscle afferent activity, feeding and pre-exercise cortisol levels.

Glucocorticoid deficiency results in an increased insulin-sensitivity, whereas glucocorticoid excess provokes insulin-resistance. In healthy subjects glucocorticoids have been reported to increase, have no effect, or decrease hepatic glucose production. Importantly, the effects on hepatic glucose production may depend on the type, quantity, and duration of steroid treatment; and the design of the study. Additionally, glucocorticoids alter insulin secretion and/or insulin clearance, which by itself, may alter hepatic glucose production, lipid, and amino acid metabolism. It is now well known that at rest cortisol plays an important role in the defense against prolonged insulin-induced hypoglycemia. Thus, it is plausible that during prolonged exercise the role of cortisol may become more important. In addition, glucocorticoids increase the physical working capacity of the chronically cortisol-deficient patient.

Animal studies indicate that during exercise glucocorticoids: (1) are likely to exert effects within the time frame of a prolonged exercise bout and suggest that the rise of plasma cortisol during exercise may be of metabolic significance, especially in the trained animal; (2) increase substrate availability, by stimulating gluconeogenesis, lipolysis and glycogenesis; (3) play a pivotal role in alanine availability and metabolism; (4) modestly increase endurance time to exhaustion, suggesting an ergogenic effect; (5) operate via gene transcription-protein synthesis; and (6) appear to mediate endurance via the glucocorticoid type II but not the type I receptor activity.

Human studies in hypopituitary and ADX subjects appear to show that these patients have proper or even exaggerated metabolic responses to moderate and heavy exercise provided that glucocorticoid and mineralcorticoid replacement is adequate. However, these studies failed to provide information on substrates or counterregulatory hormones responses when the subjects were off cortisol replacement. Little is known about the acute effects of cortisol on substrate availability and utilization during exercise. A few studies have examined the effects of exogenous glucocorticoid (Viru and Smirnova, 1982) and ACTH (Soetens et al. 1995) on performance and blood substrates. However, the results are equivocal and inconclusive.

Although, there is extensive literature on the effects of exercise on cortisol level, there is a paucity of data on hormonal regulation of glucose, lipids, and amino acids during prolonged exercise (>60 min) under low-cortisol conditions. Furthermore, very little is known concerning the physiological significance of the transient increase of cortisol levels during exercise. This lack of information is probably because most of cortisol's effects are delayed when compared to the more fast acting hormones. However, this does not negate its potential significance during exercise. In humans, no studies have clamped cortisol at different concentrations during exercise. Based on animal and human studies at rest and during exercise, it is plausible to suggest that

cortisol may be playing a more active role on substrate regulation during exercise by its apparent active effects on glucose production and uptake, proteolysis, lipolysis, and overall, by gluconeogenic enzyme induction. Current understanding of the effects of cortisol during exercise are by and large based on studies performed on animals, and hypopituitary and ADX humans. The evaluation of these findings is often difficult and may lead to erroneous conclusions because of the potential differences in exercise metabolism between humans and different animal species. Additionally, the humans studies are plagued with methodological problems (i.e., multiple hormone deficiency, chronic morphological and functional differences compared to healthy subjects). We propose to investigate the metabolic effects of an acute reduction of cortisol concentration (i.e., by metyrapone suppression) on substrate availability and fuel utilization during prolonged exercise in humans.

CHAPTER III

Methods

Subjects

Nine healthy (5 males and 4 females), non-smoking college students were recruited from the University of Tennessee-Knoxville to participate in the study. The subjects were of various fitness levels and seven of the nine subjects participated in regular but not competitive exercise activities such as cycling, running, hiking, walking or weight training. In addition one was a sedentary subject and one was an amateur triathlete. All subjects were invited to ask questions about any aspect of the study that they believed was unclear. Each subject completed a health history questionnaire (see Appendix C) and was asked to read and sign an informed consent form (see Appendix B) which was approved by the University of Tennessee's Institutional Review Board. The subjects were not taking any medication (including oral contraceptives), or reported suffering from any metabolic disorder (i.e., diabetes, thyroid gland abnormalities, etc.). Additionally, the subjects were asked to refrain from exercise, alcohol, and caffeine 24 h prior to reporting to the laboratory. The subjects were reminded to maintain consistent pre-exercise diets during the course of the experiment. The menstrual phase shows no consistent effect on substrate oxidation (Friedman and Kinderman, 1989; Kanaley et al., 1992a) and cortisol levels (Kanaley et al., 1992b; De Sousa et al., 1991) during prolonged exercise and therefore was not controlled. Each subject reported to the laboratory on 3 separate days, completing baseline measurements and a graded exercise test (GXT) on the first visit, and a submaximal exercise test on the second and third visits.

Baseline Measurements And GXT

On the first visit each subject arrived to the laboratory at approximately 0730 h, and height and weight were measured and recorded. The subject was then taken to a quiet room and seated in recumbent position, the right hand was heated with an electrically heated pad kept at 65 to 70 °C. A 21-gauge teflon catheter was placed in a retrograde fashion into a right hand dorsal vein to obtain arterialized-venous blood (MacGuire et al., 1974). Each subject remained seated for the collection of a 2-ml blood sample at 30-min intervals from 0800 to 1000 h. The IV-line was kept patent with a continuous drip of 154 nmol/L NaCl solution. Prior to the blood sample collection a 2-ml waste sample was discarded. The blood samples were used to monitor the cortisol circadian rhythm for statistical comparison between resting and exercise conditions. It has been shown that cortisol comparisons should not be made relative to the "pre-exercise" resting value, but rather to samples collected at the same time of the day on a separate day (Brandenberger and Follenius, 1975; Thuma et al., 1995).

Following the collection of the last resting blood sample the subject sat on a Monark 817E pendulum-style cycle ergometer (Varberg, Sweden) and was fitted with a 2-way breathing valve (Hans-Rudolph, Kansas City, MO), a mouthpiece, and a noseclip for the measurement of oxygen uptake (VO_2) and respiratory exchange ratio (RER). After each subject cycled at a low intensity for ~ 2 min to become familiar with the cycle ergometer, a graded exercise test was conducted to determine the subject's 2.5 mmol/L lactate threshold and peak oxygen consumption ($\text{VO}_{2\text{peak}}$). The expired gas was analyzed continuously by previously calibrated O_2 (Ametek oxygen analyzer) and CO_2 (LB-2 Beckman, model S-3A/1) analyzers. Before the test, the analyzers were calibrated with gases of known O_2 and CO_2 concentrations. An Apple II+ computer, using Rayfield REP-200C software, was interfaced with the gas analyzers and the dry gas

meter to calculate VO_2 and carbon dioxide (VCO_2) production, ventilation, respiratory rate and RER. The VO_2 and RER were measured continuously and displayed on the computer monitor.

Each subject pedaled the cycle ergometer for successive 3-min stages starting at a work rate of 60 to 105 watts depending on the subject's fitness level (as assessed by the medical history questionnaire) with subsequent 15 to 17.5 watt increments every 3 min. At the end of each stage, a 2-ml blood sample was collected from the previously inserted catheter for immediate lactate analysis. When two subsequent stages generated blood lactate levels $> 2.5 \text{ mmol/L}$, the power output was increased by 30 to 35 watts per one-minute stage until volitional fatigue. The experimental work rate was set at that power output at which the concentration of blood lactate was $\sim 2.5 \text{ mmol/L}$. After completion of the first test, the seat height of the cycle ergometer was recorded and kept constant for the two submaximal exercise sessions. The catheter was removed and the subject was fed approximately 80 gm of carbohydrate in the form of fruit juice and fruit bar, and was monitored for 15 min before being discharged from the Applied Physiology Laboratory.

Submaximal Exercise Test

Approximately one week after the first visit, each subject performed, in random order, one of two submaximal rides (metyrapone or placebo). Both exercise bouts required the subject to pedal at the power output associated with 2.5 mmol/L . In order to examine the effects of low cortisol levels on exercise performance, metyrapone or placebo was randomly self-administered (orally) by each subject at midnight. Each subject was given a dose equivalent to $30 \text{ mg} \cdot \text{kg}^{-1}$ to the nearest 250 mg and advised to take it with a glass of milk or yogurt at midnight immediately prior to sleep (metyrapone

was supplied by 2 physicians, see the acknowledgment section). Metirapone causes few side effects in healthy individuals and can be taken without hospitalization. If hypoglycemia developed while in the laboratory (blood glucose < 2.9 mmol/L), the test was to be terminated and blood glucose was to be returned to normal by means of a 50 ml bolus of 50% dextrose in water. However, no subject developed hypoglycemia at rest, during or immediately following exercise. Additionally, if any clinical symptomatology indicated the presence of adrenal insufficiency (i.e., hypotension), 100 mg of hydrocortisone was to be infused, and the test would have been terminated. Moreover, we continuously monitored the subject's state of consciousness and willingness to continue the experiment. Metirapone has not been found to have intrinsic effects on substrates or other counterregulatory hormones (Atiea et al., 1990; De Feo et al., 1987 Samra et al., 1996; Schade et al., 1978). During testing a physician was present to supervise blood glucose, blood pressure and the 12-lead electrocardiogram throughout the exercise period. To allow sufficient time for washout of the drug the submaximal exercise tests were separated by at least 4 days (4 to 10 days). Each subject arrived at approximately 0730 h and weight was measured and recorded. The subject was then taken to the testing area and was seated in a recumbent position, and the right hand was heated with an electrically heated pad kept at 65 to 70 °C. A 21-gauge teflon catheter was placed in a retrograde fashion into a right hand dorsal vein to obtain arterialized-venous blood (McGuire et al., 1974) and a constant drip ($\sim 1 \text{ ml} \cdot \text{min}^{-1}$) of 154 nmol/L NaCl solution was used to keep the catheter patent. At approximately 0800 h each subject was transferred to the cycle ergometer to pedal at a power output equal to the previously determined 2.5 mmol/L lactate threshold for a period of 120 min. At the onset of exercise each subject ingested (with water) 2 capsules of placebo or metirapone (500 mg). The exercise intensity was set relative to the fixed 2.5 mmol/l lactate threshold

to reduce intersubject blood glucose kinetics differences seen at intensities relative to % $\text{VO}_{2\text{max}}$ (Coggan et al. 1992).

VO_2 was measured from the onset of the exercise to the 8th min, and for 5 min at 25, 55, 85, and 115 min of exercise. A 12-lead electrode placement was used with a Marquette ECG-MAC II/ST electrocardiograph (Marquette Electronics Inc., Milwaukee, Wisconsin) to monitor HR and ECG every 15 min. The rating of perceived exertion (RPE) was recorded at 30, 60, 90 and 120 min of exercise (Borg, 1982). Systolic and diastolic blood pressures were measured and recorded at rest and every 15 min thereafter by an automated system (Colin STBP-780, Japan). The laboratory was maintained at 21 to 26 °C, and subjects were cooled by fans blowing from the front and back throughout exercise. Additionally, subjects drank cold water ad-libitum. At the end of the exercise bout the catheter was removed and the subject was fed approximately 80 gm of carbohydrate in the form of fruit juice and fruit bar. Additionally, the subject was given a prophylactic dose of 20 mg of cortisone and monitored for 15 min before being discharged from the Applied Physiology Laboratory.

Sample Collection And Analysis

Following the collection of a 2-ml waste sample, a 10-ml blood samples were collected at rest, 30, 60, 90 and 120 min of exercise, and a 2-ml blood sample was withdrawn and immediately analyzed for blood glucose and lactate at 15, 45, 75, and 105 min of exercise. The 10-ml blood samples were separated into 3-chilled tubes: 1 ml for glucagon analysis; 3 ml for epinephrine and norepinephrine analysis; and 6 ml for glucose, glycerol, β -hydroxybutyrate, alanine, branched chain amino acids, insulin, growth hormone and cortisol analysis. All tubes were kept on ice before collection and until later centrifugation at 4 °C. Samples from a given subject were analyzed

concurrently in duplicate to reduced variability. Plasma volume shifts were ignored because the actual plasma cortisol that affect target tissues were of interest, and because subjects served as their own controls in the repeated measures design.

Immediately after each blood sample was drawn, a 25 μ l of blood sample was aspirated into the YSI analyzer system (Yellow Spring Instruments, Yellow Springs, OH) to determine whole-blood concentrations for blood glucose and lactate. The analyzers were calibrated using known standards (glucose: 3 and 5 mmol/L; lactate: 5 and 15 mmol/L) prior to the analysis of blood samples.

A 1-ml sample for plasma glucagon was transferred to a chilled vacutainer tube containing Na₂EDTA and 25 μ l of aprotinin (1,500 kallikrein inhibitor units per milliliter of blood). The samples were kept on ice and centrifuged at 7000 rpm for 10 min at 4 °C within 15 min of their collection. The plasma was then transferred to a marked test tube and stored at -20 °C. Plasma glucagon was analyzed in duplicate by a commercially available radioimmunoassay kit obtained from ICN Biomedicals (Costa Mesa, CA).

A 3-ml of the blood sample was mixed with 60 μ l of an Ethylene Glycol-Tetraacetic Acid (EGTA) - Glutathione solution to yield a final concentration of 1.8 mg EGTA and 1.2 mg glutathione per milliliter of blood. The samples were kept on ice and centrifuged within 15 min of collection at 7000 rpm for 10 min at 4 °C. Then the plasma was transferred into a marked test tube which were frozen at -20 °C until analysis for plasma catecholamines. The plasma catecholamines were determined in duplicate by the assay of Passon and Peuler (1973), with internal 0.5458 nmol/L epinephrine and 0.5911 nmol/L norepinephrine standards. The catechol-o-methyl transferase was obtained from rat livers by the purification procedure of Axelrod and Tomchick (1958).

Six ml of the blood sample was transferred to a chilled vacutainer tube containing Na₂-EDTA and kept on ice until the end of the experiment. The blood was centrifuged at 3000 rpm for 20 min at 4 °C to obtain plasma. Plasma was aliquoted for

analysis of insulin, growth hormone and cortisol, all of which were determined in duplicate by commercially available radioimmunoassay kits obtained from ICN Biomedicals, Inc. (Costa Mesa CA). The Corti-Cote kit used to measure cortisol has only a 2.2% cross-reactivity with 11-deoxycortisol (known to be elevated during cortisol blockade).

A 10- μ l plasma sample was used to determine glycerol by a spectrophotometric method (Sigma procedure 337, St Louis, MO). For the measurement of alanine and β -hydroxybutyrate 0.5 ml of plasma was deproteinized with 0.5 ml of 1 M perchloric acid, centrifuged, neutralized with KOH, and aliquoted in microcentrifuge tubes. β -hydroxybutyrate (Williamson and Mellanby, 1974) and alanine (Lowry and Passonneau, 1973) were analyzed by enzymatic fluorometric methods. To measure total branched chain amino acids 0.1 ml of plasma was deproteinized in 1 ml of 0.3 M perchloric acid, centrifuged and stored in microcentrifuge tubes. Branched chain amino acids were determined by a referenced enzymatic fluorometric method (Glesson and Maughan, 1987).

