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The effects of chronic intermittent hypoxia on insulin and leptin homeostasis in the rat

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1.1 General Introduction

Obstructive sleep apnoea is characterised by repeated episodes of upper airway obstruction, occurring during sleep. The obstructive events are associated with a reduction in blood oxygen saturation and arousal from sleep (International Classification of Sleep Disorders, 1990). It is possible that both the hypoxia and the sleep disruption produce a sustained elevation in sympathetic nervous system activity in sleep apnoea patients, which decreases in response to treatment of the repetitive apnoeic events (Somers *et al*. 1995). This rise in sympathetic nervous system activity is associated with the development of insulin resistance, elevated plasma leptin levels (Manzella *et al*. 2002), hypertension and other cardiovascular complications (Wolk *et al*. 2003).

The insulin and leptin resistance seen in obstructive sleep apnoea patients have been attributed to obesity. However, the severity of these hormonal abnormalities are not in proportion to the degree of obesity (Yun *et al*. 2004) and non-obese sleep apnoea patients also suffer from insulin and leptin resistance (Ip *et al*. 2002; Shimizu *et al*. 2002).

There are two components of obstructive sleep apnoea which could mediate an increase in sympathetic nervous system activity; sleep fragmentation and hypoxia. It remains unclear which of these two components causes the insulin and leptin resistance, seen in obstructive sleep apnoea patients. There is no evidence to suggest that insulin resistance and increased plasma leptin levels are prevalent in other sleep disorders, where sleep is disrupted, such as insomnia and periodic limb movement disorder, therefore it is reasonable to speculate that these hormonal changes may be caused by the hypoxic element of sleep apnoea and not the sleep disruption per se.

Chronic intermittent hypoxia is used as an experimental animal model to simulate the hypoxia occurring in obstructive sleep apnoea. The majority of chronic intermittent hypoxia research has focused on the sympathetic nervous system response and cardiovascular complications associated with obstructive sleep apnoea. Animal research aimed at examining the effects of chronic intermittent hypoxia on plasma insulin and leptin levels have focused on genetically obese rodents (Polotsky *et al*. 2003) or *in vitro* studies (Ambrosini *et al*. 2002; Dionne *et al*. 1993; Meibner *et al*. 2003). At present, there is no study examining the possible change in plasma insulin and leptin levels in normal weight rats exposed to chronic intermittent hypoxia.

In previous animal models for chronic intermittent hypoxia, only the sympathetic nervous system activity has been assessed by measuring blood pressure (Fletcher *et al*. 1992) or the renin-angiotensin system activity (Fletcher *et al*. 1999). The sympathetic nervous system, however, is not the only system activated in animals exposed to a stressful environment. The hypothalamic-pituitary-adrenal (HPA) axis activity is up-regulated in response to stressful conditions (Möstl *et al*. 2002), such as exposure to chronic intermittent hypoxia. A change in HPA axis activity has been associated with insulin resistance and increased body fat mass (Wang, 2005). The relationship between HPA axis activity, as measured by excreted glucocorticoid metabolites, and insulin and leptin resistance has not been studied in rats exposed to chronic intermittent hypoxia.

The aims of this study were to (1) measure plasma insulin and leptin concentrations and (2) monitor HPA axis activity in a group of male, normal weight rats subjected to chronic intermittent hypoxia, in a setting which attempted to control for sleep disruption.

1.2 Literature Review

1.2.1 Obstructive sleep apnoea

Obstructive sleep apnoea is characterised by loud snoring, usually crescendo in nature, indicative of progressive narrowing of the upper airway until the airway becomes partially blocked, resulting in hypopnoeas, or completely blocked, resulting in apnoeas (Ballard, 1993). Hypopnoeas are defined as airflow reductions of at least 50% and apnoeas as almost complete cessations in breathing which last for more than ten seconds (Lévy *et al*. 1992).

There are three types of apnoeas: central apnoea, in which there is a cessation of airflow due to a lack of respiratory effort; obstructive apnoea, in which the cessation of airflow occurs despite ongoing respiratory effort; and mixed apnoea, in which there is an initial central apnoea followed by an obstructive component (Lévy *et al*. 1992). The severity of sleep apnoea is indicated by the apnoea-hypopnoea index (AHI), being the number of respiratory disturbances per hour. In general an AHI > 10 is considered to be abnormal (Ballard, 1993). The apnoeas result in a sudden drop in the partial pressure of arterial oxygen (PaO_2), which lasts for a few seconds. This partial pressure has a direct relationship to the saturation of haemoglobin, which is the parameter usually measured (American Academy of Sleep Medicine, 1999). An

apnoea is considered significant if the PaO_2 decreases more than 3% from baseline level (American Academy of Sleep Medicine, 1999).

The mechanism producing the obstructive sleep apnoeas is repeated collapse of the pharynx during sleep, associated with an increased breathing effort. Obstruction to the upper airway could be the result of anatomical abnormalities in structures surrounding the airway, such as nasal blockage, an enlarged or low-lying uvula and soft palate, dental overbite, enlarged tongue and a reduced retroglossal area (Bassiri & Guilleminault, 2000). Increased fat deposition around the neck is also associated with narrowing of the upper airway and a positive correlation between neck circumference and severity of obstructive sleep apnoea has been shown (Davies & Stradling, 1990).

Risk factors for the development of sleep apnoea include male gender, advancing age and obesity (Vgontas *et al*. 2000). The risk in females increases with obesity and post menopausal status (Bassiri & Guilleminault, 2000)

The patient is woken up by the inability to breathe (Tiihonen *et al*. 1993), but the awakenings are so brief that the sufferer is rarely aware of them, and may occur several hundred times a night. This disruption of sleep

has been implicated in the symptoms of daytime somnolence, cognitive impairment and mood disorders (Ballard, 1993; Douglas, 1993).

The main treatment for obstructive sleep apnoea is nasal continuous positive airway pressure (CPAP). The CPAP device pressurises room air which is then blown through a mask, which the sleep apnoeic wears over their nose. The pressurised air is then blown into the collapsing part of the pharynx and acts as a pneumatic splint to prevent the obstruction (Dickins *et al*. 1991)

1.2.2 Physiological abnormalities associated with obstructive sleep apnoea

Obstructive sleep apnoea is associated with a number of physiological complications, some more acute including increased release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin- 6 (IL-6) (Vgontas *et al.* 1997) and others more chronic including hypertension (Worsnop *et al*. 1998), myocardial ischemia (Pendlebury *et al*. 1998) and renal failure (Sica *et al*. 2000). There is also a link between obstructive sleep apnoea and hormonal abnormalities, such as an impairment of growth hormone secretion (Gianotti *et al*. 2002), reduced testosterone secretion (Semple *et al*. 1980) and insulin and leptin resistance (Manzella *et al*. 2002).

It has been suggested that the primary mechanism producing the physiological complications associated with obstructive sleep apnoea is an increase in sympathetic nervous system activity (Somers *et al*. 1995). Clinical studies have demonstrated elevated urinary and plasma catecholamine levels in obstructive sleep apnoea patients (Narkeiwicz *et al*. 1998), as well as increased muscle sympathetic nerve activity, during the day as well as at night (Somers *et al*. 1995).

As mentioned previously, there are two components of obstructive sleep apnoea which could, either singly or in combination, cause an increase in sympathetic nervous system activity namely; sleep disruption and hypoxia. Sleep disruption occurs due to the frequent arousals and the hypoxic element of sleep apnoea is caused by the sudden closure of the airway.

