

University of Warwick institutional repository: <http://go.warwick.ac.uk/wrap>

This paper is made available online in accordance with publisher policies. Please scroll down to view the document itself. Please refer to the repository record for this item and our policy information available from the repository home page for further information.

To see the final version of this paper please visit the publisher's website. Access to the published version may require a subscription.

Author(s): Alison Harte, Philip McTernan, Rajkumar Chetty, Simon Coppack, Jonathan Katz, Stephen Smith, Sudhesh Kumar.

Article Title: Insulin-Mediated Upregulation of the Renin Angiotensin System in Human Subcutaneous Adipocytes is Reduced by Rosiglitazone

Year of publication: 2005

Link to published version:

<http://www.circ.ahajournals.org/cgi/content/abstract/111/15/1954>

CIRCULATIONAHA/2004/518720

**Insulin Mediated Upregulation of the Renin Angiotensin System in Human
Subcutaneous Adipocytes is Reduced by Rosiglitazone**

¹Alison Harte*, PhD; ¹Philip McTernan*, PhD; ¹Rajkumar Chetty, MChB; ²Simon Coppack,
MD; ²Jonathan Katz, MD; ³Stephen Smith, Phd; ¹Sudhesh Kumar, MD.

* Joint first author

¹ Unit for Diabetes and Metabolism, Warwick Medical School Research Wing, Clinical
Sciences Building, UHCW Trust, Clifford Bridge Road, Walsgrave, Coventry, CV2 2DX,
UK.

² St.Bartholomew's and The Royal London School of Medicine (S.W.C.), London E1 1BB,
UK.

³ Glaxo-SmithKline, New Frontiers Science Park, Third Avenue, Harlow, CM19 5AW, UK

Corresponding Author:

Professor S Kumar, Unit for Diabetes and Metabolism, Warwick Medical School, University
of Warwick, Coventry, CV4 7AL, UK.

Tel: 00 44 (0)24 7657 4665

Fax: 00 44 (0)24 7567 4871

Email: Sudhesh.Kumar@warwick.ac.uk

Word count: 5,360

Abstract

Background: Obesity associated hypertension is likely to be due to multiple mechanisms. Identification of the renin-angiotensin system (RAS) within adipose tissue does, however, suggest a potential causal role for it in obesity-associated hypertension. Obese patients are often hyperinsulinaemic, but mechanisms underlying insulin upregulation of the RAS in adipose tissue are unclear. TNF α , an inducer of angiotensinogen in hepatocytes, is elevated in hyperinsulinaemic, obese individuals, and may provide a link in mediating insulin upregulation of the RAS in adipose tissue. Further, thiazolidinediones lower blood pressure *in vivo* and downregulation of the RAS in adipose tissue may contribute to this effect. We therefore examined the effect of rosiglitazone (RSG), on the insulin mediated upregulation of the RAS.

Methods and Results: Sera were obtained from the arterial circulation and from venous blood draining subcutaneous abdominal adipose tissue. Isolated human abdominal subcutaneous adipocytes (n=12) were treated with insulin (1-1000nM) and insulin in combination with RSG (10nM), and RSG (10nM) alone to determine angiotensinogen expression, angiotensin II, bradykinin and TNF α secretion. Subcutaneous adipocytes were also treated with TNF α (10-100ng/mL) to examine the direct effect on angiotensinogen expression and angiotensin II secretion. The findings showed that the arterio-venous difference in angiotensin II levels was significant (\uparrow 23%; $p<0.001$). Insulin increased TNF α secretion in a concentration-dependent manner ($p<0.01$) whilst RSG (10nM) significantly reduced the insulin mediated rise in TNF α ($p<0.001$), as well as AGT and angiotensin II. TNF α also increased angiotensinogen and angiotensin II in isolated adipocytes.

Conclusions: Our *in vivo* data suggest that human subcutaneous adipose tissue is a significant source of angiotensin II. This study also demonstrates a potential TNF α mediated

mechanism through which insulin may stimulate the RAS and may contribute to explain obesity associated hypertension. RSG downregulates the RAS in subcutaneous adipose tissue and this effect may contribute to the long-term effect of RSG on blood pressure.

Key words: obesity, hypertension, angiotensin II, TNF-alpha, rosiglitazone

Introduction

Hypertension is a heterogeneous condition that is positively linked with obesity, although the causative factors for this association remain unclear (1). Whilst the identification of the renin angiotensin system (RAS) has been documented within several tissues, its' presence in human adipose tissue may offer a potential link between obesity and hypertension (2). Thus, RAS activation through the effector hormone, angiotensin II (ANG II), may alter vasoconstrictive and pro-thrombotic properties associated with cardiovascular disease (3-6).

