Mechanisms of Reactive Oxygen Species–Dependent Downregulation of Insulin Receptor Substrate-1 by Angiotensin II

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Objective—Angiotensin II has been implicated in the pathogenesis of the vascular complications of insulin resistance. Recently, serine phosphorylation and degradation of insulin receptor substrate-1 (IRS-1) were shown to inhibit Akt activation and reduce glucose uptake. Therefore, we examined the effects of chronic angiotensin II treatment on IRS-1 phosphorylation and protein expression in vascular smooth muscle cells (VSMCs).

Methods and Results—Using Western analysis, we found that angiotensin II (100 nmol/L; 18 hours) caused a 61±5% degradation of IRS-1 and abolished insulin-induced activation of Akt. Phosphorylation of IRS-1 on Ser307, which leads to subsequent IRS-1 degradation, was stimulated by angiotensin II. This phosphorylation was blocked by the Src inhibitor PP1 and by the antioxidants N-acetylcysteine and ebselen. Stable overexpression of catalase abrogated angiotensin II–induced IRS-1 phosphorylation and IRS-1 degradation. Similarly, a mutant phosphoinositide-dependent kinase-1 (PDK1) that cannot associate with Src abolished IRS-1 phosphorylation and degradation induced by angiotensin II. Proteasome inhibitors also prevented IRS-1 degradation.

Conclusions—Thus, angiotensin II decreases IRS-1 protein levels in VSMCs via Src, PDK1, and reactive oxygen species (ROS) in the complications of insulin resistance.4,6,7 In vascular smooth muscle cells (VSMCs), angiotensin II (Ang II) activates NAD(P)H oxidases to produce ROS, which contribute to hypertension and atherosclerosis. Recent studies indicate that angiotensin II–induced IRS-1 phosphorylation and subsequent proteasome-dependent degradation. These events impair insulin signaling and provide a molecular basis for understanding the clinical observation that angiotensin II type 1 receptor antagonists improve insulin resistance and its associated vasculopathies. (Arterioscler Thromb Vasc Biol. 2005;25:1142-1147.)

Key Words: vascular smooth muscle ■ angiotensin II ■ insulin ■ IRS-1 ■ insulin resistance ■ phosphoinositide-dependent kinase-1

Clinical manifestations of type 2 diabetes occur when pancreatic insulin production cannot compensate for target tissue insulin resistance. In the context of the vascular system, “insulin resistance” likely does not regulate systemic glucose levels, but rather, in conjunction with the accompanying hyperglycemia, it manifests as impaired vasodilation and myogenic responsiveness,1,2 microvessel disease leading to retinopathy and nephropathy,3 enhanced atherosclerotic lesion formation,4 constrictive remodeling of small arteries,2 medial hypertrophy,2 and enhanced vascular inflammation.5

Recent work has implicated the renin-angiotensin system and reactive oxygen species (ROS) in the complications of insulin resistance.4,6,7 In vascular smooth muscle cells (VSMCs), angiotensin II (Ang II) activates NAD(P)H oxidases to produce ROS, which contribute to hypertension and atherosclerosis. Recent studies indicate that angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 (AT1) receptor blockers improve insulin resistance4,8 and diabetic vascular complications.4 ACE inhibitors restore impaired endothelium-dependent vasodilation in patients with type 2 diabetes9 and improve arterial structure in diabetic rats.10 These studies raise the provocative possibility that Ang II may directly participate in the development of insulin resistance, particularly in the vasculature.

