Angiotensin II-induced process of angiogenesis is mediated by spleen tyrosine kinase via VEGF receptor-1 phosphorylation

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Buharalioglu CK, Song CY, Yaghini FA, Ghafoor HU, Motiwala M, Adris T, Estes AM, Malik KU. Angiotensin II-induced process of angiogenesis is mediated by spleen tyrosine kinase via VEGF receptor-1 phosphorylation. Am J Physiol Heart Circ Physiol 301: H1043–H1055, 2011. First published June 3, 2011; doi:10.1152/ajpheart.01018.2010.—Spleen tyrosine kinase (Syk), expressed in endothelial cells, has been implicated in migration and proliferation and in vasculogenesis. This study was conducted to determine the contribution of Syk and the underlying mechanism to the angiogenic effect of ANG II and VEGF. Angiogenesis was determined by tube formation from the endothelial cell line EA.hy926 (EA) and human umbilical vein endothelial cells (HUVECs) and microvessel sprouting in rat aortic rings. ANG II (10 nM), EGF (30 ng/ml), and VEGF (50 ng/ml) stimulated EA cells and HUVECs to form tubular networks and increased aortic sprouting; these effects were blocked by VEGF receptor-1 and Flt-1 antibody (Flt-1/Fc) but not by the VEGF receptor-2 (Flk-1) antagonist SU-1498. ANG II increased the phosphorylation of Flt-1 but not Flk-1, whereas VEGF increased the phosphorylation of both receptors in EA cells and HUVECs. VEGF expression elicited by ANG II was not altered by Flt-1/Fc or SU-1498. EGF stimulated tube formation from EA cells and HUVECs and Flt-1 phosphorylation and aortic sprouting, which were blocked by the EGF receptor antagonist AG-1478 and Flt-1/Fc but not by SU-1498. ANG II, EGF, and VEGF-induced tube formation and aortic sprouting were attenuated by the Syk inhibitor piceatannol and by Syk short hairpin interfering (sh)RNA and small interfering RNA, respectively. ANG II, EGF, and VEGF increased Syk phosphorylation, which was inhibited by piceatannol and Syk shRNA in EA cells and HUVECs. Neither piceatannol nor Syk shRNA altered ANG II-, EGF-, or VEGF-induced phosphorylation of Flt-1. These data suggest that ANG II stimulates angiogenesis via transactivation of the EGF receptor, which promotes the phosphorylation of Flt-1 and activation of Syk independent of VEGF expression.

aortic sprouting; tube formation; transactivation of endothelial growth factor receptor; EA.hy926 cells; human umbilical vein endothelial cells

THE RENIN-ANGIOTENSIN SYSTEM is an important component of the mechanisms that regulate renal and cardiovascular homeostasis (18, 21). The increased activity of the renin-angiotensin system, mainly through the generation of ANG II, contributes to several cardiovascular diseases, including hypertension, atherosclerosis, and restenosis after vascular injury, heart failure, and myocardial infarction (1, 38). ANG II also promotes inflammation and the growth of cardiac myocytes, fibroblasts, and vascular smooth muscle cells (VSMCs) (9, 24, 34) and stimulates angiogenesis in lower concentrations but inhibits in higher concentrations (36). The process of angiogenesis involves the differentiation, proliferation, and migration of endothelial cells (ECs) and results in tubulogenesis and formation of vessels (13). ANG II promotes EC proliferation and angiogenesis through the angiotensin type 1 receptor (AT1R) (17, 31).

ANG II also stimulates VEGF synthesis in ECs (6, 23). The effects of VEGF on ECs are mediated via two receptor tyrosine kinases: VEGF receptor (VEGFR)-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) (8). VEGF binds with Flt-1 with a 50-fold higher affinity than with Flk-1, although many of the effects of VEGF (e.g., mitogenesis, chemotaxis, and morphological changes) are mediated by its interaction with Flk-1 (22). However, the angiogenic response to VEGF can be inhibited by selective blockade of the high-affinity receptor Flt-1 alone in vitro and in vivo (3, 12, 27). The reduction in Flt-1 expression by small interfering (si)RNA inhibits VEGF-induced human umbilical vein EC (HUVEC) proliferation and capillary-like tube formation to a greater extent than that obtained by a reduction in Flk-1 expression (10). ANG II has been shown to increase Flk-1 mRNA via AT1R but has no effect on tube formation and mRNA levels of VEGF and Flt-1 in bovine retinal microcapillary ECs (16). However, ANG II increased VEGF-induced growth and tube formation (32). Conversely, in EA.hy926 (EA) cells and human coronary artery ECs (HCAEC), ANG II had no effect on Flk-1 autophosphorylation (32). Moreover, it has been shown that, in HUVECs, proliferation by ANG II depends on increased expression of VEGF and is abolished by the AT1R blocker candesartan or the VEGFR inhibitor Flt-1/Fc (14). Whether VEGF expressed in ECs mediates tubulogenesis in response to ANG II via Flt-1 or Flk-1 is not known. Moreover, the signaling pathway by which ANG II stimulates angiogenesis through VEGFRs has not been elucidated.