We have previously shown that insulin upregulates the RAS system in human adipose tissue but the underlying mechanism for it was not clear (7). TNF α is implicated in the development of insulin resistance because of the multitude of effects it exerts on insulin sensitive tissues. Numerous studies have demonstrated a positive correlation between TNF α and obesity, with regard to protein and mRNA expression in adipose tissue, as well as circulating levels of TNF α in obese and type 2 diabetic subjects (8, 9). Furthermore, an association between elevated TNF α expression in adipose tissue and characteristics of insulin resistance has been described in obese and diabetic animal models, as well as humans (8, 10). TNF α can also regulate expression of AGT in hepatocytes as the AGT promoter contains a cytokine inducible enhancer known as the acute phase response element (APRE) (11). TNF α induces transcription of AGT via the transcription factor NF κ B, which is known to be involved in the production of numerous pro-inflammatory markers. This suggests a possible TNF α directed mechanism through which insulin may increase AGT and subsequent ANG II secretion.

Therefore, the aim of this study was to investigate the importance of the RAS in the pathogenesis of obesity associated hypertension both *in vivo* and *in vitro*. First, we established the importance of subcutaneous abdominal adipose tissue as a source of circulating ANG II and TNF α using an arterio-venous approach. As our previous studies had shown that insulin increases expression of the RAS, we examined the effect of insulin on the secretion of bradykinin to determine the net effect of insulin on the RAS pathway, thus delineating the potential hypertensive effect of insulin through its' effects on adipose tissue. Furthermore, we investigated whether insulin was regulating AGT through a TNF α directed mechanism by examining the effect of insulin and RSG on the secretion of TNF α from mature adipocytes. We then proceeded to determine the effect of TNF α on AGT protein expression and ANG II secretion, to elucidate a potential pathway for insulin leading to obesity associated hypertension through its' effects on adipose tissue. Lastly, because the insulin sensitiser, rosiglitazone (RSG), has been shown to lower blood pressure in animal models, patients with impaired glucose tolerance, T2DM patients and non-diabetic hypertensives (12-16), we investigated the effect of RSG on AGT expression and ANG II secretion in human adipocytes.

Materials & Methods

Subjects

Serum samples were obtained from consenting, Caucasian, non-diabetic, female subjects (age 42.3 ± 16 years (mean \pm SD); BMI 29.8 ± 5.4 Kg/m² (mean \pm SD) n=26). Arterio-venous difference studies were undertaken on 18 of the subjects. All subjects were weight-stable for at least 2 months prior to the study, and were considered to be in good health, after completing a comprehensive medical evaluation including history and physical examination, blood tests and electrocardiogram. All subjects were taking no regular medication and premenstrual female subjects were studied during the follicular phase of their cycle. All obese subjects had been weighed regularly prior to the study. The study was approved by the East London Research Ethics Committee and all subjects gave informed, written consent before their participation.

For the purposes of tissue culture, subcutaneous abdominal adipose tissue was obtained from a separate cohort of female subjects (age: 49.1 ± 9.7 years; BMI: 24.96 ± 0.70 Kg/m² n=12). All human adipose tissue was obtained through elective surgery in accordance with guidelines of the South Birmingham ethics committee.

***In vivo* Assessment of the Release of ANG II and TNF α from Subcutaneous Abdominal Adipose Tissue into the Circulation**

ANG II and TNF α levels were assayed in sera obtained from the arterial circulation and directly from venous drainage of the subcutaneous abdominal adipose tissue depot, in accordance with a previously described method (17, 18). These measurements were made on 18 subjects who had been requested to consume a diet containing 70 mmoles per day for at least

7 days prior to study. Twenty-four hour urinary collections were done to check compliance with the sodium restriction. Subjects were admitted to the Clinical Research Centre in the evening before the study. At 1800 h subjects ingested a meal containing 12 kcal/kg body weight for lean subjects and 12 kcal/kg adjusted body weight for obese subjects (adjusted body weight = ideal body weight + [(actual body weight - ideal body weight)x(0.25)]). At 2000 h, subjects ingested a defined snack containing 250 kcal, 40 g carbohydrate, 6.1 g fat, and 8.8 g protein. After consuming this snack, all subjects fasted until completion of the study the following day.

On the morning of the study, 20-gauge catheters were inserted into a forearm vein for isotope infusion and into a radial artery for arterial blood sampling. An abdominal vein draining subcutaneous abdominal adipose tissue was cannulated with a 10-20cm, 22-gauge polyurethane catheter (Hydrocath, Viggo-Spectramed, Oxnard, CA (17-19)). Blood withdrawn from such catheters represents drainage from adipose tissue and overlying skin. All vascular catheters were kept patent by continuous saline infusion. Subjects remained supine throughout the study and room temperature was kept constant at 23 °C during the entire study. Blood samples were taken into pre-chilled syringes. Samples were kept on ice and serum was separated rapidly by centrifugation at 4°C and were thereafter stored at -80°C until assay. Commercially available ELISA based colorimetric kits were used to examine levels of ANG II and TNF α in the thawed serum samples (Phoenix Pharmaceuticals, USA and R&D Systems UK, respectively).

