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G Protein Coupling and Second Messenger Generation Are Indispensable for Metalloprotease-dependent, Heparin-binding Epidermal Growth Factor Shedding through Angiotensin II Type-1 Receptor*

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A G protein-coupled receptor agonist, angiotensin II (AngII), induces epidermal growth factor (EGF) receptor (EGFR) transactivation possibly through metalloprotease-dependent, heparin-binding EGF (HB-EGF) shedding. Here, we have investigated signal transduction of this process by using COS7 cells expressing an AngII receptor, AT_1. In these cells AngII-induced EGFR transactivation was completely inhibited by pretreatment with a selective HB-EGF inhibitor, or with a metalloprotease inhibitor. We also developed a COS7 cell line permanently expressing a HB-EGF construct tagged with alkaline phosphatase, which enabled us to measure HB-EGF shedding quantitatively. In the COS7 cell line AngII stimulated release of HB-EGF. This effect was mimicked by treatment either with a phospholipase C activator, a Ca^{2+} ionophore, a metalloprotease activator, or H_2O_2. Conversely, pretreatment with an intracellular Ca^{2+} antagonist or an antioxidant blocked AngII-induced HB-EGF shedding. Moreover, infection of an adenovirus encoding an inhibitor of G_q markedly reduced EGFR transactivation and HB-EGF shedding through AT_1. In this regard, AngII-stimulated HB-EGF shedding was abolished in an AT_1 mutant that lacks G_q protein coupling. However, in cells expressing AT_1 mutants that retain G_q protein coupling, AngII is still able to induce HB-EGF shedding. Finally, the AngII-induced EGFR transactivation was attenuated in COS7 cells overexpressing a catalytically inactive mutant of ADAM17. From these data we conclude that AngII stimulates a metalloprotease ADAM17-dependent HB-EGF shedding through AT_1/G_q/phospholipase C-mediated elevation of intracellular Ca^{2+} and reactive oxygen species production, representing a key mechanism indispensable for EGFR transactivation.

Angiotensin II (AngII)[1] and its G protein-coupled receptor (GPCR), the AngII type-1 receptor (AT_1), play critical roles in mediating cardiovascular diseases such as hypertension, atherosclerosis, and restenosis after vascular injury (1, 2). It is widely believed that AngII promotes these diseases by inducing vascular remodeling that involves hypertrophy, hyperplasia, and migration of vascular smooth muscle cells (VSMCs) (3, 4). We and others have shown that AngII promotes these cellular effects by "trans"-activation of the epidermal growth factor receptor (EGFR) through the AT_1 receptor (5, 6). Similar to EGF stimulation, AngII transactivates EGFR, which recruits the adaptor proteins Shc and Grb2, leading to the activation of the extracellular signal-regulated kinase (ERK) cascade (7). Moreover, EGFR transactivation by AngII also leads to critical signaling responses such as activation of Akt/protein kinase B, p70 S6 kinase, and p38 mitogen-activated protein kinase (MAPK) in VSMCs (5, 8, 9). These data suggest that EGFR transactivation is one of the main points of convergence by which AngII induces several pathophysiological functions in its target organs (10).

Recently, several interesting observations have been made regarding the possible components involved in EGFR transactivation by GPCRs. First, EGFR transactivation by GPCRs appears to require a second messenger directly and/or signal transduction pathways operated by second messengers, such as elevation of intracellular Ca^{2+} (11), activation of protein kinase C (12), and generation of reactive oxygen species (ROS) (13). In this regard, the EGFR transactivation by AngII seems to involve elevation of intracellular Ca^{2+} concentration and production of ROS in VSMCs (7, 14–16). Second, a cytosolic nonreceptor tyrosine kinase such as Src or PYK2 may be involved in the EGFR transactivation (17, 18). Both kinases have been

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[1] The abbreviations used are: AngII, angiotensin II; AP, alkaline phosphatase; ADAM, a disintegrin and metalloprotease; APMA, *p*-aminophenylmercuric acetate; AT_1, AngII type 1 receptor; BiPS, 2*R*-[(4-biphenylsulfonyl)amino]-*N*-hydroxy-3-phenylpropionamide; $[Ca^{2+}]_i$, intracellular Ca^{2+}; dn, dominant-negative; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; HB-EGF, heparin-binding EGF; GPCR, G protein-coupled receptor; GqI, inhibitor of G_q signaling; MAPK, mitogen-activated protein kinase; m.o.i., multiplicity of infection; m-3M3FBS, 2,4,6-trimethyl-*N*-(*m*-3-trifluoromethylphenyl)benzenesulfonamide; NAC, *N*-acetylcysteine; PLC, phospholipase C; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell; WT, wild type.

shown to be activated by AngII in VSMCs (19–21). Third, an attractive mechanism for the EGFR transactivation by a GPCR was proposed recently that involves metalloprotease-dependent EGFR ligand production from its membrane-bound precursor (9, 22).

The EGF ligand family consists of EGF, heparin binding-EGF like growth factor (HB-EGF), transforming growth factor-α, epiregulin, amphiregulin, epigen, neuregulins, and betacellulin (23). Among these, HB-EGF has been most implicated in vascular remodeling because it is a potent mitogen and chemotactic factor for VSMCs and its expression is enhanced in vascular lesions such as atherosclerosis and restenosis following angioplasty (24, 25). Like other members of the EGF ligand family, HB-EGF is synthesized as a transmembrane precursor "pro-HB-EGF" that is proteolytically cleaved ("shedding") to release a biologically active soluble growth factor (26). Recently, many GPCR agonists appear to mediate EGFR transactivation through this metalloprotease-dependent HB-EGF shedding (27). We and others also showed the requirement of HB-EGF for EGFR transactivation through the AT_1 receptor (9, 28, 29). However, the identity of the metalloprotease as well as the detailed signaling mechanisms of HB-EGF shedding by AngII in relation to G protein coupling, second messengers, and upstream kinases are largely unknown.

