Lysophosphatidylcholine Inhibits Insulin-Induced Akt Activation Through Protein Kinase C-α in Vascular Smooth Muscle Cells

Evangeline D. Motley, Syeda M. Kabir, Carla D. Gardner, Kunie Eguchi, Gerald D. Frank, Toshio Kuroki, Motoi Ohba, Tadashi Yamakawa, Satoru Eguchi

Abstract—To better understand the intracellular signaling mechanism that causes the association of insulin resistance and hyperlipidemia with cardiovascular diseases, we specifically looked at the ability of lysophosphatidylcholine (lysoPC) to inhibit the Akt activation induced by insulin in cultured rat aortic vascular smooth muscle cells. LysoPC inhibited the insulin-induced phosphorylation of Akt at Ser473, and the inhibition was concentration dependent. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, inhibited the insulin-induced phosphorylation of Akt. LysoPC stimulated PKC phosphorylation at Ser660, which was inhibited by the PKC inhibitor GF109203X. The PKC-α/β-selective inhibitor Go6976 also blocked the PMA- and lysoPC-induced inhibition of Akt phosphorylation by insulin. PKC-α, but not PKC-β, is expressed in vascular smooth muscle cells, and overexpression of PKC-α, but not PKC-β or PKC-δ, inhibited insulin-induced Akt activation. LysoPC rapidly stimulated PKC-α translocation to the membrane. In contrast, pretreatment with the p42/44 mitogen-activated protein kinase kinase inhibitor PD98059 or the p38 mitogen-activated protein kinase kinase inhibitor SB203580 did not block the lysoPC-induced inhibition of Akt phosphorylation by insulin. In addition, lysoPC inhibited the insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS)-1 but not that of the insulin receptor β subunit or insulin binding. PMA treatment or PKC-α overexpression also inhibited the tyrosine phosphorylation of IRS-1. From these data, we conclude that lysoPC negatively regulates the insulin signal at the point of IRS-1 through PKC-α in the vasculature, which may explain the association of hyperlipidemia with hyperinsulinemia in cardiovascular diseases. (Hypertension. 2002;39[part 2]:508-512.)

Key Words: lysophosphatidylcholines • insulin resistance • muscle, smooth, vascular • protein kinases

Insulin resistance, which results from increased insulin concentration and decreased insulin sensitivity, is an important risk factor in the development of cardiovascular diseases, such as atherosclerosis and coronary artery disease.1,2 However, the specific role of insulin resistance in the etiology of these diseases is poorly understood. Oxidized LDL also plays a key role in the development of atherogenesis.3 Circulating levels of several forms of modified LDL are elevated in the plasma of diabetic patients.4,5 Furthermore, LDL from patients with poorly controlled insulin-dependent diabetes have an enhanced susceptibility to LDL oxidation.6,7 During the oxidation of LDL, as much as 40% of its phosphatidylcholine can be converted to lysophosphatidylcholine (lysoPC), a putative active component of oxidized LDL. LysoPC has several biological activities in the vasculature, eg, stimulation of proliferation8 and migration of vascular smooth muscle cells (VSMCs),9 induction of adhesion molecules in endothelial cells,10 and promotion of endothelial dysfunction.12 In cultured VSMCs, we have recently shown that lysoPC induces mitogen-activated protein kinase (MAPK) activation and c-fos expression and enhances activator protein-1 DNA binding activity, which requires protein kinase C (PKC).13 Although alterations in insulin action in the vasculature have been proposed to contribute to atherosclerosis and the regulation of vascular tone,14 little is known regarding the pathways of insulin signaling and their regulation in VSMCs. Activation of the insulin receptor is known to result in tyrosine phosphorylation of several cytosolic docking proteins called insulin receptor substrates (IRSs). IRSs bind to several effector molecules, including the regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase) via Src homology 2 domains. Recruitment of the catalytic subunit results in activation of PI3-kinase, which is necessary for insulin action.15,16 Akt, a Ser/Thr protein kinase (also called protein kinase B) is one of the major targets of PI3-kinase–generated

Received September 23, 2001; first decision October 29, 2001; revision accepted November 12, 2001.
From the Department of Anatomy and Physiology (E.D.M., S.M.K., C.D.G., K.E.), Meharry Medical College, and the Department of Biochemistry (G.D.F., S.E.), Vanderbilt University School of Medicine, Nashville, Tenn; the Institute of Molecular Oncology (T.K., M.O.), Showa University, Tokyo, Japan; and the Department of Endocrinology and Diabetes (T.Y.), Yokohama City University Medical Center, Yokohama, Japan.

