Expression and Mechanism of Spleen Tyrosine Kinase Activation by Angiotensin II and Its Implication in Protein Synthesis in Rat Vascular Smooth Muscle Cells*5

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Fariborz A. Yaghini1, Fang Li, and Kafait U. Malik2

From the Department of Pharmacology and Centers of Vascular Biology and Connective Tissue Diseases, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38163

Syk, a 72-kDa tyrosine kinase, is involved in development, differentiation, and signal transduction of hematopoietic and some non-hematopoietic cells. This study determined if Syk is expressed in vascular smooth muscle cells (VSMC) and contributes to angiotensin II (Ang II) signaling and protein synthesis. Syk was found in VSMC and was phosphorylated by Ang II through AT1 receptor. Ang II-induced Syk phosphorylation was inhibited by piceatannol and dominant negative but not wild type Syk mutant. Syk phosphorylation by Ang II was attenuated by cytosolic phospholipase A2 (cPLA2) inhibitor pyrrolidine-1 and retrovirus carrying small interfering RNAs (shRNAs) of this enzyme. Arachidonic acid (AA) increased Syk phosphorylation, and AA- and Ang II-induced phosphorylation was diminished by inhibitors of AA metabolism (5,8,11,14-eicosatetraynoic acid) and lipoxigenase (LO; baicalin) but not cyclooxygenase (indomethacin). AA metabolites formed via LO, 5(S), 12(S), and 15(S)-hydroxyeicosatetraenoic acids, which activate p38 MAPK, increased Syk phosphorylation. p38 MAPK inhibitor SB202190, and dominant negative p38 MAPK mutant attenuated Ang II- and AA-induced Syk phosphorylation. Adenovirus dominant negative c-Src mutant abolished Ang II- and AA-induced Syk phosphorylation. Adenovirus dominant negative c-Src mutant abolished Ang II- and AA-induced Syk phosphorylation. Ang II may also activate various signaling molecules directly or indirectly via transactivation of tyrosine kinase receptors, including platelet-derived growth factor receptor, EGFR, and insulin-like growth factor receptor (13, 14). EGFR transactivation by Ang II has been reported to be mediated through activation of c-Src and Pyk2 (13, 15).

Angiotensin II (Ang II),3 the major effector peptide of the renin-angiotensin system, regulates vascular tone, salt and water homeostasis, and stimulates growth of vascular smooth muscle cells (VSMC) (1). One or more of these effects of Ang II are mediated via an increase in cytosolic calcium (Ca2+), activation of phospholipase A2 (PLA2), phospholipase D, protein kinase C, mitogen-activated protein kinases (MAPKs) and NAD(P)H oxidase, phosphatidylinositol 3-kinase, and Akt (2–5). Activation of PLA2 by Ang II through stimulation of Ca2+/calmodulin-dependent kinase II releases arachidonic acid (AA), a polyunsaturated fatty acid from tissue phospholipids in VSMC (4). AA metabolites derived via lipoxigenase (LO) (12-hydroxyeicosatetraenoic acid (12-HETE)) and cytochrome P450A (CYP4A) (20-HETE and epoxyeicosatrienoic acids) have been implicated in the action of Ang II to activate extracellular signal-regulated kinase (ERK1/2) and p38 MAPK in VSMC (4–6). Activation of p38 MAPK via increase in phospholipase D activity and generation of phosphatidic acid causes epidermal growth factor receptor (EGFR) transactivation and phosphatidylinositol 3-kinase and Akt activation in VSMC (5). Ang II also activates intracellular tyrosine kinases c-Src, focal adhesion kinase family protein-tyrosine kinase, Pyk2, and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) that play a role in growth signaling and inflammation (3, 7). Activation of c-Src by Pyk2 promotes phosphorylation of adapter protein Shc, recruitment of Grb2 and Sos, and activation of Ras and ERK1/2 (7, 8). Src-like cytosolic protein-tyrosine kinases Lyn, Btk, or Yes are known to cause tyrosine phosphorylation of the FceR1 β and γ subunits, which in turn activates another 72-kDa cytosolic non-receptor protein-tyrosine kinase spleen tyrosine kinase (Syk) (9–12). Ang II may also activate various signaling molecules directly or indirectly via transactivation of tyrosine kinase receptors, including platelet-derived growth factor receptor, EGFR, and insulin-like growth factor receptor (13, 14). EGFR transactivation by Ang II has been generated by the American Society for Biochemistry and Molecular Biology. Printed in the U.S.A.
Syk, a non-receptor-tyrosine kinase expressed in hematopoietic cells (16), plays an important role in B cell development and differentiation and T cell antigen receptor signaling (17, 18), ERK1/2 activation, and release of allergic mediators in mast cells (19). However, Syk is expressed in many non-hematopoietic cells, including epithelial cells, hepatocytes, fibroblasts, neuronal cells, and endothelial cells and has been implicated in embryogenesis, tumor growth, and increase in intracellular calcium and activation of cPLA₂, Ras, ERK1/2, and phosphatidylinositol 3-kinase/Akt (20–22). The present study was conducted to determine whether Syk is expressed and is involved in Ang II signaling process and its relationship to EGFR in its growth promoting effects in rat VSMC. The results of our study show that Syk is expressed in VSMC and is activated by Ang II through generation of AA metabolites via LO, most likely HETEs, consequent to cPLA₂ activation. AA metabolites via p38 MAPK-activated c-Src phosphorylate and activate Syk, which contributes to Ang II-induced VSMC protein synthesis independent of EGFR transactivation.

