Role of angiotensin II and oxidative stress in vascular insulin resistance linked to hypertension

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Zhou MS, Schulman IH, Raij L. Role of angiotensin II and oxidative stress in vascular insulin resistance linked to hypertension. Am J Physiol Heart Circ Physiol 296: H833–H839, 2009. First published January 16, 2009; doi:10.1152/ajpheart.01096.2008.—In insulin activation of the phosphatidylinositol 3-kinase (PI3K) pathway stimulates glucose uptake in peripheral tissues and synthesis of nitric oxide (NO) in the endothelium. Insulin resistance (IR) and hypertension frequently coexist, particularly among individuals with salt-sensitive hypertension. The mechanisms underlying this association are poorly understood. We investigated these mechanisms in a model of salt-sensitive hypertension in which we have previously shown that endothelial dysfunction is mediated by superoxide anion ($O_2^-$) linked to local ANG II. Dahl salt-sensitive rats were fed, for 6 wk, a normal salt diet (NS; 0.5% NaCl), high-salt diet (HS; 4% NaCl), HS plus the ANG II type 1 receptor (AT1R) blocker (ARB) candesartan (10 mg·kg$^{-1}$·day$^{-1}$), or HS plus the antioxidant tempol (172 mg/l in drinking water). Hypertensive (mean arterial pressure: 145 ± 4 vs. 102 ± 5 mmHg in NS, $P < 0.05$) rats manifested increased aortic AT1R mRNA (210%) and protein (101%) expression and $O_2^-$ production (104%) and impaired endothelium-dependent relaxation (EDR) to acetylcholine [maximal response ($E_{max}$): 68 ± 9 vs. 91 ± 8% in NS, $P < 0.05$]. ARB or tempol normalized $O_2^-$ and EDR despite that they did not normalize mean arterial pressure, which was reduced only 25%. Hypertensive rats manifested metabolic IR (36% reduction in the glucose infusion rate by insulin clamp), impaired NO-mediated insulin-induced EDR ($E_{max}$: 12 ± 5 vs. 32 ± 4% in NS, $P < 0.05$), and impaired insulin activation of PI3K/endothelial NO synthase. ARB or tempol improved insulin-mediated EDR, PI3K, Akt/ endothelial NO synthase phosphorylation, and metabolic IR (all $P < 0.05$). This study provides insight into the mechanisms that underlie the association between metabolic and hypertensive cardiovascular diseases and support the notion that $O_2^-$ overproduction linked to tissue ANG II interferes with shared insulin signaling pathways in metabolic and cardiovascular tissues.

derived NO synthase (eNOS) via PI3K/Akt stimulates NO synthesis and thereby promotes vasodilatation (19, 35, 42) and contributes to the inhibition of vascular smooth muscle growth and prevention of atherogenesis as well as regulation of blood flow in the skeletal muscle (24).

Clinical and experimental studies have confirmed that in states of insulin resistance, the PI3K/Akt pathway is dysfunctional, which results in impaired glucose metabolism in peripheral tissues and reduced insulin-mediated NO synthesis in the endothelium (7, 17, 19). The aforementioned studies have also shown that in insulin-resistant states, insulin activation of the vascular MAPK pathway is maintained unopposed, and, under those conditions, insulin may contribute to pathological vascular remodeling (7, 17).

Inhibition of the renin-angiotensin-aldosterone system (RAAS) has been shown to reduce or delay the development of diabetes in some (1, 13, 16) but not all clinical trials (8a). The RAAS and ROS have been implicated in hypertension, atherosclerosis, and insulin resistance (25). In previous studies, we have demonstrated that in the Dahl salt-sensitive (DS) rat, a paradigm of salt-sensitive hypertension characterized by cardiovascular disease and insulin resistance (20, 33, 43), there is a link between tissue ANG II, increased ROS production, decreased NO bioactivity, and impaired endothelium-dependent relaxation to acetylcholine (ACh) (43, 44). Using this experimental model, we investigated the mechanisms whereby upregulation of ANG II-derived ROS may participate in the impairment of vascular insulin signaling and resistance to the metabolic actions of insulin in salt-sensitive hypertension.

