

# Mas Deficiency in FVB/N Mice Produces Marked Changes in Lipid and Glycemic Metabolism

Sérgio Henrique S. Santos,<sup>1</sup> Luciana Rodrigues Fernandes,<sup>2</sup> Érica Guilhen Mario,<sup>3</sup> Adaliene Versiani M. Ferreira,<sup>3</sup> Laura Cristina J. Pôrto,<sup>2</sup> Jaqueline Isaura Alvarez-Leite,<sup>2</sup> Leida Maria Botion,<sup>3</sup> Michael Bader,<sup>4</sup> Natalia Alenina,<sup>4</sup> and Robson Augusto S. Santos<sup>1</sup>

**OBJECTIVE**—Metabolic syndrome is characterized by the variable coexistence of obesity, hyperinsulinemia, insulin resistance, dyslipidemia, and hypertension. It is well known that angiotensin (Ang) II is importantly involved in the metabolic syndrome. However, the role of the vasodilator Ang-(1-7)/*Mas* axis is not known. The aim of this study was to evaluate the effect of genetic deletion of the G protein-coupled receptor, *Mas*, in the lipidic and glycemic metabolism in FVB/N mice.

**RESEARCH DESIGN AND METHODS**—Plasma lipid, insulin, and cytokine concentrations were measured in FVB/N *Mas*-deficient and wild-type mice. A glucose tolerance test was performed by intraperitoneally injecting D-glucose into overnight-fasted mice. An insulin sensitivity test was performed by intraperitoneal injection of insulin. Uptake of 2-deoxy-[<sup>3</sup>H]glucose by adipocytes was used to determine the rate of glucose transport; adipose tissue GLUT4 was quantified by Western blot. Gene expression of transforming growth factor (TGF)- $\beta$ , type 1 Ang II receptor, and angiotensinogen (AGT) were measured by real-time PCR.

**RESULTS**—Despite normal body weight, *Mas*-knockout (*Mas*-KO) mice presented dyslipidemia, increased levels of insulin and leptin, and an ~50% increase in abdominal fat mass. In addition, *Mas* gene-deleted mice presented glucose intolerance and reduced insulin sensitivity as well as a decrease in insulin-stimulated glucose uptake by adipocytes and decreased GLUT4 in adipose tissue. *Mas*<sup>-/-</sup> presented increased muscle triglycerides, while liver triglyceride levels were normal. Expression of TGF- $\beta$  and AGT genes was higher in *Mas*-KO animals in comparison with controls.

**CONCLUSIONS**—These results show that *Mas* deficiency in FVB/N mice leads to dramatic changes in glucose and lipid metabolisms, inducing a metabolic syndrome-like state. *Diabetes* 57: 340–347, 2008

From the <sup>1</sup>Laboratory of Hypertension, Department of Physiology and Biophysics, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; the <sup>2</sup>Laboratory of Nutritional Biochemistry, Department of Biochemistry, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; the <sup>3</sup>Laboratory of Cellular Metabolism, Department of Physiology and Biophysics, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; and the <sup>4</sup>Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany.

Address correspondence and reprint requests to Robson A.S. Santos, Laboratório de Hipertensão, Departamento de Fisiologia e Biofísica, Universidade Federal de Minas Gerais, Av Antonio Carlos 6627-ICB, 31270-901, Belo Horizonte, Minas Gerais, Brazil. E-mail: robsonsant@gmail.com.

Received for publication 17 July 2007 and accepted in revised form 13 November 2007.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 19 November 2007. DOI: 10.2337/db07-0953.

2DOG, 2-deoxy-[<sup>3</sup>H]glucose; AGT, angiotensinogen; Ang, angiotensin; AT<sub>1</sub>R, type 1 Ang II receptor; RAS, renin-Ang system; TGF, transforming growth factor; TNF, tumor necrosis factor.

© 2008 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**M**etabolic syndrome is characterized by the variable coexistence of excess of body fat, hyperinsulinemia (insulin resistance and glucose intolerance), dyslipidemia (high triglycerides and total cholesterol plasma levels), and hypertension (1,2). The clinical alterations also include a proinflammatory and prothrombotic status as well as microalbuminuria. The metabolic syndrome pathogenesis is multifactorial and is related to central obesity, a sedentary lifestyle, an unbalanced diet, and genetic predisposition. Insulin resistance is described as the central feature of the metabolic syndrome (3); however, the molecular mechanisms underlying insulin resistance in metabolic syndrome are complex and not fully understood (4).

The renin-angiotensin (Ang) system (RAS) is an important link between metabolic syndrome and cardiovascular diseases. All of the main RAS components are present in adipose tissue (4). RAS consists primarily of an enzymatic cascade in which angiotensinogen (AGT) is converted to Ang I and subsequently to Ang II by the actions of renin and ACE, respectively (5). Ang-(1-7) is primarily formed from Ang II and Ang I by ACE 2 (6–8). Ang II is a potent proinflammatory, pro-oxidant, and prothrombotic agent that interferes in several steps of intracellular insulin signaling (9). Increased levels of Ang II have been observed in both obese and diabetic patients (10). RAS components, especially AGT, found in adipose tissue, are closely related to the Ang II effects on insulin resistance (10,11). Furthermore, AGT secretion, as well as Ang II formation in adipocytes, is increased in metabolic syndrome patients, promoting adipocyte growth, which could explain the positive correlation between high blood pressure and increased adipose tissue mass in these patients (9). Additionally, the AGT gene promoter is regulated by a glucose-responsive element, and, therefore, hyperglycemia could lead to increased tissue Ang II (10).

It has been demonstrated that Ang-(1-7) opposes many of the Ang II actions (6). Ang-(1-7), acting through the G protein-coupled receptor *Mas*, releases nitric oxide through a phosphoinositide-3 kinase/Akt-dependent pathway, causing vasodilation, inhibition of cell growth, and counter-regulation of type 1 Ang II receptor (AT<sub>1</sub>R)-mediated Ang II vasoconstrictor and proliferative effects (12,13). However, the role of Ang-(1-7)-*Mas* axis in the glycemic and lipid profile is not known. Previous studies using *Mas*-deficient mice were limited by the heterogeneous genetic background of the animals. We therefore backcrossed *Mas*-deficient mice for seven generations onto the FVB/N background, a preferable background for transgenic analyses (14). We then studied the effect of *Mas* deficiency on the glycemic and lipid profile.

