A Novel Phosphoinositide 3-Kinase-dependent Pathway for Angiotensin II/AT-1 Receptor-mediated Induction of Collagen Synthesis in MES-13 Mesangial Cells*

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Chronic activation of the angiotensin II (ANG II) type 1 receptor (AT-1R) is critical in the development of chronic kidney disease. ANG II activates mesangial cells (MCs) and stimulates the synthesis of extracellular matrix components. To determine the molecular mechanisms underlying the induction of MC collagen, a mouse mesangial cell line MES-13 was employed. ANG II treatment induced an increase in collagen synthesis, which was abrogated by cotreatment with losartan (an AT-1R antagonist), wortmannin (a phosphoinositide 3-kinase (PI3K) inhibitor), an Akt inhibitor, and stable transfection of dominant negative-Akt1. ANG II induced phosphorylation of Akt and p70S6K. This effect was inhibited by cotreatment with PP2, an Src inhibitor, or AG1478, an epidermal growth factor receptor (EGFR) antagonist. The Epac (exchange protein directly activated by cAMP)-specific cAMP analog, 8-pHPT-2'-O-Me-cAMP, significantly increased PI3K activity, whereas a PKA-specific analog, 6-benzoyladenosine-cAMP, showed no effect. The ANG II-induced increase in PI3K activity was also blocked by cotreatment with PP2, an Src inhibitor, or AG1478, an epidermal growth factor receptor (EGFR) antagonist. ANG II induced phosphorylation of Akt and p70S6K and EGFR, which was abrogated by knockdown of c-Src by small interference RNA. Knockdown of Src also effectively abolished ANG II-induced collagen synthesis. Conversely, stable transfection of a constitutively active Src mutant enhanced basal PI3K activity and collagen production, which was abrogated by AG1478 but not by 2',5'-dideoxyadenosine (2',5'-DOA). Moreover, acute treatment with ANG II significantly increased Src activity, which was abrogated with cotreatment of 2',5'-DOA. Taken together, these results suggest that ANG II induces collagen synthesis in MCs by activating the ANG II/AT-1R-EGFR-PI3K pathway. This transactivation is dependent on cAMP/Epac but not on PKA. Src kinase plays a pivotal role in this signaling pathway between cAMP and EGFR. This is the first demonstration that an AT1R-PI3K/Akt crosstalk, along with transactivation of EGFR, mediates ANG II-induced collagen synthesis in MCs.

Clinical and experimental studies have identified the renin-angiotensin system as a key factor in development and progression of glomerular diseases (1–3). A chronically enhanced effect of the renin-angiotensin system is a central event in the development of proliferative and sclerosing changes in the glomeruli. The octapeptide hormone angiotensin II (ANG II),2 the major renin-angiotensin system effector, activates mesangial cells (MCs) and increases the synthesis of extracellular matrix components, including collagens, which is a hallmark of the glomerular diseases (4, 5). The physiological functions of ANG II are mediated by at least two structurally and pharmacologically distinct seven-transmembrane helices G protein-coupled receptors (GPCRs), ANG II type 1 and 2 receptors (AT-1R and AT-2R). Glomerular cells, including MCs, express primarily the AT-1R, which mediates most of the known effects of ANG II (6, 7). Pharmacological blockade of the renin-angiotensin system by angiotensin-converting enzyme inhibitors or AT-1R antagonists attenuates development and progression of glomerular diseases (8–10). Although the effects of these agents on progression in the glomerular diseases have been studied extensively, detailed cellular mechanisms of the effects have not been fully revealed.

Phosphoinositide 3-kinases (PI3Ks) phosphorylate inositol-containing lipids at the D-3 position of the inositol ring (11). The mammalian class I PI3K can be divided into three major classes (classes I–III) based upon their structure and substrate specificity (12). The mammalian class I PI3K can be further divided into two subclasses: class IA PI3K are heterodimers of a 110-kDa catalytic subunit (p110α) or p110β, and a regulatory subunit of 85 or 55 kDa (p85α/p55), whereas the class IB...

2 The abbreviations used are: ANG II, angiotensin II; MC, mesangial cell; GPCR, G protein-coupled receptor; AT-1R, -2R, ANG II type 1 and 2 receptors; PI3K, phosphoinositide 3-kinase; PtdIns, phosphoinositide; EGFR, epidermal growth factor receptor; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 8-pCPT-2'-O-Me-cAMP, 8-(4-chlorophenylthio)-2'-O-Me-cAMP, 8-(4-hydroxyphenylthio)-2'-O-Me-cAMP, 8-(4-hydroxyphenylthio)-2'-O-Metadenosine-3',5'-cyclic monophosphate; 8-(4-chlorophenylthio)-2'-O-Metadenosine-3',5'-cyclic monophosphate; siRNA, small interference RNA; 2',5'-DOA, 2',5'-dideoxyadenosine; PKA, protein kinase A; CaMK, Ca2+/calmodulin-dependent protein kinase; Epac, exchange protein directly activated by cAMP; HB-EGF, heparin-binding EGFR-like growth factor; 1,10-PTL, 1,10-phenanthroline.

