Angiotensin II-Induced Akt Activation through the Epidermal Growth Factor Receptor in Vascular Smooth Muscle Cells Is Mediated by Phospholipid Metabolites Derived by Activation of Phospholipase D<sup>3</sup>

Fang Li and Kafait U. Malik

Department of Pharmacology and Vascular Biology Center of Excellence, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee

Received August 23, 2004; accepted October 29, 2004

ABSTRACT

Angiotensin II (Ang II) activates cytosolic Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), phospholipase D (PLD), p38 mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR), and Akt in vascular smooth muscle cells (VSMC). This study was conducted to investigate the relationship between Akt activation by Ang II and other signaling molecules in rat VSMC. Ang II-induced Akt phosphorylation was significantly reduced by the PLD inhibitor 1-butanol, but not by its inactive analog 2-butanol, and by brefeldin A, an inhibitor of the PLD cofactor ADP-ribosylation factor, and in cells infected with retrovirus containing PLD2 siRNA or transfected with PLD2 the PLD cofactor ADP-ribosylation factor, and in cells infected with its inactive analog 2-butanol, and by brefeldin A, an inhibitor of several downstream effectors including Bad, forkhead transcription factor, IKKα, E2F, Gsk3, p70S6K, hTERT, eNOS, and Akt, a serine/threonine kinase, contains N-terminal pleckstrin-homology domain, a kinase domain, and a C-terminal regulatory domain (Shiojima and Walsh, 2002). Cytokines and several growth factors, including angiotensin II (Ang II), stimulate the conversion of phosphatidylinositol(4,5)bisphosphate to phosphatidylinositol(3,4,5)trisphosphate through activation of phosphatidylinositol 3-kinase (PI3K) (Shiojima and Walsh, 2002). Phosphatidylinositol(3,4,5)trisphosphate binds to the pleckstrin-homology domain of Akt, recruits Akt to the plasma membrane, and exposes Akt to phosphorylation at serine 473 in the regulatory domain and at threonine 308 in the kinase domain by 3-phosphoinositide-dependent protein kinases, which associates with protein kinase C-related kinase-2 (Shiojima and Walsh, 2002). Phosphorylated and activated Akt stimulates several downstream effectors including Bad, forkhead transcription factor, IKKα, E2F, Gsk3, p70S6K, hTERT, eNOS, and

ABBREVIATIONS: Ang II, angiotensin II; PI3K, phosphatidylinositol 3-kinase; VSMC, vascular smooth muscle cells; AT,R, angiotensin type 1 receptor; cPLA<sub>2</sub>, cytosolic Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub>; AA, arachidonic acid; PLD, phospholipase D; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; AG1478, 4(3-chlorophenylamino)-6,7-dimethoxyquinazoline; RHC 80267, 1,6-bis-(cyclohexylloximinocarbonylamino)-hexane; ETYA, 5,8,11,14-eicosatetraynoic acid; diC8-PA, 1,2-dioctanoyl-sn-glycerol-3-phosphate; SB202190, 4-(4-flurophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole; Pyrrolidine-1, a cPLA2 inhibitor, and cPLA2 siRNA decreased Akt activity and EGFR and Akt phosphorylation elicited by Ang II. Akt activity is mediated by cPLA2-dependent, p38 MAPK regulated PLD2 activation and EGFR transactivation. We propose the following scheme of the sequence of events leading to activation of Akt in VSMC by Ang II: Ang II→cPLA<sub>2</sub>→AA→p38 MAPK→PLD<sub>2</sub>→PA→EGFR→Akt.
mTOR, which regulate cell survival, cell cycle, glucose metabolism, angiogenesis, vasomotor tone, and protein synthesis (Shiojima and Walsh, 2002).

Akt has also been implicated in the action of Ang II to promote vascular smooth muscle cells (VSMC) proliferation and growth. Ang II stimulates Akt activity through the angiotensin type 1 receptor (AT1R) in VSMC (Takahashi et al., 1999). Ang II activates cytosolic Ca2+-dependent phospholipase A2 (cPLA2) and releases arachidonic acid (AA) via activation of AT1R (Rao et al., 1994; Muthalif et al., 1998). AA and its metabolite(s) 5(S)- and 12(S)-hydroxyeicosatetraenoic acid generated via lipoxigenase have been shown to increase Akt activity in VSMC or other cell types (Szekeres et al., 2002; Zeng et al., 2002). Also, Ang II increases phospholipase D (PLD) activity in VSMC through AT1R (Freeman and Talsi, 1994). PLD has been reported to be involved in Akt activation caused by sphingosine-1-phosphate in Chinese hamster ovary cells overexpressing endothelial differentiation gene (EDG3) receptor (Banno et al., 2001). It is not known whether PLD is also involved in Ang II-induced Akt activation in VSMC. Activation of PLD by Ang II is dependent upon cPLA2 activity in rabbit VSMC (Parmentier et al., 2001b). AA production from PLA2 has been shown to increase Akt activity in mesangial cells and VSMC (Gorin et al., 2001; Neeli et al., 2003). Therefore, it is possible that Ang II causes Akt activation in rat VSMC by increasing cPLA2 or PLD activity.