Statistical Analysis

The data were analyzed as a two within -subjects repeated-measures analysis of variance (treatment x time). SAS[®] general linear model procedures were employed. Specific contrasts comparing conditions at different times and comparing responses from one time to another within or average across condition were tested for significance. All data are presented as means $\pm$ SE. Significance was accepted at $P < 0.05$.

CHAPTER IV

Results

This chapter presents a summary of the results and the statistical analyses of the data. First, data on the subjects' physical characteristics and $\text{VO}_{2\text{max}}$ -LT test will be displayed. Next the cardiovascular, perceptual, and metabolic responses during the two submaximal exercise tests will be presented, with emphasis given to any differences that were detected between treatments. Finally, the effect of exercise and metyrapone treatment on the hormonal profile will be described.

Subjects' characteristics and the lactate threshold test. The characteristics of the nine subjects who participated in this study are presented in Table 2, and the means and SE for the variables measured during the $\text{VO}_{2\text{max}}$ -LT test are listed in Table 3. The subjects' $\text{VO}_{2\text{max}}$ values were typical of untrained individuals; in addition, the percent of $\text{VO}_{2\text{max}}$ at the lactate threshold ($57 \pm 2\%$) was also as expected since the subjects were not trained cyclist. The achievement of $>95\%$ of the age-predicted maximal heart rate coupled with a high RER (1.24 ± 0.02) suggests that the subjects gave a maximal effort.

Metyrapone treatment. In general, the overnight metyrapone dose was well tolerated and no serious side effects were reported by the subjects who completed the study. One of the 9 subjects reported feeling dizziness and confusion following the overnight dose, whereas another subject reported stomach discomfort and a burning sensation. However, the former subject later reported failing to ingest metyrapone as instructed (i.e., with milk/yogurt). These side effects started soon after metyrapone ingestion and lasted ~15 min. Additionally, we documented a hypotensive reaction which is reported in appendix A.

Table 2. Subjects' characteristics

Age (yr)	26.6 ± 1.1
Height (m)	1.72 ± 0.02
Weight (kg)	73.6 ± 4.3
Resting heart rate (b·min ⁻¹)	63 ± 4
Resting systolic blood pressure (mmHg)	113 ± 4
Resting diastolic blood pressure (mmHg)	65 ± 4

Table 3. Peak values during the VO_{2max}-LT test.

Percent VO _{2peak} at 2.5 mmol/L	57 ± 2
Absolute peak oxygen uptake (L·min ⁻¹)	2.99 ± 0.10
Relative peak oxygen uptake (ml·kg ⁻¹ ·min ⁻¹)	40.6 ± 1.3
Peak heart rate (b·min ⁻¹)	187 ± 2
Peak respiratory exchange ratio	1.24 ± 0.02

The additional metyrapone dose that subjects ingested at the onset of exercise caused no serious side effects. Nevertheless, two of the subjects reported mild stomach discomfort that subsided within 15 min of metyrapone ingestion. Importantly, this discomfort could have been due to the use of water to ingest the drug at the onset of exercise (rather than milk). We conclude that despite mild side effects, this drug can be used safely in exercise studies when appropriate precautions are followed.

Plasma cortisol levels : At rest (0800 h) cortisol concentrations averaged 512 ± 42 and 493 ± 44 nmol/L ($P=0.84$) on normocortisolinemia and control days, respectively; metyrapone lowered the resting cortisol concentration by 70% to 145 ± 31 nmol/L ($P<0.001$). Figure 8 shows that during the control day plasma cortisol fell rapidly ($P<0.001$) from 0800 to 0930 h (0800 to 0830 [$P<0.01$]; 0830 to 0900 [$P<0.01$]; 0900 to 0930 [$P<0.05$] and leveled off from 0930 to 1000 h ($P=0.43$). During exercise under normocortisolinemia, plasma cortisol increased 20% to 617 ± 64 nmol/L by the 120th min compared to pre-exercise values ($P<0.001$). However, when the cortisol response was compared to the same time point during the control day a 76% increase was detected at 90 min ($P<0.01$) and was further increased to 123% at 120 min ($P<0.001$). During exercise under hypocortisolinemia, cortisol remained unchanged and was significantly lower than on the rest day and the normocortisolinemic trial, providing an adequate model for the study.

%VO_{2max}, heart rate, perceived exertion and blood pressure : During both exercise trials the subjects exercised at a similar ($P= 0.91$) intensity of $\sim 60\%$ %VO_{2max} (Fig. 9), and showed similar heart rate ($P=0.82$) (Fig. 10), and perceived exertion ($P=0.82$) responses (Fig. 11). Systolic blood pressure tended to be lower during hypocortisolinemia compared to normocortisolinemia ($P= 0.11$), whereas diastolic blood pressure was similar in both trials ($P=0.98$) (Fig. 12).

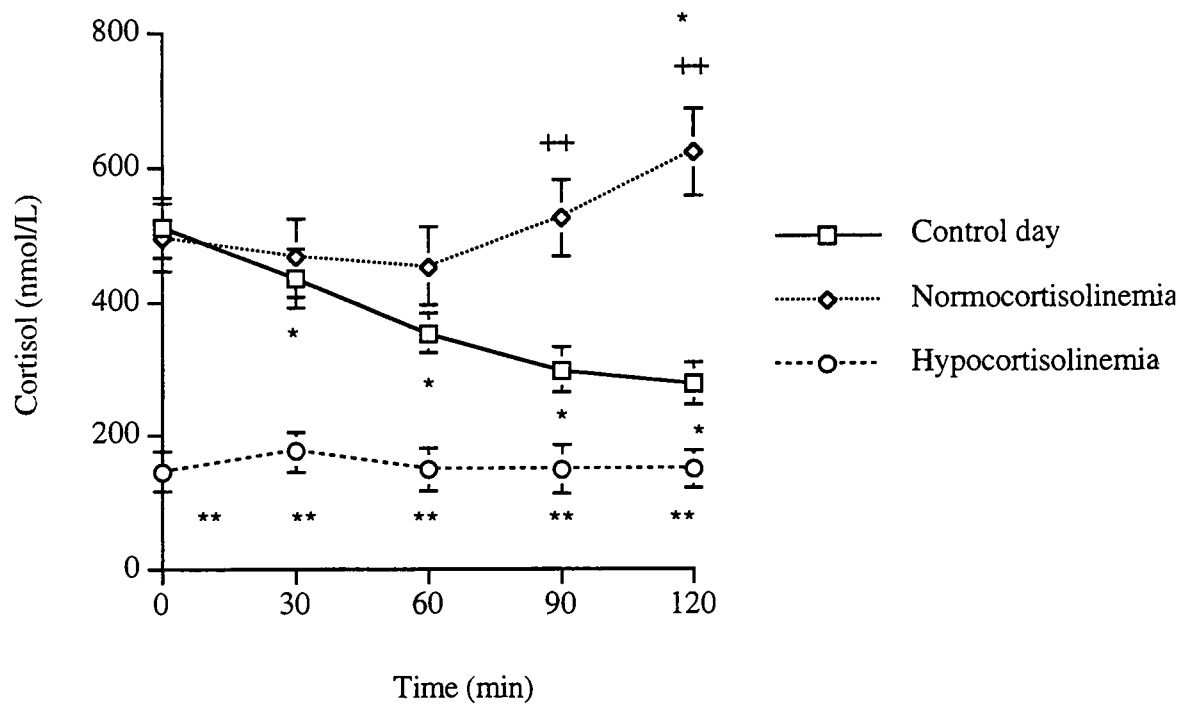


Figure 8. Plasma cortisol before and during 120 min of exercise, and during 120 min of rest on a control day. * Denotes significance compared to pre-exercise. ++ Denotes significantly higher compared to same time points during control day. ** Significantly lower compared to the same time points during normocortisolinaemia and control day

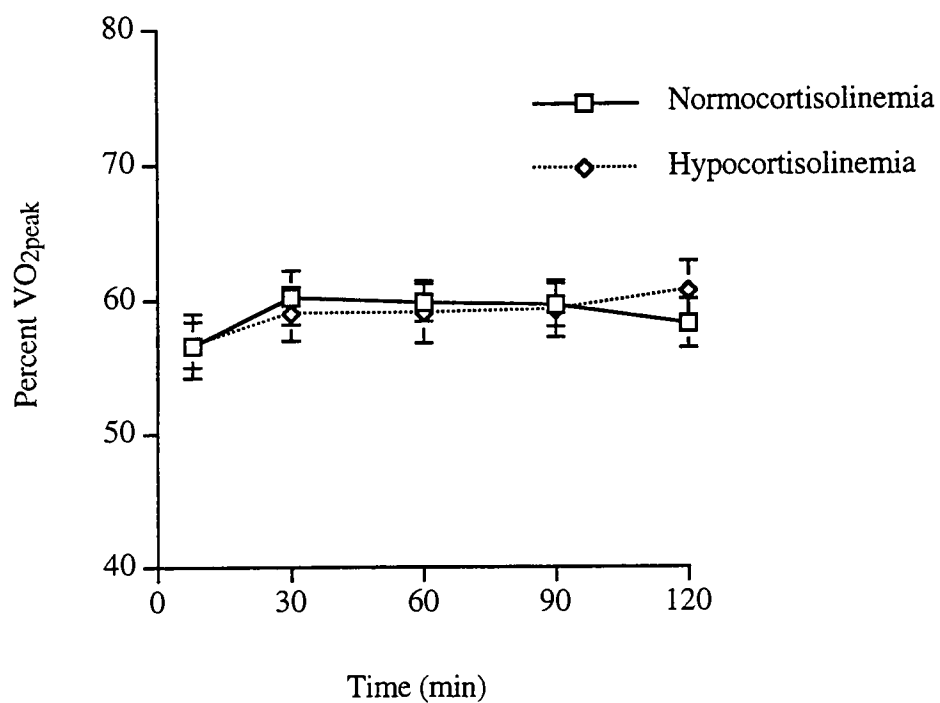


Figure 9. Percent VO_{2peak} during 120 min of exercise.

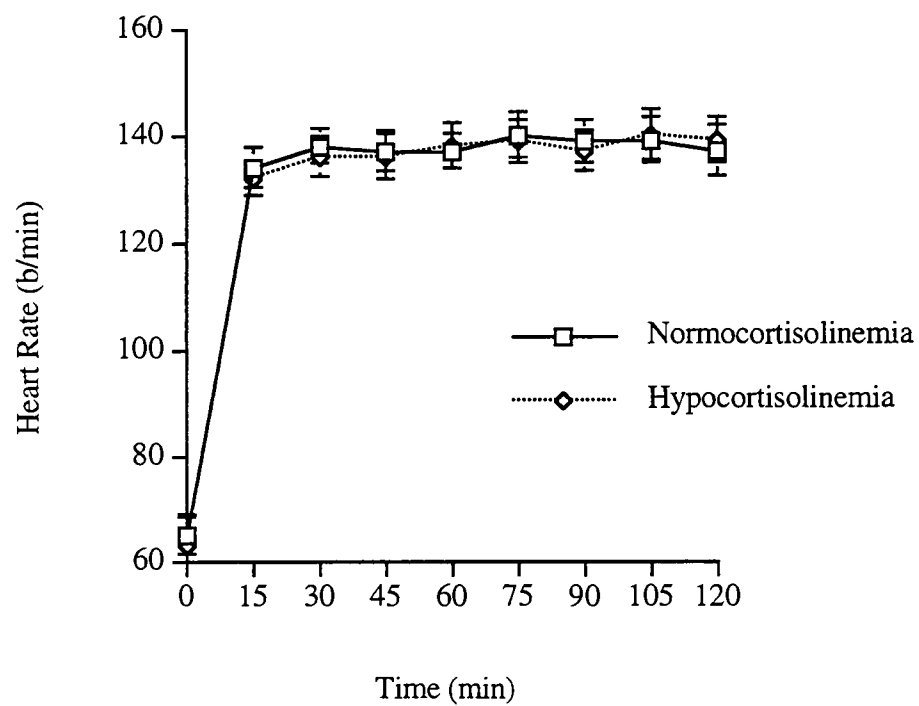


Figure 10. Heart rate at rest and during 120 min of exercise.