1.2.2a Sleep disruption

Sleep fragmentation in human subjects with no sleep disorders has been associated with increased sympathetic nervous system activity, evident by an increase in systemic blood pressure which is sustained during the daytime (Morrell *et al*. 2000). An increase in the frequency and duration of arousals are associated with reduced vigilance the following day (Bonnet, 1987).

Furthermore, experimental sleep deprivation causing sleep debt, a common effect of sleep disruption, results in raised evening and morning salivary cortisol levels (Spiegal *et al*. 1999; Ekstedt *et al*. 2004) and impaired glucose tolerance (Spiegal *et al*. 1999; Gonzalez- Ortiz *et al.* 2000).

1.2.2b Hypoxic effects of obstructive sleep apnoea

Exposure to hypoxic conditions elicits a stress response in both humans and animals (Calbet, 2003; Greenberg *et al*. 1999). An acute fall in oxygen concentration leads to activation of the sympathetic nervous system (Somers *et al*. 1995) and is associated with an increased release of catecholamines from the adrenal medulla (Bratel *et al*. 1999). The sympathetic nervous system then interacts with the HPA axis increasing corticosterones as an added hormonal component during stress (Vozarova *et al*. 2003).

Chronic intermittent hypoxia is used as an experimental animal model to simulate the hypoxia occurring in obstructive sleep apnoea. Rats exposed to chronic intermittent hypoxia showed an increased sympathetic nerve activity (Greenberg *et al*. 1999) and also developed systemic hypertension (Fletcher *et al.* 1992), an increase in the renin-angiotensin system activity (Fletcher *et al.* 1999) and other

cardiovascular complications similar to those experienced by humans with obstructive sleep apnoea (Sica *et al.* 2000).

In the canine model for obstructive sleep apnoea, it was shown that if occlusion to the upper airway was stopped immediately prior to arousal, but the hypoxia still occurred, then the obstructive apnoeic events were associated with a significant increase in mean arterial pressure (O' Donnell *et al*. 1996) and also sustained daytime hypertension (Brooks *et al*. 1997). When the obstruction was sustained until arousals occurred, there was a further increase in mean arterial pressure (O' Donnell *et al*. 1996). This suggested that intermittent hypoxia alone contributes to a sustained elevation in blood pressure but frequent arousals from sleep resulted in an additional hypertensive response.

A similar relationship between hypoxia and sleep disruption may exist to explain the metabolic abnormalities seen in patients with obstructive sleep apnoea.

1.2.3 HPA axis activity and obstructive sleep apnoea

Patients with untreated obstructive sleep apnoea demonstrated a rise in morning plasma cortisol levels compared to CPAP- treated obstructive sleep apnoea patients (Hudgel *et al*. 1995). This suggests that

obstructive sleep apnoea is associated with a rise in HPA axis activity. In humans, HPA axis activity is assessed by measuring plasma or salivary cortisol concentrations. In rodents, HPA axis activity is assessed by measuring either plasma corticosterone concentrations or faecal glucocorticoid metabolites, which are the end products of corticosterone metabolism (Wasser *et al*. 2000).

Exposure to a stressful environment, such as obstructive sleep apnoea, triggers the hypothalamus to secrete corticotropin releasing hormone (CRH), which is carried by the circulation to the anterior pituitary gland where it causes the release of adrenocorticotropic hormone (ACTH). ACTH interacts with membrane receptors on the adrenal cortex and stimulates the release of corticosterone. This glucocorticoid circulates in the bloodstream and interacts with target tissues to produce an increase in blood glucose, by increasing gluconeogenesis. Corticosterone induces protein catabolism and a decrease in protein synthesis and there may be an increase in liver glycogen formation. Corticosterone is metabolised in the liver. The metabolites are either directly excreted in the urine or accumulate in the gall bladder and are secreted with bile into the intestine and excreted in the faeces (Möstl & Palme, 2002).

An increase in the concentration of corticosterone in the blood is widely used as an indicator of stress (Möstl & Palme, 2002). Rats, as with most

animals, exhibit a circadian rhythm of corticosterone release (Robel *et al*. 1987) and therefore frequent blood sampling would be required. Frequent blood sample collections, however, increase the stress experienced by the animal directly affecting the plasma corticosterone concentration. The advantage of obtaining faecal samples is that they can be easily collected without stressing the animal and the excreted products represent an average of the circulating hormone over a period of time (Harper & Austad, 2000).

In rodents, there is evidence to suggest that there is an association between elevated plasma corticosterone levels and increased body fat mass (Mantha *et al*. 1999). Stress-induced glucocorticoid release is positively correlated with obesity, suggesting that an increase in HPA axis activity may be associated with obesity (Wang, 2005).

1.2.4 Obesity and obstructive sleep apnoea

Obesity can be defined as an accumulation of excess body fat and is becoming the most common nutritional disorder in humans (Chin *et al.* 1999) in developed countries. Approximately 40% of obese individuals develop sleep apnoea and about 70% of sleep apnoea patients are obese (Vgontzas *et al.* 1994).

Extensive research into obesity has shown that the location of body fat deposits, rather than a person's actual size, is more important in determining the risk of developing obesity-linked disorders (Bjorntorp, 1997). Visceral fat accumulation and fat deposited around the neck and abdomen are risk factors for developing obstructive sleep apnoea (Mortimore *et al.* 1998).

Approximately two thirds of middle-aged apnoeic men suffer from visceral obesity (Vgontzas *et al.* 2000). Abdominal fat accumulation leads to a reduction in lung volumes, while increased fat deposition around the pharynx directly narrows the upper airway, creating impedance to airflow (Sinha & Caro, 1998).

Weight loss is often recommended to patients with obstructive sleep apnoea since a reduction in body mass is associated with a decrease in apnoeic events (Schwatz *et al.* 1991). There is evidence, however, to suggest that obstructive sleep apnoea itself, might lead to acceleration in weight gain and impede the patients' ability to lose their excess weight. Increased levels of fatigue, caused by obstructive sleep apnoea, could result in hyperphagia, decreased desire to exercise and excessive weight gain (Wolk *et al*. 2003).

Obstructive sleep apnoea patients tend to have a history of excessive weight gain immediately prior to diagnosis (Phillips *et al.* 1999) and compared to weight-matched control subjects, sleep apnoea patients have greater subcutaneous fat deposition, as indicated by abdominal skinfold thickness (Ip *et al*. 2000). CPAP therapy has been shown to decrease visceral fat accumulation (Wolk *et al*. 2003). This suggests that there is an element of obstructive sleep apnoea which compromises weight loss. Once the sleep disorder is treated, then weight loss may be more likely.

Obesity is a common cause of insulin resistance and this correlation has been well established in humans and in experimental rodent models (Steppan & Lazar, 2004). Patients with obstructive sleep apnoea also present with insulin resistance and increased plasma leptin levels associated with leptin resistance (Ip *et al*. 2002; Shimizu *et al*. 2002). Traditionally these hormonal imbalances have been attributed to the associated obesity, however, the degree of insulin and leptin resistance seen in obstructive sleep apnoea patients is not in proportion to the degree of obesity (Yun *et al.* 2004). Furthermore, non- obese sleep apnoea patients also present with these metabolic abnormalities (Ip *et al*. 2002). This suggests that prior obesity, in the apnoeic patient, might be a contributing factor to the development of insulin and leptin resistance but it is not the only requirement.

