Angiotensin II stimulates fibronectin protein synthesis via a Gβγ/arachidonic acid-dependent pathway

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Alexander LD, Ding Y, Alagarsamy S, Cui X. Angiotensin II stimulates fibronectin protein synthesis via a Gβγ/arachidonic acid-dependent pathway. Am J Physiol Renal Physiol 307: F287–F302, 2014. First published June 11, 2014; doi:10.1152/ajprenal.00094.2014. In rabbit proximal tubular cells, ANG II type 2-receptor (AT2)-induced arachidonic acid release is PLA2 coupled and dependent of G protein βγ (Gβγ) subunits. Moreover, ANG II activates ERK1/2 and transactivates EGFR via a c-Src-dependent mechanism. Arachidonic acid has been shown to mimic this effect, at least in part, by an undetermined mechanism. In this study, we determined the effects of ANG II on fibronectin expression in cultured rabbit proximal tubule cells and elucidated the signaling pathways associated with such expression. We found that ANG II and transfection of Gβγ subunits directly increased fibronectin protein expression, and this increase was inhibited by overexpression of β-adrenergic receptor kinase (βARK)-ct or DN-Src. Moreover, ANG II-induced fibronectin protein expression was significantly abrogated by the AT2 receptor antagonist PD123319. In addition, inhibition of cystolic PLA2 diminished ANG II-induced fibronectin expression. Endogenous arachidonic acid mimicked ANG II-induced fibronectin expression. We also found that overexpression of Gβγ subunits induced c-Src, ERK1/2, and EGFR tyrosine phosphorylation, which can be inhibited by overexpression of βARK-ct or DN-Src. Gβγ also induced c-Src SH2 domain association with the EGFR. Supporting these findings, in rabbit proximal tubular epithelium, immunoblot analysis indicated that βγ expression was significant. Interestingly, arachidonic acid- and eicosatetraenoic acid-induced responses were preserved in the presence of βARK-ct. This is the first report demonstrating the regulation of EGFR, ERK1/2, c-Src, and fibronectin by Gβγ subunits in renal epithelial cells. Moreover, this work demonstrates a role for Gβγ heterotrimeric proteins in ANG II, but not arachidonic acid, signaling in renal epithelial cells.

THE SIGNALING FUNCTION OF G proteins was once attributed only to the Gα subunit. However, it is now clear that G protein βγ (Gβγ) dimer subunits of the heterotrimeric G proteins directly couple to G protein-coupled receptors (GPCRs) and many structural diverse effectors to transmit signals from GPCRs to effectors. Indeed, Gβγ subunits have been implicated in GPCR-induced transactivation of tyrosine kinase receptors. Transactivation of tyrosine kinase receptors, such as EGFR, by GPCRs has been suggested to involve signaling linked to the GTP-binding Gβγ subunits to facilitate cross talk between these distinct receptor systems. In fact, the EGFR was recently identified as a signal transducer in response to activation of the Gαq-coupled ANG II receptor or Gαi-coupled lysophosphatidic acid (LPA) receptor in cultured aortic smooth muscle cells (47) and rat-1 fibroblasts (11). In COS-7 cells, the transiently expressed Gαq-coupled M2 muscarinic acetylcholine receptor and Gαq-coupled bombesin receptor were used to demonstrate that Gαq and Gαi cooperatively mediated GPCR-induced EGFR transactivation (10). Moreover, in rat-1 fibroblast and Cos 7 cells, dominant negative mutants of the EGFR were used to demonstrate that the EGFR is important for linking GPCR activation with the activation of MAPK/p44/42 MAPK (ERK1/2) (11, 29). In fact, in many cells, the EGFR kinase inhibitor AG1478 inhibited GPCR-mediated ERK1/2 activation (6, 13, 14, 29). It is now well documented that Gβγ subunits are also able to transmit signals to the effector molecules in renal epithelial cells (19, 30). This novel discovery has raised many interesting questions that need to be answered timely and clearly. For example, how are the Gβγ subunits activated, and are there any cooperative relationships between Gα and Gβγ subunits with respect to signal transduction in renal epithelia? We have found that within the kidney, the ANG II type 2b (AT2b) receptor follows a paradigm wherein Gβγ subunits mediate PLA2 activation, arachidonic acid release, and EGFR transactivation (19). Arachidonic acid has been documented to mimic this transactivation at least in part by an undetermined mechanism. A growing body of data indicates that the Src family of protein tyrosine kinases (PTKs) functionally interacts with Gβγ subunits and a variety of nonreceptor and receptor PTKs, particularly the EGFR. For example, in smooth muscle cells, ANG II (AT1)-induced PLCγ, p21Ras, and ERK1/2 activation have been suggested to be mediated at least in part by c-Src since introduction of c-Src antisera and dominant negative mutants blocked these effects (24, 35, 40). Of interest is the fact that no direct physical association has been documented between PLCγ and c-Src following ANG II activation in smooth muscle cells. It was observed that in cells deficient in the Src-related tyrosine kinase Lyn, ERK1/2 activation by the Gαq-coupled M1 muscarinic acetylcholine receptor was blocked, whereas the Gαi-coupled M2 muscarinic acetylcholine receptor was unaffected. In cells deficient in the Src-related tyrosine kinase Syk, both M1 and M2 muscarinic acetylcholine receptors failed to stimulate ERK1/2 activation (21, 48). In this context, Luttrell et al. (33, 34) have shown that in Cos 7 cells Gβγ subunits directly activate c-Src receptors and that c-Src activation mediates Ras-dependent activation of ERK1/2. In addition, we have recently observed that ANG II induces ERK1/2 activation and transactivates EGFR in primary cultures of rabbit proximal tubule cells via an ANG II (AT2)-induced activated c-Src and Src homology 2 (SH2) domain
association with the EGFR (3). Although Gβγ seems to play an important role in both Gαq- and Gαq-mediated ERK1/2 activation in response to stimulation of GPCRs, the molecular mechanisms involved in regulating proximal tubule c-Src activation by ANG II and arachidonic remain largely unknown.