Abdominal subcutaneous adipose tissue blood flow was evaluated using the ¹³³Xe washout technique (18, 19). 40-50 mCi of ¹³³Xe dissolved in 0.1 ml of saline was slowly injected over 60 seconds into the subcutaneous abdominal adipose tissue space. The decline in ¹³³Xe was

monitored continuously from 60 to 120 min after injection with a sodium iodide scintillation detector set to measure the 81 keV ^{133}Xe photopeak.

Tissue Culture

In brief, 10-20g wet weight fresh abdominal subcutaneous adipose tissue was collected. Tissue was initially washed with 1X Hank's balanced salt solution (HBSS) containing penicillin (100U/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$). Visible blood vessels and connective tissue were removed and the tissue finely chopped. All adipose tissue was digested with the same batch of collagenase class 1 (2mg/mL, Worthington Biochemical Corporation) in 1X HBSS (Gibco, UK) for 1 h at 37°C in a water bath and shaken at 100 cycles/min at 37°C (20). The disrupted tissue was filtered through a double-layered cotton mesh and pre-adipocyte cells and adipocytes separated by centrifugation at 360g for 5 min.

Mature Adipocyte Isolation

Following centrifugation, the upper layer of mature adipocytes was removed from the collagenase-dispersed preparation, washed in phenol red-free medium DMEM:F12 twice and centrifuged at 360g for 2 min. Adipocytes were then cultured in flasks (25cm²) in phenol-red free Dulbecco's modified Eagle's (DMEM:F12) medium containing 15mmol/L glucose, penicillin (100units/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$). Aliquots of 1mL containing approximately 500,000 mature adipocytes were maintained in medium (5mL) per 25cm² flask for 48 h and treated once with insulin alone (1-1000nM; Sigma UK) insulin in combination with RSG, (Ins: 1nM-1000nM & RSG 10nM), RSG alone (10nM) and TNF α (10ng-100ng) for 48 h. Adipocytes maintained in untreated medium for 48 h were used as controls. Following incubation of adipocytes (37°C/5%CO₂) with their respective treatments, the

conditioned media and adipocytes were separated by centrifugation (360g for 2 min). The media were then removed, aliquoted and stored at -70°C.

Protein Assay

Following removal of the conditioned media, 4% SDS was added to the adipocytes. The suspension was then heated for 2 h at 95°C, until the adipocyte cells had dissolved. The resultant extracted proteins were stored at -70°C as previously described (21). Protein was extracted and quantified via the Bio-Rad DC (Detergent Compatible) protein assay kit (22). Adipocyte protein samples were assessed to determine that there was no significant statistical variation between control and treatment regimens, indicating secretion of ANG II was not due to adipocyte protein variation between samples.

Western Blotting

Western blot analysis was performed using a method previously described (23). In brief, equal amounts (20µg) of protein were loaded onto a 10% gel. Following gel electrophoresis and electroblotting, filters were incubated overnight at 4°C with continual motion, with a primary antibody of 1:250 for AGT. Detection of AGT (61Kda) and TNFα (16kDa) was achieved using horseradish peroxidase-conjugated secondary antibodies; (CalBiochem) diluted 1:40,000 in PBS-T (0.05%T). A chemiluminescent detection system ECL/ECL+ (Amersham, Little Chalfont, UK) enabled visualisation after exposure to X-ray film for 5-20 min. Autoradiographs were quantified by densitometry using a Gelbase/Gelblot programme (UVP Ltd, UK).

ANG II, Bradykinin and TNF α Assays

Secreted ANG II, bradykinin and TNF α levels from abdominal subcutaneous adipocytes were determined. For this, stored conditioned medium samples were thawed and commercially available ELISA based colorimetric kits were used to determine the quantities of ANG II, bradykinin and TNF α secreted from adipocytes (Phoenix Pharmaceuticals, USA; Bachem, UK and R&D Systems UK, respectively).

Statistical analysis

For assessment of protein expression and secretion, statistical analysis was undertaken using ANOVA for comparison of control versus treatments. The threshold for significance was $p < 0.05$. Data in the text and figures are presented as mean \pm SD or mean \pm SEM. SPSS version 12 was used to examine correlations.

For comparison of arterial and venous drainage for each subject, data were analysed using a paired t-test. Correlations were determined using a Pearson correlation. All statistics were performed on SPSS, version 12.