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PI3K (PI3Kγ) is composed of a p110γ catalytic subunit and a p101 regulatory subunit (11). The class I PI3Ks can phosphorylate phosphoinositide (PtdIns), PtdIns 4-phosphate, and PtdIns 4,5-bisphosphate in vitro. In the cells, however, class I PI3Ks preferentially convert PtdIns 4,5-bisphosphate to PtdIns 3,4,5-trisphosphate following stimulation by tyrosine kinase or PI3Ks. The p101 regulatory subunit (11). The class I PI3Ks can phosphorylate phosphoinositide-dependent kinase-1. This is followed by phosphorylation at threonine 308 by phosphoinositide-dependent kinase-1. This is coupled to GPCRs (11). This is best represented by studies performed in leukocytes (14, 15). We have recently demonstrated, however, that cardiac PI3Ks can be activated by stimulation of β-adrenergic receptor in vivo (16). It is unclear which isoform of class I PI3Ks is functionally linked to AT-1R in MCs.

Following PI3K activation, PtdIns 3,4,5-trisphosphate recruits and phosphorylates the phosphoinositide-dependent kinase-1 and Akt/protein kinase B, bringing these proteins into proximity at the plasma membrane where Akt is phosphorylated on threonine 308 by phosphoinositide-dependent kinase-1. This is followed by phosphorylation at serine 473 by a yet-to-be-identified mechanism (17). Growing evidence indicates that ANG II/AT-1R signaling induces a crosstalk to the PI3K/Akt pathway (18–21), and a few studies have shown that these changes in PI3K/Akt activities demonstrated altered phenotypes in collagen synthesis of various types of cells, including MCs (22–25). These findings suggested that ANG II/AT-1R signaling is implicated in the development and progression of various types of glomerular diseases. However, the detailed mechanisms of ANG II-induced collagen synthesis in MCs and a role of ANG II in the signaling pathway have not been fully elucidated. To explore these changes in PI3K/Akt activities and their crosstalk to the PI3K/Akt pathway (18–21), and a few studies have shown that these changes in PI3K/Akt activities demonstrated altered phenotypes in collagen synthesis of various types of cells, including MCs (22–25). These findings suggested that ANG II/AT-1R signaling is implicated in the development and progression of various types of glomerular diseases. However, the detailed mechanisms of ANG II-induced collagen synthesis in MCs and a role of ANG II in the signaling pathway have not been fully elucidated. To explore the ANG II/AT-1R to PI3K/Akt pathway in these cells, we employed an in vitro culture system by using the well characterized mouse mesangial cell line, MES-13. With pharmacological and genetic signal modification studies we demonstrated that several factors such as cAMP, Src kinase, and epidermal growth factor receptor (EGFR) play critical roles in the pathway.

EXPERIMENTAL PROCEDURES

Materials—All chemicals and reagents were obtained from Sigma unless stated otherwise.

Cell Culture—MES-13 mouse mesangial cells (ATCC® no. CRL-1927) were grown in a 3:1 mixture of Dulbecco’s modified Eagle’s and Ham’s F-12 media (Invitrogen) supplemented with 5% (v/v) fetal bovine serum containing 50 units/ml penicillin G and 50 μg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells were first grown up to 70% confluence and synchronized overnight in serum-free medium prior to treatment. For immunoprecipitation and PI3K assay, the quiescent cells were treated with vehicle or ANG II (0.1 μM) for various durations. In some experiments cells were pretreated with selective inhibitors for indicated durations before ANG II treatment.

PI3K Assays—MES-13 cell lysates were prepared as described (26), and protein concentrations were determined with the bicinchoninic acid assay (Pierce). PI3K activity was determined with in vitro immunoprecipitation lipid kinase assay as described previously (26). Briefly, cell lysates (0.5 mg) were immunoprecipitated with anti-phosphotyrosine (anti-pY) antibody (Upstate, Charlotte, VA), and i-α-phosphoinositide (Avanti Polar Lipids, Alabaster, AL) was used as the lipid substrate (2 μg/reaction). After incubation, the final extracted reaction mixtures were spotted onto silica gel-coated TLC plates (Whatman, Florham Park, NJ) and run in TLC buffer (65% n-propanol, 0.54 M acetic acid). The results were analyzed by phosphorimaging (Bio-Rad).