METHODS

Animals and experimental protocols. Animals were housed in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use Committee of the Miami Veterans Affairs Medical Center approved this study. Six-week-old male DS rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) and maintained under controlled conditions of light, temperature, and humidity. After 2 wk of accommodation to the new environment, rats were divided into four groups and treated for 6 wk as follows: fed a 0.5% NaCl diet [normal salt diet (NS) group], fed a 4% NaCl diet [high-salt diet (HS) group]; fed a 4% NaCl diet plus the ANG II type 1 receptor (AT1R) blocker (ARB) candesartan (10 mg/kg in drinking water; HS/ARB group); fed a 4% NaCl diet plus the antioxidant tempol, a SOD mimetic (172 mg/l in drinking water).

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drinking water; HS/tempol group). At the end of the study, rats were killed by decapitation, and aortas were harvested.

**Hyperinsulinemic-euglycemic clamp experiments and determination of intra-arterial pressure.** A group of rats was used to investigate metabolic insulin sensitivity by the hyperinsulinemic-euglycemic clamp method as previously described (30). Animals were fasted overnight for 16 h before the experiments were conducted. Animals were anesthetized with pentobarbital sodium (50 mg/kg ip). The right femoral vein and left femoral artery were catheterized and used for glucose and insulin infusion and blood glucose sampling, respectively. Mean arterial pressure (MAP) was measured from the left femoral artery before the insulin clamp experiments were conducted. The arterial catheter was connected to a pressure transducer, and MAP was recorded by Powerlab. After a 30-min equilibration period, MAP was continuously measured for 60 min (45). After the MAP measurement, baseline plasma samples were obtained for measurements of glucose, insulin, and C-reactive protein (CRP). Insulin (from the bovine pancreas, Sigma-Aldrich) at a constant rate of 30 µU/kg \(^{-1}\)·min \(^{-1}\) and glucose (17.5 g/100 ml saline) at varying infusion rates were continuously infused for 120 min. The blood glucose concentration, measured with an automatic blood glucose meter (Accu-Chek Advantage Blood Glucose Meter), was clamped at 5.5 mmol/l. The glucose infusion rate was adjusted according to the blood glucose levels at 5-min intervals during the first 60-min period and, once stable, at 15-min intervals during the second 60-min period. Data were obtained from the second 60-min period of insulin infusion. After the insulin clamp, rats were killed, and aortas harvested and used for immunoprecipitation and Western blot analysis to determine insulin signaling protein expression as described below.

**Determination of endothelium-dependent relaxation to insulin and ACh in the aorta.** A separate group of rats was used for these experiments. Rats were killed by decapitation, and aortas were immediately removed. Vessels were cleaned of adherent connective tissue, and rings (3 mm) were cut and used for organ chamber experiments. The remainder of the aorta was collected for the determination of superoxide anion (O\(_2^−\)) production and protein expression of AT\(_1\)Rs, ANG II type 2 receptors (AT\(_2\)Rs), and JNK, as described below. Endothelium-dependent relaxation (EDR) to ACh and insulin in aortic rings was examined using an organ chamber as we have previously described (43, 44). EDR in response to ACh (10\(^{-6}\)–10\(^{-5}\) mol/l) was assessed in aortic rings precontracted to 70% of the maximal contraction to norepinephrine. After washout, EDR to insulin (10\(^{-6}\)–10\(^{-6}\) mol/l) was assessed in aortic rings precontracted to 70% of the maximal contraction to norepinephrine in the absence or presence of a 30-min incubation with wortmannin, a specific inhibitor of PI3K (100 mol/l). Data were expressed as the maximal response (\(E_{\text{max}}\)) to ACh or insulin and the concentration of ACh or insulin required for a half-maximal response (ED\(_{50}\)).