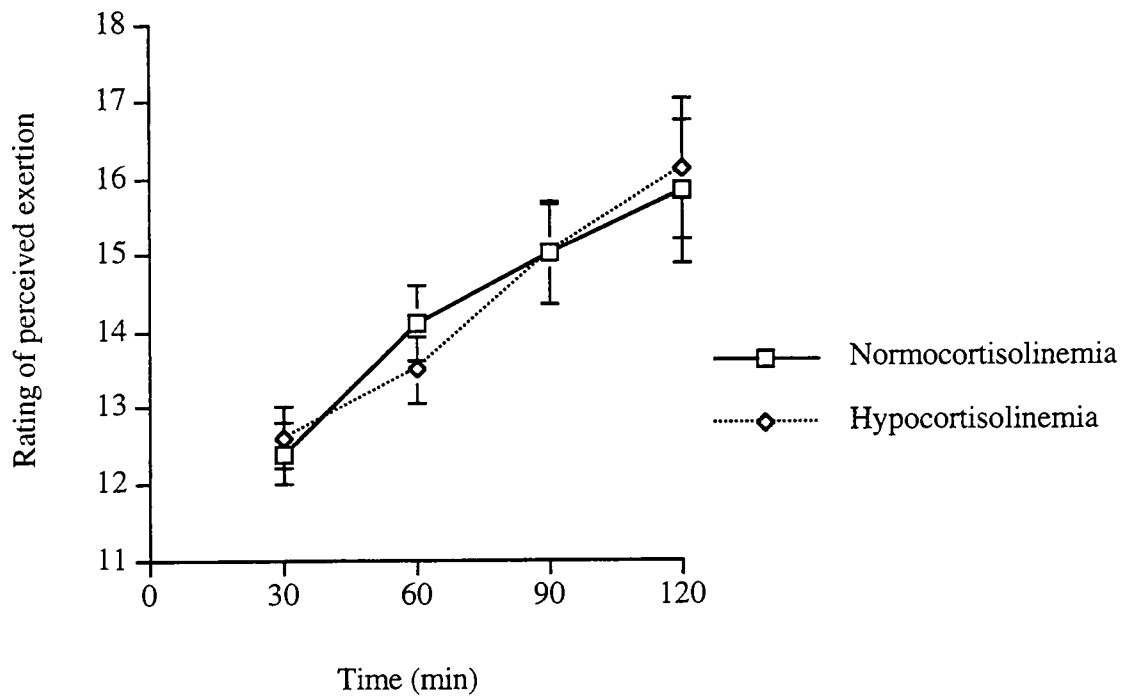


Figure 11. Rating of perceived exertion during 120 min of exercise

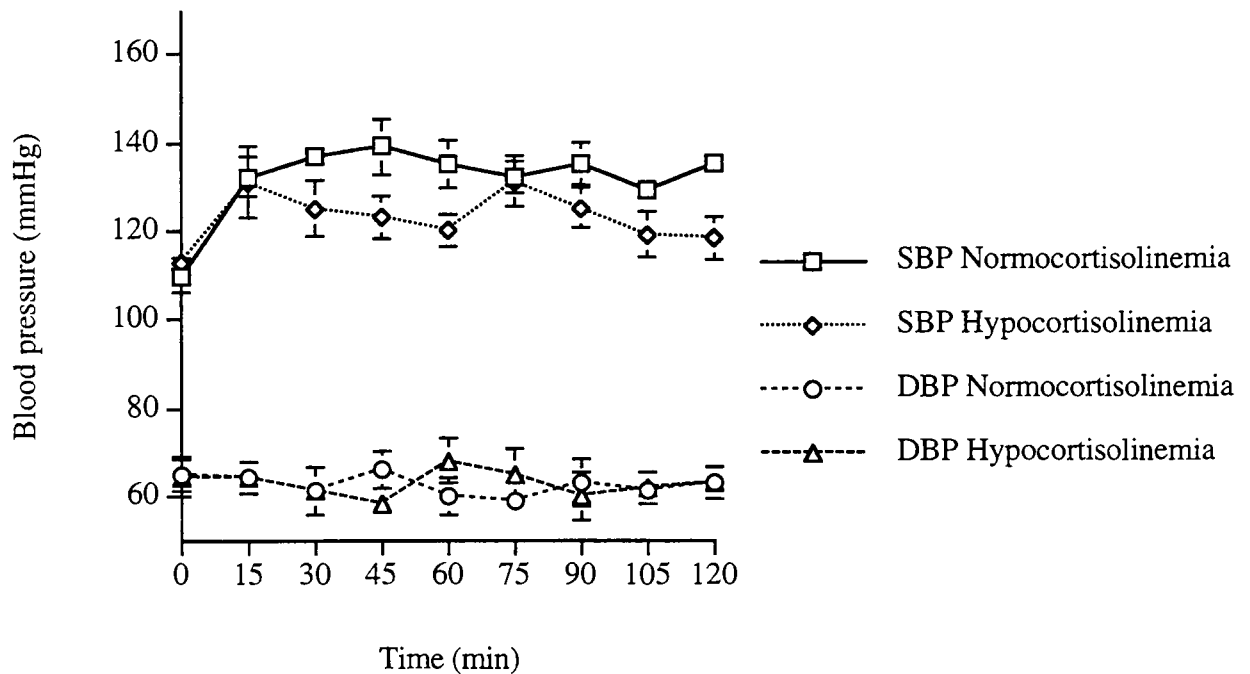


Figure 12. Blood pressure before and during 120 min of exercise. SBP: systolic blood pressure; DBP: diastolic blood pressure.

Plasma glucose and blood lactate: Pre-exercise plasma glucose was similar under normocortisolinemia (4.96 ± 0.18 mmol/L) and hypocortisolinemia (4.71 ± 0.15 mmol/L), respectively. Exercise led to a small but significant decrease in plasma glucose during both trials (Fig. 13). The decrease was first detected by the 15th min ($P < 0.01$) and reached the lowest point at 120 min ($P < 0.001$). During hypocortisolinemia plasma glucose appeared to be lower throughout the exercise period compared to normocortisolinemia, however the difference was not significant ($P = 0.18$). Pre-exercise blood lactate was similar under normocortisolinemia (0.84 ± 0.05 mmol/L) and hypocortisolinemia (0.92 ± 0.04 mmol/L), respectively ($P = 0.77$). During exercise blood lactates (Fig. 14) rose to ~ 2.5 mmol/L at 15 min under both conditions, but by the 120th min it had declined to nearly resting values. Lactate tended to be slightly elevated under hypocortisolinemic compared to normocortisolinemic conditions ($P = 0.13$).

Plasma glycerol and β -hydroxybutyrate : Pre-exercise glycerol (0.078 ± 0.014 mmol/L and 0.090 ± 0.012 mmol/L), and β -hydroxybutyrate (0.103 ± 0.012 mmol/L and 0.095 ± 0.010 mmol/L) were similar under normocortisolinemia and hypocortisolinemia, respectively. During exercise glycerol (Fig. 15) and β -hydroxybutyrate (Fig. 16) increased in both trials but showed a different rate of change for the two conditions ($P < 0.001$ and $P < 0.001$, respectively). Under hypocortisolinemia glycerol and β -hydroxybutyrate increase more slowly over time.

Plasma alanine and branched chain amino acids: Figure 17 shows that pre-exercise plasma alanine was similar ($P = 0.22$) under both experimental conditions (0.217 ± 0.009 mmol/L and 0.176 ± 0.012 mmol/L). During the first 60 min of exercise alanine increased in both trials ($P < 0.001$), but by the 120 min it had declined to nearly resting levels.

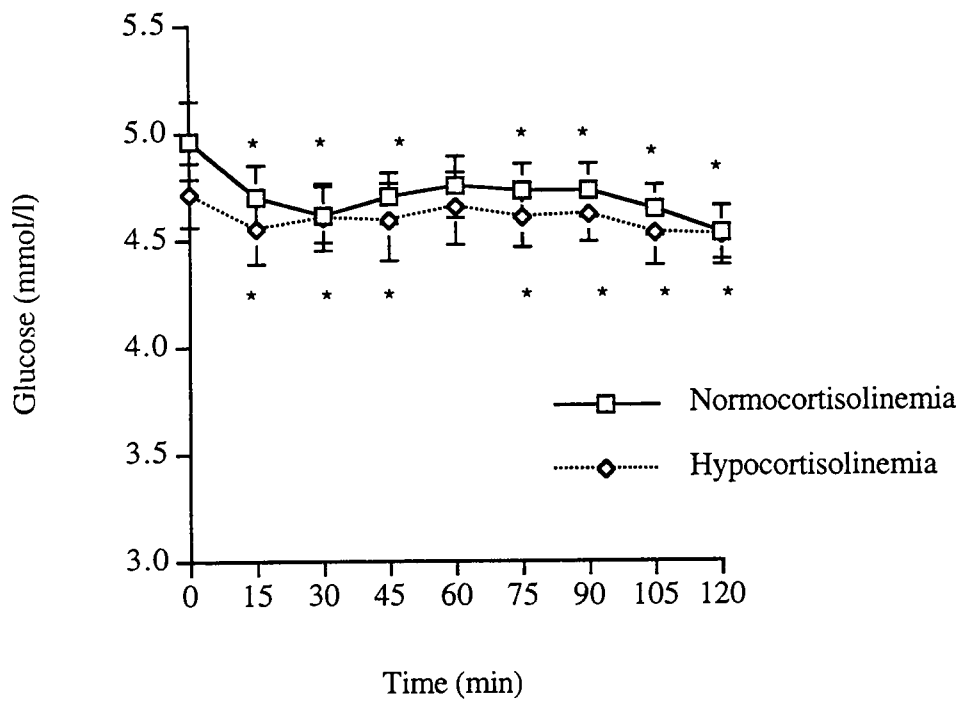


Figure 13. Plasma glucose before and during 120 min of exercise. * Denotes significant difference compared to pre-exercise ($P < 0.05$).

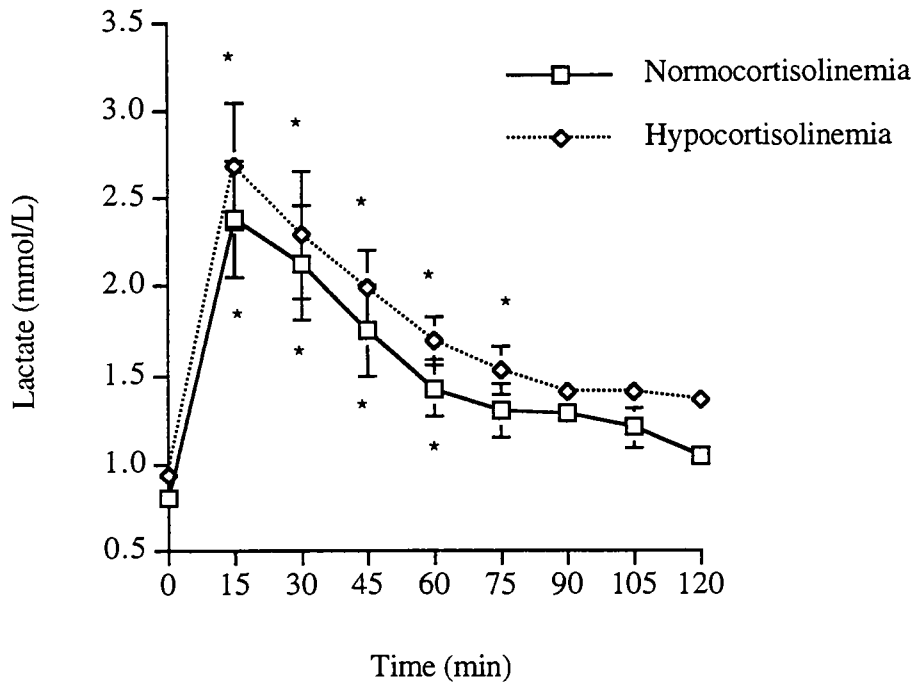


Figure 14. Blood lactate before and during 120 min of exercise. * Denotes significant difference compared to pre-exercise ($P < 0.05$).

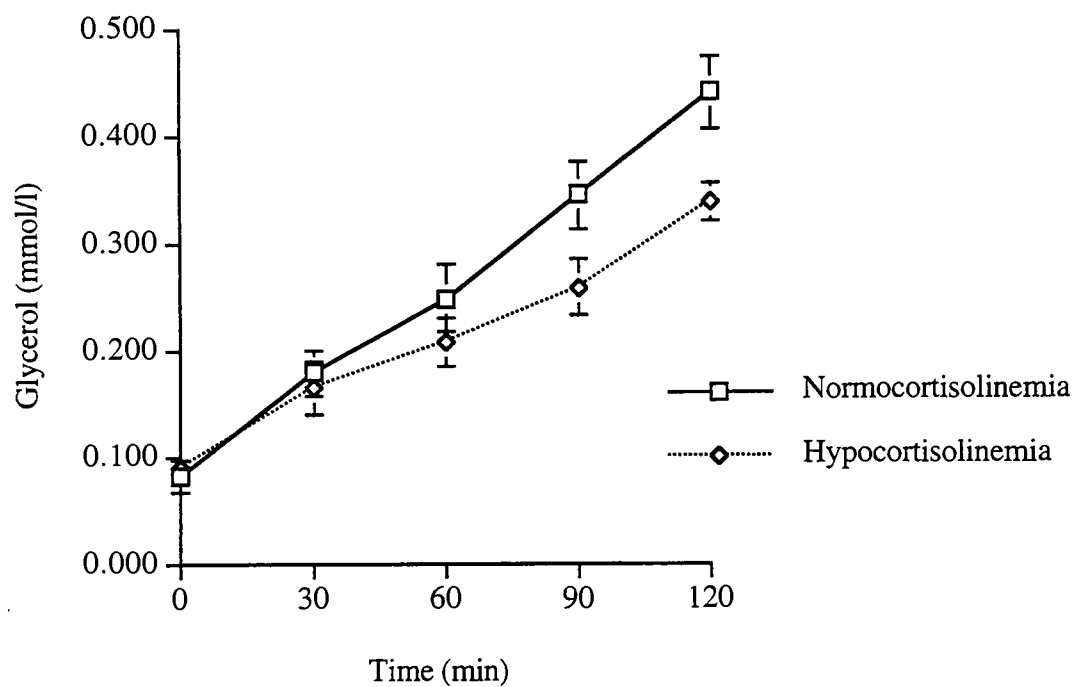


Figure 15. Plasma glycerol before and during 120 min of exercise. There was a significant difference in the rate of change between conditions ($P=0.0002$).

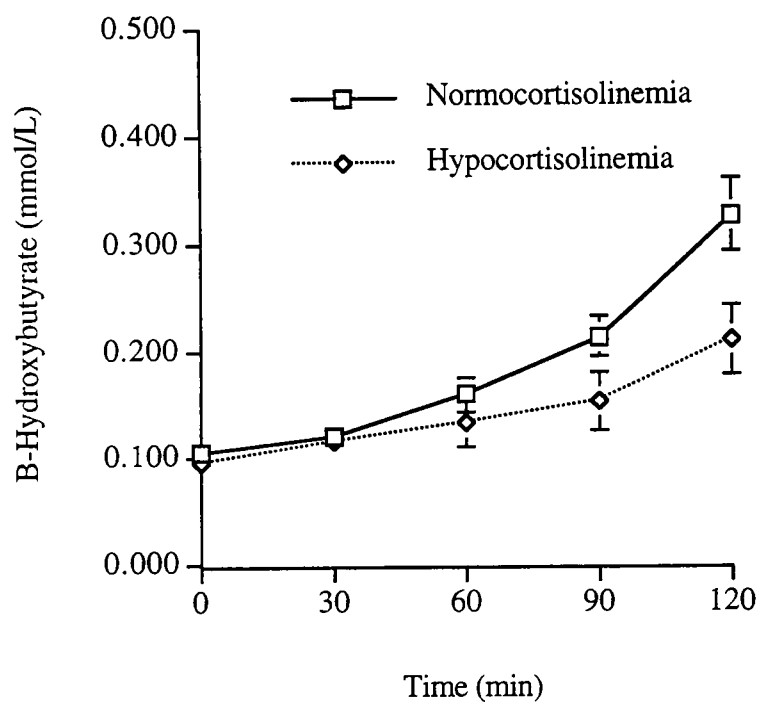


Figure 16. Plasma beta-hydroxybutyrate before and during 120 min of exercise. There was a significant difference in the rate of change between conditions ($P= 0.0002$).

