Evidence for a Causal Role of the Renin–Angiotensin System in Vascular Dysfunction Associated With Insulin Resistance

Kazuya Shinozaki, Kazuhide Ayajiki, Yoshihiko Nishio, Takeshi Sugaya, Atsunori Kashiwagi, Tomio Okamura

Abstract—Excess production of superoxide anion in response to angiotensin II plays a central role in the transduction of signal molecules and the regulation of vascular tone. We examined the ability of insulin resistance to stimulate superoxide anion production and investigated the identity of the oxidases responsible for its production. Rats were fed diets containing 60% fructose (fructose-fed rats) or 60% starch (control rats) for 8 weeks. In aortic homogenates from fructose-fed rats, the superoxide anion generated in response to NAD(P)H was more than 2-fold higher than that of control rats. Pretreatment of the aorta from fructose-fed rats with inhibitors of NADPH oxidase significantly reduced superoxide anion production. In the isolated aorta, contraction induced by angiotensin II was more potent in fructose-fed rats compared with control rats. Losartan normalized blood pressure, NAD(P)H oxidase activity, endothelial function, and angiotensin II-induced vasoconstriction in fructose-fed rats. To elucidate the molecular mechanisms of the enhanced constrictor response to angiotensin II, expressions of angiotensin II receptor and subunits of NADPH oxidase were examined with the use of angiotensin II type 1a receptor knockout (AT1a KO) mice. Expression of AT1a receptor mRNA was enhanced in fructose-fed mice, whereas expression of either AT1b or AT2 was unaltered. In addition, protein expression of each subunit of NADPH oxidase was increased in fructose-fed mice, whereas the expression was significantly decreased in fructose-fed AT1a KO mice. The novel observation of insulin resistance-induced upregulation of AT1 receptor expression could explain the association of insulin resistance with endothelial dysfunction and hypertension. (Hypertension. 2004;43:255-262.)

Key Words: insulin resistance ■ angiotensin II ■ angiotensin antagonist ■ endothelium ■ free radicals ■ blood pressure

There is a growing body of evidence demonstrating the coexistence of hypertension in the insulin-resistant states, such as obesity and type 2 diabetes. Impairment of vasomotor function in hypertension may be caused by an imbalance of vasodilating and vasoconstricting factors. We have recently reported that insulin resistance may be a pathogenic factor for endothelial dysfunction through impaired endothelial NOS (eNOS) activity caused by the enhanced formation of superoxide anion (O₂⁻), which is caused by relative deficiency of tetrahydrobiopterin (BH₄) in vascular endothelial cells. Although BH₄ supplementation results in the restoration of the endothelial function, BH₄ treatment did not normalize blood pressure and vascular oxidative stress in the insulin-resistant state. These findings suggest that an additional element to regulate vascular tone is therefore likely to be present in the insulin-resistant state.

Apart from the L-arginine–nitric oxide (NO) pathway, the vascular tone is regulated by a variety of autocrine and paracrine systems localized in the endothelium and smooth muscle cells. Blockade of the renin–angiotensin system with angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (Ang II) type 1 (AT1) receptor antagonists has been reported to have beneficial effects on patients with insulin resistance. The third Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto Miocardico (GISSI-3) study demonstrated that diabetic patients benefited more from ACE inhibitors than nondiabetic patients did, independent of other risk factors for elevated mortality rates. Recent comparative trials in patients with type 2 diabetes and hypertension have suggested that, for the prevention of cardiovascular events, ACE inhibitors may be superior to alternative antihypertensive agents. Reduction in cardiovascular events with ACE inhibitors was much greater (almost 50%) than that expected from blood pressure reduction alone compared with placebo, supporting the view that additional mechanisms contribute to the prevention of cardiovascular events with ACE inhibition.
There are two major subtypes of Ang II receptors, AT1 and AT2, and AT1 receptors are further subdivided into AT1a and AT1b receptors in the rodents. It is generally accepted that most of the well-known Ang II functions in the cardiovascular system are mediated through AT1. ACE inhibitors and AT1 receptor antagonist have been noted to improve vascular reactivity in the insulin-resistant state. We have recently reported that experiments on blood vessels from insulin resistant generate an excess O$_2^-$, which rapidly destroys NO and promotes the production of other active oxygen species, such as hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (·OH), which are responsible for lipid peroxidation and damage of cellular membranes.