EXPERIMENTAL PROCEDURES

Materials—Anti-phospho-Syk (Tyr-525/526), phosphotyrosine, phospho-cPLA₂, phospho-p38 MAPK, and phospho-c-Src antibodies were from Cell Signaling (Beverly, MA). The corresponding non-phospho antibodies for these kinases, anti-green fluorescent protein (GFP) and anti-12/15LO polyclonal antibodies, were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-actin smooth muscle antibody was obtained from Sigma-Aldrich. Anti-5-LO monoclonal antibody was purchased from R&D Systems, respectively. [3H]thymidine (20 Ci/mmol) were purchased from American Science Products, and [3H]leucine (40 Ci/ml) and [3H]thymidine (20 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO) and PerkinElmer Life Sciences, respectively. 10× lysis, 10× kinase buffer and ATP were from Promega (Madison, WI). Polyethylene glycol (PEG) was kindly provided by Dr. R. J. Davis, University of Massachusetts Medical School, Worcester, MA) using Effectene transfection reagent (Qiagen, Valencia, CA) in a ratio of 25 μl of Effectene to 1 μg of plasmid in M-199 containing 5% FBS for 48 h according to manufacturer’s instructions. Then the transfected cells were lysed and subjected to SDS-PAGE and Western blot analysis. The transfection efficiency was determined by Western blot analysis by co-transfection of plasmid containing the GFP sequence along with plasmids of interest and visualizing GFP bands.

Preparation of shRNA and Transfection of VSMC with Retroviral Gene Suppressor System—shRNA cPLA₂ was synthesized and used to transfect VSMC as previously described (5). Briefly, the Primers with forward (5′-TCGAGATCATGATTCTAAGGCTAGGGCAGTGTAGAGAA-CCACTACTGTTT-3′) and reverse (5′-CTAGAAAAAACAGTATGTTTCTACGTTCCGCTGCTGCTT-3′) sequences for cPLA₂ were synthesized by Integrated DNA Technologies (Coralville, IA). The oligonucleotides were annealed and inserted into linearized pSuppressorRetro viral vector using ready-to-go T4 DNA ligase kit (Amersham Biosciences). Competent DH5α cells (Invitrogen) were transformed with ligated plasmid DNA. After transformation, the colonies were amplified and purified using a Qiagen miniprep purification kit, and plasmid DNAs were sequenced using primer complementary to suppressor retroviral vector. Then the plasmids with correct sequences were amplified and purified with Qiagen Maxi Plasmid DNA kit and used to transfect human embryonic kidney 293 cells along with pECO packaging vector for the preparation of the retrovirus containing cPLA₂ shRNA insert as described (5). The virus was harvested by filtering the virus-containing supernatant. VSMC were made quiescent and infected with the viral supernatant in M-199 containing 8 μg/ml Polybrene and 0.1% FBS for 48 h before exposure to Ang II. The infection efficiency was con-
Contribution of Syk in Ang II Signaling in VSMC

![Image]

**FIGURE 1. Syk is expressed in rat VSMC and is phosphorylated through AT1 receptor by Ang II.** A, quiescent VSMC were treated with Ang II (200 nM) or its vehicle (Veh) for 5 min, the cells were lysed, Syk was immunoprecipitated (IP) with anti-Syk monoclonal antibody and subjected to SDS-PAGE and Western blot analysis, and the blots were probed with anti-phospho (p-Syk) (Tyr-525/526) (upper panel), anti-phosphotyrosine (middle panel), and anti-Syk (lower panel) antibodies. The bands were detected, and their density was measured as described under “Experimental Procedures.” The blot is representative of three experiments (n = 3). B and C, confluent serum-deprived VSMC lysates were treated with different concentrations of Ang II (25–200 nM) (n = 4) and with 200 nM Ang II for indicated time points or its vehicle (n = 5). D, confluent VSMC were pretreated with AT1 receptor blocker (losartan, 10 μM) or AT2 receptor antagonist (PD123319, 1 μM) for 30 min before the addition of Ang II (200 nM) for 5 min (n = 4). In all experiments (A–D), equal amounts of protein from each cell lysates were resolved by SDS-PAGE. B–D, the density of p-Syk bands were normalized to the quantity of Syk and presented as fold increase from the value obtained with vehicle alone in the absence of Ang II taken as 1. The upper panel shows a representative blot, and the lower panel shows the ratio of densitometric analysis of p-Syk and Syk. Values are the means ± S.E. The asterisk denotes a value significantly different from that obtained in the absence of Ang II.

Confirmed by β-galactosidase staining (Invitrogen) of cells infected with retrovirus containing control LacZ.