In this study we established a COS7 cell line expressing alkaline phosphatase (AP)-conjugated HB-EGF that enabled us to measure the HB-EGF shedding activity quantitatively. By using this system together with molecular and pharmacological tools including several AT_1 receptor mutants, we have elucidated the involvement of heterotrimetric G protein coupling and second messengers (Ca^{2+} and ROS) in a critical step of a metalloprotease-dependent HB-EGF production. The findings presented here will provide a novel molecular insight by which AngII contributes to cardiovascular diseases.

EXPERIMENTAL PROCEDURES

Materials—Phospho-specific antibodies for Tyr^{1068}-phosphorylated EGFR and for Tyr^{1007}-Tyr^{1008}-phosphorylated JAK2 were purchased from BIOSOURCE. Antibody against EGFR was purchased from Santa Cruz Biotechnology. Antibody against hemagglutinin was purchased from Zymed Laboratories Inc.. YM-254890 was a gift from Yamanouchi Pharmaceutical Co. AngII, *N*-acetylcysteine (NAC), and H_2O_2 were purchased from Sigma. A23187, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester, GF109203X, TMB-8, phorbol 12-myristate 13-acetate, CRM197, m-3M3FBS, *p*-aminophenylmercuric acetate (APMA), and 2*R*-[(4-biphenylsulfonyl)amino]-*N*-hydroxy-3-phenylpropionamide (BiPS) were purchased from Calbiochem. BiPS was originally described as a matrix metalloprotease-2 and matrix metalloprotease-9 inhibitor (30). However, our subsequent findings demonstrated the ability of BiPS to inhibit EGFR transactivation by AngII that was not mediated by matrix metalloproteases (31). BiPS shares its structure with CGS27023, which can also inhibit EGFR transactivation by AngII (31). CGS27023 was demonstrated to block the catalytic activity of ADAM9 and ADAM17 (32), suggesting that BiPS could act as an ADAM inhibitor as well.

Cell Lines—COS7 cells were obtained from the American Type Culture Collection and subcultured as described previously (33). COS7 cells permanently expressing HB-EGF were established by antibiotic selection after transfection with the AP-tagged HB-EGF (HBEGF-AP) plasmid (34) as described previously (33). A Chinese hamster ovary cell line stably expressing wild type rat AT_1 (AT_1WT) and its deletion mutants, AT_1-(1–309) and AT_1-(1–317), were established as described previously (35). The AT_1 receptors expressed in these cells have a comparable K_d and B_{max} (35).

Adenoviral Infection—Adenovirus constructs encoding wild type rat AT_1 receptor and a carboxyl-terminal mutant, AT_1Y319F, in which carboxyl-terminal Tyr^{319} was replaced with Phe^{319} were generated as described previously (29, 36, 37). The adenoviral vector containing the inhibitor of G_q signaling (GqI), comprised of the amino acids 305–359 of murine $G\alpha_q$, was constructed as described previously (38). Each adenovirus titer (m.o.i.) was determined by Adeno-X™ rapid titer kit (BD Biosciences). Confluent COS7 cells were infected with adenovirus at 50–100 m.o.i. for 2 days as described previously (33). Transfection efficiency was estimated to be >95% as defined by infection with adenovirus (50 m.o.i.) encoding green fluorescent protein.

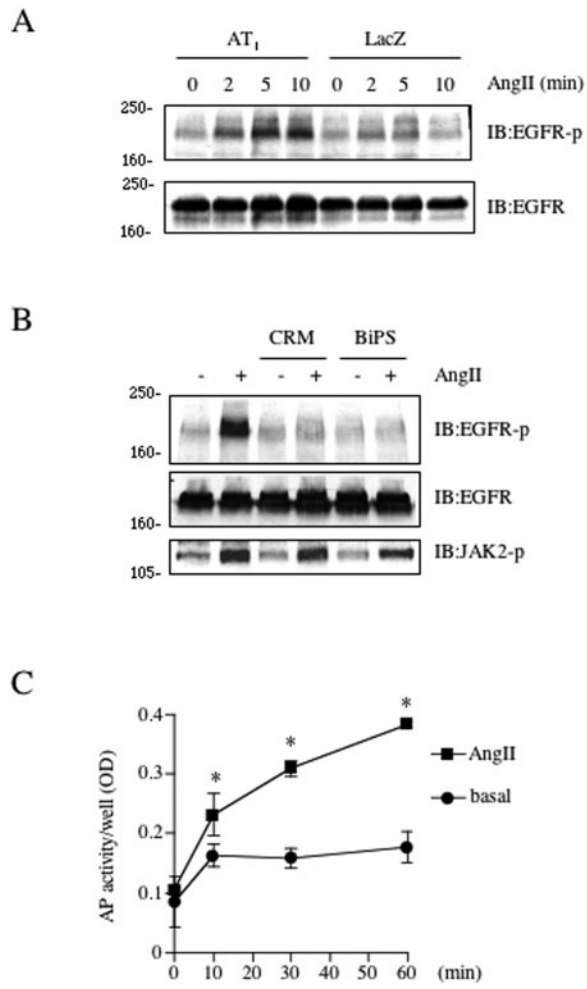

FIG. 1. **Involvement of metalloprotease-dependent HB-EGF shedding in AngII-induced EGFR transactivation in COS7 cells expressing the AT_1 receptor.** *A*, COS7 cells infected with an adenovirus (100 m.o.i.) encoding the AT_1 receptor or control LacZ were stimulated by AngII (100 nM) for the indicated time periods. Cell lysates were immunoblotted (*IB*) with antibodies against Tyr^{1068}-phosphorylated EGFR (*EGFR-p*) and total EGFR as indicated. *B*, COS7 cells infected with adenovirus encoding AT_1 receptor (100 m.o.i.) were pretreated with a selective inhibitor of HB-EGF, CRM197 (10 μg/ml), or a metalloprotease inhibitor, BiPS (10 μM), for 30 min and stimulated with AngII (100 nM) for 5 min. Cell lysates were immunoblotted (*IB*) with antibodies against Tyr^{1068}-phosphorylated EGFR (*EGFR-p*), total EGFR, and Tyr^{1007}-Tyr^{1008}-phosphorylated JAK2 (*JAK2-p*) as indicated. *C*, COS7 cells permanently expressing HBEGF-AP construct were infected with AT_1 receptor adenovirus (100 m.o.i.), and stimulated with or without AngII (100 nM) for the indicated time period. AP activity in the medium was determined. Results are the means ± S.E. ($n = 3$). *, $p < 0.05$ as compared with non-stimulated control at each time point. *basal*, control sample.

