INVESTIGATING THE CARDIOVASCULAR AND METABOLIC EFFECTS OF RESISTIN

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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DECLARATION

To the best of my knowledge, this thesis does not contain material that has been accepted for the award of any other degree or diploma in any university. I certify that except where due acknowledgement has been made the work is that of the author alone and does not contain any material which has been published or written by another person, except where due reference is made in the text of the thesis. The content of this thesis is the result of work that has been carried out since the official commencement date of the approved research program and any editorial work, paid or unpaid, carried out by a third party is acknowledged and ethics procedures and guidelines have been followed.

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Signature

Samin Kosari

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Name

…… **December, 2012** …….

Date

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2- Kosari S, Rathner JA, Chen F, Kosari S, Badoer E. Centrally administered resistin enhances sympathetic nerve activity to the hindlimb but attenuates the activity to brown adipose tissue. Endocrinology. 2011 Jul;152(7):2626-33.

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3-SFN Conference, 2011 Washington DC, Poster presentation

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"Resistin reduces thermogenesis by reducing BAT sympathetic nerve activity"

5-SOBR conference, 2011 Melbourne Brain Centre, Poster presentation

"Centrally administered resistin differentially affects sympathetic nerve activity to brown adipose tissue and to the hindlimb"

6-ANS Conference, 2011 NZ, Oral presentation

"Resistin can act in the brain to influence cardiovascular regulation"

7-ASCEPT Conference, 2010 Melbourne, Poster presentation

"Resistin can act in the brain to influence cardiovascular regulation"

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ABBREVIATIONS AND ACRONYMS

AMPK	AMP-activated kinase
ANOVA	Analysis of variance
BAT	Brown adipose tissue
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
CRP	C-reactive protein
CVD	Cardiovascular disease
DAB	3,3'-Diaminobenzidine hydrochloride
ERK	Extracellular regulated kinase
HR	Heart rate
i.v.	Intravenous
ICAM	Intracellular adhesion molecule-1
ICV	Intracerebroventricular
MAP	Mean arterial pressure
NHS	Normal horse serum
NGS	Normal goat serum
PBS	Phosphate buffered saline
PI 3-Kinase	Phosphatidylinositol-3-kinase
PVC	Polyvinyl chloride
PVN	Paraventricular nucleus
RELM	Resistin-like molecule
RT-PCR	Real-time quantitative polymerase chain reaction
SFO	Subfornical organ
SNA	Sympathetic nerve activity
SON	Supraoptic nucleus
TNF-α	Tumor necrosis factor-alpha
UCP-1	Uncoupling protein-1
VCAM	Vascular cellular adhesion molecule
WAT	White adipose tissue

Summary

Introduction

Resistin is an adipokine, originally identified in adipose tissue and its plasma levels are elevated in obesity. Resistin induces insulin resistance and affects energy homeostasis by reducing food intake. Plasma levels of resistin are correlated with the development and severity of heart failure, hypertension and myocardial infarction, suggesting that resistin has cardiovascular effects, however, the mechanisms underlying such effects are unclear. Characteristics of obesity include impaired metabolic regulation and cardiovascular dysfunction such as increased sympathetic nerve activity to the kidney and skeletal muscle vasculature. It is unknown whether resistin affects sympathetic nerve activity.

This thesis investigates (1) the effect of centrally administered resistin on (i) blood pressure and heart rate, (ii) sympathetic nerve activity targeting the kidney and skeletal muscle vasculature, (iii) sympathetic nerve activity to brown adipose tissue and thermogenesis and (2) (i) the intracellular signalling pathways mediating the changes in sympathetic nerve activity affecting the kidney and brown adipose tissue by resistin and (ii) the role of Extracellular regulated kinase-1/2 in the brain in mediating the effects of resistin on brown adipose tissue temperature, body core temperature and protein markers of thermogenesis in brown adipose tissue (uncoupling protein-1 and peroxisome proliferator-activated receptor gamma coactivator 1-α).

Methods

Overnight-fasted Sprague-Dawley rats were anesthetized (induced with isoflurane gas 2.5%-3% in O_2, and maintained with intravenous urethane, 1–1.4 g/kg initially, followed by supplemental doses of 0.05 g/kg as required). Resistin (7ug) or vehicle was administered into

the lateral cerebral ventricle. In separate groups of animals, blood pressure, heart rate, lumbar sympathetic nerve activity, renal sympathetic nerve activity, brown adipose tissue sympathetic nerve activity, brown adipose tissue temperature and body core temperature were recorded before and after the drug administration. To identify the intracellular signalling pathways mediating the effects of resistin on sympathetic nerve activity, resistin was administered in the presence of either the Extracellular Regulated Kinase-1/2 inhibitor (U0126, 7µg) or Phosphatidylinositol-3-kinase inhibitor (LY294002, 5µg) and the response on sympathetic nerve activity to the kidney or brown adipose tissue was measured in different groups of animals. In some experiments the potential central sites of action of resistin were investigated. In those experiments, brains were removed for immunohistochemical processing to detect the protein Fos, a marker of increased neuronal activation. In other experiments, brown adipose tissue was removed and mRNA was extracted to investigate the role of resistin on genetic markers of thermogenesis in brown adipose tissue. The role of Extracellular regulated kinase-1/2 in the brain in mediating the effects of resistin on thermogenesis in brown adipose tissue was also investigated.

Results

In chapter 3, intracerebroventricular resistin was found to produce long lasting significant increases in lumbar and renal sympathetic nerve activity by approximately 40% ($p<0.05$), however, it did not induce a significant change in blood pressure and heart rate. Central administration of resistin significantly increased Fos production in paraventricular nucleus, supraoptic nucleus and subfornical organ ($p<0.05$) but not in the arcuate nucleus. The findings suggest that resistin increases the sympathetic nerve activity to the kidneys and skeletal muscle vasculature, however, it may not be sufficient to increase the blood pressure. Investigating the sites of action of resistin in the brain suggests that the paraventricular

nucleus may be a key brain area mediating the effects of resistin on sympathetic nerve activity.

In chapter four, the increase in renal sympathetic nerve activity by intracerebroventricular resistin was found to be mediated by Phosphatidylinositol-3-kinase in the brain, since inhibition of Phosphatidylinositol-3-kinase by pre-treatment with LY294002 (5µg), significantly prevented the action of resistin on renal sympathetic nerve activity ($p<0.05$, compared to control). Inhibition of Extracellular regulated kinase-1/2 had no effect on the renal sympathetic nerve activity response to resistin.

In chapter 5, intracerebroventricular resistin was shown to significantly reduce brown adipose tissue sympathetic nerve activity by over 50% ($p<0.05$), brown adipose tissue temperature (1.27 ± 0.46 ºC) and body core temperature (1.32 ± 0.49 ºC). The data suggests that resistin reduces thermogenesis in brown adipose tissue. Furthermore, resistin does not have a generalised effect on sympathetic nerve activity since resistin increased lumbar and renal sympathetic nerve activity but reduced brown adipose tissue sympathetic nerve activity.

In chapter 6, the reduction in brown adipose tissue sympathetic nerve activity by resistin was found to be mediated by Extracellular regulated kinase-1/2 in the brain, since pre-treatment with U0126 (7µg), prevented the action of resistin on brown adipose tissue sympathetic nerve activity for approximately 2 hours. Inhibition of Phosphatidylinositol-3-kinase had no effect on the response induced by resistin on brown adipose tissue sympathetic nerve activity. Extracellular regulated kinase-1/2 inhibition in the brain, attenuated the effects of resistin on brown adipose tissue temperature ($p<0.05$) and body core temperature. Intracerebroventricular resistin reduced the gene expression of uncoupling protein-1, a

marker of thermogenesis in brown adipose tissue and this effect was attenuated by inhibition of Extracellular regulated kinase-1/2, although the effects were not statistically significant. Resistin had no effect on peroxisome proliferator-activated receptor gamma coactivator 1-α, a transcription factor involved in thermogenesis.

Summary and conclusion

The results show that intracerebroventricular resistin increases sympathetic nerve activity to the kidney and skeletal muscle vasculature. Elevated sympathetic nerve activity to these cardiovascular organs is observed in obesity, a condition in which resistin levels are increased. Resistin did not significantly change the blood pressure or heart rate, suggesting that increases in sympathetic nerve activity by resistin may not be generalised. This is supported by my finding that intracerebroventricular resistin reduced sympathetic nerve activity to brown adipose tissue. This was accompanied by reduction in brown adipose tissue temperature and body core temperature. The results suggest that resistin increases the sympathetic nerve activity to cardiovascular organs such as kidney and skeletal muscle vasculature, but reduces sympathetic nerve activity to metabolically active organs like brown adipose tissue. Investigating the intracellular signalling pathways mediating the effects of resistin showed that Phosphatidylinositol-3-kinase mediated the effects of resistin on renal sympathetic nerve activity and Extracellular regulated kinase-1/2 mediated the effects of resistin on brown adipose tissue sympathetic nerve activity.

The findings indicate that resistin has differential effects on sympathetic nerve activity to tissues involved in metabolic and cardiovascular regulation. The increased lumbar and renal sympathetic nerve activity and the decreased thermogenesis in brown adipose tissue elicited by resistin suggest that it may contribute to the sympathetic nerve over-activity to

cardiovascular organs and reduced energy expenditure observed in obesity and metabolic syndrome.

CHAPTER 1: Introduction

1.1 Introduction

Obesity is a major social problem, expanding rapidly from western societies to the rest of the world. The growing health concern about obesity for both developed and developing countries has prompted the World Health Organisation to declare obesity as a global epidemic. About 60% of the Australian adult population is either overweight or obese (1). The prevalence of excess weight in children and adolescents is approximately 25%, and it is estimated that within 30 years approximately 60% of children will be overweight. Obesity is a well-characterized independent risk factor for cardiovascular disease and high blood pressure (2).

The sympathetic nervous system plays a major role in homeostasis. The level of activity in sympathetic nerves innervating blood vessels and the kidneys is a major determinant of blood pressure. Increased sympathetic nerve activity to the blood vessels and kidneys are characteristic of obesity and metabolic syndrome (3, 4). In obesity, body mass index is independently correlated with sympathetic nerve activation, suggesting sympatho-stimulating effects are related to visceral adiposity (4).

Adipose tissue is not only a storage site for fat but is known to act as a major endocrine organ that synthesises and releases a variety of different hormones including leptin, adiponectin, and resistin, collectively known as adipokines. Leptin is the most intensively studied adipokine. Plasma levels of leptin are increased in obesity and it acts centrally to increase sympathetic nerve activity (SNA) to the skeletal muscle vasculature and kidneys (5-7).

Adiponectin is the only other adipokine that has been studied in relation to effects on sympathetic nerve function. Adiponectin reduces sympathetic nerve activity, thus opposing the actions of leptin. Adiponectin levels are decreased in obesity and its levels are negatively correlated to hypertension in obesity whilst leptin levels are positively correlated. The findings highlight the important role of adipokines on SNA regulating cardiovascular and metabolic functions. With the exception of leptin and adiponectin, however, little is known about the effects of other adipose-derived hormones, for example resistin.

Resistin is a newly discovered adipokine, originally identified in adipose tissue (8), but now known to be expressed in smaller amounts in a variety of tissues including the hypothalamus (9, 10). It belongs to a family of cysteine-rich proteins capable of inducing insulin resistance. The plasma levels of resistin are reported to be increased with obesity and type 2 diabetes (8, 11-16), and together with its influence on insulin sensitivity, it has been suggested that resistin may be a link between type 2 diabetes and obesity (8). Resistin also has a role in the regulation of metabolism by decreasing food intake, and its expression is influenced by dietary intake, such that the level of resistin in plasma is increased by feeding and reduced by fasting (8).

Several epidemiological studies have shown that resistin is associated with cardiovascular disease for example plasma resistin levels were reported to be correlated with the development and severity of heart failure, ischemic heart disease, hypertension and vascular disease (14, 17-25), however, its influence on SNA had not been investigated prior to my work.

This thesis investigates the role of resistin on SNA to cardiovascular tissues and metabolic organs. In this first chapter, I provide a description of resistin and its distribution, and

discuss its cardiovascular and metabolic actions and highlight the gaps in the knowledge about cardiovascular and metabolic functions of resistin.

1.2 Resistin and its structure

Resistin is a member of the resistin-like molecule (RELM) hormone family. Two other members of the RELM family include RELM-alpha and RELM-beta. All RELM family members are characterized by ten conserved cysteine residues. Resistin and RELM-beta contain an additional cysteine near their amino termini, which is conserved among species (26). The rsesistin gene is located on chromosome 19 in humans and on chromosome 8 in mice (8, 27). The mouse resistin genomic sequence displays 46% sequence identity with the human resistin sequence (27). Human and mouse resistin show 64% sequence identity at the mRNA level while the two proteins exhibit 59% identity at the amino acid levels (8, 27). Resistin is secreted as a disulphide-linked homotrimer (8) and circulates in plasma as either a trimer or a hexamer (28). Human resistin has 108 amino acids while in rodents, resistin has 114 amino acids.

The receptor for resistin has not been clearly identified to date, however, several studies have investigated the potential receptors for resistin in rodents and humans. It has been suggested that Delta Decorin, which is a cleavage product of decorin lacking the glycation site, may serve as a functional receptor for resistin in adipocyte progenitor cells in mice (29).

In 3T3-L1 cells, tyrosine kinase-like orphan receptor-1 was found to be inhibited by resistin, resulting in modulating glucose uptake and promoting adipogenesis (30). A recent study in 2013 by Benomar et al (31), identified Toll-like receptor-4 as a binding site for resistin in the

hypothalamus in rats. In human cells, Toll-like receptor-4 may serve as a receptor for the pro-inflammatory effects of resistin (32).

1.3 Tissue distribution of resistin

Resistin is found in number of different tissues in the periphery and in the brain.

1.3.1 Peripheral distribution of resistin

The plasma levels of resistin are reported to be about 7.3-14.3 ng/ml in humans (15) and 35.7-42.9 ng/ml in rats (33). In rodents, white adipose tissue is the main source of resistin. Resistin mRNA expression has been shown in white adipose tissue of mice (8) and rats (34). In mice, resistin mRNA levels vary as a function of the white adipose depot and gender, with the highest level of expression in female gonadal fat. Immunohistochemistry of epididymal white adipose tissue showed that the resistin protein was abundant in adipocyte cytoplasm (8).

In contrast to rodents, the expression level of resistin mRNA in human adipocytes is low and is about 1/250 of that in the mouse (10). Resistin in humans is mainly produced by macrophages (10). In obesity, macrophages that infiltrated into visceral white adipose tissue are the predominant source of resistin (35).

Resistin has also been detected in a variety of other tissues in the periphery including adrenal glands (36, 37), gastrointestinal tract (37), liver (38), human pancreatic islets (39), pituitary gland (9), skeletal muscle (37), bone marrow, breast, colon, heart, kidney, lung, liver, prostate, spleen, testis, thymus, thyroid, uterus (36) and synovial fluid of patients with rheumatoid arthritis and osteoarthritis (40). Resistin mRNA expression was found in human placenta where it is localised to trophoblastic cells, suggesting the possibility of a role for

resistin in modulating insulin sensitivity during pregnancy (41, 42). Finally resistin has been identified in brown adipose tissue (BAT) in rat (37), however, the role of resistin in BAT physiology is not clearly understood.

1.3.2 Central distribution of resistin

Resistin mRNA has been detected in hypothalamus in rodents (43) and in humans it has been found in the whole brain (36) and cerebrospinal fluid (44).

Hypothalamus

By using in situ hybridization, Tovar et al (43) detected mouse resistin mRNA expression in the arcuate nucleus, ventromedial nucleus and hippocampus.

They showed that following the intracerebroventricular (ICV) administration of resistin (10 micrograms) in rats, the only hypothalamic region to show resistin-induced Fos protein expression was the arcuate nucleus of fasted but not fed rats. This observation suggests that the hypothalamus may be more responsive to resistin in the fasted state.

Cerebrospinal fluid

Resistin was detected in human cerebrospinal fluid (CSF) by Kos et al (44), however, in that study, the raised circulating resistin levels did not correspond to higher CSF levels, suggesting limited uptake into the CSF, possibly by an active transport mechanism across the blood brain barrier. This, of course, does not exclude local production of resistin in the brain.

1.4 Resistin in obesity and type 2 diabetes

Resistin levels are elevated in obesity and diabetes (8, 11). An increase in serum resistin levels can increase blood glucose levels by inducing insulin resistance (45). For example in

rodents, over-expression of resistin or administration of exogenous resistin decreased insulin sensitivity and altered glucose metabolism. Blocking resistin activity by neutralising antibodies improved insulin sensitivity and restored glucose homeostasis (46). Furthermore, resistin-knockout mice have lower fasting blood glucose levels and enhanced glucose tolerance and insulin sensitivity (47). There are several studies in humans indicating a positive correlation between resistin levels and increased obesity, visceral (16, 48, 49) and abdominal fat (50), body mass index, fasting blood glucose (14, 15) and insulin resistance (51).

Although the majority of studies confirm the correlation between resistin levels and obesity and type 2 diabetes, a few authors have reported that resistin was not increased in patients with severe insulin resistance and type 2 diabetes (15, 52). These contrasting findings may be due to differences in ethnic backgrounds, average body mass index and gender of the participants. Thus the metabolic role of resistin in humans remains controversial not only due to these studies but also due to the fact that resistin in humans is mainly produced by macrophages that have infiltrated into the adipose tissue while in rodents it is produced by adipocytes.

The recent report from Lazar's lab (53) suggests a physiological role of resistin in glucose homeostasis. They created a transgenic mouse model that lacks mouse resistin but produces human resistin. When those mice were placed on a high-fat diet, there was an increase in human resistin production which induced insulin resistance. This result was similar to the reports from wild-type mice in which adipocyte-derived mouse induced insulin resistance. The findings provide strong evidence that although the site of resistin production differs between species, resistin is physiologically important in glucose homeostasis.

1.5 Cardiovascular disease in obesity

Obesity is an independent well-characterized cardiovascular risk factor. It is also linked to cardiovascular disease through diabetes, hypertension, dyslipidemia and vascular disease (2, 54, 55). Overweight or obesity is a major social problem. The growing public health concern surrounding obesity and subsequent cardiovascular or metabolic disease have focused attention on treatment and prevention of these conditions. Thus, understanding the mechanisms underlying obesity, and linking obesity with cardiovascular or metabolic disease, is crucial for designing therapeutic strategies to target obesity and its cardiovascular and metabolic complications.

1.6 Excessive sympathetic activation in obesity and metabolic syndrome

In obesity and metabolic syndrome, there is an increase in sympathetic nerve activity to the skeletal muscle blood vessels and to the kidneys (3, 4), (56). For example many studies indicate that obese humans have about 50-100% higher levels of muscle sympathetic nerve activity compared with their non-obese peers (56).

In obesity, body mass index is independently correlated with renal norepinephrine spillover rate (57) and sympathetic nerve activation (4). Interestingly, weight loss is associated with a reduction in muscle sympathetic nerve activity (58) and whole body norepinephrine spillover (59). More detailed study on fat composition showed that abdominal visceral fat plays an important role in the link between obesity and sympathetic overactivity since muscle sympathetic nerve activity has been reported to be about 40% higher in men with higher abdominal visceral fat compared with subjects with lower abdominal visceral fat but with the same total fat mass (60). The sympathetic nervous system plays an essential role in the

regulation of metabolic and cardiovascular functions. Sympathetic over-activity is characteristic of a number of cardiovascular disorders such as hypertension and heart failure (4, 61).

1.6.1 Role of adipocytokines in sympathetic overactivity

Studies have revealed that adipose tissue in addition to storing fat also produces and secretes different hormones. Some of these hormones have been implicated in cardiovascular pathophysiology such as leptin, adiponectin and resistin (2). Studies on two adipocyte-derived hormones, leptin and adiponectin, have revealed a role for them in regulating sympathetic nerve activity (6, 62-65). Resistin, a recently discovered adipocytokine elicits many metabolic effects that are similar to leptin. For example leptin and resistin levels are increased in obesity and correlate with the severity of cardiovascular disease in humans (Fig 1.1). It is unknown however, whether resistin has any direct effect on regulating sympathetic nerve activity. This is an important question because any effect of resistin on sympathetic nerve activity may contribute to the altered cardiovascular function in obesity.

1.6.2 Leptin

Leptin is the most well-known adipocytokine. It was discovered by Friedman in 1994 (66). Leptin levels are increased in obesity in proportion to fat mass and leptin is well known to act in the central nervous system to reduce food intake and increase energy expenditure by regulating neuropeptides in the hypothalamus such as neuropeptide-Y & agouti-related protein and stimulating proopiomelanocortin neurons in the arcuate nucleus of the hypothalamus (67).

Clinical studies have revealed that leptin is an independent risk factor for coronary heart disease. It induces platelet aggregation and promotes arterial thrombosis, inflammation and contributes to cardiovascular disease such as hypertension (68). Leptin is known to increase sympathetic nerve activity to the kidney, adrenal gland and skeletal muscle vasculature (5, 69) (Fig 1.2). This increased sympathetic nerve activity is now believed to be an important contributor to obesity induced hypertension in which the sympathetic nerve activity and arterial blood pressure are both increased (70, 71).

Leptin also increases sympathetic nerve activity to the BAT suggesting an important physiological role of leptin in the regulation of thermogenesis (62) (Fig 1.2). In rodents, brown adipose tissue is the primary thermogenic organ which has a significant role in regulating thermogenesis and energy expenditure (72).

1.6.3 Adiponectin

Adiponectin levels in plasma are decreased in obesity and it is the only other adipocytokine known to influence SNA (73). Adiponectin suppresses renal SNA and reduces blood pressure while increasing sympathetic outflow to brown adipose tissue (64, 65) (Fig 1.2).

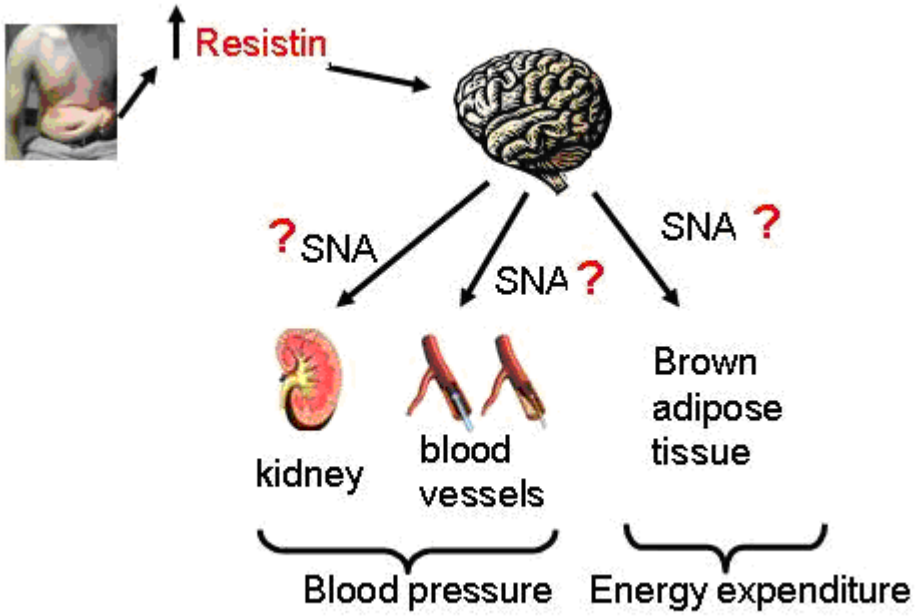

Figure 1.1 The adipokine resistin is released from adipose tissue. Effects of resistin on sympathetic nerve activity (SNA) to kidney, skeletal muscle vasculature and brown adipose tissue are not known.

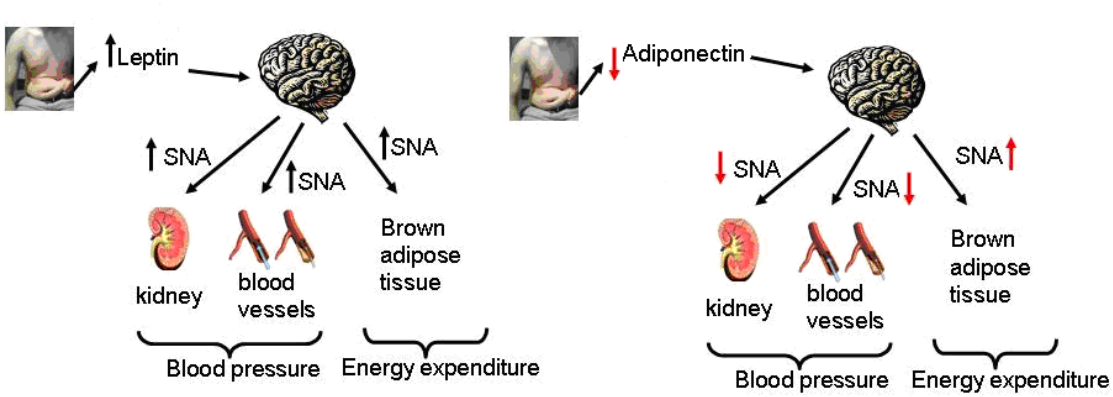

Figure 1.2

The adipokines leptin and adiponectin are released from adipose tissue. Left Panel: Leptin acts centrally to increase sympathetic nerve activity (SNA) to blood vessels, kidney and brown adipose tissue. Right Panel: Adiponectin reduces SNA to the kidneys and skeletal muscle blood vessels but increases SNA to brown adipose tissue.