Ang II has also been reported to increase Akt activity via transactivation of epidermal growth factor receptor (EGFR) in VSMC (Eguchi et al., 1999). Cholesterol depletion that disrupts the structure of caveolae inhibits tyrosine phosphorylation of EGFR and subsequent activation of Akt induced by Ang II (Ushio-Fukai et al., 1998b). EGFR overexpression or EGFR stimulation also increase PLD activity, and PLD associates with EGFR during its activation in fibroblasts (Slabay et al., 1998; Lu et al., 2000; Kim et al., 2003).

Whether Ang II-induced Akt activation is mediated by transactivation of EGFR through PLD activation or via EGFR-stimulated PLD activity in VSMC is not known. EGFR transactivation by Ang II has also been shown to cause activation of p38 mitogen-activated protein kinase (MAPK) in VSMC (Eguchi et al., 2001). Moreover, expression of an active form of Akt increases p38 MAPK and its upstream kinase MKK3/6 activity in a follicular dendritic cell-like cells (Lee et al., 2003), whereas blockade of PI3K/Akt stimulates p38 MAPK-dependent apoptosis in endothelial cells (Gratton et al., 2001). However, in some other cell types including VSMC, p38 MAPK has been reported to act upstream of Akt (Taniyama et al., 2004), where p38 MAPK is constitutively associated with Akt and activates Akt through downstream MAPK-activated protein kinase-2 in VSMC (Taniyama et al., 2004). p38 MAPK has also been shown to be upstream of PLD in VSMC (Min do et al., 2002). These observations and the demonstration that in VSMC 1) activation of p38 MAPK by norepinephrine is mediated by metabolites of AA generated through activation of cPLA2 (Kalyanakrishna and Malik, 2003) and that lipoxigenase inhibitors cinnamyl-3,4-dihydroxy-o-cyanocinnamate and baicalein attenuate p38 MAPK activation induced by Ang II in H295R adrenocortical cells (Natarajan et al., 2002), and 2) Ang II-induced PLD activation is dependent upon cPLA2 activity (Parmentier et al., 2001b) have led us to hypothesize that Ang II-stimulated Akt phosphorylation in VSMC is mediated via transactivation of EGFR by a mechanism dependent upon PLD activation by p38 MAPK, which is stimulated by AA metabolites generated via cPLA2 activation. The results of our study support this hypothesis and provide evidence that Ang II-induced Akt phosphorylation is mediated by the following sequence of activation of these signaling molecules: cPLA2→AA metabolite(s)→p38 MAPK→PLD→PA→EGFR transactivation→Akt.

Materials and Methods

Materials

Chemicals were purchased commercially from the following sources: aprotinin, dithiothreitol, phenylmethylsulfonyl fluoride, sodium orthovanadate, leupeptin, HEPES, antipain, propranolol, 1-butanol, and 2-butanol from Sigma-Aldrich (St. Louis, MO); 43-chlorophenylamino)-6,7-dimethoxyquinazoline (AG1478) from Calbiochem (San Diego, CA); 1,6-bis(cyclohexylaminocarbonylamino)-hexane (RHC 80267) and 5,8,11,14-eicosatetraynoic acid (ETYA) from BIOMOL Research Laboratories (Plymouth Meeting, PA); 1,2-dioctanoyl-sn-glycerol-3-phosphate (diC8-PA) monosodium salt from Avanti Polar Lipids (Alabaster, AL); AA from Cayman Chemical (Ann Arbor, MI); 4-(4-flurophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB202190) from Tocris Cookson Inc. (Ellisville, MO); human Ang II (H-Asp-Avg-Val-tyr-Ile-His-Pro-Phe-OH) and human EGF from Bachem Biosciences (King of Prussia, PA); anti-EGFR, anti-p-Akt, and anti-Akt antibody from Cell Signaling Technology Inc. (Beverly, MA); anti-rabbit horseradish peroxidase-conjugated Igg serum from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK); and anti-goat horseradish peroxidase-conjugated IgG (H + L) from Vector Laboratories (Burlingame, CA). Pyrrolidine-1 was kindly provided by Dr. M. H. Gelb (University of Washington, Seattle, Washington).

Anti-PLD2 antibody was kindly supplied by Dr. Sylvain Bourgin (Universite Laval, QC, Canada).

Methods

Isolation and Culture of VSMC. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 to 350 g were anesthetized with 40 mg/kg sodium pentobarbital (Abbott Laboratories, Abbott Park, IL), and the thorax was opened. The thoracic aorta was excised and rapidly removed. VSMC were isolated, cultured, and maintained under 5% CO2 in M199 medium with penicillin/streptomycin and 10% fetal bovine serum as described (Uddin et al., 1998). VSMC between the fourth and tenth passages were made quiescent for 48 h in M199 containing 0.1% fetal bovine serum before exposure to various agents. VSMC were characterized using smooth muscle specific antiactin antibody. Cell viability was determined before and after each experimental intervention by measuring trypan blue exclusion.