In the present study, to determine the role of renin–angiotensin system in the insulin-resistant state, we examined the oxidases responsible for O$_2^-$ production and Ang II receptor expression in vascular tissues and the relationship with vasoreactivity in rodent models of insulin resistance induced by feeding of high-fructose diet.

**Methods**

**Materials**

Losartan was provided by Merck (Rahway, NJ), A$^5$-nitro-l-arginine (L-NA) was purchased from Peptide Institute (Minoh, Japan). Pae- paverine hydrochloride was obtained from Dainippon (Osaka, Japan). Monoclonal antibodies against murine eNOS and p67$^{phox}$ were purchased from Transduction Laboratories (San Diego, Calif) and monoclonal antibodies against murine p22$^{phox}$ and gp91$^{phox}$ were kindly provided by D. Roos (University of Amsterdam). All other materials were purchased from Sigma Chemical (St. Louis, Mo).

**Experimental Animals**

Six-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) were divided into 4 groups and fed ad libitum one of the following diets for 8 weeks: standard chow (control rats), standard chow supplemented with 30 mg/kg per day losartan (AT1 receptor antagonist), a diet high in fructose (fructose-fed rats), or a diet high in fructose with 30 mg/kg per day losartan. In a separate study, AT1a receptor knockout (AT1a KO) mice aged 6 weeks and age-matched C57BL/6 control wild-type (WT) mice from the same genetic background were used as previously described. The two groups of mice were randomly assigned to 1 of 2 regimens for 8 weeks:

1. standard chow or
2. fructose-enriched chow.

The normal chow consisted of 58% carbohydrate, 12% fat, and 30% protein. The high-fructose diet contained 67% carbohydrate (98% of which was fructose), 13% fat, and 20% protein by calorie.

At the end of the 8-week dietary intervention, food was withdrawn for 12 hours, and the animals were weighed and anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Venous blood samples were obtained for measurement of plasma glucose, insulin (Rat Insulin RIA kit; LINCO Research, Mo), Ang II (SRL Angiotensin II set; SRL, Tokyo), total cholesterol, and triglyceride. Insulin sensitivity was measured by the steady-state plasma glucose method. Then, the aortas were isolated for the O$_2^-$ measurement or tension experiment. All procedures were approved by the animal ethics committee of Shiga University of Medical Science.

**Measurements of Ex Vivo Aortic O$_2^-$ Production and Estimation of Enzyme Sources of O$_2^-$ Production**

O$_2^-$ production in aortic segments was measured using lucigenin-enhanced chemiluminescence method as described previously.

Segments of the isolated aorta preincubated with or without the inhibitor were placed in the modified Krebs/Hepes buffer (pH 7.4) containing 0.01 mmol/L EDTA, and the chemiluminescence value was measured. The vessel was dried at 90°C for 24 hours, and then the dry weight was measured.

The vessel was homogenized in a glass homogenizer after the addition of a 400-μL homogenizing buffer (pH 7.8), and the homogenate was subjected to centrifugation (1000g) for 10 minutes. A 20-μL aliquot of the supernatant was then added to glass scintillation vials containing lucigenin (50 μmol/L) in a 2-mL homogenizing buffer and the chemiluminescence value was obtained.

**Infusion of Ang II and Measurement of Blood Pressure**

Four groups of rats aged 14 weeks were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Using sterile techniques, an incision was made in the mid-scapular region, and osmotic mini-pumps (Alzet model 2002; Alza, Palo Alto, Calif) containing Ang II (infusion rate 0.4 mg/kg per day) were implanted. Systolic and diastolic blood pressures were measured with an electrophysmomanometer after the rats were prewarmed for 15 minutes.

**Isometric Tension Studies**

Isometric tension studies were performed as previously described.

The thoracic aorta was isolated and cut into strips with special care to reserve the endothelium. The strips were exposed to cumulative concentration of L-phenylephrine (10$^{-9}$ to 3×10$^{-5}$ mol/L). Ang II (10$^{-9}$ mol/L to 10$^{-5}$ mol/L) was added directly to bathing media in single concentrations to avoid the development of tachyphylaxis. To prevent synthesis of prostaglandins, arterial strips had been treated for 20 minutes with 10 μmol/L indomethacin before test drugs were added. Finally, the strips were partially precontracted with L-phenylephrine. After the plateau was attained, the strips were exposed to calcium ionophore A23187 (10$^{-9}$ mol/L to 10$^{-7}$ mol/L) to construct dose–response curves. At the end of each experiment, 10$^{-4}$ mol/L papaverine was added to induce the maximal relaxation, which was taken as 100% for relaxation induced by agonists.