*Amplification and Transduction of Adenovirus Carrying Partially Active and DN c-Src in VSMC—*The subconfluent low passage human embryonic kidney 293 cells were infected with adenovirus stock containing partially active (AxSrcY416F/Y527F) and DN (AxSrc Kinase Dead) c-Src (kindly provided by Dr. R. Baron, Yale School of Medicine, New Haven, CT) for about 3–5 days. As the cytopathic effect was complete, the medium was collected and centrifuged at 1500 × g for 5 min, and supernatant was removed and kept in a separate sterile tube. The pelleted was subjected to freeze-thaw cycle three times, re-suspended, and centrifuged at 1500 × g for 5 min. After mixing the first and second supernatants, they were filtered through a filter-sterile bottle and stored at −80 °C. Before storing, the number of viral particles in stock was determined using the physical particles method. The optical density of viral stock in 0.05% SDS (1:50) was measured at 260 nm, and multiplicity of infection was calculated by infecting VSMC with a different volume of viral stock (24). The efficiency of infection was confirmed by increased expression of c-Src kinase in VSMC by Western blot analysis. Subconfluent VSMC were then incubated with adenovirus carrying partially active or DN c-Src (10 multiplicity of infection) in M-199 containing 5% FBS for 48 h. The cells were washed with M-199 medium and then exposed to Ang II (200 nM) or its vehicle for 5 min and lysed in lysis buffer, and an equal amount of cell lysate protein was subjected to SDS-PAGE and Western blot analysis.

**Immunoblotting and in Vitro Kinase Assay—**After various treatments, the VSMC were lysed, and equal amounts of protein from each cell lysates were incubated with the antibodies against the protein of interest at 4 °C overnight. The next day the agarose-bound protein A (Vector, Berlingame, CA) was added and incubated for 6 h at 4 °C. After centrifugation of the lysates and washing the pellet with 1× lysis buffer, 100 μl of 4× sample buffer were added to the pellet and used for Western blot analysis. For in vitro p38 MAPK kinase assay, the immunoprecipitate was washed with 1× kinase buffer and suspended in 50 μl of 1× kinase buffer containing ATP (200 μM) and the substrate (ATF-2, 1 μl) and incubated for 20 min at 37 °C, and reaction was stopped by adding 4× sample buffer. The activity of p38 MAPK was determined by measuring phosphorylation of ATF-2 by Western blot analysis. For in vitro Syk assay, the immunoprecipitate was washed with kinase buffer and suspended in 50 μl of 1× kinase buffer containing ATP (200 μM),...
[\gamma^{32}\text{P}]{\text{ATP}} (1 \mu\text{Ci, Amersham Biosciences}), and MBP (5 \mu\text{g}) and incubated at 30 °C for 20 min. The reaction was stopped by adding 4× sample buffer, and the reaction mixture was subjected to SDS-PAGE. The Syk activity was determined by measuring the density of radioactive MBP bands by autoradiography.

RNA Extraction and Real-time Quantitative PCR—5-LO primers (GenBank\textsuperscript{TM} accession number NM_012822, forward, ccccgagatatccggttaa, and reverse, aggttctcaagccgg, 12/15-LO primers (GenBank\textsuperscript{TM} accession number NM_031010, forward, aaggatggctcataatcctgaa, and reverse, tcctctgaacagtggtctg), and 18\textsuperscript{S} RNA primers (GenBank\textsuperscript{TM} accession number X01117, forward, ccgcagctaggaatatgga, reverse, ccctcttaatcatggcctca) were designed to have ~50% or less G/C content and a melting temperature less than 60 °C. RNA was extracted from quiescent VSMC and used for real-time quantitative PCR as described (25).

DNA and Protein Synthesis Determination in VSMC—DNA and protein synthesis was determined by measuring the incorporation of [\textsuperscript{3}H]\text{thymidine} or [\textsuperscript{3}H]\text{leucine}, respectively, in VSMC as described (26). Subconfluent VSMC were plated in 24-well plates for 24 h, transfected with DN or WT Syk mutant or empty vector for 48 h, and exposed to Ang II (100 ng/ml) or EGF (100 ng/ml) for the last 24 h. [\textsuperscript{3}H]\text{Leucine} (0.25 \mu\text{Ci/well}) or [\textsuperscript{3}H]\text{thymidine} (0.5 \mu\text{Ci/well}) was also added to the plates along with Ang II or EGF. The cells were washed with phosphate buffered saline, trichloroacetic acid (10%), and ethanol/ether (2:1) each three times. [\textsuperscript{3}H]-Labeled cells were collected with 0.1% SDS, 0.1 M NaOH, and radioactivity was measured using liquid scintillation spectroscopy. [\textsuperscript{3}H]\text{Leucine} and [\textsuperscript{3}H]\text{thymidine} incorporation was measured as disintegrations per min (dpm) per well and expressed as -fold change from vehicle.

To directly measure the DNA and protein content, VSMC in each plate were counted using a hemocytometer, protein content was measured by the Bradford method and DNA content was determined by a DNA extraction kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Briefly VSMC were harvested and centrifuged at 1500 rpm for 15 min. After resuspending and homogenizing the pellet, the nucleic acids were isolated by incubation with Pronase (0.44 \mu\text{g/ml}) on a shaker overnight. DNA from samples was purified by adding RNase, precipitated by ethanol, and measured using Biophotometer.