In the present study, we investigated whether arachidonic acid-induced fibronectin protein expression and c-Src, ERK1/2, and EGFR tyrosine phosphorylation are subject to G protein regulation. The data showed that stimulation of proximal tubule cells with Gβγ subunits resulted in significant fibronectin synthesis and ERK1/2 and EGFR tyrosine phosphorylation via the activation of c-Src and SH2 associations with the EGFR. In addition, both ANG II and arachidonic acid induced significant time-dependent increases in fibronectin protein expression in rabbit proximal tubule cells. Whereas ANG II appears to elicit its effects through coupling of Gβγ subunits, the mechanisms involved in the activation of the above cascade by arachidonic acid appear to be independent of Gβγ subunits. Thus a novel pathway has been identified wherein arachidonic acid induces c-Src-dependent transactivation of the EGFR, association of the EGFR with c-Src, and the sequential downstream activation of ERK1/2 and fibronectin synthesis independently of Gβγ signaling. In addition, these data demonstrate that Gβγ mediates ANG II-induced fibronectin expression and c-Src and ERK1/2 activation in a PLC- and PKC-independent manner and that c-Src acts sequentially upstream of EGFR in rabbit proximal tubule cells. Moreover, we present for the first time an alternative paradigm to Gγ-induced transactivation of a kinase receptor, whereby Gβγ subunits can activate cytoplasmic (c)PLA2 to increase the amount of arachidonic acid present, ultimately inducing fibronectin synthesis and c-Src, EGFR, and ERK1/2 activity. Thus these observations are important, as they have established an alternative signal transduction pathway between GPCRs and the activation of c-Src, EGFR, and ERK1/2, mediated by arachidonic acid, a fatty acid released on activation of a variety of signaling mediators (PLA2, PLC, PLD, etc.). In addition, the involvement of Gβγ in this AT2 signaling paradigm is novel with respect to ANG II receptor subtypes for this and other locations.

EXPERIMENTAL PROCEDURES

Materials. Cell culture media, serum, cell culture supplements, and Lipofectamine 2000 transfection reagent were purchased from Invitrogen. Arachidonic acid was purchased from MP Biomedical. ANG II, eicosatetraenoic acid (ETYA), losartan, PD123319, and GP 109203X hydrochloride were purchased from Sigma-Aldrich. [3H]arachidonic acid was purchased from American Radiolabeled Chemicals. Antibodies against EGFR, phosphotyrosine (Py20), sheep IgG horseradish peroxidase (HRP)-conjugated goat anti-mouse, rabbit IgG, and U73122 were purchased from Calbiochem. Antibodies against phospho-c-Src (Tyr416), phospho-cPLA2, (Ser505), phospho-p42MAPK (Trp202)/p44MAPK, p42MAPK (Trp204), cPLA2, and c-Src were purchased from Cell Signaling Technology. Antibodies against fibronectin, β-actin, Gβ1, Gβ2, Gβ3, Gβ4, and Gγ2 were purchased from Santa Cruz Biotechnology. Arachidonil trifluoromethyl ketone (ACOCF3), methyl arachidonyl fluorophosphonate (MAFP), oleoyloxyethyl phosphorylcholine (OPC), and bromoeno lactone (BEL) were purchased from Cayman Chemical. All other chemicals were of best available quality, usually analytic grade.

Cell culture and reagents. All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Primary culture of proximal tubule cells was isolated from male New Zealand White rabbits and cultured as described previously (2). Cells were maintained for 9–14 days in DMEM/F-12 medium (1:1) supplemented with 10% fetal calf serum (Invitrogen), 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 100 U/ml of penicillin, and 100 μg/ml streptomycin (Life Technology). Subconfluent monolayers of first-passage cells were employed for experiments. Cells were grown in 100-mm dishes (for preparation of immunoblots) and six-well plates (for arachidonic acid release assay).

Transfections. Transfection was carried out by using Lipofectamine 2000 transfection reagent (Invitrogen) using 10 μg of plasmid DNA (e.g., 5 μg β and 5 μg γ2) in 30 μl of transfection reagent/100-mm dish or six-well plate to rabbit proximal tubule cells at 75% confluence. The transfection mixture remained in the medium for 4 h. Afterward, cells were grown in standard growth medium for 20 h and subsequently serum restricted for 24 h. For mock-transfection, the pCMV5 vector (10 μg) without the cDNA inserts was employed.

Cell growth-glutathione-S-transferase-SH2 fusion protein pull-down assay. pGEX vectors containing the cDNA sequences encoding the SH2 and SH3 domains of c-Src were retrieved from chicken c-Src cDNA plasmid by PCR. The sequence of both strands was verified by Cleveland Genomic LT, with ABI PRISM, model 377. A standard protocol (Amersham Pharmacia Biotech) was employed to prepare the glutathione-S-transferase (GST) fusion protein conjugated with glutathione-Sepharose 4B beads (Sigma). Twelve microliters of GST fusion proteins noncovalently coupled to Sepharose beads were incubated with 1 μg of cell lysates prepared in SDS/RIPA buffer for 2 h at 4°C. After binding, the sample was centrifuged for 30 s at 4°C and the Sepharose beads were washed three times with lysis buffer. Bound proteins were boiled in 25 μl Laemmli’s sample buffer (2×) for 5 min and resolved by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, blocked in 5% nonfat dry milk in PBS-Tween 20 (0.1%) for 1 h at room temperature, and subsequently incubated with anti-EGFR sheep polyclonal antibody (1:1,000 dilution, Sigma) overnight. The blot was washed three times in PBS-T (0.1% Tween 20) for 30 min each and immunoblotted with horseradish peroxidase-conjugated anti-goat IgG (1:2,000 dilution, Calbiochem). The immunoreactive proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). The exposed autoradiograph was analyzed by Un-Scan-It gel version 5.1 (Silk Scientific) to obtain densitometry data. Protein contents were determined by BCA assay (Pierce).

Arachidonic acid release assay. Arachidonic acid release was determined as previously described (1). Briefly, cells grown on six-well plates to subconfluence were labeled with 0.5 μCi/ml-1 well-1 [3H]arachidonic acid (American Radiolabeled Chemicals) for 4 h before treatment. They were washed three times with DMEM containing 1 mg/ml fatty acid-free bovine serum albumin to remove free labels. After treatment of cells, the medium was removed and the released arachidonic acid was determined by scintillation counting. Each data point is the average from at least three wells repeated at least four times.

Immunoblotting. After stimulation, cells were lysed in RIPA buffer, adjusted to an equal amount of protein (30 μg) and equal volume, boiled in 2× Laemmli’s sample buffer, and protein extracts were separated by SDS-PAGE. The proteins were then transferred to a PVDF membrane by electroblotting at 300 mA for 1.5 h. After electrophoresis, nonspecific binding was blocked by incubation with 5% (w/v) or nat fat milk in PBS/T (0.1% Tween 20) for 1 h and probed with designated antibodies. After 24-h incubation at 4°C, the membrane was washed three times with PBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse (Calbiochem) or goat anti-rabbit IgG (Calbiochem). After washing, blots were developed with an ECL. Intensities of immunoreactive protein bands were quantified using Un-Scan-It gel, version
5.1, to obtain densitometry data. Protein contents were determined by BCA assay (Pierce).