Transfection of VSMC with Antisense Oligonucleotides. Phosphorothioate oligonucleotides directed against the translation initiation sites of rat cPLA2 and PLD2 were synthesized (Invitrogen, Carlsbad, CA). The sequences of oligonucleotides used in this study were: cPLA2 sense, 5’-AZF GAT CCT TAT CAC CAG FZA-3’; cPLA2 antisense, 5’-TFZ GTG CTG AT AAG AGG ATC ZPT-3’; and PLD2 sense, 5’-AZE ACT GTA ACC CAG AGG EFC-3’; PLD2 antisense, 5’-GZO CGT CTG GGT TAC AGT OFT-3’; F-3’ (F=A-phosphorothioate, O=C-phosphorothioate, E=G-phosphorothioate, and Z=T-phosphorothioate). VSMC, about 80% confluent, were transiently transfected with either sense or antisense oligonucleotides (200 nM) complexed with oligofectamine reagent according to the manufacturer’s protocol (Invitrogen) and incubated for 6 h in the medium without antibiotics.
and serum. VSMC were maintained in M199 containing 0.1% fetal bovine serum in the presence of oligonucleotides. After 48 h, the cells were washed three times with Hanks’ balanced salt solution and treated with Ang II (200 nM) or its vehicle.

**Western Blot Analysis.** The whole cell lysate from parental cells and transfected cells was prepared in lysis buffer (1% Igepal CA-630, 25 mM HEPES, pH 7.5, 50 mM NaCl, 50 mM NaF, 10 mM odacitic acid, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μM antipain, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), sonicated, centrifuged, and quantitated for protein content using the Bradford (1976) method. Equal amounts of protein (20–80 μg) were loaded onto SDS-polyacrylamide gel electrophoresis (20 mA/gel) and transferred onto nitrocellulose membrane (1 mM/m² membrane). The blots were blocked, incubated with primary antibodies (1:1000) at 4°C overnight, and then incubated with their respective secondary antibodies conjugated with horseradish peroxidase for 1 h. Blots were developed with the ECL or ECL plus Western blotting detection kit (Amersham Biosciences Inc., Piscataway, NJ). The density of bands was measured using the NIH Image 1.6 program.

**Preparation of siRNA and Transient Transfection of VSMC with Retroviral Vector Suppressor System.** The primers with forward sequences 5'-TGAGACGACGTGTTCTAGTGCC gagctag CGACGTGACACCATGTTTTTTT-3' for cPLA2; 5'-TGAGACGACGTGTTCTAGTGCC gagctag ACATGACGTGACACCATGTTTTTTT-3' for PLD2; and reverse 5'-CTAGTAGGAACATGACGTGACACCATGTTTTTTT-3' for PLD2, and reverse 5'-CTAGAGACACCATGTTTTTTT-3' for PLD2 were synthesized by Integrated DNA Technologies (Coralville, IA). Forward and reverse primers were mixed and annealed at 95°C for 10 min and then gradually cooled to room temperature. The annealed oligonucleotides were inserted into linearized pSuppressorRetro viral vector using ready-to-go T4 DNA ligase kit (Amersham Biosciences Inc.). Competent DH5α (Invitrogen) were transformed with ligated plasmid DNA. After transformation, the colonies were amplified and purified using a miniprep purification kit (QIAGEN, Venlo, The Netherlands), and plasmid DNAs were sequenced using primer complementary to pSuppressorRetro viral vector. Plasmids with correct sequences were then amplified and purified with QIAGEN Maxi Plasmid DNA kit and used to transfect HEK 293 cells using the following procedures for amplification. HEK 293 cells were grown to 30% to 50% confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The cells were transfected with the plasmid DNAs (pECKO packaging vector and pSuppressorRetro vector containing the cPLA2 or PLD2 siRNA insert) using the calcium phosphate precipitation method. The transfected cells were incubated for 3 to 4 h. Fresh medium was added and replaced on the second day. Virus was harvested by filtering the virus-containing supernatant. VSMC were made quiescent and infected with the viral supernatant in M199 containing 8 μg/ml polybrene and 0.1% fetal bovine serum for 48 h before the stimulation. The infection efficiency was confirmed by β-galactosidase staining (Invitrogen) of cells infected with retrovirus containing control LacZ.

**PLD Activity Assay.** PLD activity was measured by the method described (Parmentier et al., 2001b), which is based on the transphosphatidylation reaction between phosphatidic acid (PA) and ethanol that produces phosphatidylethanol (PtdEthanol). Briefly, VSMC transfected with cPLA2 or PLD2 sense or antisense oligonucleotides or VSMC infected with cPLA2 siRNA or PLD2 siRNA were radiolabeled with [3H]oleic acid (1 μCi/ml) in M199 containing penicillin/streptomycin and 0.1% fetal bovine serum for 18 h. The labeled cells were preincubated with ethanol (200 mM) for 10 min. For VSMC treated with various inhibitors, the inhibitors were added into cells for 30 min before adding ethanol. Cells were then exposed to 200 nM Ang II for 15 min, and the reaction was terminated by adding ice-cold methanol/2 M HCl (9:1) and 0.25 M HCl. The lipids were extracted, separated by thin-layer chromatography as described (Parmentier et al., 2001b), and visualized in iodine vapor. PtdEthanol was identified by the mobility of PtdEthanol standard and visualized with iodine vapor. Lanes containing radiolabeled PtdEthanol were moistened and scraped, and radioactivity was measured by scintillation spectroscopy. The data were expressed as the fractional [%]PtdEthanol of total [%]Ptd ethanol.

