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ORIGINAL ARTICLE

Angiotensin II receptor activation in youth triggers persistent insulin resistance and hypertension—a legacy effect?

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Although the involvement of angiotensin II (Ang II) in insulin resistance and hypertension has been established, the temporal relationships between Ang II receptor activation and changes in insulin sensitivity and blood pressure are not clear. To better understand this issue, we infused rats with Ang II (200 ng kg^{-1} min^{-1}) or vehicle for 4 weeks and assessed the residual effects after the discontinuation of the infusion on blood pressure, insulin sensitivity and tissue parameters of inflammation. Four weeks after the discontinuation of the Ang II infusion, the blood pressure was higher by 12.8 mm Hg, and insulin sensitivity as determined by a euglycemic hyperinsulinemic glucose clamp was reduced (glucose infusion rate: 11.1 ± 0.7 *vs.* 17.6 ± 0.5 mg kg^{-1} min^{-1}) in the Ang II-treated group compared with controls. The persistent hypertension and insulin resistance were associated with greater than two-fold increases in macrophage chemoattractant protein-1, tumor necrosis factor-α and thiobarbituric acid-reactive substrates in the soleus muscle. Furthermore, total and activated forms of Rac-1, a regulatory subunit of the NADPH oxidase complex, were increased by 144 ± 14% and 277 ± 82%, respectively, in the skeletal muscle of Ang II-treated rats. These residual effects after Ang II infusion were all attenuated by the co-administration of tempol, a free radical scavenger, or candesartan with Ang II. The effects of candesartan were not mimicked by hydralazine at an equidepressant dose. These findings suggest that Ang II receptor activation in youth triggers the upregulation of inflammatory cytokines and the production of reactive oxygen species, thereby inducing later insulin resistance and hypertension.
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INTRODUCTION

Evidence from patients and animal models has indicated that the angiotensin II (Ang II) type 1 receptor (AT_1 receptor) has a major role in the development of insulin resistance and hypertension.[1–4] However, the relationship between timing of the AT_1 receptor activation and the development (and persistence) of changes in insulin sensitivity and blood pressure has not been fully elucidated. Interestingly, the Trial of Preventing Hypertension Study showed that the treatment of pre-hypertensive patients with the AT_1 receptor blocker (ARB) candesartan for 2 years significantly delayed the progression from pre-hypertension to stage 1 hypertension during the following 2 years.[5] This finding suggests long-lasting effects of transitory activation of the AT_1 receptor in the development of hypertension. The results of the Trial of Preventing Hypertension Study bear certain similarities to those of a study by Holman *et al.*[6] (United Kingdom Prospective Diabetes Study), indicating the so-called 'legacy effect'. These authors found that the reduction of microvascular events continued for 10 years, in spite of the loss of differences in glycemic control after 10 years of intensive glycemic control had been discontinued. Furthermore, such 'legacy effects' have been reported for glycemic control in type 1 diabetes and for blood pressure control in hypertension.[7] Mechanisms by which the beneficial effects of glycemic control or blood pressure control persist after the discontinuation of the therapy remain unclear.

In this study, we hypothesized that the activation of the AT_1 receptor triggers chronic inflammatory reactions in skeletal muscle that mediate persistent insulin resistance and hypertension even after the cessation of AT_1 receptor stimulation. The rationale for this hypothesis is three-fold. Activation of the AT_1 receptor has been shown to activate multiple signaling pathways, leading to inflammatory reactions in blood vessels,[8,9] adipose tissue[10,11] and skeletal muscles.[12,13] Second, inflammatory reactions are often self-perpetuating[14] and are maintained even after the initial stimuli are withdrawn. Third, inflammatory cytokines and reactive oxygen species (ROS) have been shown to contribute to insulin resistance,[12,15–18] which is closely related to the development of hypertension. To test our hypothesis, we examined changes in blood pressure, insulin sensitivity and tissue levels of inflammatory cytokines and ROS production at 4 weeks after a 4-week infusion of Ang II in rats.

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METHODS

This study was approved by the Committee of Animal Experiments of the Sapporo Medical University and strictly conformed to the Guidelines of Animal Use for Scientific Research of the Sapporo Medical University.

Animals and experimental protocols

Sprague-Dawley rats (Charles River Japan Inc., Yokohama, Japan) were fed standard rat chow containing 60% vegetable starch, 5% fat and 24% protein (Oriental Yeast Co., Tokyo, Japan), and they were maintained on a 12-h light/dark cycle and given water and chow *ad libitum*.

Protocol 1

At 4 weeks of age, the rats were randomly divided into three groups. In the Ang II group (Ang II-infused rats), osmotic mini-pumps (Alzet model 2004; Durect Co., Cupertino, CA, USA) for Ang II infusion ($200\,ng\,kg^{-1}\,min^{-1}$) were implanted under anesthesia by intraperitoneal pentobarbital injection ($50\,mg\,kg^{-1}$). The pumps were removed 4 weeks later, that is, at 8 weeks of age. In the Ang II+Tempol group (Ang II group treated with tempol in drinking water), the implantation and removal of the Ang II-filled osmotic mini-pumps were performed as in the Ang II group, and tempol, a superoxide dismutase mimetic, was additionally administered in the drinking water ($1\,mmol\,l^{-1}$) during the Ang II treatment. Osmotic mini-pumps loaded with a vehicle were implanted in the Control group. Ang II infusion was commenced at 4 weeks of age in this study, because preliminary experiments had shown that the present dose of Ang II induced cachexia in rats when started at 8 weeks of age.

Four weeks after the removal of the osmotic mini-pump (that is, at 12 weeks of age), glucose clamp (GC) experiments to determine insulin sensitivity or tissue sampling for biochemical analyses (below) were performed in all of the study groups.

Protocol 2

The rats were randomly assigned to the Ang II, Ang II+ARB (Ang II group given candesartan orally), Ang II+Hyd (Ang II group given hydralazine (Hyd) orally) and Control groups at 4 weeks of age. The administration of Ang II was performed in the Ang II, Ang II+ARB and Ang II+Hyd groups as in protocol 1. In the Ang II+ARB and Ang II+Hyd groups, candesartan ($1\,mg\,kg^{-1}$ per day) or Hyd ($20\,mg\,kg^{-1}$ per day), respectively, were administered orally during the 4-week infusion of Ang II. The Control and Ang II groups received vehicle solution orally. GC was performed 4 weeks after the discontinuation of the Ang II infusion as in protocol 1.

