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

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p38 MAPK activated PLD mediates Akt phosphorylation

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Arf, ADP-ribosylation factor; AT1R, Angiotensin type 1 receptor; cPLA2, cytosolic Ca²⁺-dependent phospholipase A₂; diC₈-PA, 1,2-dioctanoyl-sn-glycerol-3-phosphate; EGFR, epidermal growth factor receptor; ETYA, 5,8,11,14-eicosatetraynoic acid; MAPK, mitogen activated protein kinase; PPH, phosphatidate phosphohydrolase; PIP₃, phosphatidylinositol (3, 4, 5) trisphosphate; PI3K, phosphatidylinositol 3-kinase; VSMC, vascular smooth muscle cells.

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Abstract

Angiotensin II (Ang II) activates cytosolic Ca\textsuperscript{2+}-dependent phospholipase A\textsubscript{2} (cPLA\textsubscript{2}), 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 analogue 2-butanol, and by brefeldin A, inhibitor of the PLD cofactor ADP-ribosylation factor, and in cells infected with retrovirus containing PLD\textsubscript{2} siRNA or transfected with PLD\textsubscript{2} antisense, but not control LacZ or sense oligonucleotide. Diacylglycerol kinase inhibitor II diminished Ang II-induced, and diC8-phosphatidic acid (PA) increased, Akt phosphorylation, suggesting that PLD-dependent Akt activation is mediated by PA. Ang II-induced EGFR phosphorylation was inhibited by 1-butanol and PLD\textsubscript{2} siRNA, and also by cPLA\textsubscript{2} siRNA. In addition, the inhibitor of arachidonic acid (AA) metabolism 5,8,11,14-eicosatetraynoic acid (ETYA) reduced both Ang II- and AA- induced EGFR transactivation. Furthermore, ETYA, cPLA\textsubscript{2} antisense and cPLA\textsubscript{2} siRNA attenuated Ang II-elicited PLD activation. p38 MAPK inhibitor SB202190 reduced PLD activity and EGFR and Akt phosphorylation elicited by Ang II. Pyrrolidine-1, a cPLA\textsubscript{2} inhibitor, and cPLA\textsubscript{2} siRNA decreased p38 MAPK activity. These data indicate that Ang II-stimulated Akt activity is mediated by cPLA\textsubscript{2} dependent, p38 MAPK regulated, PLD\textsubscript{2} 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\textsubscript{2}→AA→p38 MAPK→PLD\textsubscript{2}→PA→EGFR→Akt.
Introduction

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 (PIP3) through activation of phosphatidylinositol 3-kinase (PI3K) (Shiojima and Walsh, 2002). PIP3 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 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 (Muthalif et al. 1998; Rao et al. 1994). AA and its metabolite(s) 5(S)- and 12(S)-hydroxyeicosatetraenoic acid generated via lipoxygenase 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 Tallant, 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). Whether PLD is also involved in Ang II-induced Akt activation in VSMC is not known. 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., 2001b). EGFR overexpression or EGFR stimulation also increase PLD activity, and PLD associates with EGFR during its activation in fibroblasts (Kim et al., 2003; Lu et al., 2000; Slaaby et al., 1998). 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 M KK3/6 activity in a follicular dendritic cell-like cells (Lee et al., 2003), while 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 a) activation of p38 MAPK by norepinephrine is mediated by metabolites of AA generated through activation of cPLA₂ (Kalyankrishna and Malik, 2003) and that lipooxygenase inhibitors CDC and baicalein attenuate p38 MAPK activation induced by Ang II in H295R adrenocortical cells (Natarajan et al., 2002); and b) 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 the Sigma Chemical Company (St. Louis, MO); 4(3-chlorophenyamino)-6,7-dimethoxyquinazoline (AG1478) from Calbiochem (San Diego, CA); 1,6-bis-(cyclohexyloximinocarbonylamino)-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 Inc (Alabaster, AL); AA from Cayman Chemical (Ann Arbor, MI); 4-(4-flurophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB202190) from Tocris (Ellisville, MO); human Ang II (H-Asp-Arg-Val-tyr-Ile-His-Pro-Phe-OH) and human EGF from Bachem Bioscience (King of Prussia, PA); Anti-EGFR, Anti-p38 MAPK and Anti-Akt1 (c-20) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Anti-phospho-tyr1068-EGFR, phospho-thr180/tyr182-p38 MAPK and phospho-ser473-Akt antibodies from Cell Signaling Technology (Beverly, MA); Anti-rabbit HRP-conjugated Ig serum from Amersham Biosciences (England, UK); Anti-goat HRP 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 is kindly supplied by Dr. Sylvain Bourgoin (Universite Laval, QC, Canada).