**P38 MAPK Kinase Assay.** p38 MAPK activity was determined by measuring the phosphorylated of its substrate ATF-2. Cell lysate (30 μg of total protein) was immunoprecipitated with immobilized phospho-thr180/tyr182-p38 MAPK (monoclonal; Cell Signaling Technology Inc.). Pellet was suspended and incubated 30 min in 50 μl of kinase buffer (20 mM HEPES pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, and 2.0 mM dithiothreitol) containing 200 μM radioactive cold ATP and 2 μg of ATF-2 fusion protein at 37°C. Reaction was terminated with 25 μl of 3× SDS sample buffer subjected to SDS-polyacrylamide gel electrophoresis. p38 MAPK kinase activity was measured by probing the blots with phospho-ATF-2 antibody (1:1000, anti-rabbit).

**Akt Kinase Assay.** Akt activity was determined by measuring the phosphorylation of its substrate GSK-3α with Akt activity assay kit (Calbiochem). Cell lysate (30 μg of total protein) was rotated with an anti-Akt antibody for 45 min at room temperature and then immunoprecipitated with protein A agarose beads for 1 h. Beads were washed twice with kinase extraction buffer and one time with kinase assay buffer supplied by kit. Washed beads were then suspended and incubated 3 h in 50 μl of kinase assay buffer containing a 2-μl GSK-3α/ATP mixture at 30°C. The beads were spun down and discarded. Reaction of supernatant was terminated with 25 μl of 3× SDS sample buffer. Akt kinase activity was measured by probing with phospho-GSK-3α antibody (1:1000, anti-rabbit).

**Results**

**Ang II-Stimulated Akt Phosphorylation Is Dependent on PLD Activity.** Phosphorylation of Akt on threonine 308 in the kinase domain and serine 473 in the hydrophilic motif activates the kinase. Akt activity can be determined from the extent of phosphorylation of ser473. In cultured VSMC, Ang II promotes Akt phosphorylation through activation of AT1R (Takahashi et al., 1999). In the present study, Ang II was used at a concentration of 200 nM, which has been shown to cause the maximal increase in Akt phosphorylation (Takahashi et al., 1999). In rat VSMC, Ang II-stimulated Akt phosphorylation at ser473, which was inhibited by 1-butanol (Fig. 1A) a potent PLD inhibitor (Parmentier et al., 2001a,b). However, its inactive analog 2-butanol did not affect the phosphorylation of Akt at the same concentration (Fig. 1A). 1-Butanol, but not 2-butanol, inhibited the transphosphatidylation in the presence of ethanol catalyzed by PLD, an index of PLD activity (Fig. 1B). These observations suggest that PLD is involved in Akt activation. Ang II-induced increase in PLD activity is mainly due to activation of the PLD2 isoform in rabbit VSMC, rat renal microvascular smooth muscle cells, and A10 VSMC line (Shome et al., 2000; Andersen et al., 2001; Parmentier et al., 2001b). To exclude possible nonspecific effects of these inhibitors on Akt activation, a retrovirus containing PLD2 siRNA or PLD2 antisense oligonucleotides were used to reduce the expression and activity of PLD2 in the cultured VSMC. PLD2 siRNA and antisense decreased PLD2 protein expression and decreased Akt phosphorylation stimulated by Ang II without affecting Akt total protein expression (Fig. 1, C and D), whereas VSMC infected with control retrovirus containing LacZ or transfected with PLD2 sense oligonucleotide did not affect Ang II-induced Akt activation. PLD2 siRNA, but not LacZ, attenuated Ang II-
Fig. 1. Ang II-induced Akt phosphorylation is regulated by PLD activity. A, PLD-specific inhibitor 1-butanol but not its inactive analog 2-butanol diminished Ang II-induced Akt phosphorylation. VSMC were made quiescent by incubating in M199 containing 0.1% fetal bovine serum for 48 h. Quiescent VSMC were pretreated with 0.4% (v/v) 1-butanol or 2-butanol for 30 min before exposure to Ang II (200 nM) for 5 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-Akt and Akt protein. The bottom panel shows the quantification of phosphorylated Akt compared with Akt protein level determined by using the NIH Image 1.6 program (n = 4). B, 1-butanol but not 2-butanol decreased PLD activity stimulated by Ang II. Quiescent VSMC were loaded with [3H]oleic acid for 18 h and treated with 1-butanol or 2-butanol (0.4% v/v) for 30 min before treating with Ang II (200 nM) for 15 min. The transephosphatidylation by PLD as a measure of PLD activity is
induced increase in PLD activity (Fig. 1E), indicating that Akt phosphorylation elicited by Ang II is dependent on PLD2 activation. Moreover, PLD2 siRNA did not affect cPLA2 protein level (data not shown). These data strongly suggest that Ang II-stimulated Akt phosphorylation is PLD2-dependent. The small G protein ADP-ribosylation factor (Arf) has been reported to regulate PLD2 activity in A10 cells (Shome et al., 2000). Brefeldin A, an Arf inhibitor, diminished Ang II-induced PLD2 activation in VSMC (Shome et al., 2000; Andreisen et al., 2001). In this study, the demonstration that brefeldin A decreased Ang II-activated Akt phosphorylation (Fig. 1F, lanes 1–4) further confirmed Ang II-stimulated Akt activity is dependent on PLD2 activation.

