Angiotensin II-induced Akt activation is mediated by metabolites of arachidonic acid generated by CaMKII-stimulated Ca\(^{2+}\)-dependent phospholipase A\(_2\)

**Fang Li and Kafait U. Malik**

Department of Pharmacology, University of Tennessee Health Science Center, Memphis, Tennessee

Li, Fang, and Kafait U. Malik. Angiotensin II-induced Akt activation is mediated by metabolites of arachidonic acid generated by CaMKII-stimulated Ca\(^{2+}\)-dependent phospholipase A\(_2\). Am J Physiol Heart Circ Physiol 288: H2306–H2316, 2005. First published January 6, 2005; doi:10.1152/ajpheart.00571.2004.—Angiotensin II (ANG II) promotes vascular smooth muscle cell (VSMC) growth, stimulates Ca\(^{2+}\)-calmodulin (CaM)-dependent kinase II (CaMKII), and activates cytosolic Ca\(^{2+}\)-dependent phospholipase A\(_2\) (cPLA\(_2\)), which releases arachidonic acid (AA). ANG II also generates H\(_2\)O\(_2\) and activates Akt, which have been implicated in ANG II actions in VSMC. This study was conducted to investigate the relationship of these signaling molecules to AA activation in rat aortic VSMC. ANG II increased Akt activity, as measured by its phosphorylation at serine-473. ANG II (200 nM)-induced Akt phosphorylation was decreased by extracellular Ca\(^{2+}\) depletion and calcium chelator EGTA and inhibitors of CaM \([N\-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) and CaMKII \([(2\-[N\-(2-hydroxyethyl)]-N\-(4-methoxybenzenesulfonyl)]}almino-N\-(4-chlorocinnamyl)-N\-methylbenzylamine\)]. cPLA\(_2\) inhibitor pyrrolidine-1, antiseisme oligonucleotide, and retroviral small interfering RNA also attenuated ANG II-induced Akt phosphorylation. AA increased Akt phosphorylation, and AA metabolism inhibitor 5,8,11,14-eicosatetraenoic acid (ETYA) blocked ANG II- and AA-induced Akt phosphorylation (199.03 ± 27.91% with ANG II and 110.18 ± 22.40% with ETYA + ANG II; 405.00 ± 86.22% with AA and 153.97 ± 63.26% with ETYA + AA). Inhibitors of lipoxxygenase (cinnamyl-3,4-dihydroxy-\(\alpha\)-cyanocinnamate) and cytochrome P-450 (ketoconazole and 17-octadecynoic acid), but not cyclooxygenase (indomethacin), attenuated ANG II- and AA-induced Akt phosphorylation. Furthermore, 5(S), 12(S), 15(S), and 20-hydroxyeicosatetraenoic acids and 5,6-, 11,12-, and 14,15-epoxyeicosatrienoic acids increased Akt phosphorylation. Catalase inhibited ANG II-increased H\(_2\)O\(_2\) production but not Akt phosphorylation. Oleic acid, which also increased H\(_2\)O\(_2\) production, did not cause Akt phosphorylation. These data suggest that ANG II-induced Akt activation in VSMC is mediated by AA metabolites, most likely generated via lipoxigenase and cytochrome P-450 consequent to AA release by CaMKII-activated cPLA\(_2\) and independent of H\(_2\)O\(_2\) production.

Angiotensin II; Akt; hydroxyeicosatetraenoic acids; lipoxigenase; Ca\(^{2+}\)-dependent phospholipase A\(_2\); epoxyeicosatrienoic acid; reactive oxygen species

ANGIOTENSIN II (ANG II), the principal biologically active component of the renin-angiotensin system, contributes to the regulation of blood pressure by increasing vascular tone and salt and water retention (19). It also promotes vascular smooth muscle cell (VSMC) migration and proliferation and/or hypertherphy (5). These actions of ANG II are mediated via activation of effector systems such as Ca\(^{2+}\)-dependent phospholipase A\(_2\) (cPLA\(_2\)), NAD(P)H oxidase, phosphatidylinositol 3-kinase (PI3K), and Akt (13, 29–30, 35, 48, 57). Akt is a serine-threonine kinase with a pleckstrin homology (PH) domain. Three isoforms of Akt (Akt1, Akt2, and Akt3) have been identified in the mammalian genome. Akt isoforms contain an NH\(_2\)-terminal PH domain, a kinase domain, and a COOH-terminal regulatory domain. All three isoforms are expressed ubiquitously. When cytokines and growth factors bind to their receptors, they activate PI3K and promote the conversion of phosphatidylinositol 3,4-bisphosphate into phosphatidylinositol 3,4,5-trisphosphate. The latter binds to the PH domain of Akt, which recruits it to the plasma membrane and exposes it to phosphorylation by phosphoinositide-dependent protein kinase(s) of serine-473 in the regulatory domain and threonine-308 in the kinase domain (3, 10). Activated Akt stimulates downstream signals such as Bad, Forkhead transcription factor, I\(\kappa\)B kinase-\(\alpha\) complex (IKK\(\alpha\)), E2F, glycogen synthase kinase-3 (GSK3), p70 ribosomal protein S6 kinase (p70S6K), endothelial nitric oxide synthase (eNOS), and mammalian target of rapamycin (mTor) to regulate cell survival, cell cycle, glucose metabolism, angiogenesis, vasomotor tone, and/or protein synthesis (3, 10, 43).