Data Analysis—The densitometric analysis was performed using NIH ImageJ program. The data were analyzed by one-way analysis of variance; the unpaired Student’s t test was used to determine the difference between two groups. The values of at least three different experiments is expressed as the mean ± S.E. p values less than 0.05 were considered as statistically significant.

RESULTS

Syk Is Expressed in Rat VSMC and Phosphorylated and Activated by Ang II through AT1 Receptor—The Western blot analysis of rat VSMC lysates showed that Syk (72 kDa) is expressed in these cells (Fig. 1A). To determine the effect of Ang II on phosphorylation of Syk, the serum-deprived confluent VSMC were treated with Ang II at different concentrations (25–200 nm) and with 200 nm Ang II for different time periods (1, 2, 5, 10, and 20 min). Ang II increased Syk phosphorylation in a concentration-dependent manner with the maximal effect at 200 nm (Fig. 1B). The increase in Syk phosphorylation was evident as early as 2 min, peaked at 5 min, and was maintained in the presence of Ang II for 20 min (Fig. 1C). Therefore, VSMC were treated with 200 nm Ang II for 5 min in all subsequent experiments. Tyrosine phosphorylation of Syk was also meas-
Receptor blocker losartan (10 μM) abolished Ang II-induced Syk phosphorylation, which was reversed by add-

To further assess the effect of Ang II on Syk phosphorylation, we transiently transfected 60–70% confluent quiescent VSMC with EV, WT, or DN Syk plasmid for 48 h using Effectene reagent, as described under “Experimental Procedures.” As shown in Fig. 2A, transient transfection of VSMC with WT Syk but not EV or WT Syk plasmid significantly diminished Ang II-induced Syk phosphorylation. To determine the efficiency of transfection, the cells were co-transfected with GFP-expressing plasmid along the vectors, subjected to Western blot, and probed with GFP polyclonal antibody (Fig. 2A). Ang II-induced Syk activity was also diminished in VSMC transfected with DN Syk, but not EV or WT Syk plasmid, as determined by in vitro kinase assay by measuring the phosphorylation of MBP (Fig. 2B).

Ang II-induced Syk Phosphorylation is Dependent upon Both Extracellular Ca2+ and Intracellular Ca2+—Ang II is known to cause a biphasic [Ca2+]i response, an initial transient phase mediated by [Ca2+]i mobilization from intracellular stores, and a sustained phase caused by influx of extracellular [Ca2+]i (28, 29). To determine the contribution of extra- and intracellular [Ca2+]i to Ang II-induced Syk phosphorylation, we examined the effect of extracellular Ca2+ depletion, Ca2+ chelator (EGTA, 1 mM), and intracellular Ca2+ chelator (BAPTA-AM, 20 μM), respectively. Extracellular Ca2+ was depleted by replacing Ca2+-containing M-199 medium (1.8 mM) with the Ca2+-free Hank’s-buffered saline solution, and VSMC were exposed to Ang II (200 nM) for 5 min. Depletion of extracellular Ca2+ abolished Ang II-induced Syk phosphorylation, which was reversed by adding exogenous CaCl2 (1.8 mM) (Fig. 3A). In addition, treatment of VSMC with EGTA (1 mM) for 30 min significantly decreased phosphorylation of Syk (Fig. 3A). Because intracellular Ca2+ chelator BAPTA-AM also blocked Syk phosphorylation elicited by Ang II (Fig. 3C), it appears that increased levels of Ca2+ are sufficient to activate Syk.

**FIGURE 3. Ang II-induced phosphorylation of Syk is dependent on extra- and intracellular Ca2+.**

A. Quiescent VSMC were incubated in Ca2+-free Hank’s-buffered saline solution with or without the addition of Ca2+ (1 mM) and Ang II (200 nM) or its vehicle for 5 min (n = 4). B. VSMC were incubated with extracellular Ca2+ chelator (EGTA, 1 mM) or its vehicle for 30 min before the addition of Ang II (200 nM) or its vehicle for 5 min (n = 4). C. Cells were stimulated with Ang II (200 nM) for 5 min after exposure to intracellular Ca2+ chelator (BAPTA-AM, 20 μM) or its vehicle for 30 min (n = 4). p-Syk and Syk were detected, and the density of the p-Syk and Syk bands was calculated as described under “Experimental Procedures” and presented as -fold increase relative to vehicle in the absence of Ang II as in Fig. 1. A and B, the upper panel shows a representative blot, and lower panel shows the ratio of the densitometric analysis of p-Syk and Syk. Values are the means ± S.E. The asterisk denotes a value significantly different from corresponding value obtained in the absence of Ang II (p < 0.05).
of cytosolic Ca^{2+}, caused by influx of extracellular Ca^{2+} promotes Syk phosphorylation.