Methods:

Isolation and Culture of VSMC
Male Sprague Dawley rats (Harlan, Indianapolis, IN), weighing 250-350g were anesthetized with 40 mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL) and the thorax was opened. The thoracic aorta was excised and rapidly removed. VSMC were isolated, cultured and maintained under 5% CO₂ in M-199 Medium with penicillin/streptomycin and 10% fetal bovine serum as described (Uddin et al., 1998). VSMC between the 4th-10th 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 anti-actin 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, CA). The sequences of oligonucleotides used in this study were: cPLA₂ sense, 5’-AZF GAT CCT TAT CAG CAC FZA-3’; cPLA₂ antisense, 5’-TFZ GTG CTG ATA AGG ATC ZFT-3’; and PLD₂ sense, 5’-AZE ACT GTA ACC CAG ACG EFC-3’; and PLD₂ antisense, 5’-GZO CGT CTG GGT TAC AGT OFT-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, CA) and incubated for 6 hs in the medium without antibiotics and serum. VSMC were maintained in M199 containing 0.1% FBS in the presence of oligonucleotides. After 48 h, the cells were washed three times with HBSS 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 nM okadaic acid, 1 mM Na3VO4, 1 mM PMSF, 10 µg/ml antipain, 10 µg/ml aprotinin and 10 µg/ml leupeptin), sonicated, centrifuged and quantitated for protein content using the Bradford method. Equal amounts of protein (20–80 µg) were loaded onto SDS-PAGE gels for electrophoresis (20 mA/gel) and transferred onto nitrocellulose membrane (1 mA/mm² 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, Arlington Heights, IL). The density of bands was measured using the NIH Image 1.6 program.

Preparation of siRNA and transient transfection of VSMC with retroviral gene suppressor system

The primers with forward sequences 5’-TCGAG ACAGT AGTGG TTCTA CGTGCC gagtactg GGCAC GTAGA ACCAC TACTG TTTTTT-3’ for cPLA2; 5’-TCGAG CACAT GGAGC CAGAT GTGGTT gagtactg AACCA CATCT GGCTC CATGT GTTTTT-3’ for PLD2; and reverse 5’-CTAGA AAAAC ACATG GAGCC AGATG TGGTT cagtactc AACCA CATCT GGCTC CATGTGC-3’ for cPLA2; and 5’-CTAGA AAAAC ACATG GAGCC AGATG TGGTT cagtactc AACCA CATCT GGCTC CATGTGC-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, Piscataway, NJ). Competent DH5α cells (GibcoBRL, Carlsbad,
CA) were transformed with ligated plasmid DNA. After transformation, the colonies were amplified and purified using a miniprep purification kit (Qiagen, 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 transfected HEK 293 cells using the following procedures for amplification: HEK293 cells were grown to ~30%-50% confluence in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. These cells were transfected with the plasmid DNAs (pECO packaging vector and pSuppressorRetro Vector containing the cPLA₂ or PLD₂ siRNA insert) using the calcium phosphate precipitation method. The transfected cells were incubated for 3-4 h. Then 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, Carlsbad, CA) by 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 PA and ethanol that give phosphatidylethanol (PtdEthanol). Briefly, VSMC transfected with cPLA₂ or PLD₂ sense or antisense oligonucleotides, or VSMC infected with cPLA₂ siRNA or PLD₂ siRNA, were radio-labeled with [³H] oleic acid (1 µCi/ml) in M199 containing penicillin/streptomycin and 0.1% FBS for 18 h. The labeled cells were pre-incubated with ethanol (200 mM) for 10 min. For
VSMC treated with various inhibitors, the inhibitors were added into cells for 30 min before ethanol. Cells were then exposed to Ang II 200 nM for 15 min, and the reaction was terminated by adding ice-cold methanol/2M 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 fractionional $[^3H]$-PtdEtoH of total $[^3H]$-total lipids.

