Insulin-Induced Akt Activation Is Inhibited by Angiotensin II in the Vasculature Through Protein Kinase C-α

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Abstract—Insulin resistance is an important risk factor in the development of cardiovascular diseases such as hypertension and atherosclerosis. However, the specific role of insulin resistance in the etiology of these diseases is poorly understood. Angiotensin (Ang) II is a potent vasculotrophic and vasoconstricting factor. We hypothesize that in vascular smooth muscle cells (VSMCs), Ang II interferes with insulin action by inhibiting Akt, a major signaling molecule implicated in the biological actions of insulin. By immunoblotting with a phospho-specific antibody for Akt, we found that Ang II inhibits insulin-induced Akt phosphorylation in a time-dependent manner. The inhibitory effect of Ang II was blocked by a Ang II type 1 receptor antagonist, RNH6270. A protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate, also inhibited insulin-induced Akt phosphorylation. PKC inhibitors, including Go6976 (specific for α- and β-isoforms), blocked the Ang II– and PMA-induced inhibition of Akt phosphorylation by insulin. Moreover, overexpression of PKC-α but not PKC-β isoform by adenovirus inhibited insulin-induced Akt phosphorylation. By contrast, an epidermal growth factor receptor inhibitor (AG1478), a p42/44 mitogen-activated protein kinase (MAPK) kinase inhibitor (PD 598,059), and a p38 MAPK inhibitor (SB 203,580) did not block the Ang II–induced inhibition of Akt phosphorylation. From these data, we conclude that Ang II negatively regulates the insulin signal, Akt, in the vasculature specifically through PKC-α activation, providing an alternative molecular mechanism that may explain the association of hyperinsulinemia with cardiovascular diseases. (Hypertension. 2003;41[part 2]:775-780.)

Key Words: angiotensin II ■ insulin ■ protein kinases ■ Akt ■ muscle, vascular, smooth

Because angiotensin (Ang) II is widely believed to play a critical role in vascular remodeling associated with hypertension, atherosclerosis, and restenosis after vascular injury,1,2 there has been considerable interest in defining its signaling pathways in vascular smooth muscle cells (VSMCs). In cultured VSMCs, Gq-coupled Ang II type 1 (AT1) receptor activates phospholipase C, which initiates the generation of inositol triphosphate and diacylglycerol, causing intracellular Ca2＋ mobilization and protein kinase C (PKC) activation, respectively.3 The AT1 receptor also activates protein Ser/Thr kinases and protein tyrosine kinases in many cell types, including VSMCs.3–5 Earlier studies from our laboratory indicated that a tyrosine kinase mediates Ang II–induced p42/44 mitogen-activated protein kinase (MAPK) activation in VSMCs.6 Subsequently, we have shown that this mechanism involves the epidermal growth factor (EGF) receptor transactivation, which seems to play a critical role in Ang II–induced vascular hypertrophy.5,7

Insulin resistance and hyperinsulinemia are closely associated with several disease processes such as hypertension, non–insulin-dependent diabetes, atherosclerosis, and dyslipidemia (syndrome X).8–10 However, the pathogenic role of insulin resistance and/or hyperinsulinemia in the development of hypertension and other cardiovascular diseases is still not clear. Alterations in insulin action are mediated through the insulin receptor. Insulin binds to its cell surface transmembrane, heterotetrameric receptor stimulating receptor autophosphorylation and activation of the intrinsic tyrosine kinase activity, which results in tyrosine phosphorylation of several cytosolic docking proteins called insulin receptor substrates (IRSs). Tyrosine phosphorylation of IRS-1 and IRS-2 induces their binding to Src homology 2-domain–containing molecules, including phosphatidylinositol 3-kinase (PI3-kinase).11 The interaction between the IRS proteins and PI3-kinase results in an increase in the catalytic activity of the p110 subunit of the enzyme. Activation of PI3-kinase is necessary for many of the actions of insulin.12,13 PI3-kinase activates Akt/protein kinase B by binding phosphatidylinositol-3,4,5-trisphosphate to its pleckstrin homology domain and by its Ser/Thr phosphorylation.12–14 Two positive regulatory phosphorylation sites in Akt have been identified, Thr308 and Ser473.15 A recent study shows that prior

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activation of PKC can inhibit the subsequent ability of insulin to stimulate the enzymatic activity of Akt in 3T3-L1 adipocytes.\textsuperscript{15} Because the activation of Akt may mediate the ability of insulin to stimulate various biological responses, its negative regulation by PKC could contribute to insulin-resistant states.