Coimmunoprecipitation analysis was performed using confluent rabbit proximal tubule cells treated with vehicle or stimulated with the agonist of interest, or transfected with empty vector (mock) or Gβγ12 subunits. After stimulation, cells were lysed in RIPA buffer, adjusted to an equal amount of protein (100–500 μg) and equal volume. A designated antibody (IgG) was added, and samples were incubated overnight at 4°C. Immune complexes were recovered by the addition of 100 μl of A/G PLUS-Agarose (Santa Cruz Biotechnology). Samples were incubated for 2 h with gentle agitation at 4°C. The immunoprecipitates were washed once with lysis buffer and twice with ice-cold PBS. The immunoprecipitated proteins were eluted with 50 μl 2× Laemmli’s sample buffer (Santa Cruz Biotechnology), boiled for 5 min, subjected to SDS-PAGE, transferred to a PVDF membranes, probed with specific antibodies, and developed with ECL. The exposure autoradiograph was analyzed by Un-Scan-It gel, version 5.1, to obtain densitometry data. Protein contents were determined by BCA assay.

Drug treatments. cPLA2 inhibitors AACOCF3 and MAFP, secretory (s) PLA2 inhibitor OPC, and Ca2+-independent (i) PLA2 inhibitor BEL (Cayman Chemical) were dissolved in ethanol at 10 mM/ml for stock and used as 10 μM/ml; PLC inhibitor U73122 (Calbiochem) and PKC inhibitor GF 109203X hydrochloride (Sigma) were prepared as 10 mM/ml stocks in DMSO and finally used as 1 and 10 μM/ml, respectively.

Statistical analysis. Data expressed as means ± SE were analyzed with a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls multiple comparison post-hoc test or with a two-tailed Student’s t-test when appropriate. A value of P < 0.05 was considered significant.

RESULTS

Gβγ subunits mediate ANG II-induced c-Src, EGFR, and ERK1/2 phosphorylation. Our previous studies have shown that both ANG II and arachidonic acid induce EGFR, c-Src, and ERK1/2 phosphorylation in rabbit renal proximal tubules (3), effects which were readily abrogated by pretreatment with the Src family kinase inhibitor 3-(4-chlorophenyl)1-(1,1-dimethyl-ethyl)-1H-pyrazolo[3,4-d]pyrimidin-4amine (PP2) or by transfection of rabbit proximal tubule cells with dominant-negative (DN) c-Src (DN-Src). Previous work by Haithcock and colleagues (19) indicates that Gβ1/γ2 subunits enhance ANG II-dependent arachidonic acid release and that sequestration of endogenous free Gβγ subunits, by the carboxyl terminus of the β-adrenergic receptor kinase 1 (βARK-ct), abrogates ANG II-induced arachidonic acid release in these same cells, but whether Gβ1/γ2 subunits mediate arachidonic acid-induced EGFR, c-Src, and ERK1/2 phosphorylation is not known.

Figure 1, A, B, and C, respectively, shows that the overexpression of Gβ1/γ2 increased c-Src, ERK1/2, and EGFR activation by 2.41-, 4.53-, and 2.46-fold, respectively, compared with controls. Transfection of βARK-ct significantly reduced Gβ1/γ2-induced c-Src, ERK1/2, and EGFR activation, indicating that both exogenous and endogenous Gβγ subunits could activate c-Src, ERK1/2, and EGFR. In addition, the transfection of βARK-ct significantly attenuated ANG II-induced c-Src (3.44–1.55-fold) (Fig. 1A), ERK1/2 (5.04–1.54-fold) (Fig. 1B), and EGFR phosphorylation (3.10–1.45-fold) (Fig. 1C), indicating endogenous Gβγ regulation of ANG II-induced c-Src, ERK1/2, and EGFR phosphorylation in rabbit renal proximal tubules. By contrast, arachidonic acid-induced c-Src, ERK1/2, and EGFR phosphorylation was unaffected in the presence of βARK-ct, confirming that arachidonic acid activates c-Src, ERK1/2, and EGFR independently of Gβγ subunits. Similar results were demonstrated with 5,8,11,14-ETYA, a nonmetabolized arachidonic acid analog. This suggests that the mechanism of action for arachidonic acid is different compared with that induced by ANG II and that endogenous Gβγ subunits might be a direct effector of c-Src, ERK1/2, and EGFR activation in rabbit proximal tubular cells.

Effect of different Gβγ combinations on EGFR, c-Src, and ERK1/2 activity. Next, we studied the possibility that Gβγ dimers in addition to Gβ1/γ2 activate EGFR, c-Src, and ERK1/2. Figure 2 shows that rabbit proximal tubule epithelium expresses endogenous Gβ1, Gβ2, Gβ3, and Gβ4, of which the β1 is the predominant isoform. In addition, as a positive control for the anti-Gβ1–Gβ4, Gβ1–Gβ4 subunits were transfected into rabbit proximal tubule cells and the expression levels of Gβ1–Gβ4 were determined by Western blot analysis (Fig. 2). Moreover, results in Fig. 3 show that, in contrast to the significant stimulatory effect of Gβ1/γ2 on EGFR, c-Src, and ERK1/2 activity, these effects were not observed in proximal tubular cells transfected with equal amounts of DNA for expression of Gβ2/γ2, Gβ3/γ2, or Gβγ2. Taken together, these results indicate, by overexpression, that the stimulatory effect of Gβγ on EGFR, c-Src, and ERK1/2 activity is isoform specific in rabbit proximal tubular cells and that only Gβ1/γ2 was able to induce a significant increase in the phosphorylation of EGFR, c-Src, and ERK1/2. The activation of EGFR, c-Src, and ERK1/2 in proximal tubules by Gβγ subunits is novel.

Gβγ induces binding of EGFR with the SH2 domain GST fusion protein. To determine whether Gβ1/γ2 also induced binding of EGFR with the SH2 domain fusion protein, cell lysates were immunoprecipitated with a c-Src SH2 domain GST fusion protein and subsequently immunoblotted with monoclonal anti-EGFR antibodies. As shown in Fig. 4, ANG II, arachidonic acid, and ETYA induced an association of c-Src SH2 domain with EGFR in a manner similar to Gβ1/γ2. Overexpression of βARK-ct totally abrogated Gβ1/γ2- and ANG II-induced association of EGFR with the SH2 domain GST fusion protein, while having no effect on arachidonic acid- and ETYA-induced association of EGFR with the SH2 domain GST fusion protein.