Immunoprecipitation and Western Blotting—For immunoprecipitation, MES-13 cells were treated with ANG II (0.1 μM) for 1–30 min. Protein lysates (0.5 mg) were incubated with protein G-Sepharose beads (GE Healthcare, Piscataway, NJ) for 2 h at 4 °C. After centrifugation, the supernatant was transferred to a fresh tube and incubated with an antibody specific for EGFR (1005, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 4 h with continuous rotation. A 20-μl packed volume of protein G-Sepharose was added, and the mixture was incubated overnight at 4 °C. After washing, 45 μl of 2× Laemmli sample buffer was added. The sample was heated in boiling water for 5 min and quenched on ice for 2 min. After vortex and centrifuge, 20 μl of the supernatant was resolved on a 7.5% SDS-PAGE gel and immunoblotted with anti-pY (Santa Cruz Biotechnology). The blot was stripped and re-blotted with anti-EGFR. Collagen expressions were measured in cells treated with ANG II (0.1 μM) for 24 h by Western blotting using anti-procollagen type I (Y-18, Santa Cruz Biotechnology) and normalized with β-actin levels. Protein phosphorylation of Akt and p70S6K was measured in cells treated with ANG II (0.1 μM) for 1–30 min by Western blotting using phospho-specific antibodies against Akt (Thr-308) and p70S6K (Thr-389) and normalized with total Akt and p70S6K (Cell Signaling Technology, Beverly, MA) as previously described (26). The results were visualized by ECL chemiluminescence (Pierce).

Stable Transfection—A vector expressing dominant negative Akt1 was engineered by inserting a K179M mutant dominant negative Akt1 cDNA (27) into a multiple cloning site of an eukaryotic expression vector, pUSEamp (+) (Upstate, Charlotte, VA). A 529F mutant constitutively active Src cDNA (28) was processed similarly to engineer a vector expressing constitutively active Src. Stable transfection of the constructs in MES-13 cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Individual single cells were isolated and screened for neomycin resistance. The stably transfected cells were treated similarly as described above.

RT-PCR—Total RNA was isolated from MES-13 cells using TRIzol reagent (Invitrogen). Reverse transcription was carried out on MES-13 cell total RNA followed by PCR using the following primers pairs: collagen, type I, α1 (Coll-1α1) sense, 5’-CCT GGT AAC ACT GGT CCT CCG TGG 3’; Coll-1α1 antisense, 5’-ATC TTC ACC ACC CTT GCC ACC TTG-3’; collagen, type I, α2 (Coll-1α2) sense, 5’-GAC ATT GGT GGT GAC ACC-3’; Coll-1α2 antisense, 5’-GGA AAC CCG AAA GAG GAA G-3’; collagen, type III, α1 (Coll-3α1) sense, 5’-GTT CTA GAG GAT GGC TTG ACT AAA CAC ACC A-3’; Coll-3α1 antisense, 5’-TTG CCT TGC GTG TTT GAT ATT C-3’; collagen, type IV, α1
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(5′-TGG ACA GAA AGG TCA GAA AG-3′; Coll-4α1 antisense, 5′-TAC CAG GGA AGC CAA CTC-3′; c-Src sense, 5′-GCT ACA GAC GAT AGG AAA GG-3′; c-Src antisense, 5′-CTC CAC ACA TCA GAC TTG-3′; GAPDH sense, 5′-TGG CAA AGT GGA GAT TGT TG-3′; and GAPDH antisense, 5′-CTT CTG GGT GCC AGT GAT G-3′).

RESULTS

ANG II Induces Increases in Phosphotyrosine-associated PI3K Activity and Phosphorylation of p70S6K and Akt through AT-1R—In MES-13 cells, acute treatment with ANG II (0.1 μM) induced a significant increase in phosphotyrosine-associated PI3K activity (Fig. 1A). The increases in PI3K activity were seen because co-treatment with losartan (1 μM, Merck & Co., Inc, Whitehouse Station, NJ), an AT-1R antagonist, abrogated the effect of ANG II, whereas co-treatment with PD123319 (1 μM), an AT-2R antagonist, had no effect (Fig. 2). To assess if the downstream signaling molecules in the PI3K signaling pathway are similarly affected by ANG II, MES-13 cells were treated with ANG II for 1, 2, 5, 15, or 30 min, and phosphorylation of Akt and p70S6K was examined by Western blotting. There was a significant increase in Akt phosphorylation because co-treatment with losartan (1 μM, Merck & Co., Inc, Whitehouse Station, NJ), an AT-1R antagonist, abrogated the effect of ANG II, whereas co-treatment with PD123319 (1 μM), an AT-2R antagonist, had no effect (Fig. 2). To assess if the downstream signaling molecules in the PI3K signaling pathway are similarly affected by ANG II, MES-13 cells were treated with ANG II for 1, 2, 5, 15, or 30 min, and phosphorylation of Akt and p70S6K was examined by Western blotting. There was a significant increase in Akt phosphorylation...
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**FIGURE 2.** The effect of ANG II on PI3K activation is mediated by AT-1 receptor. MES-13 mesangial cells were stimulated with vehicle, ANG II (0.1 μM) alone, ANG II in combination with losartan (1 μM, an AT-1R-specific antagonist), or ANG II in combination with PD123319 (1 μM, an AT-2R-specific antagonist) for 2 min. The antagonists were added 1 h before ANG II treatment. Cell lysates were immunoprecipitated with an antibody specific for phosphotyrosine, and PI3K activity was determined with an in vitro lipid kinase assay. PIP (phosphoinositide 3-phosphate), the phosphorylated end-product. The bar graph shows the densitometric scanning results from three individual experiments. Data are expressed as means ± S.E. of percent change in PI3K activity relative to that of vehicle control. *, p < 0.05 versus vehicle control.

