Overproduction of Angiotensinogen from Adipose Tissue Induces Adipose Inflammation, Glucose Intolerance, and Insulin Resistance

Nishan S. Kalupahana[1–3], Florence Massiera[4,5], Annie Quignard-Boulange[6], Gérard Ailhaud[4], Brynn H. Voy[1,2], David H. Wasserman[7] and Naima Moustaid-Moussa[1,2]

Although obesity is associated with overactivation of the white adipose tissue (WAT) renin–angiotensin system (RAS), a causal link between the latter and systemic insulin resistance is not established. We tested the hypothesis that overexpression of angiotensinogen (Agt) from WAT causes systemic insulin resistance via modulation of adipose inflammation. Glucose tolerance, systemic insulin sensitivity, and WAT inflammatory markers were analyzed in mice overexpressing Agt in the WAT (aP2-Agt mice). Proteomic studies and *in vitro* studies using 3T3-L1 adipocytes were performed to build a mechanistic framework. Male aP2-Agt mice exhibited glucose intolerance, insulin resistance, and lower insulin-stimulated glucose uptake by the skeletal muscle. The difference in glucose tolerance between genotypes was normalized by high-fat (HF) feeding, and was significantly improved by treatment with angiotensin-converting enzyme (ACE) inhibitor captopril. aP2-Agt mice also had higher monocyte chemotactic protein-1 (MCP-1) and lower interleukin-10 (IL-10) in the WAT, indicating adipose inflammation. Proteomic studies in WAT showed that they also had higher monoglyceride lipase (MGL) and glycerol-3-phosphate dehydrogenase levels. Treatment with angiotensin II (Ang II) increased MCP-1 and resistin secretion from adipocytes, which was prevented by cotreating with inhibitors of the nuclear factor-κB (NF-κB) pathway or nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. In conclusion, we show for the first time that adipose RAS overactivation causes glucose intolerance and systemic insulin resistance. The mechanisms appear to be via reduced skeletal muscle glucose uptake, at least in part due to Ang II-induced, NADPH oxidase and NFκB-dependent increases in WAT inflammation.

Obesity (2012) **20,** 48–56. doi:10.1038/oby.2011.299

INTRODUCTION

The renin–angiotensin system (RAS) is classically known for its role in the regulation of blood pressure and fluid balance (1). Angiotensinogen (Agt) is cleaved by the enzymes renin and angiotensin-converting enzyme (ACE) successively, to form angiotensin II (Ang II), the main bioactive peptide of this system. In addition to Ang II, several other angiotensin peptides such as Ang 1–7 are also generated by the RAS (1). It is also well documented that other nonclassical enzymatic and nonenzymatic pathways can generate many of the RAS intermediate peptides (1). Ang II exerts its physiological actions, primarily via two G-protein coupled receptors, Ang II type 1 (AT_1R) and type 2 (AT_2R) receptors (1). Hence, ACE inhibitors and angiotensin receptor blockers (ARBs) are common drug targets for antihypertensive therapy (1). Interestingly, several clinical trials have shown that the risk for type-2 diabetes mellitus is lower in hypertensive individuals treated with ACE inhibitors or ARBs compared to those treated with other antihypertensive medications (2). Further, RAS blockade also ameliorates insulin resistance and glucose intolerance in several rodent models of obesity (3). However, the exact mechanism of modulation of insulin sensitivity via RAS blockade is not fully understood.

Adipose tissue is well recognized now as an important endocrine organ which secretes a number of bioactive peptides collectively known as adipokines. These include leptin, adiponectin, resistin, tumor necrosis factor-α, plasminogen activator inhibitor-1, monocyte chemotactic protein-1(MCP-1) and Ang II (4). Obesity leads to a chronic low-grade inflammatory state in the adipose tissue and a dysregulation of adipokine

[1]Obesity Research Center, University of Tennessee (UT), Knoxville, Tennessee, USA; [2]Department of Animal Science, University of Tennessee (UT), Knoxville, Tennessee, USA; [3]Department of Nutrition, University of Tennessee (UT), Knoxville, Tennessee, USA; [4]Université de Nice Sophia-Antipolis, CNRS, IBDC, UMR, Nice, France; [5]CEPRODI SA, Paris, France; [6]Nutrition Physiology and Ingestive Behavior, INRA-AgroParisTech, Paris, France; [7]Department of Molecular Physiology and Biophysics and Mouse Metabolic Phenotyping Center, Vanderbilt University, Nashville, Tennessee, USA. Correspondence: Naima Moustaid-Moussa (moustaid@utk.edu)

Received 3 June 2011; accepted 29 August 2011; published online 6 October 2011. doi:10.1038/oby.2011.299

secretory patterns, which is causally linked to the pathogenesis of metabolic syndrome and type-2 diabetes (4). Several lines of evidence point to the adipose RAS as a potential link between obesity and insulin resistance. Indeed, adipose tissue synthesizes and secretes the major components of RAS (5). There is also evidence for overactivation of adipose tissue RAS in obesity in rodents (6), and for a positive correlation between adipose tissue Agt levels and BMI in humans (7). Moreover, circulating levels of Agt correlate with BMI and estimated total adipose tissue-derived Agt in humans (8), suggesting an endocrine role for adipose Agt. Further, Ang II secretion from adipose tissue is increased following sympathetic stimulation in obese, but not lean, individuals (9). Conversely, plasma and adipose Agt levels are decreased following weight-loss (10). Despite this strong evidence for an association between adipose RAS overactivation and insulin resistance, it is hitherto unknown whether the former is causally linked to the latter.

More recent studies targeted manipulation of RAS (overexpression or deletion) and subsequent effects on obesity and insulin sensitivity. Loss of function in any single component of the RAS tested so far, provides protection from diet-induced obesity and insulin resistance; i.e., Agt, renin, ACE, AT_1R or AT_2R knockout rodents are lean and insulin sensitive (11–15). Systemic RAS overactivation via gene overexpression or chronic Ang II infusion also induces insulin resistance, but not necessarily obesity (16,17). Considering the relationship of adipose tissue inflammation to insulin resistance, it is possible that overactivation of adipose RAS specifically, leads to obesity and associated insulin resistance and inflammation. The hypothesis that adipose RAS overactivation will induce insulin resistance via increasing adipose tissue inflammation was tested in the present studies using a mouse model which overexpresses Agt in adipose tissue via the adipose aP2 promoter (aP2-Agt mice). These mice have elevated plasma Agt and develop hypertension (18). As previously reported, male aP2-Agt mice exhibit higher fat pad weights compared to wild-type littermates. Here, we further demonstrate that these mice also develop adipose tissue inflammation, glucose intolerance, and insulin resistance even on a low-fat (LF) diet. Moreover, the glucose intolerance was significantly improved when aP2-Agt mice were treated with an ACE inhibitor, captopril. Our studies thus demonstrate that adipose tissue RAS overactivation causes systemic insulin resistance in an Ang II-dependent manner.

