Angiotensin II Impairs the Insulin Signaling Pathway Promoting Production of Nitric Oxide by Inducing Phosphorylation of Insulin Receptor Substrate-1 on Ser$^{312}$ and Ser$^{616}$ in Human Umbilical Vein Endothelial Cells

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Abstract—It has been suggested that serine (Ser) phosphorylation of insulin receptor substrate-1 (IRS-1) decreases the ability of IRS-1 to be phosphorylated on tyrosine, thereby attenuating insulin signaling. There is evidence that angiotensin II (AII) may impair insulin signaling to the IRS-1/phosphatidylinositol 3-kinase (PI 3-kinase) pathway by enhancing Ser phosphorylation. Insulin stimulates NO production by a pathway involving IRS-1/PI3-kinase/Akt/endothelial NO synthase (eNOS). We addressed the question of whether AII affects insulin signaling involved in NO production in human umbilical vein endothelial cells and tested the hypothesis that the inhibitory effect of AII on insulin signaling was caused by increased site-specific Ser phosphorylation in IRS-1. Exposure of human umbilical vein endothelial cells to AII resulted in inhibition of insulin-stimulated production of NO. This event was associated with impaired IRS-1 phosphorylation at Tyr$^{612}$ and Tyr$^{632}$, two sites essential for engaging the p85 subunit of PI3-kinase, resulting in defective activation of PI 3-kinase, Akt, and eNOS. This inhibitory effect of AII was reversed by the type 1 receptor antagonist losartan. AII increased c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) 1/2 activity, which was associated with a concomitant increase in IRS-1 phosphorylation at Ser$^{312}$ and Ser$^{616}$, respectively. Inhibition of JNK and ERK1/2 activity reversed the negative effects of AII on insulin-stimulated NO production. Our data suggest that AII, acting via the type 1 receptor, increases IRS-1 phosphorylation at Ser$^{312}$ and Ser$^{616}$ via JNK and ERK1/2, respectively, thus impairing the vasodilator effects of insulin mediated by the IRS-1/PI 3-kinase/Akt/eNOS pathway. (Circ Res. 2004;94:1211-1218.)

Key Words: endothelium ■ angiotensin II ■ nitric oxide ■ insulin

Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis and a feature of insulin-resistant conditions, including type 2 diabetes, obesity, and hypertension.$^{1,4}$ Several preclinical and clinical studies have established the involvement of angiotensin II (AII) and its type 1 receptor (AT$_1$) in endothelial dysfunction.$^{5-7}$ Insulin promotes vasodilatation by activation of the signaling pathway involving the insulin receptor/insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI 3-kinase)/Akt and insulin signaling has been demonstrated.$^{9}$ Inhibition of RAS by angiotensin-converting enzyme inhibitors or AT$_1$ antagonists has been shown to both increase insulin sensitivity and improve endothelial function.$^{10-12}$ Evidence has been provided that AII interferes with insulin signaling in vascular cells mainly by affecting insulin-induced tyrosine phosphorylation of IRS-1 and impairing its interaction with the p85 regulatory subunit of PI 3-kinase.$^{9}$ However, it is still unclear whether AII adversely affects the downstream signaling pathway involving Akt/eNOS that controls NO production in response to insulin. A hypothesis has emerged recently that serine phosphorylation of IRS proteins decreases their ability to be phosphorylated on tyrosine, thereby attenuating insulin signaling.$^{13-17}$ Several serine residues in IRS-1 have been identified as negative regulatory sites, including Ser$^{312}$ (orthologous to Ser$^{307}$ in rat IRS-1), which is activated by c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) 1/2 activity, which was associated with a concomitant increase in IRS-1 phosphorylation at Ser$^{312}$ and Ser$^{616}$, respectively. Inhibition of JNK and ERK1/2 activity reversed the negative effects of AII on insulin-stimulated NO production. Our data suggest that AII, acting via the type 1 receptor, increases IRS-1 phosphorylation at Ser$^{312}$ and Ser$^{616}$ via JNK and ERK1/2, respectively, thus impairing the vasodilator effects of insulin mediated by the IRS-1/PI 3-kinase/Akt/eNOS pathway.
on insulin signaling is caused by an increase in site-specific serine phosphorylation in IRS-1.