**Reverse Transcription Polymerase-Chain Reaction Analysis**

Total RNA was prepared from the thoracic aorta of 4 groups of mice using the method of Chomczynski and Sacchi and treated with DNase (Takara Shuzo, Tokyo, Japan) to eliminate contamination of genomic DNA. We used a method for quantification of mRNA levels based on reverse transcription polymerase-chain reaction (RT-PCR) because of the low levels of each component of NADPH oxidase mRNAs in the mouse aorta. The oligonucleotide primers used for RT-PCR are as follows: for AT1a receptor (220 bp), 5'-TCGCCCTTGGCTGACTTT-ATGC-3' and 5'-AACCGGTGTGTCGAGCG-3'; for AT1b receptor (450 bp), 5'-CTGCCCAAGGGCTGAGGGCC-3' and 5'-GCCACTACTGGCAGCCTT-3'; for AT2 receptor (230 bp), 5'-GTTCTGTCTTGGGATGCTTCA-3' and 5'-GGTCCAGTTCGTT-AGGTTC-3'; and for GAPDH (670 bp), 5'-GGTCCGDTGAGCAAGCC- GATTGTG-3' and 5'-CTGCCCTGTTAACCCTTAC-3'. The reaction mixture was denatured at 94°C for 45 seconds, annealed at 58°C for 1 minute, and polymerized at 72°C for 1 minute. Thirty such cycles were performed, followed by a 10-minute extension at 72°C. mRNA was visualized by Kodak electrophoresis documentation and analysis system 290 and quantified by ID image analysis software (Eastman Kodak, Rochester, NY). The quantified value of each mRNA levels was standardized with that of GAPDH. Normalized values of mRNA expression in the aortas of WT mice were arbitrarily expressed as 100%.

**Western Blots**

Western blot analysis was performed as described previously.

Protein samples (50 μg) were prepared from thoracic aortas of 4 groups of mice and denatured and run on polyacrylamide gels. After transfer onto Immobilon PVDF transfer membranes, the membranes were blocked for 90 minutes in 5% nonfat milk solution. The
primary antibodies (eNOS, p22phox, gp91phox, or p67phox) were used at a 1:1000 dilution in 5% nonfat milk solution for 12 hours at 4°C. Bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG and visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Statistical Analysis
All values are presented as means±SEM. The dose-dependent vascular contraction or relaxation in response to L-phenylephrine, Ang II, or A23187 was compared among 4 groups with 2-way ANOVA with a post hoc Scheffe comparison. Comparison among those groups for metabolic data, blood pressure chemiluminescence detection system, and vascular O2− production was found in vessels incubated in the presence of EDTA. Statistical analyses among three groups were performed using multiple comparison tests using ANOVA with a post hoc Scheffe comparison. Values are expressed as means±SEM. *P<0.01, †P<0.001 vs the corresponding vessels obtained from control.

Results
Superoxide Production From Aortas With or Without Endothelium
Basal O2− production by aortic segments with endothelium from fructose-fed rats was 1.5-fold higher than that of control rats (Table 1). Endothelial removal produced a slight reduction of O2− production in vessels from control rats, whereas the marked reduction of O2− production was found in vessels without endothelium from fructose-fed rats. Thus, after removal of the endothelium, the O2− production rates among 2 groups were no longer different. Furthermore, incubation of intact aortic segments from 2 groups with either apocynin (an inhibitor of activity/assembly of the components of NADPH oxidase) or diphenylene iodonium (an inhibitor of flavin-containing enzymes inhibitor)25 markedly attenuated the lucigenin signal, respectively. In contrast, oxypurinol (an inhibitor of xanthine oxidase), rotenone (an inhibitor of mitochondrial respiration), indomethacin (an inhibitor of cyclooxygenase), nordihydroguaiaretic acid (an inhibitor of lipoxygenase), and L-NA (an inhibitor of NOS) did not affect the lucigenin signal in intact aortic segments.