ANG II also activates cPLA\(_2\), which releases arachidonic acid (AA) from tissue phospholipids (4, 29–30, 35). AA is metabolized by cyclooxygenase into prostaglandins and thromboxane A\(_2\) by lipoxigenase into leukotrienes and 5(S), 12(S), and 15(S)-hydroxyeicosatetraenoic acids (HETEs) and by cytochrome P-450 into 12(R)-, 19-, and 20-HETE and 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs) (15, 28, 33, 37–38). In mesangial cells, PLA\(_2\)-activated AA release and H\(_2\)O\(_2\) production mediate ANG II-induced Akt activation (16). Akt activation has also been shown to be dependent on Ca\(^{2+}\)-calmodulin (CaM) in endothelial cells (32). In VSMC, reactive oxygen species (ROS) play an important role in Akt activation caused by ANG II (52). However, the relationship between these signaling molecules involved in the activation of Akt by ANG II has not yet been established. We have previously shown that ANG II, by stimulating Ca\(^{2+}\)-CaM-dependent kinase II (CaMKII), activates cPLA\(_2\) and releases AA; by activating the Ras-ERK1/2 pathway, AA metabolites of lipoxygenase [12(S)- and 15(S)-HETE] and cytochrome P-450 (20-HETE) amplify cPLA\(_2\) activity (28). It is therefore possible that ANG II-induced Akt activation is mediated by CaMKII-stimulated cPLA\(_2\), release of AA, and generation of AA metabolites and ROS. To test this hypothesis, we have investigated the effect of Ca\(^{2+}\) depletion and inhibitors of CaM, CaMKII, cPLA\(_2\), and AA metabolism on ANG II-induced Akt activation in rat VSMC. The effect of ANG II and AA on ROS production and the effect of oleic acid that also generates ROS on Akt phosphorylation were determined. The results of our
study show that in rat aortic VSMC, ANG II-induced Akt activation is mediated by metabolites of AA-derived via lipoxgenase and cytochrome P-450 consequent to CaMKII-activated cPLA2 and is independent of ROS production.

**EXPERIMENTAL PROCEDURES**

**Materials**

Chemicals were purchased from the following sources: apropin, dithiothreitol, phenylmethylsulfonyl fluoride, sodium orthovanadate, leupeptin, HEPES, and antipain from Sigma-Aldrich (St. Louis, MO); halolme lactone suicide substrate (HELSS), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), ketocamozole, 5,8,11,14-eicosatetraynoic acid (ETYA), cinnamyl-3,4-dihydroxy-cyancinnamnate (CDC), and indothatcin were from BioMol Research Laboratories (Plymouth Meeting, PA); 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93) and 2-[N-(4-methoxybenzenesulfonfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-92) were from Calbiochem (San Diego, CA); 17-octadecyacenic acid (17-ODYA), (all Z)-20(HETE), 15(Δ)-HETE, 12(Δ)-HETE, 5(Δ)-HETE, 5,11,12-, and 14,15 EETs, and AA were from Cayman Chemical (Ann Arbor, MI); human ANG II (H-Asp-Arg-Val-tyr-Ile-His-Pro-Phe-OH) was from Bachem BioScience (King of Prussia, PA); anti-Akt1 (c-20) was from Santa Cruz Bio-Techology (Santa Cruz, CA); phospho-Akt (Ser473) antibody was from Cell Signaling Technology (Beverly, MA); anti-rabbit horseradish peroxidase (HRP)-conjugated Ig serum was from Amersham Bicscience (Little Chalfont, Buckinghamshire, UK); and anti-goat HRP conjugated-IgG (H + L) was from Vector Laboratories (Burlingame, CA). Pyrrolidine-1 was kindly provided by Dr. M. H. Gelb (University of Washington, Seattle, WA). Anti-PLD2 antibody was kindly supplied by Dr. Sylvain Bourgoin (Universite Laval, QC, Canada). N-hydroxy-N’-(4-butyl-2-methylphenyl)formamide (HET0016) was kindly supplied by Dr. Richard J. Roman (Medical College of Wisconsin, Milwaukee, WI).

**Methods**

**VSMC isolation and culture**. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250–350 g were anesthetized with pentobarbital sodium (Abbott, North Chicago, IL), and the thorax was opened. The thoracic aorta was excised and rapidly removed. VSMC were isolated and cultured as described (51). The cultured cells were maintained under 5% CO2 in medium 199 (M199) with penicillin-streptomycin. These cells were transfected with the plasmid DNAs (pECO packaging vector and pSuppressor-Vector containing the cPLA2 siRNA insert) using the calcium phosphate precipitation method. The transfected cells were incubated for 3–4 h. Fresh medium was then added and replaced on the second day. The virus was harvested by filtering the virus-containing supernatant. VSMC were made quiescent and infected with the supernatant in M199 containing 8 μg/ml polybrene and 0.1% fetal bovine serum for 48 h before stimulation. The LacZ retrovirus was used to check infection efficiency and to exclude any possible nonspecific effect of the virus on ANG II-induced Akt phosphorylation in VSMC. The infection efficiency in VSMC was confirmed by β-galactosidase staining (Invitrogen, Carlsbad, CA).