Materials and Methods

Materials
Monoclonal anti-phosphotyrosine antibodies and polyclonal antibodies against IRS-1, p56 subunit of PI 3-kinase, were from Upstate Biotechnology Inc (Lake Placid, NY). Polyclonal antibody against eNOS and Ser1177-eNOS were from Santa Cruz Biotechnology Inc (Santa Cruz, Calif). Polyclonal antibodies against Akt, Ser1177-Akt, anti-Thr183/Akt, ERK1/2, phosphoryl-ERK1/2 (Thr202/Tyr204), JNK, and phospho-JNK (Thr183/Tyr185) were from Cell Signaling Technology (Beverly, Mass). Polyclonal antibodies against the insulin receptor (IR), Tyr185/Tyr187/Tyr188 in the active loop of the catalytic domain of the insulin receptor, Ser112-IRS-1, Ser616-IRS-1, Tyr210-IRS-1, and Tyr611-IRS-1 were purchased from Biosource (Camarillo, Calif). All other chemicals were from Sigma Chemical Co (St Louis, Mo). Losartan was generously provided by Merck Sharp & Dohme (Whitehouse Station, NJ). A cell-permeable inhibitor of JNK and PD98059, a reversible MEK1 inhibitor, were from Calbiochem.

Phosphospecific Phosphorylation of IRS-1, Association of IRS-1 With the p56 Subunit of PI 3-Kinase, Akt, and eNOS Phosphorylation

Human umbilical vein endothelial cells (HUVECs) were cultured for 18 hours in serum-deprived medium containing 10 mmol/L glucose and incubated for 30 minutes in the presence or absence of 100 mmol/L AII followed by stimulation with 100 mmol/L insulin for the indicated periods of time. In experiments with losartan, this was added to cells 30 minutes before AII addition. HUVECs were lysed in buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X-100, 10 mmol/L NaPO4, 100 mmol/L NaF, and 2 mmol/L sodium orthovanadate supplemented with protease inhibitor cocktail. Insoluble material was removed by centrifugation and equal amounts of supernatants were incubated with anti-IRS-1 antibody. Immune complexes were collected by incubation with protein A Sepharose for 2 hours at 4°C, incubated with anti-IRS-1 antibody, and immune complexes were collected by agitation at 30°C for 45 minutes. The reaction was terminated by transferring 25-μL aliquots onto P-81 phosphocellulose paper discs and washed in 0.75% H3PO4. The discs were washed once with acetone and air-dried, and the 32P incorporated into myelin basic protein was measured by liquid scintillation counting. Specific kinase activity was determined by subtracting the radioactivity in the absence of substrate from that in the presence of substrate. Western blotting was performed on a portion of the immunoprecipitates, and the radioactivity was normalized for ERK1/2 content.

eNOS Activity

HUVECs were serum-starved for 18 hours and incubated in the presence or absence of the indicated hormones and inhibitors. The amount of NOS activity produced by HUVECs was assayed using an NOS Detection System (Sigma, Saint Louis, Mo) that measures the ability of NOS to convert L-14C-arginine (Amersham) to L-14C-citrulline, according to the manufacturer’s instructions. Data were normalized by the amount of protein and reaction time.

Statistical Analysis

All results are given as mean±SE and were analyzed with the use of the Newman-Keuls test for ANOVA for multiple comparisons. P<0.05 was considered statistically significant.