Effects of AT1 Receptor Blockade on Vascular NAD(P)H Oxidase Activity in Insulin Resistance
Consistent with increased O2− generation under ex vivo conditions, O2− production derived from either NADH or NADPH oxidases in aortic homogenates from fructose-fed rats was 2-fold higher than those from control rats (Figure 1), respectively. AT1 receptor blockade had no effect on enzyme activities in either NADH or NADPH oxidases in control rats. In fructose-fed rats, AT1 receptor blockade for 8 weeks inhibited NAD(P)H oxidase activities and significantly attenuated vascular O2− production (44.2±3.9 cpm×103/mg of dry weight of vessel in fructose-fed rats versus 10.2±2.0 cpm×103/mg of dry weight of vessel in fructose-fed rats with losartan treatment).

Effects of AT1 Receptor Blockade on Metabolic Characteristics of the Rats
All 4 treatment groups gained weight to a similar degree over the study period without any significant difference (Table 2).

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**TABLE 1. Superoxide Anion Production From Aortic Vessels Incubated in the Absence or Presence of Various Inhibitors**

<table>
<thead>
<tr>
<th>Endothelium</th>
<th>Incubation Condition</th>
<th>N</th>
<th>Control</th>
<th>Fructose</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>Buffer alone</td>
<td>4</td>
<td>10.5±1.4†</td>
<td>14.7±0.8‡</td>
</tr>
<tr>
<td>+</td>
<td>Buffer alone</td>
<td>13</td>
<td>29.4±1.8</td>
<td>44.2±3.9§</td>
</tr>
<tr>
<td>+</td>
<td>Apocynin (100 μmol/L)</td>
<td>5</td>
<td>12.2±0.8*</td>
<td>9.6±2.0</td>
</tr>
<tr>
<td>+</td>
<td>Diphenylene iodonium (10 μmol/L)</td>
<td>5</td>
<td>11.3±2.8*</td>
<td>9.0±1.4†</td>
</tr>
<tr>
<td>+</td>
<td>Oxypurinol (100 μmol/L)</td>
<td>5</td>
<td>33.4±2.0</td>
<td>46.5±5.0‡</td>
</tr>
<tr>
<td>+</td>
<td>Rotenone (100 μmol/L)</td>
<td>5</td>
<td>27.0±0.5</td>
<td>49.0±6.1§</td>
</tr>
<tr>
<td>+</td>
<td>Indomethacin (10 μmol/L)</td>
<td>5</td>
<td>26.8±1.7</td>
<td>41.5±3.9‡</td>
</tr>
<tr>
<td>+</td>
<td>Nordihydroguaiaretic acid (10 μmol/L)</td>
<td>5</td>
<td>25.4±2.1</td>
<td>42.9±3.8‡</td>
</tr>
<tr>
<td>+</td>
<td>L-NA (10 μmol/L)</td>
<td>5</td>
<td>27.7±0.9</td>
<td>37.1±4.6</td>
</tr>
</tbody>
</table>

Vascular superoxide production was measured in the presence of EDTA. Statistical analyses among three groups were performed using multiple comparison tests using ANOVA with a post hoc Scheffe comparison. Values are expressed as means±SEM. †P<0.01, ‡P<0.001 vs the corresponding vessels with endothelium incubated in buffer alone using Student unpaired t test. ¶P<0.05, §P<0.01 vs the corresponding vessels obtained from control.

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**Figure 1.** Comparison of vascular superoxide anion production from vascular homogenates in the presence of (A) 00 μmol/L NADH and (B) 500 μmol/L NADPH between control rats (n=6) and fructose-fed rats (n=6). Data are expressed as means±SEM. †P<0.001 versus control rats (C). ‡P<0.001 versus control rats with losartan treatment (C+Lo). §P<0.01 versus fructose-fed rats with losartan treatment (F+Lo).
Both high levels of fructose feeding and losartan treatment did not affect fasting plasma glucose levels. Fructose-fed rats showed significant elevations of plasma insulin, total cholesterol, triglyceride, and steady-state plasma glucose compared with control rats. Treatment with losartan partially but significantly reversed these values in fructose-fed rats, whereas losartan did not affect any of these parameters in control rats. High levels of fructose feeding tended to increase plasma Ang II levels compared with control rats. Losartan treatment showed 2.6-fold and 3.3-fold elevations of plasma Ang II compared with control rats, respectively.