Clinical and pharmacological studies showed that Ang II infusion induces insulin resistance, and Ang II converting enzyme inhibitors and AT\textsubscript{1} receptor blockers improve insulin sensitivity.\textsuperscript{16–19} Therefore, overactivity of the renin-angiotensin system is likely to impair insulin signaling and contribute to insulin resistance observed in cardiovascular diseases. However, the molecular mechanism by which Ang II inhibits the insulin signal remains unclear. In the present study, we hypothesize that in VSMCs, Ang II interferes with insulin action by inhibiting Akt, a major signaling molecule implicated in the biological actions of insulin. We found that Ang II inhibits the insulin-induced phosphorylation of Akt through PKC-\textalpha, suggesting a role for PKC in insulin resistance associated with cardiovascular diseases.

Methods

Materials

Ang II, phorbol 12-myristate 13-acetate (PMA), and insulin were purchased from Sigma Chemical. Antibodies directed to Ser\textsuperscript{473}, phosphorylated Akt and Akt were purchased from Cell Signaling. Antibodies for Tyr\textsuperscript{204}-phosphorylated p42/p44 MAPK (ERK1/2), p42 MAPK (ERK2), and IRS-1 were purchased from Santa Cruz Biotechnology. Antibodies for phospho-tyrosine and insulin receptor-\textalpha subunit were purchased from Upstate Biotechnology. Antibody directed to Tyr\textsuperscript{1158}-phosphorylated insulin receptor-\textalpha was purchased from BioSource International. PD 98059, SB203580, SP600125, AG1478, GF109203X, Go6976, Go6983, and rotterlin were purchased from Calbiochem. RNH 6270 was a gift from Sankyo Co., Ltd. (Tokyo, Japan).

Cell Culture

The thoracic aorta from 12-week-old Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass) was used to prepare VSMCs by the explant method. The cells were cultured in Dulbecco’s Modified Eagle’s Medium containing 10\% fetal calf serum.\textsuperscript{6} Subcultured VSMCs used in the experiments showed \textapprox 99\% positive immunostaining of smooth muscle \textalpha-actin antibody.\textsuperscript{6} For the experiments, cells from passage 3 to 12 at \textapprox 90\% confluence in culture were used after 3 days of serum depletion.

Immunoprecipitation

After stimulation with insulin at 37\°C, the cells were lysed with ice-cold immunoprecipitation buffer (150 mmol/L NaCl, 50 mmol/L HEPES at pH 7.5, 1\% Triton X-100, 1 mmol/L EDTA, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 2 mmol/L sodium orthovanadate 10\% (v/v) glycerol and 10 \mu mol/L of leupeptin, 10 \mu g/mL of aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). The cell lysates were centrifuged, and the supernatant was immunoprecipitated with the antibody and protein A/G plus agarose at 4\°C for 16 hours as described previously.\textsuperscript{6,7}

Western Blotting

Cell lysate or immunoprecipitation lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and was electrophoretically transferred to a nitrocellulose membrane as previously described.\textsuperscript{6} The membranes were then exposed to primary antibodies overnight at 4\°C. After incubation with the peroxidase-linked secondary antibody for 1 hour at room tempera-

Adenovirus Transfection

The generation of PKC-\alpha and PKC-\beta wild-type adenovirus was described in detail elsewhere.\textsuperscript{20} VSMCs were infected with the adenovirus for 2 days before stimulation as previously described.\textsuperscript{21}

PKC-\alpha Membrane Translocation

PKC-\alpha translocation was determined by collection of the membrane fraction, as described previously.\textsuperscript{22} After stimulation with Ang II, cells were lysed in a buffer containing 20 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EGTA, 0.1 mmol/L 4-(2-aminoethyl) benzene sulfonic fluoride, and 20 \mu mol/L leupeptin. The cell lysates were centrifuged at 100 000g for 60 minutes at 4\°C. The pellet was solubilized, subjected to SDS-PAGE, and immunoblotted with anti-PKC-\alpha antibody.

Statistical Analysis

Data were analyzed by using the Student \textit{t} test. The mean \pm SEM was measured with a significance level of \textit{P}<0.05. Results are representative of 3 separate experiments.