**FIGURE 3.** ANG II induces increases in phosphorylation of Akt and p70S6K. MES-13 mesangial cells were treated with vehicle or ANG II (0.1 μM) for the indicated durations. Western blotting was performed on cell lysates (30 μg/lane) with antibodies against phospho-Akt (Thr-308) and total Akt (A) or antibodies against phospho-p70S6K (Thr-389) and total p70S6K (B). The bar graphs show the densitometric scanning results from three individual experiments. Data are normalized with individual total protein levels and represent means ± S.E. of percent change in protein phosphorylation relative to that of vehicle control. *, p < 0.05 versus vehicle control.

(threonine 308) 1 min after ANG II treatment. The effect was peaked at 2 min then tapered down gradually thereafter (Fig. 3A). The phosphorylation of p70S6K (threonine 389) was also significantly increased with the peak effects seen at 2 min after ANG II treatment (Fig. 3B). These results demonstrate that acute AT-1R stimulation induces activation of PI3K and the downstream signaling pathway in MES-13 cells.

**AT-1R Stimulation-induced Type I Collagen Synthesis in MES-13 Cells Is Dependent on PI3K Signaling Pathway**—As mentioned above, a hallmark of the glomerular diseases is an increase in the synthesis of extracellular matrix components, including collagen. ANG II has been shown to increase the synthesis of collagen. We next examine what subtypes of collagen are affected by acute ANG II treatment. First, quiescent MES-13 cells were treated with ANG II under serum-free condition for various durations (30, 60, 90, 120, 240, and 360 min), and the gene levels of four subtypes of collagen (Coll-1α1, Coll-1α2, Coll-3α1, and Coll-4α1) were determined by RT-PCR. The quiescent cells expressed only trace amounts of collagen genes. ANG II induced a significant increase in Coll-1α1 and Coll-1α2 gene levels 90 min after initiation of treatment, and the effect was still evident after 6 h (Fig. 4A). In contrast, neither of the Coll-3α1 and Coll-4α1 gene levels was affected by ANG II.

To further examine if ANG II treatment can increase collagen protein levels and to determine if this effect is mediated by AT-1R, quiescent MES-13 cells were treated with vehicle, ANG II (0.1 μM), or ANG II in combination with an inhibitor for PI3K or AT-1R for 24 h. Western blotting results demonstrated a significant increase in the protein levels of procollagen type I in ANG II-treated cells (Fig. 4B). This effect was completely abrogated by coinuberation with either a PI3K inhibitor (wortmannin, 20 nm) or an AT-1R antagonist (losartan, 1 μM) (Fig. 4B). These results demonstrate an AT-1R-mediated, PI3K-dependent increase in collagen synthesis in MES-13 cells.

As shown in Fig. 3B, Akt phosphorylation was significantly increased by ANG II treatment. To further determine the role of Akt in the ANG II-mediated increase in collagen synthesis, MES-13 mesangial cells were stably transfected with an empty vector (pUSEamp(+)) or the vector expressing a dominant negative Akt1. As seen in nontransfected cells (Fig. 4B), ANG II...
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FIGURE 4. AT-1R-induced type I collagen synthesis in MES-13 mesangial cells is dependent on PI3K signaling pathway. A, cells were treated with saline vehicle or ANG II (0.1 μM) for the indicated durations in serum-free condition. RT-PCR for the four subtypes of collagen and GAPDH genes was performed using total RNA from the cell lysates. The pictures shown are representative images from triplicate experiments. B, cell were treated with vehicle, ANG II alone, ANG II in combination with wortmannin (20 nM), or ANG II in combination with losartan (1 μM) for 24 h. Cell lysates were subjected to Western blotting using anti-procollagen type I antibody. The bar graphs show the densitometric scanning results from three individual experiments.

Data were normalized with β-actin levels and represent means ± S.E. of percent change in protein levels relative to that of vehicle control. *, p < 0.05 versus vehicle control. C, cells were stably transfected with pUSEamp(+) plasmid or pUSEamp(−) plasmid or pUSEamp(+) containing a dominant negative-Akt1 (dominant negative Akt1) cDNA. Cells transfected with pUSEamp(+) plasmid were treated with vehicle, ANG II, or Akt inhibitor (10 μM), and cells transfected with dominantnegative Akt1 cDNA were treated with vehicle or ANG II for 24 h. Measurements of procollagen type I and β-actin were as described in B.

Treatments induced a significant increase in procollagen type I levels in cells stably transfected with pUSEamp(+) (Fig. 4C). This effect of ANG II, however, was abrogated when the same pUSEamp(+) transfected cells were co-treated with an Akt inhibitor (10 μM, Calbiochem). In cells stably transfected with dominant negative Akt1, the effect of ANG II on collagen synthesis was also abrogated (Fig. 4C). Taken together, these data suggest that the PI3K signaling pathway plays an important role in the AT-1R-mediated activation of collagen gene expression in MES-13 cells.