Results

Effects of AII on Site-Specific Serine Phosphorylation of IRS-1

Increased serine phosphorylation of IRS-1 has been shown to inhibit the ability of this substrate to be tyrosine phosphorylated by the insulin receptor and to bind and activate PI 3-kinase. Among the serine kinases that have been reported to phosphorylate IRS-1 at specific sites, it has been shown that JNK phosphorylates human IRS-1 at position Ser312 and MAPK at position Ser416. Because AII activates upstream signaling pathways, leading to activation of both JNK and MAPK, we tested whether AII would induce serine phosphorylation of IRS-1 in human endothelial cells. As shown in Figures 1A and 1B, exposure of HUVECs to AII resulted in a time-dependent increase in IRS-1 phosphorylation at both Ser312 and Ser416, respectively, with maximal effect occurring after 30 minutes of incubation. These stimulatory effects of AII were paralleled by a time-dependent increase in phosphorylation of JNK and the ERK1/2 members of the MAPK family, respectively (Figures 1C and 1D). The stimulatory effects of AII on IRS-1 phosphorylation at Ser312 and Ser416 were reversed by treatment with a cell-permeable JNK inhibitor and PD98059, a reversible MEK1 inhibitor, the enzyme that directly activates ERK1/2, respectively (Figures 2A and 2B). JNK inhibitor did not affect Ser416 phosphorylation, and PD98059 did not affect Ser312 phosphorylation, thus indicating that their inhibitory effects were specific (Figures 2A and 2B). The stimulatory effects of AII on phosphorylation of Ser312 and Ser416 were also reversed by losartan in parallel with inhibition of phosphorylation of both JNK and ERK-1/2, respectively (Figures 2A and 2B). Also, insulin was able to stimulate IRS-1 phosphorylation at both Ser312 and Ser416, although to a less extent as compared with...
AII (Figure 2C). These stimulatory effects of insulin were paralleled by phosphorylation of both JNK and ERK1/2, respectively (Figures 2D through 2F). The effects of insulin and those of AII on IRS-1 phosphorylation at either Ser312 or Ser616 were not additive. Losartan reversed the stimulatory effect of AII (Figures 2A and 2B) but not that of insulin (Figures 2C through 2F). Thus, these data indicate that AII induces IRS-1 phosphorylation at Ser312 and Ser616 by activation of JNK and ERK1/2, respectively, which can be effectively reversed by their respective inhibitors.

Effects of AII on Insulin-Stimulated Tyrosine Phosphorylation of IRS-1 and IRS-1/PI 3-Kinase Docking

Because prior studies in rat aortic smooth muscle cells have shown that treatment with AII (100 nmol/L) inhibited insulin-stimulated tyrosine phosphorylation of IRS-1 and its ability to engage PI 3-kinase,8 we tested whether AII would affect insulin signaling in human endothelial cells. As shown in Figure 3A, exposure of HUVECs to AII resulted in a time-dependent inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1, with maximal effect occurring after 30 minutes of incubation. Therefore, subsequent experiments aimed at studying the inhibitory effect of AII on insulin signaling were performed with HUVECs exposed to AII for 30 minutes, a time at which AII exerted both its maximal stimulatory effect on IRS-1 phosphorylation at both Ser312 and Ser616 and its maximal inhibitory effect on insulin-stimulated tyrosine phosphorylation of IRS-1. Losartan reversed the inhibitory effect of AII in a dose-dependent manner, with maximal effect occurring at 200 nmol/L (Figure 3B). AII did not significantly alter basal tyrosine phosphorylation of IRS-1 or levels of expressed IRS-1 (Figures 3A and 4A). Nonimmune serum did not lead to immunoprecipitation of IRS-1, thus indicating that IRS-1 immunoprecipitation by anti-IRS-1 antibody was specific (Figure 4A). Because association of the p85 regulatory subunit of PI 3-kinase with tyrosine-phosphorylated IRS-1 is essential to promote downstream signaling, the effect of AII on IRS-1/p85 docking was examined by immunoprecipitation of IRS-1 from cell lysates followed by immunoblotting with anti-p85 antibody. Insulin stimulated by 1.7-fold the binding of IRS-1 to p85 subunit (P<0.01) (Figure 4B). AII treatment reduced by 30% insulin-stimulated binding of IRS-1 to p85 subunit (P<0.02). This inhibitory effect of AII on IRS-1/p85 association was reversed by losartan (Figure 4B). There is evidence that tyrosine residues in the YXXM motifs at positions 612 and 632 (Tyr612 and Tyr632) play a major role in engaging the tandem SH2 domains of p85 subunit of PI 3-kinase, thus allowing for its full activation.20 To test the possibility that impaired IRS-1/p85 association induced by AII was related to changes in phosphorylation states of these two YXXM motifs, IRS-1 was immunoprecipitated from cell lysates and immunoblotted with phosphospecific anti-Tyr612 or anti-Tyr632 IRS-1 antibody. Insulin stimulated by 3-fold phosphorylation of Tyr612 and Tyr632 on IRS-1 (P<0.01) (Figures 4C and 4D, respectively). AII treatment reduced by 40% insulin-stimulated phosphorylation of Tyr612 and Tyr632 (P<0.02). The inhibitory effect of AII on phosphorylation of Tyr612 and Tyr632 was reversed by losartan. Thus, these data indicate that AII inhibits tyrosine phosphorylation on IRS-1 sites necessary for p85 binding. Previous studies have shown that hyper-Ser phosphorylation of IRS-1 converts IRS-1 in an inhibitor of the intrinsic insulin receptor tyrosine kinase.17 To test the possibility that increased Ser phosphorylation of IRS-1 induced by AII was associated with impaired insulin-stimulated tyrosine phosphorylation of the receptor, the insulin receptor was immunoprecipitated from cell lysates and immunoblotted with anti-Tyr1157/Tyr1162/Tyr1163 phospho-
specific antibody directed to the active loop of the catalytic domain of the insulin receptor. Insulin stimulated by 4-fold tyrosine phosphorylation of the insulin receptor (P<0.002) (Figure 4E). All treatment reduced by 65% insulin-stimulated Tyr1158/Tyr1162/Tyr1163 phosphorylation of the receptor (P<0.005). Nonimmune serum did not lead to immunoprecipitation of the insulin receptor, thus indicating that immunoprecipitation of the insulin receptor by anti-IR antibody was specific (Figure 4E). The inhibitory effect of AII on Tyr1158/Tyr1162/Tyr1163 phosphorylation of the insulin receptor was reversed by losartan.