**Western blot analysis**. VSMC were dispersed into lysis buffer [1% IGEPAL CA-630, 25 mM HEPES (pH 7.5), 50 mM NaCl, 50 mM NaF, 10 mM okadaic acid, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml antipain, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. The lysates were sonicated and centrifuged, and protein concentration was determined. Equal amounts of protein (20–80 μg) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The blots were blocked and incubated with primary antibody (1:1,000) overnight at 4°C. The blots were then exposed to their respective secondary antibodies conjugated with horseradish peroxidase and developed by using the ECL Plus Western Blot Detection kit (Amersham). The density of bands was measured using the NIH Image 1.6 program.

**cPLA2 assay**. Quiescent VSMC were stimulated with ANG II or its vehicle after pretreatment with inhibitors, and cPLA2 activity was determined as described (29). The cells were lysed and sonicated in lysis buffer (350 mM sucrose, 1 mM EGTA, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 20 μg/ml soybean trypsin inhibitor). The protein concentration was determined by the Bradford method (Bio-Rad). 1-α-1-Palmitoyl-2-arachidonoyl-1’4-phosphatidylcholine (American Radiolabeled Chemicals) was concentrated under N2 and cosonicated in substrate buffer [9 mM dioleoylglycerol, 25 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM CaCl2, 1 mM diithiothreitol, and 1 mg/ml bovine serum albumin]. The lysate (containing 30 μg protein) was incubated in substrate buffer (25 μl) at 37°C for 1 h. The reaction was stopped by the addition of Dole’s reagent (2-propanol-heptane-0.5 M H2SO4, 20:5:1 vol/vol/vol), heptane, and 20 μg/ml AA. The upper heptane phase was drawn out and passed through a Sep-Pak silica column (Waters Chromatography, Milford, MA). The eluate was collected in glass vials and air dried. The radioactivity of free AA was determined by liquid scintillation spectrometry.

**ROS determination**. ROS production was measured according to the method described (24). Briefly, quiescent VSMC were incubated with the H2O2-sensitive fluorescent probe 2’,7’-dichlorofluorescein diacetate (DCF-DA) (10 μM; Molecular Probes, Eugene, OR) for 10 min and treated with ANG II (200 nM), oleic acid (100 μM), AA (20 μM), or their vehicle for 5 or 10 min. Nonfluorescent DCF-DA is
deacetylated by cellular esterases to the polar compound 2',7'-dichlorofluorescein, which remains trapped in the cell and oxidized by oxygen radicals (H₂O₂, lipid hydroperoxides, HOO⁻, and ONOO⁻) into the highly fluorescent 2',7'-dichlorodihydrofluorescein. Relative fluorescence intensity and fluorescent images were obtained with an Eclipse TE200 inverted microscope (Nikon, Melville, NY) using a SPOT RT slider digital camera (Diagnostic Instruments, Sterling Heights, MI) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. To examine the inhibitory effect of catalase or ETYA on the production of H₂O₂, quiescent VSMC were pretreated with catalase (2,000 U/ml) for 48 h or ETYA (10 μM) for 30 min before incubation with DCF-DA and stimulated with the various agonists as described above. Treatment of VSMC with catalase for this period has been reported to increase cellular expression of catalase and reduce H₂O₂ level in rat VSMC (46).

**Cytosolic Ca²⁺ measurement.** Cytosolic Ca²⁺ concentration was measured as described (11, 18). Briefly, coverslips were washed with nitric acid and water and sterilized under UV light. Trypsinized VSMC were cultured to confluence on sterilized coverslips and then arrested overnight in serum-free and antibiotic-free M-199. Quiescent VSMC were loaded for 30 min with 5 μM fura 2-AM mixed with 0.05% bovine serum albumin and 0.025% pluronic acid in Krebs-Ringer (KB) solution (in mM: 120 NaCl, 5 KCl, 0.62 MgSO₄, 10 HEPES, 6 glucose, and 1.8 CaCl₂; adjusted to pH 7.4). Coverslips were placed in cuvettes and superfused with KB solution with or without Ca²⁺ (0.89 mM MgCl₂ and 1 mM EGTA replaced CaCl₂). After a stable basal level of cytosolic Ca²⁺ was achieved, the cells were exposed to ANG II (200 nM) in KB solution with or without Ca²⁺. Cytosolic levels of Ca²⁺ were measured using luminescence spectrometer (Perkin-Elmer LS 50B, Norwalk, CT) with an excitation wavelength alternately at 340 and 380 nm and emission wavelength 510 nm. Calibration of the Ca²⁺-dependent fluorescence (F) for each measurement was obtained by the addition of 5 μM ionomycin [maximal F (Fmax)] in the presence of 4 mM Ca²⁺, followed by the addition of 5 μM ionomycin in the presence of 4 mM EGTA in Ca²⁺-free KB solution [minimal F (Fmin)]. Intracellular Ca²⁺ concentration ([Ca²⁺]) was calculated using a previously described formula (11, 18): [Ca²⁺]i = Kd (F - Fmin)/Fmax - F, with the dissociation constant (Kd) = 224 nM, Fmax = 10.7125 (n = 4), and Fmin = 1.175 (n = 4).

**Analysis of Data**

Data were analyzed by ANOVA; the unpaired Student’s t-test was used to determine the difference between two groups. A value of P < 0.05 was considered to be statistically significant.