TABLE 1  
Primers used to perform real-time PCR

Gene	Sequence (5' to 3') forward	Sequence (5' to 3') reverse
TNF- $\alpha$	CAT CTT CTC AAA ATT CGA GTG ACA A	TGG GAG TAG ACA AGG TAC AAC CC
TGF- $\beta$	TGA CGT CAC TGG AGT TGT ACG G	GGT TCA TGT CAT GGA TGG TGC
AGT	GAT GTG ACC CTG AGC AGC CC	TGA GTC CTG CTC GTA GAT GG
AT <sub>1</sub> R	ATG GCT GGC ATT TTG TCT GG	GTT GAG TTG GTC TCA GAC AC
<i>Mas</i>	ACT GCC GGG CGG TCA TCA TC	GGT GGA GAA AAG CAA GGA GA
HPRT	GTT GGA TAC AGG CCA GAC TTT GTT	GAT TCA ACT TGC GCT CAT CTT AGG C

## RESEARCH DESIGN AND METHODS

**Animals.** To obtain *Mas*-knockout (*Mas*-KO) animals on a pure genetic background, *Mas*<sup>+/-</sup> mice (mixed genetic background, 129  $\times$  C57BL/6) (15) were bred to the FVB/N mouse line (Charles River, Sulzfeld, Germany) for seven generations at the Max Delbrück Center for Molecular Medicine. The selection for the *Mas*-KO allele was done by PCR with primers MAS12: 5'-GCC GTT GCC CTC CTG GCG CCT GGG-3' and NeoPVU: 5'-GGC AGC GCG GCT ATC GTG G-3'. Primers MAS10: 5'-GTA CAG CTT CGA AGA ATG GGA GGC CC-3' and MAS14: 5'-CCT AAC TGA GCC ACC CTC ACC-3' were used for the detection of wild-type alleles. Thereafter, F7 heterozygous males were bred with F7 heterozygous females to generate the line FVB/N *Mas*-KO.

Mice were maintained at the transgenic animal facilities of the Laboratory of Hypertension (Federal University of Minas Gerais, Belo Horizonte, Brazil) and were treated according to the international guidelines for animal care. Nine- to 10-week-old male FVB/N wild-type and FVB/N *Mas*-KO mice were used for the experiments. The experimental protocol was approved by the ethics committee in animal experimentation of the Federal University of Minas Gerais (protocol no. 006/05). The animals were maintained under

controlled light and temperature conditions and had free access to water and standard diet.

**Measurements of body weight, food intake, and tissue collection.** Nine-week-old male *Mas*-KO and wild-type mice were individually housed and weighed on the first experimental day. Food intake was measured daily for 7 days in order to obtain food efficiency (food intake divided by body weight). Mice were killed with ketamine (130 mg/kg) and xylazine (0.3 mg/kg) after anesthesia, and samples of blood and epididymal retroperitoneal white adipose tissue, muscle, and liver were collected, weighted, and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for posterior analysis.

**Determination of plasma parameters.** Serum was obtained after centrifugation (3,500 rpm for 5 min at  $4^{\circ}\text{C}$ ). Total serum cholesterol and triglycerides were assayed using enzymatic kits (Dole, Goiás, Brazil). Enzyme-linked immunosorbent assay kits were used to measure serum adiponectin (Adipo-Gen, Seoul, Korea) and leptin (Linco, St. Louis, MO) levels. Serum insulin was measured by chemiluminescence using an IRI Bayer kit (Kyowa, Tokyo, Japan) and ADVIA-Centaur equipment.

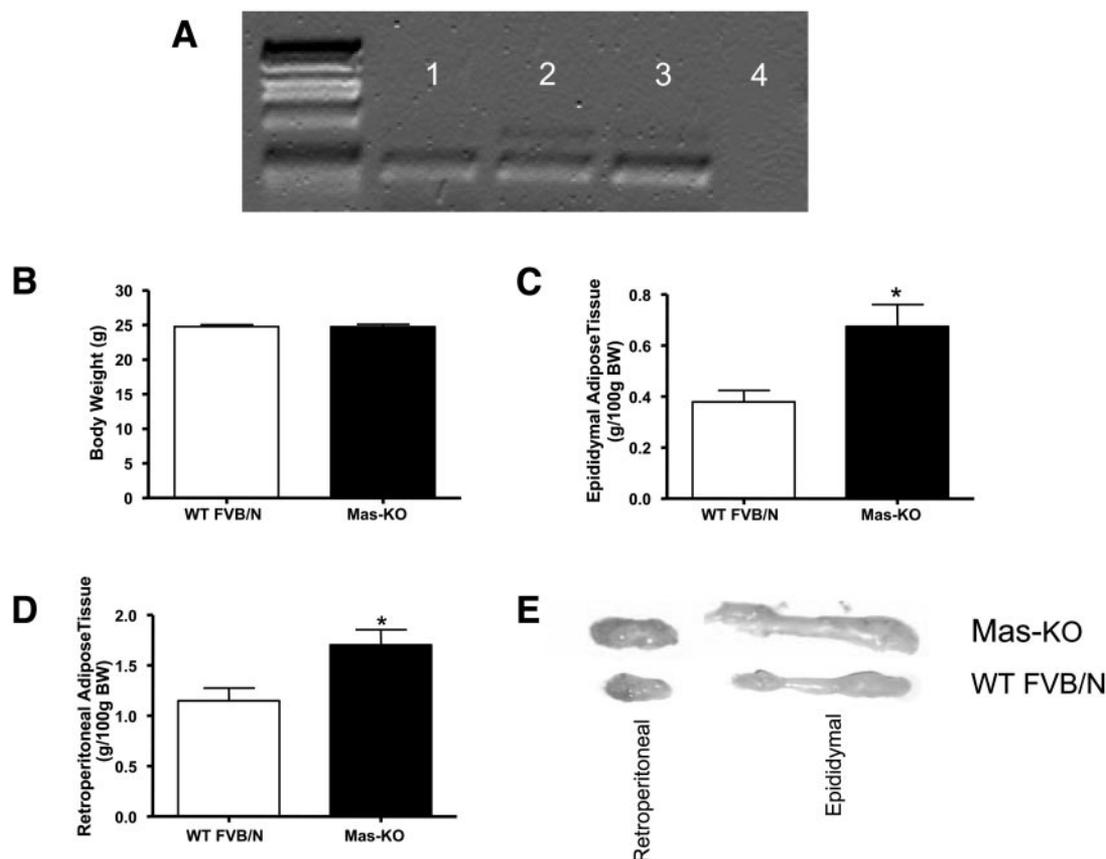


FIG. 1. *Mas* expression, body weight, and adipose tissue weight. **A:** Agarose gel image of *Mas* mRNA quantification by real-time PCR: 1, mouse epididymal adipose tissue; 2, rat epididymal adipose tissue; 3, mouse testis (positive control); 4, *Mas*-KO mouse epididymal adipose tissue. **B:** Body weight (g) of 10-week-old *Mas*-KO ( $n = 19$ ) and wild-type (WT) FVB/N ( $n = 15$ ) male mice. There was no significant difference between the groups. **C:** Epididymal adipose tissue weight from *Mas*-KO ( $n = 5$ ) and wild-type (WT) FVB/N ( $n = 5$ ) mice. In *Mas*-KO mice, the tissue weight was significantly increased ( $P < 0.05$ ) in comparison to WT FVB/N mice. **D:** Retroperitoneal adipose tissue weight from *Mas*-KO ( $n = 5$ ) and wild-type (WT) FVB/N ( $n = 5$ ) mice. In *Mas*-KO mice, the tissue weight was significantly increased ( $P < 0.05$ ) in comparison to WT FVB/N mice. **E:** Retroperitoneal and epididymal adipose tissue of *Mas*-KO and wild-type (WT) FVB/N mice.