ANG II-induced Increase in PI3K Activity Requires the Involvement of Adenylyl Cyclase, Src Family Tyrosine Kinases, and EGFR Transactivation—To investigate the intracellular signaling event responsible for ANG II/AT-1R-mediated activation of PI3K, MES-13 cells were treated with vehicle, ANG II alone, or ANG II in combination with activators or inhibitors of several signaling pathways. The effect of acute ANG II treatment on activation of PI3K activity was abolished by co-treatment of 2′,5′-dideoxyadenosine (100 μM; 2′,5′-DOA), an adenylyl cyclase inhibitor (Fig. 5A). Co-treatment with H-89 (10 μM), a cAMP-dependent kinases (PKA) inhibitor, however, failed to diminish the effect of ANG II on PI3K activity. Treatment with forskolin alone (1 μM, 5 min), an adenylyl cyclase activator, also increased PI3K activity in MES-13 cells (Fig. 5A). These results suggest that a PKA-independent, Gαs-cAMP pathway is involved in the ANG II/AT-1R-mediated increase in PI3K activity in MES-13 cells.

The Ca2+/calmodulin-dependent protein kinases (CaMKs) play important roles in controlling a variety of cellular functions in response to increases in intracellular Ca2+ and has been shown to be a factor in ANG II-induced signaling cascade (49). To explore the role of CaMK in ANG II-induced activation of PI3K, MES-13 cells were treated with the vehicle, ANG II alone, ANG II in combination with KN93 (5 μM, Calbiochem), the specific CaMK inhibitor, or ANG II in combination with KN92 (5 μM, Calbiochem), an analog of KN93 that does not affect CaMK activity. The effects of ANG II/AT-1R stimulation on PI3K activation were not significantly changed by either KN93 or KN92 (Fig. 5B). The result suggests that the cAMP-CaMK pathway is not involved in the ANG II/AT-1R-PI3K crosstalk in the MES-13 cells.

Cells were further treated with vehicle, ANG II, cAMP (1 mM, Upstate), 8-(4-hydroxyphenylthio)-2′,5′-O-methylenadenosine-3′,5′-cyclic monophosphate (8-pHPT-2′,5′-Me-cAMP, 200 μM, BIOLOG LSI, Bremen, Germany), a potent specific activator of the exchange protein directly activated by cAMP (Epac) or &-benzoyladenosine-cAMP (300 μM, BIOLOG LSI), a PKA-specific activator, for 2 min, and PI3K activity was assayed. As with ANG II, 8-pHPT-2′-O-Me-cAMP as well as cAMP induced significant increases in PI3K activity, whereas...
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We next examined other signaling pathways that may be involved in ANG II-at 1R-mediated increase in PI3K activity in MES-13 cells.

Next, we treated with GF109203X (10 M, Tocris Bioscience, Ellisville, MO), an inhibitor for PKC, or farnesylthiosalicylic acid (20 M, Calbiochem), an inhibitor for the Src-family tyrosine kinases, completely abolished the effect of ANG II on PI3K activity. In contrast, co-treatment with PP2 (10 M), an inhibitor for Ras, did not significantly alter the effect of ANG II (Fig. 6A). Moreover, co-treatment with an inhibitor for platelet-derived growth factor receptor (AG1296, 10 M, Calbiochem) or insulin-like growth factor-1 receptor (AG1024, 1 M, Calbiochem) did not alter the effect of ANG II, whereas co-treatment with an inhibitor of EGFR (AG1478, 5 M/g/ml) fully abrogated the effect of ANG II (Fig. 6B). The crosstalk between AT-1R and EGFR was further established in immunoprecipitation experiments. Quiescent MES-13 cells were treated with ANG II for 1–30 min, and cell lysates were subjected to immunoprecipitation with an antibody against EGFR (Santa Cruz Biotechnology), followed by immunoblotting using an antibody specific for EGFR or phosphotyrosine (Santa Cruz Biotechnology). In control cells, the levels of EGFR phosphorylation were only traceable. Acute ANG II treatment significantly increased tyrosine phosphorylation of EGFR (Fig. 7). This effect peaked at 2 min and disappeared at 15 min after ANG II treatment. These data establish EGFR as an important signaling player in AT-1R-mediated signaling pathway in MES-13 cells.