Expression of VEGF and capillary formation from HCAECs in response to ANG II has been reported to be mediated via actin-like scavenger receptor through the activation of NADPH oxidase/ROS and p38 and p44/42 MAPK (16). In addition to VEGF, other growth factors, including heparin-binding EGF, have been implicated in neovascularization induced by ANG II (12). However, it is not known whether EGF receptor (EGFR) transactivation in response to ANG II causes angiogenesis via VEGF expression and/or VEGFR activation.

In addition to VEGF and other growth factors, nonreceptor tyrosine kinases, including c-Src, and signal transducers and activators of transcription/JAK have also been implicated in the process of angiogenesis (7). A nonreceptor tyrosine kinase, spleen tyrosine kinase (Syk), which is ubiquitously distributed in hematopoietic cells, is expressed in ECs and epithelial cells (29). Syk has been implicated in various EC functions, including morphogenesis, growth, and survival, and contributes to the maintenance of vascular integrity in vivo (17, 39). Mouse
embryos lacking Syk develop abnormal blood-lymphatic endothelial connections (30). Recently, we (24, 38) have shown that, in VSMCs, ANG II causes the activation of Syk through p38 MAPK-activated c-Src and promotes VSMC hypertrophy and migration. These observations raised the possibility that ANG II might stimulate angiogenesis via EGFR transactivation and VEGF expression and/or Flt-1 or Flk-1 through the activation of Syk. To test this hypothesis, we investigated the effect of Syk and EGFR inhibitors and Syk gene silencing on ANG II-, EGF-, and VEGF-induced capillary tube formation in an in vitro model of ECs, EA cells and HUVECs, and in an ex vivo model of rat aortic ring sprouting. The results of our study show that ANG II promotes tubulogenesis of ECs and aortic sprouting by transactivation of EGFR, which results in the phosphorylation of Flt-1 and activation of Syk independent of VEGF expression.

**EXPERIMENTAL PROCEDURES**

**Materials.** Rabbit anti-phospho-Syk (Tyr525/Tyr526), anti-phospho-Flk-1 (Tyr1213), and anti-phospho-EGFR (Tyr1068) were obtained from Cell Signaling (Beverly, MA); rabbit anti-EGFR, mouse anti-Flk-1, and anti-β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human Sky was designed and purified (Genescript, Piscataway, NJ); rabbit anti-EGFR, anti-VEGF, rabbit anti-human Sky were obtained from Amersham Biosciences (Buckinghamshire, UK); secondary antibody of goat IgG was obtained from Vector Laboratories, (Burlingame, CA). The following reagents were used in this study: human ANG II and EGF (Bachem, King of Prussia, PA), Syk inhibitor piceatannol, Flk-1 antagonist SU-1498 (Calbiochem, San Diego, CA), AT1R antagonist losartan (LKT Laboratories, St. Paul, MN), angiotensin type 2 receptor (AT2R) antagonist PD-123319 and VEGF A (VEGF 165, Sigma-Aldrich, St. Louis, MO), and EGFR antagonist AG-1478 (Invitrogen, Carlsbad, CA). ANG II, losartan, VEGF, and EGF were dissolved in distilled water to make stock solutions. PD-123319, piceatannol, SU-1498, and AG-1478 were dissolved in DMSO. The final concentration of DMSO was <0.1% in these experiments.

**Cell culture.** HUVEC-derived EA cells (10) were kindly provided by Dr. Cora-Jean S. Edgell (University of North Carolina, Chapel Hill, NC), and HUVECs were purchased from Cell Application (San Diego, CA). EA cells were grown to 90% confluence under 5% CO2 at 37°C in DMEM (Invitrogen) supplemented with 10% FBS, 1% penicillin-streptomycin, and 0.1% amphotericin B (all from Cellgro Mediatech, Herndon, VA). HUVECs were cultured in medium 199 (Invitrogen) supplemented with 20% FBS, 4 U/ml heparin sodium, 2 mM l-glutamine, 15 mM HEPES, and 10 mg/ml EC growth supplement in 0.2% gelatin-coated plates. Rat VSMCs were isolated from Sprague-Dawley rat thoracic aortae and cultured in M199 with 10% FBS. Cells were made quiescent for 24 h with DMEM or M199 containing 1% FBS before exposure to different agents for all experiments. Experiments were performed according to the protocols approved by our Institutional Animal Care and Use Committee in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

