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Differential Pathways of Angiotensin II-Induced Extracellularly Regulated Kinase 1/2 Phosphorylation in Specific Cell Types: Role of Heparin-Binding Epidermal Growth Factor

BUKHTIAR H. SHAH, AKIN YESILKAYA, J. ALBERTO OLIVARES-REYES, HUNG-DAR CHEN, LÁSZLÓ HUNYADY, AND KEVIN J. CATT

Endocrinology and Reproduction Research Branch (B.H.S., H.-D.C., K.J.C.), National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-4510; Akdeniz Universitesi (A.Y.), Tip Fakultesi, Biyokimya Anabilim Dali, 07070 Antalya, Turkey; Departamento de Bioquímica (J.A.O.-R.), Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Apartado Postal 14-740 México; and Department of Physiology (L.H.), Faculty of Medicine, Semmelweis University, H-1444 Budapest, Hungary

Stimulation of the angiotensin II (Ang II) type 1 receptor (AT_1-R) causes phosphorylation of extracellularly regulated kinases 1 and 2 (ERK1/2) via epidermal growth factor receptor (EGF-R) transactivation-dependent or -independent pathways in Ang II target cells. Here we examined the mechanisms involved in agonist-induced EGF-R transactivation and subsequent ERK1/2 phosphorylation in clone 9 (C9) hepatocytes, which express endogenous AT_1-R, and COS-7 and human embryonic kidney (HEK) 293 cells transfected with the AT_1-R. Ang II-induced ERK1/2 activation was attenuated by inhibition of Src kinase and of matrix metalloproteinases (MMPs) in C9 and COS-7 cells, but not in HEK 293 cells. Agonist-mediated MMP activation in C9 cells led to shedding of heparin-binding EGF (HB-EGF) and stimulation of ERK1/2 phosphorylation. Blockade of HB-EGF action by neutralizing antibody or its selective inhibitor, CRM197, attenuated ERK1/2 activation by Ang II. Consistent with its agonist action, HB-EGF stimulation of these cells caused marked phosphorylation of the EGF-R and its adapter molecule, Shc, as well as ERK1/2 and its dependent protein, p90 ribosomal S6 kinase, in a manner similar to that elicited by Ang II or EGF. Although the Tyr319 residue of the AT_1-R has been proposed to be an essential regulator of EGF-R transactivation, stimulation of wild-type and mutant (Y319F) AT_1-R expressed in COS-7 cells caused EGF-R transactivation and subsequent ERK1/2 phosphorylation through release of HB-EGF in a Src-dependent manner. In contrast, the noninvolvement of MMPs in HEK 293 cells, which may reflect the absence of Src activation by Ang II, was associated with lack of transactivation of the EGF-R. These data demonstrate that the individual actions of Ang II on EGF-R transactivation in specific cell types are related to differential involvement of MMP-dependent HB-EGF release. (*Molecular Endocrinology* 18: 2035–2048, 2004)

G PROTEIN-COUPLED RECEPTORS (GPCRs) activate MAPK signaling cascades by a variety of biochemical pathways. These include the generation of second messengers [Ca^{2+}, protein kinase C (PKC), and cAMP], G protein subunit coupling to novel effectors, and activation of receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor, and IGF receptor (1, 2). MAPKs are activated by a wide spectrum of stimuli, ranging from mitogens and growth factors to cellular stress and neurotoxic factors. ERK1/2 are closely related members of the MAPK family that are predominantly activated by growth factors and GPCRs, and are important factors in cell growth, survival, motility, and secretion (3).