Results

We have previously demonstrated that insulin induces Akt activation in VSMCs.\textsuperscript{21} In the present study, we examined the effect of Ang II on insulin-induced Akt phosphorylation in VSMCs (Figure 1). Ang II pretreatment significantly inhibited insulin-induced Akt phosphorylation, with maximal inhibition occurring at 20 to 40 minutes (Figure 1A). The maximal concentration of Ang II that inhibited insulin-induced Akt phosphorylation was 1 \mu mol/L (Figure 1B). By using an AT\textsubscript{1} receptor antagonist, RNH 6270,\textsuperscript{23} we demonstrated that Ang II is acting through the AT\textsubscript{1} receptor (Figure 1B) because in the presence of this antagonist, the inhibitory effect of Ang II on insulin-induced Akt phosphorylation is totally blocked.

The G\textsubscript{q}-coupled AT\textsubscript{1} receptor activation leads to the mobilization of Ca\textsuperscript{2+} and the activation of PKC.\textsuperscript{3} We wanted to see if these second messengers were responsible for the Ang II–induced inhibition of Akt phosphorylation stimulated by insulin. In Figure 2A, A23187, a Ca\textsuperscript{2+} ionophore, did not inhibit the insulin-induced phosphorylation of Akt at the lower time points, but did show a slight inhibition at the 40-minute point. In contrast, pretreatment with a PKC activator, PMA, for 10 minutes markedly inhibited insulin-induced phosphorylation of Akt, and this inhibition was blocked by GF109203X, a PKC inhibitor (Figure 2B).\textsuperscript{6} We were further able to demonstrate that other PKC inhibitors, Go6976 and Go6983,\textsuperscript{22,24} similar to GF109203X, blocked the Ang II–induced inhibition of Akt (Figure 2C). These data suggest that PKC plays a critical role in the inhibition of Akt by Ang II in VSMCs.

Because these PKC inhibitors commonly inhibit PKC-\alpha and PKC-\beta, we examined the effects of overexpression of PKC-\alpha and -\beta isoforms on insulin-induced Akt phosphorylation (Figure 3A). We found that overexpression of PKC-\alpha isoform but not PKC-\beta isoform inhibited insulin-induced Akt phosphorylation (Figure 3A). In addition, Ang II stimulated the translocation of PKC-\alpha to the membrane of VSMCs (Figure 3B). Although GF109203X and Go6983 inhibit PKC-\delta, the PKC-\delta inhibitor rotterlin (10 \mu mol/L pretreat-
ment for 30 minutes) did not affect the Ang II-induced inhibition of Akt phosphorylation (data not shown). These data suggest that PKC-α may be the PKC isoform by which Ang II inhibits Akt in VSMCs. To determine the point at which Ang II inhibits the insulin signaling pathway, we further examined the effect of Ang II on IRS-1 and insulin receptor tyrosine phosphorylation. Figure 3C shows that Ang II inhibits IRS-1 tyrosine phosphorylation, and Figure 3D shows that neither Ang II nor PMA inhibit insulin-induced phosphorylation of the insulin receptor. Thus, Ang II appears to inhibit Akt through its effects on IRS-1.

Three major MAPKs are implicated in the inhibition of the insulin signal through IRS-1 Ser/Thr phosphorylation. Therefore, the possible involvement of MAPKs in the inhibition of Akt by Ang II was studied. In Figure 4A, the p42/44 MAPK kinase inhibitor, PD 98,059, did not block the inhibitory effect of Ang II on the insulin-induced phosphorylation of Akt. We also found that neither the p38 MAPK inhibitor, SB203580 (Figure 4B), nor the c-Jun N-terminal kinase (JNK) inhibitor (SP600125) blocked the inhibitory effect of Ang II on insulin-induced phosphorylation of Akt, suggesting that these MAPKs are not involved in the Ang II–induced inhibition of Akt.

We have demonstrated that Ang II activates Akt through Ca2+-dependent but PKC-independent EGF receptor transactivation in VSMCs. Here, we show that EGF receptor is not involved in the Ang II–induced inhibition of Akt because the EGF receptor inhibitor AG1478 does not block the inhibitory
The major findings of this study are (1) Ang II inhibited insulin-induced activation of Akt through the AT1 receptor in VSMCs by blocking IRS-1 function, and (2) the inhibition requires PKC-α activation by Ang II. These data suggest an alternate role for intracellular signaling of Ang II and other PKC activators in the development of cardiovascular diseases associated with insulin resistance. Although Folli et al.28 demonstrated in VSMCs that Ang II inhibits insulin-stimulated PI3-kinase activity through PKC activation, the current study advances this paradigm by demonstrating the requirements of the AT1 receptor and a specific PKC isoform, PKC-α.