METHODS AND PROCEDURES

Animals

Generation of transgenic (Tg) mice overexpressing Agt in adipose tissue using the adipose aP2 promoter (aP2-Agt mice) has been described previously (18). Mice used in the current study were bred on a C57BL/6J background and maintained in our animal facility at the University of Tennessee. Genotyping of the offspring was performed using PCR of genomic DNA using transgene-specific (5′-CTTTGCCTTTCTCTCCACAG-3′) and intron-specific (5′-TTATCTCGCAGGGTCTTCTC-3′) oligonucleotides. All mice were housed under 12-h light/dark cycles with free access to food and water. For the first study, mice were fed a regular LF diet from weaning. For the second study, a separate cohort of male mice were fed a high-fat (HF) diet (45, 20, and 35% of energy from fat, protein, and carbohydrate, respectively; D12451; Research Diets, New Brunswick, NJ) (19) for 12 weeks from the time of weaning. The mice on the HF diet were housed individually. Half the number of HF-fed mice was given the ACE inhibitor captopril with drinking water (30 mg/l) (20). At the end of each study, mice were feed-deprived for 6 h and then killed using the CO_2 inhalation method. Blood was collected into tubes with EDTA, kept on ice for 10 min, centrifuged at 3,000*g* for 20 min, and plasma samples were collected and stored at −80 °C for subsequent analyses. Epididymal, inguinal, retroperitoneal, and subscapular fat pads were dissected, snap-frozen in liquid N_2, and stored at −80 °C for subsequent analyses. These protocols were all approved by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville.

Glucose tolerance test

Mice were feed-deprived for 6 h with free access to water. A drop of tail blood was used to measure the blood glucose levels using the One Touch Ultra glucometer (LifeScan, Milpitas, CA). Next, 1 g/kg body weight of 20% D-glucose was injected intraperitoneally. Serial blood glucose measures were taken at 15, 30, 60, and 120 min after the injection (21).

Hyperinsulinemic euglycemic clamp

Detailed procedure has been previously reported (22). Catheters were chronically implanted in the jugular vein (for infusions) and carotid artery (for sampling) 5–7 days before clamps (n = 8–10). Insulin was continuously administered at 4 mU/kg/min. Arterial glucose levels were measured every 5–10 min and glucose infusion rates were adjusted to maintain fasting glucose. Mice were infused with (3-^{3}H)glucose at a rate of 0.4 μCi/min. Endogenous glucose appearance and disappearance rates were calculated as described previously (22). Glucose clearance was calculated by dividing the glucose disappearance by the arterial glucose concentration. To measure a tissue-specific index of glucose metabolism (Rg), mice were injected with 12 μCi of (^{3}H)-labeled 2-deoxyglucose ((2-^{3}H)DG). Arterial plasma samples were collected in intervals for 40 min before mice were anesthetized and tissues were extracted and frozen in liquid nitrogen until further analysis.

Plasma analyte measurements

Commercially available enzyme-linked immunosorbent assay kits were used to measure plasma Agt, leptin, and total adiponectin (Linco Research, Billerica, MA) concentrations, whereas a colorimetric assay was used to measure plasma nonesterified fatty acids (Wako Chemicals, Richmond, VA). Plasma insulin, MCP-1, and resistin concentrations were measured using a commercially available microsphere-based multiplexing system (Luminex xMAP; Millipore, Billerica, MA).

Adipose tissue adipokine measurements

Because the epididymal (gonadal) fat depot is known to show a pronounced dysregulation of adipo/cytokine secretory patterns in response to HF diets and other metabolic challenges, we used it to study the adipo/cytokine changes in our mice. Epididymal adipose tissue was homogenized in modified radioimmunoprecipitation assay buffer containing a cocktail of protease inhibitors for total protein extraction. The protein concentration was determined by the Bradford assay as described previously (21). Luminex xMAP was used to measure adipokines. Analyte values in the adipose protein extracts were normalized to total protein concentration.

Proteomics

Proteins were extracted from epididymal adipose tissue of aP2-Agt and control littermates matched for adipose mass, and labeled with cyanine 3 or 5 (green or red), respectively. Both samples were run on a two-dimensional gel electrophoresis (Applied Biomics, Hayward, CA). A complete analysis of all differentially expressed proteins was obtained using Decyder software from which quantitative data were derived. Spots with a volume ratio of >30% and a consistent presence in replicate gels were identified and obtained using the spot picker robot, and

Table 1 Plasma biomarkers in low-fat fed aP2-Agt mice

	Females		Males		*P* value		
	Wt	Tg	Wt	Tg	Sex effect	Genotype effect	Sex x genotype effect
Insulin (pmol/l)	131 ± 93	164 ± 67	191 ± 73	312 ± 133	0.019*	0.157	0.725
Glucose (mg/dl)	204 ± 16	183 ± 9	199 ± 15	221 ± 22	0.352	0.753	0.166
Adiponectin (mg/l)	18.2 ± 7.0	19.7 ± 6.1	9.8 ± 3.1	9.9 ± 2.9	0.001*	0.743	0.769
Leptin (μg/l)	1.6 ± 1.0	4.1 ± 2.5	2.9 ± 1.7	6.0 ± 4.6	0.204	0.046*	0.508
Agt (mg/l)	2.8 ± 0.8	3.4 ± 0.8	3.4 ± 0.5	4.4 ± 0.4	0.021*	0.022*	0.617
NEFA (mmol/l)	0.55 ± 0.19	0.70 ± 0.19	0.62 ± 0.22	0.71 ± 0.19	0.649	0.200	0.727
MCP-1 (ng/l)	18.9 ± 7.0	25.4 ± 15.0	22.6 ± 14.7	24.0 ± 9.2	0.894	0.638	0.761
Resistin (ng/l)	1,400 ± 211	1,748 ± 566	1,184 ± 279	1,032 ± 99	0.034*	0.777	0.267

Data are presented as mean ± s.d. (n = 4–9 per group).
Agt, angiotensinogen; MCP-1, monocyte chemotactic protein-1; NEFA, nonesterified fatty acid; Wt, wild-type; Tg, transgenic.
*$P < 0.05$.

proteins within each spot were enzymatically digested and analyzed by mass spectrometry. Proteins identified from this analysis were then uploaded into DAVID Bioinformatics Resources, where the functional annotation chart was used to search for significantly enriched gene ontology categories. Differential expression of these proteins was further confirmed by western blotting.