The type 1 angiotensin II (Ang II) receptor (AT_1-R) is a typical GPCR, and mediates a wide variety of physiological actions in the cardiovascular system, kidney, brain, adrenal glands, and liver (4). Ang II and the AT_1-R have critical roles in cardiovascular diseases including hypertension, atherosclerosis, and cardiac hypertrophy (5, 6). In most Ang II target cells, the

Abbreviations: Ang II, Angiotensin II; AT_1-R, Ang II type 1 receptor; C9 cells, clone 9 hepatocytes; CRM197, cross-reactive mutant of diphtheria toxin; Csk, negative regulatory Src kinase; EGF, epidermal growth factor; EGF-R, EGF receptor; FCS, fetal calf serum; Grb2, growth factor binding protein-2; GPCR, G protein-coupled receptor; HB, heparin binding; HEK, human embryonic kidney; LPA, lysophosphatidic acid; MEK, MAPK kinase; MMP, metalloproteinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]-pyrimidine; PY20, phosphotyrosine antibody; Pyk2, proline-rich tyrosine kinase; RSK-1, p90 ribosomal S6 kinase-1; RTK, receptor tyrosine kinase; Shc, Src homology and collagen domain protein; Sos, son of sevenless protein; Src, c-Src kinase; VSMC, vascular smooth muscle cell.

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AT_1-R interacts primarily with pertussis-toxin insensitive $G_{q/11}$ proteins, leading to activation of phospholipase C, generation of diacylglycerol, and activation of PKC, and inositol trisphosphate, which mobilizes Ca^{2+} from intracellular stores. However, the AT_1-R is also coupled to inhibitory G_i proteins in rat hepatocytes (7) and in rat adrenal, pituitary, and renal cells (4). AT_1-R stimulation causes activation of all types of MAPKs; ERK1/2, SAPK/JNK, and p38 MAPKs. However, there is little consensus about the mechanisms involved in Ang II-induced activation of MAPKs.

Ang II-mediated ERK1/2 activation in certain cell types [such as clone 9 (C9) hepatocytes and vascular smooth muscle cells (VSMCs)] that express endogenous AT_1-R is dependent on transactivation of the EGF-R, which activates the ras-mediated signaling pathways (7–9). In another Ang II target site, bovine adrenal glomerulosa cells, the major mechanism of Ang II-induced ERK1/2 activation is a PKC-mediated and ras-independent pathway (10). In contrast, ectopic expression of AT_1-R in other cell types is associated with a considerable degree of heterogeneity in terms of the dependence of ERK1/2 phosphorylation on EGF-R transactivation and the ensuing cellular responses. Whereas EGF-R transactivation is a major factor after agonist activation of transfected μ-opioid receptors, little or no such role has been observed for thrombin, Ang II, endothelin, and thromboxane A2 receptors expressed in human embryonic kidney (HEK) 293 cells (11–15). Interestingly, recent studies show that Ang II causes G protein-independent, β-arrestin-mediated ERK1/2 activation in HEK 293 cells (16). In contrast, ERK1/2 activation by Ang II in COS-7 cells is primarily dependent on EGF-R transactivation (17), a process that may be initiated by phosphorylation of the AT_1-R at Tyr319 (18). The reason(s) for such differential involvement of EGF-R in GPCR-mediated signaling are not known and are currently a subject of great interest.

There is substantial evidence that the kinetics and duration of ERK1/2 activation, its localization within subcellular compartments, and the ultimate changes in cellular function are highly dependent on the type of signaling molecules involved during GPCR stimulation (19–22). Thus, the signaling characteristics of endogenously expressed GPCRs not only reflect the molecular mechanisms operating in the specific cell type, but may also differ from those observed in cells containing over- or underexpressed ectopic receptors and transducing proteins (19, 23, 24). We have recently shown that Ang II-mediated ERK1/2 activation in C9 hepatocytes, which express endogenous AT_1-R, is primarily dependent on activation of PKCδ, Src/Pyk2, and EGF-R transactivation (7). In contrast, transactivation of the EGF-R in HEK 293 cells has only a minor role during Ang II action (11). The basis of these differential effects of Ang II in various cell types is not clear. The present study shows that Ang II-induced ERK1/2 phosphorylation is primarily dependent on activation of PKC in both C9 and HEK 293 cells. However, Ang II signaling bifurcates distal to PKC in these cells. ERK1/2 activation in C9 and COS-7 cells is largely dependent on the release of HB-EGF through metalloproteinase (MMP) activation, leading to EGF-R activation and subsequent phosphorylation of ERK1/2. In contrast, Ang II signaling in HEK 293 cells is largely independent of Src and EGF-R transactivation due to lack of MMP activation.