**RESULTS**

**Fig. 1.** ANG II, EGF, and VEGF stimulate tube formation from EA.hy926 (EA) cells and capillary sprouting from rat aortic rings. A and D: quiescent EA cells and rat vascular smooth muscle cells (A) and rat aortic rings (D) were incubated with vehicle (DMEM) of ANG II, EGF, or VEGF for 16 h and 3–5 days, respectively. EA cells were incubated with ANG II, EGF, or VEGF for various time periods (0, 3, 12, and 24 h) or in the presence of vehicle (Veh; B), and the length of tube structures was measured as described in EXPERIMENTAL PROCEDURES (B, bottom). Aortic rings were incubated with DMEM with an intact or denuded endothelium (D). C and E: EA cells and rat aortic rings were exposed to ANG II (0.1, 1, 10, or 100 nM), EGF (1, 3, 10, or 30 ng/ml), VEGF (1, 10, 30, or 50 ng/ml), or their vehicle for 16 h for tube formation and 3–5 days for maximal aortic sprouting. The tube formation from EA cells and aortic sprouting were measured by network formation and sprouting area, respectively, as described in EXPERIMENTAL PROCEDURES. The values for an increase in relative tube formation and aortic sprouting caused by ANG II, EGF, and VEGF were normalized to values obtained with the vehicle (taken as 100%) for tube formation (C, bottom) and aortic sprouting with endothelium (E, bottom), respectively. Values are means ± SE. *Significantly different from that obtained in the presence of vehicle at 0 h (B, C, and E, bottom) (P < 0.05); †significantly different from that obtained in the presence of vehicle. Magnification: ×100. Scale bar = 200 μm.
In vitro angiogenesis assay (capillary tube formation). EA cells or HUVECs were grown in 60-mm tissue culture dishes until 90% confluent and arrested for 24 h. In some experiments, cells were cultured in the presence or absence of different chemicals or neutralizing antibody for the indicated time periods. After trypsinization, cells were counted and resuspended in DMEM for EA cells or M199 for VSMCs and HUVECs containing 1% FBS (at 4 × 10^4 cells/ml) with the indicated concentrations of agonists. Before cells were plated, 48-well tissue culture plates were coated with growth factor-reduced Matrigel (0.1 ml/well, BD Biosciences, Bedford, MA), which was allowed to polymerize at 37°C for 30 min, according to the manufacturer’s instructions. Cell growth and three-dimensional organization were observed at different time points and photographed with an Olympus CKX41 inverted phase-contrast microscope equipped with an Altra20 CMOS digital camera (Olympus America, Melville, NY). Phase-contrast images were recorded with a video image-analysis system, Microsuite Five (Olympus America), and network formation was quantified using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij). Results are presented as total tube length as an average of three photographic fields from each well in an experiment. Each experiment was repeated three to four times on different batches of cells.

Ex vivo angiogenesis assay (aortic ring assay). Thoracic aortae were isolated from Sprague-Dawley rats (4–6 wk old, Charles Rivers, Wilmington, MA) anesthetized with pentobarbital sodium (60 mg/kg ip), cut into 1-mm-thick sections, embedded in 48-well Matrigel-coated plates, and sealed with an overlay of 50 μl Matrigel with and without the supplement of agonists as previously described (21). Inhibitors or neutralizing antibodies were added fresh 30 min before agonists were added. In some experiments, aortic rings were transfected with 20 pmol Syk siRNA (sense: 5'-CACCAUAAMAUUGCAAGGGC-3' and antisense: 5'-GACCUUGCATAAUAUUGGG-3', Accession No. NM_012758, IDT, Coralville, IA) or its scrambled siRNA (sense: 5'-CAAGGUC-3' and antisense: 5'-AACUUGCCAGCUCACAUUA-3') under transfection conditions and then embedded on Matrigel. Transfections were carried out using the manufacturer’s protocol with DharmaFECT1 transfection reagent (Dharmacon, Lafayette, CO). The experiments were repeated on at least four aortic rings from different rats, and maximal EC sprouting was observed and photographed as described above in
3–5 days. Four to seven experiments were performed for each series. For quantitative assessment of sprouting, the area of the outer aortic EC sprouting was assessed using ImageJ software.

**Western blot analysis.** EA cells or HUVECs were lysed in RIPA buffer, and equal amounts of protein were resolved by SDS-PAGE and analyzed by Western blot analysis as previously described (38). Blots were blocked and incubated with primary antibody (1:500–1,000) overnight at 4°C. Blots were then exposed to their respective secondary antibodies conjugated with horseradish peroxidase and developed using West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). The band density was calculated with the ImageJ program. Loading of equal amounts of proteins on gels was confirmed by reprobing the membranes with β-actin or corresponding nonphosphorylated antibodies.

**Transfection of EA cells and HUVECs with Syk shRNA and its control short hairpin interfering RNA.** The GeneSuppressor (IMG-808) encoding Syk short hairpin interfering (sh)RNA or its control shRNA contained in the plasmid was obtained from Imgenex (San Diego, CA). EA cells and HUVECs were transiently transfected with Syk shRNA or its control shRNA using Effectene transfection reagent (QIAGEN, Valencia, CA) at a ratio of 50 μl Effectene to 0.2 μg plasmid in DMEM containing 10% FBS for 24 h according to the manufacturer’s instructions. Briefly, EA cells and HUVECs were trypsinized, counted, plated at a density of 10^4 cells/cm^2 in six-well plates (MIDSCI, St. Louis, MO) in antibiotic-free DMEM with 10% FBS, and incubated overnight at 37°C with 5% CO₂. The next morning, the medium was replaced, and ~60–80% confluent cells were transfected with 0.2 μg plasmid of Syk shRNA or control shRNA.