In previous studies, we have shown that PKC can inhibit Akt in VSMCs29 and were further able to demonstrate that lysophosphatidylcholine, a major bioactive product of oxidized low-density lipoproteins, also inhibits Akt phosphorylation through PKC.24 Similar to lysophosphatidylcholine, in

**Discussion**

The major findings of this study are (1) Ang II inhibited insulin-induced activation of Akt through the AT1 receptor in VSMCs by blocking IRS-1 function, and (2) the inhibition requires PKC-α activation by Ang II. These data suggest an alternate role for intracellular signaling of Ang II and other PKC activators in the development of cardiovascular diseases associated with insulin resistance. Although Folli et al.28 demonstrated in VSMCs that Ang II inhibits insulin-stimulated PI3-kinase activity through PKC activation, the current study advances this paradigm by demonstrating the requirements of the AT1 receptor and a specific PKC isoform, PKC-α.
this study, we showed that Ang II inhibits Akt through PKC activation. These vascular pathogens are known to activate PKC,3,30 and we were further able to show that the PKC-α isoform inhibited insulin-induced Akt activation. Overexpression of PKC-α, and not PKC-β or PKC-δ,24 inhibited insulin-induced Akt activation. Moreover, PKC-α was translocated to the membrane in VSMCs when stimulated with Ang II. In addition, we used an AT₁ receptor antagonist, RNH 6270,23 to demonstrate that the inhibition of insulin-induced activation of Akt by Ang II was mediated by the Gₛ-coupled AT₁ receptor, which activates PKC.3 This is in good agreement with a recent publication31 showing that overexpression of constitutively active Gₛ mutant inhibits Akt in HEK 293 cells.

We have demonstrated that PKC-α overexpression inhibits IRS-1 tyrosine phosphorylation induced by insulin24; however, the exact mechanism by which this occurs requires further investigation. It has been shown that Ang II inhibits insulin-stimulated IRS-associated PI3-kinase activity in VSMCs.28,32 Ang II activates 3 major MAPKs26 that can inhibit IRS function through Ser/Thr phosphorylation in VSMCs.25 In HEK 293 cells, it was shown that PMA inhibited insulin-stimulated PI3-kinase through p42/44 MAPK-dependent IRS-1 phosphorylation.33 Studies have also shown that p38 MAPK is involved in H₂O₂-induced inhibition of insulin-stimulated glucose transport in cultured skeletal muscle cells.34 These data suggest that p42/44 MAPK or p38 MAPK may play a role downstream of PKC activation in the Ang II–induced inhibition of Akt phosphorylation. However, the p42/44 MAPK inhibitor PD 98,059 and the p38 MAPK inhibitor SB203580 had no effect on the Ang II–induced inhibition of Akt phosphorylation by insulin in VSMCs. Although JNK was shown to inhibit insulin signaling through IRS-1 phosphorylation at Ser307,35 inhibition of JNK had no effect on the Ang II–induced inhibition of Akt. As demonstrated in human kidney fibroblast 293 cells,36 direct phosphorylation of IRS-1 by PKC could be a mechanism by which Ang II inhibits IRS-1 function in VSMCs.

In summary, the present study demonstrates that Ang II inhibits insulin-induced Akt activation through PKC activation. These vascular pathogens are known to activate PKC,3,30 and we were further able to show that the PKC-α isoform inhibited insulin-induced Akt activation. Overexpression of PKC-α, and not PKC-β or PKC-δ,24 inhibited insulin-induced Akt activation. Moreover, PKC-α was translocated to the membrane in VSMCs when stimulated with Ang II. In addition, we used an AT₁ receptor antagonist, RNH 6270,23 to demonstrate that the inhibition of insulin-induced activation of Akt by Ang II was mediated by the Gₛ-coupled AT₁ receptor, which activates PKC.3 This is in good agreement with a recent publication31 showing that overexpression of constitutively active Gₛ mutant inhibits Akt in HEK 293 cells.

Perspectives

Accumulating data indicate the important pathophysiological function of insulin resistance in the vasculature.37 Insulin resistance is an important risk factor in cardiovascular diseases, and the activation of PKC is associated with many tissue abnormalities observed in diabetes. Because Ang II, a vasoactive hormone, is critically involved in the development of cardiovascular remodeling associated with hypertension and atherosclerosis, the findings presented here will provide a
potential mechanism of signal transduction associated with insulin resistance leading to cardiovascular diseases.

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References


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