There is no evidence to suggest that insulin and leptin resistance are prevalent in patients with other sleep disorders causing sleep disruption such as insomnia and periodic limb movement disorder. Therefore there appears to be a component of sleep apnoea, independent of obesity or sleep disruption, which is responsible for the development of these metabolic abnormalities.

1.2.5 Insulin resistance and obstructive sleep apnoea

Insulin is a protein hormone secreted by the pancreas that decreases the concentration of glucose in the blood by stimulating peripheral glucose uptake and suppressing glucose production in the liver (Frayn, 2001). Insulin resistance is characterised by target tissues becoming desensitised to the actions of this hormone (Vgontzas *et al.* 2000) resulting in a rise in plasma glucose concentrations, possibly leading to type-II diabetes. The rise in plasma glucose results in an increase in insulin secretion leading to sustained hyperinsulinemia.

Patients who experience even mild forms of sleep apnoea develop insulin resistance. An increase in the apnoea-hypopnoea index and increasing severity of the desaturations are associated with a decline in glucose tolerance, independent of obesity (Punjabi *et al.* 2002).

The association with the severity of desaturations suggests that the hypoxic element of sleep apnoea plays a significant role in the development of insulin resistance (Punjabi *et al.* 2002; Tiihonen *et al.* 1993). It has been shown that rats exposed to chronic intermittent hypoxia for three hours a day (with minimal sleep disruption) develop insulin resistance (Djovkar, 1983).

After three months of CPAP therapy, with resolution of the apnoeas, sleep apnoea patients demonstrate improved insulin sensitivity (Harsch *et al.* 2004). However, the effect of CPAP therapy is smaller in obese patients than in non-obese patients (Harsch *et al.* 2004).

Although increased plasma insulin concentrations seen in obstructive sleep apnoea patients can be largely predicted by the obstructive sleep apnoea- obesity relationship, there is evidence to suggest that there is an independent association between obstructive sleep apnoea, obesity and insulin resistance (Vgontzas *et al*. 2000). Insulin resistance is seen in both obese and non-obese patients with obstructive sleep apnoea (Ip *et al*. 2002).

A group of obese patients with obstructive sleep apnoea was compared to age, weight and height matched control subjects. There was a higher degree of insulin resistance in the sleep apnoea patients compared to

weight-matched nonapnoeic control subjects (Vgontzas *et al*. 2000). Furthermore, women with polycystic ovary syndrome (a condition associated with hyperandrogenism and insulin resistance) are more likely to develop obstructive sleep apnoea and daytime somnolence than women without polycystic ovary syndrome (Vgontzas *et al*. 2001). These studies suggest that the insulin resistance seen in obstructive sleep apnoea patients is not entirely caused by obesity, but is also closely related to the sleep disorder.

Not all studies support the hypothesis that there is a strong association between obstructive sleep apnoea and insulin resistance independent of obesity. A group of healthy normotensive individuals were tested for the presence or absence of insulin resistance and sleep disordered breathing. Thirty seven percent of the population studied were insulin resistant and thirty percent had significant sleep-disordered breathing (apnoea hypopnoea index > 10). The lack of correlation between the insulin resistance and sleep-disordered breathing in this group of subjects, after adjusting for obesity, suggested that the relationship between insulin resistance and obstructive sleep apnoea is entirely dependent on obesity and that insulin resistance may be independent of, rather than follow, sleep-disordered breathing (Stoohs *et al.* 1996). This does not explain why non-obese sleep apnoea patients also develop insulin resistance (Ip *et al*. 2002) or why CPAP therapy,

independent of weight loss, results in an improved glucose tolerance (Harsch *et al.* 2004).

An elevation in sympathetic nervous system activity, associated with obstructive sleep apnoea, may contribute to the development of insulin resistance. Upregulation of sympathetic nervous system activity produces elevated catecholamine levels, which stimulate glycogenolysis, gluconeogenesis and glucagon secretion. The increased levels of circulating glucose stimulate insulin release. Over the long term the increased insulin concentration results in the peripheral target tissues becoming desensitised to the actions of insulin and thus insulin resistance (Marshall *et al*. 1991; Strohl *et al*. 1993).

Furthermore, catecholamines have been shown to be insulin antagonists i.e. they compete with insulin for the same receptor binding sites, in the peripheral target tissue, thereby increasing the cells' resistance to the actions of insulin (Strohl, 1996).

We propose that the hypoxic element of sleep apnoea is the major component responsible for the development of insulin resistance and that repeated sleep fragmentation further disrupts this hormonal imbalance, as it does with hypertension.

1.2.6 Leptin resistance and obstructive sleep apnoea

Leptin is a peptide hormone, produced primarily by adipose tissue (Sinha & Caro, 1998). Leptin may also be secreted by other tissues including the placenta and stomach (Mortimore *et al*. 1998; Sobhani *et al*. 2000). In the brain leptin binds to receptors in the hypothalamus to decrease food intake (Sinha & Caro, 1998). Plasma leptin levels are positively correlated with total body fat mass (Sinha & Caro, 1998). The increase in circulating leptin levels in obese subjects should theoretically decrease food intake, suggesting that obesity is characterised by leptin resistance, although this has not been conclusively demonstrated (Maffei *et al*. 1995).

Although there is a strong relationship between leptin resistance and obesity, increased leptin levels are seen in both lean and obese patients with obstructive sleep apnoea (Shimizu *et al*. 2002) and the concentration of leptin is correlated to the frequency of apnoeas (Schafer *et al*. 2002). Like insulin, plasma leptin levels are higher in patients with obstructive sleep apnoea compared to weight-matched control subjects (Ip *et al*. 2000; Phillips *et al*. 2000). This suggests that leptin resistance is not only due to the obesity seen in obstructive sleep apnoea patients, but higher than that expected, due to the sleep disorder.

Elevated plasma leptin levels can be normalized in response to CPAP treatment (Ip *et al*. 2000). In obstructive sleep apnoea patients, CPAP therapy for three to four days resulted in reduced plasma leptin levels without a significant change in body weight (Chin *et al*. 1999), suggesting that increased plasma leptin levels associated with obstructive sleep apnoea is not solely related to body fat accumulation.

1.2.6a Leptin and respiratory depression

Leptin resistance has been associated with respiratory depression and elevated partial pressure of arterial carbon dioxide ($PaCO_2$) (O' Donnell *et al.* 2000; Phipps *et al.* 2002). Obese *ob/ob* mice which lack the gene responsible for leptin production, exhibited respiratory depression and elevated $PaCO_2$ (O' Donnell *et al.* 1999). Leptin deficient mice demonstrated impaired hypercapnic ventilatory responses during wakefulness and sleep (Fitzpatrick, 2002). Leptin replacement studies in genetically obese mice have shown improvements in minute ventilation and hypercapnic ventilatory response during wakefulness and sleep (O' Donnell *et al.* 1999; Tankersley *et al.* 1998). Leptin resistance and impaired ventilatory control could possibly be further compromised by exposure to chronic intermittent hypoxia.

1.2.6b The relationship between leptin and sympathetic nervous system activity

Obstructive sleep apnoea patients have significantly higher muscle sympathetic nerve activity than control subjects (Somers *et al.* 1995). Increased muscle sympathetic nerve activity is positively correlated with plasma leptin levels in healthy men (Chin *et al*. 1999). However, a relationship between plasma leptin levels and muscle sympathetic nerve activity in sleep apnoea patients has not been found (Ip *et al*. 2000).