1.7 Cardiovascular impact of resistin

1.7.1 Hypertension

Several studies have shown that plasma resistin levels are associated with either the presence or the development of high blood pressure in humans. A positive association between plasma resistin levels and hypertension, measured using 24 hours ambulatory blood pressure monitoring, has been observed (21, 24). In adults with hypertension, higher circulating plasma resistin levels were associated with a lower estimated glomerular filtration rate and higher albuminuria. This association highlights the possible contribution of resistin to the progression of chronic renal disease in hypertensive patients (74-76).

It is of great interest that increased plasma resistin levels has been observed in a young healthy population with a positive family history for essential hypertension (77). Findings from this study as well as a study on the population of women without previous history of hypertension or diabetes, suggested that plasma resistin levels may have a predictive value since plasma resistin levels were associated with the risk of developing hypertension over the 14 years of follow up (78).

Although the majority of studies have shown a correlation between plasma resistin levels and hypertension, the contribution of resistin to hypertension remains to be clarified as there are some studies that have not observed a significant correlation (79, 80) or found a correlation only in patients with other risk factors present such as type 2 diabetes (81).

1.7.2 Heart function

Resistin is expressed in the heart and increased expression has been found in diabetic hearts (82). This can be clinically important since resistin has been shown to reduce insulin stimulated glucose uptake in cardiomyocytes (83). This action can impair cardiac function and contribute to the incidence of cardiomyopathy in diabetic patients (84).

Resistin may also influence the function of the heart by reducing contractility, increasing apoptosis and fibrosis in the heart (82, 85). Increased fibrosis and hypertrophy are characteristics of cardiomyopathy. Resistin can also induce cardiac hypertrophy (82) and recently this has been shown to involve reduced activity of AMP-activated protein kinase and increased activity of mammalian target of rapamycin (86). These findings together with the observations that resistin increased pro-fibrotic mediators such as fibronectin and collagen 1 and 2 (85), reinforces the potential role of resistin in diabetic cardiomyopathy.

Plasma resistin levels are elevated following myocardial infarction (87). Of greater interest, are reports that plasma resistin levels are correlated with the incidence of myocardial infarction (but not ischemic stroke) in a large study of 26490 people (88) suggesting that plasma resistin levels may be predictive of myocardial infarction. More recent studies also confirm an association between plasma resistin levels and ischemic heart disease (18) and incidence of atrial fibrillation (89).

Given that myocardial infarction is a major cause of heart failure, one would expect that plasma resistin levels should correlate to heart failure. In two clinical studies of over 2000 participants each, the plasma resistin levels have indeed been correlated to the incidence of heart failure; Butler et al (19) followed elderly patients with an average age of 74 years for about 9.4 years, whilst Frankel et al (20) followed patients who were younger (average age

of 60 years) for approximately 6 years. These studies strongly suggested that plasma resistin concentrations are predictive of new incidence of heart failure, independent of other cardiovascular risk factors. More recent studies have also confirmed an association between plasma resistin levels and ischemic heart disease (18), incidence of atrial fibrillation (89) and progression of chronic heart failure (17).

1.7.3 Atherosclerosis and Inflammation

Inflammation in obesity

As obesity develops, there is a progressive infiltration of macrophages into the adipose tissue. The infiltration of macrophage causes changes in adipocyte size and function. For example, in obesity, adipocytes begin to secrete low levels of tumor necrosis factor-alpha (TNF-α) which contributes further to this inflammation. Increased secretion of leptin in combination with decreased production of adiponectin by adipocytes may also contribute to macrophage accumulation by stimulating the transport of macrophages into the adipose tissue and promoting adhesion of macrophages to endothelial cells resulting in chronic peripheral inflammation (90). This chronic peripheral inflammation observed in obesity is also accompanied by inflammation in the brain. The adipokine leptin has been shown to contribute to central inflammation by stimulating the release of interlukin-1β (IL-1β) from microglial cells in the brain (91).

Since resistin in humans is primarily produced by macrophages (10, 92, 93), many studies have confirmed the strong correlation of serum resistin levels with circulating inflammatory markers including TNF-α, IL-6 and C-reactive protein (CRP). (13, 14, 94-96). This correlation becomes important because CRP levels are associated with high blood pressure (94) and atherosclerotic cardiovascular disease in humans (97). Resistin also stimulates the

secretion of inflammatory cytokines such as TNF-α, IL-12, IL-8, IL6 and monocyte chemoattractant protein-1 (MCP-1) (98).

White adipose tissue inflammation

In 2009, using a mouse model of human hyper-resistinaemia, Qantani et al (53) demonstrated that with a high fat diet, there is an increase in human resistin expression in WAT and macrophage infiltration into the WAT, which is augmented by a resistin-dependent increase in the MCP-1 leading to further inflammation, muscle lipid accumulation and insulin resistance. This effect of macrophage-derived human resistin in WAT contributes to promoting and maintaining an inflammatory environment such as that observed in metabolic syndrome in humans.

Vascular inflammation and atherosclerosis

Plasma resistin levels have been correlated with markers of inflammation, including C-reactive protein, TNF-α and IL6 in plasma (99), and it is well known that inflammatory cytokines are key mediators in atherosclerotic plaque formation (100). Resistin has been found in atherosclerotic plaques in mice and humans, and is produced by macrophages that have infiltrated the vessel wall (101). Additionally, resistin mRNA levels in atherosclerotic lesions progressively increase during the development of atherosclerosis (51). This suggests that resistin may have a role in atherosclerosis and in the cardiovascular sequelae, and this is supported by various clinical studies. In a cross-sectional study on asymptomatic subjects with a family history of premature coronary artery disease, plasma resistin levels correlated with coronary artery calcification, a quantitative index of atherosclerosis (99). Patients diagnosed with premature coronary artery disease were also found to have higher serum levels of resistin (102). In patients with multi-vessel coronary artery disease, elevated

plasma resistin was a strong, independent predictive factor of major adverse cardiac events (103).

The mechanisms by which resistin influences atherosclerotic plaque formation are under intense investigation at present. Evidence suggests that resistin increases adhesion molecules, chemo-attractant proteins and smooth muscle cell proliferation (101, 104-106). Resistin increases both the expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1) by endothelial cells (101, 104, 105, 107), increases VLA-4 (very late antigent-4) on monocytes, increases migration of monocytes and enhances the attractant effects of MCP-1 (101). Together, these contribute to monocyte-endothelial cell adhesion and the resultant infiltration into- and retention of monocytes within the blood vessel wall. Recently, it was found that the effects of resistin on ICAM-1 VCAM-1 are mediated by p38MAPK-dependent pathways (105). Resistin has also been reported to increase lipid uptake into macrophages suggesting it can facilitate the macrophage to foam cell changes that occur in atherosclerotic plaques (108). Resistin has also been reported to alter endothelial function and increase the production of reactive oxygen species which could further contribute to vascular inflammation, atherosclerosis and the cardiovascular sequelae (109).

1.8 Metabolic actions of resistin

1.8.1 Insulin resistance and glucose homeostasis

Resistin reduces insulin sensitivity by attenuating insulin receptor phosphorylation, insulin receptor substrate 1 phosphorylation, phosphatidylinositol-3-kinase (PI 3-Kinase) activation, phosphatidylinositol triphosphate production, and activation of protein kinase B/Akt (110). Furthermore, resistin has been shown to inhibit AMP-activated kinase (AMPK) in liver and muscle (47, 111) and to activate suppressor of cytokine signalling 3 (SOCS-3) in mouse adipose tissue (110). AMPK is known to inhibit hepatic gluconeogenesis and stimulate muscle glucose uptake (112) and SOCS-3 is known to suppress insulin signalling in several tissues (113-115). These effects can explain in vivo experiments that administration of resistin to rodents either centrally (111) or peripherally (45, 111), or over-expression of resistin (116) increase glucose levels.

1.8.2 Food intake

Resistin gene expression in adipose tissue suggests that it may play a role in energy regulation because resistin mRNA levels are influenced by nutritional status, for example down-regulated by fasting and up-regulated by feeding (8).

Tovar et al (43) assessed the effects of central resistin on food intake in rats. They found that ICV injection of resistin exerted an anorectic effect on food intake. This effect was transient as it was observed only during the first 90 minutes after the ICV injection. The acute anorectic effect of resistin was associated with changes in the neuropeptide genes expression involved in the central regulation of food intake. Resistin was associated with (i) decreased neuropeptide-Y and agouti-related protein expression and (ii) increased cocaine and amphetamine-regulated transcript gene levels in the arcuate nucleus of the hypothalamus

(117). These effects are similar to leptin (67). The results suggest that resistin and leptin might regulate food intake via similar pathways.

1.8.3 Metabolism

Central administration of resistin induced the expression of fat-promoting enzymes in the liver and adipose tissue in a nutrition-dependent manner, being increased in the liver of fed and in the adipose tissue of fasted rats suggesting that hypothalamic resistin can promote fat storage by increasing the lipid synthesis in the liver and WAT (117).

Tovar et al (43) showed that although ICV resistin reduces food intake, repeated central administration of resistin over several days, did not lead to significant changes in body weight. Thus, resistin reduces energy intake by acting centrally to acutely reduce food intake but this may not be sufficient to reduce body weight, suggesting that resistin may alter energy expenditure.

One of the important components of energy expenditure is thermogenesis in brown adipose tissue (BAT). The main function of BAT is heat production by transferring energy from food into heat. This physiological activity of the tissue is under the control of norepinephrine released from sympathetic nerves, acting on adrenergic receptors, predominantly β-3. The rate of thermogenesis is centrally controlled in the hypothalamus. When there is a need to increase the rate of heat production, a signal is transmitted via the sympathetic nervous system to the brown adipocytes. The intracellular pathways involve cAMP, protein kinase A and increased the activity of uncoupling protein-1 (UCP1, thermogenin).

UCP1, a marker of thermogenesis, is a member of uncoupling proteins found in mitochondria in BAT, it allows for mitochondrial combustion of substrates, independent of

the production of ATP. The outcome is that an increased fraction of the food and the oxygen available in the blood is taken up by the tissue and combusted therein, leading to an increased heat production (118).

There has been considerable discussion lately about the role of thermogenesis and BAT in metabolic regulation in humans. Until recently, BAT was believed to be present only in infants; however, it is now recognized that BAT is present and is metabolically active in adults (119). Stimulation of thermogenesis in BAT may have dramatic effects on energy expenditure because calculations show that activation of 40–50 g of BAT in humans could result in a 20% increase in energy expenditure (120) and could have dramatic effects on weight loss.

The adipokine, leptin, is known to stimulate thermogenesis in BAT by increasing BAT SNA (63). Leptin also reduces fat tissue mass and lipid accumulation, preventing the development of obesity (121). In contrast, resistin promotes fat storage by increasing lipid synthesis (117), however, the role of resistin on energy expenditure or thermogenesis in BAT is not known (Fig 1.1).

1.9 Summary

In summary, resistin is an adipokine, originally identified in adipose tissue and its plasma levels are elevated in obesity. Resistin induces insulin resistance and affects energy homeostasis by reducing food intake. Plasma levels of resistin are correlated with the development and severity of heart failure, hypertension and myocardial infarction, suggesting that resistin may have cardiovascular effects, however, the mechanisms underlying such effects are unclear. Characteristics of obesity include impaired metabolic

regulation such as imbalance between energy intake and energy expenditure, and cardiovascular dysfunction such as increased SNA to the kidney and skeletal muscle vasculature. Prior to my work, it had not been investigated whether resistin affects SNA. This thesis investigates the effect of resistin on (i) SNA targeting the cardiovascular organs, (ii) thermogenesis in BAT and (iii) the intracellular signalling pathways mediating those effects.

1.10 AIMS

The specific aims of the present work are to investigate:

1- The role of resistin on lumbar sympathetic nerve activity and the central sites in the hypothalamus that are activated by resistin.

2- The role of resistin on renal sympathetic nerve activity and the intracellular mechanisms involved.

3- The role of resistin on brown adipose tissue sympathetic nerve activity and the intracellular mechanisms involved.

4- The effects of resistin on thermogenic markers in BAT and role of ERK1/2 in mediating the effects of resistin on thermogenesis in BAT.

Chapter 2: Materials and Methods

2.1 Instrumentation

2.1.1 Manufacture of blood vessel cannulae

To measure blood pressure and allow intravenous infusion of drugs, cannulae were manufactured to be inserted into the femoral artery and vein. To manufacture the cannulae, two different sizes of polyvinyl chloride (PVC) tubings were used. The smaller tube (catalogue number 530010 – internal diameter of 0.28 mm and outer diameter of 0.61 mm; Biocorp, VIC, Australia) was inserted approximately 20 mm into a pre-cut 20 cm length of the larger bore tubing (catalogue number 530055- internal diameter 0.80 mm and outer diameter 1.20 mm, Biocorp, VIC, Australia) and the two were connected using Araldite ® Epoxy Resin (Selleys Pty Ltd; NSW, Australia) glue. The glue was moulded into a ball to assist in tying the cannula in place following implantation. The smaller tube was trimmed at a 45 ° angle so that it extended a minimum of 30-40 mm past the ball of glue (Figure 2.1)

2.1.2 Manufacture of glass Micropipettes

Glass micropipettes were made to allow intracerebroventricular injections. To manufacture the glass micropipettes a P-97 Flaming/Brown micropipette puller (Sutter Instrument Company, CA, USA) was used. These glass micropipettes (Accu-fill 90®, Clay Adams, Becton, Dickson and Co., NJ, USA) were placed in the micropipette puller machine. The puller was programmed (Program details: Heat 740; Pull 40; Velocity 50; Time duration 110s) to produce pipettes with an external diameter of approximately 60 μm and an internal diameter of approximately 30 μm when the shaft was trimmed down to 12 mm in length (Figure 2.2).

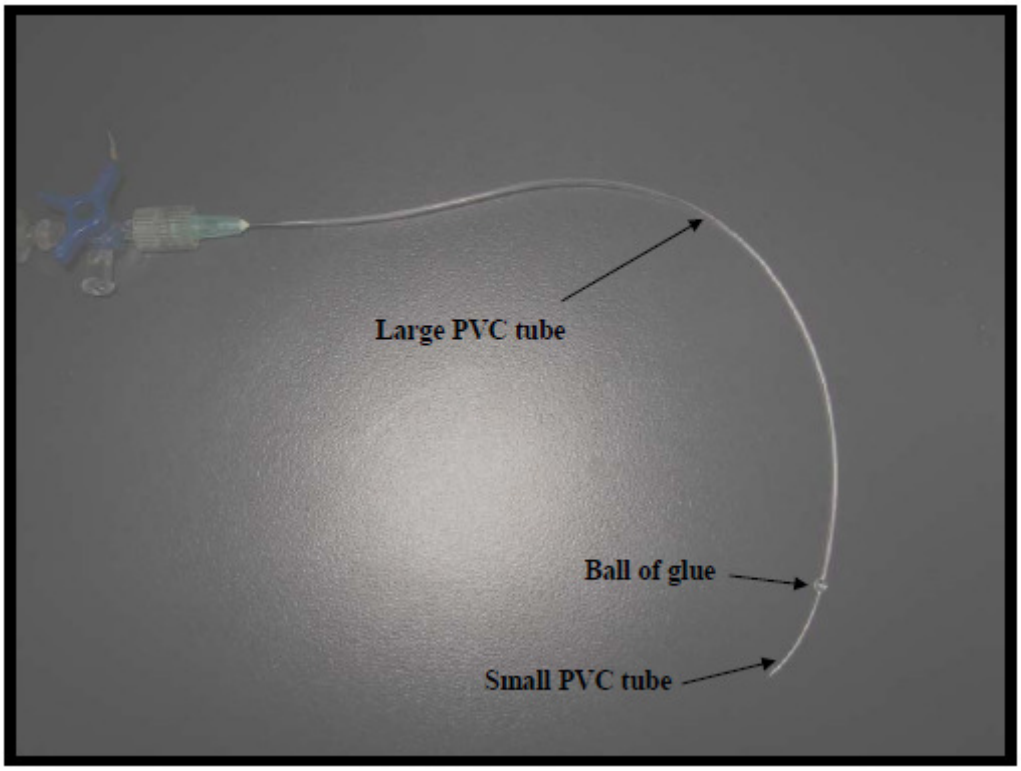

Figure 2.1 – Photo of a typical blood vessel cannula. The tube of smaller bore is inserted approximately 20 mm into the larger bore tubing and held fast by Araldite ® Epoxy Resin glue. The glue was then moulded into a ball to assist in tying the cannula in place following implantation. The smaller PVC tube was trimmed at a 45°angle so that it extended a minimum of 30-40 mm past the ball of glue.

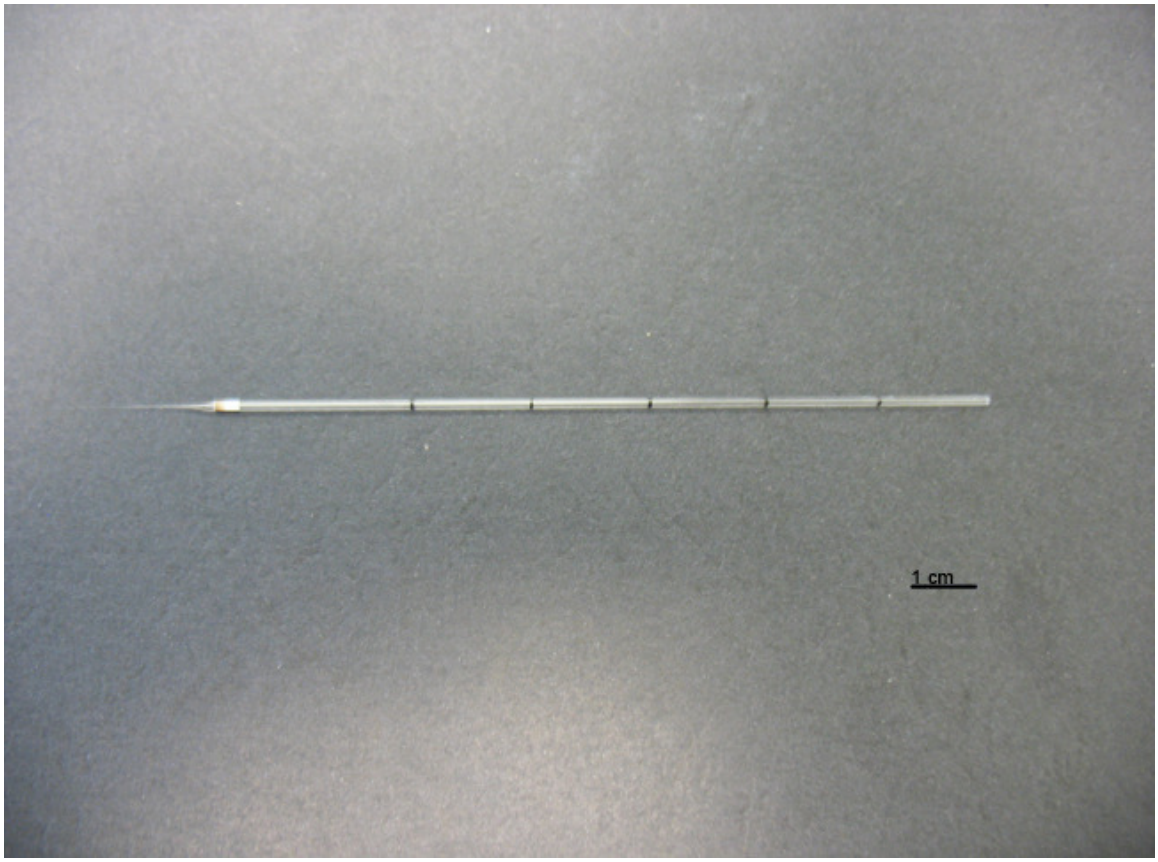

Figure 2.2- Glass micropipette used for intracerebroventricular microinjections.

2.1.3 Manufacture of electrodes

For recording sympathetic nerve activity signals, a bipolar electrode was manufactured using two Teflon-insulated stainless steel wires (catalogue number: AGT1025, World Precision Instrument Inc., FL, USA). The tips of the bipolar electrode were bared from the Teflon-insulation for about 5mm and were folded to form a hook. The electrode tips were 4 mm apart. The other end was soldered to two gold plated pins to allow connection to an amplifier. The electrode was insulated in a PVC tube (catalogue number 530055- internal diameter 0.80 mm and outer diameter 1.20 mm, Biocorp, VIC, Australia) to reduce electrical interference (Figure 2.3).

2.1.4 Thermistor calibration

Brown adipose tissue temperature was measured using a thermistor probe.

The thermistor probe was connected to a multimeter (Fluke 73III, Fluke Australia, NSW, Australia) and calibrated to accurately measure the temperature between 20°C to 40°C using a water bath and a mercury thermometer**.**

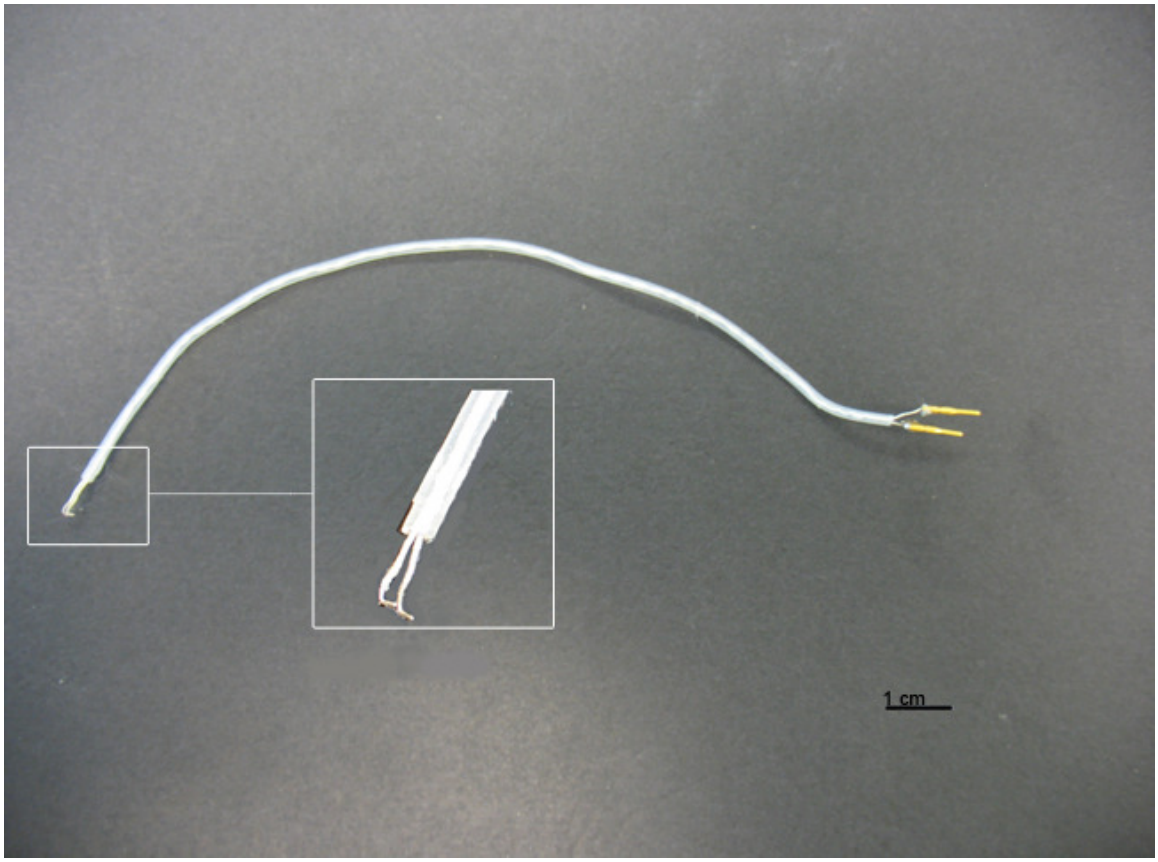

Figure 2.3- Bipolar Teflon-insulated stainless steel electrode wire insulated in a polyvinyl chloride tube.

2.2 Surgical preparations of animals

2.2.1 Animals and housing

Male Sprague-Dawley rats were obtained from Monash University Animal Services (Victoria, Australia) and ARC (animal resources centre, W.A, Australia). Animals typically had a body weight ranging between 240-320 grams on the days of the experiments. The animals were housed 2-4 in a cage in a temperature-controlled room on a 12:12 hour light / dark cycle (lights on at 7:00 A.M.) in the Animal Facility (RMIT University, Victoria, Australia), where rat chow and tap water were available *ad libitum*. Animals were acclimatized for approximately 7 days prior to the experiment.

All experimental protocols were performed in accordance with the Prevention of Cruelty to Animals Act 1986 (Australia). These protocols conform to (1) the "Guiding Principles for Research Involving Animals and Human Beings" (2) and the guidelines set out by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2007 (National Health and Medical Research Council of Australia) and were approved by the Royal Melbourne Institute of Technology (RMIT) University Animal Ethics Committee. Every attempt was made to minimize animal suffering, discomfort and reduce the number of animals needed to obtain reliable results.