Ang II-Induced PLD-Dependent Akt Phosphorylation Is Mediated by Phosphatidic Acid. PLD hydrolyzes phosphatidylcholine to PA and choline, and PA is metabolized by phosphatidate phosphohydrolase (PPH) into diacylglycerol (DAG) (Ueno et al., 2000; Parmentier et al., 2001a). Whether PA or DAG causes Akt activation is not known. DAG can be phosphorylated to PA by DAG kinase (Billah et al., 1989; Ueno et al., 2000). DAG kinase inhibitor II, which diminishes PA generation from DAG, attenuated Ang II-induced Akt activation in VSMC (Fig. 2, A and B). DAG kinase inhibitor II did not alter EGF-induced Akt phosphorylation, indicating that there is a selective contribution of PA to Ang II- but not EGF-induced Akt activation (Fig. 2A). Exogenous diC8-PA with short acyl chains that increase its water solubility and cell permeability also stimulated Akt phosphorylation (Fig. 2C). These data indicate that PA generated by PLD activation is most likely involved in Ang II-activated Akt phosphorylation. DAG formed from PA by PPH causes the prolonged activation of protein kinase C and triggers other cell signaling pathways (Billah et al., 1989), and it can also be hydrolyzed to AA by DAG lipase (Billah et al., 1989). However, propranolol, a PPI inhibitor that reduces DAG production, and RHC 80267, a DAG lipase inhibitor that reduces AA release from DAG (Parmentier et al., 2001a), did not diminish Ang II-activated Akt phosphorylation (Fig. 2D). These data suggest that PA generated from PLD activation regulates Akt activation, whereas DAG or AA generated by PLD activation is not involved in Ang II-activated Akt phosphorylation in rat VSMC.

Ang II-Stimulated Akt Phosphorylation Is Mediated through PLD-Regulated EGFR Transactivation. Ang II signal transduction, including PI3K/Akt pathway (Ushio-Fukai et al., 2001b), has been reported to be mediated in part through EGFR transactivation (Ushio-Fukai et al., 1999, 2001). PI3K activation recruits Akt to the cell membrane and allows Akt phosphorylation by 3-phosphoinositide-dependent protein kinases (Shiojima and Walsh, 2002). EGFR inhibitor AG1478 attenuated both Ang II- and EGF-induced Akt as well as EGFR phosphorylation, which suggests that Akt phosphorylation elicited by Ang II is mediated through EGFR transactivation in rat VSMC (Fig. 3A). This is consistent with previous reports (Eguchi et al., 1999; Ushio-Fukai et al., 2001b). Whether PLD-regulated Akt phosphorylation is mediated through EGFR transactivation by a sequential or a parallel pathway is not known and was investigated in the present study. 1-Butanol, an inhibitor of PLD activity, diminished Ang II- but not EGF-induced Akt phosphorylation (Fig. 3B), suggesting that PLD acts upstream of EGFR and/or independent of EGFR transactivation. Since 1-butanol inhibited EGFR phosphorylation caused by Ang II (Fig. 3C), it appears that PLD acts upstream of EGFR. PLD2 siRNA, which decreased PLD protein expression, also reduced EGFR phosphorylation elicited by Ang II (Fig. 3D). Moreover, brefeldin A reduced EGFR as well as Akt phosphorylation caused by Ang II without affecting EGF-stimulated Akt phosphorylation (Fig. 1F). These data suggest that PLD-regulated Akt activation is mediated through EGFR transactivation. The effects of DAG kinase inhibitor II to reduce Ang II- but not EGF-induced Akt phosphorylation (Fig. 2A) and the effect of diC8-PA to promote EGFR phosphorylation (Fig. 2C) indicate that PA is most likely the mediator of PLD-dependent EGFR transactivation.

Ang II-Stimulated PLD and Akt Activation and EGFR Phosphorylation Are Dependent upon cPLA2 Activity. As shown in Fig. 1D, PLD2 siRNA inhibited Ang II-induced Akt phosphorylation. PLD2 siRNA also inhibited Akt activity as measured directly by the phosphorylation of its substrate, GSK-3β (Fig. 4). Moreover, Ang II-induced Akt activation was also inhibited in VSMC infected with retrovirus containing cPLA2 siRNA, but not by LacZ (Fig. 4). cPLA2 siRNA decreased cPLA2 but not Akt protein expression. Furthermore, cPLA2 siRNA did not significantly decrease PLD2 protein expression (data not shown). These observations indicate that Ang II-stimulated Akt phosphorylation is also dependent upon cPLA2 activation. Since both cPLA2 and PLD are involved in Ang II stimulation of Akt and since PLD activity is dependent on cPLA2 stimulation in VSMC (Parmentier et al., 2001b), we determined the relationship between cPLA2 and PLD in Akt phosphorylation. cPLA2 antisense but not sense oligonucleotide or cPLA2 siRNA but not LacZ control virus attenuated Ang II-stimulated PLD activity (Fig. 5, A and B). Moreover, ETYA, an inhibitor of metabolism of AA that is generated from cPLA2 activation, also indicated by the percentage of 3H-phosphatidylethanol in total 3H-labeled lipids as described under Methods (n = 5). C, PLD2 antisense but not sense oligonucleotide inhibited Ang II-stimulated Akt phosphorylation. VSMC were transfected with sense or antisense oligonucleotide of PLD2 for 48 h before stimulation with Ang II (200 nM) for 5 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-Akt, PLD2, and Akt protein. The blots are representative of three different experiments. The quantification of Ang II-induced Akt phosphorylation or Ang II-stimulated PLD activity was normalized by the value obtained with treatment with their respective vehicles. Values are means ± S.E. *, value significantly different from the corresponding value obtained with vehicle alone or inhibitors alone (P < 0.05). †, value significantly different from the corresponding value obtained with Ang II in the absence of inhibitors (P < 0.05).
diminished PLD activity (Fig. 5C). Since 1-butanol and PLD2 siRNA inhibited EGFR transactivation, we examined EGFR phosphorylation by Ang II in cells infected with retroviral vectors containing cPLA2 siRNA and LacZ. EGFR phosphorylation elicited by Ang II was inhibited by cPLA2 siRNA but not by LacZ (Fig. 3D). Moreover, ETYA inhibited both Ang II- and AA-stimulated phosphorylation of EGFR (Fig. 6, A and B), suggesting that the metabolites of AA generated through cPLA2 activation are also involved in Ang II-induced EGFR transactivation that is mediated by PLD activation.