**Fig. 4.** ANG II-, EGF-, and VEGF-induced Flt-1 phosphorylation is inhibited by Flt-1/Fc but not by SU-1498, and VEGF-induced Flk-1 phosphorylation is inhibited by SU-1498 but not by Flt-1/Fc in EA cells. A–C: quiescent EA cells were incubated with ANG II (10 nM; A), EGF (10 ng/ml; B), and VEGF (50 ng/ml; C) for the indicated time periods (0.17, 0.5, 1, 4, and 16 h) or in the absence (vehicle) of these agents (0 h). D–F: EA cells were incubated with Flt-1 neutralizing antibody (Flt-1/Fc, 5 μg/ml), Flk-1 tyrosine kinase inhibitor SU-1498 (10 μM), or their vehicle for 30 min and then exposed to ANG II (10 nM), EGF (30 ng/ml), VEGF (50 ng/ml) (+) or to the vehicle of these agents (−). Cells were lysed and subjected to SDS-PAGE and Western blot analysis, and blots were probed with anti-phosphorylated (p-)Flt-1 and anti-Flt-1 antibodies and anti-p-Flk-1 and anti-Flk-1 antibodies. Bands were detected, and the density of bands was measured as described in EXPERIMENTAL PROCEDURES. Blots are representative of three to four experiments (A–C, top), or from that obtained in the presence of the vehicle of ANG II, EGF, or VEGF (D–F, bottom). Values are means ± SE. *Significantly different from that obtained at time 0 (A–C, bottom) and significantly different from the corresponding value obtained with the vehicle of ANG II, EGF, and VEGF (D–F, bottom) (P < 0.05); †significantly different from the corresponding value obtained with the vehicle of Flt-1/Fc or SU-1498 (D–F, bottom) (P < 0.05).
shRNA using 10 μl Effectene/well. After 24 h, cell lysates were subjected to Western blot analysis as described above to confirm the silencing effects of Syk shRNA.

Data analysis. Data were analyzed by one-way ANOVA and a Newman-Keuls multiple-comparison test. Values are expressed as means ± SE of at least four different experiments. Densitometric analysis was performed using the ImageJ program. P values of <0.05 were considered statistically significant.

RESULTS

ANG II, EGF, and VEGF stimulate tube formation from ECs and capillary sprouting from rat aortic rings. EA cells but not VSMCs plated on Matrigel exhibited distinct tube formation at 16 h (Fig. 1A). The tube-like structures started to appear within 3 h, reached a maximal level at 16 h, and maintained until 24 h (Fig. 1B). After 24 h, the tube formation declined due to detachment of cells. Exposure of EA cells to lower concentrations of ANG II (0.1–10 nM) for 16 h increased the formation of tube-like structures. The maximum capillary formation occurred at 10 nM ANG II exposure for 16 h (Fig. 1C). Higher concentrations of ANG II (100 nM) produced only a smaller increase in tube formation, which is consistent with a previous report (19). Therefore, we chose the maximal proangiogenic concentration of ANG II (10 nM) in subsequent experiments. EGF (1–30 ng/ml) caused tube formation in EA cells at 16 h. Maximal tube formation was observed at 30 ng/ml, which we used in the rest of our experiments (Fig. 1C). Treatment of ECs with VEGF (1–50 ng/ml) for 16 h also maximally stimulated the formation of capillary-like structures. The maximal effect of VEGF on tube formation was observed at 50 ng/ml, a concentration that we used in the rest of our experiments (Fig. 1C). We also examined the effect of ANG II (0.1–100 nM), EGF (1–30 ng/ml), and VEGF (1–50 ng/ml) on capillary sprouting from rat aortic rings embedded on Matrigel. Three to five days of incubation of aortic rings with but not without the endothelium resulted in capillary sprouting (Fig. 1D). Exposure to ANG II (0.1–100 nM), EGF (1–30 ng/ml), or VEGF (1–50 ng/ml) increased aortic capillary sprouting. ANG II at 10 nM, EGF at 30 ng/ml, and VEGF at 50 ng/ml produced maximal effects on aortic sprouting (Fig. 1E).

ANG II-induced tubulogenesis and rat aortic sprouting are mediated through Flt-1 but not Fk-1. To determine the subtype of VEGFR involved in ANG II-, EGF-, and VEGF-induced angiogenesis, we examined the effect of Flt-1 neutralizing antibody, Flt-1/Fc, and a selective Flk-1 tyrosine kinase inhibitor, SU-1498, on ANG II-, EGF-, and VEGF-induced tube formation in EA cells and rat aortic sprouting. Flt-1/Fc, but not SU-1498 (10 μM), inhibited the effect of ANG II, EGF, and VEGF on capillary tube formation and aortic sprouting, respectively (Fig. 2, A and B). A higher concentration of SU-1498 (25 μM) inhibited VEGF- but not ANG II- and EGF-induced tube formation (Fig. 2A). These observations indicate that ANG II- and EGF-induced angiogenesis is mediated via Flt-1 but not Fk-1, whereas VEGF stimulates angiogenesis through both Flt-1 and Fk-1.