2.2.2 Cannulation of femoral artery and vein

The cannulation of the femoral artery was performed to enable continuous monitoring of blood pressure, whilst the femoral vein was cannulated to enable infusion of urethane to maintain anaesthesia during the experiments. Under general gaseous anaesthesia (2.5–3% isoflurane in O_2), the right groin region was shaved and swabbed with 70% alcohol before a 5-7 mm incision was made through the skin over the femoral neurovascular bundle. The subcutaneous fascia was cleared by a blunt dissection to expose the underlying bundle, and the vessels separated by clearing the femoral sheath. The femoral vein was cannulated first. Two lengths of fine thread (silk 3/0, Dynek Pty, Australia) were passed under the vessel and pulled tight to elevate the vessel and temporarily stop the blood flow. A few drops of the local anaesthetic, lignocaine (Sigma Aldrich, NSW, Australia), were placed onto the vessel to be cannulated to avoid vasospasm. Using very fine McPherson-Vannas 8cm scissors (World Precision Instruments Inc., FL, USA), a small cut, about one-third through the diameter of the vessel, was made in the vessel. Closed fine tweezers # 5 (World Precision Instruments Inc., FL, USA) were inserted into the cut and expanded slightly to enable the cannula tip to be introduced into the vessel. The tweezers were then withdrawn and the cannula, filled with heparinised saline (50 U/mL) was threaded 30-40 mm along the vessel and secured around the ball of glue of the cannula. The femoral artery was cannulated using a similar procedure as described. Upon completion of the cannulation procedures, the incision was closed using stiches (silk 0-USP, Dynek Pty, Australia). The free end of the arterial cannula was connected to a transducer for recording blood pressure using a PowerLab data acquisition system (ADInstruments, NSW, Australia). The heart rate was calculated from the arterial pressure pulse. The free end of the venous cannula was used for intravenous infusion.

2.2.3 Temperature recording

BAT temperature

Interscapular brown adipose tissue (BAT) was exposed through an incision in the nape of the neck. Temperature within the interscapular BAT was recorded by placing the tip of a thermistor probe into the interscapular BAT (Fluke 73III, Fluke Australia, NSW, Australia). The thermistor probe was secured by silk suture (silk 0-USP, Dynek Pty, Australia) to the skin. The probe was calibrated in a water bath using a mercury thermometer**.**

Body core temperature

Body core temperature was measured by a thermometer placed in the rectum (Fluke 52II thermometer, Fluke Australia, NSW, Australia). The temperature was measured at 15 minute intervals.

2.2.4 Nerve dissection and recording

Lumbar nerve

After a midline abdominal incision and retraction of the intestines, the abdominal aorta and vena cava were gently pulled aside to expose the lumbar nerve. The left sympathetic trunk lies on the psoas muscle located under the aorta. The left lumbar postganglionic sympathetic nerve trunk was identified and dissected free of surrounding tissue. With the aid of an operating microscope, the nerve was placed onto the bared tips of two Teflon-coated silver wire electrodes. Lumbar nerve activity was made audible with an audio amplifier. When optimal nerve activity was confirmed by observing the rhythmic bursts of nerve traffic, the wires of the electrode and the isolated lumbar sympathetic nerve were embedded in a two-component silicone gel (Kwik-Cast Sealant, WPI) to insulate the nerve-electrode junction

from surrounding tissue. Once the silicone gel had hardened, two small pieces of gauze were placed around the electrode to support the electrode position. The incisions were sutured closed and the free end of the wire electrode was connected to the recording equipment.

Brown adipose tissue nerve

Rats were placed prone, and the head was mounted in a Stoelting stereotaxic frame (Stoelting, IL, USA). To avoid hypothermia and maintain the body core temperature, animals were placed on an insulated heat pad. Interscapular brown adipose tissue (BAT) was exposed through an incision in the nape of the neck. The fat pad was divided along the midline and reflected laterally. The postganglionic BAT sympathetic nerve which is a fine branch of the intercostal nerves that innervate the right interscapular BAT was identified and dissected free of surrounding tissue under mineral oil to prevent the nerve from dehydration. The nerve was transected where it entered BAT and placed onto the bared tips of two Teflon-coated silver wire electrodes. The nerve activity was made audible with an audio amplifier. The optimal nerve activity was confirmed by observing the rhythmic bursts of nerve traffic responding to changes in body core temperature.

Renal nerve

After a flank incision, the left kidney was exposed retroperitoneally. With the aid of retractors, the muscle and fat tissue were pulled aside. With the aid of an operating microscope, a renal nerve was carefully dissected free of surrounding tissue under mineral oil. The distal end of the nerve was cut and the nerve was placed onto the bared tips of two Teflon-coated silver wire electrodes. Renal nerve activity was made audible with an audio amplifier. When optimal nerve activity was confirmed by observing the rhythmic bursts of nerve traffic, the wires of the electrode and the isolated renal sympathetic nerve were

embedded in a two-component silicone gel (Kwik-Cast Sealant, WPI) to insulate the nerve-electrode junction from surrounding tissue.

The sympathetic nerve activity of either the lumbar, the BAT or the renal nerve was amplified using a low-noise differential amplifier (models ENG 187B and 133, Baker Institute, Victoria, Australia), filtered (band pass 100–1,000 Hz), rectified, and integrated (at 2.5 s intervals for the BAT SNA and 0.5 s intervals for lumbar and renal SNA). The signal was recorded using a PowerLab data acquisition system (ADInstruments, NSW, Australia). At the end of the experiment, nerve activity was verified as sympathetic postganglionic nerve activity by observing an elimination of activity after IV injection of a sympathetic ganglion blocker (hexamethonium, 10 mg/kg, Sigma, Australia). Background noise was determined by post-mortem measurements.

2.2.5 Microinjection into the lateral ventricle (ICV)

Each animal was placed prone, and the head was mounted in a Stoelting stereotaxic frame, such that bregma and lambda were positioned on the same horizontal plane. For exposure of the dorsal surface of the brain, a hole (4 mm diameter) centred 0.7 mm caudal and 1.8 mm lateral from bregma, was drilled into the skull. After the drilling procedure, the hole was covered with cotton wool soaked in normal saline to prevent drying of the exposed surface. Injections were made unilaterally using a fine glass micropipette (50–70 μm tip diameter) inserted into the lateral brain ventricle (stereotaxic coordinates: 0.7 mm caudal to bregma, 1.8 mm lateral to midline, and 3.7 mm ventral to the surface of the dura). After the microinjection, the micropipette was left in place for 1 min. At the end of the experiment a small amount of pontamine sky blue was microinjected using the same coordinates to confirm microinjection into the lateral ventricle (Figure 2.4).

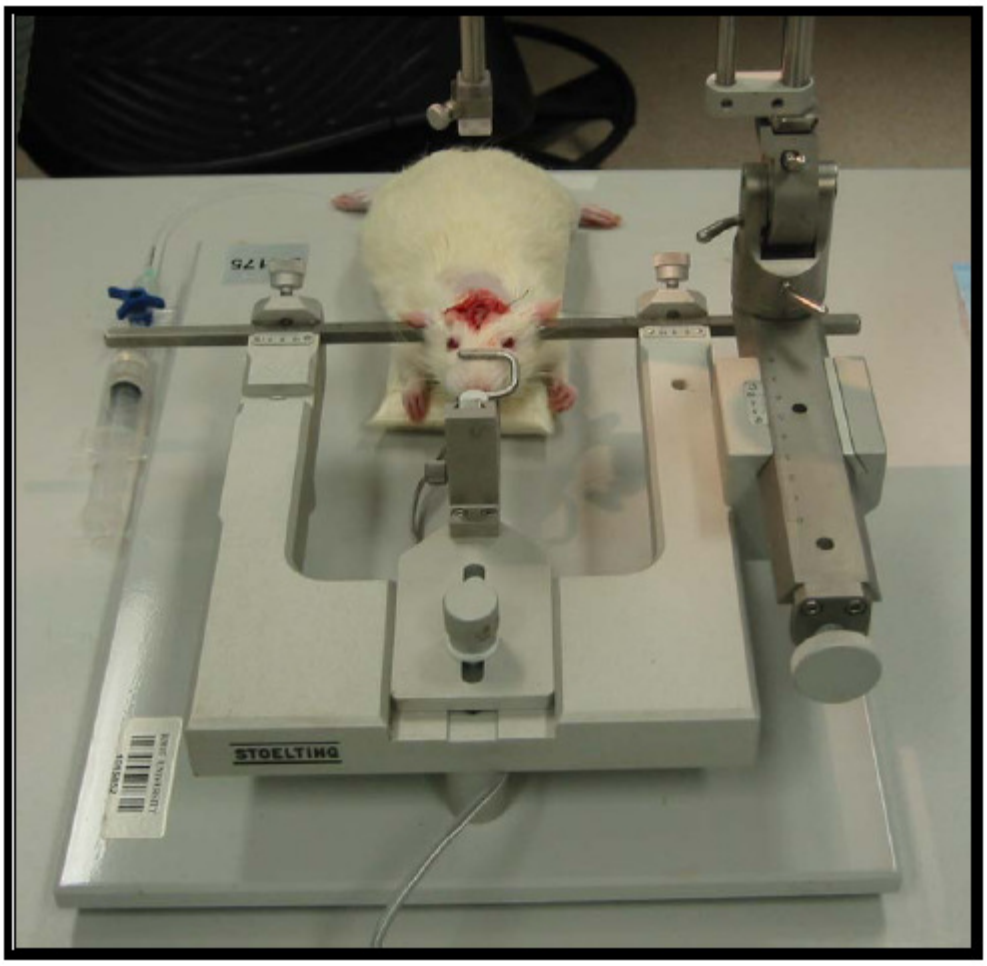

Figure 2.4 – Rats were positioned prone and the head was mounted and stabilised in a Stoelting stereotactic frame.

2.2.6 Tissue collection

At the end of the experiments, animals were euthanized and brain and brown adipose tissue were collected.

Brain

Following a midline incision of the skin extending approximately from between the eyes to the level of T2, the muscle layers were scraped away to reveal the occipital bone and vertebrae. Using rongeurs, the spongy part of the occipital bone was carefully removed to reveal the cerebellum, and then the cerebral hemispheres and brainstem. The dura mater was peeled back from the brain surface, followed by two transverse cuts, one at the level of the olfactory bulb and the other at the caudal end of the medulla. A long thin spatula was used to raise the brain and the optic nerves were cut. Then the brain was carefully removed and transferred into fixative solution (see section 2.3).

Brown adipose tissue

BAT was exposed by an incision in the nape of the neck. The fat pad was divided along the midline and reflected laterally. Several small pieces of the BAT (about 100mg) were carefully dissected and rapidly excised, frozen in liquid nitrogen and stored at -80°C.

2.2.7 Drugs

Recombinant rat resistin (lot#L16251/F, L28370,), recombinant rat leptin (lot#AQP2411031), LY294002 (lot#3-S13213W) and U0126 (lot#3-Z5175W) were purchased from Sapphire Biosciences (Australia). DMSO (lot#109K2350) was obtained from Sigma (Australia). Artificial cerebrospinal fluid contained NaCl 124mM, KCl 3.0mM, $NaH_2PO_4.2H_2O$ 1.3mM, $MgCl_2.6H_2O$ 2.0mM, $NaHCO_3$ 26mM, glucose 10mM, $CaCl_2$ 2.0mM in Milli-Q water, buffered with carbogen.

2.3 Immunohistochemistry experiments

2.3.1 Fixing the brain

After removing the brain from the skull, brains were fixed in the fixative solution, containing 4% PFA for four hours and then placed in phosphate buffered saline (PBS) containing 20% sucrose solution and stored at 4 ºC.

2.3.2 Sectioning of the rat brain

Serial coronal sections (40 µm thick) of the brain were cut using a a Leica CM 1900 cryostat (Leica Microsystems Nussloch GmbH, Nussloch, Germany). One in five sections was collected, placed onto gelatine-coated slides, dried for 2 hours at room temperature and then processed immunohistochemically to detect Fos protein using standard immunohistochemical procedures.

2.3.3 Immunohistochemical Staining procedures

To identify activated neurons by using Fos as a marker of neuronal activation, immunohistochemistry for the protein Fos was performed. The sections were incubated and processed using standard immunohistochemical procedures as follows. Sections underwent washes in PBS (3 times for 5 min duration) between each incubation. Endogenous peroxidase activity was destroyed by incubating with 0.5% H_2O_2 for 30 minutes. The sections were incubated in 10% normal goat serum (NGS) for 60 minutes prior to 0.5% Triton X-100 (10 minutes) to facilitate antibody penetration. The sections were incubated in anti-Fos primary antibody (rabbit polyclonal IgG , c-Fos (K-25): sc-253, Santa Cruz Biotechnology, CA, USA; dilution: 1:400) for 24 hours at room temperature. The sections were incubated for 1 hour with (i) biotinylated secondary antibody (1:600, Anti rabbit raised in goat, B8895, Sigma Aldrich, Australia) and subsequently (ii) Extravidin (1:400, Sigma Aldrich, Australia) for 1 hour. Both incubations were at room temperature. Then the sections were incubated in 0.05% 3,3′-diaminobenzidine hydrochloride (DAB; sigma Aldrich, Australia) in Tris buffer (0.05 M, pH 7.6) for 10 minutes. The reaction was initiated by adding 5 µl of 17.5% hydrogen peroxide and terminated by washes with fresh Tris buffer. Finally the sections were allowed to dry and were coverslipped using Depex mounting medium.

2.3.4 Photomicroscopy

Photographic images were acquired using a digital camera (Sensi Cam, PCO CCD Imaging, Kelheim, Germany) on an Olympus BX60 microscope (Olympus Inc, PA, USA). The digital images obtained were imported into Adobe Photoshop ® 7 (Adobe Systems Incorporated, CA, U.S.A) and the contrast and brightness were modified for presentation purposes.

2.4 Real time PCR

2.4.1 RNA Extraction and Quantification by real time PCR

Brown adipose tissue RNA extraction was performed using a TRIzol-based kit (Invitrogen, Melbourne, Australia, Cat No 12183-018A). Approximately 30 mg of brown adipose tissue was placed in 500 µl of TRIzol and homogenised with a handheld, motorised Teflon pestle. Samples were allowed to stand for a few minutes, after which chloroform (1/5 volume TRIzol) was added and the sample vigorously shaken. Samples were allowed to stand for 5 min and spun at 12,000g for 15 min at 4ºC after which the upper aqueous phase was transferred to a new tube. The aqueous phase was precipitated by mixing with isopropanol alcohol (1/2 volume TRIzol). Samples were incubated at room temperature for 10 min and then centrifuged at 12,000g for 15 min at 4ºC. The supernatant was removed and the resulting pellet was washed with 200 µl of 70% ethanol in diethylpyrocarbonate-treated water. After elution through a spin cartridge, RNA was extracted and then quantified using a QUANT-iT analyser kit (Invitrogen, Melbourne, Australia, Cat No Q32852). The RNA samples were diluted as appropriate to equalise concentrations, and stored at -80˚C for subsequent reverse transcription.

First-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen, Melbourne, Australia) in a final reaction volume of 20 µl. All RNA and negative control samples were reverse transcribed to cDNA in a single run from the same reverse transcription master mix. A serially diluted pooled RNA sample from the control group was produced and also included to ensure efficiency of reverse transcription and for calculation of a standard curve for real-time quantitative polymerase chain reaction (RT-PCR). Quantification of mRNA (in duplicate) was performed on a 72-well Rotor-Gene 3000 Centrifugal Real-Time Cycler

(Corbett Research, Mortlake, Australia). Taqman-FAM-labelled primer/probes for UCP1 (Cat No. Rn 00562126_m1), PGC-1α (Cat No. Rn00580241_m1) and Deiodinase Type II (Cat No. Rn00581867_m1) were used in a final reaction volume of 20 µl. PCR conditions were 2 min at 50 ºC, 10 min at 95 ºC then 40 cycles of 95 ºC for 15 s and 60 ºC for 60 s. 18S ribosomal RNA (18S rRNA) (Cat No. Hs99999901_s1) was used as a housekeeping gene to normalize threshold cycle (CT) values. The relative amounts of mRNAs were calculated using the relative quantification (ΔΔCT) method (122).

2.5 Statistical analysis

2.5.1 Mean arterial pressure and heart rate

The basal levels of MAP and HR prior to ICV injections were compared between the treatment and control groups using Student's unpaired t-test. Changes in MAP and HR were compared between groups in each experimental series by using two-way ANOVA with repeated measures. All results are expressed as means ± SE. $P<0.05$ was considered to be statistically significant.

2.5.2 Sympathetic nerve activity

The integrated SNA was calculated over a period of 1-2 minutes at each time point, subtracted from the noise level and expressed as a percentage of the resting level prior to the ICV injections. Changes in SNA were compared between groups in each experimental series by using two-way ANOVA with repeated measures. All results are expressed as means ± SE. $P<0.05$ was considered to be statistically significant.

2.5.3 BAT & Body Core Temperature

The basal levels of body core temperature and BAT temperature prior to ICV injections were compared between the groups using Student's unpaired t-test. Changes in body core temperature and BAT temperature were compared between groups in each experimental series by using two-way ANOVA with repeated measures. All results are expressed as means ± SE. $P<0.05$ was considered to be statistically significant.

2.5.4 Immunohistochemistry (FOS)

Fos-positive cell nuclei were counted unilaterally in 3 sections containing the paraventricular nucleus, (Anterior, mid and caudal levels), 2 sections containing the supraoptic nucleus (at the anterior and mid levels of the paraventricular nucleus) , 2 sections containing the arcuate nucleus (located within 0.5mm caudal to the paraventricular nucleus) and 1 section containing the subfornical organ. The overall mean number of Fos-positive nuclei in each area were calculated and compared between the resistin-treated and the control group using Student's unpaired t-test.

Chapter 3: Effects of resistin on blood pressure, heart rate, lumbar and renal sympathetic nerve activity

3.1 Introduction

Resistin has been linked to cardiovascular disease and there is emerging epidemiological evidence showing that plasma resistin levels are associated with the development and severity of heart failure (19, 20, 22, 23, 123) and hypertension (21, 24, 77, 78). A positive association between plasma resistin levels and hypertension, measured using 24 hours ambulatory blood pressure monitoring, has been observed (21, 24). Interestingly, increased plasma resistin levels has been observed in a young healthy population with positive family history for essential hypertension (77). Furthermore, a study on the population of women without previous history of hypertension or diabetes, suggested that plasma resistin levels may have a predictive value since plasma resistin levels were associated with the risk of developing hypertension over the 14 years of follow up (78).

A characteristic of both heart failure and hypertension is an elevation of sympathetic nerve activity (SNA) (4, 61). Similarly in obesity and metabolic syndrome, there is an increase in sympathetic nerve activity to the skeletal muscle blood vessels and to the kidneys (3, 4, 56). For example many studies indicate that obese humans have about 50-100% higher levels of muscle sympathetic nerve activity compared with their non-obese peers (56). In obesity, body mass index is independently correlated with renal norepinephrine spillover rate (57) and sympathetic nerve activation (4). This may contribute to the cardiovascular complications observed in obesity, which is a recognized risk factor for cardiovascular disease, hypertension and type 2 diabetes.

Leptin is another adipokine which has some similarities to resistin. Plasma levels of leptin are increased in obesity and cardiovascular disease (124). Leptin is known to increase sympathetic nerve activity to the kidney, adrenal gland and skeletal muscles vasculature (5, 69). This increased SNA is now believed to be an important contributor to sympathetic over-activity observed in obesity and obesity-induced hypertension (70, 71).

Similar to leptin, plasma levels of resistin are elevated in obesity, metabolic syndrome and cardiovascular disease (12-16, 19-21, 24, 47). Whether resistin can influence SNA to different organs has not been investigated to date. The primary aims of this study were to determine the acute effects of resistin on sympathetic nerve activity to the skeletal muscle vasculature (lumbar, SNA) and to the kidneys (renal SNA), end-organs that are important in cardiovascular regulation and in fluid and electrolyte balance. Blood pressure and heart rate were also monitored. Since the receptor for resistin is not clearly identified to date, I investigated the distribution of the protein Fos, a marker of increased neuronal activity, in the hypothalamus to gain an insight into the potential central sites of action of resistin.

3.2 Methods

3.2.1 Experimental protocols

Rats were fasted overnight before the experiment. On the day of the experiment, anesthesia was induced using isoflurane gas (2.5–3%) in O_2. The femoral vein was cannulated to enable infusion of the drugs and to maintain anesthesia (iv urethane 1–1.4 g/kg initially, followed by supplemental doses of 0.05 g/kg as required). The femoral artery was cannulated to allow the measurement of arterial pressure. Mean arterial pressure and heart rate were calculated as explained in detail in Chapter 2 (Section 2.2).

Intracerebroventricular (ICV) administration: In these experiments the mean arterial pressure, heart rate and either lumbar SNA or renal SNA were recorded for 3 to 4 hours following the ICV administration of resistin (7µg in 7µl) or vehicle (artificial cerebrospinal fluid 7µl).

Intravenous administration: In three urethane-anaesthetized rats, resistin (7µg in 7µl) was administered intravenously via a femoral vein. Mean arterial pressure, heart rate and lumbar SNA were recorded for four hours.

In all experiments, resting levels were recorded for at least 10 min prior to the injection of resistin or vehicle. Following the injections, all variables were monitored continuously and recorded every 15 minutes over the observation period.

3.2.2 Immunohistochemistry for Fos protein

On completion of the ICV administration of resistin / vehicle in the experimental series recording lumbar SNA, the animals were euthanized with an overdose of pentobarbitone

(300mg/kg, IV) and were decapitated. The brains were removed, cut and processed for immunohistochemistry to detect Fos protein as explained in Chapter 2 (Section 3).

3.2.3 Statistical analysis

The mean arterial pressure and heart rate data from the two experimental series monitoring lumbar SNA and renal SNA have been combined because no differences were observed between the experimental series in these variables. The resting levels of mean arterial pressure and heart rate before the injections were compared between the resistin and control groups using Student's unpaired t test. The integrated SNA was calculated over a period of 1-2 minutes at each time point and expressed as a percentage of the resting level prior to the injections. Changes in mean arterial pressure, heart rate, lumbar SNA or renal SNA were compared between groups in each experimental series by using two-way ANOVA with repeated measures. Quantification of Fos-positive nuclei was performed as explained in chapter 2 section 5.4. All results are expressed as means ± SE. $P < 0.05$ was considered to be statistically significant.

3.3 Results

3.31 Effect of resistin on mean arterial pressure

The resting levels of mean arterial pressure before the ICV administration of vehicle and resistin are shown in the bar graphs in Fig. 3.1. There were no significant differences in the resting levels of mean arterial pressure between the two groups (Fig. 3.1). After the administration of resistin, there was an acute increase in mean arterial pressure by approximately 5 mmHg within the first 15 minutes but returned to resting levels soon after (Fig 3.1). This effect was not significantly different from the control group (Fig. 3.1).

3.3.2 Effect of resistin on heart rate

The resting levels of heart rate before the ICV administration of vehicle and resistin are shown in the bar graphs in Fig. 1. There were no significant differences in the resting levels of heart rate between the two groups (Fig. 3.1). After the ICV injection of resistin, the effect on heart rate was quite variable over the 4-h observation period (Fig. 3.1). After vehicle administration, heart rate was variable and not markedly affected. There was no significant difference in the heart rate responses between the two groups.

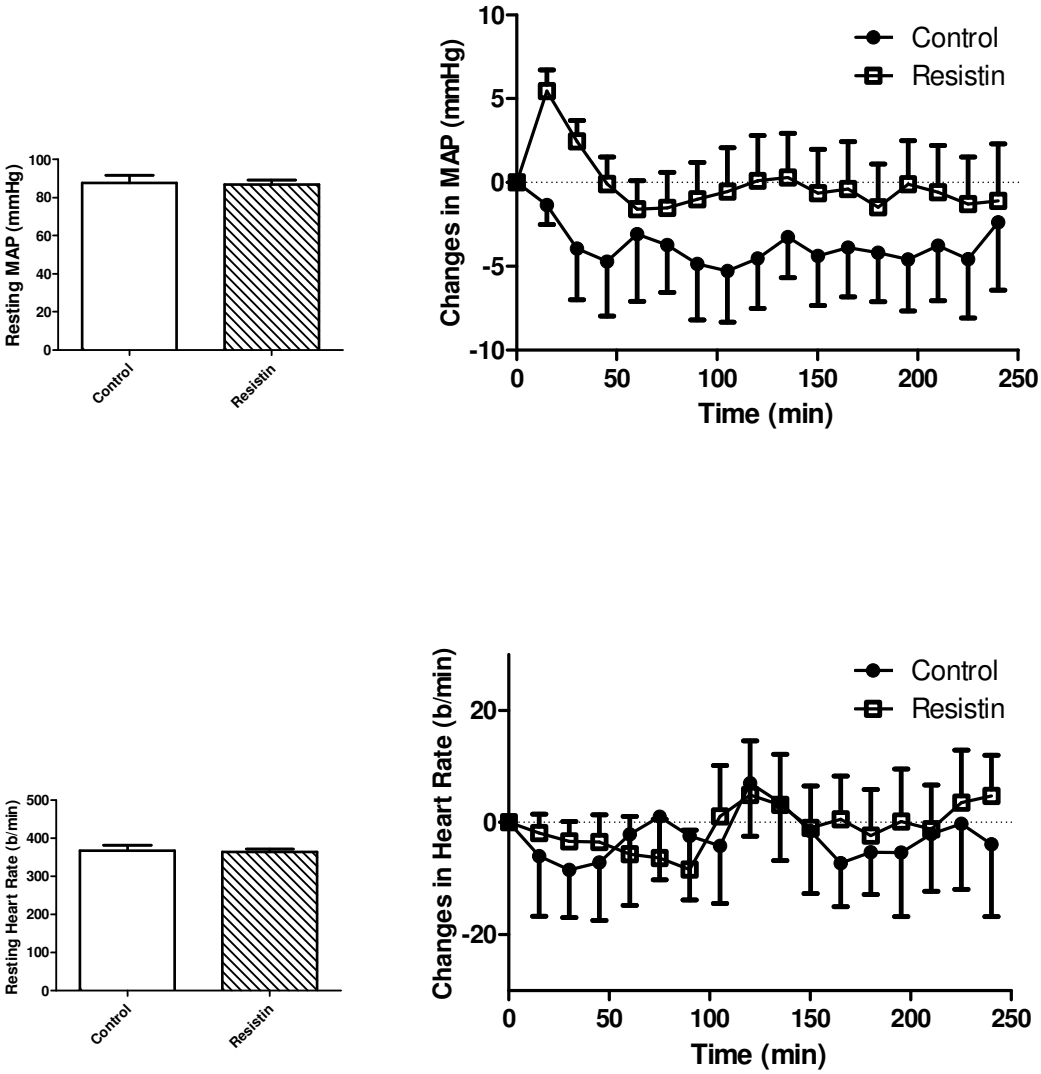

Figure 3.1

Left panels: Resting mean arterial pressure (MAP) and heart rate prior to intracerebroventricular (ICV) administration of resistin (7µg, n=15) or vehicle (artificial CSF, aCSF, n= 11) are shown in the bar graphs. Right panels: Changes in MAP and changes in heart rate induced by ICV resistin or vehicle (aCSF) over time.