**P38 MAPK kinase assay**

p38 MAPK activity was determined by measuring the phosphorylation of its substrate ATF-2. Cell lysate (~200 µg total protein) was immunoprecipitated with immobilized phosphothr180/tyr182-p38 MAPK (monoclonal, Cell Signaling, Beverly, MA). Pellet was suspended and incubated 30 min in 50 µl kinase buffer (20 mM HEPES pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 2.0 mM dithiothreitol) containing 200 µM cold ATP and 2 µg ATF-2 fusion protein at 37°C. Reaction was terminated with 25 µl 3X SDS sample buffer. P38 MAPK kinase activity was measured by probing 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, La Jolla, CA). Cell lysate (~200 µg 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 with two times 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 Kinase Assay Buffer containing 2 µl GSK-3α/ATP mixture at 30°C. The beads were spined down and discarded. Reaction of supernatant was terminated with 25 µl 3X SDS sample buffer. Akt kinase activity was measured by probing with phospho- GSK-3α antibody (1:1000, anti-rabbit).
Results

AngII-stimulated Akt phosphorylation is dependent on PLD activity

Phosphorylation of Akt on threonine 308 in the kinase domain and serine 473 in the hydrophobic 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 AT_1R (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; 2001b). 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 PLD_2 isoform in rabbit VSMC, rat renal microvascular smooth muscle cells and A10 VSMC line (Parmentier et al., 2001b; Andresen et al., 2001; Shome et al., 2000). To exclude possible nonspecific effects of these inhibitors on Akt activation, a retrovirus containing PLD_2 siRNA or PLD_2 antisense oligonucleotides were used to reduce the expression and activity of PLD_2 in the cultured VSMC; PLD_2 siRNA and antisense decreased PLD_2 protein expression and decreased Akt phosphorylation stimulated by Ang II without affecting Akt total protein expression (Fig. 1C and D), while VSMC infected with control retrovirus containing LacZ or transfected with PLD_2 sense oligonucleotide did not affect Ang II-induced Akt activation. PLD_2 siRNA, but not LacZ, attenuated Ang II-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 another phospholipase, cPLA2, protein level (Data not shown). These data strongly suggested 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 (Andresen et al., 2001; Shome et al., 2000). In this study, the demonstration that brefeldin A decreased Ang II-induced 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 phosphatidic acid (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 (Ueno et al., 2000; Billah et al., 1989). DAG kinase inhibitor II, which diminishes PA generation from DAG, attenuated Ang II-induced Akt activation in VSMC (Fig. 2A 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-induced Akt phosphorylation. DAG formed from PA by PPH causes the prolonged activation of PKC 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 PPH 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-induced Akt phosphorylation (Fig. 2D). These data suggest that PA generated from PLD activation regulates Akt activation, while DAG or AA generated by PLD activation is not involved in Ang II-induced 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 (Eguchi 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, 2001). EGFR inhibitor AG1478 attenuated both Ang II- and EGF-induced Akt phosphorylation 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-3α (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. 5A and B). Moreover, ETYA, an inhibitor of metabolism of AA that is generated from cPLA2 activation, also 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. 6A 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 a) p38 MAPK (Kalyankrishna and Malik, 2003) and PLD (Parmentier et al., 2001a; 2001b) activation in rabbit VSMC has been shown to be mediated by metabolites of AA derived via lipoxygenase and CYP450, consequent to cPLA2 activation, b) EGFR inhibitor AG1478 suppressed MAPK activation induced by AA in a renal tubular cell line overexpressing the rat nonglycosylated \( \alpha_2 \)-adrenergic receptor (Cussac et al., 2002) and c) p38 MAPK mediate 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 specific 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. 7C 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 specific 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). Since EGF stimulates p38 MAPK and cPLA₂ in VSMC (Eguchi et al., 2001; Muthalif et al., 1998), 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 metabolite(s) 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 metabolite(s) of AA generated by increased cPLA$_2$ activity (Ang II $\rightarrow$ cPLA$_2$ $\rightarrow$ AA metabolite(s) $\rightarrow$ p38 MAPK $\rightarrow$ PLD$_2$ $\rightarrow$ PA $\rightarrow$ EGFR $\rightarrow$ 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 PLD$_2$ 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 PLD$_2$ isoform predominates (Parmentier et al., 2001b; Shome et al., 2000; Andresen et al., 2001), infection of the cells with retrovirus containing PLD$_2$ siRNA inhibited Akt phosphorylation and PLD activation elicited by Ang II. Furthermore, PLD$_2$ antisense oligonucleotide reduced Akt phosphorylation stimulated by Ang II. From these observations, it follows that Ang II-induced Akt phosphorylation is dependent on PLD$_2$ activation in rat VSMC. Lysophosphatidic acid and sphingosine-1 phosphate have also been reported to activate protein translation via PI3K/Akt by a mechanism dependent on PLD (Banno et al., 2001; Kam and Exton, et al., 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 Boolag, et al., 2003). From these observations it follows that the contribution of PLD in Akt activation may depend upon the stimulus and the cell type.