Retroviral Infection—C-terminal hemagglutinin-tagged catalytically inactive/dominant negative ADAM17 (dnADAM17), in which Glu^{406} was replaced with Ala (39), was cloned into the pBM-IRES-PURO retroviral vector (40). For retroviral infection, 4×10^5 cells were seeded into 25-cm^2 tissue culture flasks and cultured for 24 h prior to infection. Cells were incubated with 5 ml of virus stock for 12 h in the presence of 4 μg/ml Polybrene and then replenished with fresh media. Cells were then grown for 48 h prior to passaging into media containing 6 μg/ml puromycin. Resistant cells were used in subsequent experiments (40).

Plasmid Transfection—Plasmids encoding rat AT_1WT and AT_1Y319F were generated as described previously (35, 41). COS7 cells were transiently transfected with the plasmids by using FuGENE6 (Roche Applied Science) for 24 h with 10% serum, and then the cells were serum-starved for 24 h before stimulation.

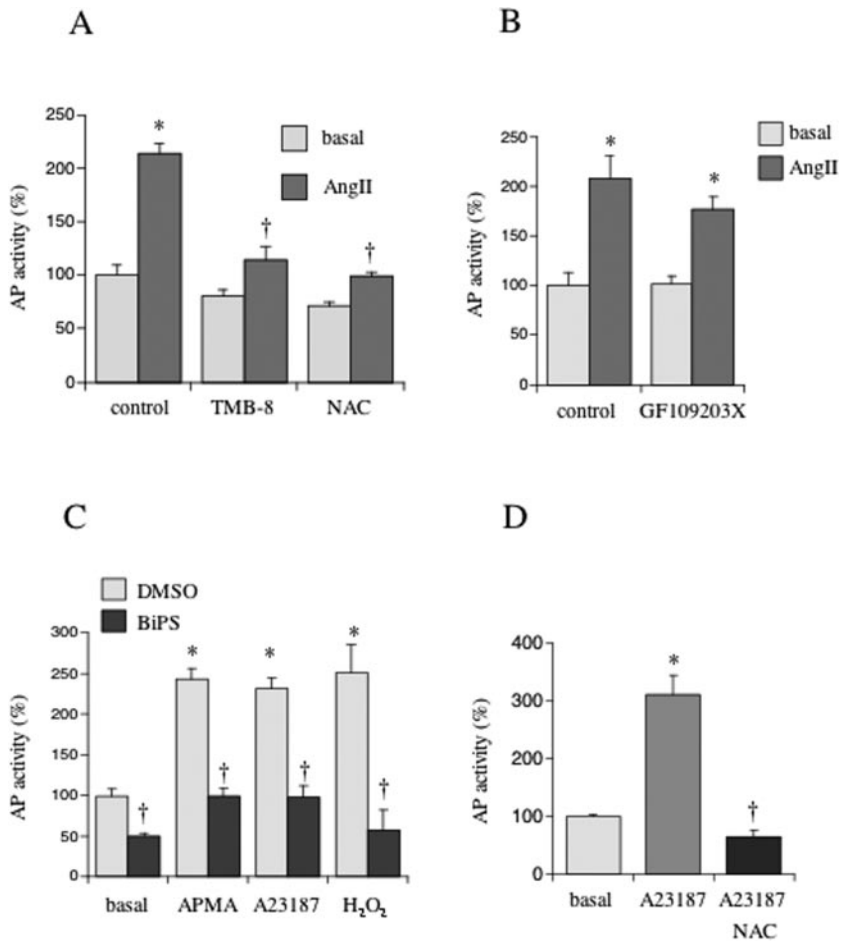

FIG. 2. **Involvement of second messengers in AT_1 receptor-mediated HB-EGF shedding.** *A* and *B*, COS7-HBEGF-AP cells were infected with AT_1 receptor adenovirus (100 m.o.i.). After pretreatment with an intracellular Ca^{2+} antagonist, TMB-8 (100 nM), or an antioxidant, NAC (20 mM), for 30 min (*A*) or with a protein kinase C inhibitor, GF109203X (2 μM) (*B*), cells were stimulated with AngII (100 nM) for 60 min, and AP activity in the medium was determined. *C*, after pretreatment with BiPS (10 μM) or its vehicle, Me_2SO (*DMSO*; 0.1%), for 30 min, COS7-HBEGF-AP cells were stimulated with a metalloprotease activator, APMA (30 μM), a Ca^{2+} ionophore, A23187 (10 μM), or H_2O_2 (1 mM) for 60 min, and AP activity in the medium was determined. *D*, COS7-HBEGF-AP cells were pretreated with or without NAC (20 mM) for 30 min, cells were stimulated with A23187 (10 μM) for 60 min, and AP activity in the medium was determined. Results are the means ± S.E. (n = 3). *, $p < 0.05$ as compared with the basal control; †, $p < 0.05$ as compared with the stimulated control.

HB-EGF Shedding Assay—48 h after AT_1 receptor transfection, COS7-HBEGF-AP cells were pre-incubated in fresh phenol red-free Dulbecco's modified Eagle's medium for 30 min in the presence or absence of inhibitors and then stimulated by agonists up to 60 min. The HB-EGF-AP secreted into the medium was assessed by measuring alkaline phosphatase activity as described previously (33).

Western Blotting—Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane as described previously (42). The membranes were then exposed to primary antibodies overnight at 4 °C. After incubation with the peroxidase-linked secondary antibody for 1 h at room temperature, the immunoreactive proteins were visualized by a chemiluminescence reaction kit (Chemicon).