Blood pressure and pulse rate measurements

The systolic blood pressure (SBP) and pulse rate were measured in conscious rats restrained in a 37 °C preheated cloth jacket, using an indirect tail-cuff method (BP-98A; Softran, Tokyo, Japan) under baseline conditions and then every 4 weeks after the implantation of the osmotic mini-pumps. The data from five recordings were averaged for each rat at each time point for hemodynamic measurements.

Euglycemic hyperinsulinemic GC technique

GC was performed by a previously reported technique.[12] Briefly, on the day before the GC experiments, the rats were anesthetized, and the right common carotid artery and the right jugular vein were cannulated with polyethylene tubes (PE50; Becton Dickinson and Co., Sparks, MD, USA) for collecting blood samples and administration of the infusion. After overnight fasting (approximately 12 h), each conscious rat was placed in a foam plastic jacket. The fasting plasma glucose levels were determined under baseline conditions, and then a loading dose of insulin ($25\,mU\,kg^{-1}$ of humalin R, U-40; Shionogi Pharmaceutical Co., Osaka, Japan) was injected as a bolus, followed by the constant infusion of insulin at a rate of $4\,mU\,kg^{-1}\,min^{-1}$ for 147 min. During the GC, a 12.5% glucose solution was infused as needed, to maintain the plasma glucose at the fasting level. Samples (10 μl) of arterial blood were obtained at 7-min intervals for the determination of plasma glucose. The average rate of the glucose infusion over the last 35 min was used as the index of insulin sensitivity (glucose infusion rate (GIR)). At the end of the GC, 1.5 ml of blood was withdrawn for the measurement of the plasma insulin level.

Determinations of tissue levels of TNF-α, MCP-1 and TBARS

Under anesthesia, the soleus muscle was excised and immediately frozen in liquid nitrogen. The tissue level of tumor necrosis factor-α (TNF-α) was determined as previously reported.[12] Briefly, the muscle tissues were homogenized with a polytron homogenizer (Kinematica, Luzern, Switzerland) in ice-cold lysis buffer. The resulting solution was centrifuged at 17 500 *g* for 30 min and the supernatant was assayed for TNF-α, using ELISA kits (Invitrogen Corporation, Carlsbad, CA, USA). Samples of the soleus muscle were used for the determinations of macrophage chemoattractant protein-1 (MCP-1) and thiobarbituric acid-reactive substrates (TBARS) with a MCP-1 ELISA kit (Pierce Biotechnology, Rockford, IL, USA) and a TBARS Assay kit (ZeptoMetrix Corporation, Buffalo, NY, USA), respectively.

Determination of tissue Rac-1 level

Rac activation assay kits (Millipore, Billerica, MA, USA) were used in a pull-down assay to measure the guanosine-5′-triphosphate (GTP)-bound Rac-1. Soleus muscles frozen immediately after sampling were powdered in liquid nitrogen and homogenized in ice-cold lysis buffer containing $125\,mmol\,l^{-1}$ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), $750\,mmol\,l^{-1}$ NaCl, 5% Igepal CA-630, $50\,mmol\,l^{-1}$ $MgCl_2$, $5\,mmol\,l^{-1}$ EDTA and 10% glycerol. Total protein of 2.5 mg from each clarified lysate was incubated with PAK-1 PBD (the p21-binding domain of human p21 protein (Cdc42/Rac)-activated kinase-1) to precipitate the GTP-bound Rac1 that was then used for immunoblotting. The lysates that were not incubated with GST-PBD were used for the determination of total Rac-1 protein levels by immunoblotting.

Assessment of Akt phosphorylation in response to insulin

Separate groups of rats were used for assessments of the response of Akt to insulin in the soleus muscle. At 12 weeks of age, the right jugular vein was cannulated with a polyethylene tube under pentobarbital anesthesia before tissue sampling. Following overnight fasting, 1 ml of normal saline (0.9% NaCl) with or without $1000\,\mu g\,kg^{-1}$ of insulin was injected intravenously. Five minutes after the injection, rats were killed by pentobarbital overdose, and their soleus muscles were immediately sampled and frozen in liquid nitrogen.

The frozen soleus muscle samples were pulverized in liquid nitrogen and then homogenized with a glass Dounce homogenizer in ice-cold lysis buffer containing $20\,nmol\,l^{-1}$ Tris-HCl (pH 7.5), $150\,nmol\,l^{-1}$ NaCl, $1\,mmol\,l^{-1}$ Na_2EDTA, $1\,mmol\,l^{-1}$ ethylene glycol tetraacetic acid, 1% Triton X-100, $2.5\,mmol\,l^{-1}$ sodium pyrophosphate, $1\,mmol\,l^{-1}$ β-glycerophosphate, $1\,mmol\,l^{-1}$ Na_3VO_4, $1\,\mu g\,ml^{-1}$ leupeptin, $50\,\mu g\,ml^{-1}$ phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Complete mini, Roche Molecular Biochemicals, Mannheim, Germany). The insoluble materials were removed by centrifugation at 16 000 *g* for 20 min at 4 °C, and the supernatants were used for immunoblotting for total and phospho-Akt (Thr308).

Immunoblotting

Equal amounts of the protein extracts were subjected to 12.5% SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking with a Tris buffered saline with Tween 20 (TBS-T) buffer containing 5% non-fat dry milk or 5% bovine serum albumin, the blots were incubated with antibodies that recognize Rac-1 (BD Biosciences, Franklin Lakes, NJ, USA), phospho-Akt (Thr308) or total Akt (Cell Signaling Technology, Beverly, MA, USA), or glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBS-T buffer, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (Sheep anti-mouse IgG and Donkey anti-rabbit IgG; GE Healthcare, Little Chalfont, Buckinghamshire, UK; Donkey anti-goat IgG: Santa Cruz Biotechnology) for 1 h. Immunoblotted proteins were visualized with an Immobilon Western detection kit (Millipore) and quantified with a lumino-image analyzer, the LAS-2000 mini (Fujifilm, Tokyo, Japan).