Results

Subcutaneous Abdominal Adipose Tissue Production of ANG II

In these subjects (age: 43 ± 15.3 years (mean \pm SD); BMI: 30.70 ± 4.83 Kg/m² (mean \pm SD); insulin: 8.1 ± 3.5 uU/mL (mean \pm SD); glucose: 5.3 ± 0.9 mmol/L; blood pressure – systolic: 129.3 ± 19.5 mmHg; diastolic: 83.2 ± 9.5 mmHg, n=18) abdominal venous ANG II levels were significantly higher than levels in the arterial circulation. ANG II levels were 23% greater in the subcutaneous abdominal venous drainage than the artery ($p < 0.001^{***}$; Table 1). The arterio-venous concentration difference across adipose tissue showed a similar trend for TNF α , although this did not achieve statistical significance ($p < 0.083$; data not shown). The mean adipose tissue plasma flow across this depot was 1.46 ± 0.91 ml/100g⁻¹/min⁻¹. The production rate for ANG II was 54 ± 56.68 pg/100g adipose tissue/min (mean \pm SD), whereas TNF α was 14 ± 26.24 pg/100g adipose tissue/min (mean \pm SD). Whole body ANG II adipose tissue production rate was 1626 ± 390.7 pg/mL (mean \pm SE).

Correlation Between Plasma ANG II Levels and BMI

Increasing BMI showed a positive correlation with increasing circulating ANG II levels in these subjects (age 42.3 ± 16 years (mean \pm SD); BMI 29.8 ± 5.4 Kg/m² (mean \pm SD) n=26; $r^2 = 0.142$; $p = 0.057$, Figure 1), although this was not significant. ANG II levels from subcutaneous venous drainage showed no correlation with increasing BMI (data not shown). Increasing BMI also showed a significant positive correlation with increasing diastolic blood pressure ($r^2 = 0.414$; $p < 0.01$, data not shown). However, no such association was observed with systolic blood pressure (data not shown).

Correlation Between Venous Adipose Tissue TNF α and ANG II Levels

TNF α and ANG II from subcutaneous adipose tissue venous drainage demonstrated a significant positive correlation with increasing levels ($r^2=0.491$; $p<0.01^{**}$, Figure 2).

The Effect of Insulin and Insulin with RSG on Secretion of TNF α

In human subcutaneous adipose cells, insulin increased TNF α secretion in a concentration-dependent manner ($p<0.01$) whilst RSG (10nM) significantly reduced the insulin mediated rise in TNF α (Control: $3.8\pm(\text{SEM}) 0.60\text{ng/mL}$; Ins 10M: $5.2\pm0.3\text{pg/mL}$; Ins 10nM + RSG: $0.9\pm0.4\text{ng/mL}$, $p<0.001^{***}$, Figure 3.). RSG alone (10nM) significantly reduced TNF α secretion compared to control, (Control: $3.8\pm(\text{SEM}) 0.60\text{ng/mL}$; RSG: $0.9\pm0.4\text{ng/mL}$, $p<0.001$, data not shown).

The Effect of TNF α on AGT Protein Expression

Western blot analysis confirmed a concentration dependent increase in AGT protein expression in subcutaneous adipocytes treated with increasing concentration of TNF α compared with control (Control: 1 ± 0.0 ; (mean \pm SE), 3.7 ± 0.5 , $p<0.001^{***}$ at 100ng TNF α , Figure 4).

The Effect of TNF α on ANG II Secretion

Human subcutaneous abdominal adipocytes treated with TNF α for 48h showed a concentration dependent increase in ANG II secretion with maximal stimulation at 100ng TNF α compared to control (Control: 237.0 ± 52 ; (mean \pm SE), TNF α 100ng: 398 ± 61 pg/mL, $p<0.05^*$; Figure 5).

The Effect of Insulin in Combination with RSG on AGT Protein Expression

Western blot analysis demonstrated that the presence of the insulin sensitiser, RSG, significantly reduced the insulin-mediated increase in AGT protein expression at all concentrations of insulin (Control: 1.0 ± 0.0 ; (mean \pm SE), protein expression measured relative to control; Ins 1nM: $2.64 \pm 0.32 \uparrow^{***}$; Ins 10nM: $3.52 \pm 0.48 \uparrow^{***}$, 100nM Ins: $4.37 \pm 0.57 \uparrow^{***}$; 1000nM Ins: $6.50 \pm 0.97 \uparrow^{***}$; $p < 0.001^{***}$; Figure 6).

The Effect of Insulin and RSG on ANG II Secretion

ANG II secretion was regulated by insulin in a concentration dependent manner, with maximal stimulation occurring at 1000nM (Figure 7). However, in the presence of RSG the insulin mediated rise in ANG II secretion was significantly reduced at all insulin concentrations ($p < 0.001^{***}$; Figure 7). RSG alone (10nM) significantly reduced ANG II secretion compared to control, (Control: $214.33 \pm (\text{SEM}) 12.34$ pg/mL; RSG: 104.44 ± 14.35 pg/mL, $p < 0.01$; data not shown).