Gβ1/γ2 induces EGFR and ERK1/2 phosphorylation in a c-Src-dependent manner. We previously showed that pretreatment of proximal tubule cells with PP2, a Src family tyrosine kinase inhibitor, or transfection of proximal tubule cells with DN-Src, did not affect the ability of EGF to stimulate EGFR and ERK1/2 activation, but significantly reduced ANG II- and arachidonic acid-induced c-Src, EGFR, and ERK1/2 activation (3). We now demonstrate that transfection of DN-Src significantly decreased Gβ1/γ2-induced c-Src, EGFR, and ERK1/2 activation (Fig. 5, A, B, and C, respectively). Collectively, these data demonstrate that, in early passages of kidney epithelial cells, Gβ1/γ2, ANG II, and arachidonic acid induce EGFR, c-Src, and ERK1/2 activation. Moreover, c-Src appears to be upstream of EGFR and ERK1/2, and the induced transactivation of EGFR appears to occur via inducing the association of EGFR with the SH2 domain of the Src protein.

cPLA2 inhibition attenuates ANG II- and Gβ1/γ2-induced arachidonic acid release and c-Src and ERK1/2 activation. The renal epithelial AT2 receptor and cPLA2 signaling complex appears to be linked to Gβγ in that overexpression of Gβ1/γ2...
augments ANG II-induced arachidonic acid release in proximal tubule cells and βARK-ct abrogates ANG II-induced arachidonic acid release under basal conditions and with overexpression of Gβ1γ2 (19). However, the mechanism of cPLA2 phosphorylation and arachidonic acid release is still unresolved. We show here that overexpression of Gβ1γ2 subunits activates cPLA2 in primary cultured renal proximal tubular cells (Fig. 6A), resulting in increased intracellular and extracellular arachidonic acid release (Fig. 7). The use of different PLA2 inhibitors revealed that Gβ1γ2-induced arachidonic acid release is mediated by activation of cPLA2, whereas iPLA2 or sPLA2 does not seem to be involved in the response to Gβ1γ2 (Fig. 7). Similarly, the
cPLA₂-specific inhibitors MAFP and AACOCF₃ prevented c-Src and ERK1/2 activation of cultured epithelial cells upon Gβ₁γ₂ stimulation, whereas inhibitors specific for iPLA₂ or sPLA₂ were without effect (Figs. 6, B and C, respectively). On the other hand, neither PKC inhibitor GF1092003 nor PLC inhibitor U73122 affected the stimulatory effect of Gβ₁γ₂ on arachidonic acid release and cPLA₂, c-Src, and ERK1/2 activity (data not shown). Accordingly, these results suggest that arachidonic acid generation by activation of cPLA₂ during ANG II stimulation, whereas inhibitors specific for iPLA₂ or sPLA₂ were without effect (Figs. 6, B and C, respectively). On the other hand, neither PKC inhibitor GF1092003 nor PLC inhibitor U73122 affected the stimulatory effect of Gβ₁γ₂ on arachidonic acid release and cPLA₂, c-Src, and ERK1/2 activity (data not shown). Accordingly, these results suggest that arachidonic acid generation by activation of cPLA₂ during ANG II stimulation plays an important role in the induction of c-Src and ERK1/2 activation in renal proximal tubular AT₂ receptor signaling that is independent of both PKC and PLC.

Gβ₁γ₂-induced EGFR, c-Src, and ERK1/2 phosphorylation is eicosanoid independent. To assess whether the stimulatory effect of Gβ₁γ₂ on EGFR, c-Src, and ERK1/2 phosphorylation was mediated by arachidonic acid metabolites, inhibitors of different arachidonic acid metabolic pathways were used. Figure 8 shows that when primary proximal tubule cells were treated with 10 μmol/l 17-ODYA (a specific inhibitor of the cytochrome P-450 pathway); it had no effect on Gβ₁γ₂-induced stimulation of Src-Src and ERK1/2 phosphorylation. In addition, pretreatment of proximal tubular cells with either 5 μmol/l NDGA (an inhibitor of the lipoxigenase pathway) or 50 μmol/l indomethacin did not affect arachidonic acid-induced c-Src and ERK1/2 phosphorylation. Thus the fact that Gβ₁γ₂-induced c-Src and ERK1/2 activation was not significantly altered in the presence of the specific cytochrome P-450 inhibitor 17-ODYA, as well as in the presence of the selective lipoxigenase and cyclooxygenase inhibitors NDGA and indomethacin, respectively, indicates that Gβ₁γ₂-induced c-Src and ERK1/2 activation is independent of eicosanoid biosynthesis.

ANG II induces fibronectin protein expression via c-Src, EGFR, and ERK1/2 in renal proximal tubular cells. ANG II has been reported to be associated with the development of renal interstitial fibrosis through the stimulation of fibronectin protein expression (8, 41, 42, 49). However, the molecular mechanisms of the ANG II-induced fibronectin in renal proximal tubule cells have not been extensively studied. Moreover, the role of G protein βγ in mediating the effects of ANG II has not been investigated. We recently reported that ANG II-induced ERK1/2 and c-Src activation in kidney epithelium occurs via AT₂ receptor-dependent signaling pathways and that ANG II evokes arachidonic acid release by stimulating cPLA₂ in rabbit proximal tubule cells (3, 12). To assess the effect of ANG II on fibronectin expression, proximal tubular cells were treated with 1 μmol/l ANG II for various time periods. ANG II induced a significant increase in fibronectin protein expression (1.41-fold compared with control) within 12 h, and the expression was sustained until 48 h (Fig. 9A). Arachidonic acid mimicked the effect of ANG II on fibronectin protein expression, in that direct stimulation of proximal tubular cells with arachidonic acid (15 μmol/l) induced the expression of fibronectin protein in a time-dependent manner, corresponding to the kinetic of fibronectin expression after exposure to ANG II (Fig. 9B). In addition, overexpression of proximal tubule cells with Gβ₁γ₂ induced fibronectin protein expression (Fig. 10A), which was inhibited by transient transfection of proximal tubular cells with βARK-ct. Transfection of βARK-ct prevented ANG II, but not arachidonic acid, upregulation of fibronectin protein expression (Fig. 10B). Furthermore, expression of a dominant negative mutant of c-Src abrogated ANG II-, arachidonic acid-, and Gβ₁γ₂-induced fibronectin protein

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expression (Figs. 11, A and B, respectively). Similar inhibition was demonstrated by the EGFR kinase inhibitor AG1478 and MEK inhibitor PD 98059 (Fig. 12, A and B, respectively).