Exposure to hypoxic conditions is associated with an increase in sympathetic nervous system activity, which could result in changes in plasma leptin concentrations. The expression of the leptin protein and mRNA in isolated human cells is up-regulated in response to hypoxic conditions (Ambrosini *et al.* 2002). An increase in leptin secretion has been shown in human placental tissue exposed to hypoxic conditions both *in vivo* and *in vitro* (Grosfeld *et al*. 2001; Sagawa *et al*. 2002). Although there is evidence to suggest that plasma leptin levels are increased in response to hypoxic conditions, there has been no study examining the effect of chronic intermittent hypoxia on plasma leptin levels, in normal weight rats.

1.2.6c The relationship between leptin and HPA axis activity

Leptin interacts with the HPA axis to maintain metabolic homeostasis (Ahima *et al*. 1998). In rats, leptin administration inhibits glucocorticoid synthesis and secretion in the adrenal cortex in response to restraint stress (Heiman *et al*. 1997). In leptin deficient mice there is a significant elevation of corticosterone levels (Ahima *et al*. 1998). Intraperitoneal administration of leptin has been shown to decrease corticosterone levels before significant weight loss occurred (Ahima *et al*. 1998). Furthermore, corticosterone release in humans and rodents peaks at night, coinciding with the leptin nadir (Ahima & Osei, 2004). This indicates that leptin may modulate HPA axis activity independently of its role in energy balance.

If HPA axis activity is found to be elevated in rats exposed to chronic intermittent hypoxia, then it is reasonable to suggest that the rise in plasma leptin levels could modulate the corticosterone concentrations.

1.2.7 Interactions between insulin and leptin

There is a strong association between insulin sensitivity and circulating plasma leptin levels. In humans, circulating plasma leptin concentrations have been shown to be proportional to the degree of insulin resistance

(DeCourten *et al*. 1997). An increased level of circulating plasma insulin stimulates leptin secretion (Meibner *et al*. 2003) and insulin is known to activate leptin transcription in human adipocytes *in vitro* (Meibner *et al*. 2003).

The effect of nocturnal chronic intermittent hypoxia on insulin sensitivity was investigated in lean and genetically obese mice, which lack circulating leptin. There was no increase in serum insulin concentrations in normal weight mice exposed to intermittent hypoxia for five days when compared to the control mice exposed to intermittent bursts of room air. However, in obese leptin deficient mice exposed to intermittent hypoxia for five days there was a significant rise in fasting serum insulin levels when compared to weight-matched leptin deficient control mice exposed to intermittent air (Polotsky, 2003). This suggests that leptin deficiency plays an important role in the development of insulin resistance even after only five days exposure to chronic intermittent hypoxia. In both leptin resistance and leptin deficiency the impact on the target cell is that of a reduction in leptin. It is possible that leptin resistance may mediate a rise in plasma insulin concentrations in response to chronic intermittent hypoxia.

In mice, leptin administration can down-regulate insulin gene transcription (Kulkarni *et al*. 1997). In isolated human pancreatic islets,

leptin administration has been shown to suppress the release of insulin by the pancreas (Seufert *et al*. 1999). Leptin administration can also stimulate peripheral tissues to increase glucose uptake (Barzilai *et al*. 1997).

1.2.8 Cytokines and obstructive sleep apnoea

Cytokines are regulatory proteins secreted by white blood cells and a variety of other cells in the body. Cytokines are involved in immune system function and are important mediators of inflammatory responses (Thomson, 1992). Several cytokines have been reported to have sleep-regulatory properties (Opp, 2004).

Two cytokines namely; TNF-α and IL-6 are associated with fatigue and insulin and leptin resistance seen in patients with obstructive sleep apnoea, independent of obesity (Vgontzas *et al.* 1997; Vgontzas *et al.* 2000). Elevated circulating IL-6 levels are associated with disrupted sleep (Mills & Dimsdale, 2004) and increased fatigue in obese patients (Vgontzas, 1997). Sleep disturbances are associated with increased daytime levels of TNF-α and IL-6 (Mills & Dimsdale, 2003).

The plasma levels of TNF-α and IL-6 are significantly higher in obstructive sleep apnoea patients than in patients with idiopathic

hypersomnia (Hatipoglu & Rubinstein, 2004). There is evidence to suggest that there is inflammation of the pharynx in obstructive sleep apnoea patients. This swelling not only aggravates the apnoea but sustains the elevation of pro-inflammatory cytokines (Carpagnano *et al*. 2002). These elevated cytokine levels may mediate the increase in sympathetic nervous system activity and increased levels of fatigue (Mills & Dimsdale, 2004). It has been shown that elevated IL-6 levels in apnoeic patients are reduced to normal in response to CPAP treatment (Hatipoglu & Rubinstein, 2004).

Pro-inflammatory cytokines such as TNF-α and IL-6 may have an impact on glucose and lipid metabolism, independent of obesity (Ciftci *et al*. 2004; Vgontzas *et al*. 1997; Vgontzas *et al*. 2000). In a study comparing 43 obese men with obstructive sleep apnoea to 22 obese non-apnoeic control subjects, it was found that sleep apnoea patients had significantly higher plasma IL-6 and TNF-α levels. Similar to insulin and leptin levels, a positive correlation was found between the apnoea hypopnoea index and plasma cytokine levels (Ciftci *et al*. 2004). The cellular mechanism therefore, connecting obesity, leptin and insulin resistance to hypoxia may be through the cytokines.

Similar to leptin, TNF-α and IL-6 are released into the interstitial fluid of adipose tissue (Gotamisligil *et al*. 1993). An excess of adipose tissue,

therefore, could lead to an increased secretion of these cytokines (Visser *et al*. 1999). In humans, plasma TNF-α levels correlate strongly with lipolysis and have been associated with marked insulin resistance and increased leptin secretion (Zumbach *et al*. 1997).

Since elevated TNF-α and IL-6 levels are associated with insulin and leptin resistance, increased levels of circulating TNF-α and IL-6 in obstructive sleep apnoea patients could explain why these patients develop abnormally high plasma insulin and leptin levels, resulting in resistance to these hormones.

The development of leptin and insulin resistance in patients with obstructive sleep apnoea, therefore, cannot be solely attributed to obesity and there is evidence to suggest that the hypoxic element of sleep apnoea may play a significant role in the development of these metabolic abnormalities. It is for this reason that we wanted to develop an animal model for obstructive sleep apnoea which would minimise factors such as stress and sleep disruption, to test the contribution of the hypoxic element of sleep apnoea in the possible development of insulin and leptin resistance, independent of obesity.

1.2.9 An animal model for obstructive sleep apnoea

Several animal models have been developed to induce chronic intermittent hypoxia in a similar fashion to the hypoxia seen in obstructive sleep apnoea patients. The majority of these models do not take possible sleep disruption into account.

Sleep disruption is likely to occur if an animal is exposed to repeated episodes of hypoxia, which are characterised by intermittent bursts of air. These air bursts are likely to disrupt the animals' sleep and create unnecessary stress for the animal. Previous models have used rats or mice housed in plexiglass cylinders and exposed to either bursts of 100% nitrogen for approximately twelve seconds (Fletcher *et al*. 1992) or bursts of hypoxic or hypercapnic air (5% oxygen) (Kanagy *et al*. 2000) followed by bursts of compressed air (21% oxygen). In a similar model chambers were flushed with 100% nitrogen for 30 seconds followed by 100% oxygen (Greenberg *et al*. 1999). In each model a device was used to dissipate the jet of air that entered the chamber.