Cell culture experiments

Adipocytes were cultured as described previously (23). Briefly, 3T3-L1 preadipocytes were cultured in six-well plates in regular growth media consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. At confluence, the cells were differentiated by the addition of growth media supplemented with 250 nmol/l dexamethasone and 0.5 mmol/l methyl isobutylxanthine for 72 h, after which regular media were added for two additional days. Subsequently, cells were placed in serum-free media (Dulbecco's modified Eagle's medium, penicillin/streptomycin, and 1% bovine serum albumin) for 18 h before applying various treatments for 24 h, as described in the results and figure legends. Secreted adipokine levels were assayed in culture media, using Luminex xMAP.

Western blotting

Total proteins were isolated from epididymal adipose tissue, homogenized in modified radioimmunoprecipitation assay buffer containing a cocktail of protease inhibitors. Twenty micrograms of total protein was loaded into each lane and separated by electrophoresis in an 8–10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane in transfer buffer, blocked overnight with 5% nonfat dry milk in Tris-buffered saline, and 0.1% Tween, incubated with polyclonal antibodies against monoglyceride lipase (MGL) and actin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by secondary antibodies containing horseradish peroxidase and finally detected by chemiluminescence. Western blots were quantified using the ImageJ software.

Statistical analysis

Plasma biomarkers were analyzed by ANOVA using the general linear models procedure, taking the effect of the genotype, sex, and interactions between these factors into account. For the diet study, a similar analysis was performed taking the effect of genotype, treatment, and interactions between these factors into account. Body and fat pad weight, area under the glucose curve, glucose infusion rate and tissue glucose uptake in the clamp studies, adipose tissue cytokine levels and MGL expression was compared using the Student's t-test. In the cell culture studies, one way-ANOVA was used. If the F-test was significant, group means were compared using the Tukey's *post hoc* test for multiple comparisons. The fold difference in protein expression between replicate gels in proteomic studies was compared using the one-sample t-test. The level of significance for all tests was $P < 0.05$. All data are expressed as mean ± s.d..

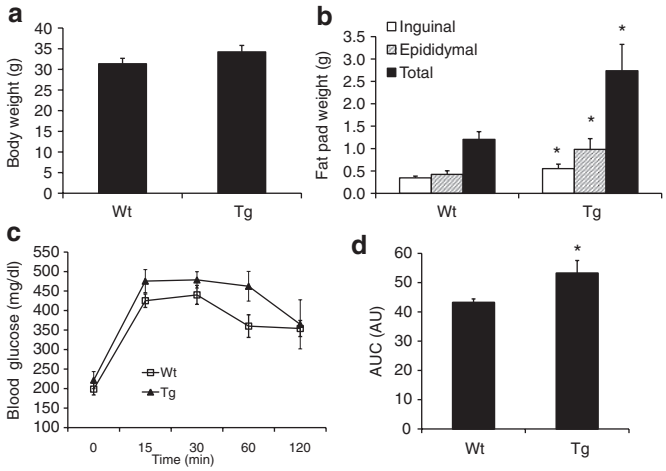

Figure 1 Adipose Agt overexpression induces adiposity and glucose intolerance. Wild-type (Wt) and transgenic (Tg) male aP2-Agt mice were fed a low-fat diet from weaning until 24-weeks of age. (**a**) Body weight and (**b**) fat pad weight are shown. At 22-weeks of age, an intraperitoneal glucose tolerance test was performed. (**c**) Change in blood glucose over time and (**d**) area under the glucose curve (AUC) are shown. Data are presented as mean ± s.e.m. (n = 6–7 per each group). *$P < 0.05$ vs. Wt. Agt, angiotensinogen; AU, arbitrary unit.

RESULTS

Adipose Agt overexpression induces higher adiposity, glucose intolerance, and insulin resistance

As expected, the aP2-Agt Tg mice had higher adipose Agt levels (5.2 ± 3.9, 16.9 ± 8.6, 15.9 ± 7.7, and 20.4 ± 10.8 μg/g protein in female wild-type (Wt), female Tg, male Wt, and male Tg respectively; $P < 0.05$ for genotype effect) at the age of 24-weeks. It is worth noting that similar results have been reported previously for Agt secretion from adipose explants (18) and adipocytes isolated from these mice (24). The transgenics also had ~20% higher plasma Agt levels (Table 1) than the Wt mice, also in agreement with previous reports (18). Because female