**Immunoprecipitation and immunoblot analysis for insulin signal- ing proteins.** Aortic tissue was harvested immediately after the hyperinsulinemic-euglycemic clamp. After homogenization and centrifugation, the supernatant (500 µg protein) was immunoprecipitated by an incubation with anti-insulin receptor substrate (IRS)-1 antibody, followed by immunoblot analysis with an antibody against the p85 subunit of PI3K. Total IRS-1, phospho-Ser\(^{1177}\)-IRS-1, phospho-Thr\(^{183}/\text{Y}^{185}\)-Akt, and phospho-Ser\(^{612}\)-IRS-1 antibodies were determined by Western blot analysis using specific polyclonal antibodies. The expression of phospho-Ser\(^{1177}\)-IRS-1 and association of the p85 subunit of PI3K with IRS-1 was normalized to total IRS-1 and further expressed as the fold increase versus the NS group. The expression of phospho-eNOS or phospho-Akt was normalized by total eNOS or Akt, respectively, and expressed as the fold increase versus the NS group. All antibodies were obtained from Cell Signaling Technology.

**Determination of plasma insulin and CRP levels.** Plasma was collected from the animals immediately before insulin clamp experiments were started. Plasma levels of insulin (Alpco Diagnostics) and CRP (BD Biosciences) were determined by an ELISA assay kit following the manufacturer’s instructions. Concentrations were calculated from a standard curve.

**Detection of aortic \(O_2^-\) generation.** \(O_2^-\) generation in intact aortic rings was determined by chemiluminescence of lucigenin (5 µM) as described previously (43, 44), and the results were expressed as counts per minute per milligram of dry tissue. Chemiluminescence of lucigenin has been validated as a method to measure \(O_2^-\) production. In the present and previous studies (43, 44), the specificity of lucigenin to assess aortic \(O_2^-\) generation was confirmed by preincubation of the aortic rings with tiron, which resulted in a 90% reduction in \(O_2^-\) measurement (data not shown).

**Determination of AT\(_1\)Rs, AT\(_2\)Rs, and JNK protein expression.** Protein expression of AT\(_1\)Rs and AT\(_2\)Rs in the aorta was determined by Western blot analysis. Briefly, after homogenization, the protein concentration was determined by the Bio-Rad assay. Protein (50 µg) was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Transferred proteins were incubated overnight with specific primary antibodies against the AT\(_1\)R or AT\(_2\)R (Santa Cruz Biotechnology, Santa Cruz, CA). After being washed, blots were incubated with the appropriate secondary antibody, and the signal was detected by luminol chemiluminescence followed by an exposure to an autoradiography film. The membrane was reblotted for β-actin as a loading control. JNK and phospho-JNK (active form of JNK-1) were determined by Western blot analysis using anti-JNK or phospho-JNK antibodies (Thr\(^{183}/\text{Y}^{185}\), Cell Signaling, Danvers, MA).

**Real-time PCR for mRNA expression of the AT\(_1\)R.** Total RNA (2 µg) was extracted from the rat aorta and reverse transcribed using the SuperScript II RT First Strand Synthesis kit (GIBCO-BRL) according to the manufacturer’s instructions. Real-time PCR for the AT\(_1\)R was performed in a 50-µl reaction mixture containing an appropriately diluted (80 ng) cDNA solution, 0.1 µmol/l of each primer, 0.2 µmol/l of probe, and the PCR Master Mix assay kit (ABI) as previously described (46). The relative expression of each mRNA was normalized by a housekeeping gene (GAPDH) and expressed as the fold increase versus the NS group.

**Data analysis.** Results are expressed as means ± SE. \(E_{\text{max}}\) values to ACh or insulin and ED\(_{50}\) values of ACh or insulin were determined from concentration-response curves using the best fit to a logistic sigmoid function. Statistical analyses were performed by ANOVA with Bonferroni’s correction for multiple comparisons followed by Scheffé’s test. Significance was assumed at \(P < 0.05\).