**Ang II-Stimulated, cPLA2-Dependent PLD Activation Leading to EGFR Transactivation and Akt Phosphorylation Is Mediated by p38 MAPK.** Ang II causes p38 MAPK activation in VSMC, which has been also been implicated in Akt activation (Taniyama et al., 2004). However, the relationship between p38 MAPK, PLD, EGFR, and Akt in VSMC has not yet been established. Since 1) p38 MAPK (Kalyankrishna and Malik, 2003) and PLD (Parmantier et al., 2001a,b) activation in rabbit VSMC has been shown to be mediated by metabolites of AA derived via lipoxigenase and P450, consequent to cPLA2 activation, 2) EGFR inhibitor AG1478 suppresses MAPK activation induced by AA in a renal tubular cell line overexpressing the rat nonglycosylated α2-adrenergic receptor (Cussac et al., 2002), and 3) p38 MAPK mediates PLD activation in VSMC (Min do et al., 2002), we determined the possible involvement of p38 MAPK in Ang II-regulated PLD activation, EGFR transactivation, and Akt phosphorylation in rat VSMC. Ang II increased p38 MAPK activity in rat VSMC (Fig. 7A), and SB202190, a selective p38 MAPK inhibitor (Fatima et al., 2001), reduced PLD activation elicited by Ang II (Fig. 7B). SB202190 also attenuated EGFR and Akt phosphorylation elicited by both Ang II and AA (Fig. 7, C and D). These data suggest that p38 MAPK acts upstream of PLD-dependent EGFR transactivation in Akt phosphorylation elicited by Ang II. The inhibitory effect of SB202190 on AA-stimulated EGFR and Akt phosphorylation implicates p38 MAPK in cPLA2-dependent PLD activation. The selective cPLA2 inhibitor pyrrolidine-1 also reduced p38 MAPK phosphorylation induced by Ang II (Fig. 8A), as did cPLA2 siRNA but not its LacZ control virus (Fig. 8B). We also measured p38 MAPK activity by examining the phosphorylation of its substrate.
ATF-2. cPLA2 siRNA inhibited ATF phosphorylation elicited by Ang II (Fig. 8C). Because EGF stimulates p38 MAPK and cPLA2 in VSMC (Muthalif et al., 1998; Eguchi et al., 2001), SB202190 and ETYA were used to determine their effect on EGF-stimulated Akt and EGFR phosphorylation. SB202190 did not inhibit EGF-stimulated Akt phosphorylation, although it significantly reduced Akt phosphorylation elicited by both Ang II and AA (Fig. 9A). ETYA, which inhibited Ang II- and AA-induced EGFR phosphorylation (Fig. 6), did not inhibit EGF-stimulated EGFR phosphorylation (Fig. 9B). These data further support our findings that p38 MAPK and AA metabolites generated by cPLA2 activation act upstream of EGFR.

**Discussion**

The present study demonstrates that in rat VSMC Ang II-induced Akt activation is mediated by PLD-regulated EGFR transactivation consequent to activation of p38 MAPK by me-
tabolite(s) of AA generated by increased cPLA2 activity (Ang II → cPLA2 → AA metabolite(s) → p38 MAPK → PLD2 → PA → EGFR → Akt). This conclusion is based on our demonstration that Ang II-induced increase in Akt activity measured by its phosphorylation at serine 473 was inhibited by 1-butanol, which reduced PLD activity. PLD activity is regulated by the small G protein Arf (Shome et al., 2000; Andresen et al., 2001). Brefeldin A, an inhibitor of Arf, inhibits Ang II-induced PLD2 activity in renal microvascular smooth muscle cells and A10 cells (Shome et al., 2000; Andresen et al., 2001). That brefeldin A also diminished Akt phosphorylation induced by Ang II supports our contention that PLD is involved in Akt activation. The selectivity of 1-butanol and brefeldin A to inhibit Ang II-induced Akt phosphorylation was indicated by our finding that these agents did not reduce Akt phosphorylation elicited by EGF. That Ang II-induced Akt phosphorylation is dependent upon PLD activity was further indicated by our demonstration that in VSMC, in which the PLD2 isoform predominates (Shome et al., 2000; Andresen et al., 2001; Parmentier et al., 2001b), infection of the cells with retrovirus containing PLD2 siRNA inhibited Akt phosphorylation and PLD activation elicited by Ang II. Furthermore, PLD2 antisense oligonucleotide reduced Akt phosphorylation stimulated by Ang II. From these observations, it follows that Ang II-induced Akt phosphorylation is dependent on PLD2 activation in rat VSMC. Lysophosphatidic acid and sphingosine-1 phosphate have also been reported to activate protein translation via PI3K/Akt by a mechanism de-
ependent on PLD (Banno et al., 2001; Kam and Exton, 2004). However, in adrenocortical carcinoma cells, Ang II-induced PLD activation is attenuated by PI3K/Akt inhibitors, suggesting that Akt acts upstream of PLD in these cells (Zheng and Bollag, 2003). From these observations, it follows that the contribution of PLD in Akt activation may be dependent upon the stimulus and the cell type.