RESULTS

C9 cells express AT_1- but not AT_2-R, and respond to Ang II with a rapid and transient increase of ERK1/2 activity that reaches a maximum at 5 min and then declines rapidly within 15–30 min (Fig. 1A). Stimulation of endogenous EGF-R with EGF (20 ng/ml) likewise resulted in transient ERK1/2 activation (Fig. 1B). In contrast, Ang II-induced ERK1/2 activation was relatively sustained in HEK 293 cells transfected with AT_1-R, reaching a maximum at 5 min and persisting for up to 30 min or longer (Fig. 1C). HEK 293 cells express receptors for tyrosine kinase-linked growth factors, such as the EGF-R and platelet-derived growth factor receptor. EGF stimulation (20 ng/ml) caused a transient increase in ERK1/2 phosphorylation to a peak at 5 min and a subsequent rapid decline (Fig. 1D).

Ang II increased tyrosine phosphorylation of the EGF-R in C9 cells, albeit of lesser magnitude than EGF (Fig. 2A). Moreover, treatment with the selective EGF-R kinase inhibitor, AG1478, abolished ERK1/2 activation induced by both Ang II and EGF in a concentration-dependent manner (Figs. 2, B–D). In HEK 293 cells, whereas EGF caused marked tyrosine phosphorylation of the EGF-R, Ang II had no such effect (Fig. 3A). Similarly, blockade of the EGF-R with its selective antagonist, AG1478, completely abolished ERK1/2 activation by EGF, but had no significant inhibitory effect after Ang II stimulation (Fig. 3, B–D). These data indicate that, in contrast to C9 cells, transactivation of the EGF-R has no significant role in Ang II-induced ERK1/2 activation in HEK293 cells.

Although the genesis of agonist-induced EGF-R transactivation is not fully understood, one major mechanism is the GPCR-induced activation of MMPs that cause the release of cytokines and growth factors such as transforming growth factor-α or -β (TGF-α or -β) and HB-EGF. To examine the extent to which this mechanism is responsible for Ang II-induced EGF-R transactivation, C9 cells were pretreated with the MMP inhibitor, GM6001, and stimulated with Ang II. In this study, GM6001 treatment markedly inhibited Ang II-induced phosphorylation of ERK1/2 and its dependent protein, p90 ribosomal S6 kinase-1 (RSK-1) (Fig. 4, A and B). In contrast, GM6001 had no effect on Ang II-induced ERK1/2 and RSK1 responses in HEK 293 cells, excluding the involvement of MMP activation in these cells (Fig. 4C). GM6001 also did not inhibit the ERK1/2 activation induced by EGF stimulation in

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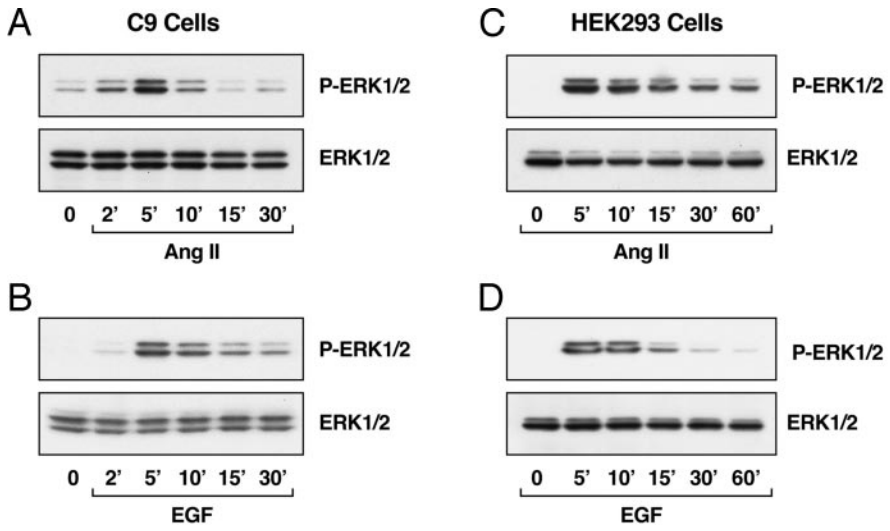


Fig. 1. Time-Course of Ang II- and EGF-Induced ERK1/2 Phosphorylation (P-ERK1/2) in C9 and HEK 293 Cells

C9 (A and B) and HEK 293 (C and D) cells were treated with Ang II (100 nM) and EGF (20 ng/ml) for the time periods indicated, washed with ice-cold PBS, lysed in Laemmli sample buffer and analyzed by SDS-PAGE for phosphorylation of ERK1/2 using phospho-specific (Thr202/Tyr204) antibodies. The blots were stripped and reprobed with ERK1/2 antibody to show total ERK1/2. The data are representative of three to four experiments.