The Effect of Insulin and Insulin with RSG on Bradykinin Secretion

Subcutaneous abdominal adipocytes treated for 48 h showed no significant change in bradykinin secretion with insulin treatment (1nM-100nM) or insulin (1-100nM) in combination with RSG (10nM) (Data not shown). RSG alone (10nM) did not alter bradykinin secretion (data not shown).

Discussion

Many studies have implicated a role for the adipose tissue RAS in the development of obesity associated hypertension. Rodent studies comparing blood pressure levels in obese Zucker rats versus genetically lean Zucker rats have shown that blood pressure is significantly higher in the obese rodents. Furthermore, the administration of an ANG II receptor antagonist to these animals significantly lowered blood pressure, thus demonstrating that ANG II contributes to the elevated blood pressure observed in these animal models (24). However, studies by Faloiu and colleagues did not demonstrate a difference in circulating components of the RAS between obese hypertensive, obese normotensive subjects and lean controls (25). Our findings indicate a positive correlation between increasing BMI and circulating ANG II levels, although this was not significant. The present study also revealed a positive association between BMI and diastolic blood pressure, whilst systolic blood pressure exhibited no correlation with BMI. These findings support the association between obesity, hyperinsulinaemia, increased ANG II production and elevated blood pressure. Furthermore, in addition to its' systemic effects, recent studies have highlighted the importance of resistance vessels within adipose tissue depots with regard to hypertension. Both structural and functional abnormalities have been identified in arterial resistance vessels extracted from adipose tissues depots in type 2 diabetic and hypertensive patients (26, 27). Therefore the local RAS may also be acting in a paracrine fashion to have a significant effect on blood pressure, as well as being a potential mediator in the observed vascular remodeling of these vessels.

Whilst the positive correlation between obesity and hypertension is apparent, the molecular mechanisms that underlie its' pathogenesis remain unclear. Hypertension and

hyperinsulinaemia/insulin resistance form part of the diagnostic criteria for the metabolic syndrome, a condition that is strongly associated with obesity. We have previously investigated the effect of increasing insulin concentration on the RAS in subcutaneous human adipocytes and subsequently demonstrated that insulin concentration increases both expression and secretion of AGT and ANG II, respectively (7). These data suggested that chronic exposure to elevated insulin levels might induce upregulation of the RAS in subcutaneous adipocytes, thus proffering a potential mechanism mediating obesity associated hypertension. Furthermore, the importance of the RAS pathway is not limited to hypertension as ANG II, the effector molecule in the pathway, has also been identified as a pro-inflammatory factor. Studies have shown that it activates expression of early response and inflammatory genes in vascular smooth muscle cells (28, 29) and regulates the production of PAI-1, which inhibits fibrinolysis (4). Obesity is associated with a state of sub-clinical inflammation so the pluripotent nature of ANG II has implications for other facets of the condition. Therefore, understanding the mechanisms through which AGT and hence ANG II are regulated may provide additional insights into the pathogenesis of the metabolic syndrome and new approaches to reducing the risk of cardiovascular disease.

In the present study we have demonstrated that subcutaneous adipose tissue is a significant site of AGT and angiotensin II production via arterio-venous measurements and through *in vitro* protein studies. Previous rodent studies have indicated the importance of adipose tissue RAS as contributing to plasma levels of AGT and ANG II, but no study to date has examined the importance of subcutaneous, abdominal, human adipose tissue (30). Our findings revealed a significant increase in ANG II levels in the subcutaneous abdominal adipose tissue effluent

of 23% ($p < 0.001$), thus affirming the relevance of studying this tissue with regard to obesity associated hypertension.

We also determined that insulin did not influence bradykinin secretion. Consequently, the possible mechanisms through which insulin increased AGT, and hence ANG II protein secretion, were examined. A potential mechanism for regulation of adipose tissue RAS was suggested by the observation that, in hepatocyte cells, the AGT promoter contains a cytokine inducible enhancer known as the acute phase response element (APRE) (11). One cytokine known to induce transcription of AGT is $\text{TNF}\alpha$ - a multifunctional cytokine produced by a variety of cells that include monocytes/macrophages, muscle cells and adipose tissue (11). $\text{TNF}\alpha$ is implicated as a pathogenic factor in the development of obesity-associated insulin resistance, as elevated levels of this cytokine in adipose tissue are associated with features of the metabolic syndrome (10). Therefore, we initially examined the levels of $\text{TNF}\alpha$ produced by subcutaneous adipose tissue. $\text{TNF}\alpha$ demonstrated a similar increase in the arterial versus venous drainage to ANG II, although this was not significant ($p = 0.083$). This finding is in agreement with previous observations by Mohammed-Ali and co-workers (19), but does not completely discount the importance of adipose tissue $\text{TNF}\alpha$, as levels may be lower due to the local uptake of this cytokine. Furthermore, our findings show that increasing $\text{TNF}\alpha$ levels from venous drainage of the subcutaneous adipose tissue depot strongly correlated with ANG II levels, thus supporting the possibility of a regulatory role for $\text{TNF}\alpha$ in the RAS pathway.