3.3.3 Effect of resistin on lumbar SNA

Original recordings of the lumbar SNA from representative animals treated with ICV resistin or vehicle are shown in Fig 3.2A. ICV resistin increased lumbar SNA gradually over the observation period and reached a maximum increase of approximately 40% (Fig 3.2B). This response was significantly different compared to the vehicle-treated group ($F(1,176) = 11.85$, $p<0.05$) (Fig 3.2).

3.3.4 Effect of resistin on Renal SNA

Original recordings of the renal SNA from representative animals treated with ICV resistin or vehicle are shown in figure 3. In the vehicle-treated group, there was a small reduction in renal SNA over time, which may be due to anaesthesia. In the treatment group, ICV resistin increased renal SNA within 15 minutes and was sustained over the observation period. The maximum increase in renal SNA was approximately 40% (Fig 3). This response was significantly greater than that observed in the vehicle-treated group ($F(1,120) = 5.932$, $P<0.05$) (Fig 3.3).

3.3.5 Effects of intravenous resistin

To determine whether leakage of resistin from the cerebral ventricles into the systemic circulation could account for the changes described, we injected resistin intravenously at the same dose (7ug) as that administered ICV. Intravenous resistin did not significantly change mean arterial pressure, heart rate or lumbar SNA compared to resting levels recorded prior to resistin (Fig 3.4).

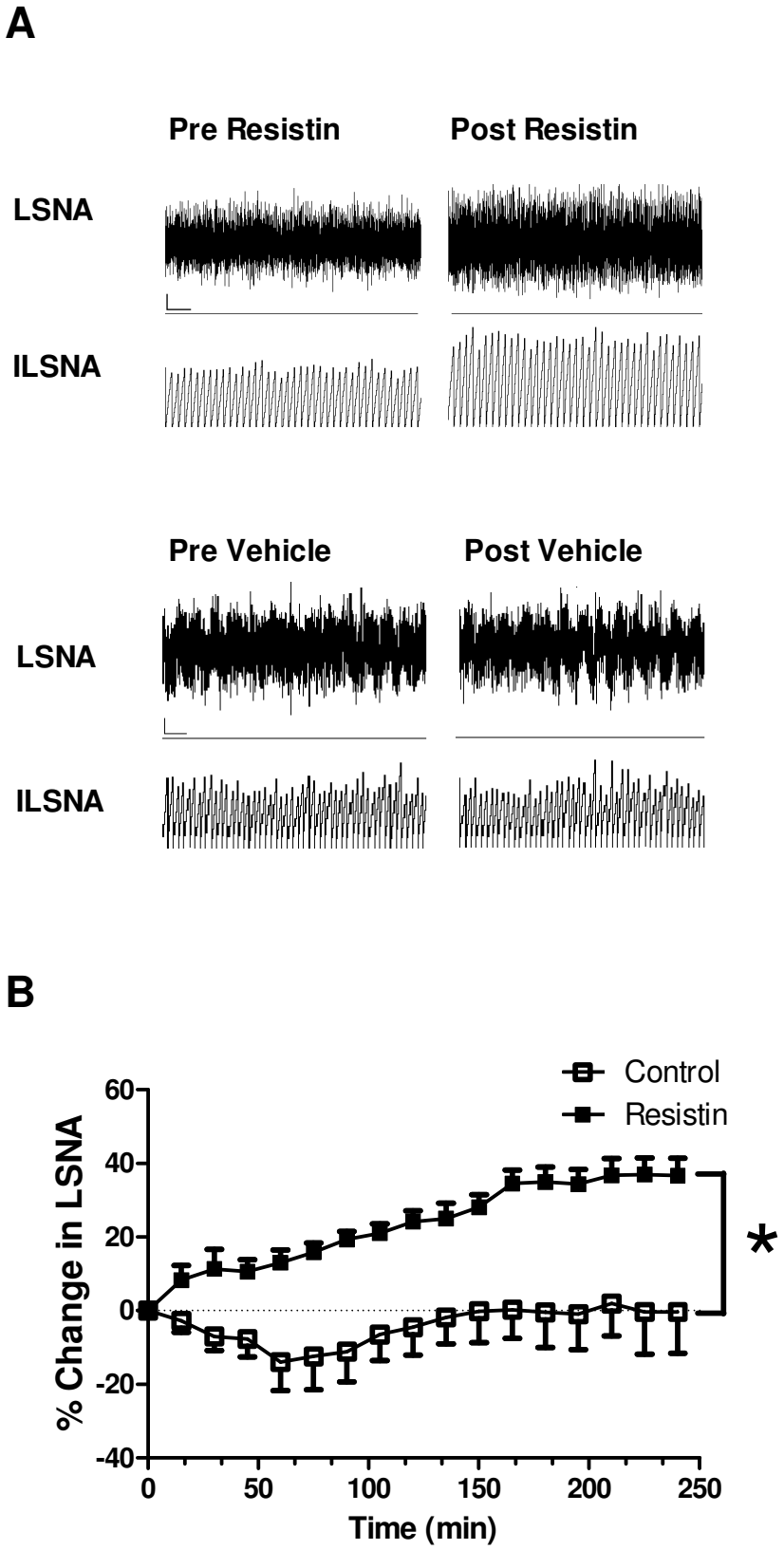

Figure 3.2 See the next page for figure legends

Figure 3.2

Panel A: Screen capture of the raw recordings of lumbar sympathetic nerve activity (lumbar SNA) and integrated lumbar SNA (ILSNA) before and after resistin (7µg) or vehicle (artificial CSF) administered into the lateral brain ventricle. ∟, horizontal bar =2 seconds, vertical bar =100mV (lumbar SNA) and 10mV.s (ILSNA). *$p<0.05$

Panel B: The percent changes in lumbar SNA from resting levels over four hours following administration of resistin (7µg; n=6) or control (artificial CSF; n=7).

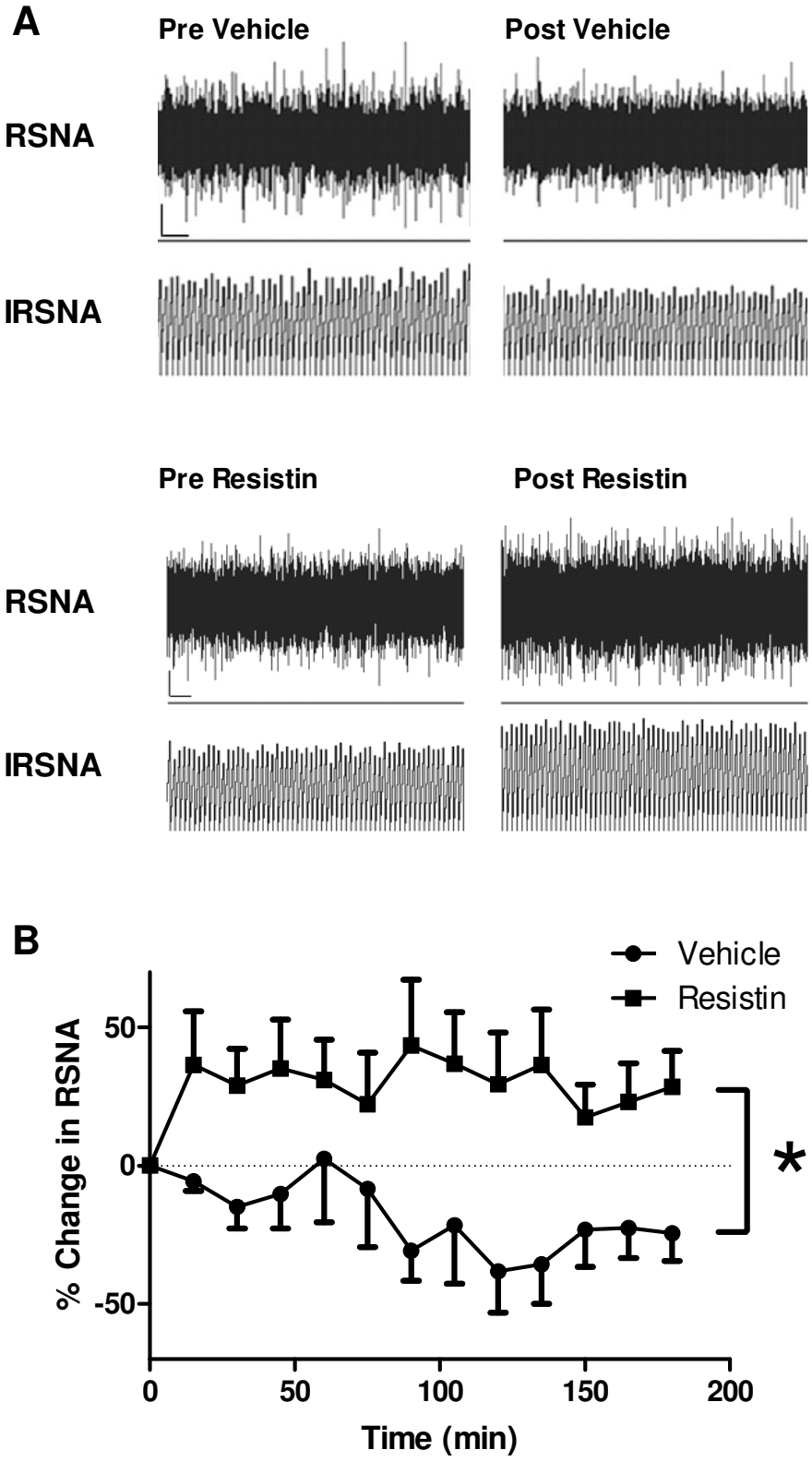

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Figure 3.3

(A) Screen capture of raw recordings of renal sympathetic nerve activity (RSNA) and integrated renal SNA (IRSNA) before and after resistin (7µg) or vehicle (artificial CSF) administered into the lateral brain ventricle. ∟, horizontal bar =2 seconds, vertical bar =200mV (RSNA) and 10mV.s (IRSNA).

(B) The percent changes in renal sympathetic nerve activity (RSNA) from resting levels over time following administration of resistin (7µg; n=7) or vehicle (artificial CSF; n=5). *$p<0.05$

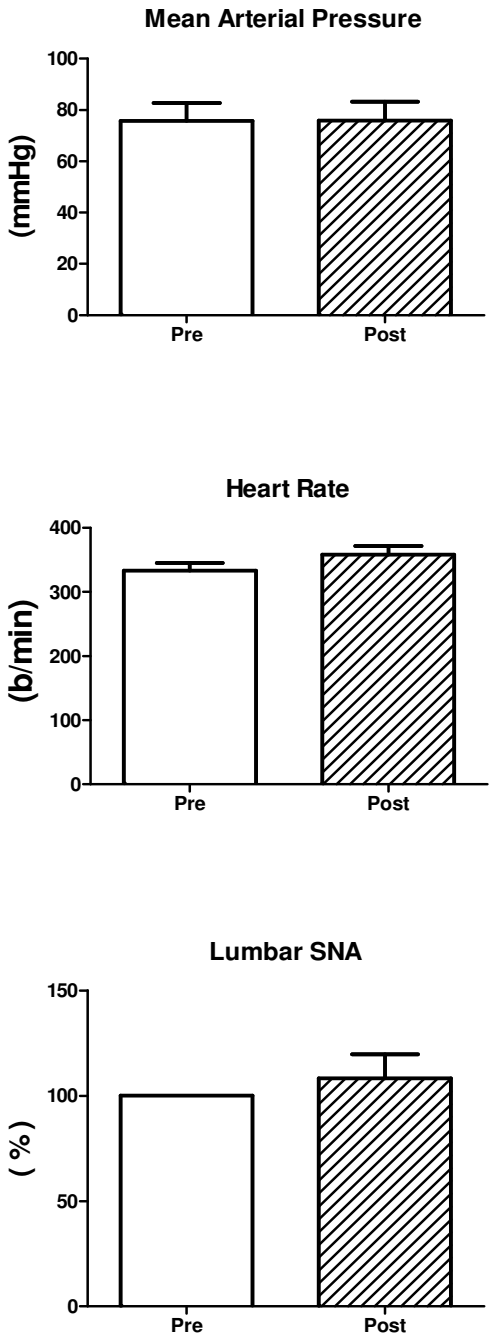

Figure 3.4

Average mean arterial pressure, heart rate and lumbar sympathetic nerve activity (SNA) pre and at 4 hours post intravenous resistin (7ug, n=3). Lumbar SNA is expressed as % of the resting level.

3.3.6 Effects of resistin on Fos, a marker of increased neuronal activation

Central administration of resistin significantly increased Fos production in most of the hypothalamic nuclei examined. In the paraventricular nucleus, Fos was detected in both the magnocellular and parvocellular subnuclei (Fig 3.5). Overall there was a 7 fold increase in the number of Fos-positive cell nuclei counted in the paraventricular nucleus following the administration of resistin compared to the vehicle-treatment (Fig 3.6). Similarly, in the supraoptic nucleus, resistin significantly increased Fos production by 7 fold (Fig 3.5 and 3.6). In the subfornical organ, the numbers of Fos-positive nuclei were increased by 30 fold with resistin treatment (Fig 3.5 and 3.6) and this was significantly different from vehicle-treated group. In the arcuate nucleus, there was no significant difference in the numbers of Fos-positive cell nuclei counted in the resistin-treated group compared to vehicle (Fig 3.5 and 3.6).

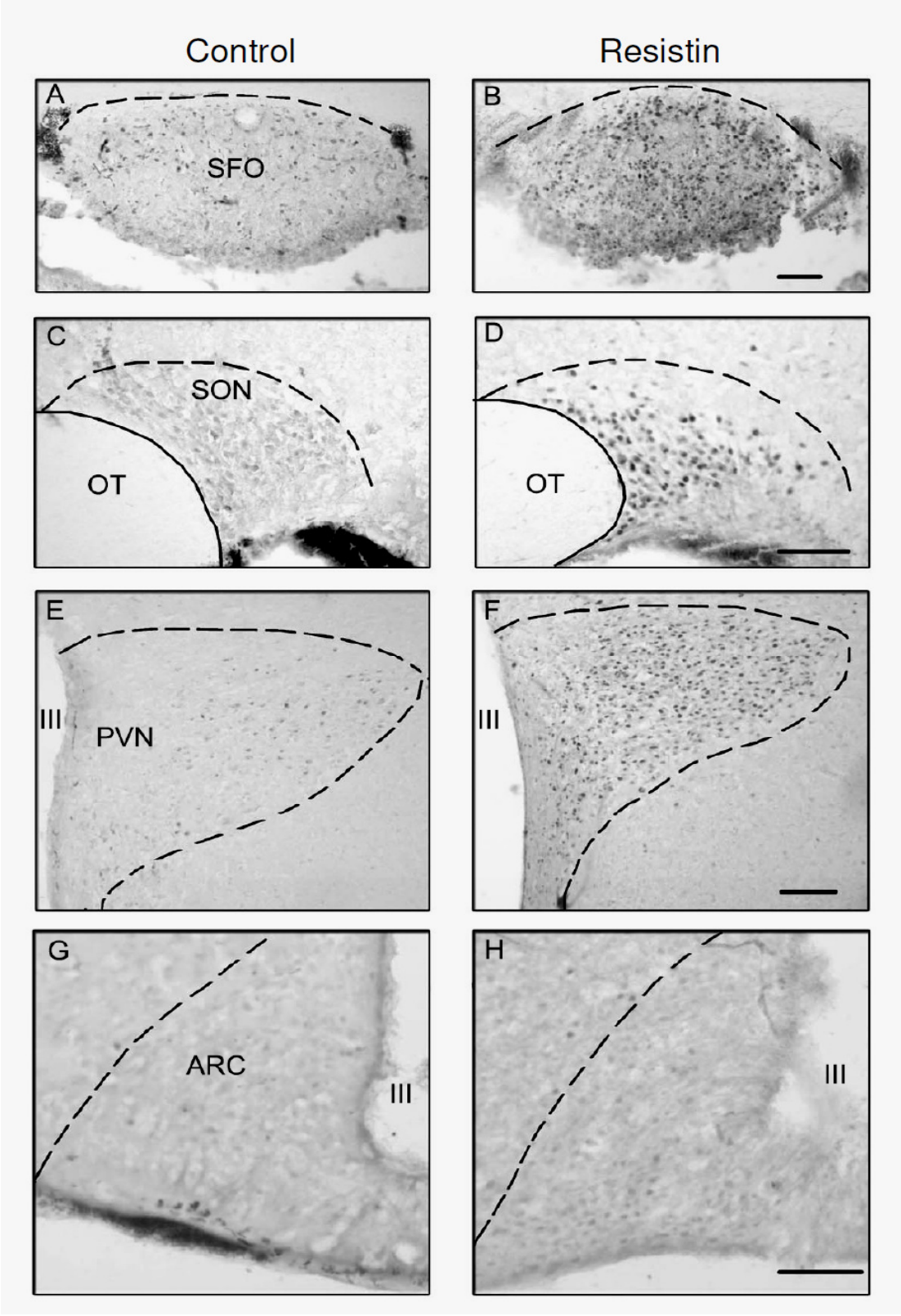

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Figure 3.5

Photomicrographs showing Fos-positive cell nuclei in hypothalamic nuclei from rats administered resistin (7µg) (B,D,F,H) or vehicle (artificial CSF) (A,C,E,G) administered into the lateral brain ventricle. Abbreviations: PVN, paraventricular nucleus; ARC, arcuate nucleus; SFO, subfornical organ; SON, supraoptic nucleus; III, third ventricle. Bar represents 100 µm.

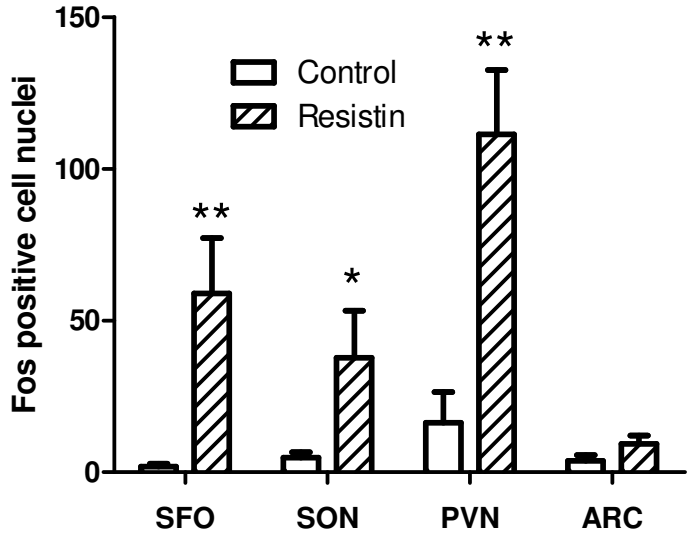

Figure 3.6

Fos-positive cell nuclei counted in the hypothalamic subfornical organ (SFO), supraoptic (SON), paraventricular (PVN) and arcuate (ARC) nuclei from brain of rats treated with resistin (7µg; n=6) or control (artificial CSF; n=6). * $P<0.05$, ** $P<0.005$ resistin vs control.

3.4 Discussion

The present study is the first to directly measure the effects of resistin administration on sympathetic nerve activity. The key findings of the study show that ICV resistin increased lumbar and renal SNA. I did not observe any significant effects on blood pressure and heart rate.

In patients and animal models of type 2 diabetes and obesity, sympathetic nerve activity to the skeletal muscle vascular beds and kidney is increased and this has been suggested to contribute to the incidence of cardiovascular disease in patients with those conditions (125), (61, 126) . Resistin has been linked to cardiovascular disease and there is emerging epidemiological evidence showing that plasma resistin levels are associated with the development and severity of heart failure (17-20, 22, 23, 123). A correlation between plasma resistin levels and hypertension has also been highlighted (21, 24, 25). Thus, resistin may contribute to cardiovascular complications but the mechanisms involved are unknown. In the present study, we found that acute intracerebroventricular administration of resistin induced a significant increase in lumbar SNA. Since muscle SNA is elevated in obesity and diabetes (61, 125, Barretto, 2008 #39), the effect of resistin we have observed may be a potential contributing factor to the cardiovascular complications associated with resistin.

An increase in lumbar SNA has also been observed with the adipokine, leptin, administered into the cerebral ventricles (127). Leptin also increased sympathetic nerve activity to other vascular beds and elicited a concomitant rise in blood pressure. We investigated lumbar SNA since muscle sympathetic nerve activity is increased in obese patients (60), a condition in which plasma resistin levels are elevated (13-16). Abdominal visceral adiposity, in

particular, is closely associated with increased muscle SNA (60). Both lumbar SNA and muscle SNA are indicative of sympathetic nerve activity to the skeletal muscle vasculature.

This study showed for the first time that resistin acts centrally to increase SNA directed at the kidney, a key organ in regulating electrolyte balance and blood pressure. There is a well-recognised link between obesity and hypertension, but the mechanisms responsible for the hypertension in obese individuals is not known. In obesity, noradrenaline spillover studies suggest there is an abnormal elevation in renal SNA. The kidney is a key organ in cardiovascular regulation and increased renal SNA increases sodium retention, alters renal haemodynamics, increases renin release and activation of the renin angiotensin system, (128). It is interesting to speculate that since resistin and leptin have sympatho-excitatory effects on renal SNA, the increased levels of resistin and leptin observed in obesity, could contribute to the elevated renal SNA and thereby to the altered fluid and electrolyte balance observed with excess weight gain (3, 129).

In the present studies, acute administration of resistin did not significantly increase blood pressure. Perhaps resistin may not elicit a generalised increase in sympathetic nerve activity to cardiovascular tissues such that the increased lumbar and renal sympathetic nerve activity is offset by reductions in other outputs. This requires further investigation. It is also possible that baroreceptor reflex responses are sufficiently activated and act to oppose an effect of resistin on blood pressure. It should also be noted that we investigated the acute effects of ICV resistin, and the effects of chronic administration of resistin on blood pressure and heart rate need to be investigated. Interestingly, the adipokine, leptin, has similar effects to resistin and significantly increases lumbar and renal sympathetic nerve activity (5, 63). Leptin has been reported to have variable effects on blood pressure when acute and chronic administrations of this adipokine were compared (130).

The effects observed following ICV resistin were centrally mediated since intravenous administration of the same dose of resistin did not have marked effects. The receptor for resistin and the central sites of action of resistin are unknown. Previous studies using Fos as a marker of increased neuronal activation suggested that the arcuate nucleus may be a site in which neurons are activated by resistin (43). This was only observed in fasted rats (48 hours). No other hypothalamic area showed increased Fos immunoreactivity in that study (43). In mice, central administration of resistin has been found to increase Fos production in the hypothalamus, namely the arcuate, paraventricular and dorsomedial hypothalamic nuclei (131). In our present work we found increases in Fos immunoreactivity in the hypothalamic paraventricular, supraoptic and subfornical nuclei but no significant increase in the arcuate nucleus and dorsomedial hypothalamus (data not shown). Our work in rats was performed in anesthetised animals and this could complicate interpretation of the results. Species differences, whether the animals were fasted, and the duration of fasting may also account for some of the differences observed between reports. Since the receptors for resistin have not been clearly identified as yet, unequivocal confirmation of the sites of action of resistin in the hypothalamus awaits further investigations.

3.5 Methodological aspects of the study

The dose of resistin (7µg) used in this study was chosen based on previous studies in which ICV resistin at a dose of 10µg significantly influenced the food intake but had no effect at lower doses (1 and 5µg) (43). A similar dose has also been used in studies investigating the effects on sympathetic nerve activity of intracebreventricular administration of leptin. I have also observed that similar doses of resistin and leptin can increase renal sympathetic nerve activity (data not shown). Whether the dose used in the present study is physiologically

relevant rather than pharmacological is pertinent since the normal plasma concentration of resistin is in the low ng/ml range rising approximately 10 fold in obese individuals (e.g. 35-40ng/ml). However, it is interesting to note that a similar range of concentrations of leptin are found in humans (15, 132), but μg doses are required to be administered intracerebroventricularly in rodents to induce changes in renal sympathetic nerve activity.