Statistical analysis

The data are expressed as the means ± s.e. The differences between treatment groups were tested by the two-way analysis of variance and the Tukey–Kramer honestly significant difference *post-hoc* test for multiple comparisons. The difference was considered significant if the *P*-value <0.05.

RESULTS

Protocol 1

Blood pressure, pulse rate and body weight. Under baseline conditions, the blood pressure, pulse rate and body weight were similar in all of the study groups (Table 1). As shown in Figure 1, during the treatment period when mini-pumps were implanted, the SBP was significantly higher in the Ang II group than in the Control group: 172.0 ± 6.3 (n=12) *vs.* 121.6 ± 2.6 mm Hg (n=15) at 8 weeks of age. The co-administration of tempol did not attenuate the elevation of the SBP by Ang II infusion: 161.8 ± 7.6 mm Hg (n=10). After removal of the mini-pumps, the SBP declined in the Ang II group; however, it remained significantly higher than the SBP in the Control group even 4 weeks after the discontinuation of the Ang II infusion: 138.5 ± 2.7 *vs.* 125.7 ± 1.5 mm Hg at 12 weeks of age. In contrast, the SBP in the Ang II+Tempol group was reduced to a level comparable to that in the Control group after the removal of the osmotic mini-pumps: 129.2 ± 3.3 mm Hg at 12 weeks of age ($P<0.05$ *vs.* Ang II group). The time courses of pulse rates and body weight gain during the 8-week experimental period were similar in the three study groups (Figures 1b and c).

Insulin sensitivity. The GIR in the GC was significantly lower (11.1 ± 0.7 *vs.* 17.6 ± 0.5 mg kg^{-1} min^{-1}) and the fasting plasma glucose level was higher (6.38 ± 0.40 and 5.23 ± 0.16 mmol l^{-1}) in the Ang II group than in the Control group (Table 2). These differences were not observed in the Ang II+Tempol group; the GIR and fasting glucose level were 18.5 ± 1.4 mg kg^{-1} min^{-1} and 5.29 ± 0.30 mmol l^{-1}, respectively (both $P<0.05$ *vs.* data in the Ang II group). We ensured that the steady-state levels of blood glucose and insulin during the GC were comparable between all study groups.

Inflammatory cytokines and indices of ROS production in skeletal muscle. Four weeks after the discontinuation of Ang II infusion, a three-fold increase in MCP-1 and a 70% increase in TNF-α were observed in the soleus muscle of the Ang II group compared with the Control group as shown in Figure 2a (MCP-1: 113 ± 14 *vs.* 42 ± 3 pg per mg protein, TNF-α: 167 ± 22 *vs.* 98 ± 16 pg per g wet tissue). However, this upregulation of these inflammatory cytokines in the soleus muscle was not detected in the Ang II+Tempol group (MCP-1: 50 ± 7 pg per mg protein, TNF-α 105 ± 8 pg per g wet tissue).

The level of TBARS, products of lipid peroxidation, in the soleus muscle was also significantly increased in the Ang II group (1.30 ± 0.12 *vs.* 0.77 ± 0.08 nmol per mg protein in the Control group), and the elevation of the TBARS level was attenuated in the Ang II+Tempol group (0.90 ± 0.16 nmol per mg protein, $P>0.05$ *vs.* Control group).

Because NADPH oxidase is a major ROS-producing oxidase, the change in the protein level of its regulatory subunit Rac-1 was examined (Figure 3). The level of Rac-1 normalized to glyceraldehyde 3-phosphate dehydrogenase was significantly elevated in the Ang II group, to 144 ± 14% of the control, and co-treatment with tempol partially suppressed the change in total Rac-1 level (120 ± 13%). Similar results were obtained in the assays of an activated form of Rac-1, GTP-bound Rac-1. GTP-bound Rac-1 in the Ang II group was significantly increased (277 ± 82% of the control), and the increase in the active form of Rac-1 was suppressed by tempol (185 ± 41% of the

Table 1 Baseline parameters at the age of 4 weeks

	Control (n=*15*)	*Ang II* (n=*12*)	*Ang II+Tempol* (n=*10*)
Body weight (g)	102.1 ± 1.3	101.4 ± 1.2	101.1 ± 1.6
Systolic blood pressure (mm Hg)	96.9 ± 2.6	99.7 ± 2.8	105.3 ± 4.6
Pulse rate (beats per min)	416.6 ± 10.5	394.8 ± 7.9	413.3 ± 13.6

Abbreviation: Ang II, angiotensin II.