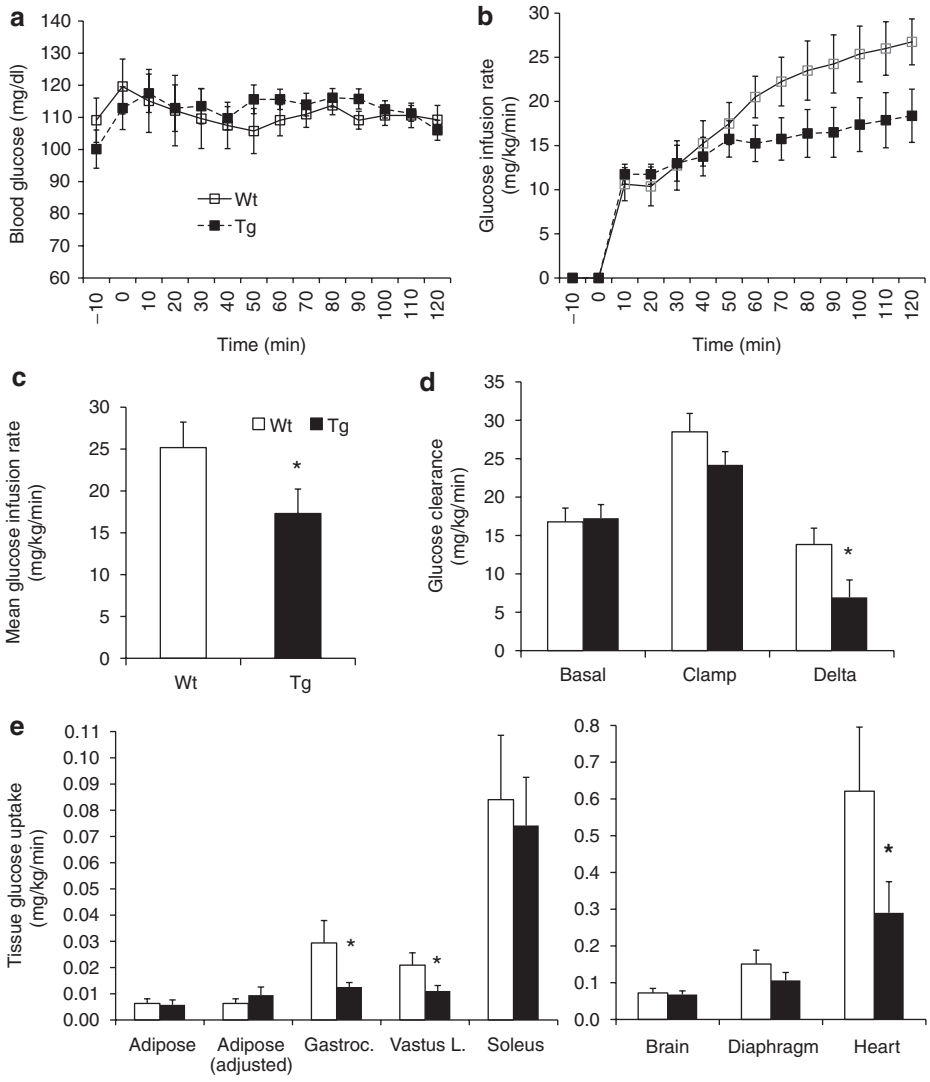

Figure 2 Adipose Agt overproduction induces insulin resistance. Low-fat diet fed, male aP2-Agt wild-type (Wt) and transgenic (Tg) mice were subjected to a hyperinsulinemic euglycemic clamp. (**a**) Change in blood glucose and (**b**) glucose infusion rate over time, (**c**) mean steady-state glucose infusion rate and (**d**) glucose clearance rate are shown. (**e**) Using (^{3}H)-labeled 2-deoxyglucose, tissue-specific index of glucose metabolism (Rg) was measured. Data are presented as mean ± s.e.m. (n = 8–10 in each group). *P < 0.05. Agt, angiotensinogen; Gastroc., gastrocnemius muscle; Vastus L., vastus lateralis muscle.

aP2-Agt mice did not show evidence of insulin resistance or glucose intolerance (data not shown), we used only male mice for the subsequent studies. Male aP2-Agt mice exhibited higher fat pad weights, whereas there was no genotype effect on body weight (Figure 1a,b). An intraperitoneal glucose tolerance test performed at 22-weeks of age showed that male Tgs were glucose intolerant as compared to Wt males, indicated by a higher area under the glucose curve (Figure 1c,d). While the Tgs also had higher plasma leptin levels compared to their Wt counterparts, as expected from fat pad weight differences, there was no significant genotype effect on plasma levels of several other adipocytokines (Table 1). Next, to determine if overexpression of Agt in adipose tissue causes insulin resistance, we performed hyperinsulinemic euglycemic clamps on male aP2-Agt mice. The Tgs had a lower steady-state glucose infusion rate compared to Wt mice, indicating lower insulin sensitivity (Figure 2b,c). The difference in glucose clearance between basal and clamp was also lower in the Tgs (Figure 2d). Isotope studies for tissue glucose uptake showed that Rg was significantly lower in the gastrocnemius and vastus muscles in the Tg mice compared to the Wt mice (Figure 2e). Interestingly, the Tgs also had lower cardiac Rg. While there was a trend for an increase in adipose tissue Rg (adjusted for adipocyte number) of transgenics, this was not significant.

Captopril attenuates glucose intolerance in HF-fed aP2-Agt and Wt mice

HF feeding is known to induce glucose intolerance and insulin resistance in C57BL/6J mice (21). To test that adipose RAS overactivation exacerbates HF diet-induced glucose intolerance, we fed Tg and Wt littermates a HF diet for 12 weeks. To determine if the glucose intolerance was Ang II-dependent, we

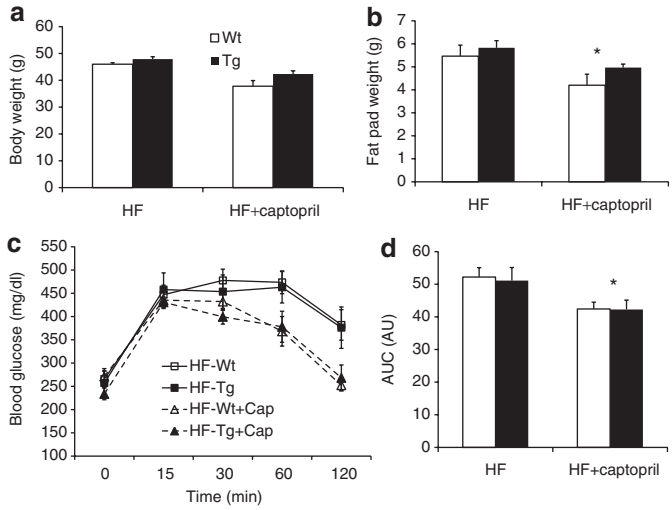

Figure 3 Captopril attenuates high-fat (HF) diet-induced adiposity and glucose intolerance. Male aP2-Agt wild-type (Wt) and transgenic (Tg) mice were fed a high-fat diet (45, 20, and 35% of energy from fat, protein, and carbohydrate, respectively) for 11 weeks. Half of each genotype was given captopril (cap–30 mg/l) in drinking water. (**a**) Body weight and (**b**) fat pad weight are shown. At 10 weeks on the HF diet, an intraperitoneal glucose tolerance test was performed. (**c**) Change in blood glucose concentration over time and (**d**) area under the glucose curve (AUC) are shown. Data are presented as mean ± s.e.m. (n = 8–12 in each group). *P < 0.05 treatment effect. Agt, angiotensinogen; AU, arbitrary unit; Cap, captopril.

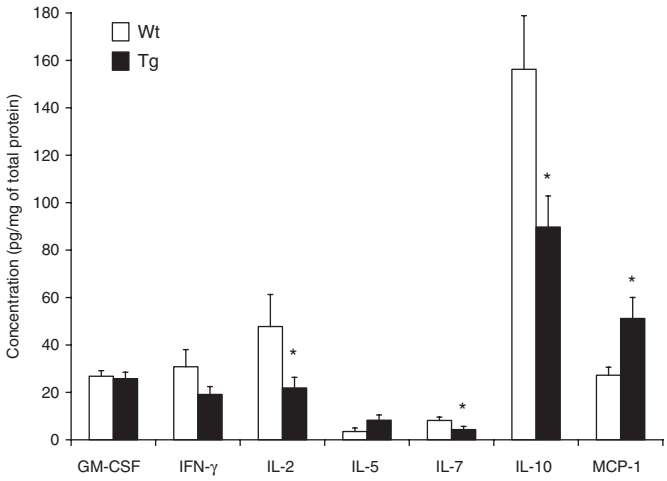

Figure 4 Adipose Agt oveexpression induces inflammatory markers in adipose tissue. Male aP2-Agt wild-type (Wt) and transgenic (Tg) mice were fed a low-fat diet from weaning until 24-weeks of age. Epididymal adipose tissue cytokine levels normalized to total protein content are shown. Data are presented as mean ± s.e.m. (n = 5 per group). *P < 0.05. Agt, angiotensinogen; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MCP-1, monocyte chemotactic protein-1.

also treated a cohort of HF-fed mice with the ACE inhibitor, captopril (30 mg/l in drinking water). In contrast to males on the LF diet (**Figure 1d**), the glucose tolerance was similar in both Tgs and Wt mice on the HF diet, as indicated by similar area under the curves (**Figure 3c,d**). Captopril prevented the HF diet induced increase in adiposity (**Figure 3a,b**) and glucose intolerance in both Tg and Wt mice (**Figure 3c,d**). Thus, while aP2-Agt mice exhibited glucose intolerance compared to control littermates when fed LF diets, HF feeding normalized these difference in glucose tolerance between genotypes. Further, ACE inhibition improved HF diet-induced glucose intolerance in both genotypes.