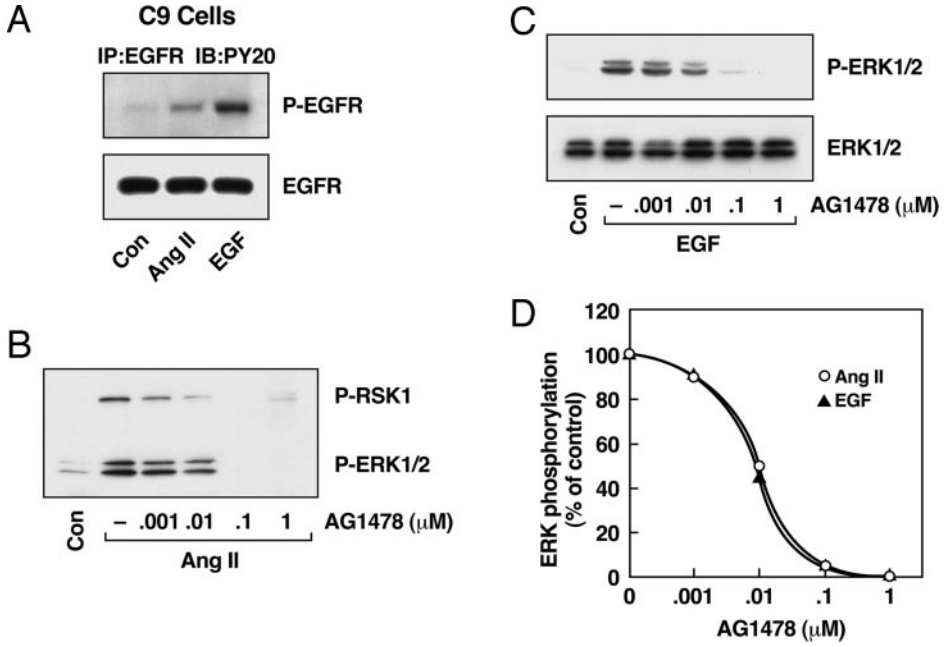


Fig. 2. Role of EGF-R Transactivation in Ang II-Induced ERK1/2 Phosphorylation (P)

A, Effects of Ang II and EGF on tyrosine phosphorylation of the EGF-R in C9 cells. After stimulation with Ang II (100 nM) or EGF (10 ng/ml) for 2 min, cells were collected in RIPA lysis buffer. Cell lysates were immunoprecipitated (IP) with anti-EGF-R antibody as described in *Materials and Methods* and immunoblotted (IB) with PY20. B and C, Concentration-dependent inhibitory effects of the selective EGF-R tyrosine kinase inhibitor, AG1478, on ERK1/2 phosphorylation by Ang II and EGF. C9 cells were treated with increasing concentrations of AG1478 for 20 min before stimulation with Ang II (100 nM) or EGF (20 ng/ml) for 5 min. Cells were washed with ice-cold PBS, lysed in Laemmli sample buffer and analyzed by SDS-PAGE. D, Quantitation of the inhibitory effects of AG1478 on ERK1/2 phosphorylation by Ang II and EGF from panels B and C, taking agonist-stimulated ERK1/2 phosphorylation as 100% ($n = 3$).

HEK 293 cells (Fig. 4D), a result similar to that observed in C9 cells (data not shown). These findings demonstrate the specificity of GM6001, and also indicate the dependence of Ang II signaling on MMP action upstream of the EGF-R in C9 cells. Furthermore, the insignificant involvement of EGF-R transactivation in HEK 293 cells correlates with the lack of MMP induction by Ang II.