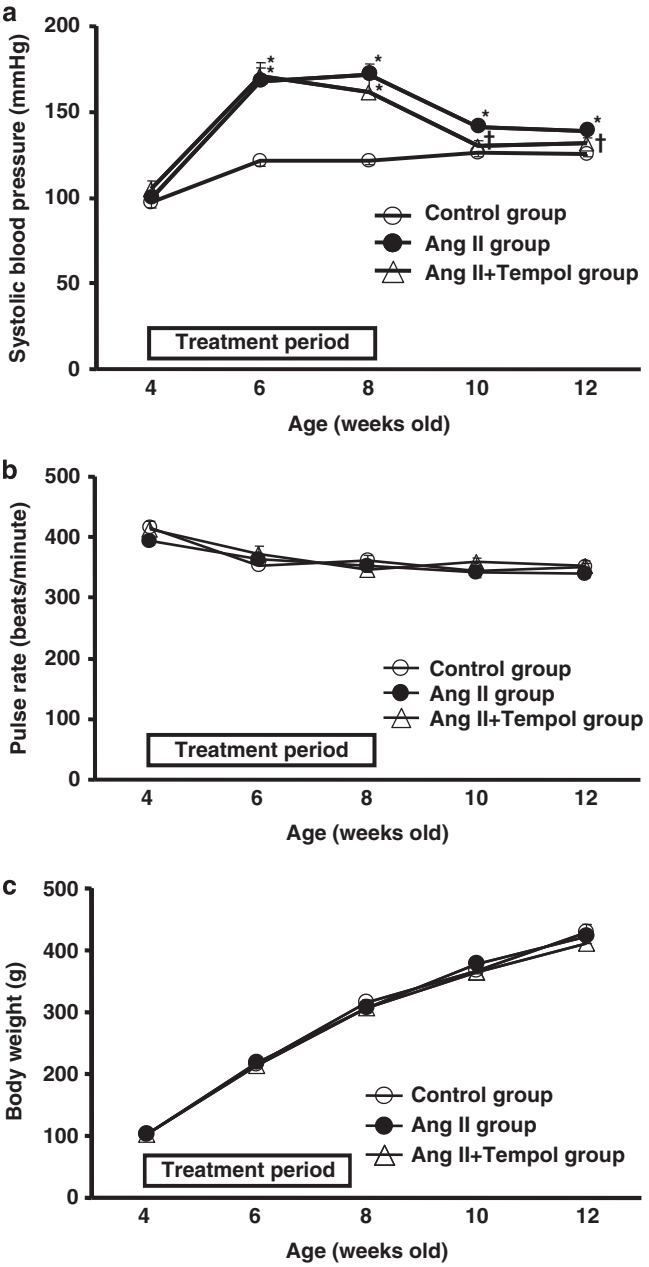

Figure 1 Time courses of (**a**) systolic blood pressure (SBP), (**b**) pulse rate and (**c**) body weight. Open circles represent values in control rats (n=15); closed circles represent Ang II rats (n=12); and open triangles represent Ang II and tempol rats (n=10). *$P<0.05$ *vs.* values in control rats; †$P<0.05$ *vs.* values in Ang II rats.

Table 2 Parameters at the age of 12 weeks

	Control (n=*15*)	*Ang II* (n=*12*)	*Ang II+Tempol* (n=*10*)
Body weight (g)	428.8 ± 12.4	422.0 ± 10.7	410.2 ± 12.0
Systolic blood pressure (mm Hg)	125.7 ± 1.5	138.5 ± 2.7	131.9 ± 3.3
Pulse rate (beats per min)	349.6 ± 7.0	338.9 ± 4.5	353.1 ± 8.7
GIR (mg kg^{-1} min^{-1})	17.6 ± 0.5	11.1 ± 0.7*	18.5 ± 1.4†
Fasting plasma glucose (mmol l^{-1})	5.23 ± 0.16	6.38 ± 0.40*	5.29 ± 0.30†
Insulin during GC (uIU ml^{-1})	100.5 ± 4.3	100.3 ± 4.9	105.6 ± 7.5
Plasma glucose level during GC (mmol l^{-1})	5.15 ± 0.04	5.23 ± 0.04	5.12 ± 0.03
Hematocrit (%)	45.9 ± 0.4	46.6 ± 0.6	47.0 ± 0.9

Abbreviations: Ang II, angiotensin II; GC, glucose clamp; GIR, glucose infusion rate.
*P<0.05 *vs.* values in control; †P<0.05 *vs.* values in Ang II.

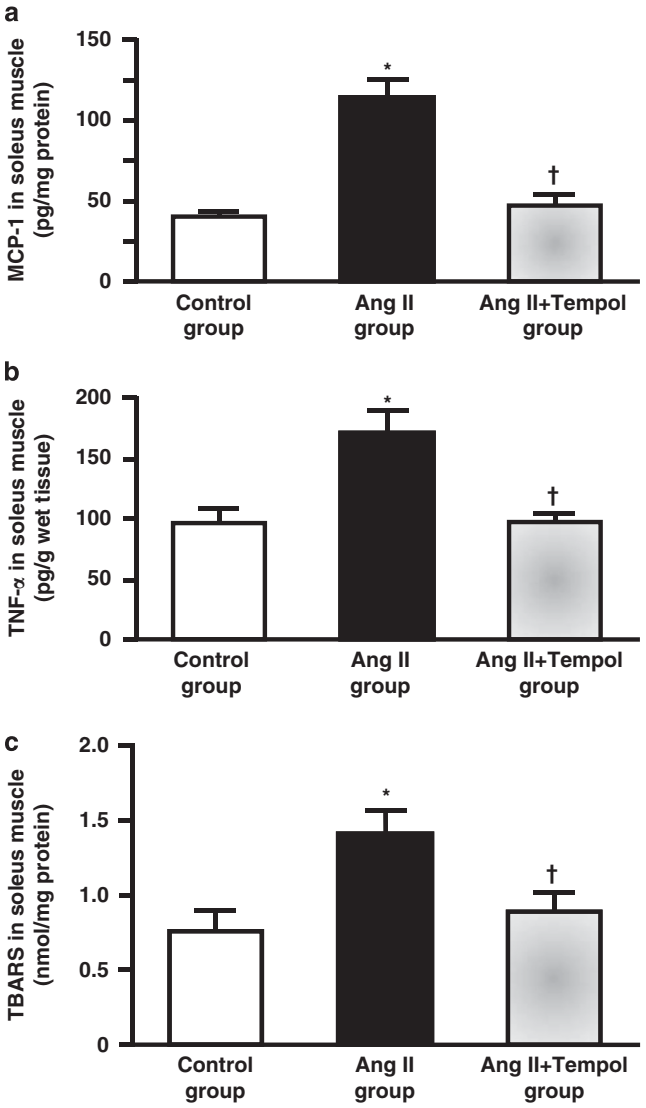

Figure 2 Ang II-triggered upregulation of inflammatory cytokines and reactive oxygen species in skeletal muscle. Levels of (**a**) macrophage chemoattractant protein-1 (MCP-1), (**b**) tumor necrosis factor-α (TNF-α) and (**c**) thiobarbituric acid-reactive substrates (TBARS) in control rats ($n \geqslant 6$), Ang II rats ($n \geqslant 6$), and Ang II and tempol rats ($n \geqslant 6$). *P<0.05 *vs.* values in control rats; †P<0.05 *vs.* values in Ang II rats. n=6–14 in each study group.