Adipose Agt overexpression increases markers of adipose inflammation

Chronic low-grade inflammation in adipose tissue is causally linked to the pathogenesis of insulin resistance in obesity. To determine if adipose inflammation is a potential mechanism for the insulin resistance in aP2-Agt mice, we measured selected markers of adipose inflammation. Proinflammatory cytokine MCP-1 was nearly twofold higher in the epididymal adipose tissue of the Tgs compared to Wt littermates (**Figure 4**). Further, expression of interleukin-10 (IL-10), an anti-inflammatory cytokine was significantly lower in the Tgs. The hematopoietic cytokine IL-7 and leukocytotrophic cytokine IL-2 were expressed lower in the Tgs, whereas there was no difference in granulocyte-macrophage colony-stimulating factor, interferon-γ and IL-5 levels between the two groups. These findings indicate that adipose Agt overexpression induces adipose tissue inflammation.

Ang II increases secretion of MCP-1 and resistin in adipocytes

Previous studies have reported that aP2-Agt mice exhibit adipocyte hypertrophy (18). Because adipocyte hypertrophy is associated with a proinflammatory adipokine secretory profile, increased MCP-1 levels in adipose tissue of Tgs could be due to either a direct action of Ang II on adipocytes, or an indirect effect due to adipocyte hypertrophy. To address this issue, we subjected 3T3-L1 murine adipocytes to a short-term (24-h) treatment of Ang II (10 nmol/l). As shown in **Figure 5**, Ang II increased the secretion of MCP-1 and resistin from adipocytes.

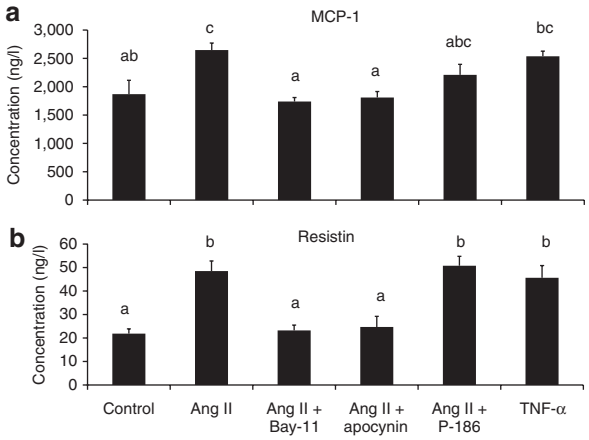

Figure 5 Ang II induces MCP-1 and resistin secretion from 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with control (DMSO), Ang II (10 nmol/l), Ang II plus NF-κB inhibitor Bay 11-7082 (5 µmol/l), Ang II plus NADPH oxidase inhibitor apocynin (200 µmol/l), Ang II plus AT_2R antagonist P-186 (100 nmol/l) or TNF-α (100 pM) for 24 h. Culture media (**a**) MCP-1 and (**b**) resistin levels are shown. Data are presented as mean ± s.e.m. ($n = 3$ per each group). Different letters indicate a significant difference ($P < 0.05$).Ang II, angiotensin II; DMSO, dimethyl sulfoxide; MCP-1, monocyte chemotactic protein-1; NADPH; nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor κB; TNF-α; tumor necrosis factor-α.

Because Ang II is known to activate the nuclear factor κ B (NF-κB) pathway, we treated the adipocytes with Ang II in the presence of an NF-κB inhibitor (Bay 11-7082). As expected, Bay 11-7082 suppressed the Ang II-induced increases in MCP-1 and resistin secretion from adipocytes. This indicates that Ang II induces MCP-1 and resistin secretion from adipocytes in an NF-κB pathway-dependent manner. To further investigate the mechanism of Ang II-mediated stimulation of the NF-κB pathway, we treated the adipocytes with Ang II in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, apocynin. As shown in **Figure 5**, apocynin also completely prevented the Ang II-induced increases in MCP-1 and resistin secretion. We have previously shown that Ang II effects in 3T3-L1 adipocytes are mediated by AT_2R (25). To test whether these changes were mediated via AT_2R, we treated the adipocytes with Ang II in the presence of an AT_2R antagonist. We found that this antagonist was able to partially prevent the Ang II-induced MCP-1, but not resistin secretion.

Adipose Agt overexpression induces MGL expression

Proteomic studies of epididymal adipose tissue were used to identify novel proteins induced by adipose Agt overexpression of adipose weight-matched Tg and Wt littermates, using 2DIGE (Applied Biomics). Several spots differentially expressed between the Wt and Tg mice were identified, quantified (**Supplementary Figure S1** online), and proteins were identified by mass spectrometry. The detailed list of proteins is given in **Table 2**. Tgs exhibited higher expression of glycerol-3-phosphate dehydrogenase, a major lipogenic enzyme and MGL, a lipolytic enzyme. Although we have previously shown that Ang II increases glycerol-3-phosphate dehydrogenase and other

Table 2 Proteins differentially expressed in adipose tissue in low-fat fed aP2-Agt mice

Protein name	GI accession no	Tg/Wt spot volume ratio[a]
Tg higher Expression than Wt		
Triosephosphate isomerase 1	6678413	1.67
Gpd1 protein (glycerol-3-phosphate dehydrogenase)	13543176	1.36
Vinculin	31543942	1.46
Isocitrate dehydrogenase 3 (NAD+)-α	18250284	1.49
Eukaryotic translation initiation factor 5A, isoform CRA_a	148680528	2.42
Vitamin D-binding protein	193446	1.40
Ehd2 protein	20072042	1.43
Medium-chain acyl-CoA dehydrogenase	6680618	1.52
Polymerase I and transcript release factor	6679567	1.59
Polymerase I and transcript release factor	6679567	1.66
Glutathione S-transferase, α-4	15215030	1.65
Fumarate hydratase 1	33859554	1.79
MgII protein (monoglyceride lipase)	34786023	2.40
Anxa1 protein	12805619	1.92
Tg lower expression than Wt		
Apolipoprotein A-I	6753096	0.17
Immunoglobulin γ-1 heavy chain	26665404	0.25
Mannose binding lectin, serum (C)	6754656	0.20
Arhgdib protein	21618829	0.26
Galactokinase 1, isoform CRA_a	148702595	0.35
Medium-chain acyl-CoA dehydrogenase	6680618	0.35
Apolipoprotein A-I precursor–mouse	109571	0.35
Dnm1l protein	51259985	0.21
Villin 2	37573976	0.41
Major urinary protein 2	47059037	0.35
Transthyretin	56541070	0.39
Proteasome β-3 subunit	6755202	0.50
DOM1	21322147	0.50
Carbonic anhydrase 3	31982861	0.20
Similar to Sly protein	38090288	0.29
Hemopexin	160358829	0.64
Catalase	157951741	0.64
Glutathione S-transferase M2	6680121	0.68
G-protein β subuit like	475012	0.68
Anti-human seminoprotein monoclonal antibody	27227449	0.25
65-kDa macrophage protein	984636	0.34