TABLE 2  
Food intake and serum parameters of fasted male wild-type FVB/N and *Mas*-KO mice

Measurements	Wild-type FVB/N mice	<i>Mas</i> -KO mice
Food intake (g/body wt)	0.176 ± 0.007	0.179 ± 0.011
Fasted glycemia (mg/dl)	56.40 ± 4.99	86.60 ± 6.45*
Leptin (ng/ml)	0.74 ± 0.17	1.28 ± 0.25*
Adiponectin (μg/ml)	5.85 ± 0.54	5.80 ± 0.44
Insulin (μU/ml)	0.097 ± 0.002	0.267 ± 0.067*

Data are means ± SE ( $n = 6$  animals in each group). \* $P < 0.05$ .

**Determination of tissue triglycerides.** Hepatic and muscle total lipids were extracted by the method of Folch et al. (16), gravimetrically quantified, and assayed for triacylglycerols using enzymatic kits (Doses).

**Histology.** Epididymal fat tissue was excised and fixed in Bouin solution and embedded in paraffin. Sections of the tissue were stained with hematoxylin and eosin. Images of nine 8-μm sections from each animal were captured, and adipocyte diameters from at least 540 cells were measured from each animal using NIH Image software using a ×20 objective.

**Glucose tolerance and insulin sensitivity tests.** For the glucose tolerance test, D-glucose (2 mg/g body wt) was intraperitoneally injected into overnight-fasted mice. Glucose levels from tail blood samples were monitored at 0, 15, 30, 60, and 120 min after injection using an Accu-Check glucometer (Roche Diagnostics, Indianapolis, IN). An insulin sensitivity test was performed on overnight-fed mice, after intraperitoneal injection of insulin (0.75 units/kg body wt; Sigma, St. Louis, MO). Tail blood samples were taken at time 0, 15, 30, and 60 min after injection for measurement of blood glucose levels.

**Glucose uptake.** Adipocytes from wild-type and *Mas*-KO mice were isolated from epididymal fat pads according to Rodbell (17). Digestion was carried out at 37°C, with constant shaking for 45 min. Cells were filtered through nylon mesh and washed three times with buffer containing (in mmol/l): 137 NaCl, 5 KCl, 4.2 NaHCO<sub>3</sub>, 1.3 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>, 0.5 MgSO<sub>4</sub>, 0.5 KH<sub>2</sub>PO<sub>4</sub>, 20 HEPES (pH 7.4), plus 1% BSA.

**Glucose transport assay.** After isolation, adipocytes were incubated for 45 min at 37°C in the presence or absence of insulin (25 ng/ml). The uptake of 2-deoxy-[<sup>3</sup>H]glucose (2DOG) was used to determine the rate of glucose transport, as previously described (18). Briefly, glucose uptake was initiated by the addition of 2DOG (0.2 μCi/tube) for 3 min. Thereafter, cells were separated by centrifugation through silicone oil and cell-associated radioactivity was determined by scintillation counting. Nonspecific association of 2DOG was determined by performing parallel incubations in the presence of 15 mmol/l phloretin, and this value was subtracted from glucose transport activity in each condition.

**Western blot analysis.** Samples of epididymal adipose tissue (~300 mg) were extracted and 30 μg of protein were resolved on SDS-PAGE (10%) and then transferred onto nitrocellulose membranes. The glucose transporter, GLUT4, was probed with a polyclonal rabbit anti-Glut4 antibody (1:5,000), and a goat anti-rabbit IgG antibody conjugated with peroxidase (1:5,000) was used as a secondary antibody. The blots were visualized using a chemiluminescence Western blotting detection reagent (ECL; Amersham Pharmacia Biotech) and revealed on a photograph film.

**Reverse transcription and real-time PCR.** Total RNA from adipose tissue was prepared using TRIzol reagent (Invitrogen, San Diego, CA), treated with DNase, and reverse transcribed with MML-V (Moloney murine leukemia virus) (Invitrogen). The endogenous HPRT-hypoxanthine guanine phosphoribosyltransferase (internal control), tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, AGT, AT<sub>1</sub>, and *Mas* cDNA were amplified using specific primers (Table 1) and SYBR green reagent (Applied Biosystems) in an ABI Prism 7000 platform (Applied Biosystems).

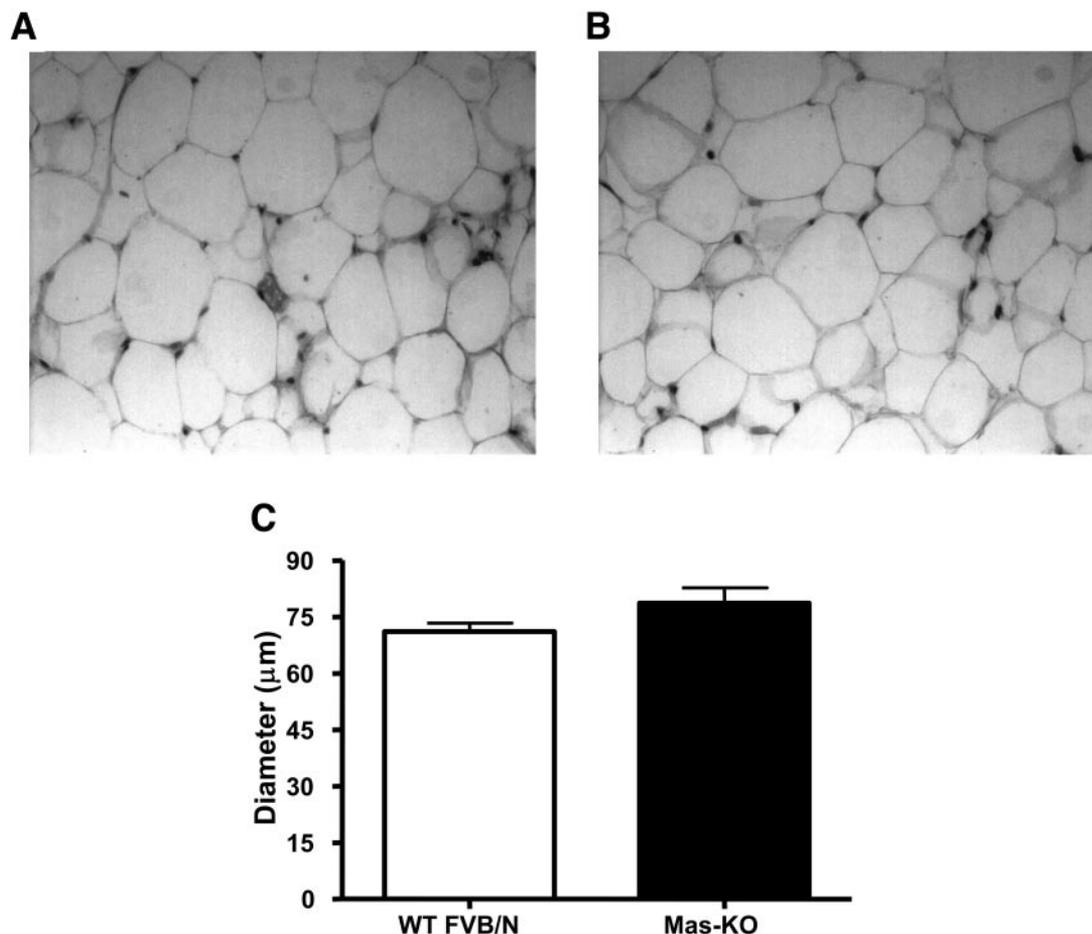


FIG. 2. Hematoxylin-eosin-stained slices of epididymal adipose tissue from *Mas*-KO ( $n = 3$ ) and wild-type (WT) FVB/N ( $n = 3$ ). The diameter of 20 adipocytes of each field was measured (three fields from each section, three sections in each slide, and three slides from each animal). A: Illustrative picture of *Mas*-KO adipocytes. B: Illustrative picture of wild-type FVB/N adipocytes. C: Adipocyte diameter. There was no significant difference between the groups.