ANG II-induced VEGF expression is not mediated via Flt-1 or Fk-1 in EA cells. ANG II is known to stimulate VEGF expression in ECs (9, 16). To determine whether the ANG

Fig. 5. ANG II-, EGF-, and VEGF-induced tube formation from EA cells and rat aortic sprouting caused by Flt-1 activation is mediated via EGFR receptor (EGFR) transactivation. A and B: quiescent EA cells and rat aortic rings were treated with the EGFR inhibitor AG-1478 (200 nM) or its vehicle for 30 min and then further exposed to ANG II (10 nM), EGF (30 ng/ml), VEGF (50 ng/ml), or their vehicle for 16 h for tube formation (A) and 3–5 days for maximal aortic sprouting (B). The value of increase in tube formation or aortic sprouting obtained in the presence of the vehicle of ANG II, EGF, or VEGF was taken as 100% for angiogenesis assays. C and D: EA cells were preincubated with AG-1478 (200 nM) or its vehicle for 30 min and then incubated with ANG II (10 nM; C) or EGF (30 ng/ml; D) (+) or their vehicle (−) for 30 min. Cells prepared for Western blot analysis were probed with p-EGFR and EGFR, p-Flt-1 and Flt-1, and p-Fk-1 and Fk-1 antibodies. The density of bands was measured as described in EXPERIMENTAL PROCEDURES. Blots are representative of three to four experiments (C and D). Densities of p-EGFR and p-Flt-1 were normalized to the quantities of EGFR and Flt-1, respectively, and presented as fold increases from the corresponding value obtained in the presence of the vehicle of ANG II or EGF, which was taken as 1 (C and D, bottom). Values are means ± SE. *Significantly different from the corresponding value obtained in the presence of the vehicle of ANG II, EGF, or VEGF; †significantly different from the corresponding value obtained in the presence of the vehicle of AG-1478 (P < 0.05).
II-induced process of angiogenesis is mediated via VEGF expression, we examined the effect of ANG II on VEGF expression in the presence of Flt-1/Fc and SU-1498 and their vehicle. ANG II initiated an increase in VEGF expression after 1 h, which became more prominent at 16 h, in EA cells (Fig. 3A). This effect of ANG II was not altered by either Flt-1/Fc or SU-1498 (Fig. 3B and C, respectively). ANG II-induced VEGF expression was blocked by AT1R (losartan) but not AT2R (PD-123319) antagonist (data not shown).

**Fig. 6. ANG II-, EGF-, and VEGF-induced spleen tyrosine kinase (Syk) phosphorylation.** A–C: quiescent EA cells were incubated with ANG II (10 nM; A), EGF (30 ng/ml; B), or VEGF (50 ng/ml; C) for various time periods (0.17, 0.5, 1, 4, and 16 h) or in the presence of the vehicle of these agents (0 h). Cells were lysed and subjected to SDS-PAGE and Western blot analysis, and p-Syk and Syk were detected on blots using anti-p-Syk and Syk antibodies as described in **EXPERIMENTAL PROCEDURES.** Blots are representative of three to four experiments (A and B, top). The density of p-Syk bands was normalized to the quantity of Syk and presented as the fold increase from the value obtained at time 0, which was taken as 1. Values are means ± SE. *Significantly different from that obtained from the corresponding value obtained at time 0 (A and B, bottom) (P < 0.05). D–F: ANG II- but not EGF- and VEGF-induced phosphorylation of Syk is mediated via angiotensin type 1 receptor (AT1R). Quiescent EA cells were incubated with ANG II (10 nM), EGF (30 ng/ml), VEGF (50 ng/ml), or their vehicles for 30 min. Losartan (10 μM) or PD-123319 (1 μM) was preincubated for 30 min. Cells were lysed and subjected to SDS-PAGE and Western blot analysis, and blots were probed with anti-p-Syk and anti-Syk antibodies and analyzed as described above. Representative blots of three to four experiments are shown in D–F (top). Values are means ± SE. *Significantly different from that obtained in the presence of the vehicle of ANG II, EGF or VEGF; †significantly different from the corresponding value obtained in the presence of the vehicle of losartan (D, bottom) (P < 0.05).
described were not performed in aortic rings because of the heterogeneity of cells in the intact tissue.