Activation of PLD promotes hydrolysis of phosphatidylcholine to PA, which is metabolized by PPH into DAG, which 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 without affecting PLD activity (Parmentier et al., 2001a), failed to alter Akt phosphorylation caused by Ang II in VSMC. Though 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 metalloproteases (Eguchi et al., 2001). EGFR activation by EGF also promotes Akt
activation (Burgering and Coffer, et al., 1995) and Ang II has been shown to cause Akt activation via EGFR transactivation in VSMC (Ushio-Fukai et al., 2001b; Eguchi et al., 1999). 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 Ca\(^{2+}\)/calmodulin have also been implicated in Ang II–induced tyrosine phosphorylation/transactivation of the EGFR (Ushio-Fukai et al., 2001a; Murasawa et al., 1998). Expression of EGFR or EGFR activation with EGF also increases PLD activity in some cell types (Kim et al., 2003; Lu et al., 2000; Slabby et al., 1998). PLD\(_2\) has been shown to bind to EGFR in HEK293 cells transiently transfected with mouse PLD\(_2\), where EGF stimulates PLD\(_2\) tyrosine phosphorylation at residue 11 (Slabby et al., 1998). This raises the possibility that EGFR transactivation-dependent Akt activation is linked to Ang II-stimulated PLD activity. Our demonstration that the inhibitors of PLD activity 1-butanol, brefeldin A and PLD\(_2\) siRNA, which diminished Akt phosphorylation, also attenuated EGFR phosphorylation, suggests that PLD\(_2\) 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, though PLD activity increases when EGFR is activated (Lu et al., 2000). Our study suggests that PA generated by PLD\(_2\) activation is involved in EGFR transactivation.