Ang II-induced Syk Phosphorylation Is Mediated by cPLA2 Activation—The increased cytosolic Ca^{2+} by binding to a Ca^{2+}-binding protein calmodulin is known to activate Ca^{2+}/calmodulin-dependent kinase II, which in turn stimulates the activity of cPLA2 (4). Therefore, to determine the possible involvement of cPLA2 in Ang II-induced Syk phosphorylation, we examined the effect of pyrrolidine-1, the inhibitor of cPLA2 (compound 4c (30)). Pyrrolidine-1 at concentrations that block cPLA2 activity in VSMC (200 nM) (31) inhibited Ang II-induced Syk phosphorylation (supplemental Fig. S2). To further assess the involvement of cPLA2 in Ang II-induced Syk phosphorylation, we investigated the effect of pyrrolidine-1 on Ang II-induced cPLA2 activity in VSMC (200 nM) (31). This inhibition of Ang II-induced cPLA2 activity in VSMC (supplemental Fig. S2) provides additional evidence for the role of cPLA2 in Ang II-induced Syk phosphorylation.

AA Promotes Syk Phosphorylation, and the AA Metabolism Inhibitor ETYA Blocks AA-induced Syk Phosphorylation—Ang II-induced activation of cPLA2 promotes release of AA from tissue phospholipids (2, 4), which in turn is metabolized into various biologically active metabolites (32–35). To determine whether AA and/or its metabolites mediate Ang II-induced phosphorylation of Syk, we examined the effect of exogenous AA and Ang II in the presence of inhibitor of AA metabolism, ETYA, and its vehicle in the rat VSMC. Exogenous AA (10 μM) but not oleic (100 μM) or linoleic acids (20 μM) (data
not shown) increased Syk phosphorylation in VSMC. Treatment with ETYA (10 μM) for 30 min inhibited the effect of both AA and Ang II to stimulate phosphorylation of Syk (Fig. 5A). Also, transfection of VSMC with DN, but not WT Syk or EV plasmid, blocked AA-induced phosphorylation of Syk in rat VSMC (Fig. 5B). These observations suggest that one or more AA metabolites but not AA per se stimulate Syk phosphorylation.

Ang II-induced Syk Phosphorylation Is Mediated by AA Metabolites Generated through the Lipoxigenase Pathway—AA is metabolized via cyclooxygenase (COX), LO, and CYP450 pathways in various cell systems (32–35). VSMC express COX and 12/15 LO (36, 37), and products of AA are generated via COX and LO (38, 39), whereas CYP 450 4A that is expressed in rat aorta and aortic VSMC is inactive (25, 40). Ang II stimulates prostaglandin synthesis in rat VSMC (41) and 12-HETE production in porcine aortic smooth muscle cells (42). In the present study 12/15 LO protein and mRNA were found to be expressed in rat VSMC (supplemental Fig. S3, A and C). To determine the possible contribution of AA metabolites generated via LO and COX, we investigated the effect of 12/15 LO inhibitor baicalein (10 μM) (43) and COX inhibitor indomethacin (50 μM) (44) on Ang II and AA-induced Syk phosphorylation. Baicalein, but not indomethacin attenuated Syk phosphorylation elicited by Ang II or AA (Fig. 6, A and B). The products of AA metabolism formed via 12/15 LO and 12(5)- and 15(5)-HETE stimulated phosphorylation of Syk in the presence of baicalein (Fig. 6). Moreover, 5(5)-HETE, formed from AA in rabbit VSMC (39) via 5-LO which was found to be expressed, both protein and mRNA, in rat VSMC (supplemental Fig S3, B and D), also increased Syk phosphorylation (Fig. 6C). 5(R)-, 12(R)-, and 15(R)-HETE, the optical antipodes of (S)-HETEs, did not stimulate phosphorylation of Syk in the VSMC (Fig. 6D). These observations suggest that Ang II-induced phosphorylation of Syk is mediated by AA metabolites most likely 5(5)-HETEs, via the 12/15-LO (46, 47), and 5-LO, subsequently to the release of AA through activation of cPLA₂.

Ang II-induced Syk Phosphorylation Is Mediated through p38 MAPK—Ang II is known to promote activation of one or more MAPKs in VSMC (14). Previously we have reported that metabolites of AA generated via LO cause activation of ERK1/2 and p38 MAPK in VSMC (45, 46). Therefore, it raised the possibility that these kinases might mediate Ang
Effect of p38 MAPK on Ang II-induced Syk phosphorylation. To test this hypothesis, we examined the effect of U0126 (10 μM) and SB202190 (10 μM), which inhibits ERK1/2 and p38 MAPK activity, respectively, in VSMC (44, 46). SB202190, which inhibited p38 MAPK activity as indicated by decreased phosphorylation of its substrate ATF-2 (supplemental Fig. S4A), but not U0126, which inhibited ERK1/2 phosphorylation (data not shown), abolished Syk phosphorylation elicited by Ang II and AA (supplemental Fig. S4B). Pretreatment with SB202190 also inhibited the effect of 5(S), 12(S), and 15(S)-HETE to promote Syk phosphorylation (Fig. 7A). Moreover, in VSMC transfected with DN but not WT p38 MAPK plasmid, which reduced p38 MAPK activity (Fig. 7B), also inhibited Ang II- and AA-induced Syk phosphorylation (Fig. 7C). Conversely, in VSMC transfected with DN Syk mutant plasmid, there was no decrease in Ang II- or AA-induced p38 MAPK phosphorylation (Fig. 7D). These observations suggest that Ang II-induced Syk phosphorylation by AA metabolites is mediated by p38 MAPK in VSMC.