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) Measurements—$[Ca^{2+}]_i$ was measured as described previously (43). VSMCs subcultured on coverslips were loaded in Hanks' balanced salt solution with 3 μM fura 2/AM at room temperature for 45 min in the dark and then washed three times with fura 2-free Hanks' balanced salt solution to allow for complete de-esterification of the dye for 15–60 min. Under these conditions, compartmentalization of the dye was minimal. The coverslips were mounted in a custom-designed bath in the stage of a S300 Axiovert Nikon inverted microscope equipped with a C & L Instruments fluorometer system. The fura 2 fluorescence was acquired at a frequency of 1 Hz, and $[Ca^{2+}]_I$ values were then obtained as described (43).

Statistic—Data were analyzed by using the Student's t test. The mean ± S.E. was determined with a significance level of $p < 0.05$. Results are representative of at least three separate experiments.

RESULTS

Metalloprotease and HB-EGF-dependent EGFR Transactivation by AngII in COS7 Cells—Using COS7 cells, we have established a system to examine the mechanism of EGFR transactivation through a GPCR, AT_1. For this purpose, COS7 cells were infected with an adenovirus encoding AT_1. Compared with the COS7 cells infected with the control adenovirus encoding LacZ, AngII stimulation resulted in marked phosphorylation of the EGFR at Tyr^{1068}, a Grb2-binding site, in COS7 cells expressing AT_1 in a time-dependent manner (Fig. 1*A*). To determine the involvement of metalloprotease activation in AngII-induced EGFR transactivation in COS7 cells, the effect of a metalloprotease inhibitor, BiPS, on EGFR phosphorylation at Tyr^{1068} was examined. As we observed previously in VSMCs (31), BiPS completely inhibited AngII-induced EGFR transactivation in COS7 cells (Fig. 1*B*). To demonstrate the critical involvement of HB-EGF in AngII-induced EGFR transactivation, the effect of a diphtheria toxin analogue, CRM197, which acts as a specific inhibitor against primate HB-EGF was examined in regard to EGFR phosphorylation. Pretreatment of CRM197 markedly inhibited AngII-induced EGFR transactivation in COS7 cells expressing AT_1 (Fig. 1*B*). In contrast, both BiPS and CRM197 did not affect JAK2 phosphorylation at Tyr^{1007}-Tyr^{1008} stimulated by AngII. These results clearly demonstrated that COS7 cells expressing AT_1 provide an interesting model for studying the mechanism of AngII-induced EGFR transactivation involving metalloprotease-dependent HB-EGF production.

To examine the detailed mechanism of metalloprotease-dependent HB-EGF shedding, we took advantage of a reporter assay system using transfection of the HB-EGF-AP plasmid, an established assay for HB-EGF shedding (34). In COS7 cells permanently expressing this plasmid, we evaluated the shedding activity of HB-EGF by measuring AP activity secreted into the medium. In these cells, there is a gradual but statistically significant accumulation of AP activity in a non-stimulated condition during pre-incubation, suggesting a presence of basal shedding activity of HB-EGF. However, basal shedding was not further enhanced up to 60 min, suggesting a saturated nature