**RESULTS**

**ANG II-Induced Akt Activation Is Dependent on Extracellular Ca²⁺**

In rat aortic VSMC, ANG II stimulated phosphorylation of Akt, as measured by phosphorylation of serine-473, in a concentration-dependent manner (Fig. 1A). The peak of Akt phosphorylation was observed at 200 nM and 5 min (Fig. 1, A and B). Therefore, in all of our experiments, VSMC were exposed to 200 nM ANG II for 5 min. ANG II-induced Akt phosphorylation was abolished by pretreating the cells with the extracellular Ca²⁺ chelator EGTA (10 mM) for 30 min (Fig. 2A). Because high concentrations of EGTA may affect [Ca²⁺]i, the effect of lower concentrations of EGTA (1 mM) in the presence and absence of BAPTA-AM (intracellular Ca²⁺ chelator) and the effect of depletion of extracellular Ca²⁺ on Akt phosphorylation were also examined. Quiescent VSMC were depleted of extracellular Ca²⁺ by replacing M199 (1.8 mM) with Ca²⁺-free Hanks’ buffered saline solution (HBSS). The cells were then stimulated with 200 nM ANG II for 5 min in Ca²⁺-free HBSS or M199 (1.8 mM Ca²⁺). Depletion of the extracellular Ca²⁺ (5 min) blocked the Akt phosphorylation induced by ANG II (Fig. 2B). However, the Akt phosphorylation was restored by the addition of Ca²⁺ (1.8 mM CaCl₂) (Fig. 2C). The addition of Ca²⁺ to the Ca²⁺-free HBSS increased both basal and ANG II-induced Akt phosphorylation (Fig. 2C). BAPTA-AM (20 μM) or EGTA (1 mM) in the presence of Ca²⁺ (M199) produced a smaller but significant decrease in ANG II-induced Akt phosphorylation (Fig. 2D). The effect of EGTA (1 mM) was not further reduced by BAPTA-AM (Fig. 2B). BAPTA-AM (20 μM) did not alter Akt phosphorylation elicited by ANG II in the presence of 1.8 mM CaCl₂ (Fig. 2C).

**Fig. 1.** Angiotensin II (ANG II) stimulates Akt activation in rat aortic vascular smooth muscle cells (VSMC). A: quiescent VSMC were stimulated with 200 nM ANG II for indicated time. **Bottom,** densitometric analysis of ANG II-induced Akt phosphorylation (n = 5). B: quiescent VSMC were stimulated with the indicated concentration of ANG II for 5 min. **Bottom,** densitometric analysis of ANG II-induced Akt phosphorylation (n = 4). Equal amounts of protein from each cell lysate sample were resolved by SDS-PAGE and transferred to nitrocellulose membranes, and total Akt and phospho-Akt were measured by Western blot analysis as described in Methods. Intensity of phospho(P)-Akt bands is normalized to the abundance of Akt and shown relative to the values obtained with vehicle alone. Values are means ± SE.

*Value significantly different from the corresponding value obtained in the absence of ANG II (vehicle alone) (P < 0.05).
increased cytosolic [Ca\(^{2+}\)] by releasing Ca\(^{2+}\) from intracellular stores. The sustained phase of cytosolic [Ca\(^{2+}\)] increase caused by ANG II was abolished in the absence of extracellular Ca\(^{2+}\) (Fig. 2D).

**ANG II-Induced Akt Phosphorylation Is Mediated by CaMKII**

Increased [Ca\(^{2+}\)] is known to activate CaM, a small and highly conserved Ca\(^{2+}\)-binding protein, which in turn activates CaMKII (28–30, 55). To assess the contribution of CaM and CaMKII to ANG II-induced Akt phosphorylation, we examined the effect of their respective inhibitors. The CaM inhibitor W-7 at 30 μM (1) reduced Akt phosphorylation, whereas its inactive analog W-5 at the same concentration had no effect (Fig. 3A). The isoquinoline sulfonamide derivative KN-93 (30 μM), an inhibitor of CaMKII (1), but not its inactive structural analog KN-92, also inhibited Akt phosphorylation elicited by ANG II (Fig. 3B).

**ANG II-Induced Akt Phosphorylation Is Mediated by cPLA2**

Activation of CaMKII as a result of increased influx of extracellular Ca\(^{2+}\) has been shown to stimulate cPLA2 activity in rabbit VSMC (28–30). Therefore, we examined the possible contribution of cPLA2 in ANG II-induced Akt phosphorylation. cPLA2 inhibitor pyrrolidine-1 (100 nM) (14, 40) and Ca\(^{2+}\)-independent PLA2 inhibitor HELSS (10 μM) (4) attenuated ANG II-induced Akt phosphorylation (Fig. 4A). Because ANG II-induced Akt phosphorylation was dependent on extracellular Ca\(^{2+}\), and pyrrolidine-1 inhibited the activity of cPLA2 as measured from the hydrolysis of its substrate arachidonyl phosphatidylcholine (Fig. 4D), the effect of ANG II on Akt phosphorylation in rat VSMC is most likely mediated by Ca\(^{2+}\)- and cPLA2. Pyrrolidine-1, at the concentration that inhibited ANG II-induced Akt phosphorylation, did not alter exogenous Ca\(^{2+}\) (1.8 mM). The results shown in traces are means ± SE (n = 4 for each group). Phospho-Akt and Akt were detected, and the intensity of phospho-Akt bands was calculated as described in Fig 1. Values are means ± SE. *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).