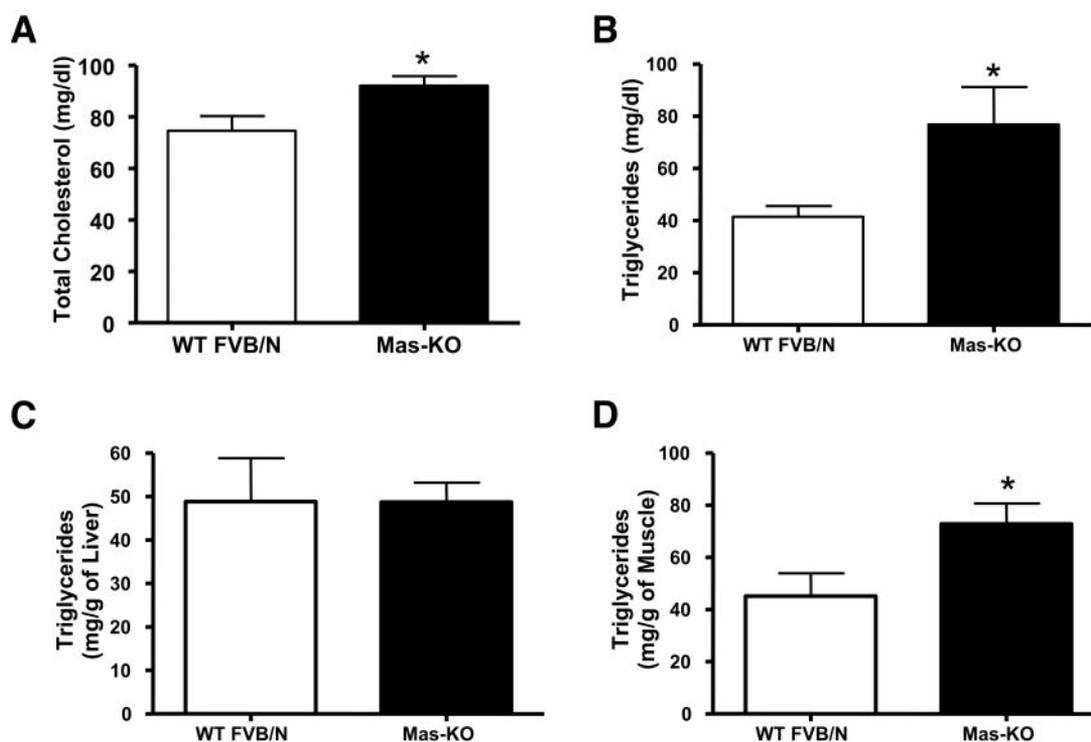


FIG. 3. Lipid profile of *Mas*-KO ( $n = 6$ ) and wild-type (WT) FVB/N ( $n = 6$ ) mice. *A*: Serum levels of total cholesterol. *B*: Serum levels of triglycerides. *C*: Hepatic triglycerides levels. *D*: Muscular triglycerides levels. Data are presented as means  $\pm$  SE. Statistically significant differences between the groups are indicated as \* $P < 0.05$ .

**Statistical analysis.** Data are expressed as means  $\pm$  SE. The statistical significance of differences in mean values between transgenic and wild-type mice was assessed by unpaired Student's *t* test, two-way ANOVA (glucose tolerance and insulin sensibility tests), and one-way ANOVA (glucose uptake test).

## RESULTS

First we analyzed *Mas* expression in adipose tissue of FVB/N mice and Sprague Dawley rats. RT-PCR revealed that the *Mas* gene is expressed in this tissue in both species (Fig. 1A), whereas the corresponding transcript was absent in *Mas*-KO adipose tissue, confirming the genetic deletion of *Mas* in this model (Fig. 1A).

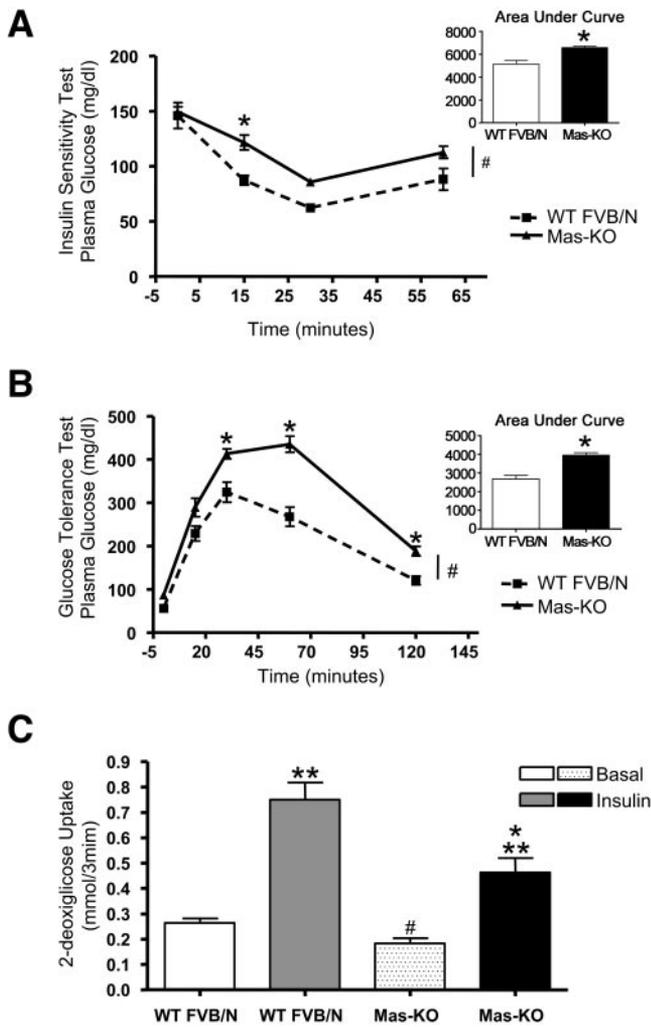
To examine the functional consequences of *Mas* deletion, we studied the relation between body and fat weight in *Mas*-KO mice. As observed in Fig. 1B, wild-type and *Mas*-KO mice did not display differences in body weight ( $24.8 \pm 0.2$  g in *Mas*-KO vs.  $24.7 \pm 0.4$  g in wild-type mice) or food intake (Table 2). However, analysis of the epididymal ( $1.70 \pm 0.15\%$  body wt in *Mas*-KO vs.  $1.15 \pm 0.13\%$  body wt in wild-type mice) (Fig. 1E) and retroperitoneal ( $0.68 \pm 0.09\%$  body wt in *Mas*-KO vs.  $0.38 \pm 0.05\%$  body wt in wild-type mice) (Fig. 1E) adipose tissue showed that *Mas*-KO mice have a substantial increase in fat mass in relation to wild-type FVB/N mice (Fig. 1C and D). Histological analyses showed a similar diameter of the adipocytes in *Mas*-KO and wild-type FVB/N mice ( $74.1 \pm 4.0$   $\mu$ m in *Mas*-KO vs.  $72.2 \pm 4.1$   $\mu$ m in wild-type mice), indicating that the increase in adipose tissue mass was not due to hypertrophy in adipocytes (Fig. 2).