In order to determine if the effects of insulin on the RAS were a result of a $\text{TNF}\alpha$ mediated pathway, the effects of insulin on $\text{TNF}\alpha$ secretion from human adipocytes were examined. The present study demonstrated that insulin stimulates $\text{TNF}\alpha$ secretion in a concentration

dependent manner in isolated subcutaneous adipocytes, following the same trend as previously observed for ANG II with increasing insulin concentration. In addition, we further examined the direct effect of TNF α concentration on AGT and ANG II stimulation. TNF α stimulated both AGT expression and ANG II secretion in a concentration dependent manner, with higher levels of TNF α (100ng) significantly stimulating ANG II. As such, our results support a TNF α mediated mechanism for the induction of AGT in human adipocytes, as observed in hepatocytes. The previously described insulin-mediated increase in AGT and ANG II may therefore be a result of TNF α activity.

Previous data have demonstrated that rosiglitazone (RSG) reduces blood pressure in animal models, type 2 diabetics and obese patients (12-16). RSG has been shown to reduce oxidative stress, thus ameliorating endothelial dysfunction and improving blood vessel elasticity (31). However, no study to date has examined the effects of RSG on the RAS in human adipocytes and obesity associated hypertension. Our present study showed that the introduction of RSG into this system dramatically reduced the insulin-mediated effect on the RAS. This is an interesting finding as the insulin sensitising action of RSG, through transactivation of responsive genes, would suggest that AGT and ANG II would increase further in the presence of this agent. These findings, however, are in accord with *in vivo* data, with RSG inducing downregulation of TNF α and the RAS in adipocytes, *in vitro*. In addition, RSG also negated the insulin mediated increase in TNF α secretion, further supporting the possibility of a TNF α regulated effect on the RAS. However, the effects of insulin and RSG were limited to the vasoconstrictive pathway in the RAS cascade, as bradykinin secretion remained unaltered by insulin in the presence of RSG.

In conclusion, exposure of adipose tissue to a hyperinsulinaemic environment may produce increased ANG II through the actions of $\text{TNF}\alpha$, which is also elevated in obese and type 2 diabetic subjects (7). Our findings demonstrate the potential of this mechanism and the contributory role that the adipose tissue RAS may have in obesity associated hypertension. RSG mitigates the insulin mediated increase in the adipose tissue RAS and therefore this finding may help to explain the long-term anti-hypertensive effects of RSG. RSG has already been shown to improve endothelial dysfunction and hence may offer additional benefits in terms of the adipose tissue RAS (31). As yet, it remains unclear as to how RSG is producing its' effects. Within liver cells it is apparent that the $\text{TNF}\alpha$ mediated induction of AGT involves the nuclear transcription factor $\text{NF}\kappa\text{B}$ - a protein linked to the regulation of many pro-inflammatory cytokines (11). This pathway has not been examined in the adipocyte with regard to the RAS. It may be the key transcription factor involved in this pathway and therefore requires further investigation.