The main difference in our model is that compressed room air continuously flowed through the plexiglass chambers at a low flow rate, with intermittent bursts of nitrogen only, as opposed to two different bursts of air being flushed into the chambers. In our model a circular

disc with multiple holes was placed at the inlet port of the chamber to dissipate the air flowing in. The nitrogen entered the chambers at a low flow rate to reduce the possibility of disrupting the rats' sleep. Therefore in our model, the "startle" response created by multiple bursts of air was reduced, thus creating less sleep disruption. The rats were adapted to the chambers for three days prior to the start of the experiment to minimise the stress experienced by the rats from being in a novel environment. It is possible that our model created less stress for the animals and thus reduced the potential for sleep disruption.

1.3 Objectives

The aims of this project were firstly, to create an animal model for obstructive sleep apnoea whereby rats receive intermittent bursts of nitrogen during their sleep phase while care was taken to reduce the potential for sleep disruption. Secondly, we wanted to determine the effects of chronic intermittent hypoxia on plasma insulin and leptin levels in normal weight rats. If rats, with no prior obesity, develop insulin and leptin resistance when exposed to chronic intermittent hypoxia, then we would be able to determine if the hypoxic element of obstructive sleep apnoea is the primary determinant for an elevation in plasma insulin and leptin concentrations. Thirdly, we wanted to assess the effects of chronic intermittent hypoxia on HPA axis activity. HPA axis activity is not only an indicator of stress in rats, but has been associated with insulin and leptin homoeostasis.

2.1 Methods and materials

2.1.1 Animals

Male Sprague Dawley rats (n = 10) were individually housed in plexiglass rat cages in a temperature controlled room (T_a = 21 ± 1° C) on a 12-hour light dark schedule (lights on at 07:00). All experimental procedures were approved by the Animal Ethics Committee, University of the Witwatersrand (2003/70/4). Standard rat chow and tap water were available to the rats *ad libitum* during the 12-hour dark phase. Body weights were measured each day throughout the study. At the start of the study the rats were randomly assigned to two weight-matched groups (n = 5/group). The experimental group of rats were exposed to chronic intermittent hypoxia (CIH group) and the control group of rats were exposed to sham hypoxia (SH group). For three days prior to any experimental procedure rats were acclimatised to sleep in plexiglass cylinders (length 300mm, diameter 90mm, volume 1.56ℓ) for seven hours per day. The cylinders had a gas inlet port at one end and an outlet port at the other end. Room air was continually pumped through the chambers at a low flow rate (0.5- 1ℓ/ min). After the three day acclimatisation period, all rats underwent surgery to insert jugular vein catheters.

2.1.2 Surgery

The rats were anaesthetized using Ketamine (Anaket 80mg/kg, im, Centaur labs, South Africa) and Xylazine (Chanazine 4mg/kg, im, Centaur labs, South Africa), for implantation of jugular vein catheters. The catheters consisted of silicon tubing (Silastic, Dow Corning, Midland, USA) with an inner diameter of 0.51mm and an outer diameter of 0.94mm.

A ventral skin incision was made in the neck above the right jugular vein and the underlying tissues were bluntly dissected to expose the jugular vein. A catheter was inserted into a small longitudinal incision made into the jugular vein and held in place with a silk suture (4.0). The jugular vein above the point of entry of the catheter was ligated to prevent bleeding. The free end of the catheter was tunnelled under the skin and exteriorised on the dorsal part of the neck. The ventral neck skin incision was sutured closed with nylon (4.0) in a simple interrupted suturing pattern. Catheters were flushed daily with heparinised saline 0.9%NaCl + 50IU heparin/ml (Bodene (Pty) Limited, South Africa) to ensure patency of the catheters. A small stopper was placed at the exterior end of the catheter to prevent infection and clogging (Silastic, Dow Corning, Midland, USA). For three days after surgery the rats were injected with

enroflaxacin subcutaneously (Baytril 5mg/day, Bayer, South Africa) to prevent possible infection.

2.1.3 Pilot study

A pilot study was done to determine the 24-hour rhythm of plasma insulin and leptin concentrations in a single male Sprague Dawley rat. The rat was housed in a standard rat cage throughout the pilot study and was not exposed to CIH or SH. Standard rat chow and water was available *ad libitum*. The rat was weighed each morning. A jugular vein catheter was implanted into the rat and was flushed daily with heparinised saline as described in section 2.1.2. After a three day recovery period from surgery, 0.8ml of blood was collected from the indwelling catheter at 09:00, 13:00, 17:00, 21:00, 01:00 and 05:00. The blood samples were centrifuged and the plasma stored at -20°C until assayed (as described in section 2.1.5). The results of the pilot study were used to determine the time points for blood collection for the experiment.

2.1.4 Experimental protocol

After a three-day recovery period from the surgery, the rats were exposed to either chronic intermittent hypoxia or sham hypoxia for fourteen consecutive days. All experimental procedures were conducted during the twelve hour light phase, the rats' inactive phase. Each morning the rats were weighed and individually placed in plexiglass cylinders for seven hours.

To induce chronic intermittent hypoxia in the CIH group, the chambers were flushed with 100% nitrogen for nine seconds every 90 seconds, while compressed air continually flowed through the chambers at a low flow rate.

A dampening device at the intake end of the chamber was used to dissipate the air stream so that no direct bursts of air disturbed the rats. The suffusion of nitrogen dropped the fraction of inspired oxygen (F_iO_2) from 20.9% to 12.1 % within eleven seconds and the F_iO_2 returned to 20.9% within 44 seconds. The change in the F_iO_2 in the chambers was assessed using an oxygen analyser (Applied Electrochemistry Inc, California, USA).

The sham hypoxia group of rats were exposed to the same sequence of events as the CIH group of rats except that compressed room air (21%

oxygen) was flushed into the chambers instead of nitrogen. The SH group of rats therefore received the same environmental cues and degree of handling as the rats exposed to chronic intermittent hypoxia. After the seven-hour exposure period each day the rats were removed from their plexiglass chambers and returned to their home cages.

2.1.5 Sample collection

Venous blood samples were collected from all rats on days one, three, five, eight and fifteen to determine plasma insulin and leptin levels. Faecal samples were collected from the rat's cages two days prior to surgery and on the days after blood samples were collected on days two, four, six, nine and fifteen. All faecal samples were collected in the mornings while the rats were in their respective cylinders, thus representing a 17 hour sample period post blood sample collection. The faecal samples were stored in specimen bottles at -20°C until extraction of corticosterones.

Blood samples

A single blood sample (0.7ml) was collected from the indwelling catheters at either, 08:00, 10:00, 12:00 or 14:00. These time points were chosen since rats exhibit a diurnal rhythm of insulin and leptin secretion (Kalsbeek *et al*. 2001; Sánchez *et al.* 2004) as shown in the results of

our pilot study. On the days when blood samples were collected, one blood sample was taken from each rat at one of the selected time points, such that a complete profile was obtained from each rat at the end of the study. On day fifteen all the rats were deeply anaesthetised Ketamine (Anaket 80mg/kg, im, Centaur labs, South Africa) and Xylazine (Chanazine 4mg/kg, im, Centaur labs, South Africa) and 1ml of blood was collected by cardiac puncture at 09:00. The rats were terminated immediately thereafter. All blood samples were centrifuged immediately after collection, the plasma collected and stored at -20ºC.

Analysis of plasma insulin concentrations

Plasma insulin concentrations were analysed using a radioimmunoassay kit 100% specific for measuring rat plasma insulin levels (Laboratory Specialities, Johannesburg, South Africa). The lowest level of rat plasma insulin concentration that could be detected by this assay kit was 0.1ng/ml when using 100 *u*l sample of plasma. In all the assay procedures we used polypropylene glass tubes. Each step was performed in duplicate to ensure accuracy of measurement.