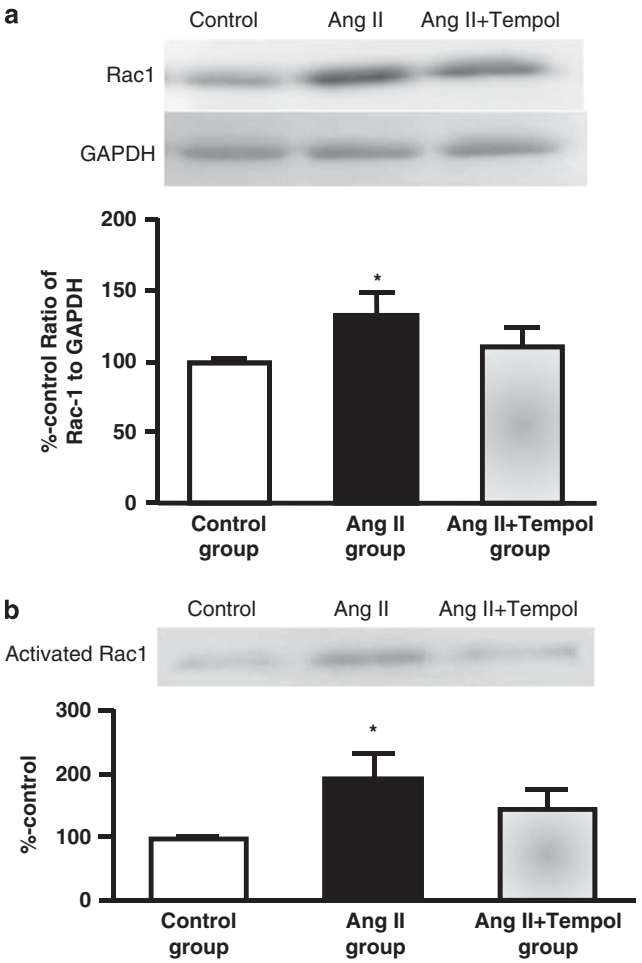

Figure 3 Ang II-triggered upregulation of total and GTP-bound Rac 1 in skeletal muscle. (**a**) Top panel: representative western blots of Rac 1 in the soleus muscle. Bottom panel: quantification of the Rac 1/glyceraldehyde 3-phosphate dehydrogenase ratio (n=9 per group). (**b**) Top panel: representative western blots of activated or GTP-bound Rac 1 in soleus muscle. Bottom panel: quantification of the GTP-bound Rac 1 (n=9 per group). *P<0.05 *vs.* values in control rats.

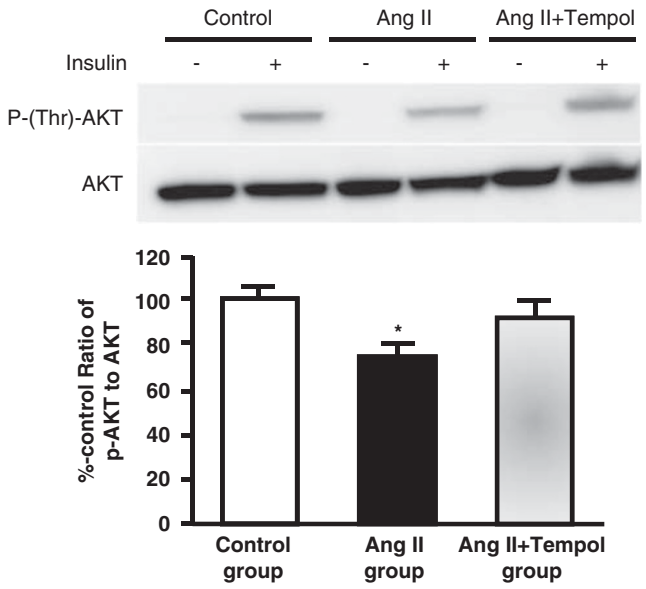

Figure 4 Ang II-triggered loss of the AKT response to insulin in skeletal muscle. Insulin (1000 μg kg^{-1}) or saline (control) was injected into the jugular vein 5 min before soleus muscles were sampled. Top panel: representative western blots of threonine-phosphorylated AKT. Bottom panel: quantification of threonine-phosphorylated AKT/AKT ratio (n=9 per group). *P<0.05 *vs.* values in control rats.

control in the Ang II+Tempol group). We also determined the protein levels of gp91-phox and p22-phox in the soleus muscle by immunoblotting, but the differences in these proteins between the three study groups did not reach statistical significance (data not shown).

The response of Akt to insulin receptor activation in skeletal muscle. As shown in Figure 4, Akt in the soleus muscle was phosphorylated at 5 min after administration of insulin. However, the level of phospho-Akt was significantly suppressed to 77 ± 7% of the control level in the Ang II group. This inhibition of phosphorylation was abrogated in the Ang II+Tempol group (96 ± 5% of the control, P>0.05 *vs.* Control group).

Protocol 2

Blood pressure, pulse rate and body weight. As in protocol 1, the blood pressure elevation was sustained after the discontinuation of the Ang II infusion in the Ang II group. The co-administration of candesartan or Hyd similarly attenuated the blood pressure elevation caused by Ang II; however, the blood pressure returned to a level comparable to the control level in the Ang II+ARB group, but not in the Ang II+Hyd group (Figure 5). There were no inter-group differences in pulse rate or body weight during the experimental period.

Insulin sensitivity and TNF-α expression in skeletal muscle. As in protocol 1, the GIR was significantly lower and the TNF-α level was elevated in the Ang II group compared with the Control group, 4 weeks after the discontinuation of the Ang II infusion (Table 3). These changes in the GIR and TNF-α level were significantly attenuated in the Ang II+ARB group (GIR=18.7 ± 1.5 mg kg^{-1} min^{-1}, TNF-α=103 ± 17 pg per g wet tissue). In contrast, the GIR and tissue TNF-α level in the Ang II+Hyd group (11.3 ± 1.8 mg kg^{-1} min^{-1}, 163 ± 15 pg per g wet tissue) were similar to those in the Ang II group.