cPLA2 inhibitors attenuated ANG II- and G\(\beta_{1,2}\)-induced fibronectin protein expression. Since we have found that the cPLA2-specific inhibitors MAFP and AACOCF3 prevented c-Src and ERK1/2 activation in these cells (Fig. 6, B and C), respectively), we were interested in examining whether PLA2 could be involved in ANG II- and G\(\beta_{1,2}\)-induced fibronectin expression. Proximal tubular cells were preincubated without or with 10 \(\mu\)mol/l of MAFP, AACOCF3, BEL, or OPC followed by 1 \(\mu\)mol/l ANG II for 48 h. Western blot analysis showed that the two cPLA2 inhibitors MAFP and AACOCF3 strongly suppressed ANG II-induced fibronectin protein expression. Conversely, the iPLA2 inhibitor BEL and sPLA2 inhibitor OPC (Fig. 13A) did not affect fibronectin expression. Moreover, the effects of both ANG II and G\(\beta_{1,2}\) on fibronectin protein expression were not attenuated by pretreatment of proximal tubular cells with 17-ODYA, NDGA, or indomethacin (data not shown). These observations document a mechanism of ANG II- and G\(\beta_{1,2}\)-induced fibronectin expression, both AT1 and AT2 receptor blockers
were used. The AT2-selective antagonist PD123319, but not the AT1-selective antagonist losartan, inhibited ANG II-induced fibronectin synthesis (Fig. 14). These results support a role for the AT2 receptor as a mediator of fibronectin synthesis. The observations with Gβγ and βARK-ct as described and discussed herein suggest a model for ANG II- and arachidonic acid-induced fibronectin synthesis and EGFR, c-Src, and ERK1/2 activation, as depicted in Fig. 15.

**DISCUSSION**

Renal fibrosis is characterized by increased synthesis and deposition of ECM proteins, which result in increased fibrotic tubular damage, an effect attributed to increased tubular fibronectin production, leading to end-stage renal disease (ESRD) (32). ANG II has important non-hemodynamic effects that have been implicated in the pathogenesis of chronic kidney disease (CKD), including stimulating the production of fibronectin (27). It has been shown in renal mesangial cells that the expression of fibronectin induced by ANG II involves both ERK1/2 and Akt/PKB pathways (5, 20) and that this effect can be blocked by the AT1 receptor antagonist losartan. Fang et al. (15) found that ANG II induced the expression of fibronectin in renal tubular cells through activation of 5′-AMP-activated protein kinase (AMPK) and cAMP pathways. Our present work documents a critical role of Gβγ, cPLA2, and c-Src as alternative mechanisms of ANG II-induced fibronectin expression in renal tubular cells. Thus inhibition of arachidonic acid release by MAPF and AACOCF3, two selective cPLA2 inhibitors, attenuated ANG II- and Gβγ-induced fibronectin expression in proximal tubular cells (Fig. 13, A and B, respectively) and arachidonic acid at low (15 μM) concentrations mimicked the effect of ANG II on fibronectin synthesis with a striking parallel in time course (Fig. 9B). Moreover, expression of Gβγ subunits stimulated expression of fibronectin in renal tubular cells to nearly the same extent as ANG II and arachidonic acid (Fig. 10A). Overexpression of the carboxy terminus of βARK-ct, an endogenous scavenger of Gβγ, resulted in inhibition of fibronectin expression induced by Gβγ (Fig. 10A) and ANG II (Fig. 10B). In contrast, overexpression of βARK-ct did not inhibit arachidonic acid-induced fibronectin expression (Fig. 10B). Furthermore, expression of a dominant negative mutant of Src markedly inhibited Gβγ, resulting in inhibition of fibronectin expression induced by Gβγ (Fig. 10A) and ANG II (Fig. 10B). In addition, our work demonstrates that ANG II-induced expression of fibronectin was inhibited by the AT2 receptor blocker PD123319, but not by the AT1 receptor blocker losartan (Fig. 14). Additional studies form this laboratory have documented that cPLA2-mediated signaling is linked to the relatively low-affinity AT2 ANG II receptor (3, 19, 25, 26) that is pharmacologically distinct from the AT2 receptor cloned previously (22, 36). Moreover, this apical AT2 receptor mediates ANG II-induced arachidonic acid release and downstream events such as Shc/Grb2/Sos and p21ras association, and c-Src and ERK1/2 activation (3, 12, 25, 26). Thus this represents an additional signaling model for AT2 receptor subtypes and G protein-coupled receptors as well.

Molecular biological observations validate the existence of two major ANG II receptor subtypes (AT1, and AT2). These receptor subtypes are pharmacologically distinct and share only ~30% sequence homology. While the AT1 receptor has been considered to subserve many of the biological functions linked to ANG II, emerging evidence indicates that the adult AT2 receptor may also mediate significant biological responses as well. Renal proximal tubular cells express a novel complement of both AT1 (basolaterally oriented) and AT2 (apically oriented) receptor subtypes that mediate many renal functions in responses to ANG II. Notable differences exist between the ANG II receptor subtypes expressed in the kidney and other locations: 1) the kidney epithelial AT2 receptor (AT2b) differs from cloned fetal AT2 (AT2α) in that G protein coupling was demonstrated pharmacologically in vitro; 2) binding affinity for losartan, PD123319, and CYP42112a differed between AT2α and AT2b; 3) signaling linked to AT2b is through a membrane-associated PLA2, arachidonic acid release, and activation of the MAPK superfamily (ERK1/2, p38 MAPK, and c-JNK), while AT2α is linked to a MAPK phosphatase (MKP) and/or PP2A and, by contrast, inhibition of MAPK phosphorylation; 4) the renal epithelial AT2 receptor-PLA2 signaling complex appears to be linked to G protein by, in that overexpression of Gβγ subunits augments ANG II-induced arachidonic acid release, and BARK-ct abrogates ANG II-induced arachidonic acid release under basal conditions and with over-
Fig. 5. Involvement of c-Src in Gβγ-induced EGFR transactivation and ERK activation in rabbit proximal tubule cells. Rabbit proximal tubule cells were transiently cotransfected with pCMV5 (empty vector) or a dominant negative Src mutant (DN-Src) in combination with Gβ1 and Gγ2. The expression and phosphorylation of EGFR, c-Src, and ERK1/2 were analyzed by Western blotting of tubular cell protein extract. One representative Western blot is shown for every experiment. A: phosphorylation of c-Src (p-c-Src; top) compared with total expression of c-Src (bottom). B: phosphorylation of EGFR (top) compared with total expression of EGFR (bottom). C: phosphorylation of ERK1/2 (p-ERK; top) compared with total expression of ERK1/2 (bottom). Bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 4 independent experiments. ***P < 0.001 indicates significant difference compared with nonstimulated control cells. +++P < 0.001 indicates significant difference compared with the respective values of cells transfected with Gβ1γ2 alone.
expression of Gβiγ2; and 5) the renal AT1 receptor differs from the vascular AT1 receptor as its signaling is linked primarily to adenyl cyclase rather than inositol-specific PLC, and receptor expression is upregulated in high ambient ANG II concentration rather than downregulated. Thus there are substantial differences in binding, G protein interaction, signaling, and MAPK regulation between the cloned AT2A and the epithelial isoform AT2B. Hence, to date, the role of the AT2 receptor in renal fibrosis remains elusive. In human renal fibroblasts, Schuttertet et al. (41) and Hua et al. (20) showed