CoA, coenzyme A; NAD, nicotinamide adenine dinucleotide; Tg, transgenic; Wt, wild-type.
[a]Spots with a volume ratio of >30% and a consistent presence in replicate gels were identified and analyzed by mass spectrometry.

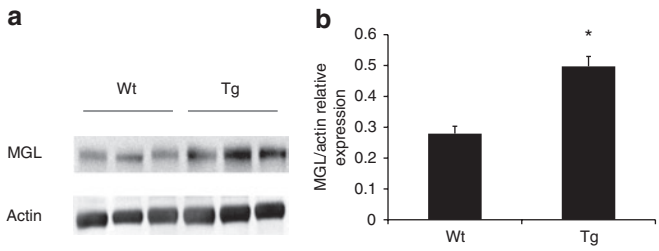

Figure 6 Adipose Agt overexpression induces monoglyceride lipase in adipose tissue. Male aP2-Agt wild-type (Wt) and transgenic (Tg) mice were fed a low-fat diet from weaning until 24-weeks of age. (**a**) Western blotting results for Wt and Tg is shown. (**b**) Epididymal adipose tissue monoglyceride lipase (MGL) expression between Wt and Tg is shown by western blot. Data are expressed as mean ± s.e.m. (n = 4–5 per group). *P < 0.05. Agt, angiotensinogen.

lipogenic activity in adipocytes (25), the differential expression of MGL here is a novel finding, which we confirmed by western blotting as shown in **Figure 6**. Proteins expressed at low levels in the Tg mice include catalase, an antioxidant enzyme.

DISCUSSION

These studies provide the first evidence that adipose RAS components directly link to insulin resistance. Male aP2-Agt mice develop glucose intolerance and systemic insulin resistance, which is at least in part due to reduced skeletal muscle glucose uptake. Moreover, the inflammatory profile of these Tg mice is characterized by elevated adipose MCP-1 levels. We further confirmed this *in vitro,* demonstrating that Ang II increases MCP-1 secretion from murine adipocytes, in an NF-κB and NADPH oxidase-dependent manner.

RAS overactivation, adiposity, and insulin resistance

In agreement with previous reports, our aP2-Agt mice developed increased adiposity even on a LF diet (18). We have previously shown that Ang II increases triglyceride content and lipogenic enzyme activity in murine adipocytes *in vitro* (25). Similarly, we found that the expression of glycerol-3-phosphate dehydrogenase, a key lipogenic enzyme, was expressed at higher levels in the adipose tissue of aP2-Agt Tgs compared to non-Tg controls. Interestingly, other models of systemic RAS overactivation such as chronic Ang II infusion (16) and overexpression of human Agt in liver (6), do not exhibit higher adiposity. The increased adiposity seen in aP2-Agt mice strongly suggests that it is due to autocrine/paracrine actions of Ang II on adipose tissue. Previous studies suggest that increased adiposity in aP2-Agt mice is in part related to reduced energy expenditure, since the aP2-Agt mice exhibited reduced locomotor activity during the light-cycle, with no difference in energy intake, when compared to Wt counterparts (18). While the aP2-Agt mice have higher adipose tissue mass, their fat-free mass is comparable to Wt littermates (18).

In addition to exhibiting higher adiposity, male aP2-Agt mice are also glucose intolerant and insulin resistant. Previous rodent models of RAS overexpression have shown that chronic systemic RAS overactivation leads to insulin resistance. These include the renin overexpressing hypertensive TG(mRen2)27 rat (26), and chronic Ang II infusion models (27). In this study we show that specific adipose Agt overexpression is sufficient to produce a level of glucose intolerance that is comparable to Wt mice on a HF diet. An important finding of these studies is that HF feeding did not further exacerbate glucose intolerance in aP2-Agt mice. This suggests that similar mechanisms operate in both instances.

Since systemic RAS blockade improves insulin resistance and glucose tolerance in numerous rodent studies (26), we tested whether blocking RAS would improve metabolic alterations in the aP2-Agt mice. As expected, treatment with ACE inhibitor captopril prevented the HF diet-induced glucose intolerance in both Tg and Wt mice. Previous studies in rodents with ACE knockout (11), renin inhibition (26), renin knockout (13), AT_1R blockade (3) or AT_2R deletion (14) have all shown improvements in insulin sensitivity and glucose tolerance. ACE2 overactivation which lowers Ang II and increases Ang (1–7) has also been shown to promote insulin sensitivity and glucose tolerance (28).

Mechanisms of insulin resistance induced by RAS overactivation

Most models of obesity and insulin resistance exhibit both muscle and adipose insulin resistance. Here, we found that while adipose tissue Rg was not impaired in the aP2-Agt mice, these mice exhibited reduced skeletal muscle and heart Rg. This could at least in part explain the whole body insulin resistance in Tg mice. Several potential factors might have caused skeletal muscle insulin resistance. We found that the level of proinflammatory cytokine MCP-1 was almost twofold higher in the adipose tissue of transgenics compared to the non-transgenic control mice, while the level of anti-inflammatory cytokine IL-10 was lower. Together, these findings indicate increased inflammation in the adipose tissue of the aP2-Agt mice. Altered adipokine secretory patterns due to adipose tissue inflammation could be a cause of skeletal muscle insulin resistance (29). It should however be noted that the plasma levels of several adipokines (with the exception of leptin) was comparable between the Wt and Tg mice. Thus, a potentially paracrine role of adipose tissue could be important in the reduced skeletal muscle and heart Rg in the aP2-Agt mice. Indeed, previous studies have linked adipose tissue distribution within skeletal muscle to insulin resistance (30). Epicardial fat is also known to produce proinflammatory cytokines, which is implicated in the postoperative insulin resistance (31). *In vitro* studies have also shown that adipokine secretion can impair insulin signaling of myocytes, suggesting cross-talk between the two tissues (32).