Briefly, assay buffer was pipetted into tubes labelled for non-specific binding (200*u*l), reference tubes (100*u*l) and all sample tubes (100*u*l). Of the sample tubes, seven tubes contained 100*u*l of standard insulin concentration solutions ranging from 0.1ng/ml to10.0ng/ml and two

tubes contained 100*u*l of quality control solutions. Hydrated ^{125}I-Insulin tracer (100*u*l) was then pipetted into all the sample tubes, including two tubes labelled total counts. A rat specific insulin antibody (100*u*l) was pipetted into all tubes except the tubes labelled total counts and non-specific binding. All the tubes were vortexed for 30 seconds each, using a single tube vortex (Heidolph, Germany). The tubes were covered and allowed to incubate overnight at 4°C.

The next morning 1ml of cold (4°C) precipitating reagent was added to all the tubes except the tubes labelled total counts. All the tubes were vortexed and allowed to incubate at 4°C for 20 minutes. All the tubes except the tubes labelled total counts were centrifuged (Sorvall, USA) at 4°C for 20 minutes. Immediately thereafter the supernatant of all the tubes, except the tubes labelled total counts were decanted. Each tube was drained for at least 20 seconds and excess liquid was blotted from the lip of the tubes. The contents of all the tubes were counted in a gamma counter for one minute. The concentration (ng/ml) of rat insulin in the unknown samples was calculated.

Analysis of plasma leptin concentrations

Plasma leptin concentrations were analysed using a radioimmunoassay kit 100% specific for measuring rat plasma leptin levels (Laboratory Specialities, Johannesburg, South Africa). The lowest level of rat plasma leptin concentration that could be detected by this assay kit was 0.5ng/ml when using 100 *u*l sample of plasma. Each step of the assay procedure was performed in duplicate to ensure accuracy of measurement.

Briefly, assay buffer was pipetted into tubes labelled for non-specific binding (300*u*l), reference tubes (200*u*l) and all sample tubes (100*u*l). Of the sample tubes, seven tubes contained 100*u*l of standard leptin concentration solutions ranging from 0.5ng/ml to 50.0ng/ml and two tubes contained 100*u*l of quality control solutions. A rat specific leptin antibody (100*u*l) was pipetted into all the tubes except the tubes labelled total counts and non-specific binding. All the tubes were vortexed for 30 seconds each, using a single tube vortex (Heidolph, Germany). The tubes were covered and allowed to incubate overnight at room temperature.

The next morning 100*u*l of ^{125}I-Rat Leptin was added to all the tubes. All tubes were vortexed and covered and allowed to incubate overnight at room temperature.

The next morning 1.0ml of cold (4°C) precipitating reagent was added to all tubes except tubes labelled total count. All the tubes were vortexed and allowed to incubate at 4°C for 20 minutes. All the tubes were then centrifuged (Sorvall, USA) at 4°C for 20 minutes and immediately thereafter the supernatent of all tubes except the tubes labelled total counts were decanted. Each tube was drained for at least 20 seconds and excess liquid was blotted from the lip of the tubes. The content of all the tubes were counted in a gamma counter for one minute. The concentration (ng/ml) of rat leptin in the unknown samples was calculated.

Faecal samples

Extraction procedure

Faecal steroid metabolites were extracted according to the method described by Good *et al*. (2003). The stored frozen samples were weighed before each sample was homogenised and 0.2g of the sample was placed in a capped tube containing 2ml 100% methanol. The samples were vortexed for 30 minutes using a multitube pulsing vortexer (Heidolph, Germany) and then centrifuged for 20 minutes at 4°C. The

supernatent was decanted and then diluted 1:10 with steroid diluent from the assay kit.

Radioimmunoassay

Analysis of faecal corticosterone concentrations were performed using a double-antibody I^{125} corticosterone radioimmunoassay kit (ICN Biomedicals, Costa Mesa, USA). Each step of the assay procedure was performed in duplicate to ensure accuracy of measurement.

Briefly, steroid diluent was added to tubes labelled non specific binding (0.3ml) and blank tubes (0.1ml). The rest of the tubes contained 0.1ml of one of the following solutions, a corticosterone calibrator of known corticosterone concentration ranging from 25ng/ml to 1 000ng/ml, quality control solutions, and diluted supernatent from the faecal samples. Corticosterone ^{125}I (0.2ml) was added to all the tubes. A rat-specific corticosterone antibody (0.2ml) was added to all the tubes except the tubes labelled non specific binding. All the tubes were vortexed (Heidolph, Germany) and allowed to incubate at room temperature for two hours. After incubation, 0.5ml of precipitant solution was added to all the tubes after which all the tubes were vortexed and centrifuged for 15 minutes. The supernatant from all the tubes was decanted and the rims of the test tubes were blotted with absorbent paper. The precipitate in

each tube was counted in a gamma counter for one minute. The concentration (ng/17 hours) of excreted corticosterones in the faecal samples was calculated.

2.1.6 Data analysis

The change in rats' body weight over the duration of the experiment was calculated by subtracting each rat's body weight before surgery (BS) from their subsequent daily body weight measurement over fourteen days. The values were averaged for each day for all rats in the CIH and SH groups. The average change in body weight between the two groups of rats was determined for each experimental day using an unpaired t-test.

A two-way ANOVA (Statistica, version 5.1, USA) was used to determine if the daily fluctuation in plasma insulin and leptin concentrations had an impact on the plasma insulin and leptin concentrations for a specific day.

Differences in plasma insulin and leptin concentrations between the CIH and SH groups over the fifteen day experimental period were determined using a one-way ANOVA with Tukey Kramer post-hoc test for multiple comparisons.

Within the CIH and SH groups of rats, plasma leptin concentrations on individual days were compared to each other using unpaired t-tests with Bonferroni correction for multiple comparisons.

Differences in faecal glucocorticoid concentrations from two days prior to surgery and over the fifteen day experimental period between the CIH and SH groups were analysed using one- way ANOVA with Tukey Kramer post-hoc test for multiple comparisons. Statistical analysis was performed using the computer software program Graphpad, Instat (GraphPad Software, version 3, California, USA). Statistical significance was set at $p \leq 0.05$ and all data are expressed as mean ± SD.

2.2 Results

2.2.1 Pilot study

Venous blood samples were collected from a single rat at six time points over a 24-hour period. Plasma insulin concentrations ranged from 1.7 ng/ml to 4.3 ng/ml over the 24-hour period (figure 1). Plasma leptin concentrations ranged from 0.7 ng/ml to 2.2 ng/ml over the 24-hour period (figure 2).

2.2.2 Body weights

There was no significant difference in the daily change in body weight between the rats exposed to CIH compared to the rats exposed to SH (figure 3) over the fourteen day exposure period (unpaired t-test $p = 0.4$). The mean starting weight for the CIH group of rats was 213 ± 7g and 215 ± 2g for the SH group of rats.

2.2.3 Plasma insulin concentrations

Differences in plasma insulin concentrations at selected time points for a specific day for each group of rats were determined (two-way ANOVA). There was no significant difference in the plasma insulin concentrations between the selected time points on any day when the blood samples were collected, in either the CIH or SH group of rats, $F(1,6) = 0.02$, $p = 0.9$. There were no significant differences in the plasma insulin concentrations between the CIH and SH groups over the fifteen day experimental period (one-way ANOVA, (figure 4).