AA metabolites generated consequent to activation of cPLA\(_2\) in rabbit VSMC mediates the Ang II-induced increase in PLD activity (Parmentier et al., 2001a). Moreover, PLA\(_2\) inhibition attenuates and AA stimulates Akt phosphorylation in mesangial cells (Gorin et al., 2001). These observations and our findings that cPLA\(_2\) 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 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 lipoxygenase and/or CYP450 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, 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) (Parmentier et al., 2001b; Muthalif et al., 2000) or a p38 MAP kinase inhibitor (SB203580) (Min do et al., 2002), though 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 ERK1/2 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 (supplementary 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 a) AA metabolites derived via lipoxygenase and cytochrome P450 activate p38 MAPK in rabbit VSMC (Kalyankrishna and Malik, 2003) and b) the AA metabolite of lipoxygenase 12-HETE 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 lipoxygenase and/or CYP450, by activating Rac, increase p38 MAPK activity, which in turn increases PLD activity in rat VSMC (Fig. 10).

Acknowledgements

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Reference


vascular smooth muscle A10 cells is mediated by small G proteins of the ADPribosylation factor family. *Endocrinology* **141**: 2200-2208.


Footnotes

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Figure Legend

**Figure 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 to 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 transphosphatidylation by PLD as a measure of PLD activity is indicated by the percentage of [3H]-phosphatidylethanol in total [3H]-labeled lipids as described in 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.

D: PLD2 siRNA but not LacZ reduced Ang II-stimulated Akt phosphorylation. VSMC were infected with retrovirus containing LacZ or PLD2 siRNA for 48 h 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, PLD2 and Akt protein. The bottom panel shows the quantification of phosphorylation of Akt compared to Akt protein level (n=7).

E: PLD2 siRNA but not LacZ reduced Ang II-stimulated
PLD activity. VSMC infected with retrovirus containing LacZ or PLD₂ siRNA were loaded with [³H] oleic acid for 18 h before treatment with Ang II (200 nM) for 15 min. The transphosphatidylation by PLD is indicated by the percentage of ³H-phosphatidylethanol in total ³H-labeled lipids as described in Methods (n=5). F: Arf inhibitor brefeldin A reduced Ang II-stimulated Akt and EGFR phosphorylation without affecting EGF-stimulated Akt phosphorylation. Quiescent VSMC were pretreated with brefeldin A (50 µg/ml) for 30 min before treating with Ang II (200 nM) or EGF (100 ng/ml) for 5 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-Akt, phospho-tyr1068-EGFR, 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 ± SE; * denotes the value significantly different from the corresponding value obtained with vehicle alone or inhibitors alone (P<0.05). † indicates the value significantly different from the corresponding value obtained with Ang II in the absence of inhibitors (P<0.05).

**Figure 2. Ang II-induced Akt phosphorylation is regulated by PA generated from PLD activation.** A: DAG kinase inhibitor II attenuated Ang II- but not EGF-stimulated Akt phosphorylation. Quiescent VSMC were pretreated with DAG kinase inhibitor II (4 µM) for 30 min before stimulation with Ang II (200 nM) or EGF (100 ng/ml) for 5 min as indicated. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-Akt and Akt protein. The blots are representative of three different experiments. B: DAG kinase inhibitor II reduced Akt phosphorylation induced by Ang II. The quantification of Ang II-
stimulated Akt phosphorylation compared to Akt protein level was measured by the NIH image 1.6 program (n=6). The quantification of Ang II-induced Akt phosphorylation was normalized to the value obtained with treatment with their respective vehicles. Values are means ± SE; * denotes the value significantly different from the corresponding value obtained with vehicle alone (P<0.05). † indicates the value significantly different from the corresponding value obtained with Ang II in the absence of DAG kinase inhibitor II (P<0.05). C: Exogenous PA stimulated Akt and EGFR phosphorylation in rat VSMC. Quiescent VSMC were treated with cell-permeable PA (diC8-PA, 200 µM) for different time periods as indicated. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-Akt, phospho-tyr1068-EGFR, and Akt protein. The blots are representative of three different experiments. D: DAG and AA generated from PLD did not affect Ang II-induced Akt phosphorylation. Quiescent VSMC were pretreated with propranolol (10 µM, upper panel) or RHC 80267 (10 µM, lower panel) for 30 min before treatment with 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 blots are representative of three different experiments.

