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

Evangeline D. Motley, Kunie Eguchi, Carla Gardner, Adrienne L. Hicks, Cherilynn M. Reynolds, Gerald D. Frank, Mizuo Mifune, Motoi Ohba, Satoru Eguchi

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- and concentration-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. A recent study shows that prior

Received September 19, 2002; first decision November 12, 2002; revision accepted November 27, 2002.
From the Department of Anatomy and Physiology, Meharry Medical College (E.D.M., K.E., C.G., A.L.H., C.M.R.), Nashville, Tenn; the Department of Biochemistry, Vanderbilt University School of Medicine (K.E., G.D.F., M.M., S.E.), Nashville, Tenn; and Institute of Molecular Oncology, Showa University (M.O.), Tokyo, Japan.
Corresponding to Evangeline D. Motley, PhD, Department of Anatomy and Physiology, Meharry Medical College, 1005 D.B. Todd Blvd, Nashville, TN 37208. E-mail emotley@mmc.edu
© 2003 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000051891.90321.12

775
activation of PKC can inhibit the subsequent ability of insulin to stimulate the enzymatic activity of Akt in 3T3-L1 adipocytes. 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 AT1 receptor blockers improve insulin sensitivity. 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-α, 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 Ser473-phosphorylated Akt and Akt were purchased from Cell Signaling. Antibodies for Tyr1158-phosphorylated insulin receptor-β and IRS-1 were purchased from Santa Cruz Biotechnology. Antibodies for phospho-tyrosine and insulin receptor-β subunit were purchased from Upstate Biotechnology. Antibody directed to Tyr118-phosphorylated insulin receptor-β 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. Subcultured VSMCs used in the experiments showed >99% positive immunostaining of smooth muscle α-actin antibody. For the experiments, cells from passage 3 to 12 at ~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, 10 mmol/L EDTA, 10 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 2 mmol/L sodium orthovanadate 10% (v/v) glycerol and 10 μg/mL of leupeptin, 10 μg/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.

**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. 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 temperature, immunoreactive proteins were visualized by ECL reagent (Amersham Life Sciences).

**Adenovirus Transfection**

The generation of PKC-α and PKC-β wild-type adenovirus was described in detail elsewhere. VSMCs were infected with the adenovirus for 2 days before stimulation as previously described.

**PKC-α Membrane Translocation**

PKC-α translocation was determined by collection of the membrane fraction, as described previously. 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 sulfonyl fluoride, and 20 μmol/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-α antibody.

**Statistical Analysis**

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

**Results**

We have previously demonstrated that insulin induces Akt activation in VSMCs. In the present study, we examined the effect of Ang II on insulin-induced Akt phosphorylation in VSMCs. We found that Ang II pretreatment significantly inhibited insulin-induced Akt phosphorylation, with maximal inhibition occurring at 20 to 40 minutes. The maximal concentration of Ang II that inhibited insulin-induced Akt phosphorylation was 1 μmol/L. We have previously demonstrated that insulin induces Akt activation in VSMCs. In the present study, we examined the effect of Ang II on insulin-induced Akt phosphorylation in VSMCs. We found that Ang II pretreatment significantly inhibited insulin-induced Akt phosphorylation, with maximal inhibition occurring at 20 to 40 minutes. We have previously demonstrated that insulin induces Akt activation in VSMCs. In the present study, we examined the effect of Ang II on insulin-induced Akt phosphorylation in VSMCs. We found that Ang II pretreatment significantly inhibited insulin-induced Akt phosphorylation, with maximal inhibition occurring at 20 to 40 minutes. We have previously demonstrated that insulin induces Akt activation in VSMCs. In the present study, we examined the effect of Ang II on insulin-induced Akt phosphorylation in VSMCs. We found that Ang II pretreatment significantly inhibited insulin-induced Akt phosphorylation, with maximal inhibition occurring at 20 to 40 minutes. The Gq-coupled AT1 receptor activation leads to the mobilization of Ca2+ and the activation of PKC. 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 Ca2+ 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 time 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). We were further able to demonstrate that other PKC inhibitors, Go6976 and Go6983, 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-α and PKC-β, we examined the effects of overexpression of PKC-α and -β isoforms on insulin-induced Akt phosphorylation (Figure 3A). We found that overexpression of PKC-α isoform but not PKC-β isoform inhibited insulin-induced Akt phosphorylation (Figure 3A). In addition, Ang II stimulated the translocation of PKC-α to the membrane of VSMCs (Figure 3B). Although GF109203X and Go6983 inhibit PKC-δ, the PKC-δ inhibitor rotterlin (10 μmol/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. Previous studies showed that Ang II activates these MAPKs in VSMCs. 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, 20 μmol/L, 30-minute pretreatment) (data not shown), 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

---

**Figure 1.** Effect of Ang II on insulin-dependent Akt activation in VSMCs. A, VSMCs were pretreated with Ang II (1 μmol/L) for the indicated durations and were stimulated with insulin (1 μmol/L) for 5 minutes. B, VSMCs were pretreated with Ang II for the indicated concentrations for 40 minutes and were stimulated with 1 μmol/L insulin for 5 minutes or pretreated with 1 μmol/L RNH 6270 for 30 minutes and stimulated with 1 μmol/L Ang II for 40 minutes and 1 μmol/L insulin for 5 minutes. Cell lysates were immunoblotted by phospho-Akt and Akt antibodies as indicated. The data are presented as mean±SEM of 3 experiments. The bar graphs show % pAkt. *P<0.05 vs basal.