*Mas*-KO mice presented a significant increase in total cholesterol ( $92.15 \pm 3.66$  mg/dl in *Mas*-KO vs.  $74.65 \pm 5.68$  mg/dl in wild-type mice) and triglyceride ( $76.77 \pm 14.45$  mg/dl in *Mas*-KO vs.  $41.45 \pm 4.08$  mg/dl in wild-type mice) serum levels, indicating a dyslipidemic state (Fig. 3A and B). Leptin levels were also increased in the serum of

*Mas*-KO mice (Table 2); however, the correlation of leptin levels with fat mass (by Pearson test) showed a similar leptin adipocyte secretion in both groups of mice. On the other hand, adiponectin levels were not altered in the serum of *Mas*-KO mice (Table 2), while the correlation of adiponectin levels with fat mass (by Pearson test) showed a decrease of adipose tissue adiponectin secretion in *Mas*-KO mice.

A low glucose tolerance and decreased insulin sensitivity were observed in *Mas*-KO when compared with wild-type FVB/N mice (Fig. 4A and B). This state was accompanied by an important increase in muscle triglyceride levels ( $45.14 \pm 8.71$  mg/g of muscle in *Mas*-KO vs.  $72.77 \pm 7.84$  mg/g of muscle in wild-type mice) in *Mas*-KO mice (Fig. 3D) without alteration in liver triglyceride levels (Fig. 3C). Additionally, fasting plasma glucose levels and insulin levels were elevated in *Mas*-KO mice (Table 2). Moreover, as shown in Fig. 5, the GLUT4 protein levels in adipose tissue were decreased in *Mas*-KO mice ( $0.336 \pm 0.054$  AU in *Mas*-KO vs.  $0.616 \pm 0.105$  AU in wild-type mice).

The 2DOG is transported, phosphorylated but not oxidized, by the adipocyte. Consequently, it accumulates as 2DOG-phosphate in the cell. Accumulated radioactivity in adipocytes was used to evaluate the capacity of glucose uptake into this cell type (Fig. 4C). Statistical analysis of glucose uptake in wild-type FVB/N and *Mas*-KO mice in basal state, utilizing a one-way ANOVA test (Fig. 4C), showed that there was no statistically significant difference. However, when tested with an independent two-sample *t* test, there was a significant difference between the groups in the basal state ( $P < 0.02$ ), with decreased glucose uptake in *Mas*-KO mice group. All other permutations of comparisons between wild-type and *Mas*-KO mice were statistically significant using ANOVA analyses ( $P <$



**FIG. 4.** Glycemic profile. *A*: Insulin sensitivity test after intraperitoneal injection of insulin (0.75 units/kg body wt) was performed on normal-diet mice. Blood samples were collected from the tail at indicated time points and analyzed for glucose concentration. Results are expressed as means  $\pm$  SE from six animals in each group. *B*: Glucose tolerance test. Overnight-fasted mice were given an intraperitoneal injection of glucose (2 mg/g body wt). Data are presented as mean of plasma glucose levels (mg/dl)  $\pm$  SE from six mice in each group. Statistically significant differences between the groups are indicated as \* $P$  < 0.05 and # $P$  < 0.01. *C*: Effects of insulin on glucose uptake. Adipocytes taken from wild-type (WT) FVB/N or *Mas*-KO mice were incubated in a media, in the presence or absence of 25 ng/ml insulin. Rates of glucose transport were determined by measuring the 2DOG uptake during a 3-min interval. The data represent means  $\pm$  SE ( $n$  = 4–5). \* $P$  < 0.05 in *Mas*-KO insulin vs. wild-type insulin; \*\* $P$  < 0.05 in insulin vs. basal (ANOVA); # $P$  < 0.02 in *Mas*-KO basal versus wild-type basal ( $t$  test).

0.05). In addition, the difference of mean glucose uptake between the basal and insulin-stimulated state was 42% lower in *Mas*-KO ( $0.28 \pm 0.06$  nmol/3 min) than in wild-type FVB/N ( $0.49 \pm 0.06$  nmol/3 min) mice. As shown in Fig. 6, real-time PCR analysis revealed a marked increase in AGT and TGF- $\beta$  expression in *Mas*-KO epididymal adipose tissue, while TNF- $\alpha$  and AT<sub>1</sub>R were not altered.

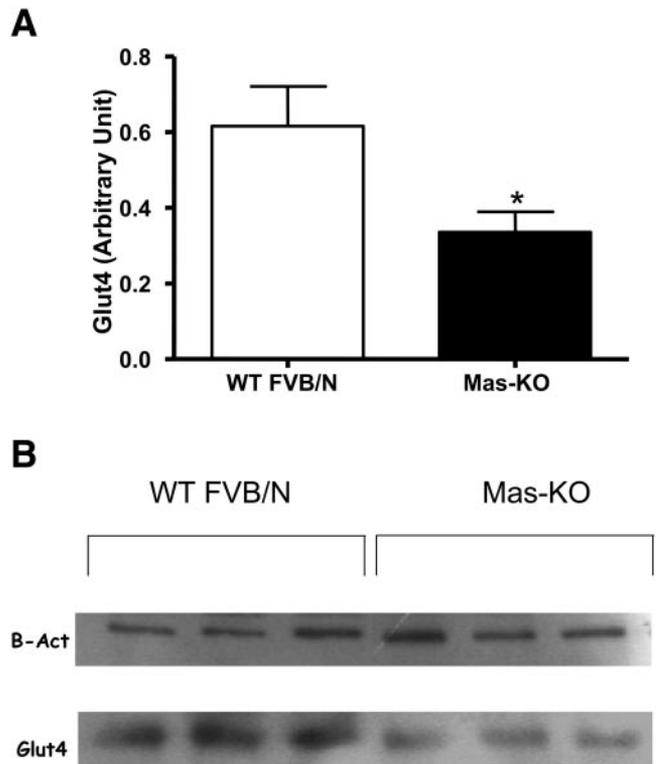
**DISCUSSION**

This study has documented for the first time that deletion of the receptor *Mas* in pure FVB/N background produced a murine model of the metabolic syndrome-like state. These animals showed an increased adipose tissue mass associated with high serum cholesterol and triglycerides

levels, enhanced fasting glycemia, hyperinsulinemia, and glucose intolerance; decreased insulin sensitivity and glucose uptake by adipocytes; elevated serum leptin levels; and reduced triglycerides in skeletal muscle. Additionally, increased AGT and TGF- $\beta$  mRNA expression, as well as decreased GLUT4 receptor expression, were observed in epididymal adipose tissue.