Figure 3. Ang II-induced Akt activation is mediated through PLD-regulated EGFR transactivation. A: AG1478 inhibited phosphorylation of EGFR and Akt stimulated by both Ang II and EGF, suggesting Ang II-stimulated Akt phosphorylation is dependent on EGFR phosphorylation. Quiescent VSMC were pretreated with EGFR specific inhibitor AG1478 (250 nM) for 1 h before exposure to Ang II (200 nM) or EGF (100 ng/ml) for 5 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-Akt, phospho-tyr1068-EGFR and Akt protein. The bottom panel shows the quantification of Akt
phosphorylation compared to Akt protein level (n=6). Values are means ± SE; * denotes the
value significantly different from the corresponding value obtained with vehicle alone (P<0.05).
† Indicates the value significantly different from the corresponding value obtained with Ang II or
EGF in the absence of AG1478 (P<0.05). B: 1-butanol decreased Ang II- but not EGF-induced
Akt phosphorylation. Quiescent VSMC were pretreated with PLD inhibitor 1-butanol (0.4% v/v)
for 30 min before stimulation with Ang II (200 nM) or EGF (100 ng/ml) for 5 min. Equal protein
loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-
Akt and Akt protein. The blots are representative of three different experiments. C: 1-butanol
inhibited Ang II-stimulated EGFR phosphorylation. Quiescent VSMC were pretreated with 1-
butanol (0.4% v/v) for 30 min before stimulation with Ang II (200 nM) for 5 min. Equal protein
loadings of each lysate sample were immunoblotted with antibody specific for phospho-tyr1068-
EGFR and EGFR protein. The blots are representative of three different experiments. D: Both
cPLA2 and PLD2 siRNA inhibited EGFR phosphorylation stimulated by Ang II. Quiescent
VSMC were infected with retrovirus containing LacZ, cPLA2 siRNA or PLD2 siRNA insert for
48 h and then stimulated with Ang II (200 nM) for 5 min. Equal protein loadings of each lysate
sample were immunoblotted with antibody specific for phospho-tyr1068-EGFR and EGFR. The
blots are representative of three different experiments.

Figure 4. cPLA2 siRNA and PLD2 siRNA inhibited Ang II-stimulated Akt kinase activity.
VSMC were infected with retrovirus containing LacZ, PLD2 siRNA or cPLA2 siRNA for 48 h
before exposure to Ang II 200 nM for 5 min. Equal amount of protein from each lysate sample
was immunoprecipitated with an anti-Akt antibody. Akt kinase activity was measured by the
phosphorylation of the substrate GSK-3α protein. Also, Equal protein loadings of each lysate
sample were immunoblotted with antibody specific for goat anti-Akt antibody. The blots are representative of three different experiments.