The aP2-Agt Tgs also exhibited higher plasma Agt levels. Higher Agt and Ang II levels were previously reported to induce skeletal muscle and vascular smooth muscle insulin resistance by several mechanisms. First, the skeletal muscle blood flow can be reduced due to the pressor effect of Ang II, resulting in lower glucose delivery. Second, Ang II can also inhibit insulin signaling in muscle (33), resulting in reduced

translocation of glucose transporter type 4, leading to reduced glucose transport. Increased Ang II levels are also known to impair glucose-stimulated insulin release from the pancreatic islets (34). Thus, the glucose intolerance in the aP2-Agt Tgs could be attributed to a combination of reduced skeletal muscle glucose uptake as well as reduced insulin response in the pancreas, which could be exacerbated by their elevated blood pressure (18). In addition, previous studies have also reported that Ang II increases hepatic glucose production, which could also contribute to glucose intolerance (27). We propose that these metabolic alterations are mediated by Ang II, as evidenced by an improvement of the glucose tolerance in the mice treated with ACE inhibitor captopril. While elevated plasma Agt levels could contribute to the systemic insulin resistance in aP2-Agt mice as outlined above, it is unlikely that it played a major role, because the difference in plasma Agt levels between the two genotypes was modest compared to circulating concentrations shown to be effective in causing insulin resistance in previous studies (27).

Ang II and adipose tissue inflammation

Adipose Agt overexpression increases markers of adipose tissue inflammation (increased MCP-1 and decreased IL-10 in this study and increased IL-6 and IL1β in previous reports ref. (24)). aP2-Agt mice also exhibit higher adiposity and adipocyte hypertrophy (18), and because the latter is associated with a proinflammatory adipokine profile, the increased MCP-1 could be a result of adipocyte hypertrophy. However, we showed that Ang II can also directly increase MCP-1 and resistin secretion from adipocytes *in vitro*. Ang II's ability to promote MCP-1 production in other cell types such as preadipocytes (35) has been reported previously. Further, RAS blockade improved plasma MCP-1 and resistin levels in humans (36).

Ang II is also documented to simulate NADPH oxidase, leading to increased production of reactive oxygen species, which can activate the NF-κB pathway and production of proinflammatory cytokines in the skeletal muscle (37). Thus, we investigated whether similar mechanisms operate in the adipose tissue. We found that Ang II-induced MCP-1 and resistin secretion from adipocytes was completely abolished by treatment with NADPH oxidase or NF-κB inhibitors.

From our proteomic studies, we found that the expression of MGL, a lipolytic enzyme, was twofold higher in the epididymal adipose tissue of Tgs. MGL cleaves 2-arachodonyl glycerol to form arachidonic acid. Activation of MGL has been shown to increase arachidonic acid levels in vascular smooth muscle (38). Since elevated arachidonic acid levels induce secretion of proinflammatory cytokines (21) and prostaglandin E2 (23) in adipocytes *in vitro,* MGL could be another mediator of Ang II-induced adipose inflammation. We have previously shown that Ang II dose-dependently increases prostaglandin E2 secretion in murine adipocytes *in vitro* (39). However, direct effects of Ang II on MCP-1 via prostaglandins remain to be tested.

In the mouse model we used for our study, the adipose-specific Agt overexpression is driven by the aP2 promoter. While several previous studies have used a similar approach (40), one recognized limitation is the macrophage expression of aP2. Whether the Agt overexpression by macrophages also contributes to adipose tissue or systemic inflammation needs further clarification, but is beyond the scope of this work. Another limitation of this study is that we did not test the effects of ACE inhibition in the LF fed aP2-Agt mice. However, we have previously shown that RAS inhibition (genetic inactivation of AT_2R) reverses metabolic alterations under HF feeding conditions (14), together indicating the importance of Ang II production and/or action in mediating the metabolic and inflammatory phenotype of the aP2-Agt mice.

Overall, the aP2-Agt Tg mouse model is a valuable model for studies of human obesity associated with insulin resistance and hypertension. This study provides for the first time evidence for a causal link between adipose tissue Agt overproduction and the generation of glucose intolerance and systemic insulin resistance. The mechanisms are at least in part due to skeletal and cardiac muscle insulin resistance, resulting from increased adipose tissue inflammation and systemic levels of Ang II.

ACKNOWLEDGMENTS

This project was supported by American Heart Association Grant In Aid # 0755626B, USDA-NIFA NRI award 2005-35200-15224, Department of Animal Science and UT AgResearch (N.M.-M.), American Heart Association predoctoral fellowship 09PRE2260238, UT Obesity Research Center (N.S.K.), and Vanderbilt Mouse Metabolic Phenotyping Center U24 DK059637 (D.H.W.). We would like to thank Taryn Stewart and Dr. Morvarid Bejnood for their assistance in the *in vivo* studies.

DISCLOSURE

The authors declared no conflict of interest.

SUPPLEMETARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/oby

REFERENCES

1. Schmieder RE, Hilgers KF, Schlaich MP, Schmidt BM. Renin-angiotensin system and cardiovascular risk. *Lancet* 2007;369:1208–1219.
2. Braga MF, Leiter LA. Role of renin-angiotensin system blockade in patients with diabetes mellitus. *Am J Cardiol* 2009;104:835–839.
3. Chu KY, Lau T, Carlsson PO, Leung PS. Angiotensin II type 1 receptor blockade improves beta-cell function and glucose tolerance in a mouse model of type 2 diabetes. *Diabetes* 2006;55:367–374.
4. Kalupahana NS, Claycombe K, Moustaid-Moussa N. (n-3) Fatty acids alleviate adipose tissue inflammation and insulin resistance: mechanistic insights. *Adv Nutr* 2011;2:304–316.
5. Engeli S, Negrel R, Sharma AM. Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* 2000;35:1270–1277.
6. Rahmouni K, Mark AL, Haynes WG, Sigmund CD. Adipose depot-specific modulation of angiotensinogen gene expression in diet-induced obesity. *Am J Physiol Endocrinol Metab* 2004;286:E891–E895.
7. Van Harmelen V, Ariapart P, Hoffstedt J *et al*. Increased adipose angiotensinogen gene expression in human obesity. *Obes Res* 2000;8:337–341.
8. Yasue S, Masuzaki H, Okada S *et al*. Adipose tissue-specific regulation of angiotensinogen in obese humans and mice: impact of nutritional status and adipocyte hypertrophy. *Am J Hypertens* 2010;23:425–431.
9. Goossens GH, Jocken JW, Blaak EE *et al*. Endocrine role of the renin-angiotensin system in human adipose tissue and muscle: effect of beta-adrenergic stimulation. *Hypertension* 2007;49:542–547.
10. Engeli S, Böhnke J, Gorzelniak K *et al*. Weight loss and the renin-angiotensin-aldosterone system. *Hypertension* 2005;45:356–362.