Chapter 4: Intracellular mechanism mediating the effects of resistin on renal sympathetic nerve activity

4.1 Introduction

Resistin acting within the brain, increases lumbar and renal sympathetic nerve activity (133), which innervate the hindlimb vasculature and the kidneys respectively. The intracellular signalling pathways mediating resistin's actions on sympathetic nerve activity are not known. This contrasts with our knowledge of the actions of resistin on cell growth, differentiation, metabolism and intracellular trafficking, which involve the enzymes phosphatidylinositol 3-kinases (PI 3-Kinase) and extracellular regulated kinases (ERK) 1/2. PI 3-Kinases are a family of cellular enzymes which have been shown to mediate resistin's effects including sprouting in murine aortic explants, migration of murine endothelial cells and the release of growth hormone from pituitary somatotropes in vitro (134, 135). ERK 1/2 has been found to mediate hypertrophy of rat neonatal myocytes, proliferation of human endothelial cells and increased expression of the transporter GLUT 1 in trophoblasts, induced by resistin (82, 136, 137). PI 3-Kinase and ERK 1/2 are involved in sympathetic nerve activity responses as shown by the critical role of PI 3-Kinase in mediating the changes in renal sympathetic nerve activity induced by central administration of leptin (Morgan et al. 2008), and the lumbar sympathetic nerve activity changes induced by insulin (138). ERK1/2 activation mediates the changes induced by leptin, and insulin, on sympathetic nerve activity to brown adipose tissue (63, 138).

The aim of the present work was to determine whether PI 3-Kinase or ERK1/2 was involved in mediating the action of resistin on renal sympathetic nerve activity.

4.2 Methods

4.2.1 Experimental protocols

Rats were fasted overnight before the experiment. On the day of the experiment, anesthesia was induced using isoflurane gas (2.5–3%) in O_2. The femoral vein was cannulated to enable infusion of the drugs and to maintain anesthesia (iv urethane 1–1.4 g/kg initially, followed by supplemental doses of 0.05 g/kg as required). The femoral artery was cannulated to allow the measurement of arterial pressure. Mean arterial pressure and heart rate were calculated as explained in details in the method chapter (Chapter 2, section 2.2).

Intracerebroventricular (ICV) administration: In these experiments the mean arterial pressure, heart rate and renal SNA were recorded. The PI 3-Kinase inhibitor (LY294002, 5 µg in 2µl) or The ERK1/2 inhibitor (U0126, 7µg in 2µl) was administered ICV followed 15 minutes later by resistin (7µg in 7µl) or vehicle (artificial cerebrospinal fluid 7µl) and the responses were monitored for 3 hours. DMSO (2µl) was used as a vehicle for LY294002 and U0126.

In all experiments, resting levels were recorded for at least 10 min prior to the injection of resistin or vehicle. Following the injections, all variables were monitored continuously and recorded every 15 minutes over the observation period.

4.2.2 Statistical analysis

The resting levels of mean arterial pressure & heart rate prior to the injections were compared between groups using Student's unpaired t-test. The integrated SNA was calculated over a period of 1-2 minutes at each time point and expressed as a percentage of the resting level prior to the injections. Changes in mean arterial pressure, heart rate and

renal SNA were compared between groups in each experimental series by using two-way ANOVA with repeated measures. All results are expressed as means ± SE. $P < 0.05$ was considered to be statistically significant.

4.3 Results

4.3.1 Role of PI 3-Kinase and ERK1/2 signalling pathways in mediating the action of resistin on blood pressure and heart rate

The resting levels of mean arterial pressure and heart rate prior to the ICV administration of resistin in the presence of PI 3-Kinase inhibitor (LY294002) or ERK1/2 inhibitor (U0126) are shown in the bar graphs in figure 4.1.

There was a statistically significant difference in baseline mean arterial pressure between the groups overall ($F(3,20) = 6.251$, $p<0.05$). Post hoc analysis detected a significant difference between the group administered vehicle + resistin compared to the group administered U0126 + resistin ($p<0.05$) (Fig 4.1). Additionally, there was a significant difference between the group administered LY294002 + resistin compared to the group administered U0126 + resistin ($p<0.05$) (Fig 4.1). There was also a statistically significant difference in baseline heart rate between the groups overall ($F(3,20) =4.253$, $p<0.05$), and post hoc analysis only detected a significant difference between the group administered LY294002 + resistin compared to the group administered vehicle ($p<0.05$) (Fig 4.1).

The effect of LY294002 or U0126 alone on mean arterial pressure and heart rate was not significantly different from vehicle (data not shown). Resistin did not elicit any significant change in mean arterial pressure or heart rate and this was not significantly affected by pre-treatment with LY294002 or U0126 (Fig 4.1).

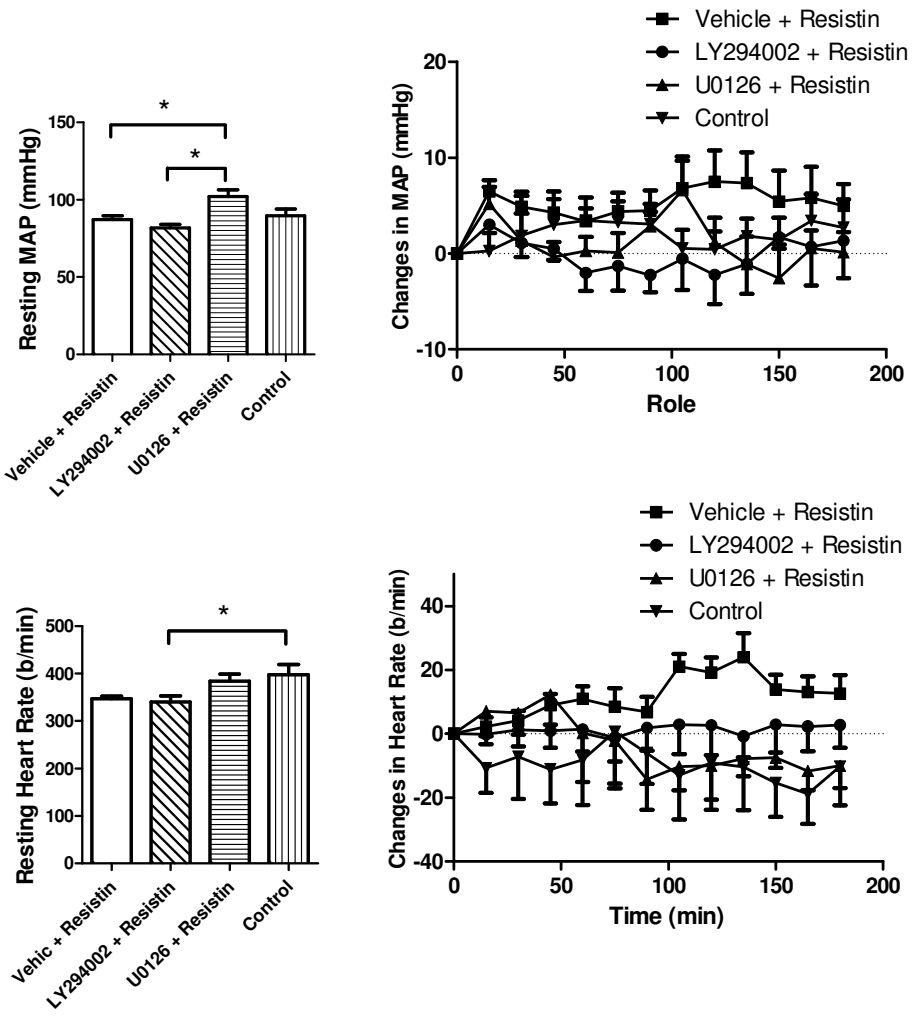

Figure 4.1

Left panels: Resting mean arterial pressure (MAP) and heart rate prior to intracerebroventricular (ICV) administration of resistin (7µg) or vehicle (artificial CSF, aCSF) are shown in the bar graphs. Right panels: Changes in MAP and changes in heart rate induced by ICV resistin or vehicle (aCSF) over time. Fifteen minutes prior to resistin administration, the rats received an ICV injection of either (i) DMSO (2µl, n=7), (ii) ERK1/2 inhibitor (U0126, 7µg, n=5) or (iii) PI 3-Kinase inhibitor (LY294002, 5µg, n=5). DMSO (2µl, n=5) was also injected ICV fifteen minutes prior to the vehicle (aCSF).

4.3.2 Role of PI 3-Kinase signalling pathway in mediating the action of resistin on renal SNA

Original recordings of the renal SNA from a representative animal treated with ICV resistin in the presence of the PI 3-Kinase inhibitor (LY294002) is shown in figure 4.2. Renal sympatho-activation induced by resistin was prevented by pre-treatment with LY294002 (Fig 4.2). The changes in renal SNA were significantly different between the groups ($F(1,120) = 7.902$, $P<0.05$) (Fig 4.3).

The effect of LY294002 alone was not different from vehicle (figure 4.4). Inhibition of ERK1/2 by U1026 did not attenuate the excitatory effect on RSNA induced by resistin (Fig 4.4).

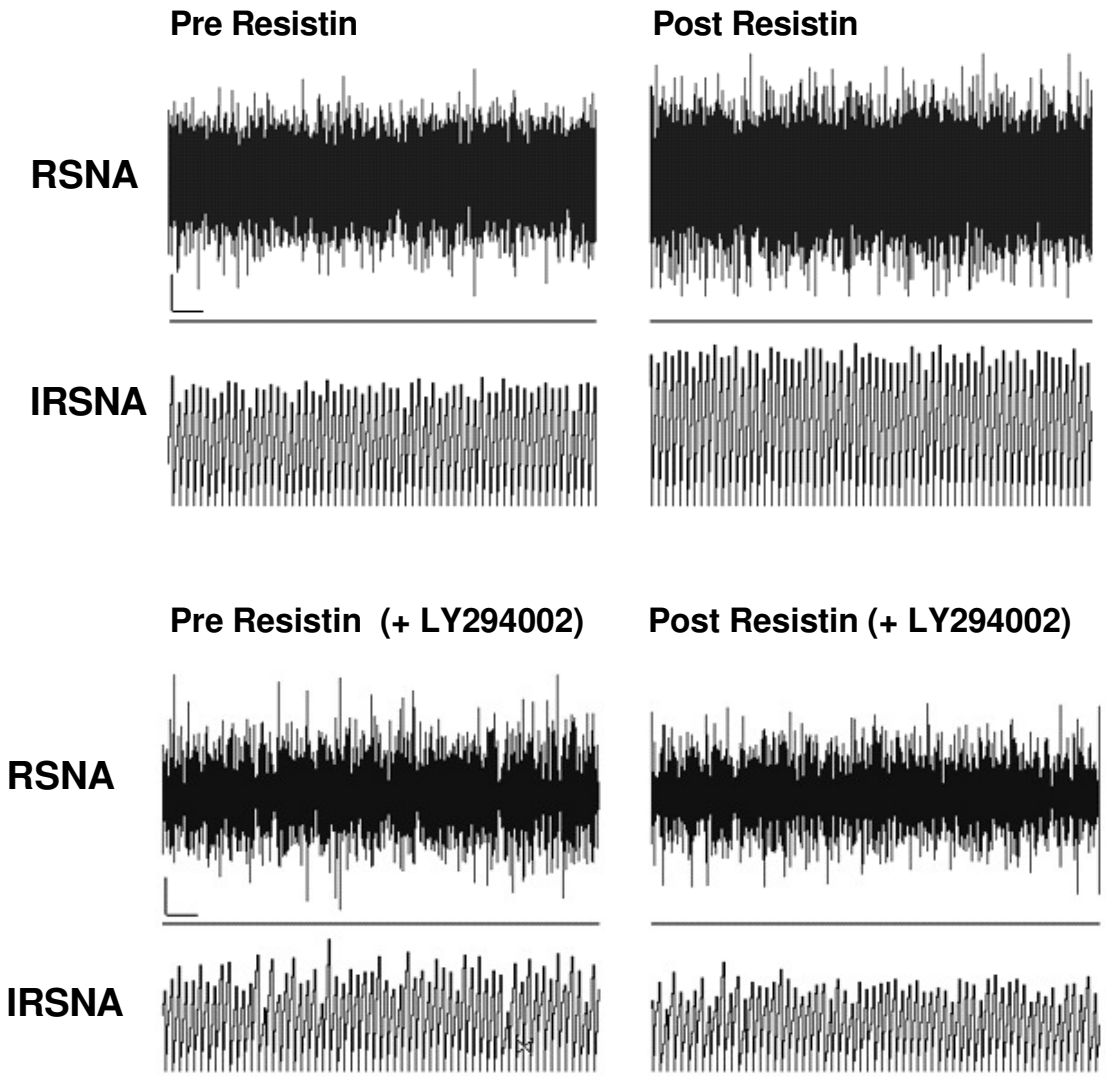

Figure 4.2

Screen capture of raw recordings of renal sympathetic nerve activity (RSNA) and integrated renal SNA (IRSNA) before and after resistin (7µg) in the presence of the PI 3-Kinase inhibitor, LY294002 (5µg) administered into the lateral brain ventricle. ∟, horizontal bar =2 seconds, vertical bar =200mV (RSNA) and 10mV.s (IRSNA).

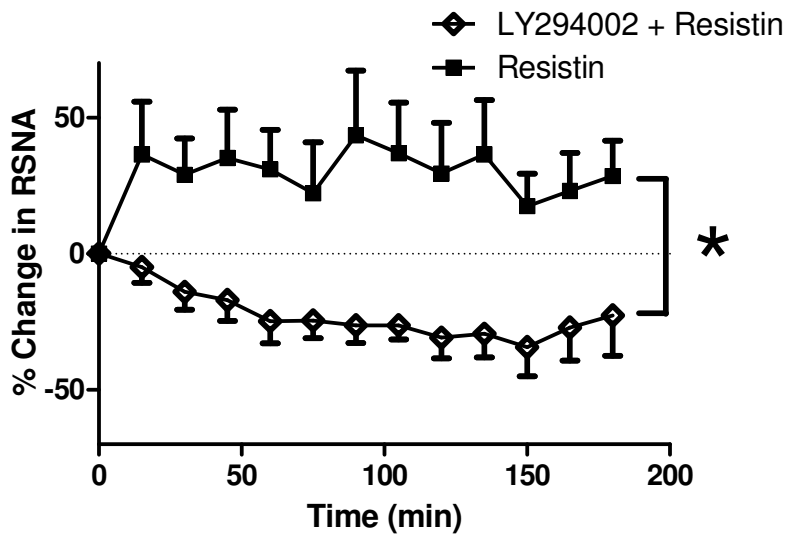

Figure 4.3

The percent changes in renal sympathetic nerve activity (RSNA) from basal levels over time following the administration of resistin (7µg) in the presence of LY294002 (5µg, n=5) or vehicle (DMSO; n=7). *$p<0.05$

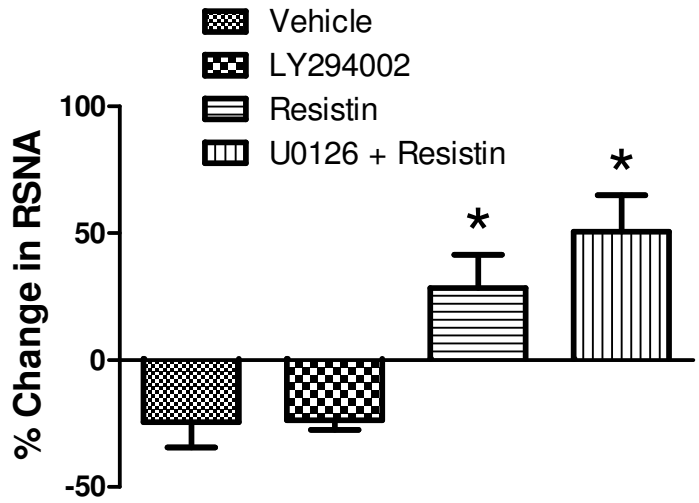

Figure 4.4

The percent change in renal sympathetic nerve activity (RSNA) from basal levels 3h post injection of vehicle (n=5), LY294002 (5µg, n=3), resistin (7µg, n=7) alone, and resistin (7µg) in the presence of U0126 (7µg, n=3). *$p<0.05$ compared to the vehicle group.

4.4 Discussion

The main finding in the present study showed that resistin increased the renal SNA via PI 3-Kinase but not through ERK1/2. Pre-treatment with the PI 3-Kinase inhibitor (LY294002) prevented the renal sympatho-excitatory action of resistin. Leptin, too, increases renal SNA via the PI 3-Kinase signalling pathway (63, 139), suggesting both adipokines act through similar mechanisms to elicit renal sympatho-excitation. Presumably, this means that there is a common nucleus in the brain mediating the increased renal SNA, but this key common site is not yet identified.

Since we used intracerebroventricular injections, it is important to note that the site of action of inhibition of PI 3-Kinase may be different from that at which the adipokines directly act. To investigate this possibility will require direct microinjections into specific nuclei in the brain. The central sites directly activated by resistin are not yet clearly elucidated. Studies using the protein Fos as a marker of increased neuronal activity, suggest the paraventricular nucleus is a potential site of action of resistin (43, 131, 133). Furthermore, it is known that stimulation of neurons in the paraventricular nucleus results in increased renal SNA (140). Thus, since studies show that resistin activates neurons in the paraventricular nucleus and increases renal SNA, the results suggest the paraventricular nucleus may be important in mediating the action of resistin on renal SNA. This, however, requires confirmation. The effect of resistin on renal SNA observed in this experiment did not involve ERK1/2, since inhibition of ERK1/2 by U1026 did not attenuate the excitatory effect on renal SNA induced by resistin. This effect is similar to leptin, further supporting similar pathways are utilised by resistin and leptin to increase renal SNA.

In-vitro studies and cell based assays have shown that U0126, the ERK1/2 inhibitor used in this study, specifically blocks ERK pathways and LY294002 is one of the most specific inhibitors of PI 3-Kinase. It does, however, also inhibit Casein Kinase 2 signalling pathways in-vitro (141). Whether this occurs in-vivo has not been investigated. Nonetheless, this PI 3-Kinase inhibitor (LY294002) is commonly used in in-vivo experiments (127, 138, 139). Data from studies investigating PI 3-Kinase role on renal sympatho-excitation induced by leptin, together with the present in-vivo work suggest that PI 3-Kinase mediates resistin effect on renal SNA. The current work also clearly shows that ERK1/2 is not involved in the action of resistin on renal SNA.

Chapter 5: Effects of resistin on sympathetic nerve activity to brown adipose tissue and thermogenesis.

5.1 Introduction

Obesity is a complex condition in which there may be metabolic dysfunction and an imbalance between energy intake and expenditure. Brown adipose tissue (BAT) is important in thermogenesis and energy expenditure in animals and in neonates and recent studies have now highlighted considerable functional deposits of BAT in adult humans (119, 142). The activity of brown adipose tissue can be dramatically influenced by alterations in sympathetic nerve activity to brown adipose tissue(118).

The adipokine, leptin, is known to increase energy expenditure and stimulate thermogenesis in BAT by increasing BAT SNA (63). Leptin also reduces fat tissue mass and lipid accumulation, preventing the development of obesity (121). Resistin also, has important effects on energy metabolism (143). Similar to leptin, resistin reduces energy intake by reducing food intake and the expression of key neurotransmitters in the hypothalamus involved in central dietary pathways (117), however, the role of resistin on energy expenditure via thermogenesis in BAT is not known.

The aims of the present study were to determine the acute effects of resistin on thermogenesis in BAT, thus I investigated the effects of ICV administration of resistin on BAT temperature, body core temperature and sympathetic nerve activity to the BAT.

5.2 Methods

5.2.1 Experimental protocols

Rats were fasted overnight before the experiment. On the day of the experiment, anesthesia was induced using isoflurane gas (2.5–3%) in O_2. The femoral vein was cannulated to enable infusion of the drugs and to maintain anesthesia (iv urethane 1–1.4 g/kg initially, followed by supplemental doses of 0.05 g/kg as required). The femoral artery was cannulated to allow the measurement of arterial pressure. Mean arterial pressure and heart rate were calculated as explained in details in the Chapter 2 (Section 2.2).

Intracerebroventricular administration: In one series of experiments the mean arterial pressure, heart rate, body core temperature and BAT temperature were recorded. In these experiments the animals were placed on a constant temperature heating pad but no attempt was made to maintain body core temperature. In a separate series of experiments mean arterial pressure, heart rate, body core temperature and BAT SNA were measured. In these experiments the animals were placed on a heating pad and the body core temperature was maintained constant by altering the temperature of the heating pad. At normal body core temperature, BAT SNA is virtually non-existent. In the present work, the resting body core temperature was lowered to approximately 35ºC where it was maintained. At this body core temperature, resting BAT SNA was clearly observable.

Intravenous administration: In three urethane-anaesthetized rats, resistin (7µg in 7µl) was administered intravenously via a femoral vein. BAT temperature and body core temperature were recorded for four hours.

In all experiments, resting levels were recorded for at least 10 min prior to the injection of resistin (7μg, n=3 to 8 per group), or vehicle (artificial cerebrospinal fluid, n=4 to 8 per group). Following the injections, all variables were monitored continuously and recorded every 15 minutes over the next 4 hours.

5.2.2 Statistical analysis

The resting levels of body core temperature and BAT temperature prior to the injections were compared between the resistin treated and vehicle injected (control) groups using Student's unpaired t-test. The integrated SNA was calculated over a period of 1-2 minutes at each time point and expressed as a percentage of the resting level prior to the injections. Changes in body core temperature, BAT temperature, and BAT SNA were compared between groups in each experimental series by using two-way ANOVA with repeated measures. All results are expressed as means ± SE. $P<0.05$ was considered to be statistically significant.

5.3 RESULTS

5.3.1 Effects of resistin on BAT temperature and body core temperature

Resting BAT temperatures prior to ICV resistin or vehicle treatments were not significantly different between the two groups. Similarly resting body core temperatures prior to resistin or vehicle were not significantly different (Fig 5.1).

ICV administration of resistin induced a marked reduction in BAT temperature (1.27 $\pm$ 0.46 ºC) and this was significantly different from the vehicle-treated group (Fig 5.1). The ICV administration of resistin also elicited a gradual steady fall in body core temperature (1.32 $\pm$ 0.49 ºC). This was not observed in the vehicle-treated group (Fig 5.1), although the difference between the groups, did not attain statistical significance.

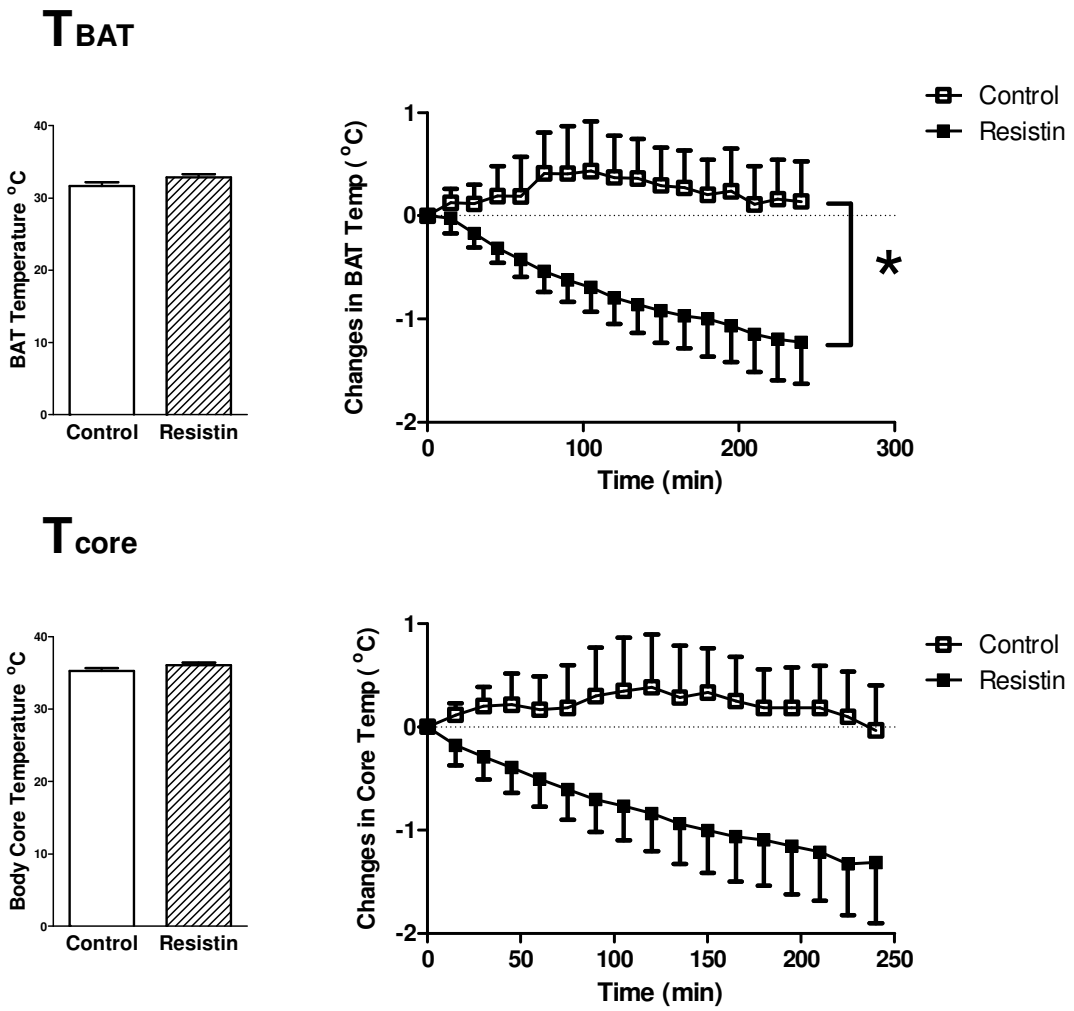

Figure 5.1

Temperature in brown adipose tissue (Tbat) and the body core temperature (Tcore) prior to intracerebroventricular administration of resistin (7µg; n=8) or control (artificial CSF; n=8, n=6 for Tcore) are shown in the bar graphs. The changes in Tbat and Tcore from resting levels over four hours following administration of resistin or artificial CSF (control) are shown in the right panels. *$p<0.05$; $F(1,224) = 5.815$, resistin vs control.