Fig. 2. ANG II-induced Akt phosphorylation is dependent on extracellular Ca\(^{2+}\); A: quiescent VSMC were treated with EGTA (10 mM) for 30 min before addition of ANG II (200 nM) as indicated. Bottom, densitometric analysis of ANG II-induced Akt phosphorylation (n = 4). B: quiescent VSMC were depleted of Ca\(^{2+}\) by replacing medium 199 (M199) with Ca\(^{2+}\)-free Hanks’ buffered saline solution (HBSS), or pretreated with EGTA (1 mM) for 30 min or BAPTA-AM (20 μM) alone, or combined with Ca\(^{2+}\)-depletion-EGTA (1 mM) for 30 min before stimulation with ANG II (200 nM) in the presence of Ca\(^{2+}\) or in the absence of Ca\(^{2+}\) as indicated. Bottom, densitometric analysis of ANG II-induced Akt phosphorylation (n = 4; C: quiescent VSMC were stimulated with ANG II (200 nM) in Ca\(^{2+}\)-free HBSS with or without exogenous Ca\(^{2+}\) (1.8 mM) as indicated, or VSMC were stimulated with ANG II (200 nM) in Ca\(^{2+}\)-free HBSS containing BAPTA-AM (20 μM) with or without exogenous Ca\(^{2+}\) (1.8 mM). The blots are representative of three different experiments. D: cytosolic levels of Ca\(^{2+}\) [intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{i}\)]) in VSMC stimulated with ANG II (200 nM) in the presence or absence of Ca\(^{2+}\). The results shown in traces are means ± SE (n = 4 for each group). Phospho-Akt and Akt were detected, and the intensity of phospho-Akt bands was calculated as described in Fig 1. Values are means ± SE. *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).

corresponding value obtained with ANG II in the absence of inhibitors (not by its inactive analog 2-N-(4-chloro-(4-methoxybenzenesulfonyl)]amino-ANG II (200 nM) for 5 min as indicated. Top alone or inhibitors alone (significantly different from the corresponding value obtained with vehicle treated with KN-92 or KN-93 (30-methylbenzylamine) (KN-92). Quiescent VSMC were pretreated with W-7 or W-5 (30-N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) but not by its inactive analog N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5). Quiescent VSMC were pretreated with W-7 or W-5 (30 μM) for 30 min before stimulation with ANG II (200 nM) for 5 min as indicated. Top, Western blot analysis; bottom, densitometric analysis of ANG II-induced Akt phosphorylation (n = 6). B: ANG II-induced Akt phosphorylation in rat aortic VSMC is inhibited by CaMKII inhibitor 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-93) but not by its inactive analog 2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (KN-92). Quiescent VSMC were pretreated with KN-92 and KN-93 (30 μM) for 30 min before stimulation with ANG II (200 nM) for 5 min as indicated. Top, Western blot analysis; bottom, densitometric analysis of ANG II-induced Akt phosphorylation (n = 5). Phospho-Akt and Akt were detected, and the intensity of phospho-Akt bands was calculated as described in Fig 1. Values are means ± SE. *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).

Phosphorylation of Akt in VSMC (Fig. 1C). Furthermore, cPLA2 siRNA inhibited cPLA2 activity in VSMC (Fig. 1E). cPLA2 siRNA did not alter Akt protein levels and produced only a slight decrease in PLD2 protein levels, indicating the specificity of its effect (Fig. 1C). These data indicate that ANG II-induced Akt phosphorylation is mediated by cPLA2, which is activated by CaMKII (28–30).

ANG II-Induced Akt Phosphorylation Is Independent of H2O2 Production

Activation of cPLA2 promotes the release of AA, which is metabolized via cyclooxygenase (lipoxygenase) and cytochrome P-450 into various biologically active products (15). Exogenous AA has been reported to increase Akt phosphorylation in mesangial cells and VSMC (16, 31). To assess the contribution of endogenous AA released by activation of cPLA2 by ANG II, we examined the effect of the inhibitor of AA metabolism ETYA on the action of ANG II and exogenous AA on Akt phosphorylation. Exogenous AA (20 μM) caused phosphorylation of Akt in VSMC (Fig. 5A). ETYA (10 μM) blocked this effect of exogenous AA as well as ANG II-induced Akt phosphorylation (Fig. 5B). It has been reported that ANG II-induced Akt activation in mesangial cells is mediated by ROS production consequent to Rac-1-regulated NAD(P)H oxidase (Nox4) stimulated by AA released via cPLA2 activation (17). Moreover, inhibition of H2O2 production reduces ANG II-induced Akt phosphorylation (17, 52). ROS can stimulate or inhibit AA metabolism under different conditions (2, 45). To assess the relative contribution of ROS and of AA metabolites to Akt activation, we measured the production of H2O2 in response to ANG II and to exogenous AA in rat VSMC treated with the inhibitor of AA metabolism ETYA and the ROS production inhibitor catalase. In VSMC exposed to ANG II (200 nM) for 5 min, there was a significant increase in H2O2 production. Exposure of the cells to exogenous AA (20 μM) for 10 min also markedly increased the production of H2O2. However, treatment of cells with oleic acid (100 μM) for 10 min, which also increased catalase-reducible H2O2 production (Fig. 6A), failed to increase the phosphorylation of Akt (Fig. 6B). ETYA, which inhibited ANG II- and AA-induced Akt phosphorylation, did not reduce but rather increased ROS production that is reducible by catalase, although it has been reported that ETYA inhibits oxidase activity (58). Moreover, ETYA produced an additive effect on stimuli-induced ROS production (Fig. 6A). ETYA by itself did not exhibit any autofluorescence (data not shown). The effect of ANG II to increase ROS production was not altered in VSMC infected with cPLA2 siRNA (http://ajpheart.physiology.org/cgi/content/full/0571.2004/DC1), suggesting that endogenous AA does not contribute to ANG II-induced ROS production. Pretreatment of cells with exogenous catalase for 48 h, which is known to increase expression of catalase (46), inhibited both ANG II- and AA-induced ROS production (Fig. 6A). Akt phosphorylation elicited by AA, but not that produced by ANG II, was reduced by catalase (Fig. 6C). These observations suggest that H2O2 does not mediate ANG II-induced phosphorylation of Akt in rat VSMC. However, in VSMC cultured in DMEM medium containing high glucose, both the basal and ANG II-induced ROS production was increased (Fig. 6E). Moreover, in this high-glucose medium, both ANG II and AA-induced Akt phosphorylation were inhibited by catalase (Fig. 6D).