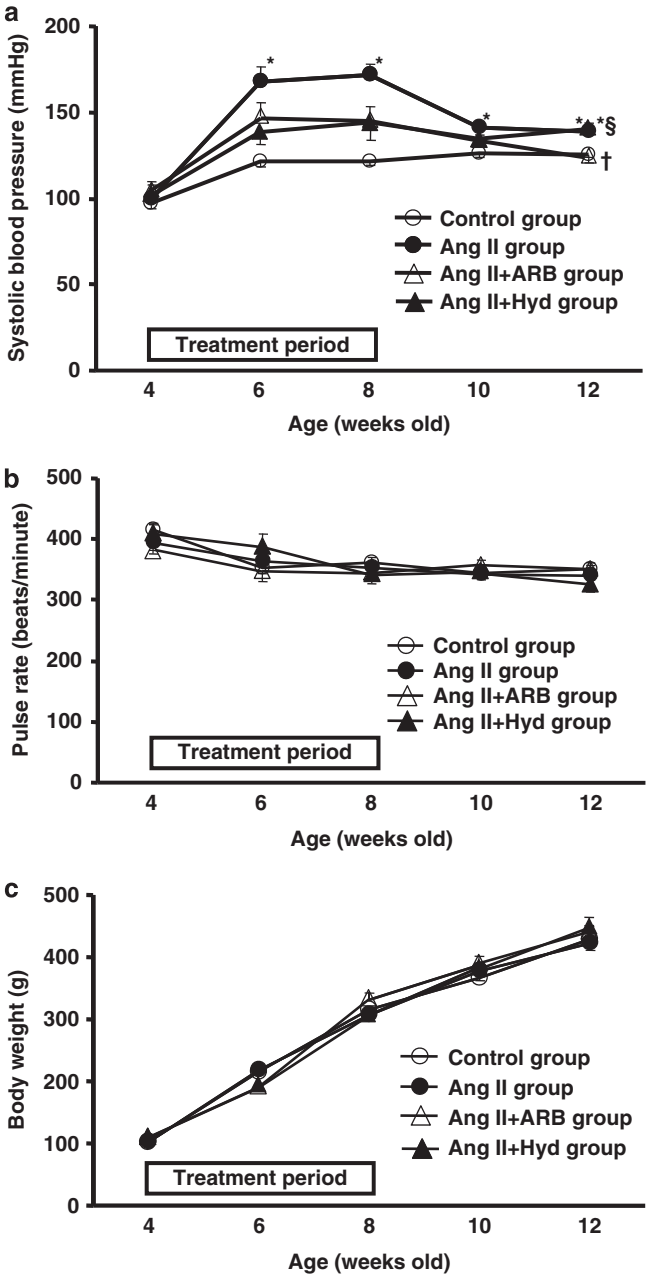

Figure 5 The effects of candesartan (an ARB) or hydralazine (Hyd) on time courses of (**a**) systolic blood pressure (SBP), (**b**) pulse rate and (**c**) body weight. Open circles represent values in control rats (n=9); closed circles represent Ang II rats (n=9); open triangles represent Ang II+ARB rats (n=10); and closed triangles represent Ang II+Hyd (n=8). *P<0.05 *vs.* values in control rats; †P<0.05 *vs.* values in Ang II rats; §P<005 *vs.* values in Ang II+ARB.

DISCUSSION

Activation of the renin–angiotensin system (RAS) has been shown to upregulate the expression of inflammatory cytokines and the ROS-producing enzyme NADPH oxidase in skeletal muscle and smooth muscle cells.[8–14,16] However, how the upregulation of these proteins decays after the withdrawal of RAS activation has remained unclear. In this study, we showed for the first time that tissue levels of MCP-1, TNF-α and activated Rac-1 remained elevated in skeletal muscle, even 4 weeks after the cessation of Ang II infusion. Consistent with the

Table 3 Effects of ARB and hydralazine on parameters at the age of 12 weeks

	Control (n=*9*)	*Ang II* (n=*9*)	*Ang II+ARB* (n=*10*)	*Ang II+Hyd* (n=*8*)
Body weight (g)	418.5 ± 8.8	411.1 ± 13.9	406.2 ± 10.5	412.1 ± 3.0
Systolic blood pressure (mm Hg)	124.8 ± 2.5	135.8 ± 1.0	124.7 ± 2.3	138.6 ± 2.9
Pulse rate (beats per minute)	341.6 ± 7.0	338.5 ± 5.6	349.4 ± 7.8	325.4 ± 9.5
GIR (mg kg^{-1} min^{-1})	17.7 ± 0.8	11.4 ± 0.9*	18.7 ± 1.5†	11.3 ± 1.8*§
Fasting plasma glucose (mmol l^{-1})	5.03 ± 0.24	6.39 ± 0.66*	4.96 ± 0.21†	6.39 ± 0.46*§
Insulin during GC (uIU ml^{-1})	108.8 ± 3.3	107.8 ± 6.3	110.4 ± 3.6	110.7 ± 6.2
Plasma glucose level during GC (mmol l^{-1})	5.13 ± 0.06	5.18 ± 0.07	5.29 ± 0.04	5.18 ± 0.09
Hematocrit (%)	46.0 ± 0.6	47.4 ± 0.7	47.7 ± 0.6	47.7 ± 0.9

Abbreviations: ARB, AT1 receptor blocker; GC, glucose clamp; GIR, glucose infusion rate; Hyd, hydralazine.
$^{*}P<0.05$ *vs.* values in control; $^{\dagger}P<0.05$ *vs.* values in Ang II; $^{\S}P<0.05$ *vs.* in Ang II+ARB.

upregulation of activated Rac-1, the tissue level of TBARS was increased by 170%, indicating an increased ROS production. ROS are known to induce inflammatory cytokines and, conversely, the cytokines also increase the ROS generation, thereby potentially forming a vicious cycle.[8–14,16,19–21] Furthermore, a recent study by Banday and Lokhandwala[22] indicates that ROS increase the expression of Ang II receptors. Interestingly, the suppression of ROS production during the Ang II infusion almost completely abolished the persistent changes in the cytokines, Rac-1 and TBARS, following the discontinuation of Ang II infusion (Figures 2 and 3). Taken together, these findings suggest that the ROS generated subsequent to the activation of AT_1 receptors during the 4 weeks of the Ang II infusion were primarily responsible for the upregulation of NADPH oxidase and inflammatory cytokine expression, thereby forming a self-perpetuating ROS-generating system in the skeletal muscle.