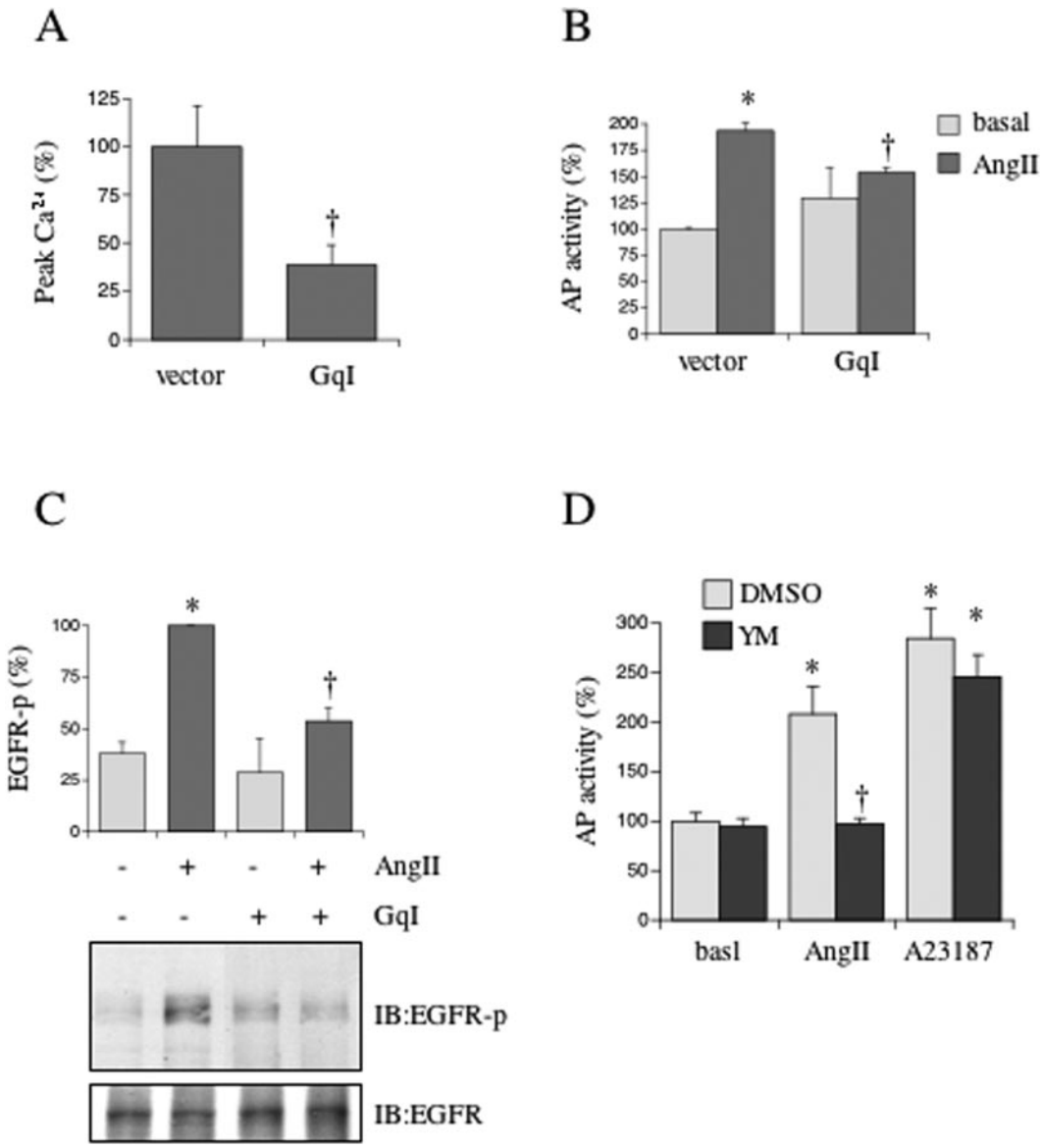

FIG. 3. **Requirement of G_q activation for HB-EGF shedding and EGFR transactivation by AngII.** *A*, COS7 cells were infected with adenovirus encoding AT_1 receptor (100 m.o.i.) together with GqI (50 m.o.i.) or control vector (50 m.o.i.). Cells were stimulated with 100 nM AngII, and peak stimulation of intracellular Ca^{2+} concentration was determined. *B*, COS7-HBEGF-AP cells were infected with adenovirus encoding AT_1 receptor (100 m.o.i.) together with GqI (50 m.o.i.) or control vector (50 m.o.i.). The cells were stimulated with AngII (100 nM) for 60 min, and AP activity in the medium was determined. *C*, COS7 cells with the infection described above were stimulated with 100 nM AngII for 5 min. Cell lysates were immunoblotted (*IB*) with antibodies against Tyr^{1068}-phosphorylated EGFR (*EGFR-p*) and total EGFR as indicated. *D*, COS7-HBEGF-AP cells were infected with AT_1 receptor adenovirus (100 m.o.i.). After pretreatment with a selective Gq inhibitor, YM-254890 (*YM*; 10 μM), or its vehicle, Me_2SO (*DMSO*; 0.1%), for 10 min, cells were stimulated with AngII (100 nM) for 60 min, and AP activity in the medium was determined. Results are the means ± S.E. ($n = 3$). *, $p < 0.05$ as compared with the basal control; †, $p < 0.05$ as compared with the stimulated control.

of the basal shedding activity. In contrast, a marked and time-dependent enhancement of AP activity was observed when cells were treated with AngII, thus demonstrating the ability of AngII to stimulate HB-EGF shedding. In addition, AngII-induced HB-EGF shedding was completely inhibited by BiPS (data not shown). These findings further indicated that the HB-EGF-AP assay system in a cell line expressing AT_1 would be an ideal tool to study the signal transduction mechanism of metalloprotease-dependent HB-EGF shedding and subsequent EGFR transactivation by AngII.

AngII Stimulates HB-EGF Shedding through Intracellular Ca^{2+} Elevation and ROS Production—Previous studies have shown the involvement of Ca^{2+} and ROS as critical signal intermediates in EGFR transactivation through AT_1 in VSMCs (7, 14, 15). Also, protein kinase C may exist upstream of the ROS production (16). In COS7 cells, AngII-induced HB-EGF shedding was completely blocked by pretreatment with TMB-8, an intracellular Ca^{2+} antagonist, as well as by NAC, a potent antioxidant (Fig. 2*A*). In contrast, a protein kinase C inhibitor, GF109203X, had no significant effect on AngII-induced HB-EGF shedding (Fig. 2*B*), whereas it markedly inhibited 100 nM phorbol 12-myristate 13-acetate-induced HB-EGF shedding in the COS7 cells (data not shown). Stimulation with a metalloprotease activator, APMA, a Ca^{2+} ionophore, A23187, and H_2O_2 resulted in enhanced HB-EGF shedding that was inhibited by pretreatment with BiPS. The basal shedding activity was also partially inhibited by BiPS (Fig. 2*C*). In addition, we confirmed that APMA and A23187 as well as H_2O_2 induced EGFR transactivation in COS7 cells (data not shown). Moreover, HB-EGF shedding stimulated by A23187 was completely blocked by NAC (Fig. 2*D*), whereas H_2O_2-induced HB-EGF shedding was minimally affected by TMB-8 (data not shown).

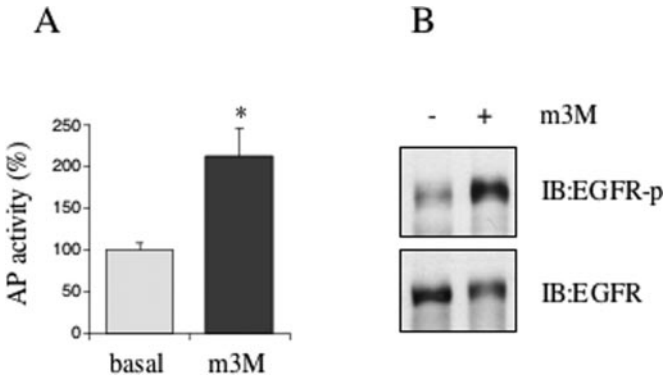

FIG. 4. **A selective PLC agonist stimulates HB-EGF shedding and EGFR transactivation.** *A*, COS7-HBEGF-AP cells were stimulated with a selective PLC agonist, m-3M3FBS (*m3M*; 50 μM), for 60 min, and AP activity in the medium was determined. *B*, COS7 cells were stimulated with m-3M3FBS (*m3M*; 50 μM) for 10 min. Cell lysates were immunoblotted with antibodies against Tyr^{1068}-phosphorylated EGFR (*EGFR-p*) and total EGFR as indicated. Results are the means ± S.E. ($n = 3$). *, $p < 0.05$ as compared with the basal control.

These results suggest that, in COS7 cells, intracellular Ca^{2+} elevation and subsequent ROS production are required for a metalloprotease activation that is responsible for EGFR transactivation through AT_1.