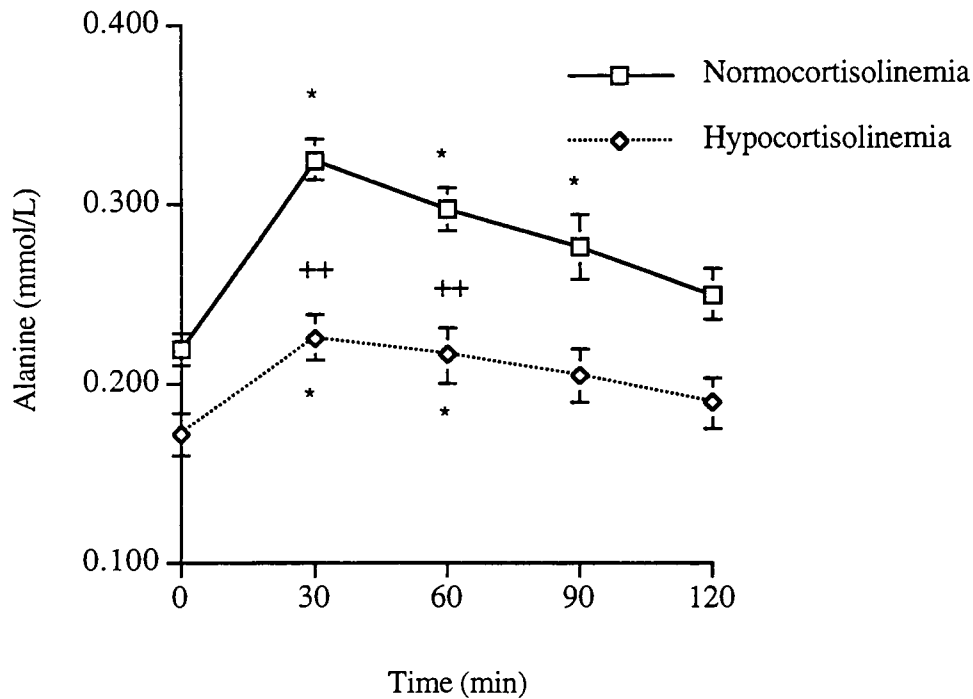


Figure 17. Plasma alanine before and during 120 min of exercise. * Denotes significantly different than pre-exercise ($P < 0.05$). ++ Denotes significantly different between conditions ($P < 0.05$).

However, the initial increase was attenuated at 30 min ($P < 0.05$), and 60 min ($P < 0.05$) under hypocortisolinaemia compared to normocortisolinaemia. Prior to exercise and at 30 min of exercise branched chain amino acids (Fig. 18) tended to be lower under hypocortisolinaemia compared to normocortisolinaemia ($P = 0.07$ and $P = 0.06$, respectively). The difference between treatments became significant at 60 min and thereafter ($P < 0.05$). Furthermore, during exercise under normocortisolinaemia branched chain amino acids increase at 90 ($P < 0.01$) and 120 min ($P < 0.0001$), whereas under hypocortisolinaemia a decrease in branched chain amino acids was detected by 120 min compared to pre-exercise ($P < 0.01$).

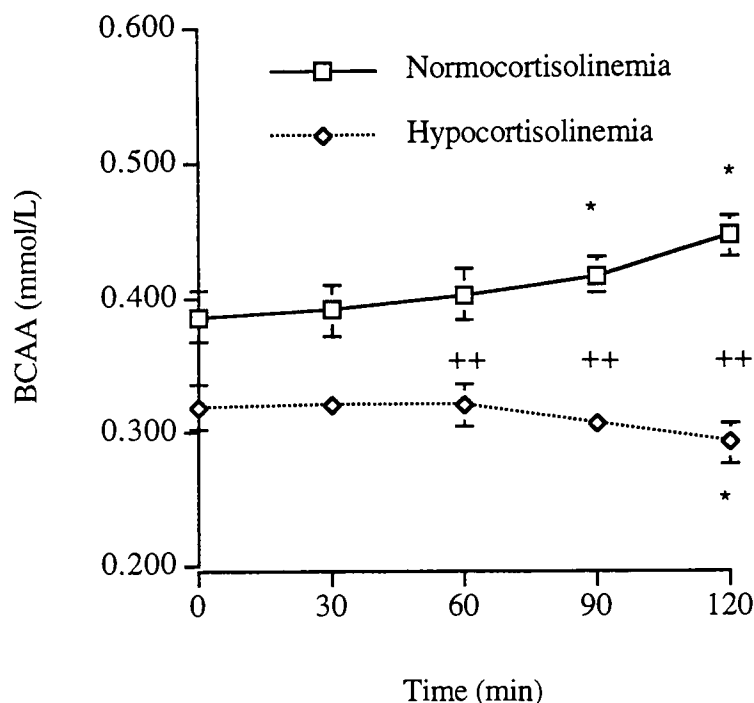


Figure 18. Plasma branched chain amino acids (BCAA) before and during 120 min of exercise. * Denotes significance from pre-exercise during normocortisolinemia ($P < 0.001$) and hypocortisolinemia ($P < 0.01$). ++ Denotes significance between conditions ($0.001 < P < 0.05$).

Plasma insulin, glucagon and growth hormone : Pre-exercise plasma insulin (86.1 ± 10.6 and 78.6 ± 11.4 pmol/L), glucagon (41.4 ± 5.5 pmol/L and 43.3 ± 5.3 pmol/L), and growth hormone (3.04 ± 0.58 μ g/L and 2.25 ± 0.61 μ g/L) were not different between treatments. Plasma insulin (Fig. 19) declined over time under both conditions, however insulin was reduced to a greater extent under hypocortisolinemia compared to normocortisolinemia ($P = 0.0002$). This effect became significant at the 30th min ($P = 0.05$) and continued until the 120th min. Plasma glucagon (Fig. 20) rose ($P < 0.01$) over time to a similar extent under both conditions, peaking at 57.8 ± 7.6 pmol/L and 58.1 ± 8.4 pmol/L for normocortisolinemia and hypocortisolinemia, respectively. However, the increase became significant earlier during normocortisolinemia (90 min) than hypocortisolinemia

(120 min). Growth hormone (Fig. 21) increased over time under both conditions ($P < 0.0001$). However, the increase was detected earlier under hypocortisolemia (30 min) compared to normocortisolemia (60 min). Furthermore, the growth hormone response was characterized by two distinct peaks, compared to the gradual increase seen during normocortisolemia. The growth hormone response tended to be higher during hypocortisolemia at 30 min ($P = 0.0559$) and at 120 min ($P = 0.067$).

Plasma epinephrine and norepinephrine: Pre-exercise plasma epinephrine and norepinephrine were similar during normocortisolemia (0.407 ± 0.031 pmol/L and 2.42 ± 0.23 pmol/L) and hypocortisolemia (0.436 ± 0.039 pmol/L and 2.44 ± 0.29 pmol/L) ($P = 0.93$ and $P = 0.97$), respectively. During exercise epinephrine (Fig. 22) rose over time in both trials ($P < 0.0001$), and tended to be higher ($P = 0.07$) during hypocortisolemia. Similarly, plasma norepinephrine (Fig. 23) rose over time ($P < 0.0001$) in both trials, and showed a tendency ($p < 0.10$) to be higher during hypocortisolemia.

RER: The RER (Fig. 24) decreased ($P < 0.0001$) over time, indicating an increase in lipid oxidation with no difference between treatments ($P = 0.37$). The decrease was significant by the 30th min of exercise ($P < 0.0001$)

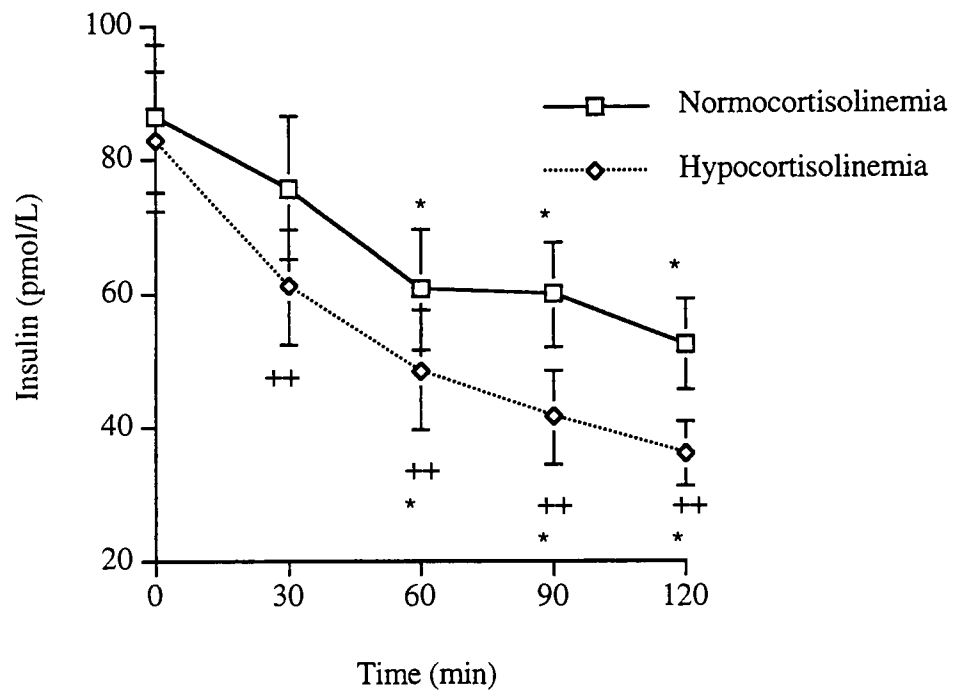


Figure 19. Plasma insulin before and during 120 min of exercise.
 *Denotes significant difference from pre-exercise. ++ Denotes significant difference between conditions ($P < 0.05$).

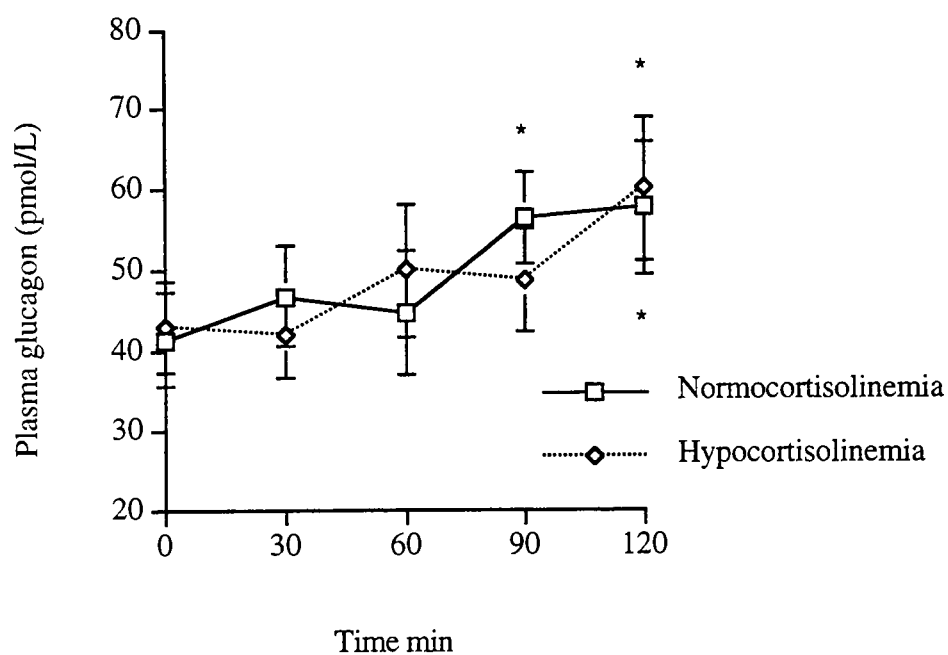


Figure 20. Plasma glucagon before and during 120 min of exercise. * Denotes significance from pre-exercise ($P < 0.01$).

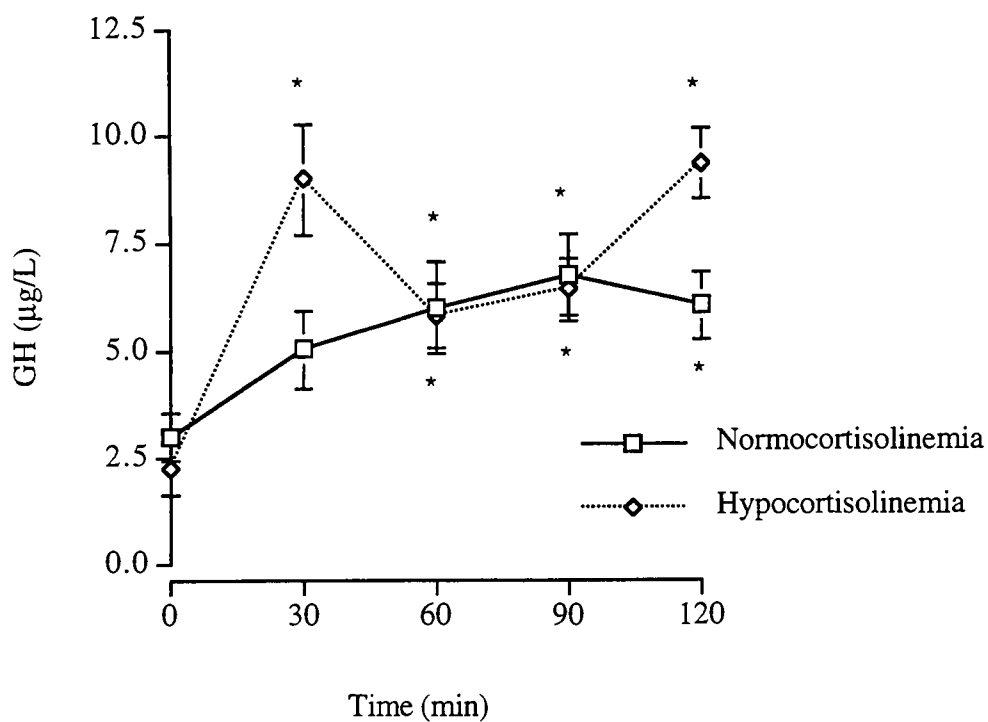


Figure 21. Plasma GH before and during 120 min of exercise. * Denotes significance compared to pre-exercise ($0.02 < P < 0.004$ and $0.005 < P < 0.0001$ during normocortisolinaemia and hypocortisolinaemia, respectively). The treatment effect approached significance at 30 and 120 min ($0.05 < P < 0.10$)