Despite an unchanged food intake and body weight, the knockout animals exhibited a considerable increase in abdominal fat mass (epididymal and retroperitoneal adipose tissue). Alterations were not observed in TNF- $\alpha$  expression or in the apparent number of macrophages in *Mas*-KO fat tissue histology, suggesting the absence of inflammation in adipose tissue. Signs of adipocyte hypertrophy were not observed in histological studies, suggesting that the increase in fat mass occurred by increased proliferation of adipocytes (hyperplasia). This observation was corroborated by the increase in TGF- $\beta$  expression, an important cytokine involved in tissue proliferation (19). On the other hand, the absence of alterations in food intake indicates that the changes in fat mass were not a consequence of metabolic changes induced by increased appetite. Although we have shown that *Mas* is expressed in fat, the changes in this tissue are not necessarily the result of a direct effect of *Mas* deficiency in adipocytes. It may also be secondary to hormonal changes induced by the lack of *Mas* in endocrine glands, including testis, where *Mas* is highly expressed.

Adipose tissue is a complex and important endocrine



**FIG. 5.** GLUT4 Western blotting analyses of epididymal adipose tissue in *Mas*-KO ( $n$  = 4) and wild-type (WT) FVB/N ( $n$  = 3) mice. *A*: Western blot for GLUT4 protein *Mas*-KO and wild-type FVB/N mice. *B*: Illustrative picture of Western blotting gel from *Mas*-KO and wild-type FVB/N mice. After incubation with primary and secondary antibody for GLUT4 detection, the membrane was stripped and incubated with primary and secondary antibody used to detect  $\beta$ -actin. Data are presented as means  $\pm$  SE. Statistically significant differences between the groups are indicated as \* $P$  < 0.05.

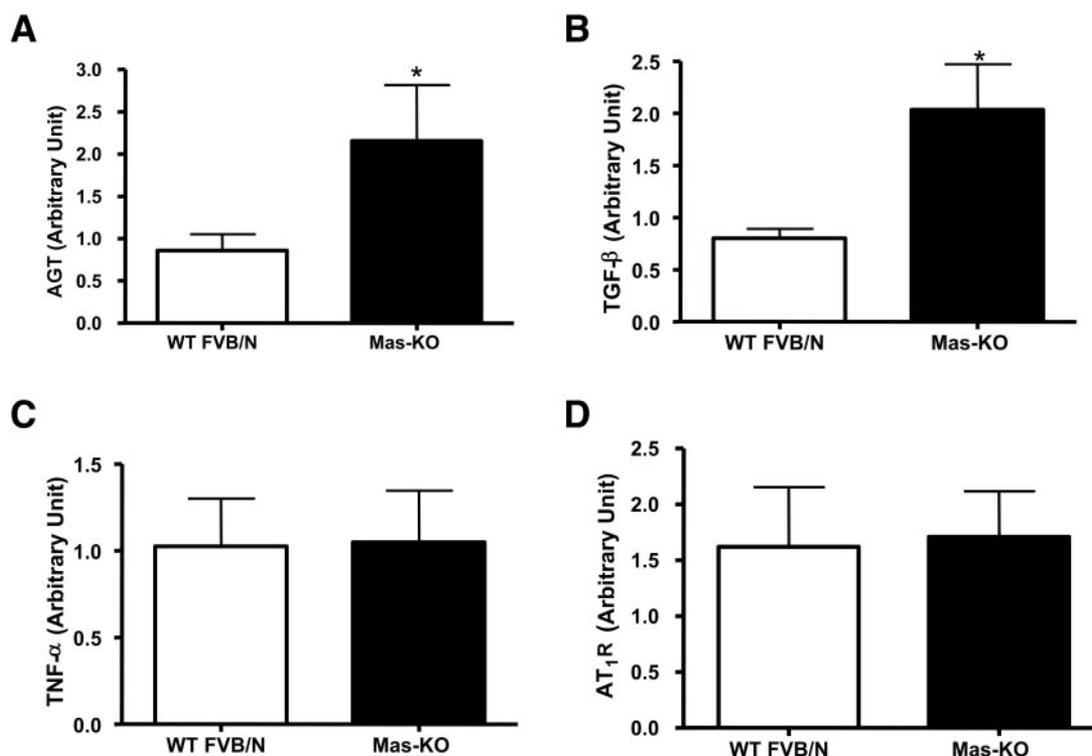


FIG. 6. Adipose tissue mRNA expression performed by real-time PCR in *Mas*-KO ( $n = 6$ ) and wild-type (WT) FVB/N ( $n = 6$ ) mice. A: Expression of AGT. B: Expression of TGF- $\beta$ . C: Expression of TNF- $\alpha$ . D: Expression of AT<sub>1</sub>R. \* $P < 0.05$  in comparison to the wild-type FVB/N group.

organ and plays an essential role in lipid and glucose metabolism (15). Many RAS components are found in the adipose tissue (20). The increased expression of AGT is an important characteristic of preadipocyte differentiation, especially because Ang II has a fundamental role in this process. It was previously demonstrated that AGT expression is elevated in obesity (20,21). Zucker rats show an increased expression and secretion of AGT in adipocytes (22). Our results are in agreement with these data showing an elevated expression of AGT in adipose tissue of *Mas*-KO mice.

It has been shown that glucose and insulin have opposite effects on AGT expression in the liver and adipose tissue of Sprague Dawley rats. While glucose augments AGT level, insulin promotes a decrease in its expression. These effects were found in the insulin-resistant state of obese rats (23). Our results indicate that *Mas*-KO mice exhibit an insulin-resistant state as evidenced by the decrease in insulin sensitivity and glucose tolerance, hyperinsulinemia, and, more importantly, by a decrease in insulin-induced glucose uptake in adipose tissue. In keeping with these findings, a decreased amount of GLUT4 protein was observed in the adipose tissue of *Mas*-KO mice. It is well established in the literature that high triglyceride muscle levels are closely related to metabolic syndrome, which is a known mechanism of muscular insulin resistance (24). Our results are in keeping with these findings, showing an important increase in muscular triglycerides. Therefore, the deficiency of insulin action followed by high glucose plasma levels could be involved in the augmentation of AGT expression in *Mas*-KO mice.

It has also been shown that AGT expression is upregulated by fatty acids (25). Our data demonstrated that triglyceride plasma levels are increased in *Mas*-KO mice, suggesting that this could be an additional underlying

mechanism involved in the increased AGT expression in *Mas*-KO adipose tissue. The increase in local AGT may lead to an increase in Ang II tissue levels and overactivity of the Ang II/AT<sub>1</sub> axis, despite normal AT<sub>1</sub>R expression. Future studies with measurement of Ang II levels, which is lacking in this study, will be helpful to confirm this possibility. The high AGT expression in adipocytes associated with increased fat mass could explain, at least in part, the higher blood pressure levels in FVB/N *Mas*-KO mice ( $118 \pm 1$  mmHg vs.  $108 \pm 2$  mmHg in wild-type mice) ( $P < 0.001$ ) (26). Moreover, the loss of counter-regulatory actions (vasodilatation) of the Ang-(1-7)/*Mas* axis, which are abolished in this model, could also contribute to the moderate hypertensive state of these animals (26).