References

1. Must A, Spandano J, Coakley EH, Field AE, Colditz G, Dietz WH. The disease burden associated with overweight and obesity. *JAMA* 1999; 282: 1523-9.
2. Karlsson C, Lindell K, Ottosson M, Sjoström L, Carlsson B, Carlsson LM. Human adipose tissue expresses angiotensinogen and enzymes required for its conversion to Angiotensin II. *J Clin Endocrinol Metab* 1998; 83: 3925-3929.
3. Nakamura S, Nakamura I, Ma L, Vaughan DE, Fogo AB. Plasminogen activator inhibitor-1 expression is regulated by the angiotensin type 1 receptor in vivo. *Kidney Int* 2000; 58 (1): 460-461.
4. Ridker PM, Gaboury CL, Conlin PR, Seely EW, Williams GH, Vaughan DE. Stimulation of plasminogen activator inhibitor in vivo by infusion of angiotensin II. *Circulation* 1993; 87: 1969-73.
5. Intengan HD, Shiffren EL. Vascular remodelling in hypertension: roles of apoptosis, inflammation and fibrosis. *Hypertension* 2001; 38 (pt 2): 581-587.
6. Linz W, Wiemer G, Schlkens BA. Beneficial effects of bradykinin on myocardial energy metabolism and infarct size. *Am J Cardiol* 1997; 80: 118A-1123A.
7. Harte AL, McTernan PG, McTernan CL, Crocker J, Starcynski J, Barnett AH, Matyka K, Kumar S. Insulin increases angiotensinogen expression in human abdominal subcutaneous adipocytes. *Diabetes Obes Metab*. 2003 Nov 5 (6):462-7.
8. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose Expression of Tumour Necrosis Factor-alpha: Direct Role in Obesity-Linked Insulin Resistance. *Science* 1993; 259: 87-90.
9. Winkler G, Salamon F, Salamon DGS, Simon K, Cseh K. Elevated serum tumour necrosis factor-alpha levels can contribute to the insulin resistance in type II (non-insulin-dependent) diabetes and in obesity. *Diabetologia* 1998; 41: 860-861.
10. Miyazaki Y, Pipek R, Mandarino LJ, DeFronzo RA. Tumour necrosis factor alpha and insulin resistance in obese type 2 diabetic patients. *Int J Obes Relat Metab Disord* 2003; 27 (1): 88-94.
11. Brasier AR & Li J. Mechanisms for inducible control of angiotensinogen gene transcription. *Hypertension* 1996; 27: 465-475.
12. Walker AB, Chattington PD, Buckingham RE, Williams G. The thiazolidinedione rosiglitazone (BRL-49653) lowers blood pressure and protects against impairment of endothelial function in Zucker fatty rats. *Diabetes* 1999; 48: 1448-1453.
13. Bakris GL, Dole JF, Porter LE, Huang C, Freed M. Rosiglitazone improves blood pressure in patients with type 2 diabetes mellitus. *Diabetes* 2000; 49 (Suppl 1): A96.

14. Bennett SMA, Agrawal A, Elasha H, Heise M, Jones NP, Walker M, Wilding JPH. Rosiglitazone improves insulin sensitivity, glucose tolerance and ambulatory blood pressure in subjects with impaired glucose tolerance. *Diabetic Medicine* 2004; 21: 415-422.
15. St John Sutton M, Rendell M, Dandona P, Dole JF, Murphy K, Patwardhan R, Patel J, Freed M. A comparison of the effects of rosiglitazone and glyburide on cardiovascular function and glycaemic control in patients with type 2 diabetes. *Diabetes Care* 2002; 25: 2058-2064.
16. Raji A, Seely EW, Bekins SA, Williams GH, Simonson DC. Rosiglitazone improves insulin sensitivity and lowers blood pressure in hypertensive patients. *Diabetes Care* 2003; 26: 172-178.
17. Frayn KN, Coppack SW, Humphries SM, Whyte PL. Metabolic characteristics of human adipose tissue in vivo. *Clin. Sci (Colch)* 1989; 76: 509-516.
18. Klein S, Coppack SW, Mohamed-Ali V, Landt M. Adipose tissue leptin production and plasma leptin kinetics in humans. *Diabetes* 1996; 45: 984-987.
19. Mohamed-Ali V, Goodrick S, Bulmer K, Holly JM, Yudkin JS, Coppack SW. Production of soluble tumor necrosis factor receptors by human subcutaneous adipose tissue in vivo. *Am J Physiol.* 1999; 277 (6 Pt 1):E971-5.
20. Rodbell M. Metabolism of isolated adipocytes. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* 1964; 239: 375-380.
21. Anwar A, McTernan PG, Anderson LA, Askaa J, Moody CG, Barnett AH, Eggo MC, Kumar S. Expression and Regulation of Oestrogen Receptor alpha and beta in human adipose tissue. *Diabetes, Obesity & Metabolism* 2001; 3: 338-349.
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochem* 1976; 72: 807-811.
23. McTernan PG, Sheppard MC, Williams GR. Hormone-induced changes in nuclear receptor stoichiometry in HL60 cells correlate with induction of monocyte or neutrophil differentiation. *J Endocrinol* 1998; 156:135-148.
24. Alonso-Galicia M, Brands MW, Zappe DH, Hall JE. Hypertension in obese Zucker rats. Role of angiotensin II and adrenergic activity. *Hypertension.* 1996; 28 (6):1047-54.
25. Faloia E, Gatti C, Camilloni MA, Mariniello B, Sardu C, Garrapa GG, Mantero F, Giacchetti G. Comparison of circulating and local adipose tissue renin-angiotensin system in normotensive and hypertensive obese subjects. *J Endocrinol Invest.* 2002; 25 (4):309-14.
26. Schofield I, Malik R, Izzard A, Austin C, Heagerty A. Vascular structural and functional changes in type 2 diabetes mellitus: evidence for the roles of abnormal myogenic responsiveness and dyslipidemia. *Circulation.* 2002; 106(24):3037-43.