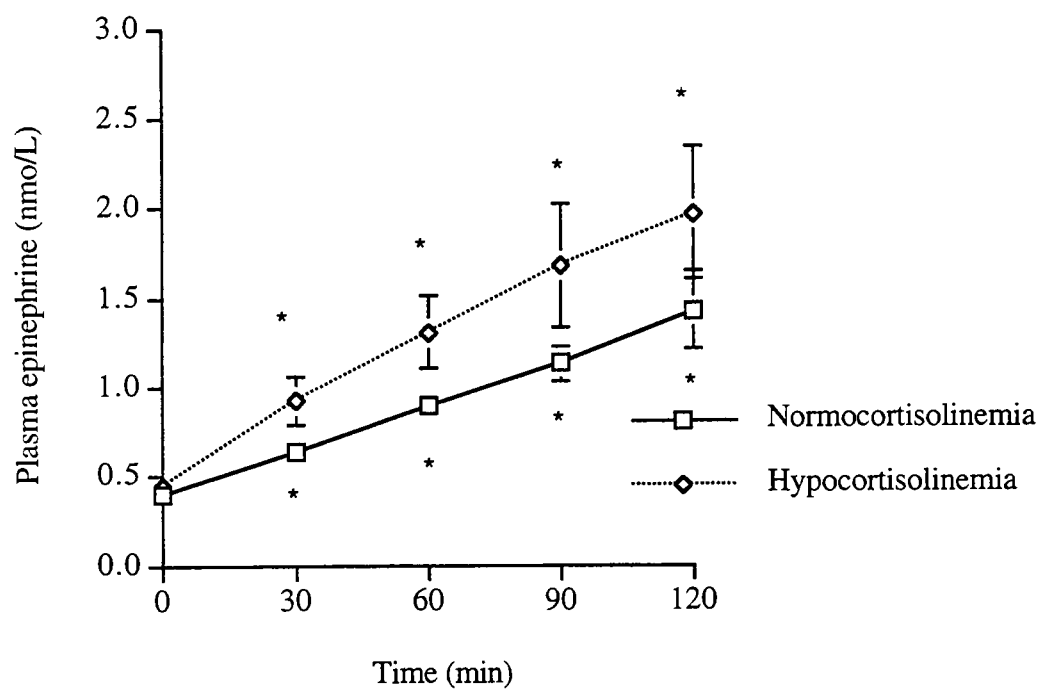


Figure 22. Plasma epinephrine before and during 120 min of exercise.
 *Denotes significant difference from pre-exercise ($0.03 < p < 0.0001$). The treatment effect approached significance ($0.05 < P < 0.10$)

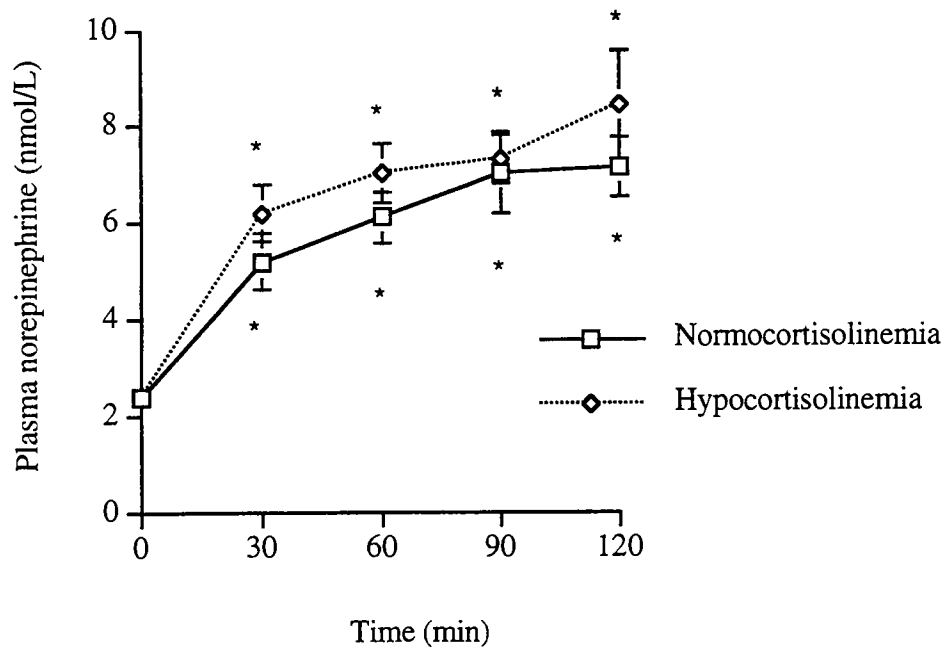


Figure 23. Plasma norepinephrine before and during 120 min of exercise.
 *Denotes significant difference from pre-exercise ($P < 0.0001$). The treatment effect approached significance ($0.05 < P < 0.10$)

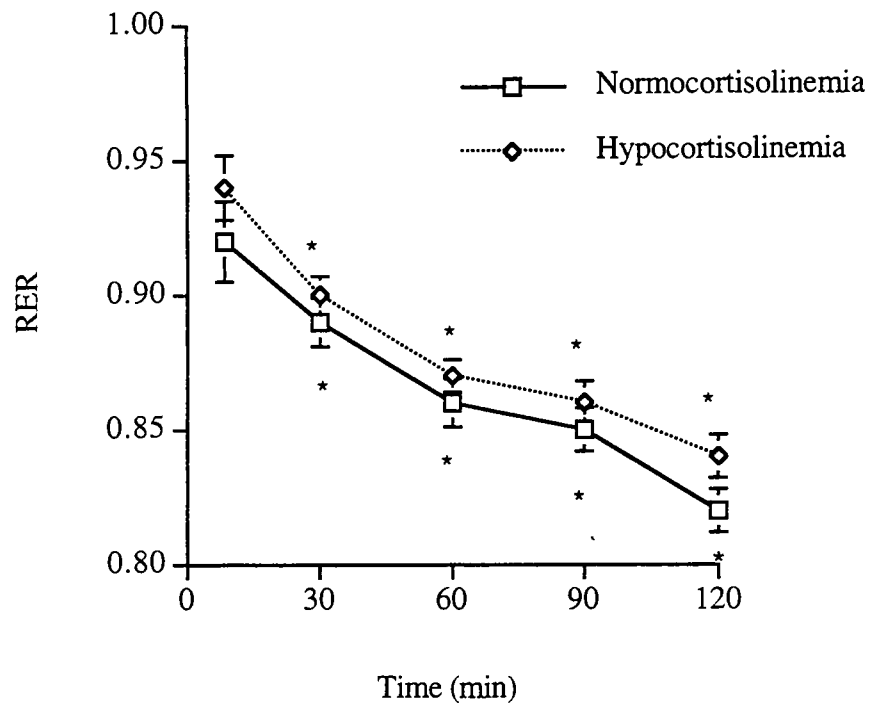


Figure 24. Respiratory exchange ratio during 120 min of exercise. Denotes a significant decrease compared to the 8th min of exercise ($P < 0.0001$) with no difference between conditions.

CHAPTER V

Discussion

This chapter will discuss the findings of the study and relate them to the literature. First, the objective will be reiterated, and will be followed by the effects of the treatment on plasma cortisol. Second, the effect of the treatment on performance, cardiovascular and perceptual responses will be discussed. Third and foremost, the chapter will cover the effects of the treatment on plasma glucose homeostasis, lipolysis ketogenesis, and proteolysis. Fourth, the effect of the treatment on carbohydrate and lipid oxidation will be described. Finally, a possible gender effect will be discussed and the conclusions of the study will be stated.

The objective of this study was to evaluate the effect of different cortisol levels on: (1) the concentration of plasma substrates; (2) whole-body substrate utilization; (3) substrate-regulating hormones; and (4) selected cardiovascular responses during prolonged exercise. To accomplish this objective, we produced an acute pharmacological-adrenalectomy by administering metyrapone (i.e., an agent that suppresses cortisol production) and compared that treatment to a control condition in which the subjects received a placebo (allowing the normal morning cortisol peak).

Metyrapone has not been found to have intrinsic effects on substrates and counterregulatory hormones, and has been extensively used in metabolic studies with (De Feo et al., 1989; Dennen et al., 1993; Dennen et al., 1995; Samra et al., 1996) and without (Atiea et al., 1990; Samra et al., 1996; Schade et al., 1978) peripheral cortisol replacement. The results of the present study showed that metyrapone suppressed cortisol by ~ 70% during exercise ranging from ~ 50% to 70% $\text{VO}_{2\text{max}}$. When compared to pre-exercise values, cortisol levels increased by ~ 20% at the 120th min of exercise under

normocortisolism. However, when the exercise responses were compared relative to those measured during the control day, the increase was detected earlier (i.e., 90 min of exercise) and was of greater magnitude (80%). Moreover, the magnitude of difference noted at the 120th min reached 120%. These results confirm and extend the findings of others (Brandenberger et al., 1982; Thuma et al., 1995) who have recommended that exercise-induced cortisol responses be compared to values measured at the same time points of a rest day, rather than to a pre-exercise value.

In both trials the subjects exercised at similar heart rates and $\%VO_{2max}$. Furthermore, the perception of effort was similar during both experimental conditions. These similarities allowed us to make appropriate comparisons between trials. In addition, these results suggest that an acute reduction in the plasma cortisol level has little bearing on tolerance to prolonged exercise of moderate intensity. On the other hand, it is known that chronic adrenocortical insufficiency limits work capacity and tolerance to prolonged exercise in animals (Tharp 1975; Viru, 1985) and in humans (Mier et al., 1988; Zelinsky et al., 1987). These studies have suggested that glucocorticoid deficiency limits exercise tolerance by inducing cardiovascular instability, and muscle weakness and pain. To our knowledge this is the first study to demonstrate that an acute cortisol deficit does not alter the ability to complete a prolonged exercise bout, at least, in healthy men and women. Interestingly, we noticed a trend ($P=0.11$) towards lower systolic blood pressure during hypocortisolism. Coincidentally, a similar tendency has been previously reported during submaximal exercise in a group of subjects who had undergone bilateral adrenalectomy (i.e., for Cushing's disease or bilateral pheochromocytoma) and were receiving basal corticosteroids (Hoelzer et al., 1986). Consistent with this observation, others have reported significant drops in systolic blood pressure in glucocorticoid-mineralcorticoid deficient individuals during maximal and submaximal exercise which are reversed upon chronic glucocorticoid and mineralcorticoid replacement (Zelinsky et al.,

1987). Based on the values observed for blood pressure, heart rate, and rating of perceived exertion, it is suggested that an acute reduction in plasma cortisol does not alter cardiovascular and perceptual responses to prolonged exercise in humans.

The hypothalamic-pituitary-adrenal axis is activated during intense (Elias et al., 1991) and prolonged (Tabata et al., 1991) exercise and as a consequence, cortisol levels increase. To date, however, little is known about the physiological significance of cortisol during exercise in humans. Recent studies on cortisol, the chief glucocorticoid produced by humans, reveal that it plays an important counterregulatory role by augmenting glucose production, decreasing glucose utilization, and accelerating lipolysis, ketogenesis and proteolysis following an overnight fast, during the morning postprandrial period, and prolonged hypoglycemia, (De Feo et al., 1989a; Deneen et al., 1993; Denneen et al., 1995).

Blood glucose is tightly regulated during exercise and seldom does overt hypoglycemia develop. It is well known that when one or more of the glucoregulatory hormones is pharmacologically suppressed there is a compensatory response by one or more of the others. This defense of the glucoregulatory control system is based on redundant mechanisms (Cryer, 1993; Vranic, 1991). In keeping with this concept, our results suggests that blood glucose is tightly regulated during exercise independent of cortisol levels. This is in contrast to previous studies in humans at rest (De Feo et al., 1989; Deenen et al., 1993; Dennen et al., 1995; Garrel et al., 1995) which have consistently demonstrated an attenuating effect of hypocortisolemia in plasma glucose, and hepatic glucose production/gluconeogenesis. The discrepancy may be explained, at least in part, by the fact that in those studies longer experimental protocols and more sophisticated designs were used (i.e., hormone clamps with fixed peripheral replacement and multiple tracers). In the present study metyrapone suppressed cortisol but allowed the other glucoregulatory hormones to change. Insulin levels were consistently lower during

the hypocortisolinemic trial, while growth hormone ($P=0.055-0.067$), epinephrine ($P=0.07$) and norepinephrine ($P=0.10$) tended to increase, thus providing reasonable evidence of a compensatory response. Particularly, the lower insulin and the higher epinephrine are well-known regulators of hepatic glucose production during exercise (Sigal et al., 1996; Wasserman, 1995). Interestingly, plasma glucagon did not differ between trials.

We believe that during prolonged exercise the glucoregulatory hormones compensated for the acute cortisol deficit. This suggests that during exercise cortisol is probably involved in maintaining glucose homeostasis because its suppression drives a modest yet rapid glucoregulatory adjustment. However, the fact that plasma glucose was similar in both exercise trials does not automatically rule out alterations in hepatic glucose production and peripheral glucose utilization. For instance, at rest moderately low cortisol levels (~ 230 nmol/L) have been associated to both a decrease in hepatic glucose production, and an increase in peripheral glucose utilization (De Feo et al., 1989a). Conversely, recent studies have demonstrated that the morning rise in cortisol increases gluconeogenesis and reduces postprandial glucose uptake whether measured isotopically or as the arteriovenous difference across the forearm (Dinneen et al., 1993; Dinneen et al., 1995). Interestingly, whether this glucose sparing effect is present during exercise is not known.

In a recent study, Tayek and Katz (1997) have demonstrated a very close correlation between plasma cortisol concentration and the Cori cycle ($r=0.963$) and gluconeogenesis ($r=0.861$). Similarly, Lecavalier et al. (1990) found that cortisol stimulates lactate-gluconeogenesis in humans. The present study did not find a treatment effect on glucose and lactate concentrations, or the RER. However, these are gross non-invasive estimates of whole-body carbohydrate metabolism during exercise. Thus, we can not rule out quantitative and qualitative alterations in hepatic glucose production, tissue glucose uptake, and muscle glycogen utilization. Finally, during the post-exercise period

elevated cortisol levels could be involved in stimulating glycogen synthesis in liver (Goldstein et al., 1993). Our findings indicate that when the glucoregulatory hormones are allowed to change glucose homeostasis is preserved independent of 3-to 4-fold differences in plasma cortisol. This is consistent with the findings of others who selectively impaired the normal response of one glucose-regulating hormone (i.e., insulin, glucagon, GH, and catecholamines) during exercise and found no consistent hypoglycemic response (Bjorkman et al., 1981; Hirsch et al., 1991; Marker et al., 1991; Wolfe et al., 1986). Thus, to better isolate the effects of cortisol on glucose homeostasis a more elaborate methodology is in order. For instance, the pituitary-adrenal-pancreatic clamp in which the release of glucoregulatory hormones is blocked and peripherally replaced at a fixed rate (De Feo et al., 1987) and the use of tracers would provide adequate models to examine the effects of cortisol on substrate kinetics during exercise.

The present study examined the effect of cortisol levels on lipid catabolism. Our findings indicate that cortisol alters lipid availability during exercise. We found that cortisol had no significant effect on baseline plasma glycerol and β -hydroxybutyrate concentrations, whereas during exercise the rate at which glycerol and β -hydroxybutyrate increased over time was attenuated under hypocortisolemia compared to normocortisolemia. However, the magnitude of the attenuating effects on lipid catabolism was modest (~ 20-35% by 120 min) and did not affect fat utilization. At rest, it is well known that lipid catabolism is affected by glucocorticoids. For instance, it has been reported that physiological hypercortisolemia (~970 nmol/L) increases palmitate turnover within 2 h of hydrocortisone infusion (Divertie et al., 1991). Similarly, Schade et al. (1978) blocked endogenous secretion of cortisol with metyrapone and cortisol was either not replaced or replaced to bring cortisol to normal and high levels, respectively. The suppression of cortisol resulted in a 60% and 25% reduction in the plasma concentrations of ketone bodies and free fatty acids, respectively. When cortisol levels were replaced to normal levels,

plasma concentrations of ketone bodies and NEFA returned to control values, whereas when cortisol was replaced to high levels hyperketonemia and elevated levels of NEFA were observed. In agreement with Divertie et al. (1991) and Schade et al. (1978), our findings suggest that during exercise there is a direct and positive relationship between plasma cortisol levels and lipid catabolism. Furthermore, studies in rodents have reported that inhibition of the rise of glucocorticoids significantly depressed plasma free fatty acids at exhaustion (Sellers et al. 1988). This study as well as ours suggests that glucocorticoids may exert their effects within the time frame of a prolonged exercise bout.