Despite intensive research, the cellular mechanisms responsible for insulin resistance remain incompletely understood. Impaired insulin responsiveness is linked to serine phosphorylation of insulin receptor substrate-1 (IRS-1),11 a protein that is tyrosine phosphorylated by the activated insulin receptor and coordinates downstream signaling events by binding and activating proteins such as phosphatidylinositol 3-kinase (PI3-K) and Src family kinases.12 Phosphorylation of rodent IRS-1 on Ser612 (human Ser616) causes dissociation of the p85 subunit of PI3-K from IRS-1,13 inhibiting further signaling. In contrast, phosphorylation of IRS-1 on Ser307 (human Ser312) results in its dissociation from the insulin receptor14 and triggers proteasome-dependent degradation.15,16 also impairing insulin signaling. Several serine/threonine kinases, including c-Jun NH2-terminal kinase (JNK),17 inhibitor κB kinase-β,18 and mam-
malian target of rapamycin (mTOR), phosphorylate IRS-1. It was also reported that degradation of IRS-1 by insulin requires the phosphoinositide-dependent kinase-1 (PDK1) pathway, suggesting an involvement of PDK1 in insulin resistance. IRS-1 phosphorylation seems to be agonist, site, and tissue specific, making it imperative to study these signaling pathways in the vasculature.

Although Ang II is known to increase ROS production, a relationship between Ang II and oxidative stress in mediating insulin resistance has not been established. Folli et al and Motley et al have clearly shown that acute short-term treatment with Ang II interferes with insulin signaling in VSMCs on multiple levels, including phosphorylation of the insulin receptor IRS-1 and p85 on serine residues. From the clinical point of view, the chronic effects of Ang II and oxidative stress may be of even more importance to the development of insulin resistance. We hypothesized that long-term treatment with Ang II interferes with insulin signaling by phosphorylating IRS-1 on Ser307 in a ROS-sensitive manner, leading to IRS-1 degradation. Our data support this hypothesis and suggest a mechanism whereby Ang II and ROS converge to mediate insulin resistance in the vasculature.

**Materials and Methods**

**Materials**

Anti–IRS-1, anti-PDK1, and anti-phosphotyrosine antibodies were from Upstate Biotechnology. Phospho–IRS-1 (Ser307), phospho-Akt (Ser473), Akt, and Myc-tag antibodies were from Cell Signaling Technology, Inc. Anti–IRS-1 monoclonal antibody was from Transduction Laboratories. Valsartan was provided by Novartis. All other chemicals and reagents, including DMEM with 25 mmol/L Hepes and 4.5 g glucose, were from Sigma. The Y9F-PDK1 adenovirus was described previously.

**Cell Culture**

VSMCs were isolated from rat thoracic aorta by enzymatic digestion and grown in DMEM as described previously. Cells were quiesced in 0.1% calf serum for 24 to 48 hours and used at confluence. In some experiments, VSMCs stably transfected with human catalase were used and maintained in selection medium until plating for experiments. Infection with recombinant adenoviruses to express Y9F-PDK1 or green fluorescent protein (GFP) was performed as described previously.

**Immunoprecipitation and Western Analysis**

Cells at 80% to 90% confluence were made quiescent by incubation with DMEM containing 0.1% calf serum for 24 hours. Cells were stimulated with agonist at 37°C in serum-free DMEM, lysed as described previously, and solubilized proteins were isolated by centrifugation and quantified by the Bradford assay. For immunoprecipitation, cell lysates were incubated with mouse anti–IRS-1 or anti-PDK1 antibody overnight, and the immunocomplex was collected with protein A/G-agarose beads. Proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes. After blocking, blots were incubated with primary antibodies, and proteins were detected by enhanced chemiluminescence (Amersham Pharmacia). Band intensity was quantified by densitometry of immunoblots using NIH Image.

**Measurement of Glucose Uptake**

Serum-starved VSMCs were stimulated with vehicle or Ang II (100 nmol/L) for 18 hours in the presence or absence of inhibitors. Cells were then incubated in Krebs–Ringer-Hepes buffer (in mmol/L: 15 Hepes, pH 7.4, 105 NaCl, 5 KCl, 1.4 CaCl2, 1 KH2PO4, 1.4 MgSO4, and 10 NaHCO3) for 2 hours. Next, cells were incubated with insulin (100 nmol/L) for 30 minutes, and 0.2 mmol/L 2-deoxy-D-glucose containing 1 μCi/mL 2-deoxy-D-[3H]glucose was added for an additional 30 minutes. Transport was stopped by removal of the buffer, followed by 3 washes with ice-cold PBS. Cells were disrupted with 0.4 mol/L NaOH, neutralized with HCl, and the amount of labeled glucose taken up was determined by scintillation counting.