Activation of PLD promotes hydrolysis of phosphatidylcholine to PA, which is metabolized by PPH into DAG, that in turn can be phosphorylated by DAG kinase to reform PA or hydrolyzed by DAG lipase to generate AA (Ueno et al., 2000; Parmentier et al., 2001a). One or more of these products could contribute to Akt phosphorylation caused by Ang II. Our findings that the DAG kinase inhibitor II diminished Ang II induced and the cell permeable diC8-PA increased Akt phosphorylation suggest that PA generated by PLD activation mediates Akt phosphorylation in VSMC. Accumulation of PA by overexpression with exogenous Streptomyces chromofuscus PLD also results in an increase in PI3K activity and the phosphorylation of Akt in Chinese hamster ovary cells overexpressing EDG3 (Banno et al., 2001). The selectivity of the DAG kinase II inhibitor to decrease Ang II-induced Akt activation was indicated by our observation that this agent did not alter EGF-induced Akt activation. DAG or AA generated by PLD activation does not appear to be involved in Ang II-induced PLD activation because propranolol, an inhibitor of PPH, and RHC 80267, a DAG lipase inhibitor, which decrease DAG and AA generation respectively without

Fig. 7. p38 MAPK mediates Akt phosphorylation through PLD and EGFR transactivation. A, Ang II activated p38 MAPK in a concentration-dependent manner. Quiescent VSMC were treated with different concentrations of Ang II for 5 min. Equal protein loadings of each lysate sample were immunoblotted with anti-phospho-thr180/tyr182-p38 and anti-p38 antibody. B, p38 MAPK inhibitor SB202190 diminished Ang II-stimulated PLD activity. Quiescent VSMC were loaded with [3H]oleic acid for 18 h and pretreated with SB202190 (20 μM) for 30 min before exposure to Ang II (200 nM) for 15 min. The transphosphatidylation by PLD, as a measure of PLD activity, is indicated by the percentage of [3H]-phosphatidylethanol in total [3H]-labeled lipids as described under Methods (n = 5). C, EGFR phosphorylation induced by Ang II or AA is attenuated by p38 MAPK inhibitor SB202190, suggesting that p38 MAPK mediates Ang II-stimulated EGFR transactivation. Quiescent VSMC were pretreated with 20 μM SB202190 for 30 min before treatment with Ang II (200 nM) for 5 min or AA (20 μM) for 10 min as indicated. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-tyr1068-EGFR and EGFR. The bottom panel shows the quantification of EGFR phosphorylation compared with EGFR protein level (n = 5). D, Akt phosphorylation elicited by Ang II or AA is attenuated by p38 MAPK inhibitor SB202190, suggesting that p38 MAPK mediates Ang II-stimulated Akt activation. Quiescent VSMC were pretreated with 20 μM SB202190 for 30 min before exposure to Ang II (200 nM) for 5 min or AA (20 μM) for 10 min as indicated. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-Akt and Akt. The bottom panel is the quantification of Akt phosphorylation compared with Akt protein level (n = 5). The quantification of Ang II-stimulated Akt phosphorylation and PLD activity was normalized to the value obtained with vehicle. Values are means ± S.E., value significantly different from the corresponding value obtained with vehicle alone or SB202190 alone (P < 0.05). †, value significantly different from the corresponding value obtained with Ang II or AA in the absence of SB202190 (P < 0.05).
affecting PLD activity (Parmentier et al., 2001a), failed to alter Akt phosphorylation caused by Ang II in VSMC. Although activation of both PLD and cPLA2 releases AA, it appears that AA and its metabolites generated consequent to the activation of cPLA2 but not PLD promotes Akt phosphorylation. This could be due to compartmentalization of AA or the quantity of AA generated by these lipases.