**Effects of AII on Insulin-Stimulated Activation of Akt and eNOS**

Evidence has been provided indicating that insulin regulates NO production via a pathway involving PI-3 kinase–dependent activation of Akt, which in turn leads to phosphorylation of eNOS on serine 1177.21,22 Therefore, we tested whether AII would affect insulin-stimulated Akt/eNOS activation. Insulin stimulated by 3-fold Ser473 Akt phosphorylation (P<0.01) and by 2.5-fold Thr308 Akt phosphorylation (P<0.01) (Figures 5A and 5B). All treatment reduced by 60% insulin-stimulated Ser473 Akt activation (P<0.01) and by 85% Thr308 activation. These inhibitory effects of AII were reversed by losartan (Figures 5A and 5B). AII did not affect Akt expression, as detected by immunoblotting (Figures 5A and 5B). Insulin increased by 3-fold phosphorylation of eNOS on Ser1177 (P<0.01) (Figure 5C). AII treatment resulted in a significant decrease (45%) of insulin-stimulated Ser1177 eNOS phosphorylation (P<0.01), whereas it did not affect eNOS expression (Figure 5C). The inhibitory effect of AII was reversed by losartan.

**Effects of AII on Insulin-Stimulated NO Production**

To additionally demonstrate that JNK and ERK1/2 play a negative role in insulin-stimulated NO production and that their activation is required for AII-mediated insulin resistance, we decided to determine whether JNK and MEK1 inhibitors can reverse AII-induced impairment in both activation of the Akt/eNOS pathway and NO production in response to insulin. As shown in Figures 6A and 6B, treatment of HUVECs with JNK inhibitor or PD98059 reversed the inhibitory effects of AII on insulin-stimulated phosphorylation of both Akt at Ser472 and eNOS at Ser1177. Insulin-stimulated NO production was reduced by AII in a
The inhibition effect of AII on insulin-stimulated NO production was reversed by losartan (Figure 6C). Treatment of HUVECs with JNK inhibitor or PD98059 reversed the inhibitory effect of AII, causing an increase of up to 80% and 55%, respectively, of NO production stimulated by insulin in the absence of AII, whereas simultaneous incubation with both inhibitors completely restored the stimulatory effects of insulin (Figure 6C). These data are consistent with the idea that AII-induced inhibition of the stimulatory effects of insulin on NO production is mediated, at least in part, through IRS-1 phosphorylation at Ser312 and Ser636 induced by JNK and ERK1/2, respectively, which negatively affects the downstream signaling pathway involving PI 3-kinase/Akt/eNOS.