**ANG II-induced tubulogenesis and rat aortic sprouting caused by Flt-1 activation are mediated via EGFR transactivation.** ANG II is known to produce its effect on VSMC migration and proliferation through the transactivation of EGFR (5, 35), and heparin-binding EGF has been implicated in neovascularization induced by ANG II (2). Therefore, we investigated the effect of the EGFR blocker AG-1478 on ANG II-, EGF-, and VEGF-induced tube formation and aortic sprouting and phosphorylation of EGFR and VEGFRs in EA cells. AG-1478 (200 nM) inhibited ANG II- and EGF- but not VEGF-induced tube formation and aortic sprouting (Fig. 5, A and B). It also blocked ANG II- and EGF-induced phosphorylation of EGFR and Flt-1 (Fig. 5, C and D). VEGF did not stimulate the phosphorylation of EGFR, and phosphorylation of Flt-1 and Flk-1 caused by VEGF was not altered by AG-1478 (data not shown). ANG II did not cause associations of EGFR and Flt-1, Flt-1 and Syk, Flt-1 and Syk, or VEGFR and Flt-1 in response to VEGF, as determined by coimmunoprecipitation of these molecules (data not shown).

**Piceatannol and Syk shRNA inhibit ANG II-, EGF-, and VEGF-induced tubulogenesis and Syk phosphorylation in EA cells and rat aortic ring sprouting.** To investigate the contribution of Syk to ANG II-, EGF-, and VEGF-stimulated tube formation from EA cells and aortic sprouting, we examined the effect of these agents on Syk activation by following its phosphorylation and modulation by ANG II receptor and Flt-1 and Flk-1 blockers. Moreover, we examined the effect of Syk inhibitors on tube formation from EA cells and aortic sprouting. We used the Syk inhibitor piceatannol (31) and Syk shRNA plasmid designed against the human sequence for experiments in EA cells and siRNA Syk designed against the rat sequence for experiments in aortic rings. ANG II (10 nM), EGF (30 ng/ml), and VEGF (50 ng/ml) increased maximal Syk phosphorylation within 10–30 min (Fig. 6, A–C, respectively). ANG II-induced Syk phosphorylation in these cells was inhibited by AT1R (losartan) but not AT2R (PD-123319) blocker (Fig. 6D). ANG II receptor blockers did not alter EGF- or VEGF-induced Syk phosphorylation in EA cells (Fig. 6, E and F). ANG II-, EGF-, and VEGF-induced tube formation from EA cells and aortic sprouting was inhibited by piceatannol (5 μM; Fig. 7, A and B, respectively). ANG II-, EGF-, and VEGF-induced Syk phosphorylation but not Flt-1 in EA cells was inhibited by piceatannol (Fig. 7, C–E). The effect of ANG II, EGF, and VEGF to stimulate tube formation in EA cells and aortic sprouting in aortic rings was abolished after transient transfection with Syk shRNA but not its control shRNA and by Syk siRNA but not its scrambled siRNA, respectively (Fig. 8, A and B). Transient transfection of EA cells with Syk shRNA but not its control shRNA reduced Syk protein levels and ANG II-, EGF-, and VEGF-induced Flt-1 phosphorylation (Fig. 8, C–E). Syk phosphorylation elicited by ANG II, EGF, and VEGF was blocked by Flt-1/Fc but not by the Flk-1 blocker.
SU-1498 at 10 μM (Fig. 9, A–C) or 25 μM (data not shown) in EA cells. ANG II- and EGF- but not VEGF-induced Syk phosphorylation was inhibited by AG-1478 (Fig. 9, D–F). These data suggest that ANG II-, EGF-, and VEGF-induced Syk phosphorylation in EA cells is mediated via Flt-1 but not Flk-1 and that ANG II stimulates tube formation from EA cells and aortic sprouting via EGFR transactivation and Syk activation.

ANG II also stimulates tube formation from HUVECs via EGFR transactivation through Flt-1 phosphorylation and Syk activation. Studies conducted in EA cells and aortic sprouting were presented at the American Heart Association meeting in Chicago, IL, in 2009, and some of our colleagues questioned if the signaling pathway described for the ANG II-induced process of angiogenesis also occurs in HUVECs that are derived from EA cells. Therefore, we conducted several of the above experiments in HUVECs. ANG II-, EGF-, and VEGF-induced tube formation and Flt-1 phosphorylation were inhibited by Flt-1/Fc but not by SU-1498 (Fig. 10, A–D), and VEGF-induced Flk-1 phosphorylation was inhibited by SU-1498 in these cells (Fig. 10E). ANG II- and EGF-induced tube formation and EGFR and Flt-1 phosphorylation were inhibited by AG-1478 in HUVECs. AG-1478 did not alter VEGF-induced tube formation and Flt-1 phosphorylation, and VEGF did not affect EGFR phosphorylation (Fig. 11). ANG II-, EGF-, and VEGF-induced tube formation and Syk but not Flt-1 phosphorylation were inhibited by the Syk inhibitor piceatannol in HUVECs (Fig. 12, A–D). Syk shRNA but not control shRNA inhibited ANG II-, EGF-, and VEGF-induced tube formation but not Flt-1 phosphorylation in these cells (Fig. 12, E–H). ANG II- and EGF-induced Syk phosphorylation were inhibited by Flt-1/Fc and AG-1478 but not by SU-1498. VEGF-induced Syk phosphorylation was inhibited by Flt-1/Fc but not by SU-1498 and AG-1478 (Fig. 13). ANG II-induced tube formation and Syk phosphorylation in HUVECs were inhibited by AT1R (losartan) but not AT2R (PD-123319) blocker (data not shown).