Obesity can induce insulin resistance and, consequently, lead to increased plasma glucose and insulin production and release, which are characteristics of type 2 diabetes (27). Moreover, it is clear now that circulating leptin levels correlate with body fat mass (9,28). Accordingly, in *Mas*-KO mice the increase in adipose mass is closely related to an increase in leptin levels. The reduction in adiponectin secretion in *Mas*-KO mice can be related to the lower insulin sensibility observed in these animals. This adipocytokine is an important agent in tissue sensitization facilitating the action of insulin (29).

The role of Ang II in obesity is still controversial since it can stimulate synthesis or lysis of fat tissue, depending on the experimental conditions (30–33). It has been shown, in rats and humans that ACE inhibitors can decrease body weight (34–36). Furthermore, many studies have shown that Ang-(1-7) can counter-regulate Ang II actions and participate in the ACE inhibitor effects (37,38). Our data showing an increased fat mass in *Mas*-KO mice suggest that the Ang-(1-7)/*Mas* axis is crucial in the control of fat accumulation and lipolysis.

Measurements of Ang II levels and blockade of AT<sub>1</sub>R in this model, in future studies, will be helpful to ascertain whether the metabolic changes observed are, at least partially, due to increased Ang II.

Ang II decreases phosphorylation of insulin-stimulated tyrosine, increasing insulin receptor substrate-1 receptor serine phosphorylation (39). As a consequence, insulin intracellular signaling, as well as its activity, are decreased (40). These alterations are also correlated with an increase in AGT and Ang II levels during insulin resistance. Taken together, these studies suggest that Ang II decreases insulin metabolic effects. Considering the opposite role of Ang-(1-7) in the RAS, the lack of Ang-(1-7)/Mas axis activity, apart from its direct effect, could be also involved in both insulin resistance and glucose intolerance mechanisms due to an exacerbation of Ang II-mediated effects in Mas-KO mice. Concerning the direct metabolic effects of Ang-(1-7)/Mas axis, the phosphoinositide-3 kinase/Akt pathway, which is activated by Ang-(1-7), at least in endothelial cells (13) and in the heart (41), might be implicated.

One question that arises from our study is whether the phenotypic changes observed in FVB/N Mas-deficient mice are also observed in other genetic backgrounds. Ongoing experiments in our laboratory suggest that the sensitivity to genetic deletion of Mas is higher in FVB/N mice than in C57BL/6 mice. This is particularly true for blood pressure, which is increased in FVB/N and normal in C57BL/6 Mas-KO mice (26). In ongoing studies concerning metabolism, we have not observed changes in blood glucose, plasma triglycerides, and total plasma cholesterol in young C57BL/6 mice, while a significant increase in triglycerides in mice was observed in older animals (R.A.S.S., S.H.S.S., unpublished observations). These differences, which may be related to the fact that C57BL/6 presents only one renin gene compared with two genes of other mice strains, including FVB/N, are in keeping with other observations (42). It has been reported that genetic background strongly influences the severity of diabetes and insulin resistance in *ob/ob* mice (43). C57BL/6 mice had much milder hyperglycemia and less whole-body and muscle insulin resistance than FVB/N mice, despite having a similar degree of obesity and hyperinsulinemia. Likewise, in the lipotrophic model A-Zip/F-1 mice, the C57BL/6 background produced milder hyperglycemia and dislipidemia than the FVB/N background, while liver insulin resistance and liver fatty acid deposition showed the opposite trend (44). Whether a repartitioning of triglycerides from the muscle and other tissues to the liver is contributing to the apparent differences between C57BL/6 and FVB/N Mas-KO mice remains to be established.

In summary, the results obtained in this study show that genetic deletion of Mas in FVB/N mice produced a devastating effect on lipid and glucose metabolism, leading ultimately to a metabolic syndrome-like state (45). The primary mechanisms involved in this effect appear to include an increase in leptin, AGT, and TGF- $\beta$  expression. The development of a murine model for a metabolic syndrome-like state, linked to the deficiency of the vasodilator/antiproliferative arm of the RAS, will contribute to the elucidation of interactions between the Ang-(1-7)/Mas and the Ang II/AT<sub>1</sub> axis in hypertension and lipid and glucose homeostasis dysregulation.

## ACKNOWLEDGMENTS

S.H.S.S. was a recipient of a CNPq master fellowship at the Post-Graduation Program in Biological Science: Physiology and Pharmacology, Biological Sciences Institute, Federal University of Minas Gerais. We also thank CNPq, PRONEX, CAPES, and FAPEMIG for financial support.

The authors thank Ilma M. da Silva for the valuable technical assistance.

## REFERENCES

1. Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C, the National Heart, Lung, and Blood Institute: American Heart Association definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on scientific issues related to definition. *Circulation* 109:433–438, 2004
2. Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults: Executive summary of the third report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 285:2486–2496, 2001
3. Park YW, Zhu S, Palaniappan L, Heshka S, Carnethon MR, Heymsfield SB: The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988–1994. *Arch Intern Med* 163:427–436, 2003
4. Strazzullo P, Galletti F: Impact of the renin-angiotensin system on lipid and carbohydrate metabolism. *Curr Opin Nephrol Hypertens* 13:325–332, 2004
5. Engeli S, Schling P, Gorzelniak K, Boschmann M, Janke J, Ailhaud G, Teboul M, Massiera F, Sharma AM: The adipose-tissue renin-angiotensin-aldosterone system: role in the metabolic syndrome? *Inter Jour Bioch Cell Biol* 35:807–825, 2003
6. Santos RAS, Ferreira AJ, Pinheiro SV, Sampaio WO, Touyz R, Campagnole-Santos MJ: Angiotensin-(1–7) and its receptor as a targets for new cardiovascular drugs. *Expert Opin Investig Drugs* 14:1019–1031, 2005
7. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Wolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S: A novel angiotensin-converting enzyme related carboxypeptidase (ACE 2) converts angiotensin I to angiotensin 1–9. *Circ Res* 87:1–9, 2000
8. Crackower MA, Sarao R, Oudit GY, Yagil C, Koziarzdzki I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, Scholze J, Ferrario CM, Manoukian AS, Chappell MC, Backx PH, Yagil Y, Penninger JM: Angiotensin converting enzyme 2 is an essential regulator of heart function. *Nature* 417:822–828, 2002
9. Prasad A, Quyyumi A: Renin-angiotensin system and angiotensin receptor blockers in the metabolic syndrome. *Circulation* 110:1507–1512, 2004
10. Giacchetti G, Sechi LA, Rilli S, Carey RM: The renin-angiotensin-aldosterone system, glucose metabolism and diabetes. *Tre Endocri Metab* 16:120–126, 2005
11. Boustany CM, Bharadwaj K, Daugherty A, Brown DR, Randall DC, Cassis LA: Activation of the systemic and adipose renin-angiotensin system in rats with diet-induced obesity and hypertension. *Am J Phys Reg* 287:R943–R949, 2004
12. Santos RAS, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T: Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A* 100:8258–8263, 2003
13. Sampaio WO, Santos RAS, Faria-Silva R, Mata Machado LT, Schiffrin EL, Touyz RM: Angiotensin-(1–7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* 49:185–192, 2007
14. Taketo M, Schroeder AC, Mobraaten LE, Gunning KB, Hanten G, Fox RR, Roderick TH, Stewart CL, Lilly F, Hansen CT: FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc Natl Acad Sci U S A* 88:2065–2069, 1991
15. Walther T, Balschun D, Voigt JP, Fink H, Zuschratter W, Birchmeier C, Ganten D, Bader M: Sustained long-term potentiation and anxiety in mice lacking the Mas protooncogene. *J Biol Chem* 273:11867–11873, 1998
16. Folch J, Lees GH, Sloane-Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497–509, 1957
17. Rodbell M: Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* 239:375–380, 1964
18. Green A: Adenosine receptor down-regulation and insulin resistance