**Figure 5. Ang II-induced PLD activation is regulated by cPLA2 activity.** A: cPLA2 antisense inhibited Ang II-stimulated PLD activity. VSMC transfected with cPLA2 sense or antisense oligos were loaded with [3H]-oleic acid for 18 h 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 in Methods (n=4). B: cPLA2 siRNA inhibited Ang II-induced PLD activation. VSMC infected with retroviral vector containing LacZ or cPLA2 siRNA insert were loaded with [3H]-oleic acid for 18 h before treatment with Ang II (200 nM) for 15 min. The transphosphatidylation by PLD is indicated by the percentage of 3H-phosphatidylethanol in total 3H-labeled lipids as described in Methods (n=4). C: AA metabolism inhibitor ETYA attenuated Ang II-stimulated PLD activity. Quiescent VSMC were loaded with [3H]-oleic acid for 18 h and pretreated with ETYA (10 µM) for 30 min before the stimulation with Ang II (200 nM) for 15 min. The transphosphatidylation by PLD is indicated by the percentage of 3H-phosphatidylethanol in total 3H-labeled lipids as described in Methods (n=5). The quantification of Ang II-stimulated PLD activity was normalized by the value obtained with the vehicle. Values are means ± SE; * denotes the value significantly different from the corresponding value obtained with vehicle alone or inhibitors alone (P<0.05). † Indicates the value significantly different from the corresponding value obtained with Ang II in the absence of the inhibitors (P<0.05).
Figure 6. Ang II- and AA-stimulated EGFR transactivation are inhibited by AA metabolism inhibitor ETYA. A: AA metabolism inhibitor ETYA attenuated EGFR phosphorylation stimulated by Ang II or AA. Quiescent VSMC were pretreated with ETYA (10 µM) for 30 min and then stimulated with Ang II (200 nM) for 5 min or AA (20 µM) for 10 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-tyr1068-EGFR and EGFR. B: The quantification of EGFR phosphorylation compared to EGFR protein level was measured by using the NIH image 1.6 program (n=6). Values are means ± SE; * denotes the value significantly different from the corresponding value obtained with vehicle alone (P<0.05). † Indicates the value significantly different from the corresponding value obtained with Ang II or AA in the absence of ETYA (P<0.05).

Figure 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]-phosphatidyethanol in total [3H]-labeled lipids as described in 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 SB202190 20 µM 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 to 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 SB202190 20 µM 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 to 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 ± SE; * denotes the value significantly different from the corresponding value obtained with vehicle alone or SB202190 alone (P<0.05). † Indicates the value significantly different from the corresponding value obtained with Ang II or AA in the absence of SB202190 (P<0.05).

Figure 8. p38 MAPK activation by Ang II is dependent on the cPLA₂ activity. A: cPLA₂ specific inhibitor pyrrolidine-1 attenuated Ang II-stimulated p38 MAPK phosphorylation. Quiescent VSMC were pretreated with pyrrolidine-1 (100 nM) for 30 min before treatment with Ang II (200 nM) for 5 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-thr180/tyr182-p38 and p38 MAPK protein. B: cPLA₂ siRNA inhibited Ang II-stimulated p38 MAPK phosphorylation. VSMC were infected with retrovirus containing LacZ or cPLA₂ siRNA for 48 h before exposure to Ang II 200 nM for 5 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-
thr180/tyr182-p38 MAPK and p38 MAPK. C: cPLA2 siRNA inhibited Ang II-stimulated p38 MAPK kinase activity. VSMC were infected with retrovirus containing LacZ or cPLA2 siRNA for 48 h before exposure to Ang II 200 nM for 5 min. Equal amount of protein from each lysate sample was immunoprecipitated with immobilized phospho-thr180/tyr182-p38 MAPK antibody. p38 MAPK kinase activity was measured by the phosphorylation of the substrate ATF-fusion protein. The blots shown in each panel are representative of three different experiments.

Figure 9. cPLA2 and p38 MAPK regulate Akt phosphorylation through EGFR transactivation. A: p38 MAPK inhibitor SB202190 did not affect EGF-stimulated Akt phosphorylation. Quiescent VSMC were pretreated with p38 MAPK specific inhibitor SB202190 (20 µM) for 30 min before treatment with Ang II (200 nM), EGF (100 ng/ml) for 5 min, or AA (20 µM) for 10 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-ser473-Akt and Akt protein. B: AA metabolism inhibitor ETYA did not affect EGF-stimulated EGFR phosphorylation. Quiescent VSMC were pretreated with ETYA (10 µM) for 30 min before stimulation with EGF (100 ng/ml) for 5 min or AA (20 µM) for 10 min. Equal protein loadings of each lysate sample were immunoblotted with antibody specific for phospho-tyr1068-EGFR and EGFR protein. The blots shown in each panel are representative of three different experiments.