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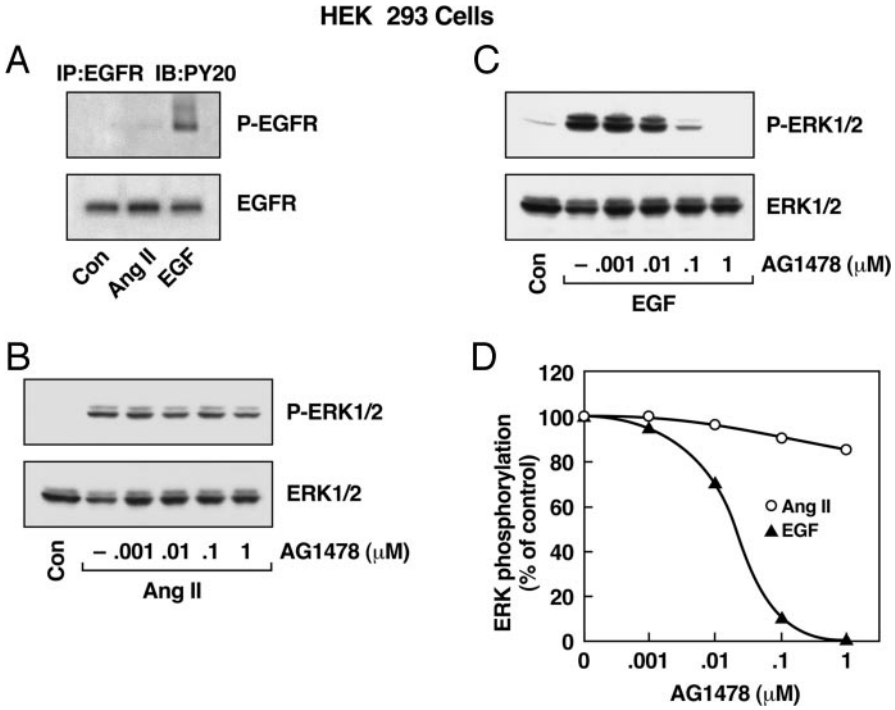


Fig. 3. Absence of EGF-R Transactivation during Ang II-Induced ERK1/2 Phosphorylation (P) in HEK 293 Cells

A, Effects of Ang II and EGF on tyrosine phosphorylation of the EGF-R in HEK 293 cells. Cells were stimulated with Ang II (100 nM) or EGF (10 ng/ml) for 5 min and collected in RIPA lysis buffer. Cell lysates were immunoprecipitated (IP) with anti-EGF-R antibody as described in *Materials and Methods* and immunoblotted (IB) with PY20. B and C, Effect of EGF-R kinase inhibition on agonist-induced ERK1/2 activation. HEK 293 cells expressing the AT_1-R were treated with increasing concentrations of AG1478 for 15 min followed by stimulation with 100 nM Ang II (B) and 20 ng/ml EGF (C) for 5 min. Cell lysates were analyzed by SDS-PAGE for ERK1/2 phosphorylation. D, Quantitation of the inhibitory effects of AG1478 on ERK1/2 phosphorylation by Ang II and EGF from panels B and C, taking agonist-stimulated ERK1/2 phosphorylation as 100% (n = 3).

Recent studies have shown that the soluble EGF-like ligand, HB-EGF, is generated by proteolytic processing of the pro-HB-EGF precursor by MMPs in response to external stimuli, and causes phosphorylation of the EGF-R (2, 25, 26). To evaluate this pathway in C9 cells, we determined whether Ang II-induced ERK1/2 activation results from the release of HB-EGF through proteolytic processing by MMPs. Addition of anti-HBEGF antibody inhibited EGF-R phosphorylation and ERK1/2 activation induced by HB-EGF and Ang II, but not by EGF. Moreover, CRM, a diphtheria toxin mutant that selectively binds to and inactivates HB-EGF (27, 28), also attenuated the effects of HB-EGF and Ang II, but not those of EGF (Fig. 5A, B). In contrast to these observations in C9 cells, neither HB-EGF antibody nor CRM inhibited ERK1/2 activation in HEK 293 cells stimulated by Ang II (Fig. 5C) or EGF (data not shown). In accordance with the involvement of MMP, Ang II stimulation increased MMP activity as measured by cleavage of a fluorescent MMP substrate (Fig. 5D). Moreover, immunoprecipitation of the supernatant and cell lysates, and immunoblotting with anti-HB-EGF, revealed a significant increase of HB-EGF levels in Ang II-stimulated cells (Fig. 5E). Taken together, these data demonstrate that Ang II-induced ERK1/2 activation through transactivation of the EGF-R in C9 cells requires the formation of HB-EGF through GM6001-sensitive MMP(s). Furthermore, the absence of EGF-R transactivation in HEK 293 cells is due to lack of MMP induction during Ang II action.