Requirement of G_q Coupling for HB-EGF Shedding through AT_1—In addition to G_q, AT_1 has been shown to couple G_i, G_{12}, and/or G_{13}, depending on cell type (44, 45). Although intracellular Ca^{2+} elevation through AT_1 is primarily believed to require $G\alpha_q$/PLC-β, additional mechanisms involving $G\alpha_{12/13}$, $G\beta\gamma$, PLC-γ, and/or L-type Ca^{2+} channel have been proposed (44). Activation of G_q has been proposed to participate in a GPCR-induced EGFR transactivation (46), whereas possible participation of the $G\beta\gamma$ subunit was reported in a metalloprotease/EGFR-dependent ERK activation by α_{2A}-adrenergic receptor in human embryonic kidney cells (47). Therefore, we examined the contribution of G_q signaling in HB-EGF shedding induced by AngII by using an adenovirus encoding the C-terminal fragment of $G\alpha_q$ termed GqI, a selective G_q inhibitor (48), as well as a novel G_q selective pharmacological inhibitor, YM-254890 (49). GqI markedly inhibited intracellular Ca^{2+} elevation (Fig. 3*A*), HB-EGF shedding (Fig. 3*B*), and EGFR transactivation (Fig. 3*C*) induced by AngII through AT_1. YM-254890 also inhibited HB-EGF shedding induced by AngII but not by A23187 (Fig. 3*D*). In addition, a selective PLC activator, m-3M3FBS (50), stimulated HB-EGF shedding (Fig. 4*A*) as well as EGFR transactivation (Fig. 4*B*) in COS7 cells. These data suggest the requirement of G_q-dependent PLC-β activation for the metalloprotease activation involved in EGFR transactivation operated through AT_1.

Effect of AT_1 Mutants on HB-EGF Shedding—Analysis of AT_1 mutation has been proven to be a useful tool for studying the mechanistic insights not only of ligand or G protein interaction but also of receptor internalization/desensitization as well as downstream signal transduction (10, 51). By using several AT_1 receptor truncation mutants, a previous study demonstrated that a particular cytosolic carboxyl-terminal region (310–317) of the AT_1 receptor is essential for G protein coupling and activation of G_q (35). We found that AngII-induced HB-EGF shedding remained intact in cells stably expressing a truncated mutant that still contains G protein coupling, AT_1-(1–317), but was abolished in cells stably expressing AT_1-(1–309), which lacks G protein coupling (Fig. 5*A*). The AT_1 receptors expressed in these cells have a comparable K_d and B_{max} (35). We also confirmed that AngII-induced EGFR transactivation remained intact in COS7 cells expressing AT_1-(1–317) (data not shown). However, recent studies using distinct mutants of AT_1 demonstrated G protein-independent activation of tyrosine kinases (36, 37, 52). In particular, it has been reported that in a mutant of a conserved YIPP motif in the C terminus of AT_1, AT_1Y319F, EGFR transactivation was attenuated, whereas G_q coupling remained intact (37). By contrast, we observed comparable HB-EGF shedding by AT_1Y319F in COS7 cells (Fig. 5*B*). We also confirmed that AT_1Y319F was able to stimulate EGFR transactivation (Fig. 5*C*) and intracellular Ca^{2+} elevation (Fig. 5*D*). Thus, these results support our notion that a metalloprotease responsible for HB-EGF shedding and subsequent EGFR transactivation by AT_1 is triggered through the interaction between G_q and AT_1 upon AngII binding.

Involvement of ADAM17 in EGFR Transactivation through AT_1—Several ADAM family metalloproteases have been implicated in the HB-EGF shedding required for EGFR transactivation by distinct GPCR agonists (53–56). Because ADAM17 has been critically implicated in HB-EGF shedding (57, 58), we have examined the involvement of this metalloprotease in EGFR transactivation induced by AngII. As shown in Fig. 6*A*, transfection of dnADAM17 but not control vector by retrovirus significantly inhibited EGFR transactivation induced by AngII through AT_1. Also, EGFR transactivation induced by A23187 and H_2O_2 but not stimulation by EGF was blocked by dn-ADAM17 (Fig. 6*B*). Taken together, ADAM17 represents a metalloprotease sensitive to AT_1/G_q-operated second messenger signals, leading to the EGFR transactivation.

DISCUSSION

In the present study we have established a cell system that enabled us to elucidate a signal transduction mechanism of metalloprotease-dependent HB-EGF shedding by AngII, an indispensable step for EGFR transactivation via the AT_1 receptor. Although there are several reports thus far describing the role of metalloproteases in mediating EGFR transactivation and subsequent functions induced by many GPCRs (53–56), very few of them have focused on the immediate signal transductions (G protein-coupling and second messengers) required for the metalloprotease activation by a GPCR agonist. We found that G_q-mediated intracellular Ca^{2+} mobilization and ROS production are essential for metalloprotease-dependent HB-EGF production by a GPCR, AT_1.

In the EGF ligand family, HB-EGF has been intensively reported to be a critical EGFR ligand for EGFR transactivation (22, 26, 27). However, there is the possibility for the participation of other EGF ligands such as betacellulin, which promotes VSMC proliferation (59, 60) and migration (61) and is expressed in VSMCs (61) and atherosclerotic lesions (60, 62). However, in our system EGFR transactivation by AngII was completely inhibited by pretreatment with CRM197. These results suggest that HB-EGF production may largely participate in EGFR transactivation within COS7 cells stimulated through the AT_1 receptor. This notion is in good agreement with similar findings in cultured VSMCs (7), neonatal myocytes (29, 53), and endothelial cells (28), where AngII physiologically transactivates EGFR. Thus, our system in COS7 cells could represent a useful model to study the molecular mechanism of the EGFR transactivation.