**Discussion**

AII has been reportedly involved in the development of both insulin resistance and endothelial dysfunction in patients with essential hypertension.5–7 It has been suggested that crosstalk between AII- and insulin-signaling pathways may underlie AII-induced insulin resistance at a vascular level.9 Vasodilator effects of insulin are mediated by the signaling pathway involving IRS-1/PI-3 kinase/Akt/eNOS that leads to increased NO production by endothelium.8 In this study, we addressed the question of whether AII-induced alterations in insulin signaling contribute to impair endothelial insulin action. We demonstrate that exposure of HUVECs to AII resulted in inhibition of insulin-stimulated production of NO. This event was associated with impaired tyrosine phosphorylation of IRS-1 and its corresponding association with the SH2 domains of the p85 subunit of PI 3-kinase. A growing body of evidence indicates that serine phosphorylation of IRS-1 is a major role in the binding of IRS-1 with the SH2 domains of the p85 regulatory subunit of PI 3-kinase. AII has been shown to activate Akt via the AT1 receptor in various cell types, including vascular smooth muscle cells.23,24 However, we did not observe AII-induced Akt activation in our study. This discrepancy probably results from differences in the experimental conditions. Indeed, time-course experiments have repeatedly shown that AII-stimulated Akt phosphorylation is rapid, peaking at 5 to 10 minutes and returning to the basal level within 30 minutes.23–25 Furthermore, a recent study carried out in vascular smooth muscle cells has shown that AII does not inhibit Akt phosphorylation when it is added at the same time as insulin.26 However, pretreatment for 5 minutes with AII is sufficient to inhibit insulin-stimulated phosphorylation of Akt. Thus, it is possible that the activation mechanism of Akt in AII may be overridden by inhibitory mechanisms of Akt by AII if followed by insulin stimulation as in the present study. Moreover, a selective impairment of insulin-stimulated activation of the IRS-1/PI 3-kinase pathway has been reported in aortas of animal models of insulin resistance, such as obese Zucker rats and spontaneously hypertensive rats.27,28 These results obtained in vivo support the likelihood that our findings in cultured endothelial cells may have pathophysiological meaning and that impairment of this insulin signaling may be important for the development of endothelial dysfunction.

The impaired activation in response to insulin of the IRS-1/PI 3-kinase/Akt/eNOS signaling pathway in HUVECs exposed to AII was not attributable to alterations in IRS-1, p85, Akt, or eNOS protein levels but rather to a reduced phosphorylation of IRS-1 at tyrosine 612 and 632, which play a major role in the binding of IRS-1 with the SH2 domains of the p85 regulatory subunit of PI 3-kinase. A growing body of evidence indicates that serine phosphorylation of IRS-1 induced by a variety of factors interferes with the ability of this substrate to be tyrosine phosphorylated on insulin stimulation and reduces its ability to engage the p85 subunit of PI 3-kinase. More recently, several specific serine phosphorylation sites in IRS-1 and the corresponding activating kinases have been identified as responsible for these inhibitory effects.13–17 Activation of JNK has been shown to result in stimulation of Ser612 of IRS-1, whereas activation of ERK1/2 has been shown to result in an increased phosphorylation of Ser632. Because AII activates both ERK1/2 and JNK in cultured vascular smooth muscle cells as well as in intact arteries,18,19 we examined the possibility that AII-induced phosphorylation at Ser612 and Ser632 of IRS-1 mediated by JNK and ERK1/2, respectively, may account for the inhibitory effects of AII on insulin signaling pathway involved in NO production. We found that HUVECs exposed to AII exhibited increased JNK and ERK1/2 activity, which was associated with a concomitant increase in IRS-1 phosphorylation.
losartan inhibited the stimulatory effects of AII on JNK and ERK1/2 activity and reverted the enhanced Ser\(^{312}\) and Ser\(^{616}\) phosphorylation of IRS-1 stimulated by AII. We additionally demonstrated the cause-effect relationship between these two events by using inhibitors of JNK and MEK1. Indeed, we found that inhibition of JNK and MEK1 activity partly reversed the negative effects of AII on insulin-stimulated NO production, whereas the combined inhibition of JNK and MEK1 activity fully restored the stimulatory effects of insulin. Obviously we cannot exclude the possibility that other serine kinases may phosphorylate IRS-1 under the conditions used in the present study, leading to impairment in the activation of downstream events of insulin signaling pathway. Furthermore, it is possible that the inhibitory effects of AII on insulin-stimulated NO production is independent of IRS-1 serine phosphorylation and is partially related to an ERK1/2-dependent eNOS phosphorylation, leading to inhibition of the enzyme, as suggested by a recent study. Notwithstanding these possibilities, the present results suggest that AII-induced activation of JNK and ERK1/2 might be an important negative regulator for the insulin pathway involved in NO production.