In parallel to the tissue levels of MCP-1, TNF-α and TBARS, the blood pressure remained elevated after the withdrawal of Ang II receptor stimulation (Figure 1a). The persistent hypertension was associated with significant insulin resistance and a blunted response of Akt to insulin receptor activation in skeletal muscle. In a previous study, we showed a significant inverse correlation between the TNF-α level in skeletal muscle and insulin sensitivity, determined by a GC in fructose-fed rats in a model of the metabolic syndrome.[12] Studies by Hotamisligil *et al.*[15,16] indicated that signaling downstream of receptors of TNF-α and other inflammatory cytokines interferes with the signaling from activated insulin receptors, causing insulin resistance. Several lines of evidence also suggest that insulin resistance is involved in the development of hypertension.[23,24] Hyperinsulinemia associated with insulin resistance enhances activities of the sympathetic nervous system[25] and RAS.[26,27] Treatment with agents that improve insulin sensitivity reduced the blood pressure in fructose-fed rats[28,29] and in human hypertensive patients.[30] Conversely, diet-induced insulin resistance increased the blood pressure.[2–3,12,18] These observations support the notion that the persistent insulin resistance induced by upregulated TNF-α and MCP-1 underlies the persistent elevation of blood pressure observed after the cessation of Ang II infusion in the present experiments.

Because the co-administration of tempol with the Ang II infusion significantly attenuated the elevation of blood pressure by Ang II, we could not exclude the possibility that the reduction of blood pressure *per se* rather than the scavenging of ROS was responsible for the reduction of tissue inflammation triggered by Ang II. To address this issue, we examined the effects of blood pressure reduction by the use of candesartan and Hyd during the Ang II infusion on insulin resistance and inflammatory reactions (protocol 2). As shown in Table 3, the upregulation of TNF-α expression by Ang II was inhibited by candesartan, but not by Hyd, although the effects on blood pressure during the Ang II infusion was similar between the two agents. Similarly, the persistence of hypertension after the discontinuation of the Ang II infusion was abolished by candesartan, but not by Hyd (Figure 5). Together with the data from protocol 1, these results indicate that the ROS production induced by the AT_1 receptor activation and not the elevation of blood pressure *per se* induces persistent inflammatory reactions in skeletal muscle, insulin resistance and hypertension.

In this study, significant changes in blood pressure and insulin resistance were detected 4 weeks after the discontinuation of the Ang II infusion. How long such residual effects of temporary Ang II infusion persist remains unclear at present. However, in an extension of our experiments, we found that the SBP remained higher (142.4 ± 3.9 *vs.* 131.0 ± 2.9 mm Hg, $P<0.05$) and that the GIR was lower (10.1 ± 1.6 *vs.* 16.9 ± 1.7 mg kg^{-1} min^{-1}, $P<0.05$) in the Ang II group (n=9) than in the Control group (n=11) 8 weeks after the discontinuation of the Ang II infusion. These findings indicate that a 'legacy effect' induced by our protocol of Ang II receptor activation persists at least 2 months in the rat.

There are several limitations in the present study. First, it remains unclear whether insulin resistance is the only mechanism of persistent elevation of blood pressure after the discontinuation of Ang II infusion. We cannot exclude the possibility that Ang II-induced renal damage[31] and/or the direct effects of ROS on vascular tone[32] were involved in the persistent elevation of blood pressure after Ang II cessation. In fact, in preliminary analyses of the kidney, CD68-positive cells (mainly macrophages) appeared to be increased in the Ang II group, but not in the Ang II+tempol and Control groups at 4 weeks after the Ang II infusion. Second, the entire time course of the 'legacy effect' of Ang II on blood pressure and insulin sensitivity was not characterized. Third, because the effects of the Ang II infusion on the plasma levels of RAS components were not determined, the involvement of the plasma RAS system in this 'legacy effect' remains unclear. Another limitation of this study is lack of information regarding the specific cell types in which MCP-1 and/or TNF-α were upregulated by Ang II. Because we used the whole soleus muscles for protein analyses, it is unclear whether the data represent changes in myocytes, satellite cells, fibroblasts and/or vascular cells. However, the total volumes of non-myocyte components are small in the soleus, and earlier studies using immunohistochemistry have shown that protein levels of MCP-1 and TNF-α were increased by 1.5-fold in myocytes.[33] Therefore, we speculate that the induction of the inflammatory cytokines in response to the Ang II infusion occurred mainly in myocytes.

In summary, this study demonstrated that the tissue levels of inflammatory cytokines and ROS in the skeletal muscle remained