Because previous studies suggested the involvement of second messengers in AngII-induced EGFR transactivation in VSMCs (5, 16, 63), we have further investigated their relation toward the metalloprotease activation. Our results presented here suggest that G_q/PLC-mediated intracellular Ca^{2+} elevation and ROS production but not protein kinase C activation are required for HB-EGF shedding mediated through AT_1. However, taken together with the previous finding that HB-

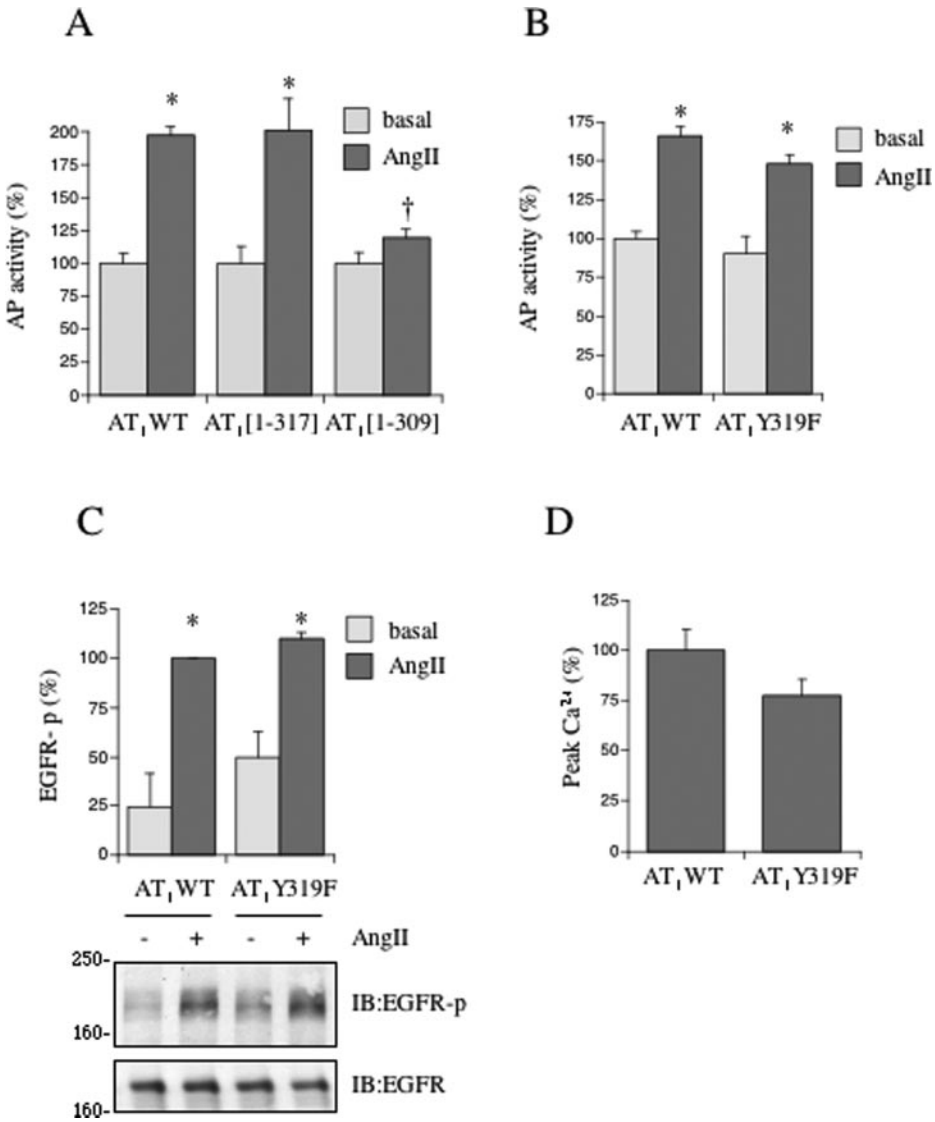

FIG. 5. **Effect of AT[1] receptor mutation on AngII-induced HB-EGF shedding and EGFR transactivation.** *A*, Chinese hamster ovary cells stably expressing AT_1WT, AT_1-(1–309), or AT_1-(1–317) were transiently transfected with the HBEGF-AP cDNA construct for 48 h and stimulated with AngII (100 nM) for 60 min. AP activity in the medium was determined. *B*, COS7-HBEGF-AP cells were transfected with AT_1WT or AT_1Y319F and stimulated with AngII (100 nM) for 60 min. AP activity in the medium was determined. *C*, COS7 cells infected with adenovirus encoding AT_1WT (100 m.o.i.) or AT_1Y319F (100 m.o.i.) were stimulated with AngII (100 nM) for 5 min. Cell lysates were immunoblotted (*IB*) with antibodies against Tyr^{1068}-phosphorylated EGFR (*EGFR-p*) and total EGFR as indicated. *D*, COS7 cells infected with above adenovirus were stimulated with AngII (100 nM), and peak stimulation of intracellular Ca^{2+} concentration was determined. Results are the means ± S.E. ($n = 3$). *, $p < 0.05$ as compared with the basal control; †, $p < 0.05$ as compared with the stimulated control.

EGF shedding can be induced by activation of protein kinase C (24), elevation of intracellular Ca^{2+} (64), or ROS generation (33, 65) in distinct cell systems, it is likely that one or more metalloprotease(s) might be responsible for EGFR transactivation by extracellular stimuli depending on the type of cells or tissues utilized.

By using deletion mutants of the AT_1 receptor, we have demonstrated the requirement of a C-tail structure between residues 310–317 for HB-EGF shedding by AngII, whereas additional C-tail sequences are dispensable. Previous studies with AT_1 receptor mutants showed that these C-tail lesions close to the seventh transmembrane region is essential for G_q coupling (35), thus confirming the important role of G_q-mediated second messengers for AT_1-operated HB-EGF shedding. The HB-EGF shedding was further blocked by selective G_q inhibitors in the present study. In conflict with our findings, several recent reports have argued against the requirement of G protein-derived second messengers for tyrosine kinase activation thorough the AT_1 receptor (36, 37, 52). Seta and Sadoshima showed that phosphorylation of the AT_1 receptor at Tyr^{319} is a prerequisite for EGFR transactivation because it can provide a docking site for protein-protein interaction, a proposed mechanism for the transactivation (37). However, this mutant is able to stimulate HB-EGF shedding and EGFR transactivation in our study. The mutant also stimulated ERK in COS7 cells that was blocked by an EGFR kinase inhibitor or a metalloprotease inhibitor (66). The discrepancy may be due to the different nature of the cells utilized together with distinct experimental strategies (Seta and Sadoshima used cells expressing an exogenous EGFR gene) (37).