Metalloproteinases have been shown to play important roles in the conversion of heparin-binding EGF-like growth factor (HB-EGF) to activate EGFR. To determine if HB-EGF is involved in ANG II-induced activation of PI3K, quiescent MES-13 cells were preincubated with a nonspecific matrix metalloproteinase inhibitor, 1,10-phenanthroline (1,10-PTL, 300 M) or heparin (10 or 100 M/g/ml), which inhibits the HB-EGF activity (29), for 30 min before acute treatment with ANG II. Neither 1,10-PTL nor heparin altered the outcome of ANG II-induced increases in PI3K activity (Fig. 8A) or EGFR phosphorylation (Fig. 8B). The results suggest that HB-EGF is not a factor in the AT-1R/EGFR/PI3K crosstalk in MES-13 cells.

c-Src Is Critical for AT-1R-mediated Induction of Collagen Synthesis and Functions Upstream of EGFR and PI3K—We have shown that pharmacological inhibition of Src-family tyrosine kinases by PP2 effectively abolished the ANG II-mediated increases in PI3K activity (Fig. 6A) and that EGFR plays an important role in AT-1R-mediated signaling in MES-13 cells (Figs. 6B and 7). To strengthen these data, we next investigated if acute ANG II treatment can alter Src activity. Acute stimulation of ANG II/AT-1 (0.1 M for 2 min) induced a significant increase in Src activity in MES-13 cells, and the effect was totally abolished by pretreatment of the cells with 2',5'-cAMP (100 M) for 1 h (Fig. 9). These results provide strong evidence...
To further establish the critical role of Src in the AT-1R-PI3K signaling pathway and to determine if EGFR functions downstream of Src in this crosstalk, we applied the following two different strategies. First, the Src gene was knocked down in MES-13 cells by stable transfection of a mouse c-Src siRNA. MES-13 cells transfected with a scrambled oligonucleotide were used as a control. Knockdown of c-Src at the gene and protein levels in the c-Src siRNA-transfected cells was confirmed with RT-PCR and Western blotting, respectively. After 25 PCR reaction cycles, there was visible c-Src transcript of predicted size (522 bp) in the scrambled oligonucleotide-transfected cells but not the c-Src siRNA-transfected cells (Fig. 10A). The gene levels of GAPDH were similar between these two transfected cells. Western blotting using an antibody specific for c-Src (N-16, Santa Cruz Biotechnology) showed a significant down-regulation of c-Src protein level in the c-Src siRNA-transfected cells. The protein levels of \( \alpha \)-H3252-actin were not different between these cells (Fig. 10A). These transfected cells were treated with ANG II for 2–15 min, and the cell lysates were immunoprecipitated with an antibody against EGFR (Santa Cruz Biotechnology), followed by immunoblotting using anti-EGFR or an antibody specific for phosphotyrosine (Santa Cruz Biotechnology). As seen earlier in non-transfected MES-13 cells (Fig. 7), in cells transfected with a scrambled oligonucleotide tyrosine phosphorylation of EGFR was induced 2 min after ANG II treatment (Fig. 10B). In contrast, Src knockdown by siRNA completely abrogated the effect of ANG II. Second, MES-13 cells were stably transfected with an empty vector (pUSEamp(+) or pUSEamp(−)) containing a constitutively for ANG II-induced, cAMP-dependent activation of Src in MES-13 cells.
active Src cDNA (Y529F mutant). As expected, cells transfected with the empty vector had a significant increase in PI3K activity after acute treatment with ANG II (Fig. 11). The basal PI3K activity in the constitutively active Src-transfected cells was about 7- and 2-fold that of the non-treated and ANG II-treated empty vector-transfected cells, respectively. This high basal PI3K activity was, however, greatly reduced by treatment with an EGFR inhibitor (AG1478) but not by an adenylyl cyclase inhibitor (2',5'-O-[P(O)]-DOA) (Fig. 11). We have shown that Src functions downstream of cAMP (Fig. 9). These results further establish that Src is a critical factor in AT-1R-mediated activation of PI3K by functioning downstream of adenylyl cyclase and upstream of the EGFR.

To examine if Src is also critical in AT-1R-mediated increases in collagen synthesis, the c-Src siRNA or scrambled oligonucleotide-transfected cells were cultured with or without ANG II for 24 h, and type I collagen products were evaluated by Western blotting as described previously. As shown in Fig. 12A, ANG II induced type I collagen production in scrambled oligonucleotide-transfected control cells. In contrast, acute ANG II treatment failed to induce synthesis of collagen in c-Src siRNA-transfected MES-13 cells. Treatment with either ANG II alone or an adenylyl cyclase activator (forskolin) alone significantly increased the protein levels of procollagen type I (Fig. 12B). Co-treatment with PP2 abolished the effect of forskolin. In cells stably transfected with the constitutively active Src mutant, the basal levels of procollagen were 5-fold that of cells transfected

![FIGURE 8. ANG II-induced increases in PI3K activity and EGFR phosphorylation are HB-EGF-independent. A, MES-13 mesangial cells were treated for 2 min with vehicle, ANG II (0.1 μM) alone, or ANG II plus pretreatment for 30 min with 1,10-PTL (300 μM) or heparin (100 μg/mL). Cell lysates were subjected to PI3K assay as described. PIP (phosphoinositide 3-phosphate), the phosphorylated end-product. The bar graphs show the densitometric scanning results from three individual experiments. Data represent means ± S.E. of PI3K activity relative to that of vehicle control. *, p < 0.05 versus vehicle control. B, MES-13 mesangial cells were treated for 2 min with vehicle, ANG II (0.1 μM) alone, or ANG II plus pretreatment for 30 min with 1,10-PTL (300 μM) or heparin (100 μg/mL). Cell lysates were immunoprecipitated with an antibody specific for EGFR and immunoblotted with an antibody specific for phosphotyrosine. The same membrane was stripped and re-blotted with an anti-EGFR antibody for a loading control. The bar graphs show the densitometric scanning results from three individual experiments. Data are normalized with the levels of EGFR and represent means ± S.E. of percent change in tyrosine phosphorylation relative to that of vehicle control. *, p < 0.05 versus vehicle control.]