significantly elevated 4 weeks after the discontinuation of a 4-week protocol of Ang II infusion. The upregulation of the cytokines/ROS was associated with persistent insulin resistance and hypertension, and co-administration of a ROS scavenger with Ang II prevented both the upregulation of the cytokines and the changes in insulin sensitivity and blood pressure. These results support our hypothesis that temporary activation of the AT_1 receptor triggers, via ROS, chronic inflammatory reactions in the skeletal muscle that mediate persistent insulin resistance and hypertension.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Moan A, Risanger T, Eide I, Kjeldsen SE. The effect of angiotensin II receptor blockade on insulin sensitivity and sympathetic nervous system activity in primary hypertension. *Blood Press* 1994; **3**: 185–188.
2 Shimamoto K, Matsuda K, Takizawa H, Higashiura K, Ura N, Iimura O. Effects of an angiotensin II receptor antagonist, TCV-116, on insulin sensitivity in fructose-fed rats. *Blood Press* 1994; **5**: S113–S116.
3 Iimura O, Shimamoto K, Matsuda K, Masuda A, Takizawa H, Higashiura K, Miyazaki Y, Hirata A, Ura N, Nakagawa M. Effects of angiotensin receptor antagonist and angiotensin converting enzyme inhibitor on insulin sensitivity in fructose-fed hypertensive rats and essential hypertensives. *Am J Hypertens* 1995; **8**: 353–357.
4 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.
5 Julius S, Nesbitt SD, Egan BM, Weber MA, Michelson EL, Kaciroti N, Black HR, Grimm Jr RH, Messerli FH, Oparil S, Schork MA. Feasibility of treating prehypertension with an angiotensin-receptor blocker. *N Engl J Med* 2006; **354**: 1685–1697.
6 Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HAW. 10-year follow-up of intensive glucose control in type 2 diabetes. *N Engl J Med* 2008; **359**: 1577–1589.
7 The Diabetes Control Complications Trial/Epidemiology of Diabetes Interventions Complications (DCCT/EDIC) Study Group. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *N Engl J Med* 2005; **353**: 2643–2653.
8 Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 2002; **91**: 406–413.
9 Takahashi M, Suzuki E, Takeda R, Oba S, Nishimatsu H, Kimura K, Nagano T, Nagai R, Hirata Y. Angiotensin II and tumor necrosis factor-alpha synergistically promote monocyte chemoattractant protein-1 expression: roles of NF-kappaB, p38, and reactive oxygen species. *Am J Physiol Heart Circ Physiol* 2008; **294**: H2879–H2888.
10 Asamizu S, Urakaze M, Kobashi C, Ishiki M, Norel Din AK, Fujisaka S, Kanatani Y, Bukahari A, Senda S, Suzuki H, Yamazaki Y, Iwata M, Usui I, Yamazaki K, Ogawa H, Kobayashi M, Tobe K. Angiotensin II enhances the increase in monocyte chemoattractant protein-1 production induced by tumor necrosis factor-alpha from 3T3-L1 preadipocytes. *J Endocrinol* 2009; **202**: 199–205.
11 Maehaut N, Van de Voorde J. Regulation of vascular tone by adipocytes. *BMC Med* 2011; **16**: 9–25.
12 Togashi N, Ura N, Higashiura K, Murakami H, Shimamoto K. The contribution of skeletal muscle tumor necrosis factor-alpha to insulin resistance and hypertension in fructose-fed rats. *J Hypertens* 2000; **18**: 1605–1610.
13 Wei Y, Sowers JR, Clark SE, Li W, Ferrario CM, Stump CS. Angiotensin II-induced skeletal muscle insulin resistance mediated by NF-kappaB activation via NADPH oxidase. *Am J Physiol Endocrinol Metab* 2007; **294**: E345–E351.
14 Polla BS, Cossarizza A. Stress proteins in inflammation. *EXS* 1996; **77**: 375–391.
15 Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. *Proc Natl Acad Sci USA* 1994; **91**: 4854–4858.
16 Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. *Nature* 2002; **420**: 333–336.
17 Wei Y, Whaley-Connell AT, Chen K, Habibi J, Uptergrove GM, Clark SE, Stump CS, Ferrario CM, Sowers JR. NADPH oxidase contributes to vascular inflammation, insulin resistance, and remodeling in the transgenic (mRen2) rat. *Hypertension* 2007; **50**: 384–391.
18 Yamaguchi K, Ura N, Murakami H, Togashi N, Hyakukoku M, Higashiura K, Shimamoto K. Olmesartan ameliorates insulin sensitivity by modulating tumor necrosis factor-alpha and cyclic AMP in skeletal muscle. *Hypertens Res* 2005; **28**: 773–778.
19 Bae YS, Lee JH, Choi SH, Kim S, Almazan F, Witztum JL, Miller YI. Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2. *Circ Res* 2009; **104**: 210–218.
20 Moe KT, Yin NO, Naylynn TM, Khairunnisa K, Wutyl MA, Gu Y, Atan MS, Wong MC, Koh TH, Wong P. Nox2 and Nox4 mediate tumor necrosis factor-alpha-induced ventricular remodeling in mice. *J Cell Mol Med* 2011; in press.
21 Khaper N, Bryan S, Dhingra S, Khaper N, Bryan S, Dhingra S, Singal R, Bajaj A, Pathak CM, Singal PK. Targeting the vicious inflammation-oxidative stress cycle for the management of heart failure. *Antioxid Redox Signal* 2010; **13**: 1033–1049.
22 Banday AA, Lokhandwala MF. Oxidative stress-induced renal angiotensin AT1 receptor upregulation causes increased stimulation of sodium transporters and hypertension. *Am J Physiol Renal Physiol* 2008; **295**: F698–F706.
23 Falkner B, Hulman S, Tannenbaum J, Kushner H. Insulin resistance and blood pressure in young black men. *Hypertension* 1990; **16**: 706–711.
24 Hirata A, Shimamoto K, Masuda A, Miyazaki Y, Fukuoka M, Iimura O. Influence of aging on insulin sensitivity in essential hypertensives and normotensives. *Hypertens Res* 1995; **18**: 307–311.
25 Rahmouni K, Morgan DA, Morgan GA, Liu X, Sigmund CD, Mark AL, Haynes WG. Hypothalamic PI3 kinase and MAP kinase differentially mediate regional sympathoactivation to insulin. *J Clin Invest* 2004; **114**: 652–658.
26 Shimamoto K, Hirata A, Fukuoka M, Higashiura K, Miyazaki Y, Shiiki M, Masuda A, Nakagawa M, Iimura O. Insulin sensitivity of the effects of insulin on renal sodium handling and pressor systems in essential hypertensive patients. *Hypertension* 1994; **23**: S129–S133.
27 Kamide K, Hori MT, Zhu JH, Barrett JD, Eggena P, Tuck ML. Insulin-mediated growth in aortic smooth muscle and the vascular renin-angiotensin system. *Hypertension* 1998; **32**: 482–487.
28 Li Y, Higashiura K, Ura N, Torii T, Agata J, Wang L, Togashi N, Shimamoto K. Effects of the Chinese medicine TSJN on insulin resistance and hypertension in fructose-fed rats. *Hypertens Res* 2000; **23**: 101–107.
29 Dobrian AD, Schriver SD, Khraibi AA, Prewitt RL. Pioglitazone prevents hypertension and reduces oxidative stress in diet-induced obesity. *Hypertension* 2004; **43**: 48–56.
30 Ogihara T, Rakugi H, Ikegami H, Mikami H, Masuo K. Enhancement of insulin sensitivity by troglitazone lowers blood pressure in diabetic hypertensives. *Am J Hypertens* 1995; **8**: 316–320.
31 Lombardi D, Gordon KL, Polinsky P, Suga S, Schwartz SM, Johnson RJ. Salt-sensitive hypertension develops after short-term exposure to angiotensin II. *Hypertension* 1999; **33**: 1013–1019.
32 Paravicini TM, Touyz RM. NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* 2008; **32**: S170–S180.
33 Wei Y, Sowers JR, Nistala R, Gong H, Uptergrove GM, Clark SE, Morris EM, Szary N, Manrique C, Stump CS. Angiotensin II-induced NADPH oxidase activation impairs insulin signaling in skeletal muscle cells. *J Biol Chem* 2006; **281**: 35137–35146.