To determine whether HB-EGF causes ERK1/2 activation through phosphorylation of the EGF-R, we examined the signaling pathways activated by HB-EGF. Treatment of C9 cells with HB-EGF caused significant but transient phosphorylation of EGF-R (Y1173) and ERK1/2, and these effects were concentration-dependent (Fig. 6, A–C). Phosphorylation of the EGF-R by HB-EGF was abolished by the selective EGF-R kinase antagonist, AG1478, but not by the selective inhibition of MAPK kinase (MEK) by PD98059, MMP(s) by GM6001, PKC by Go6983, and Src by PP2 (Fig. 6D).

Agonist-induced tyrosine phosphorylation and activation of the EGF-R in C9 cells leads to the recruitment of adapter molecules including Shc, Grb2, and Sos, which mediate ERK1/2 activation through the Ras/Raf/MEK pathway (7). Consistent with involvement of the EGF-R, Ang II stimulation caused rapid phosphorylation of Shc that was dependent on MMP induction and EGF-R activation (Fig. 7, A and C). As expected, HB-EGF also caused Shc phosphorylation that was

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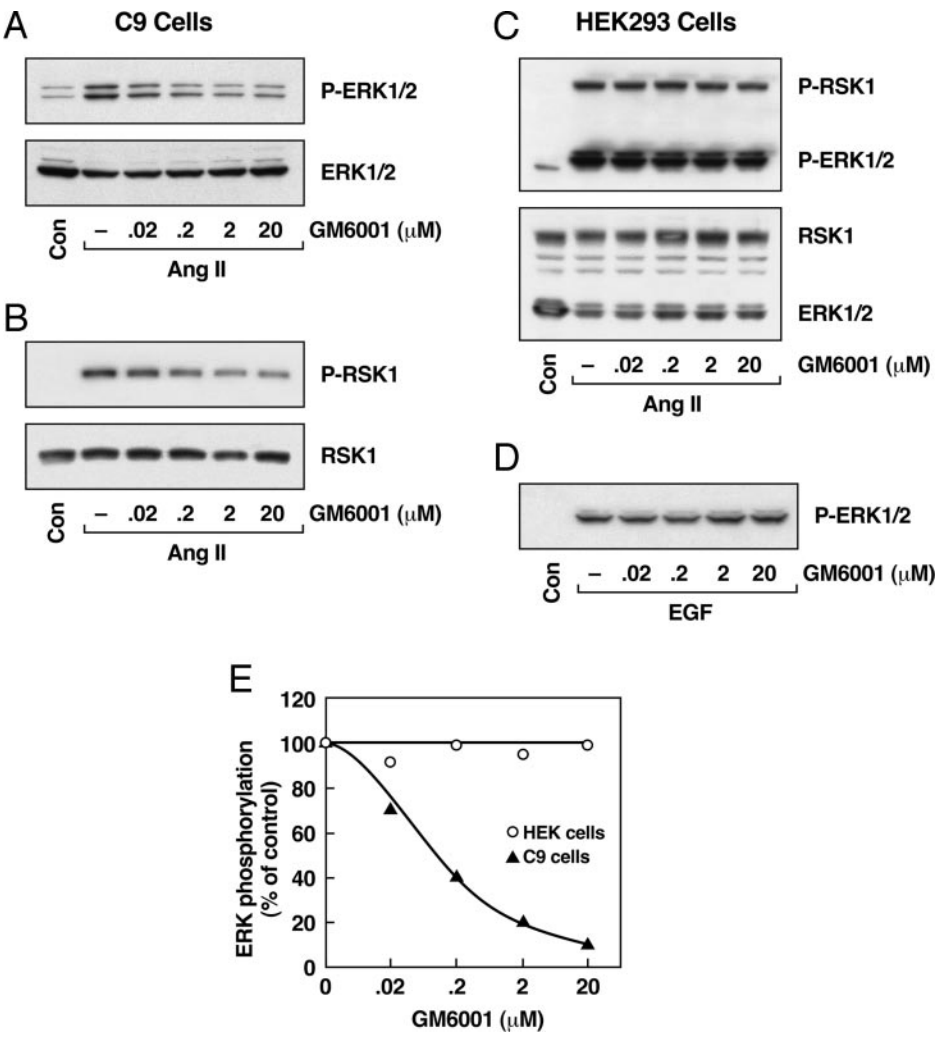