ANG II-Induced Akt Phosphorylation Is Mediated by AA Metabolites

ETYA inhibited both ANG II- and AA-stimulated Akt phosphorylation (Fig. 5B). This suggests that AA metabolites released by CaMKII-activated cPLA2 promote Akt phosphorylation. To determine whether AA metabolites generated via cyclooxygenase, lipoxygenase, and/or cytochrome P-450 contribute to the phosphorylation of Akt elicited by ANG II, we examined the effect of inhibitors of these enzymes on ANG...
II-induced Akt phosphorylation. The inhibitors of lipoxygenase CDC (10 \mu M) and cyclooxygenase (indomethacin) (35), but not cytochrome P450 inhibitors 17-ODYA (27, 37, 60) and ketoconazole (6, 37), which inhibit both cytochrome P450 \omega-hydroxylase and epoxygenase (6, 27, 37, 60), also diminished ANG II- and AA-induced Akt phosphorylation (Fig. 7, A–C). However, HET0016, a selective inhibitor of \omega-hydroxylase (22, 26), did not alter ANG II- and AA-induced Akt phosphorylation in VSMC (Fig. 7C). The metabolism of AA via lipoxygenase results in generation of 5(S), 12(S), and 15(S)-HETE and via cytochrome P450.
AA by a mechanism independent of H$_2$O$_2$ production in rat VSMC. ETYA (10$^{-6}$M) abolished Akt phosphorylation stimulated by exogenous AA (20 µM) for 10 min as indicated. Top, Western blot analysis; bottom, densitometric analysis of ANG II-induced Akt phosphorylation ($n = 4$). *Value significantly different from that obtained in the absence of AA (vehicle alone). B: Akt phosphorylation induced by ANG II and AA is inhibited by inhibitor of AA metabolism 5,8,11,14-eicosatetraynoic acid (ETYA). Quiescent VSMC were pretreated with cPLA$_2$ metabolism inhibitor ETYA (10 µM) for 30 min before stimulation with ANG II (200 nM) for 5 min or AA (20 µM) for 10 min as indicated. Top, Western blot analysis; bottom, densitometric analysis of ANG II or AA-induced Akt phosphorylation ($n = 4$). Phospho-Akt and Akt were detected, and the intensity of phospho-Akt bands was calculated as described in Fig. 1. Values are means ± SE. *Value significantly different from the corresponding value obtained with vehicle alone or ETYA alone. †Value significantly different from the corresponding value obtained with ANG II or AA in the absence of ETYA ($P < 0.05$).

omega-hydroxylase 20-HETE and epoxygenase EETs (37). All of these metabolites (500 nM) enhanced the phosphorylation of Akt (Fig. 7, D and E). These data indicate that one or more AA metabolites, generated via lipoxigenase (HETEs) and cytochrome P-450 (EETs), mediate Akt phosphorylation elicited by ANG II in rat VSMC.

**DISCUSSION**

This study demonstrates that ANG II-induced Akt activation is mediated by CaMKII-activated cPLA$_2$ and metabolites of AA by a mechanism independent of H$_2$O$_2$ production in rat VSMC. ANG II increased Akt phosphorylation, which was abolished by extracellular Ca$^{2+}$ depletion and by Cu$^{2+}$ chelator EGTA (10 mM). ANG II causes a rapid and transient increase in [Ca$^{2+}$], due to its release from intracellular sites followed by a sustained increase by extracellular Ca$^{2+}$ influx (9, 20, 50). Because BAPTA-AM, an intracellular Ca$^{2+}$ chelator, inhibits Akt phosphorylation, [Ca$^{2+}$] mobilized by ANG II has been implicated in Akt activation (48). However, in the present study, BAPTA-AM only partially reduced ANG II-induced Akt phosphorylation. Furthermore, in the absence of extracellular Ca$^{2+}$, ANG II caused a rapid rise in cytosolic [Ca$^{2+}$], but it failed to cause Akt phosphorylation. Moreover, when Akt phosphorylation was partially reduced by a lower concentration of EGTA (1 mM), the addition of BAPTA-AM did not further attenuate the effect of ANG II on Akt phosphorylation. From these observations, it follows that Akt phosphorylation by ANG II requires a sustained increase in cytosolic [Ca$^{2+}$], caused by influx of extracellular Ca$^{2+}$ rather than mobilization from intracellular sites, which is either insufficient or inaccessible to the signaling molecules involved in Akt phosphorylation.