**Table 1. Body weights, MAP, fasting glucose and fasting insulin levels, plasma CRP levels, and \(E_{\text{max}}\) and ED\(_{50}\) values of rats in the NS, HS, HS/ARB, and HS/tempol groups**

<table>
<thead>
<tr>
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<th>NS</th>
<th>HS</th>
<th>HS/ARB</th>
<th>HS/tempol</th>
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<tr>
<td>Body weight, g</td>
<td>407±9</td>
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<td>407±9</td>
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<tr>
<td>MAP, mmHg</td>
<td>102±5</td>
<td>102±5</td>
<td>102±5</td>
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<tr>
<td>Fasting glucose, mg/dl</td>
<td>90±5</td>
<td>90±5</td>
<td>90±5</td>
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<tr>
<td>Fasting insulin, mg/ml</td>
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<td>3.5±0.9</td>
<td>3.5±0.9</td>
<td>3.5±0.9</td>
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<tr>
<td>Plasma CRP, µg/ml</td>
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<td>261±16</td>
<td>261±16</td>
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<tr>
<td>E(_{\text{max}}), %relaxation</td>
<td>91±8</td>
<td>68±9*</td>
<td>94±10†</td>
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<tr>
<td>E(_{\text{max}}), log M</td>
<td>7.3±0.1</td>
<td>6.9±0.1*</td>
<td>7.4±0.2</td>
<td>7.2±0.1†</td>
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Values are means ± SE; \(n = 6–7\). Rats were divided into the following four groups: normal salt diet (NS), high-salt diet (HS), HS diet with ANG II type 1 blocker treatment (HS/ARB), and HS diet with tempol treatment (HS/tempol). MAP, mean arterial pressure; CRP, C-reactive protein; \(E_{\text{max}}\), maximal relaxation. *\(P < 0.05\) vs. the NS group; †\(P < 0.05\) vs. the HS group.
RESULTS

Mean blood pressure and body weight. DS rats in the HS group had significant increases in MAP (145 ± 4 vs. 102 ± 5 mmHg in NS, P < 0.05), as assessed by direct intra-arterial measurements. In rats receiving HS, treatment with either the ARB candesartan (HS/ARB group) or antioxidant tempol (HS/temp group) resulted in modest reductions (~25%) in blood pressure; the rats, however, remained severely hypertensive (Table 1). Hypertensive DS rats manifested a tendency toward decreased body weight gain compared with NS rats, but this did not reach statistical significance (P = 0.09). Neither ARB nor tempol treatment affected body weight (Table 1).

Metabolic insulin sensitivity. The glucose infusion rate, an index of metabolic insulin sensitivity, was determined by hyperinsulinemic-euglycemic clamp. As shown in Fig. 1, the glucose infusion rate necessary to maintain plasma glucose at 5.5 mmol/l during the insulin infusion period was significantly reduced in hypertensive DS rats and significantly improved with ARB or tempol treatment. Rats were divided into the following four groups: normal salt diet (NS), high-salt diet (HS), HS with ARB treatment (HS/ARB), and HS with tempol treatment (HS/temp). Data are expressed as means ± SE; n = 6–7. *P < 0.05 vs. the NS group; †P < 0.05 vs. the HS group.

EDR to ACh and insulin. As in our previous studies (43, 44), EDR to ACh was significantly attenuated in aortic rings of hypertensive DS rats, as demonstrated by $E_{\text{max}}$ and $E_{D50}$ (Table 1). Treatment with either the ARB candesartan or tempol significantly improved EDR to ACh (Fig. 2 and Table 1). Furthermore, preincubation with wortmannin, a specific inhibitor of PI3K, almost completely inhibited insulin-induced vasorelaxation in all groups (Fig. 3B), suggesting that insulin signaling via the PI3K pathway is essential for insulin-mediated vasorelaxation in this animal model.