In summary, we show that AII acting via the AT\(_1\) receptor exerts an inhibitory effect on the insulin signaling pathway involved in NO production and, for the first time, correlate these changes with activation of JNK and ERK1/2. Our data suggest that the uncoupling of IRS-1 and PI 3-kinase may be linked to an increased phosphorylation at Ser\(^{312}\) and Ser\(^{616}\) of IRS-1 mediated by JNK and ERK1/2, respectively. These changes are associated with a concomitant reduction in phosphorylation of Tyr\(^{612}\) and Tyr\(^{632}\) in two YXXM motifs essential for engaging p85 regulatory subunit of PI 3-kinase, resulting in impairment in activation of IRS-1-associated PI 3-kinase and sequential activation of the Akt/eNOS pathway.

In conclusion, increasing evidence suggests that the vasculature is an insulin-responsive tissue and that one of the
major vascular actions of insulin is its vasodilatory effect, which is mediated by enhanced production of NO. AII-induced insulin resistance in endothelial cells may play an important role in the pathophysiology of cardiovascular disease associated with hypertension and insulin resistance. The characterization of the molecular mechanism involved in AII-induced insulin resistance in the endothelium provides an important mechanistic link implicating JNK and ERK1/2 in the inhibitory effect of AII on insulin vascular action and may help to design efficacious pharmacological molecules to treat endothelial dysfunction associated with insulin resistance states.

Figure 5. Effects of All on insulin-stimulated activation of Akt and eNOS in HUVECs. A, Ser\textsuperscript{473} Akt phosphorylation. B, Thr\textsuperscript{308} Akt phosphorylation. C, Ser\textsuperscript{1177} eNOS phosphorylation. To normalize the blots for protein levels, after being immunoblotted with anti-phosphospecific antibodies, the blots were stripped and reprobed with anti-Akt or anti-eNOS antibodies. Each bar represents the mean±SD of 3 independent experiments, and autoradiographs of a representative experiment are shown. *P<0.01 vs control; #P<0.01 vs insulin; §P<0.02 vs All+insulin; †P<0.01 vs All+insulin by ANOVA.

Figure 6. Reversibility of the negative effect of AII on insulin-stimulated Akt and eNOS activation and NO production by JNK and MEK1 inhibitors in HUVECs. A, Effects of JNK and MEK1 inhibitors on AII-induced inhibition of Ser\textsuperscript{473} Akt phosphorylation. B, Effects of JNK and MEK1 inhibitors on AII-induced inhibition of Ser\textsuperscript{1177} eNOS phosphorylation. C, Effects of JNK and MEK1 inhibitors on AII-induced inhibition of NO production. D, Dose-response analysis for AII-induced IRS-1 phosphorylation at Ser\textsuperscript{312} and Ser\textsuperscript{616}. To normalize the blots for protein levels, after being immunoblotted with anti-phosphospecific antibodies, the blots were stripped and reprobed with anti-Akt, anti-eNOS, or anti-IRS-1 antibodies. Each bar represents the mean±SD of 3 independent experiments, and autoradiographs of a representative experiment are shown. *P<0.01 vs control; #P<0.01 vs insulin; §P<0.02 vs All+insulin; †P<0.01 vs All+insulin; ‡P<0.01 vs AII+insulin; §§P<0.01 vs AII+insulin by ANOVA.
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