5.3.2 Effects of intravenous resistin

To determine whether leakage of resistin from the cerebral ventricles into the systemic circulation could account for the changes described, we injected resistin intravenously at the same dose (7ug) as that administered ICV. Intravenous resistin did not significantly change BAT temperature or body core temperature compared to resting levels recorded prior to resistin (Fig 5.2).

5.3.3 Effects of resistin on BAT SNA

Original recordings of BAT SNA from animals treated with ICV resistin or vehicle are shown in figure 5.3. ICV resistin reduced BAT SNA by over 50%. This occurred within 2 hours of the injection of resistin and BAT SNA remained reduced for the duration of the observation (Fig 5.4). This response was significantly different from the vehicle-treated group in which BAT SNA was slightly elevated over time (Fig 5.4). In these experiments the body core temperature was carefully maintained constant.

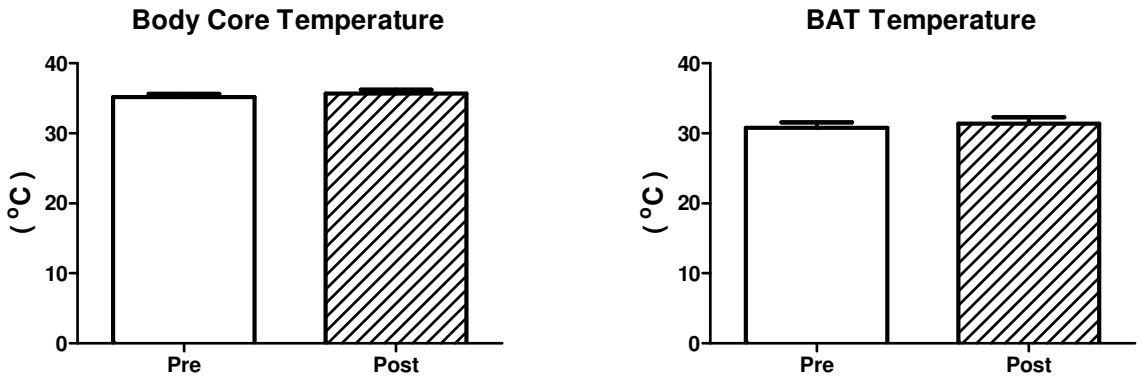

Figure 5.2

Average brown adipose tissue (BAT) temperature and body core temperature pre and at 4 hours post intravenous resistin (7ug, n=3).

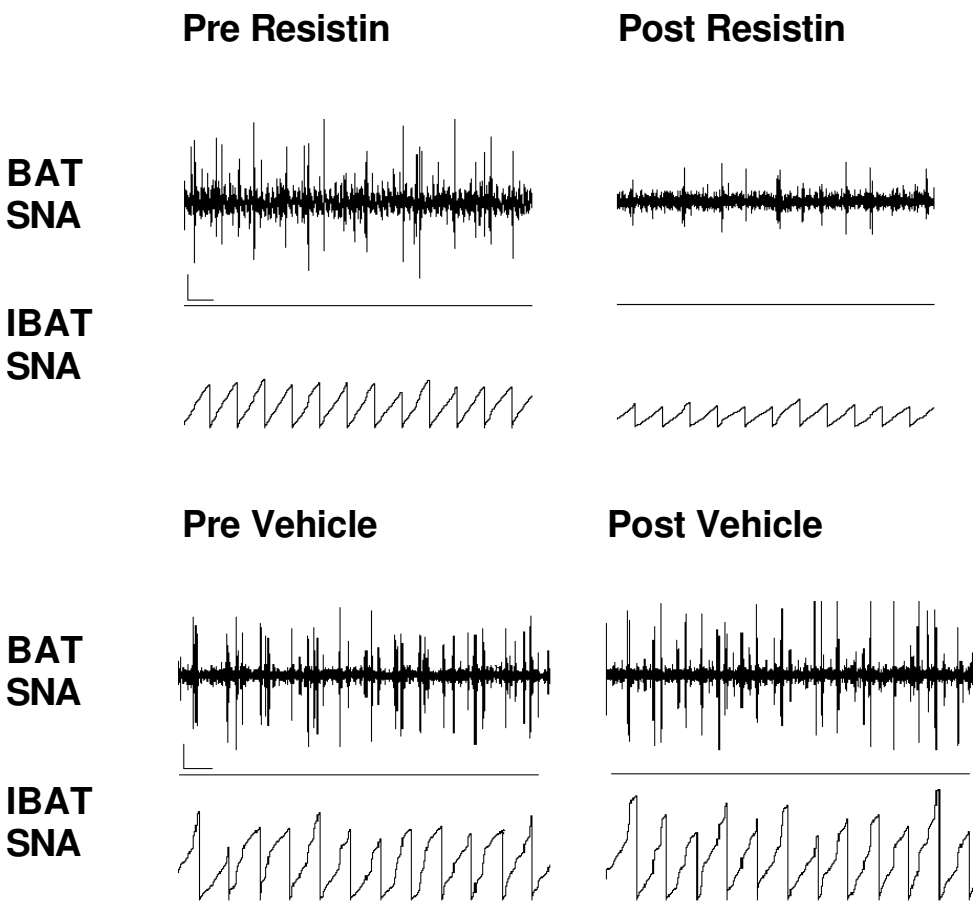

Figure 5.3

Screen capture of the raw recordings of brown adipose tissue sympathetic nerve activity (BAT SNA) and integrated BAT SNA (IBAT SNA) before and after resistin (7µg) or vehicle (artificial CSF) administered into the lateral brain ventricle. ∟, horizontal bar =2.5 seconds, vertical bar =100mV (BAT SNA) and 25mV.s (IBAT SNA).

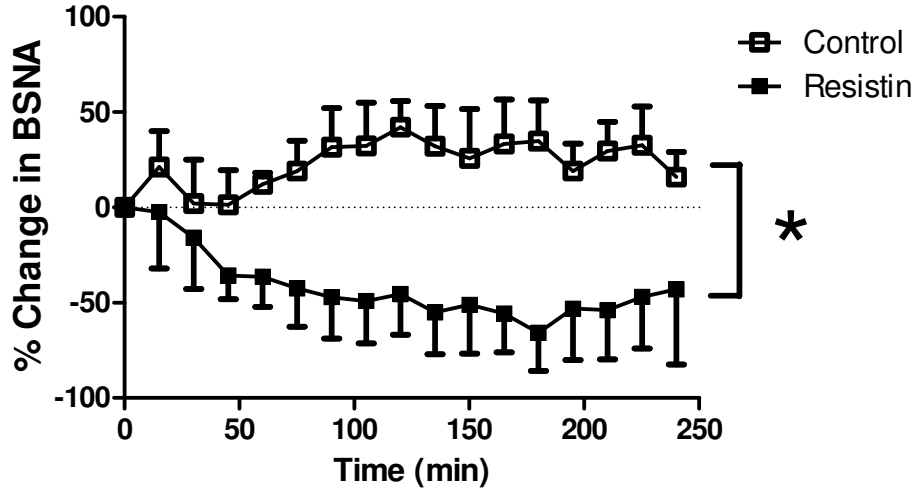

Figure 5.4

The percent changes in brown adipose tissue sympathetic nerve activity (BAT SNA) from resting levels over four hours following intracerebroventricular administration of resistin (7µg; n=5) or control (artificial CSF; n=4). *P<0.05; F(1,112) = 6.211, resistin vs control.

5.4 Discussion

The present study is the first to directly measure the effects of resistin administration on thermogenesis in BAT. The key findings of the study show that intracerebroventricular resistin reduced brown adipose tissue sympathetic nerve activity. As a likely consequence of that effect, the temperature of brown adipose tissue decreased and there was a decrease in body core temperature.

We found that ICV resistin administration induced a significant decrease of over 50% in BAT SNA. This indicates that resistin reduces thermogenesis and this is supported by our finding, in separate animals, of a reduction in BAT temperature and a concomitant decrease in body core temperature. The ability of resistin to act centrally to reduce thermogenesis contrasts with the actions of leptin which increases BAT SNA (62, 63), an action mediated via the hypothalamic arcuate nucleus (63). Thus, leptin's actions of reducing food intake and increasing thermogenesis result in an increased energy output and resultant weight loss. By contrast, it appears that resistin can decrease food intake and thermogenesis, thus counteracting the reduced dietary intake with mechanisms designed for the preservation of energy. This could contribute to the lack of effect on body weight reported following resistin administration (43, 47).

There has been considerable discussion lately about the role of thermogenesis and BAT in metabolic regulation in humans. Until recently, brown adipose tissue was believed to be present only in infants, however, it is now recognized that brown adipose tissue is present and is metabolically active in adults (119). Stimulation of thermogenesis in BAT may have dramatic effects on energy expenditure since calculations show that activation of 40-50 g of BAT in humans could result in a 20% increase in energy expenditure (120). This could have

dramatic effects on weight loss. Thus, antagonists to resistin may elicit increases in BAT SNA which could be an efficient therapeutic mechanism to reduce body weight.

The effects observed following ICV resistin were centrally mediated since intravenous administration of the same dose of resistin did not have marked effects on BAT or body core temperature. In chapter 3 of this thesis, it was shown that ICV resistin administration increases the number of Fos positive cell nuclei in the hypothalamic paraventricular, supraoptic and subfornical nuclei, but there was no significant increase in the arcuate nucleus (133). Our data are consistent with the findings that activation of the paraventricular nucleus inhibits BAT SNA (144) since we found that resistin increased activation in neurons in paraventricular nucleus and inhibited BAT SNA.

In conclusion, the present findings in addition to the findings reported in previous chapters indicate that acute administration of resistin has differential effects on sympathetic nerve activity to tissues involved in metabolic and cardiovascular regulation. The decreased BAT SNA and the increased lumbar and renal SNA elicited by resistin may contribute to the metabolic and cardiovascular dysfunction observed in obesity and diabetes.

Chapter 6: Intracellular mechanism mediating the effects of resistin on brown adipose tissue thermogenesis

6.1 Introduction

Resistin can act centrally to influence energy balance. It reduces food intake and the expression of key neurotransmitters in the hypothalamus involved in central dietary pathways (117). In chapter 5, it was shown that resistin reduced brown adipose tissue (BAT) and body core temperatures and sympathetic nerve activity (SNA) to BAT, resulting in reduced thermogenesis in BAT and reduced energy expenditure (133). BAT is an important organ for thermogenesis and energy expenditure in animals and in neonates and recent studies have now highlighted considerable functional deposits of brown adipose tissue in adult humans (119, 142).

BAT function is tightly regulated by the sympathetic nervous system through activation of adrenoreceptors that ultimately activate UCP1 and other important thermogenic genes. The transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) is abundantly expressed in BAT and can also activate many aspects of the adaptive thermogenic program (145). In this regard, PGC-1α has been shown to increase the transcriptional activity of several prominent nuclear ligand-binding receptors that can collectively stimulate mitochondrial biogenesis (146).

Recently I have shown that resistin reduces thermogenesis in BAT by decreasing the sympathetic nerve activity to BAT and reducing BAT temperature and body core temperature (133, 147), however, the intracellular signalling pathways mediating resistin's actions on sympathetic nerve activity to BAT are not known. Phosphatidylinositol 3-kinases

(PI 3-Kinase) and extracellular regulated kinases (ERK) 1/2 are known to mediate the actions of resistin on different cellular migration, hypertrophy and proliferation (82, 136, 137). PI 3-Kinase and ERK1/2 within the brain are known to be involved in sympathetic nerve activity responses, as shown by the critical role of PI 3-Kinase in mediating the changes in renal sympathetic nerve activity induced by central administration of leptin (139, 147) and resistin (139, 147), and in the lumbar sympathetic nerve activity changes induced by insulin (138). ERK1/2 activation mediates the changes induced by leptin and insulin, on sympathetic nerve activity to brown adipose tissue (63, 138).

In the present study, the role of PI 3-Kinase and ERK1/2 in the brain in mediating the effects of resistin on BAT SNA, was investigated. Based on the findings, I further explored the role of ERK1/2 in the brain in mediating the reduction in BAT temperature induced by centrally administered resistin and on the changes in the expression of UCP1 and PGC-1α, genetic markers of BAT thermogenesis.

6.2 Methods

6.2.1 Experimental protocols

Rats were fasted overnight before the experiment. On the day of the experiment, anesthesia was induced using isoflurane gas (2.5–3%) in O_2. The femoral vein was cannulated to enable infusion of the drugs and to maintain anesthesia (iv urethane 1–1.4 g/kg initially, followed by supplemental doses of 0.05 g/kg as required). The femoral artery was cannulated to allow monitoring of arterial pressure as explained in detail in Chapter 2 (Section 2.2).

Recording of BAT SNA

In these experiments the animals were placed on a heating pad and the body core temperature was maintained constant by altering the temperature of the heating pad. At normal body core temperature, BAT SNA is virtually non-existent. In the present work the resting body core temperature was lowered to approximately 35ºC where it was maintained. At this body core temperature, resting BAT SNA was clearly observable.

In these experiments, resting levels were recorded for at least 10 min prior to the ICV injections. The PI 3-Kinase inhibitor (LY294002, 5 µg in 2µl of DMSO) or the ERK1/2 inhibitor (U0126, 7µg in 2µl of DMSO) was administered ICV followed 15 minutes later by resistin (7µg in 7µl) or vehicle (artificial cerebrospinal fluid 7µl) and the responses were monitored continuously and recorded every 15 minutes for 3 hours.

Recording of body core temperature and BAT temperature

In a separate series of experiments body core temperature and BAT temperature were recorded in anaesthetised rats. In these experiments the animals were placed on a constant temperature heating pad but no attempt was made to maintain body core temperature. In these experiments, resting levels of BAT temperature and body core temperature were recorded for at least 10 minutes prior to the ICV injections. The changes in temperature were monitored for 3-4 hours after ICV resistin in rats pre-treated with the ERK1/2 inhibitor, U0126 (7 µg in 2 µl, n=5) (administered 20-30 minutes earlier) or vehicle (DMSO, n=8). In separate groups, vehicle (n=7) or U0126 (n=5) were administered alone.

BAT sampling and processing

3-4 hours after the ICV injections, animals were euthanized; BAT was rapidly excised, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction. Samples were processed for PCR experiments as explained in detail in Chapter 2 (Section 2.4). Due to technical problems, mRNA levels could not be measured in BAT from all animals.

6.3 Results

6.3.1 Role of ERK1/2 signalling pathway in mediating the action of resistin on BAT SNA

Original recordings of the BAT SNA from representative animals treated with ICV resistin in the presence or absence of the ERK1/2 inhibitor (U0126) are shown in figure 6.1. Resistin significantly reduced BAT SNA which reached a maximum reduction within about 120 minutes and then remained at that plateau level for the duration of the observation period (Fig 6.1).

Pre-treatment with U0126 prevented the inhibitory action of resistin on BAT SNA for the first 150 minutes. Thereafter however, BAT SNA fell to levels seen in the group administered resistin in the absence of U0126 (Fig 6.1). U0126 alone had no significant effect on BAT SNA (Fig 6.2). The reduction in BAT SNA induced by resistin was not prevented by inhibition of PI 3-Kinsae using LY294002 at any time during the observation period (Fig 6.2).

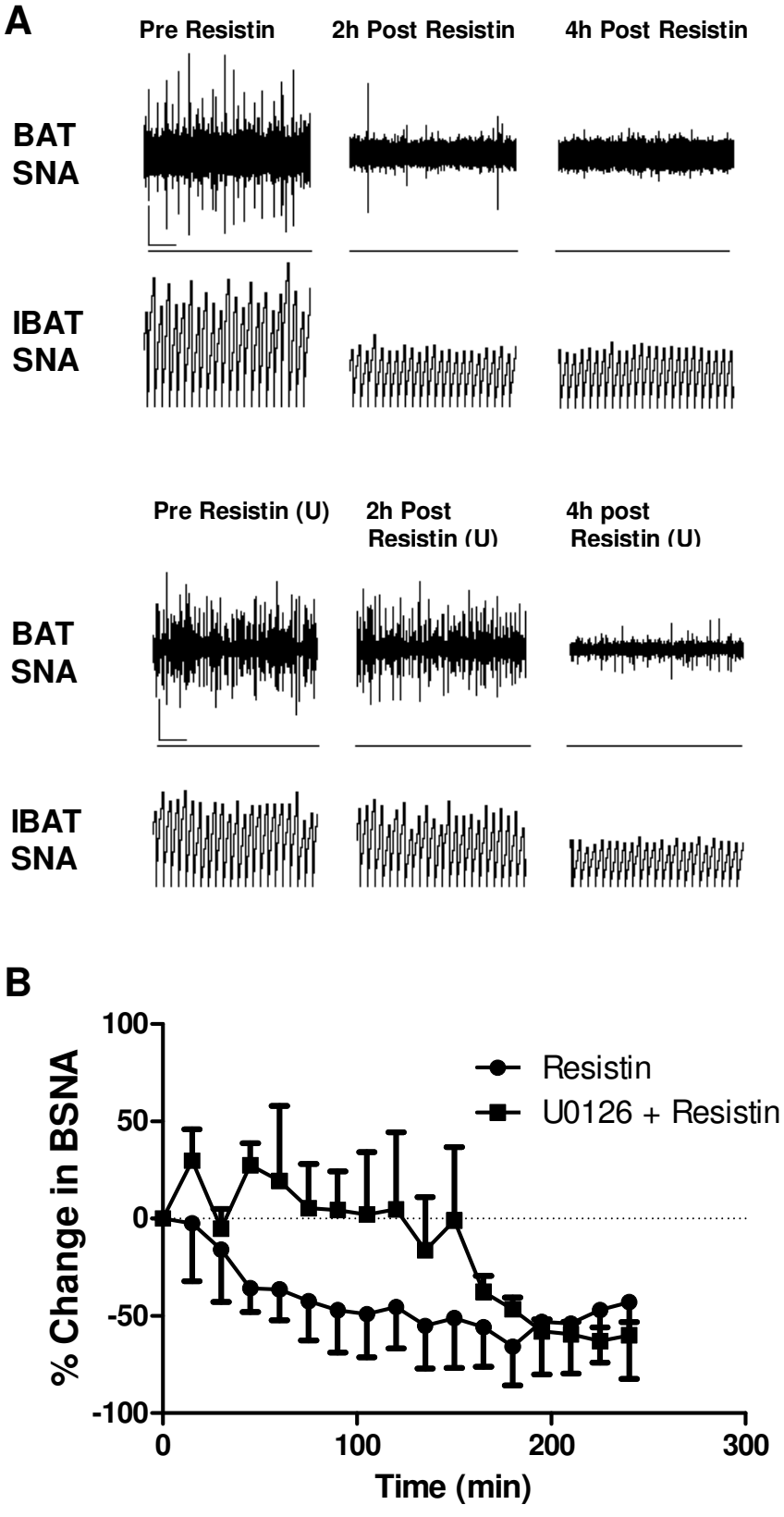

Figure 6.1 See the next page for figure legends

Figure 6.1

(A) Screen capture of raw recordings of brown adipose tissue sympathetic nerve activity (BAT SNA) and integrated BAT SNA (IBAT SNA) before, 2 hours and 4 hours after resistin (7µg) in the presence of vehicle (Panel A) or ERK1/2 inhibitor (U0126, 7µg) (panel B) administered into the lateral brain ventricle. ∟, horizontal bar =2 seconds, vertical bar =200mV (BAT SNA) and 10mV.s (IBAT SNA).

(B) The percent changes in BAT sympathetic nerve activity (BSNA) from baseline levels over time following administration of resistin (7µg) in the presence or absence of U0126 (7µg, n=4).

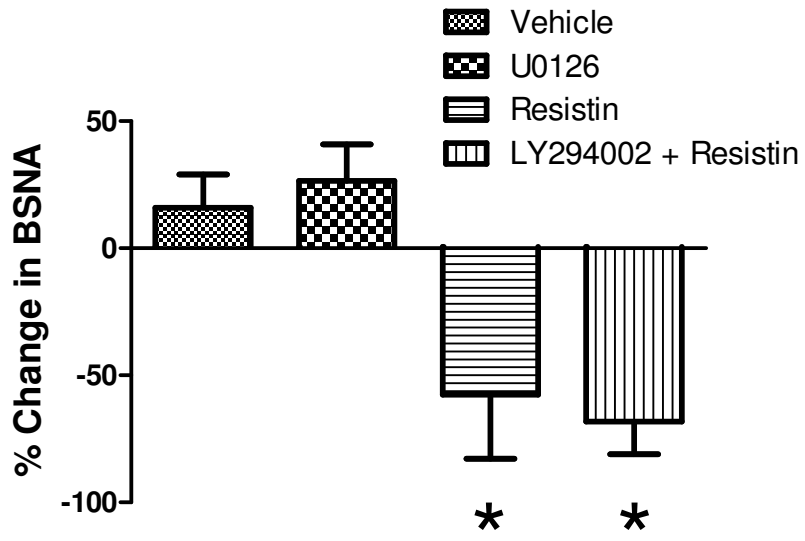

Figure 6.2

(C) The percent change in BAT sympathetic nerve activity (BSNA) from basal levels 4h post injection of vehicle (n=4), U0126 (7µg, n=5), resistin (7µg, n=5) alone, and resistin (7µg) in the presence of LY294002 (5µg, n=3). *$p<0.05$ compared to the vehicle group.

6.3.2 BAT temperature and body core temperature and effects of ERK1/2 inhibition

Resting levels of BAT and body core temperatures before ICV injection of the drugs are shown in Figure 6.3. Compared with the control group, there was a small but statistically significant difference in resting BAT temperature in groups that received resistin. Resting body core temperatures before ICV injection of resistin were not different between the groups (Fig 6.3).

ICV injection of resistin markedly reduced both BAT temperature ($P < 0.005$) and body core temperatures (Fig 6.3). In rats pre-treated with the ERK1/2 inhibitor, U0126, resistin's effect on BAT temperature was significantly attenuated by over 60% ($P < 0.05$) (Fig 6.3). U0126 alone did not significantly change BAT temperature (Fig 6.3). Resistin decreased body core temperature by 1.09 ± 0.44 ºC from resting levels, and although U0126 attenuated this effect by over 60%, the change was not statistically significant (Fig 6.3).

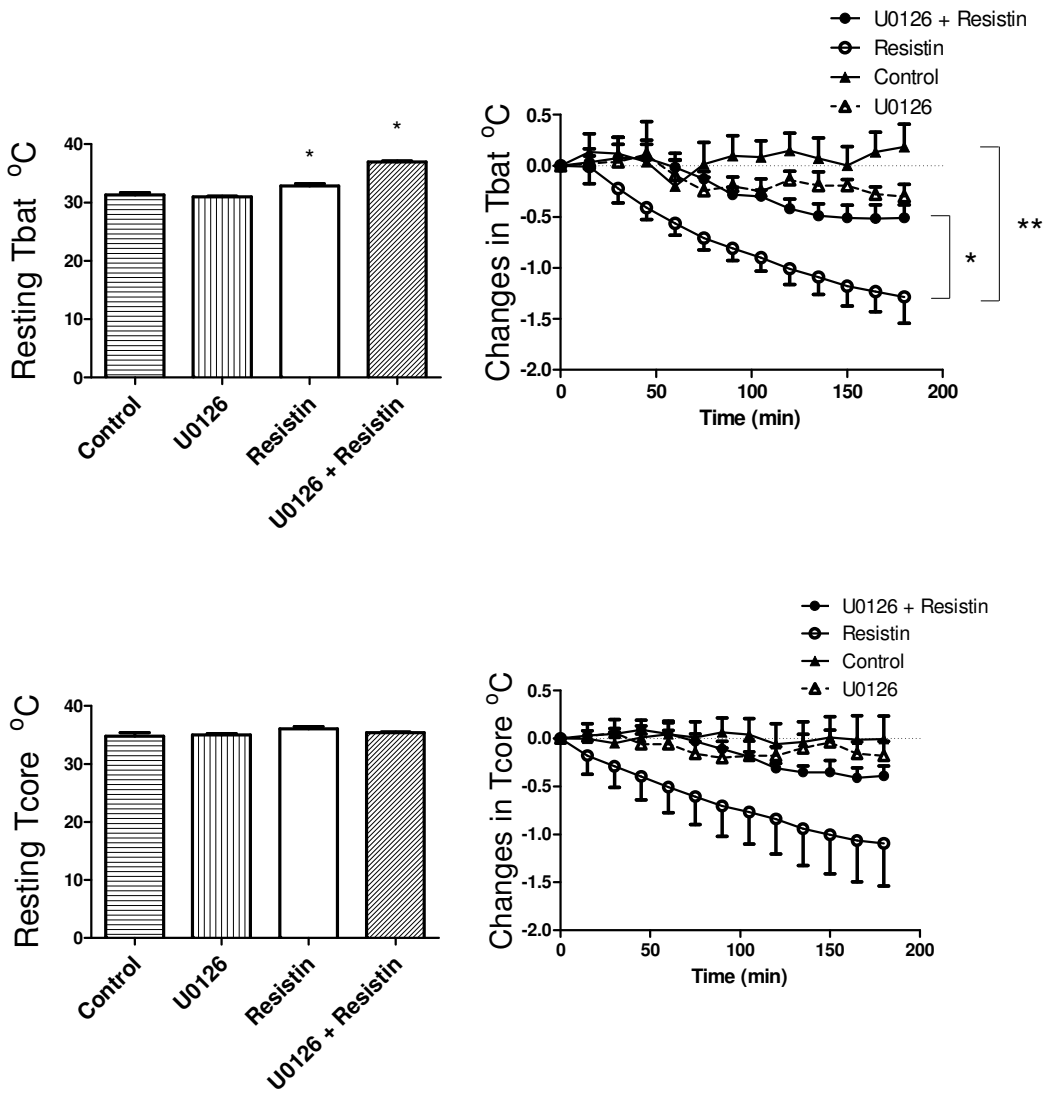

Figure 6.3

Bar graph shows brown adipose tissue temperature (Tbat) and body core temperature (Tcore) before intracerebroventricular administration of (i) resistin alone (7 µg, n=7 for Tbat, n=8 for Tcore), (ii) resistin in the presence of U0126 (7 µg in 2 µL, n=5), (iii) U0126 alone (n=6) or (iv) vehicle (Control, n=8). In the right panels, changes in Tbat and Tcore from resting levels over time in response to treatments are shown. *$p < 0.05$: resistin alone vs resistin in the presence of U0126. **$p< 0.005$, resistin alone vs control.