**Statistical Analysis**

Results are expressed as mean±SE. Statistical significance was assessed by ANOVA, followed by Bonferroni’s test. A value of P<0.05 was considered statistically significant.

**Results**

**Effect of Ang II on IRS-1 Protein Expression**

In addition to transient activation of kinase cascades, Ang II regulates cell function by changing the expression profile of signaling proteins. We have shown previously that ROS mediate many of the early signaling events in Ang II–treated cells over a period of 1 to 30 minutes, but their role in longer-term responses is unclear. Because IRS-1 is central to insulin signaling, we hypothesized that prolonged treatment with Ang II alters IRS-1 protein expression in an ROS-sensitive manner. As shown in Figure 1a, Ang II induced a time-dependent decrease in IRS-1 protein levels, with the maximal effect occurring at 18 hours (61±5%) but did not affect β-actin levels. The role of ROS in this response was investigated using VSMCs stably overexpressing catalase, in which Ang II–stimulated H2O2 production is abolished. Downregulation of IRS-1 by Ang II was significantly inhibited in these cells, suggesting that H2O2 is required for the decrease in IRS-1 levels (Figure 1b). Furthermore, IRS-1 downregulation was completely abolished by valsartan (Figure I, available online at http://atvb.ahajournals.org), indicating that this response is mediated by the AT1 receptor.

**Src-Dependent Phosphorylation of IRS-1 on Ser307 by Ang II**

One mechanism leading to IRS-1 degradation is phosphorylation of Ser307 on IRS-1. Therefore, we examined the effect of Ang II on IRS-1 phosphorylation at this site. Ang II induced a robust phosphorylation of IRS-1 at Ser307 in a time-dependent manner (maximum at 30 minutes; 13±0.8-fold; Figure 2a). We next investigated the possible involve-
ment of c-Src, a tyrosine kinase required for generation of ROS,28 in IRS-1 phosphorylation using its specific inhibitor PP1 (20 μmol/L), which effectively attenuates Src activation in these cells.29 Pretreatment of VSMCs with PP1 markedly inhibited the ability of Ang II to induce IRS-1 phosphorylation at Ser307 (81 ± 4% inhibition; Figure II, available online at http://atvb.ahajournals.org).

ROS Sensitivity of Ang II–Induced IRS-1 Phosphorylation

To determine whether ROS are required for IRS-1 phosphorylation as well as degradation, we next examined the effects of antioxidants on Ang II–induced phosphorylation of IRS-1 at Ser307. The glutathione peroxidase mimic ebselen and N-acetylcysteine (NAC) significantly inhibited Ang II–induced phosphorylation of IRS-1 at Ser307 (Figure 2b). Furthermore, Ang II–induced IRS-1 phosphorylation at Ser307 was almost completely inhibited in catalase-overexpressing cells (Figure 2b), indicating that H2O2 production is crucial for Ang II–induced phosphorylation of IRS-1 at Ser307.

Involvement of PDK1 in Ang II–Induced IRS-1 Phosphorylation and Degradation

A major target of Src in Ang II–stimulated VSMCs is PDK1,23 a serine/threonine kinase reported to be involved in IRS-1 phosphorylation.16 Src phosphorylates Tyr9 of PDK1, inducing a conformational change and providing binding sites for association with other proteins.23 Thus, we investigated the ROS sensitivity of tyrosine-phosphorylated PDK1 and its involvement in Ang II–induced IRS-1 phosphorylation. As shown in Figure 3a, Ang II–stimulated tyrosine phosphorylation of PDK1 was completely inhibited by NAC, suggesting that it is downstream of ROS production. To determine whether tyrosine-phosphorylated PDK1 is required for IRS-1 phosphorylation, we infected VSMCs with an adenovirus expressing Y9F-PDK1, a mutant form that cannot be phosphorylated by Ang II.23 As shown in Figure 3b, Ang II–induced IRS-1 phosphorylation was abolished in Y9F-PDK1–infected VSMCs but was unaltered in vector (AdGFP)-containing cells. Furthermore, IRS-1 degradation was significantly attenuated in Y9F-PDK1–expressing cells (Figure 3c). These data clearly show that ROS-dependent tyrosine phosphorylation of PDK1 plays a critical role in IRS-1 phosphorylation and degradation by Ang II.