Fig. 8. Syk short hairpin interfering (sh)RNA and Syk small interfering (si)RNA inhibit ANG II-, EGF-, and VEGF-induced tube formation and aortic sprouting, respectively. A and B: quiescent EA cells transfected with human Syk shRNA, its control shRNA, or vehicle and rat aortic rings transfected with rat Syk siRNA, its scrambled (SCR) control, or vehicle were then exposed to ANG II (10 nM), EGF (30 ng/ml), VEGF (50 ng/ml), or their vehicle for 16 h for tube formation (A) and 3–5 days for maximal rat aortic sprouting (B). The value of increase in tube formation and aortic sprouting obtained in the presence of the vehicle of ANG II, EGF, or VEGF was taken as 100% for angiogenesis assays. C–E: EA cells were transfected with human Syk shRNA, its control shRNA, or vehicle and exposed to ANG II (10 nM), EGF (30 ng/ml), VEGF (50 ng/ml) (+), or their vehicle (−) for 30 min. Cells were then exposed to ANG II, EGF, or VEGF for 30 min. Cells were lysed and subjected to SDS-PAGE and Western blot analysis, and blots were then probed with anti-p-Flt-1, anti-Flt-1, and anti-Syk antibodies. Bands were detected, and the density of bands was measured as described in EXPERIMENTAL PROCEDURES. Blots are representative of three to four experiments (C–E, top). The density of p-Flt bands was normalized to the quantity of Flt-1 and presented as the fold increase from the corresponding value obtained in the presence of the vehicle, which was taken as 1. Values are means ± SE. *Significantly different from the corresponding value obtained in the presence of the vehicle of ANG II, EGF, or VEGF; †significantly different from the corresponding value obtained in the presence of the vehicle of Syk shRNA (C–E, bottom) (P < 0.05).
**DISCUSSION**

This study demonstrates that ANG II promotes the process of angiogenesis via EGFR transactivation, resulting in the phosphorylation of Flt-1 and Syk activation independent of VEGF expression. ANG II in low concentrations (10 nM) stimulates the process of angiogenesis, as shown by increased EC proliferation and capillary tube formation from vascular ECs in vitro and ex vivo capillary sprouting from aortic rings via AT1R (2, 19, 28). ANG II stimulates VEGF expression in ECs (9, 16), and ANG II-induced HCAEC proliferation is inhibited by VEGF neutralizing antibody against Flt-1 (14). In the present study, we confirmed that ANG II-induced tube formation from EA cells and HUVECs and capillary sprouting from rat aortic rings are blocked by AT1R (losartan) but not AT2R (PD-123319) blocker (data not shown). Our finding that ANG II-, EGF-, and VEGF-induced tube formation from EA cells and HUVECs and aortic sprouting were inhibited by Flt-1 neutralizing antibody Flt-1/Fc, but not by Flk-1 blocker SU-1498, suggest that these effects of ANG II, EGF, and VEGF are mediated via the activation of Flt-1. Since SU-1498 at higher concentrations (25 μM) inhibited VEGF but not ANG II- and EGFR-induced tube formation from EA cells, it appears that VEGF stimulates tube formation also via Flk-1. ANG II also stimulates VEGF expression, and VEGF has been reported to stimulate angiogenesis mainly through Flt-1 (8). This suggests that VEGF might mediate ANG II-induced angiogenesis. In our study, ANG II also increased VEGF expression and caused the phosphorylation of Flt-1 but not Flk-1. Although we cannot exclude the involvement of VEGF in ANG II-induced tube formation from EA cells, it appears to be unlikely for the following reasons. First, in these cells, ANG II caused the phosphorylation of Flt-1 within 5 min, which reached a maximum at 4 h, whereas the expression of VEGF was slightly increased at 1 h and reached a maximum at 16 h. Second, ANG II caused the phosphorylation of Flt-1 but not Flk-1, whereas VEGF caused the phosphorylation of both Flt-1 and Flk-1. Finally, ANG II in high concentrations (100 nM) also increased VEGF expression in EA cells (data not shown), but its effect on tube formation was less than that at 10 nM.