C-Src Mediates p38 MAPK-stimulated Syk Phosphorylation in Response to Ang II—Syk contains a C-terminal domain and tandem N-terminal SH2 domain and the linker region referred to as immunoreceptor-tyrosine-based activation motifs (47). In immune cells after receptor engagement, these motifs are phosphorylated by Src family kinases, which promote the recruitment and activation of Syk that in turn phosphorylates and activates various downstream signal molecules (48). The Src kinase family, mainly c-Src, has been implicated in some actions of Ang II in VSMC (14). Therefore, we examined the possible involvement of c-Src in Ang II-induced Syk phosphorylation in the rat VSMC. In VSMC treated with PP2, a c-Src inhibitor (10 μM), or in VSMC transduced with adenovirus carrying DN c-Src but not partially active c-Src mutant, Ang II-induced Syk phosphorylation was abolished (Fig. 8 and supplemental Fig. S5). The DN or WT Syk mutant did not alter phosphorylation of c-Src (data not shown). These observations indicate that c-Src mediates Syk phosphorylation elicited by Ang II.

Ang II-induced c-Src Phosphorylation Is Mediated by p38 MAPK in VSMC—To determine whether c-Src mediates Ang II-induced Syk phosphorylation, we examined the effect of p38 MAPK inhibitor, SB202190, on c-Src phosphorylation. SB202190 (10 μM) inhibited the phosphorylation of c-Src elicited by Ang II (supplemental Fig. S6). Also, transfection of VSMC with DN but not WT p38 MAPK for 48 h blocked Ang
II-induced phosphorylation of c-Src (Fig. 9). These data suggest that p38 MAPK mediates Ang II-induced c-Src phosphorylation, which in turn stimulates Syk phosphorylation.

VSMC Protein Synthesis Caused by Ang II Is Dependent on Activation of Syk and Independent of EGFR Transactivation—Ang II is known to stimulate protein synthesis in VSMC (49). Therefore, to determine the functional significance of Syk activation in VSMC protein synthesis, we examined the effect of DN and WT Syk on VSMC protein and DNA synthesis elicited by Ang II. The VSMC protein synthesis in response to Ang II, measured by increase [3H]leucine incorporation, was inhibited in cells transfected with DN but not WT Syk mutant or EV (Fig. 10A). Ang II-induced [3H]thymidine incorporation, an index of DNA synthesis, was increased in VSMC transfected with EV, WT, or DN Syk mutants (Fig. 10B). We also measured the DNA and protein content in response to Ang II. Ang II increased both the protein and DNA synthesis, but the ratio of DNA to protein content expressed per 10^6 cells was reduced, and DN Syk but not wt mutant blocked this effect of Ang II (Fig 10C). Because Ang II is known to cause activation of one or more signaling molecules and VSMC proliferation via transactivation of EGFR (50), we also examined the effect of EGF and EGFR blocker, AG1478, on the action of Ang II and EGF on [3H]leucine and [3H]thymidine incorporation. EGF failed to stimulate Syk phosphorylation or [3H]leucine incorporation, and also the DN Syk did not alter Ang II-induced EGFR phosphorylation in VSMC (data not shown). EGFR blocker AG1478 in concentrations that inhibited EGF- and Ang II-induced EGFR phosphorylation (1 μM) did not alter Ang II-induced [3H]leucine incorporation in VSMC (data not shown). On the other hand Ang II- or EGF-induced [3H]thymidine incorporation was blocked by AG1478 (1 μM) (supplemental Fig. S7). However, AG1478 failed to inhibit Ang II-induced Syk phosphorylation (data not shown). These observations suggest that activation of Syk mediates protein synthesis in rat VSMC caused by Ang II and that this effect of Ang II is independent of EGFR transactivation.

DISCUSSION

This study demonstrates for the first time that Syk, a non-receptor-tyrosine kinase, is expressed in VSMC and is activated in response to Ang II by its phosphorylation through a mechanism that is dependent on generation of AA consequent to activation of cPLA2. AA in turn, through its metabolism by LO into most likely HETEs, by causing p38 MAPK-mediated activation of c-Src, promotes Syk phosphorylation. Moreover, Syk activa-
tion mediates Ang II-induced protein synthesis in VSMC by a mechanism independent of EGFR transactivation.

In rat VSMC, Ang II stimulated phosphorylation of Syk on a tyrosine residue(s) as detected by specific phospho-Syk (Tyr-525/526) antibody. Supporting this view was our demonstration that (a) Syk immunoprecipitated from lysates of VSMC exposed to Ang II and probed with phosphotyrosine antibody showed an increase in Syk phosphorylation, (b) a tyrosine kinase inhibitor piceatannol that blocks Syk activation in T cells, neutrophils, and macrophages (22) attenuated Ang II-induced Syk phosphorylation. That Ang II stimulates Syk phosphorylation was further supported by our demonstration that the transfection of VSMC with DN but not WT Syk mutant or EV inhibited Ang II-induced phosphorylation of Syk. Phosphorylation of Syk by Ang II was associated with an increase in its activity as shown by increased phosphorylation of MBP by Syk immunoprecipitated from lysates of VSMC exposed to Ang II. These observations strongly suggest that Syk expressed in VSMC is phosphorylated at tyrosine residue (525/526) and activated by Ang II. The effect of Ang II to increase Syk phosphorylation was mediated through AT1 and not AT2 receptor because AT1 receptor blocker losartan but not AT2 receptor blocker PD123319 inhibited Ang II-induced Syk phosphorylation.