27. Rizzoni D, Palombo C, Porteri E, Muiesan ML, Kozakova M, La Canna G, Nardi M, Guelfi D, Salvetti M, Morizzo C, Vittone F, Rosei EA. Relationships between coronary flow vasodilator capacity and small artery remodelling in hypertensive patients. *J Hypertension* 2003; 21 (3):625-31.
28. Taubman MB, Berk BC, Izumo S, Tsuda T, Alexander RW, Nadal-Ginard B. Angiotensin II induces c-fos mRNA in aortic smooth muscle. Role of Ca²⁺ mobilization and protein kinase C activation. *J Biol Chem.* 1989; 5;264(1):526-30.
29. Luft FC. Workshop: Mechanisms and cardiovascular damage in hypertension. *Hypertension* 2001; 37 (2 Part 2): 594-598.
30. Massiera F, Bloch-Faure M, Ceiler D, Murakami K, Fukamizu A, Gasc JM, Quignard-Boulangé A, Negrel R, Ailhaud G, Seydoux J, Meneton P, Teboul M. Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. *FASEB J* 2001; 15 (14)272-9.
31. Da Ros R, Assaloni R, Ceriello A. The preventative anti-oxidant action of thiazolidinediones: a therapeutic prospect in diabetes and insulin resistance. *Diab Med.* 2004; 21(11): 1249-52.

Acknowledgements

This work was supported by the West Midlands NHS Research Scheme and GlaxoSmithKline, through a research grant. Arterio-venous studies were supported by The Wellcome Trust. We thank all the operative surgeons and theatre staff at both the University Hospital Trust and the Women's Hospital Trust, Birmingham, who aided in these studies. We also thank Mr Levick for kindly providing consented samples from his plastic surgery operations at the Priory Hospital, Birmingham, UK.

Table 1. The individualised subject data points of arterial and venous drainage and the net arterio-venous concentration differences for ANG II, as well as the mean \pm SD for each grouping (n=18, p-value: $p<0.001^{***}$).

Figure 1. The relationship between circulating ANG II levels and BMI (n=26, $p=0.057$).

Figure 2. The relationship between subcutaneous venous drainage TNF α and ANG II levels (n=15; $p<0.01^{**}$) as demonstrated by Pearson Correlation,

Figure 3. TNF α secretion (mean \pm SEM ng/mL) from untreated (control) and treated adipocytes, comparing the effect of insulin (Ins: 1-1000nM) vs insulin in combination with RSG (10nM) on the secretion of TNF α in adipocyte cells over 48 h (n=12; p-values: $p<0.001^{***}$).

Figure 4. The mean relative protein expression of AGT (\pm SEM) with TNF α treatment (TNF α : 10, 50 and 100ng/mL) compared to control (untreated cells) in isolated mature subcutaneous adipocytes (n=3, p-value: $p<0.001^{***}$).

Figure 5. The mean levels of ANG II (\pm SEM) secreted from untreated subcutaneous adipocytes (control) and adipocytes treated with increasing TNF α concentration (10, 50 and 100ng/mL; n=12, p-value : $p<0.05^*$).

Figure 6. The mean relative protein expression (\pm SEM) of AGT (61kDa) in isolated mature adipocytes compared to control (untreated cells). Statistical analysis compared expression of

AGT in cells treated with insulin alone (Ins: 1-1000nM) to adipose cells treated with insulin (Ins: 1-1000nM) in combination with RSG (10nM) (n=3, p-values, p<0.01**, p<0.001***).

Figure 7. The release of ANG II (mean \pm SEM pg/mL) from insulin and RSG treated adipocytes (n=12). The statistical analysis compared the effect of insulin (Ins: 1-1000nM) versus insulin in combination with RSG (Ins: 1-1000nM & RSG: 10nM) on the secretion of ANG II (p-value: p<0.001***).

Table 1.

Subcutaneous Venous Drainage ANG II Levels (pg/mL)	Arterial Circulation ANG II Levels (pg/mL)	Individualised arterio-venous differences in ANG II Levels (Subcutaneous Venous – Arterial Circulation; pg/mL)
194.300	126.200	68.100
345.100	258.900	86.200
140.000	121.400	18.600
281.300	181.100	100.200
96.500	106.100	-9.600
115.900	113.100	2.800
174.300	141.000	33.300
145.000	101.500	43.500
197.000	168.400	28.600
205.500	206.400	-0.900
397.100	395.000	2.100
283.400	209.700	73.700
216.600	150.300	66.300
165.800	133.000	32.800
359.100	358.000	1.100
205.200	177.200	28.000
160.900	100.000	60.900
212.289	177.922	34.367
Mean ± SE =216.4 ± 19.99	Mean ± SE =179.2 ± 19.74	Mean ± SE = 37.2±7.67***

p<0.001***