Ang II causes transactivation of EGFR by releasing soluble heparin binding EGF through activation of metalloproteinases (Eguchi et al., 2001). EGFR phosphorylation activity by EGFR also promotes Akt activation (Burgering and Coffer, 1995), and Ang II has been shown to cause Akt activation via EGFR transactivation in VSMC (Eguchi et al., 1999; Ushio-Fukai et al., 2001b). In our study, an EGFR tyrosine kinase inhibitor AG1478 also diminished Ang II-stimulated Akt phosphorylation. However, in addition to metalloproteinase-sensitive EGFR transactivation, other molecules including reactive oxygen species and Ca2+/calmodulin have also been implicated in Ang II-induced tyrosine phosphorylation. Our demonstration that the inhibitors of PLD activity 1-butanol, brefeldin A, and PLD2 siRNA, which diminished Akt phosphorylation, also attenuated EGFR phosphorylation suggests that PLD2 mediates EGFR transactivation, which in turn promotes Akt phosphorylation in response to Ang II. That 1-butanol and brefeldin A did not reduce Akt phosphorylation caused by EGF suggests that PLD acts upstream of EGFR in rat VSMC, although PLD activity increases when EGFR is activated (Lu et al., 2000). Our study suggests that PA generated by PLD2 activation is involved in EGFR transactivation.

AA metabolites generated consequent to activation of cPLA2 in rabbit VSMC mediates the Ang II-induced increase in PLD activity (Parmentier et al., 2001a). Moreover, cPLA2 inhibition attenuates and AA stimulates Akt phosphorylation in mesangial cells (Gorin et al., 2001). These observations and our findings that cPLA2 antisense and siRNA and the inhibitor of AA metabolism ETYA attenuated PLD activity and EGFR phosphorylation elicited by Ang II suggest that metabolites of AA generated via activation of cPLA2 by in-
increasing PLD activity promote EGFR transactivation and phosphorylation of Akt. That ETYA inhibited EGFR phosphorylation elicited by Ang II and AA but not by EGF, indicates that AA metabolites act upstream of EGFR in the regulation of Akt activity most likely by promoting PLD activation.

The mechanism by which AA metabolites increase PLD activity in VSMC is not known. AA and/or its metabolites (hydroxyeicosatetraenoic acids) generated via lipoxigenase and/or P450 have been shown to increase p38 MAPK activity in VSMC (Kalyankrishna and Malik, 2003). In the present study, cPLA2 siRNA decreased both p38 MAPK phosphorylation and activation elicited by Ang II. Moreover, the cPLA2-specific inhibitor pyrrolidin-1 also decreased the phosphorylation of p38 MAPK. PLD activation by norepinephrine in VSMC is partially inhibited by either a MEK1 inhibitor (PD98059) (Muthalif et al., 2000; Parmentier et al., 2001b) or a p38 MAP kinase inhibitor (SB203580) (Min do et al., 2002), although SB203580 did not inhibit PLD activity in endothelial cells (Natarajan et al., 2001). However, Ang II-stimulated PLD activity has been reported to be independent of extracellular signal-regulated kinase 1/2 MAPK in VSMC derived from hypertensive rats (Wilkie et al., 1996). Moreover, the MEK-1/2 inhibitor U0126 also failed to inhibit Ang II-induced PLD-regulated Akt phosphorylation (Supplemental Data). MAPK-activated protein kinase-2, a kinase regulated by p38 MAPK in VSMC, stimulates the phosphorylation of Akt (Taniyama et al., 2004). Our finding that the specific p38 MAP kinase inhibitor SB202190 (Fatima et al., 2001) attenuated PLD activity and Akt phosphorylation elicited by Ang II or AA, suggests that p38 MAPK mediates cPLA2-dependent activation of PLD and consequently EGFR transactivation and Akt phosphorylation. Ang II has also been shown to cause activation of p38 MAPK through EGFR transactivation in VSMC (Eguchi et al., 2001). However, p38 MAPK activated via EGFR transactivation does not appear to be involved in Ang II-induced Akt phosphorylation because p38 MAPK inhibitor SB202190 did not decrease EGF-induced Akt phosphorylation. From these observations, it follows that in rat VSMC p38 MAPK acts upstream of EGFR and mediates Ang II-induced Akt activation by stimulating PLD-dependent EGFR transactivation.

In conclusion, our study demonstrates that Ang II stimulates PLD activity in rat VSMC via p38 MAPK activated by one or more AA metabolite(s) generated consequent to cPLA2 activation. The increased PLD activity by generating PA promotes EGFR transactivation, which in turn causes phosphorylation of Akt (Fig. 10). Further studies are required to elucidate the mechanism by which AA metabolite(s) cause activation of p38 MAPK and PA promotes EGFR transactivation. In view of the demonstration that 1) AA metabolites derived via lipoxigenase and cytochrome P450 activate p38 MAPK in rabbit VSMC (Kalyankrishna and Malik, 2003) and 2) the AA metabolite of lipoxigenase 12-hydroxyeicosatetraenoic acid increase the activity of small G protein Rac, which is an upstream activator of p38 MAPK (Wen et al., 1996), it is possible that AA metabolite(s) generated via lipoxigenase and/or P450 by activating Rac increase p38 MAPK activity, which in turn increases PLD activity in rat VSMC (Fig. 10).

Acknowledgments
We thank Anne Estes for technical assistance and Dr. Lauren Cagen for editorial comments. We gratefully acknowledge Dr. M. H. Gelb for generously supplying us with pyrrolidine-1 and Dr. Sylvain Bourgojn for providing anti-PLD2 antisera.

References
Li and Malik


Address correspondence to: Dr. Kafait U. Malik, Professor of Pharmacology, Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, Room 115, Crowe Building, 874 Union Avenue, Memphis, TN 38163. E-mail: kmalik@utmem.edu