Pressor Response to Ang II Infusion and AT1 Receptor Blockade

Ang II infusion caused a progressive increase in mean blood pressure from 113±2 mm Hg to 147±3 mm Hg by day 4 in control rats (Figure 2). Fructose-fed rats showed significant elevations of basal mean blood pressure (127±4 mm Hg) compared with control rats. Increment of blood pressure in response to Ang II was markedly increased in fructose fed rats (184±6 mm Hg by day 4) compared with control rats. Losartan prevented the increase in blood pressure in response to Ang II infusion in either control or fructose-fed rats. There was no significant difference in heart rate among the 4 groups of rats.

Vascular Reactivity of Aortic Strips in Response to L-Phenylephrine, Ang II, and A23187

The contractile responses of aortic strips to L-phenylephrine and Ang II are shown in Figure 3. The contraction induced by L-phenylephrine in the fructose-fed rats was similar to that in control rats. Losartan treatment did not affect L-phenylephrine-induced contraction in control and fructose-fed rats. By contrast, contraction in response to Ang II was markedly increased in fructose-fed rats compared with control rats. Losartan treatment elicited a lesser contractile response to Ang II in either control or fructose-fed rats.

The addition of A23187 produced a dose-dependent relaxation in intact strips (Figure 3). The maximal response (70.7±3.0%) was significantly reduced and the ED50 was increased in fructose-fed rats as compared with those in control rats (83.1±4.4%), whereas the curve from fructose-fed rats was significantly improved by the losartan treatment (84.7±2.1%).

RT-PCR Analysis of AT1 and AT2 Receptor mRNA in Murine Aortas

Expressions of AT1a, AT1b, and AT2 in the aorta were compared among WT and AT1a KO mice with normal and fructose diets (Figure 4). Although AT1a gene was expressed abundantly in WT aortas, AT1a was not amplified by 30-cycle PCR in aortas of AT1a KO mice. The mRNA levels of AT1a were markedly enhanced (1.6-fold) in fructose-fed mice compared with WT mice. AT1b and AT2 were expressed in 4 groups of mice at almost the same levels.

Protein Expression of eNOS and Subunits of NADPH Oxidase

Expression of eNOS did not differ among 4 groups of mice. Western blot analysis of either p22phox or gp91phox expression of aortic tissue was markedly increased in fructose-fed mice compared with WT mice. In addition, p67phox expression was also upregulated in fructose-fed mice compared with WT mice. In contrast, expression of p23phox, gp91phox, or p67phox was downregulated in AT1a KO mice with high levels of fructose feeding. These data are shown in Figure 5.

Discussion

In the present study, we demonstrated that insulin-resistant state is associated with AT1a receptor upregulation and
increased endothelial $\mathrm{O}_2^-$ production caused by an activation of NAD(P)H oxidase. The treatment with the AT1 receptor blocker normalized the activity of the oxidase, improved endothelial function, and effectively prevented the Ang II-induced pressor and contractile responses in the insulin-resistant rats. In addition, expression of AT1a receptor and all subunits of NADPH oxidase was upregulated in these states. All these findings suggest a pathogenic role of the renin–angiotensin system in the development of insulin-resistance-induced hypertension.

An increasing body of evidence suggests that vascular oxidative stress is involved in the pathogenesis of many cardiovascular disorders in the insulin-resistant states, including diabetes, hypertension, and atherosclerosis. Among many enzymatic systems producing reactive oxygen species, NAD(P)H oxidase and uncoupled eNOS have been extensively studied in vascular cells. In the present study, we measured the vascular $\mathrm{O}_2^-$ production using intact aortic segments. As a result, aortic segments in the absence of EDTA showed a 1.7-fold ($18.4 \pm 3.2 \text{ cpm}^{10}/\text{mg of dry weight of vessel}$) elevation of the basal $\mathrm{O}_2^-$ production compared with those in the presence of EDTA. Thus, calcium-independent and calcium-dependent (primarily eNOS-derived) enzymatic systems are likely to be sources of $\mathrm{O}_2^-$ production in these states.