Figure 2.

Ang II–induced phosphorylation of Ser307 on IRS-1. a and b, VSMCs were lysed and immunoprecipitated with anti–IRS-1 antibody. Western analysis was performed using phospho-Ser307–IRS-1 (top panels) or total IRS-1 (bottom panels) antibodies. Bar graphs represent means ± SE (n=3 to 4), expressed as fold increases in phosphorylation over that in unstimulated cells. *P<0.001 vs control; #P<0.001 vs Ang II alone. a,VSMCs were incubated with 100 nmol/L Ang II for the indicated times. b,VSMCs were preincubated with either ebselen (40 μmol/L) or NAC (10 mmol/L) for 30 minutes and then treated with 100 nmol/L Ang II for 30 minutes, or else stably transfected with either empty vector or catalase before Ang II stimulation. IP indicates immunoprecipitation; IB, immunoblot.

Figure 3.

Ang II–induced phosphorylation of PDK1 is ROS dependent and mediates IRS-1 phosphorylation. a, VSMCs were incubated with NAC (10 mmol/L) for 30 minutes and then 100 nmol/L Ang II for 30 minutes. After immunoprecipitation with anti-PDK1 antibody, Western analysis was performed using phospho-tyrosine (top) or total PDK1 (bottom) antibodies. Bar graph represents means ± SE (n=3) expressed as fold increases in phosphorylation over that in unstimulated cells. *P<0.001 vs control; #P<0.001 vs Ang II alone. b, VSMCs were infected with adenoviruses to overexpress either GFP or an Myc-tagged Y9F-PDK1 mutant. Cells were stimulated with Ang II (100 nmol/L) for 30 minutes, immunoprecipitated with anti–IRS-1 antibody, and Western blotted with antibodies against phospho–Ser307–IRS-1 (top), total IRS-1 (middle), or Myc-tag (bottom). Bar graph represents means ± SE (n=4) expressed as fold increases in phosphorylation over that in unstimulated cells. *P<0.01 vs control; #P<0.001 vs Ang II alone. c, VSMCs were infected with adenoviruses to overexpress either GFP or Y9F-PDK1, stimulated with Ang II (100 nmol/L) for 18 hours and immunoblotted using anti–IRS-1 (top), β-actin (loading control; middle), or Myc-tag (bottom) antibodies. Bar graph represents means ± SE (n=4). *P<0.05 vs control. IP indicates immunoprecipitation; IB, immunoblot.
Proteasome-Dependent Degradation of IRS-1 by Ang II
The observed decrease in IRS-1 protein by Ang II could occur by inhibiting protein synthesis, increasing protein degradation, or both. Prolonged treatment with Ang II had no effect on IRS-1 mRNA expression over 24 hours (data not shown), suggesting that the effect of Ang II is post-translational. In other cell types, IRS-1 is degraded by a ubiquitin–proteasome pathway in response to insulin. 60 Inhibition of the 26S proteasome with lactacystin dose-dependently attenuated the ability of Ang II to downregulate IRS-1 (Figure 4). Other proteasome inhibitors, including 1 μmol/L MG132 and 100 μmol/L N-acetyl-leucyl-leucyl-norleucinal (ALLN), also attenuated the IRS-1 degradation by Ang II (Ang II −61±5%; Ang II + MG132 −10±7%; Ang II + ALLN −3±8%; n=4; P<0.01).

Effects of IRS-1 Downregulation by Ang II on Insulin Signaling
Finally, to test whether IRS-1 downregulation by Ang II affects insulin signal transduction, we examined insulin-induced Akt phosphorylation and glucose uptake. As shown in Figure 5a, insulin increased Akt phosphorylation by 5±1-fold. However, when VSMCs were pretreated with Ang II for 18 hours, a time at which Ang II-stimulated Akt phosphorylation is back to baseline, insulin was no longer able to stimulate Akt phosphorylation. Furthermore, Ang II pretreatment decreased insulin-stimulated glucose uptake by 50%, and this decrease was reversed by the Src inhibitor PP1 and Y9F-PDK1 (Figure 5b). Thus, downregulation of IRS-1 by Ang II has profound effects on the cellular response to insulin.