Fig. 4. Differential Involvement of MMPs in Agonist-Stimulated C9 and HEK 293 Cells

A–D, Concentration-dependent effects of the MMP inhibitor, GM6001, on agonist-induced phosphorylation (P) of ERK1/2 and RSK-1. C9 or HEK 293 cells were treated with increasing concentrations of GM6001 for 15 min and then stimulated with Ang II (100 nM) or EGF (20 ng/ml) for 5 min. Cell lysates were analyzed by SDS-PAGE for phosphorylation of ERK1/2 and RSK-1 (Thr359/Ser363). E, Quantitated effects of GM6001 on Ang II-induced ERK1/2 activation in C9 (A) and HEK 293 cells (C) (n = 3).

blocked by AG1478 but not by inhibition of PKC, MMPs, Src, and MEK1/2 (Fig. 7D). However, reprobing the same blot for phospho-ERK1/2 revealed that its phosphorylation by HB-EGF was dependent on activation of the EGF-R and MEK1/2 (Fig. 7E). Similar results were obtained with EGF (data not shown). These data indicate that the effects of HB-EGF are mediated by activation of the EGF-R, and that HB-EGF release is responsible for Ang II-induced transactivation of the EGF-R in C9 cells. Moreover, MMP inhibition has no effect on HB-EGF after its release by Ang II.

Our results show that Ang II-mediated shedding of HB-EGF by MMP activation is necessary to cause transactivation of the EGF-R in C9 but not in HEK 293 cells. To explore the cause(s) of the differential signaling of Ang II in these two cell types, we examined the mechanism of Ang II-induced ERK1/2 activation. PKC inhibition by Go6983 inhibited ERK1/2 activation by Ang II as well as phorbol 12-myristate 13-acetate (PMA) in both cell types (Fig. 8, A and D). The incomplete inhibition of Ang II-mediated ERK1/2 activation in HEK 293 cells may be attributable to the G protein-independent, β-arrestin-dependent ERK1/2 activation recently reported in this cell line (16). Moreover, depletion of PKC by prolonged treatment with PMA (1 μM for 16 h) similarly decreased ERK1/2 phosphorylation induced by Ang II, but not by EGF, in both C9 and HEK 293 cells (data not shown), consistent with a major role of PKC in Ang II signaling. These data indicate that PKC is a common upstream mediator of Ang II-induced ERK1/2 activation in both cell types. GPCR-mediated activation of PKC has been shown to stimulate MMP activity (25), inhibition of which by GM6001 reduced ERK1/2 phosphorylation by PMA in C9 but not HEK 293 cells (Fig. 8, B and E). Consistent with