Our present findings are in accordance with previous observations showing that insulin resistance is associated with increased vascular $\mathrm{O}_2^-$ production and endothelial dysfunction. In fructose-fed rats, vascular $\mathrm{O}_2^-$ production measured in the presence of EDTA was increased 1.5-fold compared with that of control rats. Incubation of vessels with either apocynin or diphenylene iodonium markedly attenuated the $\mathrm{O}_2^-$ production, whereas oxyipurinol, rotenone, indomethacin, nordihydroguaiaretic acid, and 1-NA were effective. Therefore, it appears that NAD(P)H oxidase is a major source of $\mathrm{O}_2^-$ generation in these vessels, whereas the yield of $\mathrm{O}_2^-$ and lipid hydroperoxide from lipoxygenase and/or cyclooxygenase pathway is much less than that from NAD(P)H oxidase. NAD(P)H oxidase has recently been demonstrated to be the predominant $\mathrm{O}_2^-$ source in endothelial and smooth muscle cells. Endothelial removal produced no significant reduction in NAD(P)H oxidase activities in homogenates from control rats, whereas there was marked reduction of the enzyme activities in homogenates from fructose-fed rats. These findings indicate that insulin-resistant state results in enhanced NAD(P)H oxidase activities only in the presence of endothelium.

G-protein-coupled membrane oxidases are responsible for the generation of $\mathrm{O}_2^-$ at the cell surface, such as activation of NADPH oxidase by an amyloidogenic peptide in neurons or by Ang II in vascular endothelial and smooth muscle cells. To gain insight into how insulin resistance increases the oxidase activity, Ang II-induced vasoconstricting responses were assessed in vivo and in vitro. We found that the contractile response to Ang II was enhanced in the fructose-fed rats compared with the control rats. One may consider that the greater contractile response to Ang II is a manifestation of attenuated NO in these strips. However, we could not find any difference in vasoconstrictor response to L-phenylephrine. In addition, in the present study, AT1 receptor blockade inhibited NAD(P)H oxidase activities and the Ang II-induced vasoconstriction, and in parallel improved endothelial dysfunction in the insulin-resistant state. Thus, it is reasonably speculated that insulin-resistant state is related to overfunction of Ang II, probably caused by upregulation of AT1 receptor number and affinity for the agonist. Heart rate was not significantly altered in the fructose-fed rats. Similar observation has been reported by Kamide et al, suggesting that cardiac $\alpha_1$, but not $\beta$, receptor density was increased by elevated Ang II in fructose-fed rats.

Because the lack of discriminatory pharmacological antagonists made it impossible to define the individual functions of the two AT1 receptor subtypes (AT1a and AT1b), we examined the pathophysiological roles of AT1 receptor using AT1a KO mice. Fructose-fed control mice showed the similar metabolic characteristics and vascular hyperreactivity to Ang II as seen in fructose-fed rats (Shinozaki et al, 2003, unpublished observations). We found that AT1a receptor expression was dramatically increased in aortic tissues from insulin-resistant mice. The increase in Ang II receptor expression was caused by an increase in AT1a expression, because expression of AT1b and AT2 receptors was unaltered. Pueyo et al have characterized the Ang II receptor subtypes in rat endothelial and smooth muscle cells in primary culture, showing that endothelial cells express AT1a and AT1b, whereas smooth muscle cells express only AT1a. In the vascular wall, Ang II induces vasoconstriction by a direct action on smooth muscle cells, but this effect might be modulated by Ang II interaction with endothelial cells. In the present study, we demonstrated that endothelial removal produced a slight reduction of $\mathrm{O}_2^-$ levels in vessels from control rats but marked reduction of $\mathrm{O}_2^-$ production in vessels from fructose-fed rats. In addition, endothelial removal produced no significant reduction in NAD(P)H oxidase activities.
in homogenates from control rats but marked reduction of those enzyme activities in homogenates from fructose-fed rats. These data indicate that insulin-resistant state may stimulate the generation of \( \text{O}_2^- \) through the activation of NAD(P)H oxidase in aortic endothelial cells in in vivo conditions.

In the present study, losartan normalized NAD(P)H oxidase activities but significantly inhibited (30% of the control rats) vascular \( \text{O}_2^- \) production in control and fructose-fed rats. In addition, the AT1 receptor blockade showed markedly elevated plasma Ang II compared with control rats, suggesting the interference with the negative feedback of Ang II on the synthesis and release of renin from the kidneys. Recent in vitro study has shown that AT2 receptor functionally antagonizes the AT1 receptor-induced endothelial \( \text{O}_2^- \) production by a pathway involving tyrosine phosphatases. Consequently, increased stimulation of AT2 receptors during AT1 receptor blockade may inhibit endothelial \( \text{O}_2^- \) production in these rats.