Our demonstration that inhibitors of CaM, W-7, CaMKII, and KN-93, but not their inactive structural analogs, W-5 and KN-92, attenuated ANG II-induced Akt phosphorylation suggests that increased cytosolic [Ca$^{2+}$], promotes Akt phosphorylation by activating CaMKII in rat VSMC. CaMK kinase, which activates CaMK I and IV (39), has also been implicated in Akt activation in neuroblastoma cells (56). Moreover, mepracine and aristolochic acid, nonselective inhibitors of PLA$_2$, block Akt activation elicited by ANG II (16) and exogenous AA mimics this effect of ANG II in mesangial cells (16). In our study, selective cPLA$_2$ inhibitor pyrrolidine-1 and Ca$^{2+}$-independent PLA$_2$ inhibitor HELSS decreased the phosphorylation of Akt in rat VSMC. Because EGTA (10 mM), an extracellular Ca$^{2+}$ chelator, or depletion of extracellular Ca$^{2+}$ abolished ANG II-induced phosphorylation of Akt, it appears that Ca$^{2+}$-dependent cPLA$_2$ is involved in Akt phosphorylation elicited by ANG II. Therefore, HELSS appears to inhibit ANG II-induced Akt phosphorylation by a nonselective effect, because in renal proximal tubule cells, it also inhibits dopamine-stimulated PI3K activity (8), which regulates intracellular Ca$^{2+}$ mobilization through extracellular calcium-sensing receptor (53). Because Ca$^{2+}$-independent PLA$_2$ (iPLA$_2$) has been implicated in store-operated Ca$^{2+}$ entry (44), it is possible that HELSS by inhibiting iPLA$_2$ reduces Ca$^{2+}$ entry and subsequently reduces Akt phosphorylation. The involvement of cPLA$_2$ in ANG II-induced Akt phosphorylation was further supported by our demonstration that cPLA$_2$ antisense, but not sense oligonucleotide, and cPLA$_2$ siRNA inhibited Akt phosphorylation elicited by ANG II. These observations strongly suggest that ANG II-induced Akt activation is mediated via cPLA$_2$, which is stimulated by CaMKII (28–30).

Activation of cPLA$_2$ in response to ANG II releases AA (35); ANG II and exogenous AA promote Akt activation via generation of H$_2$O$_2$, independent of its metabolism in mesangial cells (16). ANG II stimulated the production of ROS, which have been implicated in Akt phosphorylation in rat VSMC (52). However, it is not known whether AA released by ANG II in VSMC phosphorylates Akt directly or through ROS production and/or through the action of its metabolites generated by cyclooxygenase, lipooxygenase, and/or cytochrome P-450. In the present study, the concentration of ANG II (200 nM) that caused the maximal increase in Akt phosphorylation also produced ROS. However, treatment with catalase, which increased intracellular catalase expression (46) and reduced H$_2$O$_2$ production, inhibited Akt phosphorylation stimulated by exogenous AA but not by ANG II. Moreover, our demonstration that the inhibitor of AA metabolism ETYA, which
stimulated ROS production by itself and had an additive effect on ANG II-stimulated ROS production, which was reducible by catalase. Quiescent VSMC were pretreated with ETYA (10 μM) for 30 min or catalase (2,000 IU/ml) for 48 h and then incubated with the H$_2$O$_2$-sensitive fluorescent probe 2',7'-dichlorofluorescin diacetate (DCF-DA, 10 μM) for 10 min. ANG II (200 nM), AA (20 μM), oleic acid (100 μM), or their vehicles were added to the cells for 5 or 10 min. Intensity of fluorescent is shown relative to control (vehicle alone). Bottom, representative fluorescent images of cells treated with different agents. B: oleic acid did not increase Akt phosphorylation. Quiescent VSMC were treated with oleic acid (100 μM) for 5 and 10 min or AA (20 μM) for 10 min. Blots are representative of three different experiments. C: catalase inhibits AA-, but not ANG II-induced, Akt phosphorylation in normal glucose medium (5.5 mM). Quiescent cells were pretreated with catalase (2,000 IU/ml) for 48 h before stimulation with ANG II (200 nM) for 5 min or AA (20 μM) for 10 min in M199. Bottom, densitometric analysis of ANG II- or AA-induced Akt phosphorylation (n = 4). D: catalase inhibited both ANG II and AA-induced Akt phosphorylation in DMEM medium with high glucose (27.5 mM). Quiescent cells were pretreated with catalase (2,000 IU/ml) for 48 h before stimulation with ANG II (200 nM) for 5 min or AA (20 μM) for 10 min in DMEM with high glucose. E: high glucose increases basal ROS production as well as ANG II-induced ROS production. VSMC were cultured with M199 and DMEM (high glucose) containing 10% fetal bovine serum and penicillin-streptomycin for 2 passages and then arrested with M199 and DMEM (high glucose) containing 0.1% fetal bovine serum. Quiescent VSMC were then incubated with the H$_2$O$_2$-sensitive fluorescent probe DCF-DA (10 μM) for 10 min before stimulation with ANG II (200 nM) in consequent phenol-free M199 and DMEM (high glucose). Fluorescent images were acquired and quantified. Phospho-Akt and Akt were detected, and the intensity of phospho-Akt bands was calculated as described in Fig. 1. Values are means ± SE. *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, AA, or oleic acid in the absence of inhibitors (P < 0.05).
phorylation in rat VSMC cultured in M199 that contain normal glucose (5.5 mM), it is possible that the higher level of ROS produced by exogenous AA potentiates the effect of one or more of its metabolites on Akt activation but is unable to activate Akt alone (Fig. 8). Supporting this view was our finding that the ETYA, which also stimulated and further increased ROS production caused by ANG II, did not activate but rather attenuated ANG II-induced Akt phosphorylation by inhibiting the metabolism of endogenous AA metabolism stimulated by ANG II. The lack of contribution of ROS to ANG II-induced Akt phosphorylation in our study seems controversial to previous reports (36, 52). This could be related to the difference in the medium used to culture the rat VSMC. For example, ANG II causes Akt phosphorylation in DMEM containing high glucose (27.5 mM) but not in DMEM containing lower glucose (5.5 mM) (23), which is equivalent to M199 used in our study. Rat VSMC cultured in high glucose (25 mM) show significant increases in basal and ANG II-induced ROS production and proliferative activities compared with VSMC cultured in normal glucose (5.5 mM) (41). Moreover, high glucose increases Akt activity as well as ROS production in mesangial cells (42). Therefore, it is possible that ROS-mediated Akt phosphorylation by ANG II is dependent on the experimental conditions, especially the glucose level. Supporting this view, we found that in VSMC cultured in DMEM with high glucose concentration (27.5 mM), there was an increase in the production of ROS and, under this condition catalase inhibited ANG II-induced Akt phosphorylation. Therefore, it appears that high levels of ROS act as a permissive factor in