Fig. 6. Cystolic (c) PLA2 is involved in Gβγ-induced c-Src and ERK activity. Rabbit proximal tubule cells were treated with 2 cPLA2 inhibitors, arachidonyl trifluoromethyl ketone (AACOCF3; 10 μmol/l) and methyl arachidonyl fluorophosphate (MAFP; 10 μmol/l), iPLA2 inhibitor oleyloxyethyl phosphorylcholine (OPC; 10 μmol/l), and sPLA2 inhibitor bromoenol lactone (BEL; 10 μmol/l) for 15 min or the vehicle DMSO, then transfected with Gβiγ2 or empty vector (mock) for 24 h. At the end of incubation, the expression and phosphorylation of c-Src and ERK1/2 were analyzed by Western blotting of tubular cell protein extract. One representative Western blot is shown for every experiment. A: phosphorylation of c-PLA2 (p-cPLA2; top) compared with total expression of c-PLA2 (bottom). B: phosphorylation of c-Src (p-c-Src; top) compared with total expression of c-Src (bottom). C: phosphorylation of ERK1/2 (p-ERK, top) compared with total expression of ERK1/2 (bottom). Bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 4 independent experiments shown as fold-increase. *P < 0.001, compared with nonstimulated control (mock) cells. **P < 0.01, compared with the respective values of cells transfected with Gβiγ2 alone.
that ANG II-mediated effect on fibronectin synthesis was diminished in the presence of the AT1 receptor blocker losartan, while no inhibition was observed using the AT2 receptor blocker PD123319. Ray et al. (38), in human fetal mesangial cells, obtained similar results. Based on the data presented in this paper, we propose a role for the AT2 receptor in the synthesis of fibronectin, in that the increased fibronectin response to ANG II was completely inhibited by the AT2 receptor antagonist PD123319, while the AT1 receptor antagonist losartan had no effect. Despite the fact that several studies have suggested that ANG II-induced fibronectin synthesis is mediated by AT1 rather than AT2 signaling, the interpretation relied on use of pharmacological concentrations of losartan that can compete at high concentrations for AT2 receptor sites (38, 41, 51). Moreover, this is the first study to investigate the role of ANG AT1 and AT2 receptors in ANG II-induced fibronectin synthesis in cultured proximal tubular epithelial cells. Furthermore, ANG II-AT2-induced fibronectin synthesis in rabbit proximal tubular cells appears to be mediated at least in part by EGFR, c-Src, and ERK1/2 activation, since introduction of pharmacological inhibitors and dominant negative mutants blocked this effect. While these data suggest that ANG II, operating through the AT2 receptor, exerts an antifibrotic effect on the kidney in vitro, they do not negate the beneficial effects of losartan and other AT1 receptor antagonists on cells other than rabbit proximal tubule or different models of renal injury. The composite mechanism and relationship to AT2 receptor and downstream events leading to fibronectin synthesis is depicted in Fig. 15.

Previous studies have documented that the Gβγ subunits mediate β2-adrenergic- and lysophosphatidic acid LPA-induced activation of c-Src in transfected Cos-7 cells as a mechanism of Gβγ-coupled receptor recruitment of the Shc-Grb2-Sos complex and activation of members of MAPK, including the ERK1/2 pathway (33). These observations have relevance to our current kidney epithelial cell signaling model in that we have documented that an apical AT2-receptor subtype mediates ANG II-induced ERK1/2 activation by transactivation of EGFR (13). Moreover, this AT2 signaling involves Gβγ-mediated PLA2 activation, arachidonic acid release, and downstream events linked to Shc-Grb2-Sos and p21Ras rather than PKC, as reported previously for ANG II receptors (19, 26). In addition, ANG II activates c-Src and in these epithelial

Fig. 7. Effects of PLA2 inhibitors on AA release. Subconfluent proximal tubular cells were labeled with 0.5 μCi/ml·well−1 [3H]AA for 4 h before treatment. They were treated with 10 μmol/l AACOCF3, MAFP, OPC, or BEL for 15 min or the vehicle DMSO, then transfected with Gβ1γ2 or empty vector (mock) for 24 h. At the end of incubation, an aliquot of medium was removed and counted for radioactivity. Values are mean ± SE of 4 independent experiments shown as fold-increase. *P < 0.01, compared with nonstimulated control (mock) cells. **P < 0.001, compared with the respective values of cells transfected with Gβ1γ2 alone.

Fig. 8. Gβγ2-induced c-Src and ERK phosphorylation is eicosanoid independent. Serum-starved proximal tubular cells were transfected with Gβ1γ2 or empty vector (mock) for 24 h. Cell lysates were immunoblotted with anti-phospho-c-Src-Tyr416 (A, top) or anti-phospho-ERK1/2 (p44/42 MAPK)-Thr202/Tyr204 (B, top) antibodies, and membranes were stripped and reprobed with anti-c-Src (A, bottom) or anti-ERK1/2 antibodies (B, bottom). Bar graphs depict the quantitative densitometry analysis for Western blot densitometry data. Values are means ± SE of 4 independent experiments. *P < 0.05 indicates a significant effect of Gβ1γ2 compared with empty vector (mock)-treated cells.
cells (3). The involvement of Gβγ subunits in this AT2 receptor signaling paradigm is novel with respect to ANG II receptor subtypes, for this and other locations, as other studies have focused on a linkage to G protein subunits including Gαq, Gα2, and Gα3 (45, 50). Even more important, the involvement of arachidonic acid in this AT2 receptor signaling paradigm is unique from the perspective that this common lipid second messenger mimics all ANG II events evaluated to date in these epithelial cells. The activation of c-Src is no exception, as it too is activated by arachidonic acid, analogous to ANG II (3). However, unresolved questions persist as to the mechanism(s) whereby arachidonic acid, a fatty acid, is linked to the receptor tyrosine kinase and non-tyrosine kinase pathways. In the present study, we further extend our previous findings by demonstrating that exogenously expressed Gβγ2 subunits lead to tyrosine phosphorylation of both c-Src and EGFR, association of EGFR with the SH2 domain of c-Src, stimulation of the ERK1/2 signaling cascade, and fibronectin production in rabbit proximal tubule cells. Conversely, reducing free endogenous Gβγ concentrations by exogenous expression of βARK-ct, which contains only the C-terminal Gβγ-binding domain of βARK (28, 37), significantly inhibits Gβγ2 subunit-induced phosphorylation of c-Src, EGFR, and ERK1/2 and