11. Jayasooriya AP, Mathai ML, Walker LL *et al*. Mice lacking angiotensin-converting enzyme have increased energy expenditure, with reduced fat mass and improved glucose clearance. *Proc Natl Acad Sci USA* 2008;105:6531–6536.
12. Kim S, Soltani-Bejnood M, Quignard-Boulange A *et al*. The adipose renin-angiotensin system modulates systemic markers of insulin sensitivity and activates the intrarenal renin-angiotensin system. *J Biomed Biotechnol* 2006;2006:27012.
13. Takahashi N, Li F, Hua K *et al*. Increased energy expenditure, dietary fat wasting, and resistance to diet-induced obesity in mice lacking renin. *Cell Metab* 2007;6:506–512.
14. Yvan-Charvet L, Even P, Bloch-Faure M *et al*. Deletion of the angiotensin type 2 receptor (AT2R) reduces adipose cell size and protects from diet-induced obesity and insulin resistance. *Diabetes* 2005;54:991–999.
15. Massiera F, Seydoux J, Geloen A *et al*. Angiotensinogen-deficient mice exhibit impairment of diet-induced weight gain with alteration in adipose tissue development and increased locomotor activity. *Endocrinology* 2001;142:5220–5225.
16. Brink M, Wellen J, Delafontaine P. Angiotensin II causes weight loss and decreases circulating insulin-like growth factor I in rats through a pressor-independent mechanism. *J Clin Invest* 1996;97:2509–2516.
17. Henriksen EJ. Improvement of insulin sensitivity by antagonism of the renin-angiotensin system. *Am J Physiol Regul Integr Comp Physiol* 2007;293:R974–R980.
18. Massiéra F, Bloch-Faure M, Ceiler D *et al*. Adipose angiotensinogen is involved in adipose tissue growth and blood pressure regulation. *FASEB J* 2001;15:2727–2729.
19. Kalupahana NS, Voy BH, Saxton AM, Moustaid-Moussa N. Energy-restricted high-fat diets only partially improve markers of systemic and adipose tissue inflammation. *Obesity (Silver Spring)* 2011;19:245–254.
20. Mattson DL. Comparison of arterial blood pressure in different strains of mice. *Am J Hypertens* 2001;14:405–408.
21. Kalupahana NS, Claycombe K, Newman SJ *et al*. Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation. *J Nutr* 2010;140:1915–1922.
22. Ayala JE, Bracy DP, McGuinness OP, Wasserman DH. Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. *Diabetes* 2006;55:390–397.
23. Wortman P, Miyazaki Y, Kalupahana NS *et al*. n3 and n6 polyunsaturated fatty acids differentially modulate prostaglandin E secretion but not markers of lipogenesis in adipocytes. *Nutr Metab (Lond)* 2009;6:5.
24. Yvan-Charvet L, Massiéra F, Lamandé N *et al*. Deficiency of angiotensin type 2 receptor rescues obesity but not hypertension induced by overexpression of angiotensinogen in adipose tissue. *Endocrinology* 2009;150:1421–1428.
25. Jones BH, Standridge MK, Moustaid N. Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology* 1997;138:1512–1519.
26. Lastra G, Habibi J, Whaley-Connell AT *et al*. Direct renin inhibition improves systemic insulin resistance and skeletal muscle glucose transport in a transgenic rodent model of tissue renin overexpression. *Endocrinology* 2009;150:2561–2568.
27. Rao RH. Pressor doses of angiotensin II increase hepatic glucose output and decrease insulin sensitivity in rats. *J Endocrinol* 1996;148:311–318.
28. Bindom SM, Hans CP, Xia H, Boulares AH, Lazartigues E. Angiotensin I-converting enzyme type 2 (ACE2) gene therapy improves glycemic control in diabetic mice. *Diabetes* 2010;59:2540–2548.
29. Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. *J Clin Invest* 2008;118:2992–3002.
30. Goodpaster BH, Thaete FL, Kelley DE. Thigh adipose tissue distribution is associated with insulin resistance in obesity and in type 2 diabetes mellitus. *Am J Clin Nutr* 2000;71:885–892.
31. Kremen J, Dolinkova M, Krajickova J *et al*. Increased subcutaneous and epicardial adipose tissue production of proinflammatory cytokines in cardiac surgery patients: possible role in postoperative insulin resistance. *J Clin Endocrinol Metab* 2006;91:4620–4627.
32. Dietze D, Koenen M, Röhrig K *et al*. Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. *Diabetes* 2002;51:2369–2376.
33. Folli F, Kahn CR, Hansen H, Bouchie JL, Feener EP. Angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels. A potential role for serine phosphorylation in insulin/angiotensin II crosstalk. *J Clin Invest* 1997;100:2158–2169.
34. Lau T, Carlsson PO, Leung PS. Evidence for a local angiotensin-generating system and dose-dependent inhibition of glucose-stimulated insulin release by angiotensin II in isolated pancreatic islets. *Diabetologia* 2004;47:240–248.
35. Tsuchiya K, Yoshimoto T, Hirono Y *et al*. Angiotensin II induces monocyte chemoattractant protein-1 expression via a nuclear factor-kappaB-dependent pathway in rat preadipocytes. *Am J Physiol Endocrinol Metab* 2006;291:E771–E778.
36. Soejima H, Ogawa H, Yasue H *et al*. Angiotensin-converting enzyme inhibition reduces monocyte chemoattractant protein-1 and tissue factor levels in patients with myocardial infarction. *J Am Coll Cardiol* 1999;34:983–988.
37. Wei Y, Sowers JR, Clark SE *et al*. Angiotensin II-induced skeletal muscle insulin resistance mediated by NF-kappaB activation via NADPH oxidase. *Am J Physiol Endocrinol Metab* 2008;294:E345–E351.
38. Tang X, Edwards EM, Holmes BB, Falck JR, Campbell WB. Role of phospholipase C and diacylglyceride lipase pathway in arachidonic acid release and acetylcholine-induced vascular relaxation in rabbit aorta. *Am J Physiol Heart Circ Physiol* 2006;290:H37–H45.
39. Kim S, Whelan J, Claycombe K, Reath DB, Moustaid-Moussa N. Angiotensin II increases leptin secretion by 3T3-L1 and human adipocytes via a prostaglandin-independent mechanism. *J Nutr* 2002;132:1135–1140.
40. Koliwad SK, Streeper RS, Monetti M *et al*. DGAT1-dependent triacylglycerol storage by macrophages protects mice from diet-induced insulin resistance and inflammation. *J Clin Invest* 2010;120:756–767.