- following prolonged incubation of adipocytes with an A1 adenosine receptor agonist. *J Biol Chem* 262:15702–15707, 1987
19. Uhal BD, Kim JK, Li X, Molina-Molina M: Angiotensin-TGF-beta1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages *Curr Pharm* 13:1247–1256, 2007
  20. Giacchetti G, Faloia E, Mariniello B, Sardu C, Gatti C, Camilloni MA, Guerrieri M, Mantero F: Overexpression of the renin-angiotensin system in human visceral adipose tissue in normal and overweight subjects. *Am J Hypertens* 15:381–388, 2002
  21. Rankinen T, Gagnon J, Perusse L, Rice T, Leon AS, Skinner JS, Wilmore JH, Rao DC, Bouchard C: Body fat, resting and exercise blood pressure and the angiotensinogen M235T polymorphism: the Heritage Family Study. *Obes Res* 7:423–430, 1999
  22. Hainault I, Nebout G, Turban S, Ardouin B, Ferré P, Quignard-Boulangé A: Adipose tissue-specific increase in angiotensinogen expression and secretion in the obese (fa/fa) Zucker rat. *Am J Physiol Endocrinol Metab* 282:E59–E66, 2002
  23. Gabriely I, Yang XM, Cases JA, Ma XH, Rossetti L, Barzilay N: Hyperglycemia modulates angiotensinogen gene expression. *Am J Physiol* 281: R795–R802, 2001
  24. Pan DA, Lillioja S, Kriketos AD, Milner MR, Baur LA, Bogardus C, Jenkins AB, Storlien LH: Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 46:983–988, 1997
  25. Brun RP, Tontonoz P, Forman BM, Ellis R, Chen J, Evans RM, Spiegelman BM: Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev* 10:974–984, 1996
  26. Moura MM, Santos RAS, Bader M, Alenina N, Haibara AS: Angiotensin-(1–7) receptor Mas modulates cardiovascular reflex responses (Abstract). *Hypertension* 48:LB26, 2006
  27. Rader DJ: Effect of insulin resistance, dyslipidemia, and intra-abdominal adiposity on the development of cardiovascular disease and diabetes mellitus. *Am J Med* 120 (Suppl. 1):S12–S18, 2007
  28. Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS: Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med* 1:1311–1314, 1995
  29. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA: Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 86:1930–1935, 2001
  30. Katsuya T, Horiuchi M, Chen Y, Koike G, Pratt RE, Dzau VJ, Reaven GM: Relations between deletion polymorphism of the angiotensin-converting enzyme gene and insulin resistance, glucose intolerance, hyperinsulinemia, and dyslipidemia. *Arterioscler Thromb Vasc Biol* 15:779–782, 1995
  31. Ueda S, Elliott HL, Morton JJ, Connell JM: Enhanced pressor response to angiotensin I in normotensive men with the deletion genotype (DD) for angiotensin-converting enzyme. *Hypertension* 25:1266–1269, 1995
  32. Nagi DK, Foy CA, Mohamed-Ali V, Yudkin JS, Grant PJ, Knowler WC: Angiotensin-I-converting enzyme (ACE) gene polymorphism, plasma ACE levels, and their association with the metabolic syndrome and electrocardiographic coronary artery disease in Pima Indians. *Metabolism* 47:622–626, 1998
  33. Jones BH, Standridge MK, Moustaid N: Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology* 138:1512–1519, 1997
  34. (U.K.) Enalapril in Hypertension Study Group: Enalapril in essential hypertension: a comparative study with propranolol. *Br J Clin Pharmacol* 18:51–56, 1984
  35. McGrath BP, Matthews PG, Louis W, Howes L, Whitworth JA, Kincaid-Smith PS, Fraser I, Scheinkestel C, MacDonald G, Rallings M: Double-blind study of dilevalol and captopril, both in combination with hydrochlorothiazide, in patients with moderate to severe hypertension. *J Cardiovasc Pharmacol* 16:831–838, 1990
  36. Campbell DJ, Duncan AM, Kladis A, Harrap SB: Converting enzyme inhibition and its withdrawal in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 26:426–436, 1995
  37. Ferreira AJ, Santos RAS: Cardiovascular actions of angiotensin-(1–7). *Braz J Med Biol Res* 38:499–507, 2005
  38. Janke J, Engeli S, Gorzelnik K, Luft FC, Sharma AM: Mature adipocytes inhibit in vitro differentiation of human preadipocytes via angiotensin type 1 receptors. *Diabetes* 51:1699–1707, 2002
  39. Velloso LA, Folli F, Sun XJ, White MF, Saad MJ, Kahn CR: Cross-talk between the insulin and angiotensin signaling systems. *Proc Natl Acad Sci U S A* 93:12490–12495, 1996
  40. Folli F, Saad MJ, Velloso L, Hansen H, Carandente O, Feener EP, Kahn CR: Crosstalk between insulin and angiotensin II signaling systems. *Exp Clin Endocrinol Diabetes* 107:133–139, 1999
  41. Giani JF, Gironacci MM, Muñoz MC, Peña C, Turyn D, Dominici FP: Angiotensin-(1 7) stimulates the phosphorylation of JAK2, IRS-1 and Akt in rat heart in vivo: role of the AT1 and Mas receptors. *Am J Physiol Heart Circ Physiol* 293:H1154–H1163, 2007
  42. Hansen PB, Yang T, Huang Y, Mizel D, Briggs J, Schnermann J: Plasma renin in mice with one or two renin genes. *Acta Physiol Scand* 181:431–437, 2004
  43. Haluzik M, Colombo C, Gavriloova O, Chua S, Wolf N, Chen M, Stannard B, Dietz KR, Le Roith D, Reitman ML: Genetic background (C57BL/6J versus FVB/N) strongly influences the severity of diabetes and insulin resistance in ob/ob mice. *Endocrinology* 145:3258–3264, 2004
  44. Colombo C, Haluzik M, Cutson JJ, Dietz KR, Marcus-Samuels B, Vinson C, Gavriloova O, Reitman ML: Opposite effects of background genotype on muscle and liver insulin sensitivity of lipoatrophic mice: role of triglyceride clearance. *J Biol Chem* 278:3992–3999, 2003
  45. Rodríguez A, Catalán V, Gómez-Ambrosi J, Frühbeck G: Visceral and subcutaneous adiposity: are both potential therapeutic targets for tackling the metabolic syndrome? *Curr Pharm Des* 13:2169–2175, 2007