![Graph A](image1.png)

**Fig. 9.** ANG II and AA induce fibronectin protein expression in rabbit proximal tubular epithelial cells. *A*: serum-starved proximal tubular cells were incubated in the absence (open bar) or presence (closed bars) of 1 μmol/l ANG II for the indicated times, cell lysates were fractionated on SDS-PAGE, and fibronectin protein expression was assessed by direct immunoblotting of cell lysates (top). B: serum-starved proximal tubular cells were incubated in the absence (open bar) or presence (closed bars) of 15 μmol/l AA for the indicated times, cell lysates were fractionated on SDS-PAGE, and fibronectin protein expression was assessed by direct immunoblotting of cell lysates (top). Actin was included as a control for loading and the specificity of change in protein expression. A representative blot from 6 independent experiments is shown. Bar graph at the bottom represents the ratio of the intensity of the fibronectin band quantified by densitometry factored by the densitometric measurements of the actin band. Values are means ± SE from 6 independent experiments. **P < 0.01 and *P < 0.05 vs. control.

![Graph B](image2.png)

**Fig. 10.** Gβγ subunits mediate ANG II-induced fibronectin expression. βARK-ct or empty vector (mock) plasmids (10 μg each) were transfected into rabbit proximal tubule cells 24 h before assay. Proximal tubule cells were treated with control [0.1% DMSO (vol/vol)], ANG II (1 μmol/l), AA (15 μmol/l), or plasmids encoding βγ and γ2, (5 μg each) for 48 h. Cell lysates were fractionated on SDS-PAGE, and fibronectin protein expression was assessed by direct immunoblotting of cell lysates (top). Actin was included as a control for loading and the specificity of change in protein expression. A representative blot from 6 independent experiments is shown. Bar graph at the bottom represents the ratio of the intensity of the fibronectin band quantified by densitometry factored by the densitometric measurements of the actin band. Values are means ± SE from 6 independent experiments. *P < 0.05, ***P < 0.001 vs. control. #P < 0.001 vs. βARK-ct.
fibronectin protein expression. Inhibition of endogenous Src family kinase activity by overexpression of a dominant negative kinase-inactive mutant of c-Src inhibits Gβ1γ2 subunit-induced phosphorylation of EGFR, c-Src, and ERK1/2 and fibronectin production. Although we show that Gβγ is required for the transactivation of EGFR, activation of c-Src and ERK1/2, and expression of fibronectin by ANG II, we also demonstrate that Gβγ subunits do not significantly

Fig. 11. ANG II-, AA-, and Gβ1γ2-induced fibronectin expression is dependent on c-Src. Cells were transfected (10 μg) with a plasmid carrying a c-Src dominant negative (DN-Src) construct or an empty vector before incubation with or without ANG II (1 μmol/l) or AA (15 μmol/l; A) or before transfection with plasmids encoding β1 and γ2 (5 μg each; B) for 48 h. Cell lysates were fractionated on SDS-PAGE, and fibronectin protein expression was assessed by direct immunoblotting of cell lysates (top). Actin was included as a control for loading and the specificity of change in protein expression. A representative blot from 4 independent experiments is shown. Bar graph at the bottom represents the ratio of the intensity of the fibronectin band quantified by densitometry factored by the densitometric measurements of the actin band. Values are means ± SE from 4 independent experiments. ***P < 0.001 vs. control. *P < 0.001 vs. DN-Src.

Fig. 12. EGFR and ERK mediate ANG II-, AA-, and Gβ1γ2-induced fibronectin expression. Serum-starved proximal tubule cells were incubated with ANG II (1 μmol/l) or AA (15 μmol/l; A) or plasmids encoding β1 and γ2 (5 μg each; B) for 48 h in the absence or presence of MAP kinase kinase (MEK) inhibitor PD98059 (50 μmol/l) or EGFR kinase inhibitor AG1478 (100 nmol/l) for 48 h. Cell lysates were fractionated on SDS-PAGE, and fibronectin protein expression was assessed by direct immunoblotting of cell lysates (top). Actin was included as a control for loading and the specificity of change in protein expression. A representative blot from 4 independent experiments is shown. Bar graph at the bottom represents the ratio of the intensity of the fibronectin band quantified by densitometry factored by the densitometric measurements of the actin band. Values are means ± SE from 4 independent experiments. ***P < 0.001 vs. control. *P < 0.001 vs. ANG II-, AA-, or Gβ1γ2-treated cells.

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contribute to the transactivation of EGFR, activation of c-Src and ERK1/2, and expression of fibronectin by arachidonic acid.

Membrane phospholipids, acted upon by PLA2 or PLC, form arachidonic acid, an essential, unsaturated, 20-carbon fatty acid, which can be further metabolized by one of three enzyme pathways into various prostaglandins (by cyclooxygenase), leukotrienes (by lipoxigenase), or epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acid (HETE; by cytochrome P-450), which themselves have potent biological activity. The latter constitutes the main pathway of arachidonic acid metabolism in renal proximal tubular epithelium. It is now well documented that arachidonic acid activates many protein kinases in a variety of cells and tissues and that this action can be either dependent or independent of cyclooxygenase-, lipoxigenase-, and cytochrome P-450-derived eicosanoids. Specifically, in the kidney, Chen et al. (7) reported that exogenous exposure of the eicosanoid 14,15-EET activates phosphoinositide 3-kinase, ERK1/2, and c-Src in rat glomerular mesangial cells (7). However, arachidonic acid was without effect. In contrast, Gorin et al. (18) showed that arachidonic acid-induced PKB/Akt activation in mesangial cells is independent of eicosanoid metabolism. By comparison, our previous studies demonstrated a role for arachidonic acid and other fatty acids in signaling linked to the MAPK superfamily and c-Src in rabbit proximal tubular epithelium without the necessity of conversion to cytochrome P-450 metabolites (2).