Discussion
It is well known that Ang II exacerbates insulin resistance in experimental animals and patients, 4,6,8,31,32 but the underlying mechanisms are poorly understood, especially in the vasculature. In this study, we show that Ang II profoundly decreases IRS-1 protein levels via ROS-mediated phosphorylation of IRS-1 on Ser307 and subsequent proteasome-dependent degradation. This leads to impaired insulin signaling and provides a basis for understanding how Ang II contributes to insulin resistance.

Previous work showed that acute administration of Ang II interferes with insulin signaling at multiple levels. Thus, Ang II phosphorylates the insulin receptor IRS-1 and the p85 subunit of PI3-K in VSMCs, leading to dissociation of IRS-1 and PI3-K and impaired insulin-stimulated Akt phosphorylation. 21,22,33 Although these studies provide insight into the initial steps leading to insulin resistance, they may not fully explain the effects of chronic exposure to Ang II as occurs in vivo. In fact, insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1 is enhanced after Ang II infusion, even though tissues from these animals remain refractory to insulin-induced glucose uptake. 34 Our finding that IRS-1 is degraded after long-term exposure to Ang II provides a novel mechanism to explain the insulin resistance observed in such animal models. Indeed, preliminary studies in Ang II–infused mice suggest that IRS-1 downregulation occurs in vivo as well (Y. Taniyama and K. Griendling, unpublished observations, 2004).

IRS-1 downregulation may also explain the known role of ROS in the development of insulin resistance and complications of diabetes. 7 Treatment of cells with H2O2 or glucose/glucose oxidase attenuates insulin-induced PI3-K activity associated with IRS-1, phosphorylation of Akt and insulin receptor autophosphorylation. 35,36 However, as with reports on Ang II, these in vitro studies focused on the short-term effects of oxidative stress. Our data suggest that ROS mediate the chronic suppression of insulin signaling as well (Figures 1 and 5). Furthermore, this effect on IRS-1 degradation by Ang II may be a general mechanism by which ROS induce insulin resistance. Diabetes is accompanied by a sustained increase in ROS levels, decreased tissue glutathione, and increased NAD(P)H oxidase activity. 16,37 In nonobese rats, insulin resistance induced by glucose infusion is prevented by coadministration of the antioxidant NAC or taurine, implicating oxidative stress in the development of insulin resistance. 38
gether with the present results, it seems likely that one mechanism whereby prolonged oxidative stress causes insulin resistance is induction of IRS-1 degradation.

The mechanism by which Ang II and ROS induce IRS-1 degradation appears to involve serine phosphorylation of IRS-1 and targeting to the proteasome. Agonists target 3 major serine residues on rat IRS-1: Ser612, Ser302, and Ser307. In other cell types, phosphorylation of Ser612 and Ser302 is involved in dissociation of IRS-1 from the p85 subunit of PI3-K, whereas Ser307 phosphorylation inhibits the binding of IRS-1 to the insulin receptor and triggers IRS-1 degradation. Thus, phosphorylation of Ser612 or Ser302 phosphorylation may be responsible for the known ability of acute Ang II stimulation to cause dissociation of IRS-1 from PI3-K. Our current data are consistent with the concept that phosphorylation of Ser307 of IRS-1 (Figure 2a) is involved in targeting it to the 26S proteasome degradative pathway (Figure 4), resulting in a prolonged loss of IRS-1 protein and refractoriness to insulin signaling (Figures 1 and 5). However, at this time, we cannot rule out other additional potential inhibitory effects of IRS-1 serine phosphorylation.