During exercise, hypocortisolemia caused alterations in plasma insulin, GH, and epinephrine concentrations. Together, the lower insulin, higher catecholamines and GH concentrations would have favored lipolysis. However, even in the presence of this hormonal milieu hypocortisolemia led to a decrease in the rate of change for glycerol and β -hydroxybutyrate. The lower response of glycerol and β -hydroxybutyrate presumably reflects decreased lipolysis and ketogenesis. Consistent with that, it has been reported that a 2-fold increase in plasma cortisol caused by an ACTH injection increased the exercise-induced plasma NEFA 3-to 5-fold more than when the subjects received a placebo injection (Soetens et al., 1995). Recently, Samra et al. (1996) studied the effects of cortisol on lipolysis in the subcutaneous adipose tissue of the anterior abdominal wall by arteriovenous differences. The researchers reported that when the morning rise of cortisol was prevented by metyrapone the venoarterialized differences for NEFA and glycerol were decreased. Moreover, the mechanism of action is attributable, at least in part, to a decrease lipoprotein lipase and in hormone-sensitive lipase action.

Amino acids can participate in energy production by entering as intermediates of the tricarboxylic acid cycle, being oxidized directly by the working muscle (i.e., leucine, isoleucine and valine) or entering gluconeogenesis (i.e., glucose-alanine cycle). Amino acid turnover is modulated by cortisol, among other hormones. Thus, we measured the

plasma concentration of branched chain amino acids and alanine before and during exercise under hypocortisolemia and normocortisolemia. We found that the pre-exercise plasma alanine concentration was similar in both experimental conditions. However, during exercise the alanine peak was attenuated by ~ 25% at the 30th and 60th min under hypocortisolemia compared to normocortisolemia. At rest, branched chain amino acids tended to be lower under hypocortisolemia compared to normocortisolemia ($P=0.07$). However, the difference between both exercise-trials became significant at 60 min and thereafter. Interestingly, during the second hour of normocortisolemia plasma branched chain amino acids increased, whereas at the end of the second hour of hypocortisolemia the plasma concentration of these amino acids dropped slightly, but significantly. Cortisol is known to stimulate proteolysis, and to attenuate or stimulate protein synthesis depending on the tissues. For instance, cortisol induces amino acid cycling by inducing peripheral proteolysis (i.e., muscle) and splanchnic (i.e., liver) protein synthesis (see [De Feo, 1996] for a recent review). Consistent with our findings, Garrel and associates (1995) infused cortisol to increase cortisol levels to ~ 870 nmol/L and reported increases in leucine concentration, and its Ra and oxidation. Others have reported that when the morning rise of cortisol is prevented by administering metyrapone and replacing cortisol peripherally at a basal rate the postprandrial alanine concentration is attenuated (Dinneen et al., 1993). These studies (Dinneen et al., 1993; Garrel et al., 1995) demonstrate that cortisol is involved in amino acid metabolism at rest. With respect to exercise, Viru and associates (1994) evaluated the interaction of exercise-induced increases in alanine and glucocorticoids on adrenalectomized and normal rats. In normal rats a 3-h swim induced increases in alanine levels in blood, quadriceps muscle and liver, whereas adrenalectomy blunted these effects. Similarly, the increase in muscle alanine aminotransferase activity was prevented by adrenalectomy. However, when glucocorticoids were replaced the exercise-induced increases in alanine levels, and alanine aminotransferase activity were restored. These

results coupled with our findings support the view that glucocorticoids promote alanine supply and utilization during exercise.

With regards to our measurements of proteolysis, a change in the alanine concentration could be due to a change in *de novo* alanine synthesis from pyruvate, a decrease in alanine utilization, and or a release during proteolysis (Haymond and Miles, 1982). However, during exercise under hypocortisolemia, a slightly higher ($P=0.12$) blood lactate concentration, a rough index of muscle glycolytic flux, argues against substantial differences in *de novo* synthesis from pyruvate. In addition, the lower BCAA during hypocortisolemia than hypercortisolemia could have contributed to lower alanine levels (i.e., perhaps less nitrogen was transferred from the BCAA to alanine because of a less active alanine aminotransferase). A possible mechanism to explain the differences in BCAA concentrations could be the cortisol-induction of branched chain alpha ketoacid dehydrogenase, a key enzyme in branched chain amino acid catabolism (Block and Buse 1990). Taken together, the lower alanine and BCAA concentrations found in the present study suggest an attenuation of proteolysis. During exercise, hypocortisolemia caused alterations in plasma insulin and GH concentrations, both of which are known to affect amino acid turnover (De Feo, 1996). The lower insulin concentrations would have favored proteolysis, whereas the higher GH could have counteracted this effect.

Importantly, despite the modest differences in substrate concentrations, no differences were observed in calculated rates of substrate oxidation between the hypocortisolemic and normocortisolemic trials. The present results suggest that during exercise, carbohydrate and lipid oxidation (as measured by indirect calorimetry) are not affected by acute changes in cortisol levels. This finding is consistent with studies conducted at rest in which researchers found that acute changes in the cortisol concentration have no effect on the RER (Brillon et al., 1995; Garrel et al., 1995). With respect to exercise, indirect calorimetry is an overall whole-body estimate of substrate oxidation and

we cannot rule out the potential for alterations in lipid metabolism that are not apparent at the level of whole-body oxidation. In agreement with our findings, Hoelzer et al. (1986) reported that adrenalectomized subjects showed similar metabolic responses to a control group during 60 min of cycling exercise at 60% $\text{VO}_{2\text{max}}$. Specifically, no differences were found in the plasma glucose concentration, and glucose production and utilization rates. In contrast to our findings, they found that glycerol, β -hydroxybutyrate, and alanine concentrations were not distinguishable from the control group. This discrepancy may be attributed, at least in part, to the shorter exercise duration and the fact that the adrenalectomized group was glucocorticoid-replaced. For instance, with the exception of plasma alanine most of the effects reported in the present study were accentuated during the second hour of exercise.

The present experiment indicates that a low plasma cortisol concentration, despite the counterregulatory hormone adjustments, influenced substrate concentrations during prolonged exercise. On the one hand, it attenuated glycerol, β -hydroxybutyrate, alanine and BCAA concentrations, whereas glucose ($P=0.18$) and lactate ($P=0.12$) were not significantly affected. By design these metabolic changes were seen when cortisol was suppressed and the other glucoregulatory hormones were allowed to change. Thus, to better isolate the effects of cortisol on substrate homeostasis a more elaborate methodology is needed.

Our study included a small number of males and females. There is no conclusive evidence that gender affects substrate availability or use (Kanaley et al., 1991a; Friedman and Kinderman, 1989). Furthermore, the cortisol response to exercise is independent of the menstrual phase status (Kanaley et al., 1991b; Loucks et al., 1989). However, despite a reduction in sample size ($n=5$) the effect of cortisol on glycerol rate of change was of greater statistical significance (i.e., lower P values) when females were excluded from the analysis, whereas the female group ($n=4$) did not show any effects. This suggests that

despite similar cortisol concentrations, males and females may show different metabolic responses to changes in the plasma cortisol concentration. A larger study will be needed to examine whether subtle differences exist. No gender effects were seen in the lactate, glucose, and amino acid concentrations.

Importantly, all the studies reported here measured total cortisol concentration. Rowbottom et al. (1995) measured total and free cortisol concentration before and after an exercise bout and found that the increase in free cortisol was disproportionate, and of much greater relative magnitude (344%), to the increase in total cortisol concentration (49%). The authors suggested that the direct measurement of free cortisol should be considered in future work. Furthermore, the use of absolute cortisol values to assess its metabolic effects ignores any differences in glucocorticoid sensitivity which might exist between individuals. To date, however, there is a lack of any robust marker of glucocorticoid activity, making it difficult to assess glucocorticoid sensitivity (Howlett, 1997).

We studied the effect of low cortisol levels (suppressed by metyrapone ingestion) and compared it to a control study in which a placebo was administered, allowing the normal morning cortisol peak. Despite counterregulatory hormonal adjustments under low cortisol, the rates of lipolysis, ketogenesis and proteolysis normally seen during prolonged exercise were attenuated by ~15 to 35%. These findings are consistent, but somewhat lower in magnitude than previous hypocortisolinemic studies during normoglycemia (Garrel et al. 1995), prolonged insulin-induced hypoglycemia (De Feo et al. 1989), and following meal ingestion (Dinneen et al. 1993; Dinneen et al. 1995). As with all experiments, the current study has limitations. First, we did not block cortisol on both studies and peripherally replace it at a fixed high and low rate. This approach would have allowed us to further control cortisol levels and to standardize the metyrapone treatment. However, we feel that the study provides a more profound blockade without replacement, maximizing the effects of hypocortisolinemia. Second, we did not clamp insulin, glucagon

and GH which resulted in alterations in the glucoregulatory hormone profile. However, this could have, if anything, underestimated the suppressive effects of hypocortisolinemia on substrate availability. Despite these limitations we conclude that during exercise: 1) cortisol normally plays a demonstrable role by accelerating lipolysis, ketogenesis and proteolysis; 2) hypocortisolinemia drives glucoregulatory adjustments to maintain glucose homeostasis; 3) despite modest changes in substrate availability, hypocortisolinemia does not alter whole-body substrate utilization; and 4) under acute hypocortisolinemia does not impair the ability to complete prolonged exercise bouts. Additionally, it is recommended that the cortisol response to prolonged exercise be compared to a rest day.

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APPENDICES

Appendix A

Hypotensive response

A 29 year-old novice bodybuilder reported to the laboratory the morning after the overnight metyrapone treatment and reported no side effects. Upon arrival at the laboratory (0740 h) the subject was seated, electrodes were placed for a 12-lead ECG, and a catheter was placed in a superficial distal wrist vein. Initially, we were unable to draw blood and the subject developed hypotension (97/54) (the 12 lead ECG was normal). The subject was then moved to a supine position and infused 250 ml of 0.9 % saline IV which improved the subject's sense of well being but failed to normalize blood pressure (85/66). However, when infused with 100 mg IV of Hydrocortisone sodium succinate (Solu-Cortef) the blood pressure increased to 97/58 within a few minutes. Twenty minutes later the blood pressure had increased to 121/68. The subject then ingested 20 mg of cortisol (Corfef) and was then allowed to leave the applied physiology laboratory. The subject chose not to continue with the study. This was the only subject (1/12) who experienced hypotension at rest. It is possible that hypocortisolism (166 nmol/L compared to 552 nmol/L on a control day) and the emotional stress of inserting the catheter might have precipitated the hypotension, which was rapidly corrected by increasing the (1932 nmol/L) cortisol levels.

Appendix B
Metabolic Effects of Cortisol During Exercise in Humans
Consent Form

You are invited to participate in a research study to examine the effects of reduced cortisol levels on energy sources. Cortisol is a hormone that is indirectly related to the supply of fat and carbohydrate. The findings of this study will help us understand the function of this hormone during exercise. In addition, it will provide us with information to understand the low exercise tolerance seen in persons with Addison's disease (chronic low cortisol levels).

First Visit

As a subject, you will make three separate visits to the laboratory. On each visit you will report to the laboratory after an overnight fast at 7:40 AM, be seated, and a catheter will be placed in a hand vein for the collection of blood samples. On your first visit, a 2 ml blood sample will be taken from 8:00 AM to 10:00 AM at 30 minute intervals. During this period of time, you can read and write but conversation will be minimized. At 10:00 you will undergo an exercise test in which the intensity will increase every 3 minutes until the 12th minute and every minute thereafter until fatigue (15 to 20 minutes). Respiratory gases and blood will be collected at the end of each stage. A total of 20 ml (<1 oz) of blood will be collected during the first visit. The blood will be analyzed for lactic acid to determine the exercise intensity to be used for your next 2 exercise sessions.

Second and third visit

Upon arrival and placement of the catheter, you will exercise for a maximum of 120 minutes on a stationary bicycle ergometer at a moderate intensity (based on your lactic acid test) from 8:00 to 10:00. A 8-ml blood sample will be taken every 30 minutes. During the exercise period, medical personnel from The University of Tennessee Medical Center will be present to monitor your cardiovascular system, and blood sugar. In addition we will monitor your oxygen uptake, heart rate, and rating of perceived exertion. In essence the second and third visits will be conducted in the same manner. However, they will differ in that one will be done under drug treatment and the other under placebo. In order to examine the effects of reduced cortisol levels in exercise, the drug metyrapone will be used to lower

your cortisol levels. The metyrapone or placebo will be self administered (orally) according to body weight the midnight before reporting to the laboratory, and immediately prior to the test (1/5 -1/7 of the night dose). A total of 1.25 oz of blood will be collected in each 120 minute test.

Potential Risks and Discomfort

The risks involved with graded exercise include abnormal blood pressure responses, fainting, muscle soreness and in very rare instances heart attacks. Risks for the blood sampling may include infection and internal bleeding. Metyrapone is a drug used to reduce cortisol levels. Although side effects are infrequent in healthy subjects, metyrapone administration may cause nausea, sedation, low blood pressure, dizziness and mild facial flushing. Side effects can be minimized by ingesting it with milk or yogurt. At the end of the test your cortisol levels will be boosted up by the administration of 20 mg dose of cortisone (approximately equivalent to the 24 hour need of the body). *Importantly*, there is only a minor chance that serious complications will occur. In the unlikely event that physical injury occurs as a result of the study, financial compensation is not available and medical treatment will not be provided free of charge. If physical injury is suffered in the course of research, please notify Pedro Del Corral or Dr. Edward Howley.

Benefits from this study include the determination of your aerobic capacity and anaerobic threshold, both of which are useful information to understand your fitness for certain recreational activities. In addition, the study will provide you with information on the percentage of fat and carbohydrate used during exercise.

The information in this study will be provided to you and will be used in research reports and/or presentations. You are free to decide whether or not to participate in this study and are free to withdraw at any time without penalty or prejudice. Before you sign this form, please ask questions about any aspects of the study which are unclear to you.