To elucidate the mechanism by which Ang II through AT1 receptor promotes phosphorylation of Syk, we first investigated the contribution of extra- and intracellular Ca$^{2+}$/H$^{+}$ because Ang II has been shown to activate several signaling molecules by increasing levels of cytosolic Ca$^{2+}$ (51, 52). Our finding that depletion of extracellular Ca$^{2+}$/H$^{+}$ or extra- or intracellular Ca$^{2+}$/H$^{+}$ chelators, EGTA and BAPTA-AM, respectively, blocked Ang II-induced Syk phosphorylation suggests that both extra- and intracellular Ca$^{2+}$/H$^{+}$ are involved in this action of Ang II. Previous studies from our laboratory have shown that increased cytosolic Ca$^{2+}$/H$^{+}$ via stimulation of Ca$^{2+}$/H$^{+}$/calmodulin-dependent kinase II activates cPLA2 and releases AA, which in turn through its metabolites promotes activation of ERK1/2 and p38 MAPK (45, 46). AA and/or its metabolites have also been shown to activate one or more tyrosine kinase (52–55). Our demonstration that pyrrolidine-1, which inhibits cPLA2 activity (31) or transfection of VSMC with retrovirus cPLA2 shRNA but not Lac Z blocked Ang II-induced Syk phosphorylation, suggests that AA/and or its metabolites mediate the effect of Ang II on phosphorylation of Syk. This is in contrast to the findings reported in the mast cell line, RBL-2H3, where antigen stimulated immunoglobulin E receptor (FcεR1) or in rat peritoneal mast cells stimulated by a G$i$ protein-coupled receptor activator c48/80, increased cPLA2 activity, and/or AA release through activation of Syk (12, 55). Therefore, it appears that Syk plays a distinct role in cell signaling by acting either upstream or downstream of cPLA2 in different cell types. In the present study AA released by cPLA2 in response to Ang II could activate Syk directly and/or through its metabolites. Our finding that AA increased Syk phosphorylation and that the inhibitor of AA metabolism, ETYA, blocked the effect of both AA and Ang II on Syk phosphorylation suggests that this effect of Ang II is mediated through AA metabolites. AA is metabolized in VSMC by COX and LO (38, 39), whereas CYP450 4A, which also metabolizes AA (40) and is

FIGURE 10. Transfection of VSMC with DN but not WT Syk mutant plasmid inhibited Ang II-induced protein synthesis. The subconfluent VSMC were transfected with EV, WT, or DN Syk mutants for 48 h using Effectene reagent as described under "Experimental Procedures." Ang II was added during the last 24 h. A and B, data are expressed as-fold increase [3H]leucine (n = 12) or [3H]thymidine (n = 6) incorporation in VSMC treated with vehicle (514 ± 27 and 6188 ± 1020 dpm/well, respectively) as described under "Experimental Procedures." C, after determining number of VSMC and quantity of protein in each plate, content of DNA was measured in each sample, as described under "Experimental Procedures." The results are expressed as the ratio of DNA content to the amount of protein per 10$^6$ cells in each sample (arbitrary unit (A.U.)) (n = 6). Values are the means ± S.E. The asterisk denotes a value significantly different from corresponding value obtained in the absence of Ang II (p < 0.05).
expressed in aortic VSMC, has been found to be inactive (25). Therefore, AA metabolites generated via a COX or LO pathway could mediate Ang II-induced Syk phosphorylation in VSMC. Our demonstration that the inhibitor of LO baicalein, but not COX inhibitor indomethacin, attenuated Ang II- and AA-induced Syk phosphorylation suggests that the LO metabolites mediate this effect of Ang II. Supporting this conclusion was our finding that the AA metabolites formed via LO in VSMC, 5(S)-, 12(S)-, and 15(S)-HETE (39), also increased Syk phosphorylation. Because 20-HETE, a metabolite formed through CYP450 4A, also increased Syk phosphorylation, it is possible that 20-HETE generated in smaller blood vessels (40, 56) may cause Syk activation. Moreover, our study does not exclude the activation of Syk by AA metabolites formed through other CYP isoforms in VSMC.