2.2.4 Plasma leptin concentrations

Differences in plasma leptin concentrations at selected time points for a specific day for each group of rats were determined (two-way ANOVA). There was no significant difference in the plasma leptin concentrations between the selected time points on any day when the blood samples were collected, in either the CIH or SH group of rats, $F(1,6) = 0.1$, $p = 0.6$. There was a significant increase in plasma leptin concentrations in both the CIH and SH groups on day fifteen, after the fourteen day exposure period (one way ANOVA, $p \leq 0.01$).There were no significant differences in the plasma leptin concentrations between the two groups for the first eight days of the experiment. Within both groups there was a

significant difference in the plasma leptin concentrations between day five and day fifteen however there was no difference between any other day and day fifteen (unpaired t-test, (figure 5).

2.2.5 Faecal glucocorticoid metabolites

Faecal samples were collected two days prior to surgery and on days subsequent to those when blood samples were collected. There was a significant increase in the levels of excreted corticosterones in the CIH group after two days exposure to chronic intermittent hypoxia (figure 6) when compared to the SH group ($p \leq 0.05$). From day four to day fourteen there was no significant difference in the levels of excreted glucocorticoid metabolites between the CIH and SH groups.

Figure 1

24-hour profile of the plasma insulin concentration (ng/ml) for a male Sprague Dawley rat (n = 1). A blood sample was collected at 09:00, 13:00, 17:00, 21:00, 01:00 and 05:00.

Figure 2

24-hour profile of the plasma leptin concentration (ng/ml) for a male Sprague Dawley rat (n = 1). A blood sample was collected at 09:00, 13:00, 17:00, 21:00, 01:00 and 05:00.

Figure 3

Change in body weight of rats exposed to either chronic intermittent hypoxia (CIH, ◇ ,n = 5) or sham hypoxia (SH, □ ,n=5). Data are expressed as the mean ± SD daily gain in body weight (g). D1-D14 represents days of exposure. Between the starting point (0) and D1 there was a four-day period covering surgery and recovery from surgery.

Figure 4

Plasma insulin concentrations for rats exposed to either chronic intermittent hypoxia (CIH, black bars, n = 5) or sham hypoxia (SH, white bars. n = 5). Data are expressed as mean ± SD.

Figure 5

Plasma leptin concentrations for rats exposed to either chronic intermittent hypoxia (CIH, black bars, n = 5) or sham hypoxia (SH, white bars. n = 5). Data are expressed as mean ± SD. * indicates plasma leptin concentrations in the CIH group significantly different compared to day five. † indicates plasma leptin concentrations in the SH group significantly different compared to day five (unpaired t-test, with Bonferroni correction for multiple comparisons, $p \leq 0.05$).

Figure 6

Mean faecal glucocorticoid metabolite concentrations for rats exposed to either chronic intermittent hypoxia (CIH, black bars, n = 5) or sham hypoxia (SH, white bars. n = 5). BS represents mean faecal glucocorticoid metabolite concentrations over two days before surgery. Data are expressed as mean ± SD. * indicates excreted glucocorticoid metabolites in the CIH group significantly different than in the SH group on day two (one-way ANOVA, Tukey Kramer post hoc test $p \leq 0.05$).

2.3 Discussion

There was no difference in the daily change in body weight between rats exposed to CIH when compared to rats exposed to SH. Both groups of rats gradually gained weight throughout the experiment, suggesting that the potentially stressful experimental conditions they were exposed to did not affect body weight. The starting body weights of the rats (214 ± 4g) suggest that they were still within the growth phase. In male Sprague Dawley rats, weight gain tends to slow down once the rats reach approximately 350g (personal communication with the Central Animal Services, University of the Witwatersrand). It is possible that the experimental procedures would have had a more marked effect on body weight if the rats used for the experiment were older and no longer in their growth phase.

Plasma insulin and leptin levels were determined at select time points (08:00, 10:00, 12:00, 14:00) on days one, three, five, eight and fifteen of the experimental protocol. The values obtained from the different time points for a specific day were pooled for that day. There was no change in the plasma insulin concentrations between rats exposed to CIH when compared to rats exposed to SH. This finding indicates that in normal weight rats, exposure to chronic intermittent hypoxia for fourteen days does not alter insulin sensitivity. In a pilot study, the 24-hour plasma

insulin profile from a single rat showed that plasma insulin concentrations tend to fluctuate during the time period when the blood samples were collected. Statistical analysis showed there was no significant difference between the four selected time points and that these plasma insulin fluctuations did not have a significant impact on the plasma insulin concentrations for a given day. The results suggest that in rats, exposure to CIH or SH for fourteen days do not affect insulin levels.

After fourteen days exposure to either CIH or SH, there was a significant increase in plasma leptin levels in both groups of rats on day fifteen. It is interesting to note that there was a significant difference within both the CIH and SH groups between day five and day fifteen, but not between day eight and day fifteen. It is possible that plasma leptin concentrations were starting to rise by day eight, but this increase was not significantly different from day five. The 24-hour plasma leptin profile determined from a single rat in a pilot study showed that plasma leptin concentrations tend to be lower during the daytime when samples were collected, followed by an increase during the nightime. Statistical analysis of the data from the CIH and SH groups showed that there was no significant difference between the plasma leptin concentrations at the four specified time points for a given day. The results suggest that the changes in plasma leptin concentrations between 08:00 and 14:00 did

not have an impact on the plasma leptin concentrations, for a given day. The lack of difference in body weight between the rats exposed to CIH compared to rats exposed to SH, suggests that over the fifteen-day experimental period, the rise in plasma leptin concentrations were not altering eating behaviour. The significant increase in plasma leptin concentrations on day fifteen could be associated with the rats' gradual increase in body weight throughout the experiment, though this is unlikely. The rats' fat weight was not specifically measured, and therefore the rise in plasma leptin concentrations in both groups of rats cannot be ascribed to an increase in body fat. The mean weight of the CIH rats on day fifteen was 314.8 and mean weight off the SH rats on day fifteen was 306.4.The rats could have been starting to slow down growth on day fifteen which could explain the rise in plasma leptin levels on that day.

A sustained elevation in plasma leptin levels may result in the target tissues becoming desensitised to the actions of leptin leading to leptin resistance (Maffei *et al*. 1995). It is therefore reasonable to hypothesise that leptin resistance seen in patients with obstructive sleep apnoea might not be the result of obesity, but rather the result of intermittent hypoxia or sleep disruption. Our results indicate that plasma leptin levels were significantly increased in rats exposed to CIH and SH by day fifteen. It is reasonable to speculate, therefore, that it was not only the

hypoxia that was contributing to the rise in plasma leptin levels in both groups of rats but rather an element of sleep disruption, since the rats exposed to SH also received intermittent bursts of room air. There is, however, no direct evidence linking leptin resistance to sleep disruption. However, sleep disruption is associated with a rise in sympathetic nervous system activity and increased sympathetic nervous system activity has been associated with a rise in leptin secretion in humans (Chin *et al*. 1999).