Signature_____

Date_____

Witness_____

Date_____

Principal Investigator:

Pedro Del Corral

Exercise Science Unit

HPER Building room 306

974-5091 (W)

Faculty Supervisor:

Dr. Edward Howley

Exercise Science Unit

HPER Building room 327

974-1294 (W)

Appendix C

Personal History Form

Name _____ Age _____ Date _____

<u>Have you ever had:</u>	Yes	No
Free or easy bleeding (hemophilia)	()	()
Rheumatic Fever	()	()
Heart Murmur	()	()
High Blood Pressure	()	()
Varicose Veins	()	()
Lung disease	()	()
Irregular heartbeat	()	()
Bronchitis	()	()
Emphysema	()	()
Diabetes or Endocrine disorders	()	()
Kidney or liver disease	()	()
Hay fever	()	()
Anemia	()	()

<u>Have you recently had</u>		
Chest pain	()	()
Shortness of breath	()	()
Heart palpitations	()	()
Leg or ankle swelling	()	()
Coughing of blood	()	()
Low blood sugar	()	()
Blood in the urine	()	()
Frequent urination	()	()
Fainting	()	()
Headache	()	()
Weakness in arms or legs	()	()
Do you smoke	()	()
Are you currently taking medications	()	()

Have taken oral contraceptives in the last three months

() ()

Have you been menstruating regularly () ()

Has your doctor ever told you that you can't exercise

() ()

Describe your exercise habits including activity, frequency and duration _____

APPENDIX D

β -Hydroxybutyrate analysis

Principle

β -hydroxybutyrate + NAD -----> Acetoacetate + NADH

At an alkaline pH of 8.5 and in the presence of hydrazine to trap the acetoacetate as the hydrazone, the reaction goes to completion.

Procedure

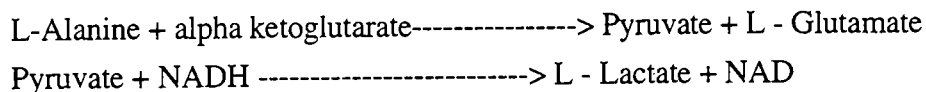
1. The procedure described is for the spectrophotometer but can be adapted to the fluorometer. Serum or plasma can be deproteinized and neutralized before use. One ml of serum can be deproteinized in about 1 ml of perchloric acid (7%), centrifuge hard, decanted and neutralized in a total volume of 2 ml with KOH.
2. The reagent cocktail is made in 20 ml batches. Prepare fresh.
 - 14 ml of 0.1 M Tris, pH 8.5
 - 5 ml of 1 N HCL
 - 1 ml of 20 M Hydrazine (Hydrazine hydrate)
 - 20 mg EDTA
 - 5 mg NAD
 - Adjust pH to 8.5
3. Pipet 0.1 ml of the deproteinized and neutralized serum extract into 10 x 75 mm borosilicate glass culture tubes. Prepare a water blank. The NADH calibration is used as a standard.
4. Add 1 ml of reagent cocktail.
5. Read initial fluorescence.
6. Add 5 μ l β -hydroxybutyrate dehydrogenase to each tube (Stock HBDH of 12.5 U/ml) (Sigma H-4132). Mix.
7. Let react for 2 h.

This assay may have to be run on range #3 for normal values and range #1 for elevated values. Extract volumes can be varied as necessary, and the assay can be run on spectrophotometer against a reagent blank. The calculations from the fluorometer are made in much the usual way from the NADH calibration of the instrument after subtracting the fluorescence of the blank from the sample. The normal fasting range is 0.05-0.2 mM.

APPENDIX E

Plasma alanine analysis

Principle



The decrease in the fluorescence of NADH is used to calculate the amount of alanine reacted.

Reagent (pH 8.1)	Stock	Final	Volume to make 25 ml
Tris base	1 M	0.05 M	1.25 ml
HCL	1 N	0.025 M	0.625
α -Ketoglutarate	0.05 M	0.05 mM	0.025
NADH	up-scale a consistent amount to between 80 and 90 units		
LDH	500 U/ml	0.2 U/ml	0.01 ml
GPT (ALT)	500 U/ml	8 U/ml	15 μ l per tube

Procedure

1. Serum or plasma is deproteinized and neutralized as for β -hydroxybutyrate.
2. Once the pH of the reagent is set (Tris base + HCL) bring volume to 25 ml with distilled water.
3. Pipet 0.1 ml of extract into 10 x 75 mm borosilicate glass culture tubes. Run a water blank and an L-alanine standard (~ 50 μ M)

4. Add 1 ml of reagent cocktail, which has been carefully adjusted with NADH to read up-scale to 80-90 units. The NADH should be 20-70% in excess of the expected alanine concentration (0.2-10 μ M/test tube).

5. Let this react for a few minutes to run the pyruvate through. (LDH is in the cocktail.)

6. Take the initial fluorescence.

7. Start the reaction with 15 μ l of GPT and let react for 1 h

8. Read the final fluorescence. Subtract the change in the fluorescence of the blank from the change in the fluorescence of the sample and standard and calculate the results from the standard or the NADH calibration of the fluorometer.

$(\Delta F_{\text{sample}} / \Delta F_{\text{std}}) \times \text{mM conc. std} = \text{mM concentration of the sample}$

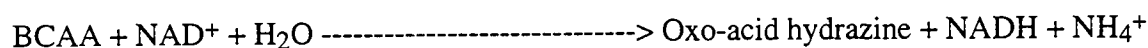
normal levels are 0.200-0.500 mM

APPENDIX F

Branched chain amino acid analysis

Principle

This method is based on the enzymatic oxidative deamination of L-leucine, L-isoleucine, and L-valine (BCAA) by leucine dehydrogenase. The NADH which is formed in stoichiometric quantities is estimated fluorometrically.



Reagents

EDTA Na₂ -- Mallinkrodt 4931

L-Leucine (F.W.=131.2) -- Mallinckrodt

NAD (F.W.=663.4) --- Sigma #N-7004 - free acid grade II (98%)

Perchloric acid (F.W.=100.46) Baker 9652-01 - 70% solution

Hydrazine hydrate (F.W.= 50.06) -- Sigma #H-0883

Hydrazine sulfate (F.W.= 130.1) -- Sigma #H-3376

phosphate buffer (K₂HPO₄/K₂PO₄, 10 mmol/L pH 7.2) Mallinckrodt.

Leucine dehydrogenase (25U) -- Sigma #L-5135

Solutions

-Leucine dehydrogenase solution (stable for 2 months @ 4°C): dissolve 25 U/ml of the enzyme in 1 ml phosphate buffer.

-NAD (5 mmol/L) (stable for at least 2 weeks @ 4 °C) 165.9 mg of NAD with distilled water to 50 ml .

-Hydrazine buffer (stable for several months @ 4 °C) (hydrazine 1.1 mol/L, pH 9.0). 1.3 g hydrazine sulfate, 5.0 g hydrazine hydrate, 0.7 EDTA Na₂ with distilled water to 100 ml

-Standards (stable for 2 weeks at 4 °C). Five mmol/L stock solutions of L-Leucine in distilled water (dissolve 328 mg of L-leucine up to 500 ml with distilled water). Dilute each day with 0.3 mol/L perchloric acid to make working standards containing 0, 150, 300, 450, 600 µmol/L.

150 µmol/L-- 3 ml stock up to 100 ml with water.

300 µmol/L-- 6 ml stock up to 100 ml with water.

450 µmol/L-- 9 ml stock up to 100 ml with water.

600 µmol/L-- 12 ml stock up to 100 ml with water.

Procedure

1. Collect blood in tube containing EDTA
2. Centrifuge @ room temp at 11000 g for 1 min to get plasma
3. Immediately deproteinize 20 µl of plasma with 200 µl of ice-cold perchloric acid (0.3 mol/L)

4. Separate the precipitate through centrifugation at 11,00 g for 1 min.

5. Prepare reaction mixture immediately before analysis (for 50 tubes)

10 ml of hydrazine buffer

500 µl of NAD (2.5 mmol/L final concentration)

100 µl of leucine dehydrogenase (235 U/L final concentration)

6. Add 20 µl of blank, standard, and sample in each tube.

7. Place 200 µl of the freshly prepared reaction mixture in the fluorometer tube and mix.

8. Incubate in water bath for 120 min @ 37 °C

9. Add 1 ml of distilled water to each tube and mix thoroughly.

10. Read Fluorescence @ 340 nm (excitation) and 460 nm (emission)

Normal values (resting postabsorptive): 250-600 $\mu\text{mol /L}$

Appendix G

Plasma Catecholamine analysis

Catechol-O-Methyl transferase Purification

Axelrod, J. and R. Tomchich. Enzymatic O-methylation of epinephrine and other catechols. J. Biol. Chem. 233: 702-705, 1958.

1. Reagents

- a) KCL- Fisher SP138-500
- b) Acetic acid, Glacial - Fisher A-38
- c) Ammonium Sulfate -Fisher A-702
- d) KPO₄ monobasic - Specialty Chemicals 2130
- e) KPO₄ dibasic - Specialty Chemical 2131
- f) THAM - Fisher T-395
- g) Dialyzer Tubing - Fisher 8-667B

2. Preliminary Set-up

- a) Prepare the day before about 1000 ml of isotonic KCL.
(11.63 g of KCL made up to 1000 ml with distilled water) and place in the refrigerator so it will be less than 5 °C in the morning. 5.82 g rat liver/500 ml isotonic KCL (need 400 ml per 100 g of rat liver).
- b) Prepare 1.0 N acetic acid solution by taking 4.0 ml of 17.5 N acetic acid (normal concentrated acetic acid) and adding it slowly to 66.0 ml of distilled water.
- c) Make certain the 250 ml centrifuge tubes are clean (will need at least 4 of them). Alternatively, 25 ml centrifuge tubes can be used.

3. Rat Liver Process and General Set-up

- a) On returning to the lab with the rat liver, remove the liver from the ice bucket and cut-off about 100 g of liver and put it into a beaker in the ice bucket. Put the bucket in the refrigerator at 5 °C or less to keep the liver cold before use. Take the remainder, wrap in plastic, and freeze it for future use (good for at least 3 months).

b) A large tray is filled with crushed ice and then tap water is added. Next a quantity of table salt (50-100) is added and stirred into solution. This will be used to keep the samples cooled between procedures if they are over a couple of minutes long.

4. Extraction

- a) Remove the liver from the beaker in the ice bucket and cut up the 100 g of liver (need about 6 rats) with scissors. Then place it in the blender (or homogenizer).
- b) Add 400 ml of the cold isotonic KCL and homogenize until there is a homogenous mixture.
- c) Centrifuge: 1) Pour the 500 ml of total homogenate equally into centrifuge tubes and place all tubes in the centrifuge; 2) Spin the tubes first at 12000 rpm at 2 °C for 30 min; 3) pour the supernatant into new tubes, equalize the volume by eye, and recentrifuge at 12000 rpm at 2 °C for 1 h.
- d) Decant the supernatant into a 500 ml beaker, add a magnetic stirring bar and using the 1.0 acetic acid previously prepared adjust the pH to 5.0 (will use about 10-15 ml of acetic acid). Let stand for 10 min in refrigerator.
- e) Centrifuge again at 12000 rpm at 2 °C for 30 min.
- f) Decant the supernatant into a 500 ml graduate cylinder (to get the volume) and then into a 500 ml beaker. Add the magnetic stirring bar and place on the stirrer in the refrigerator.

5. Ammonium Sulfate $(\text{NH}_4)_2 \text{SO}_4$ Fractionation.

- a) Add 0.173 g of $(\text{NH}_4)_2 \text{SO}_4$ per ml of supernatant into the 500 ml beaker, stir with the magnetic stirrer for 1 h (e.g., if 350 ml of supernatant, one needs $350 \times 0.173 = 60.55$ g of $(\text{NH}_4)_2 \text{SO}_4$)
- b) Pour the beaker's contents equally into centrifuge tubes and spin at 12000 rpm at 2 °C for 30 min.

c) Decant the supernatant from the tubes into the 500 ml graduated cylinder again (to get the volume) and then into the 500 ml beaker. Add a magnetic stirring bar and place on the stirrer in the refrigerator.

d) While stirring, add 0.127 g of $(\text{NH}_4)_2\text{SO}_4$ per ml of supernatant. Stir in the refrigerator for 1 h (you loose about 4-5% activity here).

e) Pour the beakers content s equally into centrifuge tubes and spin at 12000 rpm at 2 °C for 30 min.

f) The supernatant is discarded and about 10 ml of distilled water is added to each tube to dissolve the precipitate. More distilled water is added from a squeeze bottle just enough to dissolve the precipitate.

g) After the precipitant is dissolved, add it to a graduate cylinder to measure its volume (so that proper length of dialysis can be used).

6. Dialysis

a) Prepare 1.0 mM potassium phosphate buffer from 0.22823 g of KPO_4 dibasic stock per 1000 ml of distilled water and tritriated to a pH of 7.0 with monobasic KPO_4

b) Using Fisher Scientific Dialyzer tubing (catalog #8-667B: size 0.16 cm diameter inflated and 0.25 cm flat width), calculate the necessary length of tube to hold the volume of solution obtained in step (5 g) above. $\text{Length} = \text{volume} / (0.5 \text{ diameter} \times \pi^2)$. Cut off the necessary length giving several inches extra.

c) Prepare the sac by placing the dialyzer tubing into the beaker containing about 10 g of sodium bicarbonate (or baking soda) / L of distilled water (amount is not critical). This solution is brought to a boil. The sac will float to top when boiling, push it back down. When the tubing opens, remove it from the beaker (being careful not to let it dry) and rinsing it (with deionized water, and then) with distilled water.

d) One end of the tubing is folded several times, length wise, and knot tied.

e) The sac is then filled from the other end with the dissolved precipitate (5 g) from above. Tie end.

f) The dialysis tubing is placed in the 1.0 mM KPO₄ buffer at 5 °C in the refrigerator for 19 h with four changes of solution (about every 4 h).

g) Centrifuge the dialyzed preparation at 18000 rpm x g and remove the supernatant.

h) Adjust supernatant to pH 8.1 with 2 M THAM, pH 8.5 (2 M THAM = 24.2 g + 50 ml water, add concentrated HCL slowly until pH=8.5. Then make up to 100 ml. You actually use only about 2 ml to adjust the pH, so you should only make 10 ml (2.42g/ 10 ml).

Radioenzymatic Catecholamine Assay

Passon, P. G. and J. D. Peuler. A simplified radiometric assay for plasma norepinephrine and epinephrine. Anal. Chem 51: 618-631.

COMT

1. Catecholamines + ³H S-adenosyl methionine----> ³H methoxyderivatives

Epinephrine and norepinephrine are converted to ³H metanephrine and ³H normetanephrine, respectively, during incubation with catechol-O-methyl-transferase (COMT) and ³H S adenosyl methionine (³H-SAM). The corresponding metanephrines are extracted into acetic acid and washed with a series of toluene isoamyl washes. The metanephrines and normetanephrines are separated by thin layer chromatography (TLC). They are eluted from the TLC silica gel and oxidized to vanillin with sodium periodate. The vanillins corresponding to epinephrine and norepinephrine are extracted into toluene- liquifluor solvent and counted in a scintillation counter. The output is proportion to the level of catecholamines.