Correspondence 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

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signals by insulin. Recent studies have shown that a vasoactive hormone, angiotensin II, impairs insulin stimulation of IRS-1 tyrosine phosphorylation and coupling of the insulin receptor pathway to PI3-kinase in cultured VSMCs. These data indicate that cultured VSMCs are an interesting model to use for the study of the mechanism of insulin resistance, which is possibly induced by vasoactive substances.

In the present study, we have examined our hypothesis that lysoPC inhibits insulin-induced Akt activation through PKC activation in VSMCs. We demonstrated that lysoPC does inhibit insulin-induced Akt activation in VSMCs and were further able to show that this inhibitory mechanism involves PKC-α. Our results suggest a role for lysoPC in vascular insulin resistance and may contribute to a better understanding of the pathogenesis of cardiovascular diseases.

**Methods**

**Materials**

LysoPC, phorbol 12-myristate 13-acetate (PMA), and insulin were purchased from Sigma Chemical Co. PD98059, SB203580, wortmannin, LY294002, GF109203X, Go6976, Go6983, and rottlerin were from Calbiochem. LysoPC and insulin were dissolved in 100% ethanol and 0.02% acetic acid, respectively. All other agonists and inhibitors were dissolved in 100% dimethyl sulfoxide. Antibodies were purchased from the following sources: Ser473-phosphorylated Akt, Akt and Ser660-phosphorylated PKC (cell signaling), PKC-α, PKC-βΙΙ, PKC-βΙ, PKC-δ, Tyr204-phosphorylated p42/44 MAPK (ERK1/2), p42 MAPK (ERK2), and IRS-1 were from Santa Cruz Biotechnology; phosphotyrosine and insulin receptor β subunit were from Upstate Biotechnology; and Tyr1158-phosphorylated insulin receptor was from BioSource International. VSMCs were prepared from the thoracic aortas of Sprague-Dawley rats by the explant method. VSMCs from passages 3 to 12 were made quiescent by incubation with serum-free medium for 3 days. The responses of VSMCs to insulin and lysoPC were consistent during these passages.

**Immunoprecipitation and Immunoblotting**

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% [vol/vol] glycerol, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonfly fluoride). The cell lysates were centrifuged, and the supernatant was immunoprecipitated with the antibody and protein A/G plus agarose at 4°C for 1 hour with minimal phosphorylation occurring at 25 μmol/L (Figure 1B). To investigate whether PI3-kinase is involved in the insulin-induced activation of Akt, the effects of 2 structurally unrelated PI3-kinase inhibitors, wortmannin and LY294002, were examined (Figure 1C). Both inhibitors markedly attenuated the insulin-induced Ser473 phosphorylation of Akt.

To examine the effect of lysoPC on Akt activation by insulin, we pretreated VSMCs with lysoPC and determined Akt phosphorylation. LysoPC (25 μmol/L) inhibited insulin-induced phosphorylation of Akt at Ser473 as early as 5 minutes and maximally at 10 minutes (Figure 2A). The inhibition was concentration dependent, with maximal phosphorylation occurring at 1 mmol/L and maximal phosphorylation occurring at 1 μmol/L (Figure 1B). To investigate whether PI3-kinase is involved in the insulin-mediated signal transduction in VSMCs, the effect of insulin on Akt activity was examined by using an antibody that selectively recognizes Ser473-phosphorylated Akt. The Ser473 phosphorylation by insulin was first detected at 2 minutes and peaked at 5 to 10 minutes (Figure 1A). The Akt phosphorylation by insulin was concentration dependent, with minimal phosphorylation occurring at 1 mmol/L and maximal phosphorylation occurring at 1 μmol/L (Figure 1B). To investigate whether PI3-kinase is involved in the insulin-induced activation of Akt, the effects of 2 structurally unrelated PI3-kinase inhibitors, wortmannin and LY294002, were examined (Figure 1C). Both inhibitors markedly attenuated the insulin-induced Ser473 phosphorylation of Akt.