There is a strong association between insulin sensitivity and plasma leptin levels (DeCourten *et al*. 1997). In humans, plasma leptin concentrations have been shown to be directly proportional to the degree of insulin resistance (DeCourten *et al*. 1997). Obese leptin deficient mice exposed to chronic intermittent hypoxia for five days developed abnormally high plasma insulin levels, when compared to obese mice exposed to intermittent air and normal weight control mice exposed to intermittent hypoxia (Polotsky *et al*. 2003). In mice, leptin administration can down-regulate insulin gene transcription (Kulkarni *et al*. 1997). In isolated human pancreatic islets, leptin administration has been shown to suppress the release of insulin by the pancreas (Seufert *et al*. 1999). Leptin administration can also stimulate peripheral tissues to increase glucose uptake (Barzilai *et al*. 1997). It is reasonable to speculate that the increase in plasma leptin levels in rats exposed to

CIH and rats exposed to SH in our study may have prevented any potential rise in insulin levels resulting from either sleep disruption or exposure to chronic intermittent hypoxia. A rise in plasma insulin might have become evident had the experiment continued for a longer period of time.

There is an association between plasma leptin concentrations and exposure to hypoxic conditions (Ambrosini *et al*. 2002). The expression of the leptin protein and mRNA, in isolated human cells is up-regulated in response to hypoxic conditions (Ambrosini *et al*. 2002). An increase in leptin secretion has been shown in human placental tissue exposed to hypoxic conditions both *in vivo* and *in vitro* (Grosfeld *et al*. 2001; Sagawa *et al*. 2002). A rise in plasma leptin levels in response to hypoxic conditions could explain the increase in plasma leptin levels in the CIH group of rats but it does not explain the rise in plasma leptin levels in the SH group of rats. Since plasma leptin levels were increased both in rats exposed to CIH and rats exposed to SH, it is unlikely that hypoxia was primarily responsible for mediating a rise in plasma leptin in rats exposed to CIH.

Faecal glucocorticoid metabolites were significantly increased in rats after two days exposure to CIH when compared to rats exposed to SH, which returned to the same level as the SH group by day four. The

results indicate that HPA axis activity might have been elevated initially in response to chronic intermittent hypoxia but after day four there was no more evidence of increased HPA activity in the CIH rats. This indicates therefore, that after two days the rats exposed to CIH were probably not experiencing more stress than the rats exposed to SH.

There was no significant change in the concentration of faecal glucocorticoid metabolites in the rats exposed to SH over the experimental period, suggesting that the intermittent bursts of room air did not elicit a sustained HPA axis stress response in this group of rats.

Clinical studies have demonstrated an elevation in sympathetic nervous system activity in obstructive sleep apnoea patients (Narkeiwicz, 1998; Somers, 1995). An elevation in sympathetic nervous system activity is associated with a rise in HPA axis activity, which results in an increase in corticosterones in rodents (Bornstein *et al*. 1997). In rats, leptin administration inhibits glucocorticoid synthesis and secretion in the adrenal cortex in response to restraint stress (Heiman *et al*. 1997). Our results indicate a transient elevation in HPA axis activity in rats exposed to CIH for two days, compared to rats exposed to SH.

In this study we found an elevation in plasma leptin concentrations both in the rats exposed to CIH and SH, but only a transient elevation in

faecal glucocorticoid metabolites in the rats exposed to CIH. As the groups were matched in every other aspect (weight, sex and degree of handling) this transient rise in HPA activity in the rats exposed to CIH could be ascribed to the hypoxia. It cannot be concluded that the rise in plasma leptin concentration in both groups of rats on day fifteen, was mediated by either the intermittent hypoxia or the potential sleep disruption produced by intermittent bursts of air. A second control group, where weight matched rats are placed in similar plexiglass cylinders but only receive a continuous flow of room air at a low flow rate, is required to control for the possible sleep disruption caused by intermittent bursts of room air. The possibility that intermittent bursts of air result in sleep disruption, could have implications for the results of previous studies using the chronic intermittent hypoxia model, as possible sleep disruption was not controlled for.

We acknowledge that the sample size (n = 5) in both groups of rats were small and the length of the experimental protocol was relatively short, compared to other studies where rats were exposed to chronic intermittent hypoxia for thirty days (Greenberg *et al*. 1999) or thirty five days (Bao *et* al. 1997, Fletcher *et al*. 1999) . More definitive results with respect to plasma insulin and leptin concentrations might be obtained with a bigger sample size and if the experiment was allowed to continue for a longer period of time. Another possible limitation of this study is

that blood samples were collected from the rats via jugular vein catheters for the first eight days of the experiment and via cardiac puncture on day fifteen. The anaesthetics administered to the rats on day fifteen could possibly have had an effect on plasma insulin and leptin concentrations for that day.

Our results suggest that the hypoxic component of obstructive sleep apnoea could be mediating a transient increase in HPA axis activity. We are not able to conclude from our results whether hypoxia or sleep disruption could be inducing the observed rise in plasma leptin levels in rats exposed to either CIH or SH.

3.1 Conclusion

In this study we have measured plasma insulin and leptin concentrations and faecal glucocorticoid metabolite concentrations as a marker of HPA axis activity, in a group of normal weight male rats subjected to chronic intermittent hypoxia or sham hypoxia, in a setting which attempted to control for sleep disruption.

The results indicate that normal weight rats exposed to either chronic intermittent hypoxia or sham hypoxia for fourteen days developed elevated levels of plasma leptin. Rats exposed to chronic intermittent hypoxia developed a transient activation of HPA axis activity after two days. There was no difference in the daily weight gain and plasma insulin concentrations between the two groups of rats.

We have set up an animal model of chronic intermittent hypoxia to simulate the hypoxia occurring in obstructive sleep apnoea. We wanted to determine whether it is the hypoxic element of obstructive sleep apnoea, independent of obesity, which is the primary mediator of insulin and leptin resistance. Insulin and leptin resistance are found in patients with obstructive sleep apnoea. We cannot confirm this hypothesis, however, since all the rats developed increased plasma leptin levels. The possibility that non-obese sleep apnoea patients could develop

leptin resistance due to aspects of the sleep disorder resulting in weight gain, which in turn would worsen the sleep apnoea, cannot be excluded. It is possible that patients, who suffer from other sleep disorders with repeated arousals, may develop leptin resistance although this does not seem to be the case.

Hypertension, insulin resistance, hyperleptinemia and central obesity are not only characteristic of obstructive sleep apnoea but also of the metabolic syndrome. Snoring has been associated with the metabolic syndrome in both men and women (Leineweber *et al*. 2003). The metabolic syndrome is associated with chronic activation of the HPA axis and increased cortisol release. Elevated cortisol levels have been shown to induce visceral fat accumulation and induce insulin resistance (Bjorntorp, 2001).

There is a strong association between the metabolic syndrome and obstructive sleep apnoea, considering that both syndromes include obesity, insulin and leptin resistance and elevated cortisol levels in humans. It is possible that obstructive sleep apnoea is an underlying cause of the metabolic syndrome and the chronic intermittent hypoxia rat model could be utilised to test this hypothesis.

Future studies will include determining how prior obesity in rats would contribute towards the development of insulin and leptin resistance

when compared to normal weight rats. The obese rats would be exposed to either chronic intermittent hypoxia or sham hypoxia (with no intermittent bursts of room air) for a longer period of time. By eliminating the intermittent bursts of air in the sham hypoxia group the effects of sleep disruption could be further diminished. Furthermore, measuring plasma levels of tumor necrosis factor-alpha and interleukin-6 in the rats, would clarify the association between these cytokines and insulin and leptin resistance.

In conclusion, short term exposure to chronic intermittent hypoxia or sham hypoxia produces a rise in plasma leptin levels and a transient increase in HPA axis activity in rats. The change in plasma leptin concentrations could be attributed to either intermittent hypoxia or sleep disruption and is not dependent upon prior obesity.

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