6.3.3 Effects of resistin on UCP1 and PGC-1α mRNA expression

UCP1 mRNA expression was lower in the resistin-treated group (0.11±0.01) than in the control group (1.24±0.85), but this difference was not statistically significant. Inhibiting ERK1/2 in the brain blunted resistin's effect on UCP1 expression, however, this effect was not statistically significant (Fig 6.4A). Figure 6.4B shows the levels of mRNA expression for PGC-1α in BAT. There were no statistically significant differences in PGC-1α expression between groups.

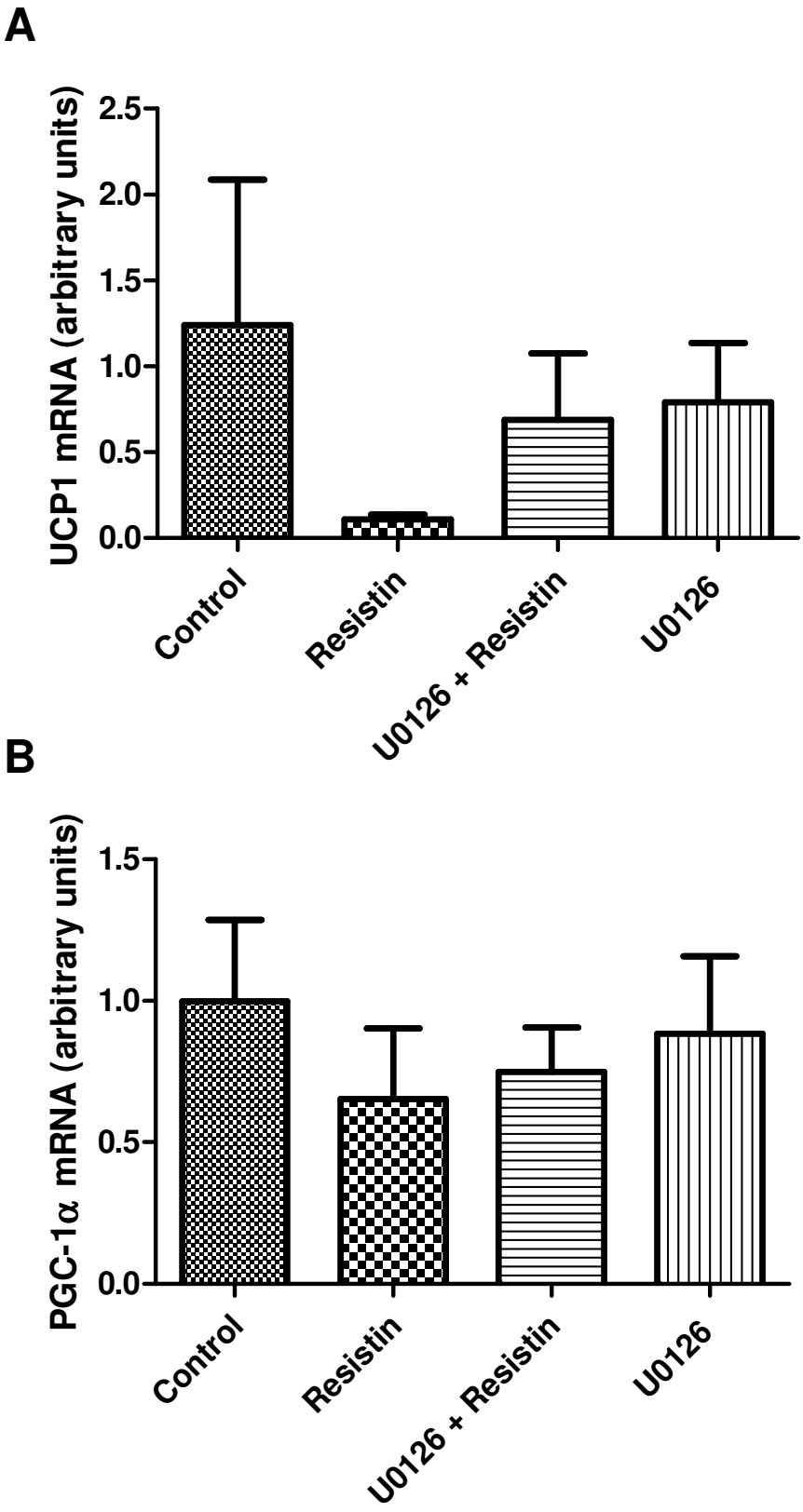

Figure 6.4 See the next page for figure legends

Figure 6.4

(A) UCP1 and (B) PGC-1α mRNA abundance in brown adipose tissue from control (n= 4 for UCP1, n=6 for PGC-1α), resistin (n=5), U0126 (ERK1/2 inhibitor) + resistin (n=4) and U0126 (n=3) groups. Values are expressed relative to 18S ribosomal RNA and presented in arbitrary units (mean ± SEM).

6.4 DISCUSSION

The present study is the first to report on (i) the effects of central ERK1/2 inhibition in the brain on thermogenesis induced by central resistin and (ii) the effects of resistin on genetic markers of thermogenesis in BAT. The findings show that ERK1/2 in the brain is involved in the action of resistin on thermogenesis in BAT.

Metabolic conditions like obesity are characterised by an imbalance between energy intake and expenditure. One mechanism to increase energy expenditure is to increase thermogenesis in BAT and is well recognised in rodents. There is considerable BAT in human newborns which plays an important role in temperature regulation and non-shivering thermogenesis and recent work has now identified considerable functional BAT deposits in adult humans suggesting an important role in energy expenditure in adults (142). The mechanism involved in the inhibition of BAT SNA by resistin was investigated in the present study. I found that blocking ERK1/2 prevented the inhibitory action of resistin on BAT SNA for approximately 150 minutes. To account for this finding, it is possible that the effect of U0126 either wore off after that time and this is consistent with the findings of previous work that showed the half-life of U0126 injected into the brains of finches was about 2 hours (148). Alternatively, other intracellular mechanisms may contribute to the actions of resistin on BAT SNA. In rats, in which ERK1/2 inhibition prevented the response induced by leptin and insulin on BAT SNA (63, 138), the duration of action of U1026 was at least 6 hours, supporting the idea of alternative mechanisms. The mechanism by which ERK1/2 can mediate the reduction in BAT SNA induced by resistin and the opposing increase induced by leptin is unclear. It is likely, however, that different cell groups mediate the actions of the two adipokines on BAT SNA.

The attenuation of the resistin response by inhibition of ERK1/2 on BAT SNA observed in this study was selective, since inhibition of PI 3-Kinase did not affect the inhibitory action of resistin on BAT SNA. Furthermore, inhibition of ERK1/2 did not affect the sympatho-excitatory action of resistin on renal nerve activity, suggesting that the dose of U0126 used in this study to block ERK1/2 pathways in the brain, did not act non-specifically to influence SNA.

In the present study, acute ICV injections of resistin markedly decreased BAT temperature, and this effect was significantly attenuated when central ERK1/2 was inhibited. The fall in BAT temperature was most likely responsible for the resistin-induced decrease in body core temperature. When central ERK1/2 was inhibited, the decrease in body core temperature induced by resistin was blunted (in excess of 60%) but the effect was not statistically significant, probably due to the greater variance seen in the control group. Nonetheless, the present results are in agreement with my earlier finding, showing that central resistin reduces BAT SNA (Chapter 4), and that the effect was mediated by central ERK1/2 (Present work).

In this study, central resistin treatment reduced UCP1 mRNA expression in BAT, which is consistent with the findings that resistin reduces BAT temperature and BAT SNA. The present data suggests this response is blunted when central ERK1/2 signalling pathways are inhibited, however, due to the variability in UCP1 expression in the control group and the limited sample size, these changes were not statistically significant. UCP1 is present on the inner mitochondrial membranes of adipocytes in BAT. It is responsible for uncoupling ATP production from oxidative phosphorylation, by reducing the proton gradient, which generates heat (118). This is important for non-shivering thermogenesis and maintaining normal body temperature. It is also believed UCP1 may play a vital role in managing body weight through its ability to expend energy. Indeed, Feldmann and colleagues recently

demonstrated UCP1 ablation induced obesity in mice fed a control diet under thermoneutral conditions (149). To our knowledge, no other study has investigated the effects of centrally administered resistin on UCP1 expression in BAT. The resistin-induced UCP1 reduction observed in this study is consistent with reduced thermogenic capacity and energy expenditure.

PGC-1α belongs to a family of nuclear transcriptional cofactors that regulate mitochondrial biogenesis and are involved in the regulation of brown adipocyte-specific genes (146). PGC-1α putatively controls UCP1 expression although this role remains controversial since studies have shown that PGC-1α and UCP1 gene expression in brown adipocytes are not necessarily correlated (150, 151). In the current study, the average PGC-1α mRNA abundance was lower in the resistin treated group, however, it did not reach statistical significance. Nonetheless, it is noteworthy that PGC-1α did follow the same expression pattern between groups as UCP1.

In conclusion, the main finding of this study highlights the role of ERK1/2 signalling pathways in the brain in mediating the action of resistin on thermogenesis in BAT. Moreover, centrally administered resistin decreased the expression of UCP1, which is in agreement with the finding.

Chapter 7: General discussion and conclusion

This study showed for the first time that the adipokine resistin acts centrally to influence sympathetic nerve activity (SNA). Prior to this study, leptin and adiponectin were the only adipokines known to influence the SNA. In the present work, I found that centrally administered resistin increased lumbar and renal SNA and reduced brown adipose tissue (BAT) SNA. Therefore resistin has a non-generalised effect on SNA. It is not uncommon to observe differential responses in SNA. An increase in sympathetic drive to the cardiovascular tissues could contribute to increased vascular resistance. A reduction in BAT SNA induces a reduction in thermogenesis and reduced energy expenditure. Such actions could contribute to the undesirable cardiovascular and metabolic abnormalities characteristic of metabolic syndrome, obesity and diabetes.

Increased SNA to the kidney and muscle vasculature is observed in obesity, indeed, the level of sympathetic nerve activity to the skeletal muscle vasculature in obese individuals is correlated to abdominal visceral adiposity (60). The findings of this thesis show that resistin increases renal and lumbar SNA, suggesting that resistin could contribute to the SNA disturbances observed in obesity, but this needs to be investigated in future studies. In hypertension, SNA is elevated (3, 56, 152, 153), however, we did not observe any significant increase in blood pressure following the acute administration of resistin. The effects of long-term central resistin administration on blood pressure, however, have yet to be studied. Nonetheless, in obesity, both resistin and leptin are elevated, thus the possibility exists that these two adipokines may act synergistically to increase renal and lumbar SNA. Since increased renal SNA can induce sodium retention and alter renal haemodynamics, and thereby influence long term blood pressure regulation, the two adipokines may act in concert

to contribute to obesity-induced hypertension. Further studies in animal models of obesity are needed to explore this suggestion.

The other major finding of this work was that centrally administered resistin reduced thermogenesis in BAT. The present work is the first to directly measure BAT SNA following administration of resistin. I found there was a significant decrease of over 50% in BAT SNA following resistin administration. This indicates that resistin reduces thermogenesis and this is supported by the finding, in separate animals, of a reduction in BAT temperature and a concomitant decrease in body core temperature. The ability of resistin to act centrally to reduce thermogenesis contrasts with the actions of leptin which increases BAT SNA (62, 63), an action mediated via the hypothalamic arcuate nucleus (63). Thus, leptin's actions of reducing food intake and increasing thermogenesis result in an increased energy output and resultant weight loss. By contrast, resistin can decrease thermogenesis as well as food intake. Thus, the reduced dietary intake is counteracted with mechanisms designed for the preservation of energy.

Consistent with the finding that resistin reduces thermogenesis in BAT, in the present study, I also found that central resistin treatment reduced the expression of uncoupling protein-1 (UCP1) mRNA, a genetic marker of thermogenesis in BAT. UCP1 is positioned on the inner mitochondrial membrane of brown adipocytes and is responsible for uncoupling the production of ATP from oxidative phosphorylation, thereby generating heat (118). While maintaining normal body temperature, it is also postulated UCP1 may play a vital role in body weight management through its ability to expend energy. Indeed, Feldmann and colleagues recently demonstrated UCP1 ablation to induce obesity in mice feed control diets under thermoneutral conditions (149). The reductions in UCP1 abundance observed in the

current study in response to resistin treatment indicate a reduction in thermogenic capacity and subsequent potential for energy expenditure. Such actions could contribute to metabolic dysfunctions characteristic of disease states such as obesity and diabetes.

Prior to this work, the intracellular signalling pathways mediating resistin's actions on sympathetic nerve activity were not known. It was known that phosphatidylinositol 3-kinases (PI 3-Kinase) and extracellular regulated kinases (ERK) 1/2 were involved in the actions of resistin on cell growth, differentiation, metabolism and intracellular trafficking. PI 3-Kinase and ERK 1/2 are involved in sympathetic nerve activity responses as shown by the critical role of PI 3-Kinase in mediating the changes in renal sympathetic nerve activity induced by central administration of leptin (139), and the lumbar sympathetic nerve activity changes induced by insulin (138). ERK1/2 activation mediates the changes induced by leptin, and insulin, on sympathetic nerve activity to brown adipose tissue (63, 138).

In the present work, it was demonstrated that PI 3-Kinase but not ERK1/2 mediated the sympatho-excitatory action of resistin on renal SNA. Leptin, too, increases renal SNA via the PI 3-Kinase signalling pathway (63, 139), suggesting both adipokines act through similar mechanisms to elicit renal sympatho-excitation. Presumably, this means that there may be a common nucleus in the brain mediating the increased renal SNA, but this key common site is not yet identified. Since we used intracerebroventricular injections, it is important to note that the site of action of inhibition of PI 3-Kinase may be different from that at which the adipokines directly act. The central sites directly activated by resistin are not yet clearly identified. Studies including the present work, using the protein, Fos, as a marker of increased neuronal activity, suggest the paraventricular nucleus as a potential site (43, 131, 133).

The mechanism involved in the inhibition of BAT SNA by resistin was also investigated in the present work. I found that ERK1/2 pathway but not PI 3-Kinase was involved in the action of resistin on BAT SNA. This was confirmed by finding that acute intracerebroventricular administration of resistin induced a marked decrease in BAT and body core temperature but this was attenuated by central inhibition of ERK1/2. Moreover, resistin was shown to reduce UCP1 expression which is a marker of thermogenesis in BAT. A trend suggesting attenuation of this response was observed by inhibition of ERK1/2, which further highlights the role of ERK1/2 in mediating the effects of central resistin on thermogenesis in BAT.

ERK1/2 is also known to mediate the action of central leptin on increasing thermogenesis in BAT. The mechanism by which ERK1/2 can mediate the reduction in BAT SNA induced by resistin and the opposing increase induced by leptin is unclear. It is likely, however, that different cell groups mediate the actions of the two adipokines on BAT SNA.

In conclusion, this study showed that in contrast to renal and lumbar SNA, resistin and leptin have opposing actions on the activity of sympathetic nerves innervating BAT. Taken together, the results suggest that the two adipokines have opposing actions on thermogenesis but similar actions on sympathetic nerves innervating cardiovascular organs. This suggests that in conditions in which plasma levels of both adipokines are elevated, for example obesity, the two could interact to contribute to the cardiovascular and metabolic disturbances that are observed in those metabolic conditions.

References

1. AIHW 2011. Key indicators of progress for chronic disease and associated determinants: data report. Canberra2011 [cited 2012 16/11/2012]; Cat. no. PHE 142:[

2. Marinou K, Tousoulis D, Antonopoulos AS, Stefanadi E, Stefanadis C. Obesity and cardiovascular disease: From pathophysiology to risk stratification. Int J Cardiol. 2009 Apr 25;138(1):3-8.

3. Hall JE. The kidney, hypertension, and obesity. Hypertension. 2003 Mar;41(3 Pt 2):625-33.

4. Grassi G, Seravalle G, Quarti-Trevano F, Scopelliti F, Dell'Oro R, Bolla G, et al. Excessive sympathetic activation in heart failure with obesity and metabolic syndrome: characteristics and mechanisms. Hypertension. 2007 Mar;49(3):535-41.

5. Dunbar JC, Hu Y, Lu H. Intracerebroventricular leptin increases lumbar and renal sympathetic nerve activity and blood pressure in normal rats. Diabetes. 1997 Dec;46(12):2040-3.

6. Haynes WG, Morgan DA, Walsh SA, Mark AL, Sivitz WI. Receptor-mediated regional sympathetic nerve activation by leptin. J Clin Invest. 1997 Jul 15;100(2):270-8.

7. Rahmouni K, Haynes WG, Mark AL. Cardiovascular and sympathetic effects of leptin. Curr Hypertens Rep. 2002 Apr;4(2):119-25.

8. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, et al. The hormone resistin links obesity to diabetes. Nature. 2001 Jan 18;409(6818):307-12.

9. Morash BA, Willkinson D, Ur E, Wilkinson M. Resistin expression and regulation in mouse pituitary. FEBS Lett. 2002 Aug 28;526(1-3):26-30.

10. Yang RZ, Huang Q, Xu A, McLenithan JC, Eisen JA, Shuldiner AR, et al. Comparative studies of resistin expression and phylogenomics in human and mouse. Biochem Biophys Res Commun. 2003 Oct 24;310(3):927-35.

11. Rajala MW, Qi Y, Patel HR, Takahashi N, Banerjee R, Pajvani UB, et al. Regulation of resistin expression and circulating levels in obesity, diabetes, and fasting. Diabetes. 2004 Jul;53(7):1671-9.

12. McTernan CL, McTernan PG, Harte AL, Levick PL, Barnett AH, Kumar S. Resistin, central obesity, and type 2 diabetes. Lancet. 2002 Jan 5;359(9300):46-7.

13. de Luis DA, Gonzalez Sagrado M, Conde R, Aller R, Izaola O, Perez Castrillon JL, et al. Relation of resistin levels with cardiovascular risk factors and insulin resistance in non-diabetes obese patients. Diabetes Res Clin Pract. 2009 May;84(2):174-8.

14. Piestrzeniewicz K, Luczak K, Komorowski J, Maciejewski M, Jankiewicz Wika J, Goch JH. Resistin increases with obesity and atherosclerotic risk factors in patients with myocardial infarction. Metabolism: clinical and experimental. 2008 Apr;57(4):488-93.

15. Azuma K, Katsukawa F, Oguchi S, Murata M, Yamazaki H, Shimada A, et al. Correlation between serum resistin level and adiposity in obese individuals. Obes Res. 2003 Aug;11(8):997-1001.

16. Degawa-Yamauchi M, Bovenkerk JE, Juliar BE, Watson W, Kerr K, Jones R, et al. Serum resistin (FIZZ3) protein is increased in obese humans. J Clin Endocrinol Metab. 2003 Nov;88(11):5452-5.

17. Bobbert P, Jenke A, Bobbert T, Kuhl U, Rauch U, Lassner D, et al. High leptin and resistin expression in chronic heart failure: adverse outcome in patients with dilated and inflammatory cardiomyopathy. Eur J Heart Fail. 2012 Jul 4;14(11):1265-75.

18. Yaseen F, Jaleel A, Aftab J, Zuberi A, Alam E. Circulating levels of resistin, IL-6 and lipid profile in elderly patients with ischemic heart disease with and without diabetes. Biomark Med. 2012 Feb;6(1):97-102.

19. Butler J, Kalogeropoulos A, Georgiopoulou V, de Rekeneire N, Rodondi N, Smith AL, et al. Serum resistin concentrations and risk of new onset heart failure in older persons: the health, aging, and body composition (Health ABC) study. Arterioscler Thromb Vasc Biol. 2009 Jul;29(7):1144-9.

20. Frankel DS, Vasan RS, D'Agostino Sr RB, Benjamin EJ, Levy D, Wang TJ, et al. Resistin, Adiponectin, and Risk of Heart Failure: The Framingham Offspring Study. Journal of the American College of Cardiology. 2009;53(9):754-62.

21. Papadopoulos DP, Perrea D, Thomopoulos C, Sanidas E, Daskalaki M, Papazachou U, et al. Masked hypertension and atherogenesis: the impact on adiponectin and resistin plasma levels. J Clin Hypertens (Greenwich). 2009 Feb;11(2):61-5.

22. Pischon T, Bamberger CM, Kratzsch J, Zyriax BC, Algenstaedt P, Boeing H, et al. Association of plasma resistin levels with coronary heart disease in women. Obes Res. 2005 Oct;13(10):1764-71.

23. Takeishi Y, Niizeki T, Arimoto T, Nozaki N, Hirono O, Nitobe J, et al. Serum resistin is associated with high risk in patients with congestive heart failure--a novel link between metabolic signals and heart failure. Circ J. 2007 Apr;71(4):460-4.

24. Thomopoulos C, Daskalaki M, Papazachou O, Rodolakis N, Bratsas A, Papadopoulos DP, et al. Association of resistin and adiponectin with different clinical blood pressure phenotypes. J Hum Hypertens. 2010 Mar 4;25(1):38-46.

25. Gupta V, Singh AK, Kumar S, Srivastava N, Jafar T, Pant AB. Association of circulating resistin with metabolic risk factors in Indian females having metabolic syndrome. Toxicol Int. 2011 Jul;18(2):168-72.

26. Steppan CM, Lazar MA. Resistin and obesity-associated insulin resistance. Trends Endocrinol Metab. 2002 Jan-Feb;13(1):18-23.

27. Ghosh S, Singh AK, Aruna B, Mukhopadhyay S, Ehtesham NZ. The genomic organization of mouse resistin reveals major differences from the human resistin: functional implications. Gene. 2003 Feb 13;305(1):27-34.

28. Patel SD, Rajala MW, Rossetti L, Scherer PE, Shapiro L. Disulfide-dependent multimeric assembly of resistin family hormones. Science. 2004 May 21;304(5674):1154-8.

29. Daquinag AC, Zhang Y, Amaya-Manzanares F, Simmons PJ, Kolonin MG. An isoform of decorin is a resistin receptor on the surface of adipose progenitor cells. Cell Stem Cell. 2011 Jul 8;9(1):74-86.

30. Sanchez-Solana B, Laborda J, Baladron V. Mouse resistin modulates adipogenesis and glucose uptake in 3T3-L1 preadipocytes through the ROR1 receptor. Mol Endocrinol. 2012 Jan;26(1):110-27.

31. Benomar Y, Gertler A, De Lacy P, Crepin D, Ould Hamouda H, Riffault L, et al. Central resistin overexposure induces insulin resistance through Toll-like receptor 4. Diabetes. 2013 Jan;62(1):102-14.

32. Tarkowski A, Bjersing J, Shestakov A, Bokarewa MI. Resistin competes with lipopolysaccharide for binding to toll-like receptor 4. J Cell Mol Med. 2010 Jun;14(6B):1419-31.

33. Yang G, Li L, Fang C, Zhang L, Li Q, Tang Y, et al. Effects of free fatty acids on plasma resistin and insulin resistance in awake rats. Metabolism: clinical and experimental. 2005 Sep;54(9):1142-6.

34. Kim KH, Lee K, Moon YS, Sul HS. A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. J Biol Chem. 2001 Apr 6;276(14):11252-6.

35. Curat CA, Wegner V, Sengenes C, Miranville A, Tonus C, Busse R, et al. Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. Diabetologia. 2006 Apr;49(4):744-7.