To determine the target of lysoPC in insulin signaling of VSMCs, the effects of lysoPC on IRS-1 and insulin receptor tyrosine phosphorylation were examined. As shown in Figure 2C, lysoPC markedly inhibited insulin-induced IRS-1 tyrosine phosphorylation. By contrast, the insulin receptor tyrosine phosphorylation induced by insulin was not inhibited by lysoPC (Figure 2D). Also, lysoPC had no effect on insulin binding to its receptor (authors’ unpublished data, 2001). The data suggest that lysoPC inhibits IRS-1 tyrosine phosphorylation, which results in the inhibition of insulin-induced Akt activation in VSMCs.

Because lysoPC significantly potentiates PKC-mediated cellular responses, we wanted to determine whether a PKC...
activated, PMA, had an effect similar to that of lysoPC on insulin-induced Akt phosphorylation. The PMA pretreatment (10 minutes) inhibited insulin-induced Akt phosphorylation in a concentration-dependent manner (Figure 3A). PMA also inhibited insulin-induced IRS-1 tyrosine phosphorylation in VSMCs (Figure 3B). To test whether PKC is responsible for the inhibition of Akt by lysoPC, we examined the effect of PKC inhibitors GF109203X, Go6976, and Go6983 on the lysoPC- and PMA-induced inhibition of Akt activation. Figure 3C shows that these PKC inhibitors block the inhibitory effects of PMA and lysoPC on insulin-induced Akt phosphorylation in VSMCs. Also, these PKC inhibitors had no effect on either the basal or insulin-induced Akt phosphorylation (authors’ unpublished data, 2001). In addition, lysoPC enhanced the PKC autophosphorylation at Ser660 that was enhanced by PMA, which is consistent with the previous report.26,27 Recently, it has been shown that activation of PKC can inhibit insulin-induced Akt activation in 3T3-L1 adipocytes.27 In the major findings of the present study are (1) lysoPC inhibited insulin-induced Akt activation, and (2) the inhibition requires PKC-α activation by lysoPC. Thus, our findings suggest a role for lysoPC in the development of vascular insulin resistance through PKC-α activation.

Activation of PKC results in the inhibition of insulin signaling in several cell types, including human fibroblasts.26 Activation was shown to be similar to that of the PKC activator, PMA, with no effect on either the basal or insulin-induced Akt phosphorylation (authors’ unpublished data, 2001). In addition, lysoPC enhanced the PKC autophosphorylation at Ser660 that was enhanced by PMA, which is consistent with the previous report.26,27 Recently, it has been shown that activation of PKC can inhibit insulin-induced Akt activation in 3T3-L1 adipocytes.27 In the present study, we observed that the inhibition of Akt by lysoPC, we examined the effect of PKC inhibitors GF109203X, Go6976, and Go6983 on the lysoPC- and PMA-induced inhibition of Akt activation. Figure 3C shows that these PKC inhibitors block the inhibitory effects of PMA and lysoPC on insulin-induced Akt phosphorylation in VSMCs. Also, these PKC inhibitors had no effect on either the basal or insulin-induced Akt phosphorylation (authors’ unpublished data, 2001). In addition, lysoPC enhanced the PKC autophosphorylation at Ser660 that was inhibited by GF109203X (Figure 3D). Although these PKC inhibitors inhibited the α, β, and δ isoforms of PKC, the selective PKC-δ inhibitor, rottlerin, could not block the inhibitory effect of lysoPC (Figure 3E), and we could not detect any PKC-β (I or II isoform) expression in VSMCs (authors’ unpublished data, 2001).

By contrast, PKC-α overexpression resulted in concentration-dependent inhibition of insulin-induced Akt activation (Figure 4A). It also inhibited insulin-induced IRS-1 tyrosine phosphorylation (Figure 4B). However, PKC-β or PKC-δ overexpression had no effect on insulin-induced Akt phosphorylation (Figure 4C). Also, lysoPC rapidly stimulated PKC-α translocation to the membranes (Figure 4D). To determine whether p42/44 MAPK or p38 MAPK was involved in the lysoPC-induced inhibition of Akt, the p42/44 MAPK kinase inhibitor PD98059 or the p38 MAPK inhibitor SB203580 was used. Pretreatment with PD98059 (Figure 4E) or SB203580 (authors’ unpublished data, 2001) for 1 hour did not affect the inhibition of insulin-induced Akt activation induced by lysoPC. In contrast, the lysoPC-induced p42/44 MAPK activation was markedly inhibited by PD98059 (Figure 4E).