ANG II stimulates the proliferation and migration of VSMC through EGFR transactivation (24), and heparin-binding EGF has been shown to be involved in neovascularization induced...
by ANG II (12). Our data showed that 1) EGF increases tubulogenesis from EA cells and HUVECs and aortic sprouting, 2) EGF and ANG II stimulated EGFR phosphorylation in EA cells and HUVECs, and 3) these effects of EGF and ANG II were inhibited by the EGFR blocker AG-1478, suggesting that ANG II-induced tubulogenesis and aortic sprouting are mediated through EGFR transactivation. Because 1) EGF-induced tubulogenesis from EA cells and HUVECs and aortic sprouting were blocked by VEGF neutralizing antibody Flt-1/Fc but not by the Flk-1 antagonist SU-1498; 2) EGF increased the phosphorylation of Flt-1 but not Flk-1, and it was inhibited by Flt-1/Fc but not SU-1498; and 3) VEGF-induced tubulogenesis and aortic sprouting as well as phosphorylation of Flt-1 and Flk-1 were not blocked by the EGFR blocker AG-1478, it appears that ANG II stimulates angiogenesis via EGFR transactivation by the phosphorylation of Flt-1 independent of VEGF expression.

To further elucidate the mechanism by which ANG II via EGFR transactivation stimulates the process of angiogenesis, we investigated the role of Syk, which is expressed in ECs and is involved in the morphogenesis, growth, and survival of ECs and contributes to the maintenance of vascular integrity in vivo (17, 39). Our findings that 1) ANG II, EGF, and VEGF increased Syk phosphorylation; 2) ANG II- but not EGF- and VEGF-induced Syk phosphorylation was blocked by AT1R (losartan) but not AT2R (PD-123319) blocker; and 3) the Syk inhibitor piceatannol blocked ANG II-, EGF-, and VEGF-induced tube formation and Syk phosphorylation in EA cells and HUVECs as well as capillary sprouting from aortic rings suggest that Syk mediates ANG II-induced angiogenesis. Further supporting this conclusion was our demonstration that transfection of EA cells with human Syk siRNA but not its control shRNA reduced tube formation and phosphorylation of Syk in EA cells and HUVECs, and transfection of aortic rings with rat Syk siRNA inhibited capillary sprouting in response to ANG II. Because 1) the Syk inhibitor piceatannol and Syk shRNA also blocked EGF- and VEGF-induced tubulogenesis and phosphorylation of Syk in EA cells and HUVECs; 2) piceatannol and Syk siRNA inhibited capillary sprouting of aortic rings stimulated by EGF and VEGF; 3) ANG II-induced Syk phosphorylation in EA cells and HUVECs was inhibited by AT1R (losartan) but not AT2R (PD-123319) blocker; 4) Flt-1/Fc but not SU-1498 blocked ANG II-, EGF-, and VEGF-induced Syk phosphorylation in EA cells and HUVECs; and 5) piceatannol or Syk shRNA did not alter EGF- and VEGF-induced phosphorylation of Syk in EA cells and HUVECs, and 6) VEGF-induced Syk phosphorylation in EA cells and HUVECs was not altered by the EGFR blocker AG-1478, it appears that the ANG II-induced process of angiogenesis is mediated via AT1R through EGFR transactivation, which promotes the phosphorylation of Flt-1 and activation of Syk. We (29) have previously reported that the effect of ANG II to stimulate VSMC proliferation is mediated via EGFR transactivation, which promotes the phosphorylation of Flt-1 and activation of Syk. Therefore, it appears that distinct signaling mechanisms are involved in Syk-dependent ANG II-induced protein synthesis in VSMCs and tubulogenesis in ECs.
or stimulate directly Flt-1, and other signaling molecules, including p38 MAPK, p44/42 MAPK, and PKC-δ, might be involved in the activation of Flt-1 by EGFR and/or of Syk by Flt-1. p38 MAPK is known to mediate transactivation of EGFR and EGFR activation, resulting in the activation of p38 MAPK and p44/42 MAPK in VSMCs (24), and both kinases have been shown to be involved in the process of angiogenesis (19). ANG II is also known to cause the activation of PKC isotypes, including PKC-δ, in VSMCs (25), and PKC-δ has been reported to mediate thrombin-induced ICAM-1 expression by increasing the transcriptional capacity of NF-κB via activation of Syk in HUVECs (4). Whether ANG II-induced activation of Syk by Flt-1 receptor and angiogenesis are mediated by PKC-δ or other isotypes of PKC (37) implicated in angiogenesis remains to be determined. Moreover, further studies are required to determine the relationship of VEGF-induced Flk-1 phosphorylation and the signaling mechanism of tube formation via Flk-1 by VEGF that is independent of Syk activation.

In conclusion, our study demonstrates (Fig. 14) that ANG II stimulates the process of angiogenesis, tube formation from EA cells and HUVECs, and capillary sprouting from rat aortic rings via transactivation of EGFR, which, in turn, promotes the phosphorylation of Flt-1, resulting in the activation of Syk, most likely independent of VEGF expression (ANG II → EGFR → Flt-1 → Syk).

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Fig. 14. Proposed mechanism of ANG II-induced angiogenesis. ANG II stimulates angiogenesis via transactivation of EGFR, which promotes the phosphorylation of Flt-1 and activation of Syk independent of VEGF expression. HB-EGF, heparin-binding EGF.