![FIGURE 9. ANG II induces Src activity in MES-13 mesangial cells. Cells were treated with saline vehicle or ANG II (0.1 μM) alone for 2 min. Some sets of cells were preincubated with 2',5'-DOA (100 μM) for 1 h and then treated with saline vehicle or ANG II (0.1 μM) for 2 min. Cell lysates (1 mg) were immunoprecipitated with anti-c-Src antibody. Kinase activity was determined by measuring [γ-32P]-labeled phosphorylation of a specific substrate (KVEKIGEGTVGVYK). The bar graphs show the average CPM measurements from three individual experiments. Data represent means ± S.E. of CPM. *, p < 0.05 versus all other groups.]

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with the empty vector (Fig. 12B). The enhanced collagen levels disappeared when cells were treated with AG1478 but not with 2',5'-DOA. These findings demonstrate Src as a critical signaling molecule in AT-1R-PI3K-mediated increase in collagen synthesis in MES-13 cells by acting between cAMP and EGFR.

FIGURE 10. c-Src knockdown by siRNA abolishes ANG II-mediated induction of EGFR phosphorylation in MES-13 mesangial cells. A, cells were stably transfected with pScilencer™ 5.1-H1 Retro vector ligated with a c-Src siRNA or scrambled oligonucleotides as described under “Experimental Procedures.” A, c-Src knockdown was confirmation with RT-PCR and Western blotting. GAPDH and β-actin were used as the loading control in PCR and Western blotting, respectively. B, cells were treated with vehicle or ANG II (0.1 μM) for the indicated durations. Tyrosine phosphorylation of EGFR was detected with immunoprecipitation and Western blotting as described in Fig. 7 legend. The bar graphs show the densitometric scanning results from three individual experiments. Data represent means ± S.E. of percent change in protein phosphorylation relative to that of individual control. *, p < 0.05 versus individual control.

FIGURE 11. Src-dependent increase in PI3K activity is abolished by EGFR inhibition but not by adenylyl cyclase inhibition. MES-13 mesangial cells were stably transfected with an empty vector (pUSEamp(+)) or a constitutively active Src cDNA (YS29F mutant). The pUSEamp(+)-transfected cells were treated with vehicle or ANG II (0.1 μM) for 2 min. The constitutively active Src-transfected cells were treated with vehicle, 2',5'-DOA (100 μM), or AG1428 (5 μg/ml) for 2 min. Cell lysates were subjected to PI3K assay as described. The bar graphs show the densitometric scanning from three individual experiments. Data represent means ± S.E. of percent change in PI3K activity relative to that of individual control. *, p < 0.05 versus pUSEamp(+), **, p < 0.05 versus Y529F mutant control.

DISCUSSION

Regardless of etiology, most end-stage glomerular diseases are characterized by accumulation of extracellular matrix proteins, including collagens, in mesangium and other areas in glomeruli. The mechanisms underlying this abnormal accumulation of extracellular matrix have not been fully elucidated. ANG II has been implicated in the development of chronic progressive glomerular diseases, and the precise mechanism of this effect has been a subject to be investigated clinically and experimentally. It has been suggested that ANG II induces glomerular injuries through renal hemodynamic effects and stimulation of growth and extracellular matrix production by glomerular cells (30). Several studies on this subject have been published (4, 31–33), and several factors such as TGF-β and endothelin-1 have been identified as crucial components in the mechanism, but the detailed signaling mechanism responsible for the effects are largely unknown. PI3K, in the mesangial context, has been reported to induce proliferation (34), enhance collagen expression (24), and prevent apoptosis (36, 37).

During the last decade, crosstalk between ANGII/AT-1R and PI3K has been demonstrated in vascular smooth muscle cells, cardiomyocytes, brain neurons, and choroideremia cells (38–42). In the present study, we set out to delineate the sig-
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FIGURE 13. A novel signaling pathway model for ANG II-induced collagen synthesis in MES-13 mesangial cells. Stimulation of AT-1R by ANG II leads to sequential activation of adenyl cyclase/cAMP/Epac, Src, and EGFR and results in transactivation of PI3K signaling pathway. Activation of PI3K and its downstream kinases, including Akt and p70S6K, results in increased collagen synthesis.