Previous studies from our laboratory have shown that the metabolites of AA formed via LO cause activation of ERK1/2, a MAPK, and p38 MAPK (45, 46). Therefore, we examined the possible involvement of these kinases in Ang II-induced Syk phosphorylation. Our observations that p38 MAPK inhibitor SB202190, which reduced p38 MAPK activity, but not U0126, that inhibited ERK1/2 phosphorylation, blocked Ang II- and AA-induced Syk phosphorylation suggests that p38 MAPK mediates Ang II- and AA-induced Syk phosphorylation. Further supporting this view was our observation that in VSMC transfected with DN but not WT p38 MAPK mutant that reduced p38 MAPK activity, also inhibited Ang II- and AA-induced Syk phosphorylation. The finding that AA metabolites 5(S)-, 12(S)-, and 15(S)-HETE generated via 5 and 12/15 LO that are expressed in rat VSMC increases p38 MAPK activity together with our demonstration that HETEs stimulate Syk phosphorylation was attenuated by p38 MAPK inhibitor SB202190 suggest that AA metabolites generated via LO, most likely HETEs, through p38 MAPK activation mediate Ang II-induced Syk phosphorylation. This is the first demonstration that in VSMC p38 MAPK acts as an upstream activator of Syk because in hematopoietic and some non-hematopoietic cells Syk is upstream and activates not only p38 MAPK but also ERK1/2 and C-Jun terminal kinase (JNK) (22, 57–60). However, there is one study in rat mucosal type mast cell line RBL-2H3, in which direct phosphorylation and activation of Syk by ERK1/2 in vitro has been reported (61). In this study the authors found that antigen-induced phosphorylation of Syk and MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) inhibitors diminished its activation as well as degranulation of cells. In our study p38 MAPK but not ERK1/2 increased Syk phosphorylation, which could be caused directly or indirectly via one or more kinases.

In immune cells Syk-tyrosine kinase, receptor engagement is phosphorylated by Src-family kinases, which promote its recruitment and activation that in turn phosphorylates and activates various downstream signal molecules (48). Also, Src kinase family, mainly c-Src, has been implicated in some actions of Ang II in VSMC (15). These observations raised the possibility that c-Src might mediate Ang II-induced Syk phosphorylation. Our finding that PP2, an Src inhibitor, and the adenovirus DN, but not partially active c-Src mutant, blocked Ang II-induced Syk phosphorylation supports this view. Our demonstration that the p38 MAPK inhibitor SB202190 blocked c-Src phosphorylation suggests that p38 MAPK stimulates Syk phosphorylation via activation of c-Src. This is the first demonstration indicating that p38 MAPK-activated c-Src regulates Syk phosphorylation elicited by Ang II in VSMC. Our recent studies have shown that Ang II-induced activation of p38 MAPK promotes transactivation of EGFR through generation of phosphatidic acid subsequent to activation of phospholipase D activity (5). However, inhibition of phospholipase D activity that blocks EGFR transactivation failed to alter Ang II-induced Syk phosphorylation. Therefore, it appears that p38 MAPK-c-Src-activated Syk phosphorylation is independent of EGFR transactivation in VSMC. The mechanism by which p38 MAPK activates c-Src remains to be elucidated.

Ang II is known to stimulate protein synthesis in VSMC (49). Also, Syk has been implicated in differentiation of hematopoietic and some non-hematopoietic cells adipogenesis and endothelial cell morphogenesis, proliferation, migration, and survival (17, 18, 62). Therefore, we investigated the functional significance of Syk in VSMC protein and DNA syntheses elicited by Ang II. Our demonstration that DN but not WT Syk mutant or EV inhibited Ang II-induced [3H]leucine incorporation and prevented the Ang II-induced decrease in the ratio of the DNA to protein suggests that Syk mediates Ang II-induced protein production in VSMC. Although Ang II activates various signaling molecules via EGFR transactivation, its effect on VSMC protein synthesis was independent of EGFR transactivation for the following reasons. First, EGF did not increase [3H]leucine uptake. Second, DN Syk failed to inhibit Ang II-induced EGFR phosphorylation. Third, AG1478, which blocked Ang II-induced EGFR phosphorylation, did not affect Ang II-induced [3H]leucine incorporation in VSMC. In our study Ang II and EGF increased [3H]thymidine uptake, which

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4 F. A. Yaghini and K. U. Malik, unpublished data.
was not altered by DN Syk mutant. However, EGFR blocker AG1478 abolished both the Ang II- and EGF-induced increase in [3H]thymidine incorporation in VSMC. The effect of AG1478 to block Ang II-induced [3H]thymidine incorporation has been previously demonstrated in VSMC (50). From these observations it follows that Ang II stimulates protein production in VSMC by a mechanism dependent on Syk activation but independent of EGFR transactivation. Moreover, the increase in DNA synthesis, measured by [3H]thymidine incorporation caused by Ang II, is mediated through EGFR transactivation but independent of Syk. The mechanism and the downstream signaling events involved in Syk-activated VSMC growth in response to Ang II remains to be determined.

In conclusion, this study demonstrates that rat VSMC express Syk that is activated by Ang II through a Ca2+-dependent cPLA2 stimulation and generation of AA metabolites, most likely HETEs, via LO. The AA metabolites, through stimulation of p38 MAPK, results in an increase in c-Src kinase activity, which promotes phosphorylation, and activation of Syk, which in turn promotes protein synthesis in VSMC by a mechanism independent of EGFR transactivation (Fig. 11). Moreover, Ang II also promotes VSMC proliferation via EGFR transactivation independent of Syk activation.

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