Several ADAM family metalloproteases have been identified as mediating EGFR ligand shedding and/or EGFR transactivation in response to distinct GPCR agonists (53–56). Our data using a dnADAM17 mutant suggest that ADAM17, at least in part, mediates AngII-induced HB-EGF shedding and subsequent EGFR transactivation in COS7 cells. However, Yan *et al.* have reported that a GPCR agonist, bombesin, induces ADAM10-dependent HB-EGF shedding and EGFR transactivation in COS7 cells (67). Although bombesin receptors expressed in mammalian cells mainly coupled to G_q (68), no information was provided regarding the upstream signal transduction pathway leading to ADAM10 activation by bombesin except for an inducible association event between CD9, ADAM10, and HB-EGF in response to bombesin (67). Thus far, only one paper has reported the identity of ADAM responsible for HB-EGF shedding and EGFR transactivation induced by AngII, and it appears to be ADAM17 but not ADAM10 in a kidney carcinoma cell line, ACHN (69). However, this study also lacks any information regarding the upstream events of the ADAM17 activation. Recent reports also suggest ADAM17 as the major sheddase of HB-EGF, whereas ADAM10 is suggested to be the major sheddase of EGF and betacellulin (40, 70, 71), thus

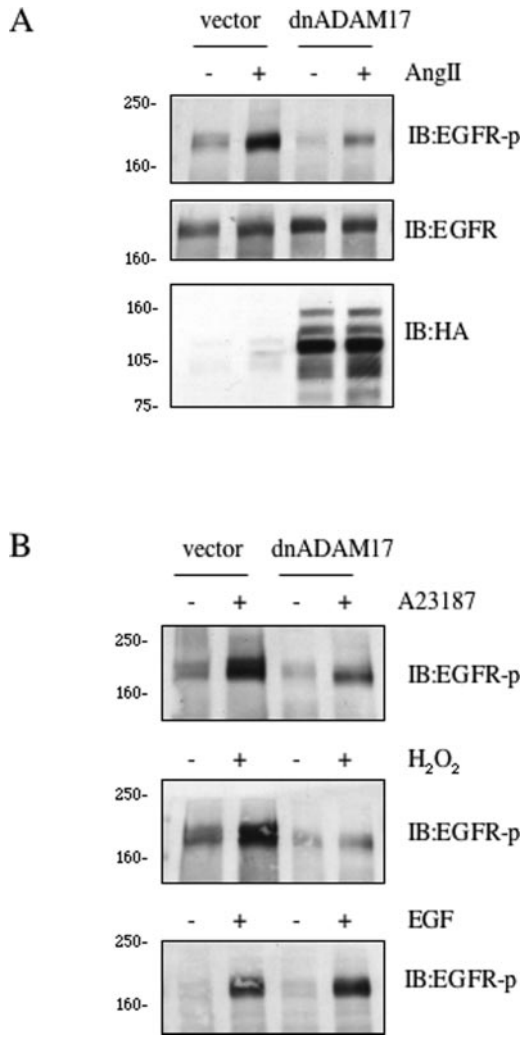

FIG. 6. **Role of ADAM17 in AngII-induced EGFR transactivation.** *A*, COS7 cells stably expressing control vector or dnADAM17 were infected with adenovirus (100 m.o.i.) encoding the AT_1 receptor and stimulated with 100 nM AngII for 5 min. Cell lysates were immunoblotted (*IB*) with antibodies against Tyr^{1068}-phosphorylated EGFR (*EGFR-p*), total EGFR, and hemagglutinin (*HA*) as indicated. *B*, COS7 cells stably expressing control vector or dnADAM17 were stimulated with A23187 (10 μM), H_2O_2 (1 mM), or EGF (10 ng/ml). Cell lysates were immunoblotted (*IB*) with antibodies against Tyr^{1068}-phosphorylated EGFR (*EGFR-p*) as indicated.

supporting the role of ADAM17 in mediating AngII-induced EGFR transactivation.

How does the upstream signal transduction pathway(s) activate ADAM17 in response to AngII? We have proposed the role of ROS production as a critical step of the HB-EGF shedding and EGFR transactivation in COS7 cells as well as in VSMCs (5, 63). Our data presented here further suggest that ROS production by AngII could lead to ADAM17 activation. ADAM could be activated by phosphorylation and/or association with Src homology 3 domain-containing adaptors (26, 72). Therefore, AngII-produced ROS may activate ROS-sensitive protein kinases to phosphorylate an intracellular region of ADAM17- and/or ADAM-associated adaptor proteins that trigger the catalytic activation of ADAM17. To support this theory, Fisher *et al.* recently demonstrated that H_2O_2-induced EGFR transactivation was blocked by a p38 MAPK inhibitor (65). However, the requirement of p38 MAPK for AngII-induced activation of ADAM17 is unlikely, because we have shown that p38 MAPK exists downstream of the metalloprotease-dependent EGFR transactivation induced by AngII (9). In fact, we could not block HB-EGF shedding induced by AngII in COS7 cells by using a p38 MAPK inhibitor,[2] suggesting the presence of other ROS-sensitive kinase(s) required for ADAM17 activation. This discrepancy may be due to the difference between the extracellular addition of ROS and intracellular production of ROS by AngII under the experimental conditions. In this regard, Fisher *et al.* showed that not only ADAM17 but also ADAM10 is involved in H_2O_2-induced EGFR transactivation (65). The other candidate kinase could be a Src family kinase. c-Src was implicated in a metalloprotease/EGFR-dependent ERK activation by α_{2A}-adrenergic receptor (47). Alternatively, ROS could directly activate ADAM17 by oxidizing electrophilic thiol groups critical for ADAM17 activity, such as through regulation of the chaperone or inhibitory functions of the prodomain (73–75).

In summary, we have demonstrated that AT_1-mediated G_q activation and resultant intracellular Ca^{2+} elevation and ROS generation are essential for AngII-induced HB-EGF shedding. Because HB-EGF-dependent EGFR transactivation participates in cardiovascular remodeling, our data presented here will provide novel therapeutic targets, such as second messenger-sensitive ADAM17, for treatment and/or prevention of cardiovascular diseases.

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G Protein Coupling and Second Messenger Generation Are Indispensable for Metalloprotease-dependent, Heparin-binding Epidermal Growth Factor Shedding through Angiotensin II Type-1 Receptor

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