Figure 10. The proposed pathway of Ang II-induced Akt activation in rat aortic VSMC. Ang II stimulates cPLA2 activity and releases AA. AA metabolite(s) stimulate the Rac/MLK/p38 MAPK pathway; the p38 MAPK activates PLD and results in PA production, which stimulates EGFR transactivation. The activated EGFR promotes Akt phosphorylation and activation.
**Fig. 2.**

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**B:**

Phosphorylation of Akt

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**DiC8-phosphatidic acid**

**Propranolol**

**RHC 80267**
**Fig. 3.**

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**Phosphorylation of Akt**

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- + - + - - Ang II
- - + - + EGF
- + - + - 1-butanol
Fig. 4.

- 37 kDa
- 60 kDa

- Polybrene
- LacZ
- ePLA2 siRNA
- PLD2 siRNA

P-GSK-3α
Akt
Ang II
Fig. 5.

A:

\[
\text{Phospholipase D activity (arbitrary units, } \beta\text{H}-\text{phosphatidyethanol)}
\]

- + - + - + Ang II
- - + + - - cPLA\(_2\) sense
- - - - + + cPLA\(_2\) antisense

B:

\[
\text{Phospholipase D activity (arbitrary units, } \beta\text{H}-\text{phosphatidyethanol)}
\]

- + - + - + Ang II
- - + + - - LacZ
- - - - + + cPLA\(_2\) siRNA

C:

\[
\text{Phospholipase D activity (arbitrary units, } \beta\text{H}-\text{phosphatidyethanol)}
\]

- + - + - + Ang II
- - + + - + ETYA
Fig. 6.

A: kDa

180 [Image of gel bands labeled P-EGFR and EGFR with conditions: Ang II - + - + - + + AA, ETYA - - + + - + + ETYA]

B: Phosphorylation of EGFR/total EGFR

[Bar graph showing phosphorylation levels with conditions: Ang II - + - - + - + AA, ETYA - - + + - - + ETYA]
Fig. 7.

**A:**

A gel showing the Phosphorylation of EGFR/Total EGFR with Ang II, AA, and SB202190 treatments. The graph indicates a significant increase in phosphorylation with Ang II and SB202190

**B:**

A bar graph showing Phospholipase D activity (arbitrary unit, pHl-phospholipidethanol) with Ang II and SB202190 treatments. The graph shows a significant increase in activity with Ang II and SB202190.

**C:**

A gel showing the Phosphorylation of EGFR/Total EGFR with Ang II, AA, and SB202190 treatments. The graph indicates a significant increase in phosphorylation with Ang II and SB202190.

**D:**

A gel showing the Phosphorylation of Akt/Total Akt with Ang II, AA, and SB202190 treatments. The graph indicates a significant increase in phosphorylation with Ang II and SB202190.
Fig. 8.

A: $kDa$
- $38\quad -\quad +\quad -\quad +\quad \text{Ang II}$
- $-\quad -\quad +\quad +\quad \text{Pyrrolidine-1}$

B: $kDa$
- $38\quad -\quad +\quad -\quad +\quad \text{Ang II}$
- $-\quad -\quad +\quad +\quad \text{LacZ}$
- $-\quad -\quad -\quad +\quad \text{cPLA}_2\text{ siRNA}$

C: $kDa$
- $35\quad -\quad +\quad -\quad +\quad \text{Ang II}$
- $-\quad +\quad +\quad -\quad \text{LacZ}$
- $-\quad -\quad -\quad +\quad \text{cPLA}_2\text{ siRNA}$
**Fig. 9.**

**A:**

- kDa
- 60

- P-Akt

- Akt

- Ang II
- AA
- EGF
- SB202190

**B:**

- kDa
- 180

- P-EGFR

- EGFR

- AA
- EGF
- ETYA
Fig. 10.
Supplementary Data. Ang II-stimulated Akt phosphorylation is not regulated by MEK/ERK. Quiescent VSMC were pretreated with 4 µM DAG kinase inhibitor II or MEK1 inhibitor U0126 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 blots are representative of three different experiments.