2. Reagents

- a) THAM - Fisher T- 395
- b) EGTA- Na_2 - Sigma E-4378
- c) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ - Fisher M-33
- d) Glutathione - Sigma G-4251
- e) Boric Acid - Fisher A-73
- f) EDTA-Na - Mallinckrodt 4931
- g) Metanephrine HCL - Sigma M-8625
- h) Normetanephrine HCL - Sigma N-7127
- i) Toluene - Mallinckrodt 8608
- J) Isoamyl Alcohol - Fisher A - 393
- k) Glacial Acetic Acid A - 38
- l) Sodium Periodate - Fisher S - 398
- m) Glycerine - Fisher G - 33
- n) Ammonium Hydroxide - Fisher A - 669
- o) Hydrochloric Acid (HCL) - Fisher A - 144
- p) Epinephrine (100 $\mu\text{g}/\text{ml}$) - Sigma E-4375
- q) Norepinephrine (Arteronol) (100 $\mu\text{g}/\text{ml}$) - Sigma A-9512
- r) ^3H -SAM - New England Nuclear (5-15 μCi)
- s) Ethanol
- t) Methanol - Fisher A-412
- u) Silica Gel Plates (10 x 20 cm 25 μ) - Analtech
- v) Benzene - Fisher B - 245
- w) Tertiary Amyl Alcohol - Fisher A - 730
- x) Methylamine (40%) - Fisher M - 223
- y) Liquiflour - New England Nuclear NEF 903
- z) Sulfuric Acid (H_2SO_4) - Mallinckrodt 2876.

3. Sample collection

- a) EGTA-Glutathione solution. Put 90 mg of EGTA and 60 mg of glutathione to 1 ml of distilled water in a test tube. Vortex vigorously. Use 20 μl of this solution per ml of blood. If undissolved try adjusting pH of the solution between 6.0 and 7.4 (a drop or two of NaOH should be enough). This will give a final concentration of 1.8 mg of EGTA and 1.2 of glutathione per ml of blood.

- b) Place a 3 ml sample in a cold test tube containing 60 μ l of EGTA-glutathione solution. Immediately invert tube slowly and careful several times to mix blood with preservatives. Do not shake.
- c) Immediately place the tube in an ice bath.
- d) Separate the plasma through centrifugation in the cold as soon as possible and store frozen at - 70 $^{\circ}$ C or - 20 $^{\circ}$ C until analysis.

4. Working Solutions

- a) Assay Buffer (Tris). Add 6.05 of THAM, 1.90 g of EGTA- Na_2 , and 3.05 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to approximately 20 ml of distilled water. Adjust pH to 8.3 with NaOH or HCL while stirring. The initial pH is close to this value. Bring final volume up to 25 ml. This solution can be prepared in advance and aliquots can be stored frozen.
- b) Glutathione Solution (0.1 Molar). Add 30.7 mg of reduced glutathione to a small test tube and add 1 ml of water. Vortex lightly and keep on ice. Prepare fresh daily.
- c) Stopping Solution (1.0 M Boric Acid + 0.2 M EDTA- Na_2) Add 1.55 g of boric acid and 0.93 of 0.2 M EDTA- H_2O - Na_2 to 20 ml water. Adjust pH to 11.1 (may have to use NaOH pellets to bring pH up to 11.1). Bring to 25 ml with water. This solution can be prepared in advance and aliquots can be stored frozen.
- d) Metanephrine & Normetanephrine (0.2 μ moles/50 μ l of borate buffer or 0.935 mg/ml of Met and 0.88 mg/ml of Normet). Add 9.34 mg of metanephrine and 8.8 mg of normetanephrine for every 10 ml of the stopping solution and vortex. Prepare fresh daily.
- e) Toluene-Isoamyl Alcohol (3:2). Combine 300 ml of toluene and 200 ml of isoamyl alcohol.
- f) Acetic Acid. Bring 2.5 ml of glacial acetic acid up to 500 ml with distilled water.

g) Sodium Periodate Solution (4% w/v NaIO_4). Bring 1.0 g of NaIO_4 up to 25 ml with water. Prepare fresh daily.

h) Ammonium Hydroxide Solution (0.05 M NH_4OH). Bring 1.5 ml of NH_4OH up to 450 ml with distilled water.

i) HCL (1.0 N). Bring 8.3 ml of concentrated HCL up to 100 ml with water.

For 0.1 N HCl, 10 ml of 1.0 N HCl to 100 ml.

For 0.01 N HCl, 1.0 ml of 1.0 N HCl to 100 ml.

For 0.001 N HCl, 1.0 ml of 1.0 N HCl to 1000 ml.

J) Epinephrine and Norepinephrine Standards (100 pg/10 μl). 1) Bring 91 mg of epinephrine bitartrate up to 50 ml with 0.1 N HCl. This will give 1000 $\mu\text{g/ml}$ of epinephrine (epinephrine stock); 2) bring 99.5 mg of norepinephrine up to 50 ml with 0.1 N HCl. this will give 1000 $\mu\text{g/ml}$ of norepinephrine (norepinephrine stock); 3) take 10 ml of epinephrine stock up to 100 ml with 0.1 N HCl. This is the working standard (100 $\mu\text{g/ml}$). Do the same with norepinephrine stock; 4) take 100 μl of epinephrine working standard (100 $\mu\text{g/ml}$) into a 50 ml volumetric flask. Bring up to 50 ml with 0.01 N HCl; 5) Bring 5 ml of this solution to 100 ml with 0.001 N HCl. 10 μl of this solution will yield 100 pg of epinephrine and norepinephrine. Prepare fresh daily.

k) COMT Enzyme. Prepared according to the procedure of Axelrod and Tomchick. This is the rat liver preparation.

l) H_2SO_4 -Ethanol solution (9:1). Add concentrated solution to water (250 ml) until pH= 2 (this will require a drop or two). Use 9 ml of this for every 1 ml of ethanol (95%).

m) Toluene-Liquifluor (1000:50 v/v). Add 50 ml of liquifluor to 1000 ml of toluene.

5. Reaction

a) Turn on water bath and set to 37 °C

b) Prepare two sets of test tubes (13 x 100 mm). The first set is for reaction and incubation and should and should be kept in ice bath. The second set is for extraction Each sample and internal standard should be done in duplicate. Therefore you need four tubes per sample (2 for samples and 2 for internal standard). Label tubes with permanent marker.

c) Remove enzyme, sample, assay buffer, and standard from freezer. Thaw and keep on ice. Thaw ^3H SAM only before use.

d) Prepare the enzyme cocktail in a round bottom 13 x 100 tube immediately before addition. Add in the following order in amounts sufficient for the assay plus 4 (see below: 1) assay buffer, glutathione, and ^3H -SAM & H_2SO_4 -ethanol. Vortex lightly. Add COMT enzyme. Cover with parafilm and gently invert several times to mix. Keep on ice.

# Assay	1	20	50	100	200
Assay Buffer	5 μl	100 μl	250 μl	500 μl	1000 μl
Glutathione	1 μl	20 μl	50 μl	100 μl	200 μl
^3H -SAM	5 μl	100 μl	250 μl	500 μl	1000 μl
H_2SO_4 -	5 μl	100 μl	250 μl	500 μl	1000 μl
Ethanol *					
Enzyme	25 μl	500 μl	1250 μl	2500 μl	5000 μl

*This solution is used to dilute the isotope. It is the solvent vehicle for the ^3H -SAM. If higher counts are required for the assay, this should be omitted and the quantity of ^3H -SAM indicated on the chart should be doubled.

e) Incubation mixture. The first set of test tubes will received the following:

1) Blanks

i) 50 μl of sample vehicle (water mixed with EGTA-glutathione in the same concentration as blood samples: 60 μl EGTA-glutathione solution added to 3 ml of water).

ii) 40 μl of enzyme cocktail.

iii) 10 μl of 0.001 N HCL

2) Internal standards

- i) 50 µl of sample plasma
- ii) 40 µl of enzyme cocktail
- iii) 10 µl of 100 pg Epinephrine & Norepinephrine standard

3) Plasma samples

- i) 50 µl of plasma sample
- ii) 40 µl of enzyme cocktail
- iii) 10 µl of 0.001 N HCL

- Plasma sample can be pipetted with the fixed volume 50 µl positive displacement pipette. The tip does not need to be replaced after each sample.

- The enzyme cocktail, 0.001 N HCL, epinephrine and norepinephrine standards can be pipetted with the eppendorf repeater pipette. Note the correct setting and adapter size for volume desired.

f) Vortex all tubes lightly and centrifuge at 2000 rpm at room temperature

g) Incubate for 75 min in the oscillating water bath at 37 °C

h) Stopping the reaction. Place reaction tubes in ice bath and add 50 µl of the borate stopping solution that contains metanephrine and normetanephrine carriers (see sections 4-c & 4d).

Vortex is not necessary. Just gently shake tube rack.

6 Extraction

The second set of test tubes will be used here. They will each receive 100 µl of 0.1 N acetic acid (see section 4-f)

a) Add 2 ml of toluene-isoamyl alcohol (3:2) to incubation tubes. Vortex well and centrifuge for about two min at 2000 rpm at room temperature.

b) Quick freeze

i) Prepare an ice bath of methanol and dry ice. Methanol level should only be deep enough to cover the bottom of test tubes (0.5 to 1 inch). Best to use a 1 L beaker filled with dry ice and place a 100 ml beaker inside of the larger beaker. Place a piece of dry ice and methanol inside of this beaker to do the actual freezing.

ii) Submerge tube to level of aqueous phase.

iii) Blot outside the tube. Decant the organic phase into its corresponding tube containing the 0.1 N acetic acid.

iv) Rhythm: Decant one tube while the next tube is in the ice bath. Be consistent 15 to 25 seconds should be enough time. Twenty seconds for each sample works very nicely.

c) Vortex the acetic acid-organic solution and centrifuge for 2 min as above.

d) Quick freeze (15 seconds works well) with tubes held at an angle. Aspirate off organic phase without touching suction pipette to pellet.

e) Add 1 ml of toluene-isoamyl alcohol to thawed aqueous phase.

f) Vortex and centrifuge 2 min as above.

g) Quick freeze with tubes held at an angle, aspirate and discard organic phase as in (d) above.

* The assay may be stopped at this point. Cap tubes and stored at - 20 °C. Tubes may be kept several days.

7. Thin Layer Chromatography

a) Number either side of score on the TLC plate to identify spot.

b) Add 100-150 μ l of ethanol to the thawed acetic acid from section 6-g above. Volume of ethanol is not critical. However, since the spotting syringe hold only

250 µl, 150 µl is usually the maximum possible. The normal number of tubes in one spotting run is 14 (both of the outside strips are omitted). Vortex and centrifuge.

c) Clear spotting syringes of air bubbles by pulling ethanol through the tips.

d) slowly pull acetic acid-ethanol solution into syringe. Do not pull excess air into syringe. Should have small bubble on the end of plunger.

e) Turn on dryer and spotter. Dryer should be placed approximately 4.5 cm from plates and set temperature at 70 °C.

f) Position syringes so that tips touch TLC plates. Set plunger speed to slowest possible speed. Spotting run will take 20-30 min. When run is over, turn spotter off and removed arm back by hand. Turn spotter back on, but do not hit start until ready to begin next run.

g) Prepare 44 ml of developing solution (6:2:3) in a 50 ml graduate cylinder and mix gently (solution should be clear). Prepare fresh before each development.

i) 24 ml of tertiary amyl alcohol

ii) 8 ml of benzene

iii) 12 ml of methylamine

h) Put developing solution in a developing tank immediately before use. Carefully place plates in tank (2 plates per tank). Develop until solvents run the entire plate (about 40-50 min). Dispose of solvent

i) Dry plates (approximately 30 min) and visualize spots under ultraviolet light. Using scapel, carefully outline the spots. Best separation is obtained when plates are still damp.

* The assay may be stopped at this point as it is not critical that the plates be scrapped immediately.

j) Scrape spots into scintillation vials using a squared-edge scapel (ANALTECH). The metanephrines are the top zones and the normetanephrine are the bottom zone.

8. Oxidation

- a) Add 1 ml of 0.05 M NH_4OH to vials. Vortex vigorously.
- b) Line up vials and add 50 μl of 4% periodate solution at timed intervals. Vortex vigorously.
- c) Stop reaction by adding 50 μl of 10% (v/v) glycerol solution (1.26 g/ml) at exactly 5 min after step b). Maintain order and timing of step b).
- d) Add 1 ml of 0.1 N acetic acid to each scintillation vial and vortex.
- e) Add 10 ml of toluene liquifluor (1000:50 v/v) to each scintillation vial and shake by hand vigorously for 10 seconds.
- f) Count each vial for 2-5 min in scintillation counter.

9. Calculations

- a) Locate blanks and obtain mean radioactivity counts (CPM) for epinephrine blank and norepinephrine blanks.
- b) Subtract blanks from epinephrine and norepinephrine sample counts to obtain Sample Net. Obtain means for each sample. $\text{Sample Net} = \text{Sample CPM} - \text{Blank CPM (mean)}$
- c) Locate standards and obtain Standard Net by subtracting Sample CPM. $\text{Standard Net} = \text{standard cpm} - \text{sample cpm (mean)}$
- d) Divide Sample Net (mean cpm) by Standard Net (mean cpm) for that sample and multiply by 2000 to obtain concentration in pg/ml.
$$\text{Concentration (pg/ml)} = (\text{Sample Net} / \text{Standard Net}) \times (\text{pg standard} / \text{ml sample volume})$$

* for this assay, pg standard is 100 pg, and ml sample volume is 0.05 ml.

VITA

Pedro Del Corral was born in Stockholm, Sweden on June 19, 1967. He attended Champagnat Elementary School and Liceo Los Arcos Junior High and High School in Caracas, Venezuela. Upon high school graduation he attended Central University of Venezuela in Caracas where he completed the first two years of Dental School. In the fall of 1989 he came to the United States of America and transferred to University of Iowa, Iowa City, IA, where he latter received a Bachelor of Arts in Interdepartmental Studies with an emphasis in Exercise Science. Following graduation in the summer of 1991 he started graduate study in the Human Performance Laboratory and the Department of Physiology and Health Sciences at Ball State University. In August 1993 he obtained a double major Master of Science degree in exercise physiology and physiology. In August, 1993 he entered American University of The Caribbean as a medical student. In March, 1995 he took a leave of absence from the medical school to enter The University of Tennessee-Knoxville. While in Tennessee Pedro served as a Graduate Teaching Associate and as a Graduate Research Assistant in the Exercise Science Unit and the Department of Nutrition, respectively. In December, 1997 he received a Doctor of Philosophy degree in Education with an emphasis in exercise physiology. Starting from January, 1998 he will return to complete the medical degree at The American University of The Caribbean.