Because the stimulation of AT1a receptor by Ang II leads not only to direct activation of the \( \text{O}_2^- \)-generating NAD(P)H oxidase but also to an enhanced expression of essential subunits (p22\(^{phox}\), gp91\(^{phox}\), p67\(^{phox}\)) of this enzyme, the decreased expression of these subunits in the aorta of fructose-fed AT1a KO mice may contribute to the observed reduction of vascular \( \text{O}_2^- \) production and NAD(P)H oxidase activity. Using molecular biological approaches, the presence of mRNAs for gp91\(^{phox}\), p22\(^{phox}\), p47\(^{phox}\), and p67\(^{phox}\) has been demonstrated in endothelial cells and in adventitial cells. Vascular smooth muscle cells appear to express p22\(^{phox}\), p47\(^{phox}\) and p67\(^{phox}\) but not gp91\(^{phox}\). Indeed, gp91\(^{phox}\) appears to be crucial for the endothelial \( \text{O}_2^- \) production, because KO mice with the gp91\(^{phox}\) gene abolished \( \text{O}_2^- \) production in endothelium-intact aortic segments and exhibited a more pronounced endothelium-dependent relaxation than that observed in aorta from WT mice. Therefore, our findings in rodent vessels support a potential mechanistic relationship.

**Figure 4.** Angiotensin II receptor mRNA levels in the aorta of wild-type (WT) and AT1a knockout (AT1a KO) mice. RT-PCR analysis was performed to evaluate AT1a, AT1b, and AT2 mRNA levels in the WT and AT1a KO mice feeding with either normal diet or high levels of fructose. Amplification of GAPDH is shown to demonstrate that equal amounts of mRNA were used for RT-PCR. The normalized values in the WT mice with normal diet are arbitrarily expressed as 100%. *\( P < 0.01 \), †\( P < 0.001 \) versus WT mice with normal diet. ‡\( P < 0.001 \) versus WT mice with fructose diet.
between upregulation of AT1 receptors and NAD(P)H oxidase-dependent endothelial O$_2^-$ production, proposed on the basis of similar findings in human blood vessels from diabetic patients.\textsuperscript{39}

The precise cellular mechanisms by which AT1a receptor expression is upregulated in the insulin-resistant state are not clear. We have recently reported that lipid peroxide content was markedly increased and DNA binding activities of 2 redox-sensitive transcription factors (nuclear factor-$\kappa$B and activating protein-1) were significantly increased in the fructose-fed rats.\textsuperscript{6} In addition, clinical and experimental studies have shown that NO synthesis in the insulin-resistant state is inhibited in the insulin-resistant state.\textsuperscript{2,3,5,6} Interestingly, recent reports have shown that chronic inhibition of NO synthesis and/or increment of reactive oxygen species may directly upregulate AT1 receptor expression.\textsuperscript{40} Nickenberg et al have reported that Ang II receptor gene expression is upregulated in rat aortic tissues by LDL.\textsuperscript{41} Nuclear factor-$\kappa$B-responsive elements have also been found in the promoter region of the AT1 receptor gene.\textsuperscript{42} Thus, roles of oxidative stress and/or redox-sensitive transcription factor in the expression of AT1 receptors remain to be elucidated.

The present study shows that treatment with AT1 receptor antagonist losartan partially prevents the elevation in plasma insulin levels and triglyceride in fructose-fed rats. In addition, losartan treatment improved insulin sensitivity in these rats. Consistent with our results, clinical and pharmacological studies have shown that Ang II infusion induces insulin resistance and that ACE inhibitors and AT1 receptor antagonists improve the insulin sensitivity.\textsuperscript{5,34} Therefore, overactivity of renin–angiotensin system is likely to impair insulin signaling and contribute in part to the pathogenesis of insulin resistance observed in hypertensive states.

**Perspectives**

Our findings provide evidence of a novel regulatory pathway by which local renin–angiotensin system modulates vascular function in vivo in the insulin-resistant state. The current study suggests that Ang II, through AT1a receptor, plays a critical role in the vascular dysfunction associated with insulin resistance. Furthermore, AT1 receptor antagonist losartan, besides having antihypertensive effects, could be an important therapeutic tool to reduce the development of vascular disorders in the insulin-resistant state.

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