Discussion
The major findings of the present study are (1) lysoPC inhibited insulin-induced activation of Akt in VSMCs by blocking IRS-1 function, and (2) the inhibition requires PKC-α activation by lysoPC. Thus, our findings suggest a role for lysoPC in the development of vascular insulin resistance through PKC-α activation.

Activation of PKC results in the inhibition of insulin signaling in several cell types, including human fibroblasts.26 Recently, it has been shown that activation of PKC can inhibit insulin-induced Akt activation in 3T3-L1 adipocytes.27 In the...
We have demonstrated that lysoPC activates p42/44 MAPK through PKC activation in VSMCs. In the present study, we have shown that IRS-1 is the target by which lysoPC inhibits Akt activation. PKC was proposed to stimulate a kinase that can phosphorylate IRS-1 at Ser612, resulting in inhibition of insulin signaling. In HEK 293 cells, PMA inhibits insulin-stimulated PI3-kinase through p42/44 MAPK–dependent IRS-1 phosphorylation. These data suggest that p42/44 MAPK may play a role at the downstream of PKC in lysoPC-induced inhibition of Akt activation in VSMCs. However, in the present study, the MAPK kinase inhibitor PD98059 did not have any effect on the lysoPC-induced inhibition of Akt activation, even though it inhibited p42/44 MAPK activation induced by lysoPC. LysoPC has also been shown to activate p38 MAPK in VSMCs. By using SB203580, p38 MAPK appears to be involved in H₂O₂-induced inhibition of insulin-stimulated glucose transport in cultured skeletal muscle cells. The H₂O₂-induced PKC-ι inhibition could be rescued by the PKC inhibitor Go6976 in other cells. However, the same p38 MAPK inhibitor did not reverse the lysoPC-induced inhibition of insulin-induced Akt activation in VSMCs. In addition, lysoPC stimulates c-Jun N-terminal kinase (JNK) phosphorylation in several cell types. Recently, tumor necrosis factor-α was shown to inhibit insulin signaling through JNK-induced IRS-1 phosphorylation at Ser307. However, it is unlikely that JNK contributes to the PKC-dependent inhibition of Akt by lysoPC because PMA and lysoPC (authors’ unpublished data, 2001) could not activate JNK in our VSMCs. Taken together, these data suggest that these MAPK pathways are not involved in the lysoPC-induced inhibition of IRS-1 function in VSMCs. Thus, direct phosphorylation of IRS-1 by PKC-α, which leads to inhibition of IRS-1 function, needs to be considered.

It is possible that the concentration of insulin used in the present study likely activates the IGF-1 receptor that is expressed in VSMCs in addition to the insulin receptor. Although insulin receptor tyrosine phosphorylation and concentration-dependent Akt phosphorylation by insulin clearly demonstrate the existence of a specific insulin receptor in VSMCs, lysoPC could also inhibit IGF-1 receptor–mediated Akt activation in our VSMCs.

In summary, the present study demonstrates that lysoPC inhibits insulin-induced Akt activation through PKC-α activation in VSMCs. Because accumulating data point out the important pathophysiological function of insulin resistance in the vasculature, our findings will provide alternative interventions for the cardiovascular diseases associated with the progression of insulin resistance and hyperlipidemia.

Acknowledgments
This research was supported in part by grants from the National Institutes of Health (NIH)–National Heart, Lung, and Blood Institute (HL-03320 and HL-58205) and by a grant from NIH–National Center for Research Resources (2G12 RR-03032). Dr Frank was supported by a United Negro College Fund/Merck Postdoctoral Science Research Fellowship. Dr Eguchi was supported by a Scientist Development Grant from the American Heart Association and the Diabetes Center Pilot and Feasibility Program of Vanderbilt University.
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Hypertension. 2002;39:508-512
doi: 10.1161/hy02t2.102907

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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