---

**Figure 2.** Roles of calcium and PKC on Ang II–induced inhibition of Akt activation in VSMCs. A, VSMCs were pretreated with A23187 (10 μmol/L) for the indicated time periods and stimulated with 1 μmol/L insulin for 5 minutes. B, VSMC were pretreated with GF109203X (2 μmol/L) for 30 minutes and PMA (1 μmol/L) for 10 minutes and stimulated with 1 μmol/L insulin for 5 minutes. C, VSMCs were pretreated with 2 μmol/L each of GF109203X, Go6976, and Go6983 for 30 minutes and Ang II (1 μmol/L) for 40 minutes, and were stimulated with 1 μmol/L insulin for 5 minutes. Cell lysates were immunoblotted by phospho-Akt and Akt antibodies as indicated. The data are presented as mean±SEM of 3 experiments. *P<0.05 vs insulin stimulation.
effect of Ang II on insulin-induced Akt phosphorylation (Figure 5A). We also confirmed that Ang II stimulates Akt phosphorylation in a time-dependent manner, with maximum phosphorylation occurring at 5 minutes (Figure 5B). In Figure 5C, Ang II does not inhibit Akt phosphorylation if it is added at the same time as insulin. However, pretreatment for 5 minutes with Ang II is sufficient to inhibit insulin-induced phosphorylation of Akt. Thus, these data suggest that the activation mechanism of Akt by Ang II may be overridden by the inhibitory mechanism of Akt by Ang II if followed by insulin stimulation.

**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.

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
In this study, we showed that Ang II inhibits Akt through PKC activation. These vascular pathogens are known to activate PKC, 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, 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, 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. This is in good agreement with a recent publication showing that overexpression of constitutively active Gₛₘₐₜ mutant inhibits Akt in HEK 293 cells.

We have demonstrated that PKC-α overexpression inhibits IRS-1 tyrosine phosphorylation induced by insulin; 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. Ang II activates 3 major MAPKs that can inhibit IRS function through Ser/Thr phosphorylation in VSMCs. In HEK 293 cells, it was shown that PMA inhibited insulin-stimulated PI3-kinase through p42/44 MAPK-dependent IRS-1 phosphorylation. Studies have also shown that p38 MAPK is involved in H₂O₂-induced inhibition of insulin-stimulated glucose transport in cultured skeletal muscle cells. 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, inhibition of JNK had no effect on the Ang II–induced inhibition of Akt. As demonstrated in human kidney fibroblast 293 cells, 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, 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, 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, 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. This is in good agreement with a recent publication showing that overexpression of constitutively active Gₛₘₐₜ mutant inhibits Akt in HEK 293 cells.

Perspectives

Accumulating data indicate the important pathophysiological function of insulin resistance in the vasculature. 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

---

Figure 5. Effect of EGF receptor inhibitor on Ang II–induced inhibition of Akt activation by insulin. A, VSMCs were pretreated with the EGF receptor inhibitor AG1478 (250 nmol/L) for 1 hour and then treated with Ang II (1 μmol/L) for 40 minutes. The cells were then stimulated with insulin (1 μmol/L) for 5 minutes. B, VSMCs were stimulated with Ang II (1 μmol/L) for the indicated durations or stimulated with insulin (1 μmol/L) for 5 minutes. C, VSMCs were stimulated with Ang II (1 μmol/L) and insulin (1 μmol/L) for 5 minutes at the same time or Ang II was added 5 minutes before insulin. Cell lysates were immunoblotted by phospho-Akt and Akt antibodies as indicated. The data are presented as mean±SEM of 3 experiments. *P<0.05 vs insulin stimulation.

Figure 6. Proposed scheme of Ang II–induced inhibition of Akt activation by insulin. This scheme shows vascular agonist, Ang II activating PKC, which in turn, leads to the inhibition of IRS-1 tyrosine phosphorylation, PI3-kinase activation, and Akt activation. Ang II also transactivates the EGF receptor through intracellular calcium-elevation, which directly activates PI3-kinase and Akt. This is an independent pathway of the PKC activated pathway that causes insulin resistance.
potential mechanism of signal transduction associated with insulin resistance leading to cardiovascular diseases.

Acknowledgments

This research was supported in part by National Institutes of Health–National Heart, Lung, and Blood Institute grant HL-03320, National Institutes of Health–National Center for Research Resources grant 2G12RR03032, and the American Heart Association’s Southeast–Affiliate Grant. Dr. Frank has been supported by the United Negro College Fund/merck Postdoctoral Science Research Fellowship. Dr. Eguchi has been supported by the American Heart Association’s Scientist Development Grant and Vanderbilt University Diabetes Center Pilot and Feasibility Program.

References

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

Evangeline D. Motley, Kunie Eguchi, Carla Gardner, Adrienne L. Hicks, Cherilynn M. Reynolds, Gerald D. Frank, Mizuo Mifune, Motoi Ohba and Satoru Eguchi

*Hypertension*. 2003;41:775-780; originally published online January 13, 2003; doi: 10.1161/01.HYP.0000051891.90321.12

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/41/3/775

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Hypertension* is online at:
http://hyper.ahajournals.org/subscriptions/