Protein expression of insulin signaling molecules. Insulin induces vasorelaxation and glucose transport via the activation
of the IRS-1/PI3K/Akt pathway (35). As shown in Fig. 4, the expression of total IRS-1 was not significantly different among the NS group, HS group, and HS groups treated with ARB or tempol. However, phospho-Ser612-IRS-1 as well as the ratio of phospho-Ser612-IRS-1 to IRS-1 were significantly increased in the HS group compared with the NS group and was normalized by ARB or tempol treatment. It has been shown that the phosphorylation of IRS-1 on Ser612 causes dissociation of the p85 subunit of PI3K, inhibiting further signaling. Consistent with this finding, downstream insulin signaling, including the association of IRS-1 with the p85 subunit of PI3K and the phosphorylation (activation) of Akt at Ser473 and eNOS at Ser1177, was significantly reduced in the aorta of hypertensive DS rats and restored in rats treated with ARB or the antioxidant tempol (Fig. 4).

**Serum CRP, aortic O$_2^-$ production, and aortic phospho-JNK protein expression.** Systemic inflammation, oxidative stress, and ANG II play a causal role in various settings of insulin resistance, including obesity and type 2 diabetes (8, 32, 41). Serum levels of CRP, an index of systemic inflammation, were slightly but significantly increased in hypertensive DS rats compared with normotensive DS rats and were normalized by treatment with ARB or tempol (Table 1). We (43, 44) have previously shown that hypertensive DS rats manifest increased vascular O$_2^-$ production, as determined by lucigenin and confocal fluorescence microscopy. Consistent with our previous findings, in the present study, hypertensive DS rats manifested a significant increase in aortic O$_2^-$ production compared with normotensive DS rats. Treatment with ARB or the antioxidant tempol significantly reduced vascular O$_2^-$ production (Fig. 5).

**Expression of AT$_1$Rs and AT$_2$Rs.** Aortic mRNA and protein expression of the AT$_1$R were determined by real-time PCR and Western blot analysis, respectively. As shown in Fig. 7, mRNA and protein expression of the AT$_1$R were significantly increased in aortas of hypertensive DS rats. Treatment with ARB or tempol did not affect the protein expression of the AT$_1$R. There were no significant differences in the protein expression of aortic AT$_2$Rs between normotensive and hypertensive DS rats. Treatment with ARB or tempol did not affect the protein expression of the AT$_2$R (Fig. 7C).

**DISCUSSION**

Clinically, hypertension and insulin resistance frequently coexist, and this association, which fosters cardiovascular disease (18, 21), is particularly prevalent in individuals with salt sensitivity of hypertension (9, 11, 34). The mechanisms that
participate in the coupling of hypertension and insulin resistance are incompletely understood. We investigated these mechanisms in an experimental model of low renin, salt-sensitive hypertension (43, 44). We demonstrated that ROS originating from local activation of the RAAS contribute to the development of metabolic insulin resistance and impaired insulin-mediated vascular NO synthesis and relaxation as well as vascular activation of JNK, a proinflammatory cytokine.

Insulin plays an important role in the regulation of metabolic and hemodynamic homeostasis (6, 24). Insulin stimulates NO production, via activation of the PI3K pathway, in the vascular endothelium, which may contribute to the vasodilatory, anti-inflammatory, and antithrombotic effects of insulin. Therefore, under physiological conditions, the constitutive stimulation of NO production by insulin may play a crucial role in the maintenance of vascular health. Diminished insulin sensitivity through PI3K may contribute to the induction of deleterious changes in the vascular endothelium, which foster the progression of vascular disease. Our study demonstrates that hypertensive DS rats have impaired insulin-mediated vasorelaxation and reduced whole body glucose disposal, supporting the notion that insulin resistance may be playing a role in the pathogenesis of cardiovascular disease in this model of hypertension.