Normal Glucose (5.5 mM)

\[
\text{ANG II} \rightarrow \text{ROS} \rightarrow \text{Akt} \rightarrow \text{AA and its metabolites} \rightarrow p-Akt
\]

High Glucose (27.5 mM)

Fig. 8. Proposed mechanism of ANG II on Akt phosphorylation in VSMC cultured in M199 containing normal glucose (5.5 mM). Release of endogenous AA is required for ANG II-induced Akt phosphorylation, and high concentration of ROS generated such as by high glucose (27.5 mM) can act as a permissive factor to potentiate Akt phosphorylation.
potentiating Akt activation caused by endogenous AA released by ANG II (Fig. 8).

Our findings that inhibitors of lipoygenase [CDC (54)] and cytochrome P-450 [ketoconazole (6, 37) and 17-ODYA (27, 37, 60)], but not cyclooxygenase (indomethacin) (35), attenuated ANG II- and AA-induced Akt phosphorylation suggest that AA metabolites derived through lipoygenase and cytochrome P-450 mediate Akt activation in response to ANG II. Supporting this view was our demonstration that AA metabolites derived via lipoygenase [5(S)-, 12(S)-, 15(S)-HETE] and via cytochrome P-450 (20-HETE, and 5,6-, 11,12-, and 14,15-EET) increased Akt phosphorylation. AA metabolites derived via lipoygenase [5(S)-, 12(S)-, and 15(S)-HETE (37, 38)] and through cytochrome P-450 [EETs (33, 37, 38)] have been shown to increase Akt phosphorylation (7, 12, 25, 34, 47, 59) in various cell systems. Because ketoconazole is more selective for cytochrome P-450 epoxygenase (6, 37) and 17-ODYA (27, 37, 60) inhibits both cytochrome P-450 ω-hydroxylase and epoxygenase activity, we examined the effect of HET0016, a selective ω-hydroxylase inhibitor (22, 26) on ANG II-induced Akt phosphorylation. HET0016 failed to alter ANG II-induced Akt phosphorylation, suggesting that AA metabolites generated via cytochrome P-450 epoxygenase (EETs), but not ω-hydroxylase (20-HETE) (27, 38) are involved in ANG II-induced Akt phosphorylation in rat VSMC. In a study conducted in rabbit VSMC, we have found that they are capable of generating EETs (our unpublished work). However, further studies on the identification of AA metabolites generated via cytochrome P-450 ω-hydroxylase and epoxygenase and lipoxygenase and the use of specific molecular tools to inhibit these pathways are required to address this issue. The lack of effect of AA metabolism inhibitors on ANG II- and AA-induced Akt activation reported in mesangial cells (16) could be due to differences in AA metabolism and the mechanism of Akt activation in these distinct cell types. Phosphorylation of Akt stimulated by HETEs and EETs could be mediated through p38MAPK because these metabolites of AA have been reported to increase p38MAPK activity (21, 34), and inhibition of p38MAPK activity blocks ANG II-induced Akt phosphorylation in rat VSMC (49).

In conclusion, this study demonstrates that in rat VSMC, ANG II stimulates Akt phosphorylation by a mechanism that is dependent on extracellular Ca\(^{2+}\). ANG II-induced increase in cytosolic [Ca\(^{2+}\)] by stimulating CaMKII activates cPLA\(_2\) and releases AA; AA metabolites generated via lipoxygenase and cytochrome P-450, possibly HETEs and EETs, respectively, by activating p38MAPK, phosphorylates Akt by a mechanism independent of H\(_2\)O\(_2\) in rat VSMC.

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