The renal epithelial AT2 receptor and PLA2 signaling complex appears to be linked to Gβγ in that overexpression of Gβ1γ2 subunits augments ANG II-induced arachidonic acid release in mouse proximal tubule cells and overexpression of βARK-ct abrogates ANG II-induced arachidonic acid release under basal conditions and with overexpression of Gβγ (19). However, the mechanism of cPLA2 phosphorylation and arachidonic acid release is still unresolved. In the present study, we have used MAFF and AACOCF3, two known inhibitors of cPLA2 phosphorylation and activation in the kidney (1, 2), to study the mechanism of arachidonic acid release and c-Src and ERK1/2 activation and fibronectin synthesis in rabbit proximal tubular cells. Previous results obtained with these same cells, within this laboratory, have revealed that Gβγ is a potent stimulator of arachidonic acid release (19); however, the relative roles of different PLA2 isoforms under these conditions have not been analyzed in detail. Therefore, to gain further insight into the mechanism of Gβγ-mediated arachidonic acid release, activation of c-Src and ERK1/2, and fibronectin synthesis, the relative roles of different PLA2 isoforms were analyzed. Here, we show that transfection with Gβγ resulted in the phosphorylation and activation of cPLA2 and increased arachidonic acid release in rabbit proximal tubule cells. Moreover, the existence of other PLA2 family members, such as sPLA2 and iPLA2, could not be detected in these cells by immunoblotting (data not shown). In addition, βARK-ct markedly suppressed Gβ1γ2-enhanced cPLA2 phosphorylation and arachidonic acid release. In addition, Gβ1γ2-stimulated arachidonic acid release, c-Src and ERK1/2 activation, and fibronectin expression were significantly inhibited by the cPLA2 inhibitors MAFF and AACOCF3. By contrast, BEL and OPC, inhibitors of iPLA2...
and sPLA₂, respectively, had no inhibitory effect on arachidonic acid release, c-Src and ERK1/2 activity, and fibronectin expression, suggesting the PLA₂ activity was attributable to cPLA₂. In addition, the Gβγ subunit is involved in activation of arachidonic acid release by ANG II stimulation of AT₂B receptors in mouse renal tubular epithelial cells (19). These observations have been supported herein utilizing early passed proximal tubule epithelial cells employing ANG II and/or arachidonic acid (without the necessity of overexpression of the Gβγ subunit or stimulation with ANG II and arachidonic acid significantly increased c-Src activity in these cells. Moreover, expressing βARK-ct inhibited Gβγ- and ANG II-stimulated tyrosine phosphorylation of EGFR and c-Src SH2 domain fusion protein and their accompanied increased association. A critical contrast relates to the observation that coexpression of βARK-ct abrogated Gβγ- and ANG II-induced association of c-Src SH2 domain with EGFR and c-Src and ERK1/2 activation, but had no effect on arachidonic acid- and 5,8,11,14-ETYA-induced association and c-Src and ERK1/2 activation. In addition, the effects of Gβγ on c-Src and ERK1/2 activity and fibronectin synthesis were significantly blocked by overexpression of the dominant negative c-Src mutant (c-Src K298M). While our data with ANG II AT₂ receptor signaling support the original model linking the GPCR, βγ, and ERK1/2 pathway, our data with arachidonic acid and its analogs document for the first time that arachidonic acid and ETYA

**Fig. 14. ANG II induces fibronectin expression via the AT₂ receptor. Rabbit proximal tubule cells were treated with losartan (1 μmol/l), an AT₁ receptor antagonist, or PD123319 (10 μmol/l), an AT₂ receptor antagonist, for 60 min or the vehicle DMSO, then stimulated with ANG II (1 μmol/l) for 48 h. At the end of incubation, cell lysates were fractionated on SDS-PAGE, and fibronectin protein expression was assessed by direct immunoblotting of cell lysates (top). Actin was included as a control for loading and the specificity of change in protein expression. A representative blot from 4 independent experiments is shown. Bar graph at the bottom represents the ratio of the intensity of the fibronectin band quantified by densitometry factored by the densitometric measurements of the actin band. Values are means ± SE from 4 independent experiments. ***P < 0.001 vs. control. *P < 0.001 vs. ANG II- treated cells.**

**Fig. 15. Schematic diagram outlines a signaling pathway that may mediate ANG II- and AA-induced ERK activation and fibronectin synthesis from G protein-coupled receptors and receptor tyrosine kinase in rabbit proximal tubule cells consistent with our present and previous observations. Both ANG II and AA induce tyrosine phosphorylation of the autoprophosphorylation site and the SH2 domain of c-Src. However, ANG II, but not AA, leads to Gβγ-dependent c-Src activation, leading to association of Src kinase with EGFR via the SH2 domain. The association of c-Src (through Src SH2 domain) with EGFR results in the stimulation of c-Src catalytic activity and enhanced phosphorylation of Src/Crb/2>Sos and p21Ras, which leads to the activation of ERK and increased fibronectin protein synthesis.**
transactivates EGFR via inducing association of the SH2 domain of c-Src with EGFR and induces ERK1/2 activation independent of Gβγ subunits. Moreover, our studies have the potential to provide a novel signaling paradigm for transactivation of kinase receptors following GPCR activation, which may apply to activation of a variety of phospholipases that release fatty acids. The observations with Gβγ and βARK-ct as described and discussed herein suggest a model for ANG II- and arachidonic acid-induced EGFR, c-Src, and ERK1/2 activation via arachidonic acid independently of a Gα subunit of Gi2 and Gi3 proteins by basic secretagogues induces exocytosis and fibronectin synthesis, as depicted in Fig. 15.

In summary, the present studies present interrelated, yet independent mechanisms that mediate the effects of ANG II vs. arachidonic acid on fibronectin synthesis, EGFR transactivation, and c-Src and ERK1/2 activation. Herein, we propose two independent mechanisms for the activation of c-Src to mediate ERK1/2 activation and fibronectin expression, one through Gβγ and the other through GPCR-independent pathways. We implicate Gβγ subunits as targets of ANG II in EGFR, c-Src, and ERK1/2 signaling pathways, whereas arachidonic acid-initiated signaling to EGFR transactivation and c-Src and ERK1/2 pathways is independent of these proteins. Collectively, we were able to show that renal the epithelial AT2 receptor has novel binding and signaling properties that involve Gβγ subunits, cPLA2, and arachidonic acid as critical upstream mediators. In addition, we show for the first time that EGFR, c-Src, and ERK1/2 are directly activated by a mechanism involving cPLA2-mediated arachidonic acid release initiated by the release of βγ dimers in rabbit proximal tubule cells. Moreover, our results identify a new and novel alternative mechanism involving arachidonic acid-induced transactivation of EGFR via inducing association of the SH2 domain of c-Src with EGFR, independently of Gβγ subunits. This reprents an important alternative paradigm whereby GPCRs linked to phospholipases may activate a tyrosine kinase pathway through arachidonic acid independent of a Gβγ subunit.

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