36. Nohira T, Nagao K, Kameyama K, Nakai H, Fukumine N, Okabe K, et al. Identification of an alternative splicing transcript for the resistin gene and distribution of its mRNA in human tissue. Eur J Endocrinol. 2004 Jul;151(1):151-4.

37. Nogueiras R, Gallego R, Gualillo O, Caminos JE, Garcia-Caballero T, Casanueva FF, et al. Resistin is expressed in different rat tissues and is regulated in a tissue- and gender-specific manner. FEBS Lett. 2003 Jul 31;548(1-3):21-7.

38. Szalowska E, Elferink MG, Hoek A, Groothuis GM, Vonk RJ. Resistin is more abundant in liver than adipose tissue and is not up-regulated by lipopolysaccharide. J Clin Endocrinol Metab. 2009 Aug;94(8):3051-7.

39. Minn AH, Patterson NB, Pack S, Hoffmann SC, Gavrilova O, Vinson C, et al. Resistin is expressed in pancreatic islets. Biochem Biophys Res Commun. 2003 Oct 17;310(2):641-5.

40. Schaffler A, Ehling A, Neumann E, Herfarth H, Tarner I, Scholmerich J, et al. Adipocytokines in synovial fluid. JAMA. 2003 Oct 1;290(13):1709-10.

41. Yura S, Sagawa N, Itoh H, Kakui K, Nuamah MA, Korita D, et al. Resistin is expressed in the human placenta. J Clin Endocrinol Metab. 2003 Mar;88(3):1394-7.

42. Lappas M, Yee K, Permezel M, Rice GE. Release and regulation of leptin, resistin and adiponectin from human placenta, fetal membranes, and maternal adipose tissue and skeletal muscle from normal and gestational diabetes mellitus-complicated pregnancies. J Endocrinol. 2005 Sep;186(3):457-65.

43. Tovar S, Nogueiras R, Tung LY, Castaneda TR, Vazquez MJ, Morris A, et al. Central administration of resistin promotes short-term satiety in rats. Eur J Endocrinol. 2005 Sep;153(3):R1-5.

44. Kos K, Harte AL, da Silva NF, Tonchev A, Chaldakov G, James S, et al. Adiponectin and resistin in human cerebrospinal fluid and expression of adiponectin receptors in the human hypothalamus. J Clin Endocrinol Metab. 2007 Mar;92(3):1129-36.

45. Rajala MW, Obici S, Scherer PE, Rossetti L. Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. J Clin Invest. 2003 Jan;111(2):225-30.

46. Lazar MA. Resistin- and Obesity-associated metabolic diseases. Horm Metab Res. 2007 Oct;39(10):710-6.

47. Banerjee RR, Rangwala SM, Shapiro JS, Rich AS, Rhoades B, Qi Y, et al. Regulation of fasted blood glucose by resistin. Science. 2004 Feb 20;303(5661):1195-8.

48. Habib SS. Serum resistin levels in patients with type 2 diabetes mellitus and its relationship with body composition. Saudi Med J. 2012 May;33(5):495-9.

49. Pagano C, Marin O, Calcagno A, Schiappelli P, Pilon C, Milan G, et al. Increased serum resistin in adults with prader-willi syndrome is related to obesity and not to insulin resistance. J Clin Endocrinol Metab. 2005 Jul;90(7):4335-40.

50. McTernan PG, McTernan CL, Chetty R, Jenner K, Fisher FM, Lauer MN, et al. Increased resistin gene and protein expression in human abdominal adipose tissue. J Clin Endocrinol Metab. 2002 May;87(5):2407.

51. Burnett MS, Devaney JM, Adenika RJ, Lindsay R, Howard BV. Cross-sectional associations of resistin, coronary heart disease, and insulin resistance. J Clin Endocrinol Metab. 2006 Jan;91(1):64-8.

52. Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV, et al. Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. Diabetes. 2001 Oct;50(10):2199-202.

53. Qatanani M, Szwergold NR, Greaves DR, Ahima RS, Lazar MA. Macrophage-derived human resistin exacerbates adipose tissue inflammation and insulin resistance in mice. J Clin Invest. 2009 Feb 2;119(3):531-9.

54. Grundy SM. Obesity, metabolic syndrome, and coronary atherosclerosis. Circulation. 2002 Jun 11;105(23):2696-8.

55. Frayn KN. Obesity and metabolic disease: is adipose tissue the culprit? The Proceedings of the Nutrition Society. 2005 Feb;64(1):7-13.

56. Davy KP, Orr JS. Sympathetic nervous system behavior in human obesity. Neuroscience and biobehavioral reviews. 2009 Feb;33(2):116-24.

57. Vaz M, Jennings G, Turner A, Cox H, Lambert G, Esler M. Regional sympathetic nervous activity and oxygen consumption in obese normotensive human subjects. Circulation. 1997 Nov 18;96(10):3423-9.

58. Grassi G, Seravalle G, Colombo M, Bolla G, Cattaneo BM, Cavagnini F, et al. Body weight reduction, sympathetic nerve traffic, and arterial baroreflex in obese normotensive humans. Circulation. 1998 May 26;97(20):2037-42.

59. Schwartz RS, Jaeger LF, Veith RC, Lakshminarayan S. The effect of diet or exercise on plasma norepinephrine kinetics in moderately obese young men. Int J Obes. 1990 Jan;14(1):1-11.

60. Alvarez GE, Beske SD, Ballard TP, Davy KP. Sympathetic neural activation in visceral obesity. Circulation. 2002 Nov 12;106(20):2533-6.

61. Barretto AC, Santos AC, Munhoz R, Rondon MU, Franco FG, Trombetta IC, et al. Increased muscle sympathetic nerve activity predicts mortality in heart failure patients. Int J Cardiol. 2008 Jun 25;135(3):302-7.

62. Hausberg M, Morgan DA, Mitchell JL, Sivitz WI, Mark AL, Haynes WG. Leptin potentiates thermogenic sympathetic responses to hypothermia: a receptor-mediated effect. Diabetes. 2002 Aug;51(8):2434-40.

63. Rahmouni K, Sigmund CD, Haynes WG, Mark AL. Hypothalamic ERK mediates the anorectic and thermogenic sympathetic effects of leptin. Diabetes. 2009 Mar;58(3):536-42.

64. Tanida M, Shen J, Horii Y, Matsuda M, Kihara S, Funahashi T, et al. Effects of adiponectin on the renal sympathetic nerve activity and blood pressure in rats. Exp Biol Med (Maywood). 2007 Mar;232(3):390-7.

65. Masaki T, Chiba S, Yasuda T, Tsubone T, Kakuma T, Shimomura I, et al. Peripheral, but not central, administration of adiponectin reduces visceral adiposity and upregulates the expression of uncoupling protein in agouti yellow (Ay/a) obese mice. Diabetes. 2003 Sep;52(9):2266-73.

66. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994 Dec 1;372(6505):425-32.

67. Bjorbaek C, Kahn BB. Leptin signaling in the central nervous system and the periphery. Recent Prog Horm Res. 2004;59:305-31.

68. Amasyali B, Kose S, Kursaklioglu H, Kilic A, Isik E. Leptin in acute coronary syndromes: has the time come for its use in risk stratification? Int J Cardiol. 2008 Nov 12;130(2):264-5.

69. Haynes WG. Interaction between leptin and sympathetic nervous system in hypertension. Curr Hypertens Rep. 2000 Jun;2(3):311-8.

70. Rahmouni K, Correia ML, Haynes WG, Mark AL. Obesity-associated hypertension: new insights into mechanisms. Hypertension. 2005 Jan;45(1):9-14.

71. Rahmouni K, W GH. Leptin and the central neural mechanisms of obesity hypertension. Drugs Today (Barc). 2002 Dec;38(12):807-17.

72. Collins S, Kuhn CM, Petro AE, Swick AG, Chrunyk BA, Surwit RS. Role of leptin in fat regulation. Nature. 1996 Apr 25;380(6576):677.

73. Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G. Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. Diabetes. 2003 Jul;52(7):1779-85.

74. Ellington AA, Malik AR, Klee GG, Turner ST, Rule AD, Mosley TH, Jr., et al. Association of plasma resistin with glomerular filtration rate and albuminuria in hypertensive adults. Hypertension. 2007 Oct;50(4):708-14.

75. Dimitriadis K, Tsioufis C, Selima M, Tsiachris D, Miliou A, Kasiakogias A, et al. Independent association of circulating resistin with glomerular filtration rate in the early stages of essential hypertension. J Hum Hypertens. 2009 Oct;23(10):668-73.

76. Tsioufis C, Dimitriadis K, Selima M, Miliou A, Toutouzas K, Roussos D, et al. Association of resistin with urinary albumin excretion in nondiabetic patients with essential hypertension. Am J Hypertens. 2010 Jun;23(6):681-6.

77. Papadopoulos DP, Makris TK, Perrea D, Papazachou O, Daskalaki M, Sanidas E, et al. Adiponectin--insulin and resistin plasma levels in young healthy offspring of patients with essential hypertension. Blood Press. 2008;17(1):50-4.

78. Zhang L, Curhan GC, Forman JP. Plasma resistin levels associate with risk for hypertension among nondiabetic women. J Am Soc Nephrol. 2010 Jul;21(7):1185-91.

79. Zhang J, Qin Y, Zheng X, Qiu J, Gong L, Mao H, et al. [The relationship between human serum resistin level and body fat content, plasma glucose as well as blood pressure]. Zhonghua Yi Xue Za Zhi. 2002 Dec 10;82(23):1609-12.

80. Olszanecka A, Posnik-Urbanska A, Kawecka-Jaszcz K, Czarnecka D, Fedak D. Adipocytokines and blood pressure, lipids and glucose metabolism in hypertensive perimenopausal women. Kardiol Pol. 2010 Jul;68(7):753-60.

81. Takata Y, Osawa H, Kurata M, Kurokawa M, Yamauchi J, Ochi M, et al. Hyperresistinemia is associated with coexistence of hypertension and type 2 diabetes. Hypertension. 2008 Feb;51(2):534-9.

82. Kim M, Oh Jk, Sakata S, Liang I, Park W, Hajjar RJ, et al. Role of resistin in cardiac contractility and hypertrophy. Journal of Molecular and Cellular Cardiology. 2008;45(2):270-80.

83. Graveleau C, Zaha VG, Mohajer A, Banerjee RR, Dudley-Rucker N, Steppan CM, et al. Mouse and human resistins impair glucose transport in primary mouse cardiomyocytes, and oligomerization is required for this biological action. J Biol Chem. 2005 Sep 9;280(36):31679-85.

84. McTernan PG, Fisher FM, Valsamakis G, Chetty R, Harte A, McTernan CL, et al. Resistin and type 2 diabetes: regulation of resistin expression by insulin and rosiglitazone and the effects of recombinant resistin on lipid and glucose metabolism in human differentiated adipocytes. J Clin Endocrinol Metab. 2003 Dec;88(12):6098-106.

85. Chemaly ER, Hadri L, Zhang S, Kim M, Kohlbrenner E, Sheng J, et al. Long-term in vivo resistin overexpression induces myocardial dysfunction and remodeling in rats. J Mol Cell Cardiol. 2011 Apr 23;51(2):144-55.

86. Kang S, Chemaly ER, Hajjar RJ, Lebeche D. Resistin Promotes Cardiac Hypertrophy via the AMP-activated Protein Kinase/Mammalian Target of Rapamycin (AMPK/mTOR) and c-Jun N-terminal Kinase/Insulin Receptor Substrate 1 (JNK/IRS1) Pathways. J Biol Chem. 2011 May 27;286(21):18465-73.

87. Lubos E, Messow CM, Schnabel R, Rupprecht HJ, Espinola-Klein C, Bickel C, et al. Resistin, acute coronary syndrome and prognosis results from the AtheroGene study. Atherosclerosis. 2007 Jul;193(1):121-8.

88. Weikert C, Westphal S, Berger K, Dierkes J, Mohlig M, Spranger J, et al. Plasma resistin levels and risk of myocardial infarction and ischemic stroke. J Clin Endocrinol Metab. 2008 Jul;93(7):2647-53.

89. Rienstra M, Sun JX, Lubitz SA, Frankel DS, Vasan RS, Levy D, et al. Plasma resistin, adiponectin, and risk of incident atrial fibrillation: the Framingham Offspring Study. Am Heart J. 2012 Jan;163(1):119-24 e1.

90. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest. 2003 Dec;112(12):1785-8.

91. Pinteaux E, Inoue W, Schmidt L, Molina-Holgado F, Rothwell NJ, Luheshi GN. Leptin induces interleukin-1beta release from rat microglial cells through a caspase 1 independent mechanism. Journal of neurochemistry. 2007 Aug;102(3):826-33.

92. Jung HS, Park KH, Cho YM, Chung SS, Cho HJ, Cho SY, et al. Resistin is secreted from macrophages in atheromas and promotes atherosclerosis. Cardiovascular research. 2006 Jan;69(1):76-85.

93. Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, et al. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. Biochem Biophys Res Commun. 2003 Jan 10;300(2):472-6.

94. Mirjanic-Azaric B, Deric M, Vrhovac M, Males-Bilic L. [Correlation between C-reactive protein levels with leading risk factors for cardiovascular disease in men]. Med Pregl. 2008 Mar-Apr;61(3-4):164-8.

95. Won JC, Park CY, Lee WY, Lee ES, Oh SW, Park SW. Association of plasma levels of resistin with subcutaneous fat mass and markers of inflammation but not with metabolic determinants or insulin resistance. J Korean Med Sci. 2009 Aug;24(4):695-700.

96. Shetty GK, Economides PA, Horton ES, Mantzoros CS, Veves A. Circulating adiponectin and resistin levels in relation to metabolic factors, inflammatory markers, and vascular reactivity in diabetic patients and subjects at risk for diabetes. Diabetes Care. 2004 Oct;27(10):2450-7.

97. Nakou ES, Liberopoulos EN, Milionis HJ, Elisaf MS. The role of C-reactive protein in atherosclerotic cardiovascular disease: an overview. Curr Vasc Pharmacol. 2008 Oct;6(4):258-70.

98. Barnes KM, Miner JL. Role of resistin in insulin sensitivity in rodents and humans. Curr Protein Pept Sci. 2009 Feb;10(1):96-107.

99. Reilly MP, Lehrke M, Wolfe ML, Rohatgi A, Lazar MA, Rader DJ. Resistin is an inflammatory marker of atherosclerosis in humans. Circulation. 2005 Feb 22;111(7):932-9.

100. Wilcox JN, Nelken NA, Coughlin SR, Gordon D, Schall TJ. Local expression of inflammatory cytokines in human atherosclerotic plaques. J Atheroscler Thromb. 1994;1 Suppl 1:S10-3.

101. Cho Y, Lee SE, Lee HC, Hur J, Lee S, Youn SW, et al. Adipokine resistin is a key player to modulate monocytes, endothelial cells, and smooth muscle cells, leading to progression of atherosclerosis in rabbit carotid artery. Journal of the American College of Cardiology. 2011 Jan 4;57(1):99-109.

102. Burnett MS, Lee CW, Kinnaird TD, Stabile E, Durrani S, Dullum MK, et al. The potential role of resistin in atherogenesis. Atherosclerosis. 2005 Oct;182(2):241-8.

103. Krecki R, Krzeminska-Pakula M, Peruga JZ, Szczesniak P, Lipiec P, Wierzbowska-Drabik K, et al. Elevated resistin opposed to adiponectin or angiogenin plasma levels as a strong, independent predictive factor for the occurrence of major adverse cardiac and cerebrovascular events in patients with stable multivessel coronary artery disease over 1-year follow-up. Med Sci Monit. 2011 Jan;17(1):CR26-32.

104. Verma S, Li SH, Wang CH, Fedak PW, Li RK, Weisel RD, et al. Resistin promotes endothelial cell activation: further evidence of adipokine-endothelial interaction. Circulation. 2003 Aug 12;108(6):736-40.

105. Hsu WY, Chao YW, Tsai YL, Lien CC, Chang CF, Deng MC, et al. Resistin induces monocyte-endothelial cell adhesion by increasing ICAM-1 and VCAM-1 expression in endothelial cells via p38MAPK-dependent pathway. J Cell Physiol. 2011 Aug;226(8):2181-8.

106. Calabro P, Samudio I, Willerson JT, Yeh ET. Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways. Circulation. 2004 Nov 23;110(21):3335-40.

107. Skilton MR, Nakhla S, Sieveking DP, Caterson ID, Celermajer DS. Pathophysiological levels of the obesity related peptides resistin and ghrelin increase adhesion molecule expression on human vascular endothelial cells. Clin Exp Pharmacol Physiol. 2005 Oct;32(10):839-44.

108. Lee TS, Lin CY, Tsai JY, Wu YL, Su KH, Lu KY, et al. Resistin increases lipid accumulation by affecting class A scavenger receptor, CD36 and ATP-binding cassette transporter-A1 in macrophages. Life Sci. 2009 Jan 16;84(3-4):97-104.

109. Chen C, Jiang J, Lu JM, Chai H, Wang X, Lin PH, et al. Resistin decreases expression of endothelial nitric oxide synthase through oxidative stress in human coronary artery endothelial cells. American journal of physiology. 2010 Jul;299(1):H193-201.

110. Steppan CM, Wang J, Whiteman EL, Birnbaum MJ, Lazar MA. Activation of SOCS-3 by resistin. Mol Cell Biol. 2005 Feb;25(4):1569-75.

111. Muse ED, Lam TK, Scherer PE, Rossetti L. Hypothalamic resistin induces hepatic insulin resistance. J Clin Invest. 2007 Jun;117(6):1670-8.

112. Winder WW, Hardie DG. AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. Am J Physiol. 1999 Jul;277(1 Pt 1):E1-10.

113. Senn JJ, Klover PJ, Nowak IA, Zimmers TA, Koniaris LG, Furlanetto RW, et al. Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. J Biol Chem. 2003 Apr 18;278(16):13740-6.

114. Rui L, Yuan M, Frantz D, Shoelson S, White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem. 2002 Nov 1;277(44):42394-8.

115. Ueki K, Kondo T, Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol. 2004 Jun;24(12):5434-46.

116. Rangwala SM, Rich AS, Rhoades B, Shapiro JS, Obici S, Rossetti L, et al. Abnormal glucose homeostasis due to chronic hyperresistinemia. Diabetes. 2004 Aug;53(8):1937-41.

117. Vazquez MJ, Gonzalez CR, Varela L, Lage R, Tovar S, Sangiao-Alvarellos S, et al. Central resistin regulates hypothalamic and peripheral lipid metabolism in a nutritional-dependent fashion. Endocrinology. 2008 Sep;149(9):4534-43.

118. Cannon B, Nedergaard J. Brown adipose tissue: Function and physiological significance. Physiological Reviews. 2004 Jan;84(1):277-359.

119. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. Am J Physiol Endocrinol Metab. 2007 Aug;293(2):E444-52.

120. Rothwell N, Stock M. Luxuskonsumption, diet-induced thermogenesis and brown fat: the case in favour. Clin Sci (Lond). 1983;64(1):19-23.

121. Gallardo N, Bonzon-Kulichenko E, Fernandez-Agullo T, Molto E, Gomez-Alonso S, Blanco P, et al. Tissue-specific effects of central leptin on the expression of genes involved in lipid metabolism in liver and white adipose tissue. Endocrinology. 2007 Dec;148(12):5604-10.

122. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.

123. Ohmori R, Momiyama Y, Kato R, Taniguchi H, Ogura M, Ayaori M, et al. Associations between serum resistin levels and insulin resistance, inflammation, and coronary artery disease. Journal of the American College of Cardiology. 2005 Jul 19;46(2):379-80.

124. Lieb W, Sullivan LM, Harris TB, Roubenoff R, Benjamin EJ, Levy D, et al. Plasma leptin levels and incidence of heart failure, cardiovascular disease, and total mortality in elderly individuals. Diabetes Care. 2009 Apr;32(4):612-6.

125. Fagius J. Sympathetic nerve activity in metabolic control--some basic concepts. Acta Physiol Scand. 2003 Mar;177(3):337-43.

126. Masuo K, Rakugi H, Ogihara T, Esler MD, Lambert GW. Cardiovascular and renal complications of type 2 diabetes in obesity: role of sympathetic nerve activity and insulin resistance. Curr Diabetes Rev. 2010 Mar;6(2):58-67.

127. Rahmouni K, Haynes WG, Morgan DA, Mark AL. Intracellular mechanisms involved in leptin regulation of sympathetic outflow. Hypertension. 2003 Mar;41(3 Pt 2):763-7.

128. DiBona GF. Neurogenic regulation of renal tubular sodium reabsorption. Am J Physiol. 1977 Aug;233(2):F73-81.

129. Davy KP, Hall JE. Obesity and hypertension: two epidemics or one? Am J Physiol Regul Integr Comp Physiol. 2004 May;286(5):R803-13.

130. Beltowski J. Role of leptin in blood pressure regulation and arterial hypertension. J Hypertens. 2006 May;24(5):789-801.

131. Singhal NS, Lazar MA, Ahima RS. Central resistin induces hepatic insulin resistance via neuropeptide Y. J Neurosci. 2007 Nov 21;27(47):12924-32.

132. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med. 1996 Feb 1;334(5):292-5.

133. Kosari S, Rathner JA, Chen F, Badoer E. Centrally administered resistin enhances sympathetic nerve activity to the hindlimb but attenuates the activity to brown adipose tissue. Endocrinology. 2011 Jul;152(7):2626-33.

134. Robertson SA, Rae CJ, Graham A. Induction of angiogenesis by murine resistin: putative role of PI3-kinase and NO-dependent pathways. Regul Pept. 2009 Jan 8;152(1-3):41-7.

135. Rodriguez-Pacheco F, Vazquez-Martinez R, Martinez-Fuentes AJ, Pulido MR, Gahete MD, Vaudry H, et al. Resistin regulates pituitary somatotrope cell function through the activation of multiple signaling pathways. Endocrinology. 2009 Oct;150(10):4643-52.

136. Di Simone N, Di Nicuolo F, Sanguinetti M, Castellani R, D'Asta M, Caforio L, et al. Resistin regulates human choriocarcinoma cell invasive behaviour and endothelial cell angiogenic processes. J Endocrinol. 2006 Jun;189(3):691-9.

137. Mu H, Ohashi R, TYan S, Chai H, Yang H, Lin P, et al. Adipokine resistin promotes in vitro angiogenesis of human endothelial cells. Cardiovascular research. 2006;70:146-57.

138. Rahmouni K, Morgan DA, Morgan GM, Liu X, Sigmund CD, Mark AL, et al. Hypothalamic PI3K and MAPK differentially mediate regional sympathetic activation to insulin. J Clin Invest. 2004 Sep;114(5):652-8.

139. Morgan DA, Thedens DR, Weiss R, Rahmouni K. Mechanisms mediating renal sympathetic activation to leptin in obesity. Am J Physiol Regul Integr Comp Physiol. 2008 Dec;295(6):R1730-6.

140. Kannan H, Hayashida Y, Yamashita H. Increase in sympathetic outflow by paraventricular nucleus stimulation in awake rats. Am J Physiol. 1989 Jun;256(6 Pt 2):R1325-30.

141. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J. 2000 Oct 1;351(Pt 1):95-105.

142. Enerback S. Human brown adipose tissue. Cell Metab. 2010 Apr 7;11(4):248-52.

143. Ahima RS, Lazar MA. Adipokines and the peripheral and neural control of energy balance. Mol Endocrinol. 2008 May;22(5):1023-31.

144. Madden CJ, Morrison SF. Neurons in the paraventricular nucleus of the hypothalamus inhibit sympathetic outflow to brown adipose tissue. Am J Physiol Regul Integr Comp Physiol. 2009 Mar;296(3):R831-43.

145. Seale P. Transcriptional control of brown adipocyte development and thermogenesis. Int J Obes. 2010;34(S1):S17-S22.

146. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1. Cell. 1999;98(1):115-24.

147. Kosari S, Rathner JA, Badoer E. Central Resistin Enhances Renal Sympathetic Nerve Activity via Pi3k but Reduces the Activity to Brown Adipose Tissue via Erk1/2. J Neuroendocrinol. 2012 Nov;24(11):1432-9.

148. London SE, Clayton DF. Functional identification of sensory mechanisms required for developmental song learning. Nat Neurosci. 2008 May;11(5):579-86.

149. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 Ablation Induces Obesity and Abolishes Diet-Induced Thermogenesis in Mice Exempt from Thermal Stress by Living at Thermoneutrality. Cell Metabolism. 2009;9(2):203-9.

150. Lehr L, Canola K, Asensio C, Jimenez M, Kuehne F, Giacobino J, et al. The control of UCP1 is dissociated from that of PGC-1α or of mitochondriogenesis as revealed by a study using β-less mouse brown adipocytes in culture. FEBS Letters. 2006;580(19):4661-6.

151. Puigserver P, Spiegelman BM. Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC-1α): Transcriptional Coactivator and Metabolic Regulator. Endocrine reviews. 2003 February 1, 2003;24(1):78-90.

152. Schlaich MP, Lambert E, Kaye DM, Krozowski Z, Campbell DJ, Lambert G, et al. Sympathetic augmentation in hypertension: role of nerve firing, norepinephrine reuptake, and Angiotensin neuromodulation. Hypertension. 2004 Feb;43(2):169-75.

153. Esler M, Rumantir M, Wiesner G, Kaye D, Hastings J, Lambert G. Sympathetic nervous system and insulin resistance: from obesity to diabetes. Am J Hypertens. 2001 Nov;14(11 Pt 2):304S-9S.