To elucidate the mechanisms underlying the impairment in insulin-mediated vasorelaxation in hypertension, we investigated insulin stimulation of the IRS-1/PI3K/Akt/eNOS signaling pathway in the aorta. Insulin signaling is initiated by circulating insulin binding to its receptor (24, 36). The insulin receptor after insulin binding undergoes a rapid tyrosine autophosphorylation that activates the receptor kinase and allows transient interaction with IRS-1. Tyrosine-phosphorylated IRS-1 binds to PI3K and results in PI3K activation, which subsequently stimulates the phosphorylation of Akt at Ser473 and eNOS at Ser1177, leading to NO synthesis (2, 24). In contrast, the phosphorylation of IRS-1 at serine residues inhibits insulin signaling and results in PI3K inactivation. It has been shown that proinflammatory cytokines, oxidative stress, or free fatty acids induce serine phosphorylation of IRS-1 and subsequently inhibit insulin signaling (4, 18, 26). In the present study, we demonstrated that the impairment of insulin signaling in the vasculature of hypertensive DS rats was linked to increased serine phosphorylation of IRS-1, which was associated with inhibition of downstream molecules of insulin signaling, including the association of IRS-1 with the p85 subunit of PI3K, phospho-Ser473-Akt, and phospho-Ser1177-eNOS.

There is increasing evidence indicating that ANG II and oxidative stress contribute to insulin resistance and other components of cardiometabolic syndrome (27, 38), including hypertension, dyslipidemia, central fat deposition, and chronic kidney disease (29). It has been shown that ANG II inhibits insulin activation of the PI3K signaling pathway in the vascular endothelium as well as in skeletal muscle cells (10, 37) and that the underlying mechanisms may involve an increase in ROS.
and proinflammatory molecule expression, such as JNK and TNF-α (2, 5, 14). These proinflammatory factors increase serine phosphorylation of IRS-1, thereby inhibiting insulin signaling. We (43, 44) have previously shown that in hypertensive DS rats, upregulation of vascular ANG II action contributed to impaired vasorelaxation to ACh (endothelial dysfunction) and increased vascular proinflammatory gene expression, which are mechanistically linked to increased ROS production. Here, we demonstrated that mRNA and protein expression of the AT1Ra was significantly increased in aortas of hypertensive DS rats, accompanied with increased aortic ROS production and activation of the proinflammatory signaling molecule JNK-1. Treatment with either the ARB candesartan or the antioxidant tempol significantly improved insulin-induced vasorelaxation and increased insulin-induced activation of PI3K and phosphorylation of Akt and eNOS, accompanied with a reduction in vascular ROS generation as well as JNK phosphorylation. These findings support the notion that the impairment in vascular insulin signaling observed in salt-sensitive hypertension is due, at least in part, to ANG II stimulation of ROS.

Of note, blood pressure was slightly reduced in the rats that received ARB or tempol treatment. These findings are consistent with our previous studies and those of others showing that inhibition of ANG II or ROS produces a minimal reduction in blood pressure in this animal model (43, 44). However, the rats remained severely hypertensive, and, therefore, it is unlikely that the observed improvements in vascular insulin signaling and metabolic insulin sensitivity are secondary to these modest changes in blood pressure. In addition, clinical studies have shown that other antihypertensive agents, such as diuretics and β-blockers, do not improve insulin sensitivity or reduce plasma glucose, despite their significant blood pressure-lowering effect (3, 22, 23).

There is accumulating evidence showing that insulin resistance occurs not only in typical metabolic tissues, such as skeletal muscle, fat, and the liver, but also in cardiovascular tissues (6, 36). NO production in response to insulin plays an important role in the maintenance of vascular homeostasis as well as glucose metabolism (24, 36). Impairments in insulin action in the vasculature may contribute to the development of vascular injury in hypertension, obesity, and type II diabetes (18). Our results suggest that in hypertension, ANG II stimulation of ROS plays a key role in vascular and metabolic insulin resistance as well as endothelial dysfunction.

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GRANTS

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