FIGURE 12. The ANG II-induced Src-dependent increase in type I collagen synthesis is abolished by EGFR inhibition but not by adenyl cyclase inhibition. A, MES-13 mesangial cells were stably transfected with a c-Src siRNA or scrambled oligonucleotides. The cells were treated with vehicle or ANG II (0.1 μM) 24 h. Cell lysates were subjected to Western blotting using anti-procollagen type I antibody. The bar graphs show the densitometric scanning results from three individual experiments. Data were normalized with β-actin levels and represent means ± S.E. of percent change in protein levels relative to that of vehicle control. *, p < 0.05 versus vehicle control. B, cells were stably transfected with an empty vector (pUSEamp(+)) or a constitutively active Src cDNA (Y529F mutant). The pUSEamp(+)-transfected cells were treated with vehicle, ANG II (0.1 μM) alone, forskolin (1 μM) alone, or forskolin in combination with PP2 (10 μM) for 24 h. The constitutively active Src-transfected cells were treated with vehicle, 2′,5′-DOA (100 μM), or AG1428 (5 μg/ml) for 24 h. Cell lysates were subjected to Western blotting using an antibody against procollagen type I or β-actin. The bar graphs show the densitometric scanning results from three individual experiments. Data are normalized with β-actin levels and represent means ± S.E. of percent change in protein levels relative to that of pUSEamp(+)-control control. *, p < 0.05 versus pUSEamp(+)-control. **, p < 0.05 versus Y529F control.

In response to binding of GPCR to its ligand, the heterotrimeric G protein complex separates into two subunits, Gs and Gαi, with Gs protein, one of the four subfamilies of Gα protein, being activated by hormone receptors, odor receptors, and taste receptors. Gαs-mediated signaling involves adenyl cyclase in all known cases (43). In the present study, we demonstrated that inhibition of adenyl cyclase by 2′,5′-DOA abolished ANG II-induced increase in PI3K activity, whereas H89, a PKA-specific inhibitor, showed no significant effects. These results suggest that ANG II-induced PI3K activation in MES-13 cell is mediated by cAMP and does not require the involvement of PKA. Starting from the 70s and until a few years ago, it was believed that most cAMP effects were mediated by the activation of PKA. Recently, it has become increasingly apparent that PKA are not the only intracellular receptors involved in cAMP signaling in eukaryote (44–46). Multiple investigations have identified novel cAMP-dependent but PKA-independent signaling pathways in the last several years (47–49). In addition, novel cAMP analogs such as 8-pHPT-2′-O-Me-cAMP and 8-pCPT-2′-O-Me-cAMP, have been experimentally tested and shown to activate downstream effectors such as CaMK and Epac, but not PKA (35, 48, 50). We used CaMK inhibitor KN93 and Epac-
specific cAMP analog 8-pHPT-2’-O-Me-cAMP to evaluate the involvement of these two factors and found that the Epac but not CaMK was playing critical roles in the ANG II/AT-1R transactivation of PI3K. Our study is the first to demonstrate such a PKA-independent effect of cAMP in a mesangial context.

Activation of AT-1R induces tyrosine phosphorylation and stimulates various downstream kinases, leading to cell proliferation (51, 52). How the AT-1R, which lacks intrinsic tyrosine kinase activity, induces these effects is not fully elucidated. Recent evidence suggests that transactivation of EGFR by GPCRs plays a significant role in this signaling mechanism (53). In accordance with this notion, we demonstrated that AT-1R stimulation induces EGFR phosphorylation and that the activation of EGFR is crucial in PI3K transactivation and collagen synthesis in MES-13 cells. Transactivation of EGFR by GPCRs, including AT-1R has been widely established in a variety of cells (54–56). The mechanisms by which ANG II/AT-1R transactivate EGFR, however, are not well understood. Recent studies have suggested the important role of metalloproteases in the enzymatic conversion of the HB-EGF precursor to soluble ligand that activates EGFR through autocrine or paracrine mechanisms (57, 58). In the present study, we used the non-specific metalloprotease inhibitor 1,10-phenanthroline and heparin, which inhibits the HG-EGF activity competitively (29), to investigate the involvement of HB-EGF in our model. Neither inhibitor, however, abolished the effect of ANG II on inducing PI3K activity, suggesting HB-EGF is not involved in this signaling pathway in MES-13 cells.

Functional signaling interactions between EGFR and non-receptor tyrosine kinase Src have been documented (59, 60). We extensively studied the role of Src in the AT-1R/EGFR/PI3K signaling pathway in MES-13 cells with four different approaches. First, pretreatment with an Src inhibitor, PP2, completely abolished the effect of acute ANG II treatment on increasing PI3K activity. Second, we measured Src activity and completely abolished the effect of acute ANG II treatment on increasing PI3K activity, suggesting HB-EGF is not involved in this signaling pathway in MES-13 cells.

In summary, we have investigated the crosstalk of ANG II/AT-1R and PI3K as a crucial factor in inducing collagen synthesis in mesangial cells. We demonstrated that EGFR transactivation is involved in this novel pathway. We further established that Src played a pivotal role in the signaling pathway by functioning between cAMP and EGFR. Although there are other components needed to be identified in this signaling pathway, our findings nonetheless provide important information for a largely unknown mechanism of ANG II-mediated pathogenesis in glomerular diseases. Elucidating further details in this signaling pathway will benefit future choices of treatment for diseases.

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