Activation of the 26S proteasome pathway and IRS-1 degradation was shown previously for insulin in other cell types but has never been demonstrated for heterologous agonists. In general, protein degradation by the 26S proteasome is initiated by protein ubiquitination. Prevention of IRS-1 downregulation by the specific proteasome inhibitors lactacystin, MG132, and ALLN suggests that a similar mechanism exists in Ang II–treated VSMCs. Although protein expression can also be regulated by transcriptional or post-transcriptional mechanisms, the contribution of these pathways to Ang II–induced IRS-1 downregulation is little, if any, because the mRNA levels of IRS-1 are unchanged by Ang II. These data agree with previous observations that insulin-stimulated IRS-1 downregulation is not attributable to transcriptional or post-transcriptional mechanisms. Decreased protein synthesis is also unlikely to explain the observed IRS-1 downregulation because blocking protein degradation with lactacystin completely reverses the effect of Ang II on IRS-1.

Our data also provide insight into the signaling pathways leading to Ser307 phosphorylation of IRS-1 by Ang II, the event that apparently triggers protein degradation. We found that c-Src inhibition nearly abolishes Ang II–induced IRS-1 Ser307 phosphorylation (Figure 2). In VSMCs, a major consequence of Src activation is stimulation of NAD(P)H oxidases to produce ROS. Indeed, reduction of ROS levels with catalase overexpression, Ang II is no longer able to phosphorylate IRS-1 (Figure 2b) or to induce protein degradation (Figure 1).

In Ang II–stimulated VSMCs, a major target of Src and ROS is PDK1, a serine/threonine kinase that was originally found to mediate activation of Akt. Src phosphorylates PDK1 on Tyr9, creating binding sites for PDK1 substrates. In the present study, we found that Tyr9-PDK1 abolishes the ability of Ang II to phosphorylate and degrade IRS-1 (Figure 3), in agreement with the work of Greene et al, who found that degradation of IRS-1 protein by insulin requires the PDK1 pathway. Because PDK1 is a serine/threonine kinase, it may be able to phosphorylate IRS-1 itself. However, Ang II has very little effect on the kinase activity of PDK1. Alternatively, Tyr9 phosphorylation of PDK1 may promote the association of IRS-1 with another immediate upstream kinase, such as JNK, inhibitor kappa B kinase-β, or mTOR. The precise interaction of PDK1 and IRS-1 awaits further investigation.

In summary, we show for the first time that an agonist other than insulin, Ang II, induces IRS-1 degradation. This event is dependent on Src-, PDK1-, and ROS-mediated phosphorylation of IRS-1 on Ser307, which leads to degrada-

tion in the 26S proteasome (Figure 6). These observations provide novel insight into the mechanisms of Ang II–induced insulin resistance and suggest a basis for the clinical observation that AT1 receptor antagonists and ACE inhibitors mitigate diabetic cardiovascular complications.

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References


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Figure I. Ang II-induced IRS-1 downregulation is mediated by AT₁ receptors.
VSMCs were pretreated with valsartan (10 µmol/L) prior to Ang II stimulation. Bar graphs represent means±SE (n=4) expressed as percent decrease compared to unstimulated cells. *, p<0.05 vs. control Ang II alone.

Figure II. Ang II-induced phosphorylation of Ser307 on IRS-1 is mediated by Src.
VSMCs were preincubated with the Src inhibitor PP1 (20 µmol/L, 30 min) and treated with 100 nmol/L Ang II for 30 min. Cells were lysed and immunoprecipitated with anti-IRS-1 antibody. Western analysis was performed using phospho-Ser307-IRS-1 (top panels) or total IRS-1 (lower panels) antibodies. Bar graphs represent means±SE (n=3-4), expressed as fold increases in phosphorylation over that in unstimulated cells. *, p<0.001 vs control; #, p<0.001 vs. Ang II alone. IP=immunoprecipitation; IB=immunoblot.
Valsartan (μM) 0 10
Ang II – + – +
IB: IRS-1

Fig. I
Fig. II

IRS-1 phosphorylation (fold)

Ang II: -  +  
PP1: -  +  
IP: IRS-1
IB: p-IRS-1 (pS307)
IP: IRS-1
IB: IRS-1

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