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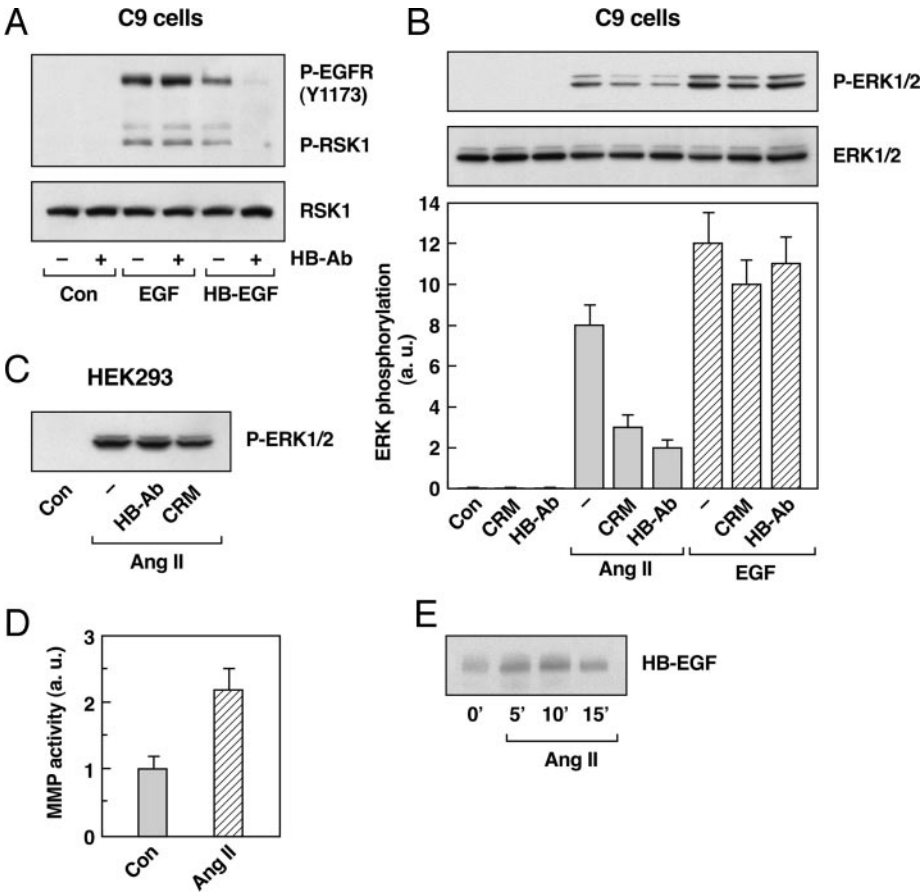


Fig. 5. Ang II-Induced Phosphorylation (P) of EGF-R and ERK1/2 in C9 Cells Is Mediated by HB-EGF Production through MMP Activation

A, Effects of neutralizing HB-EGF antibody on EGF-R and RSK phosphorylation induced by EGF and HB-EGF. C9 cells were pretreated with anti-HB-EGF antibody (10 μg/ml) and stimulated with EGF or HB-EGF for 5 min. Cell lysates were analyzed for EGF-R (Tyr1173) and RSK-1 (Thr359/Ser363) phosphorylation. *Lower panel* shows the total RSK-1 levels. B, C9 cells were treated with CRM (10 μg/ml) to inactivate HB-EGF, or with an HB-EGF antibody (HB-Ab; 10 μg/ml) for 20 min before stimulation with Ang II (100 nM) and EGF (20 ng/ml) for 5 min (n = 3). C, HEK 293 cells were treated with anti-HB-EGF or CRM as above and stimulated with Ang II for 5 min. D, Effects of Ang II on MMP activity in C9 cells as measured by cleavage of fluorescent MMP substrate. E, Time course of HB-EGF shedding by stimulation of C9 cells with Ang II. C9 cells were stimulated for indicated time periods, then cell lysates and supernatants were immunoprecipitated with and immunodetected with antibody against HB-EGF.

this, ERK1/2 activation by PMA (100 nM) was significantly attenuated by AG1478 in C9 cells, but not in HEK 293 cells (Fig. 8, C and F). These data indicate that Ang II-mediated PKC activation occurs upstream of HB-EGF generation in C9 cells, and that the bifurcation of Ang II-mediated ERK1/2 signaling is distal to PKC in C9 and HEK 293 cells.

Because Src activation has been shown to stimulate GPCR-mediated MMP induction and EGF-R transactivation (29), we next examined whether differential activation of Src is responsible for the distinctive signaling pathways in C9 and HEK 293 cells. Inhibition of Src by the selective Src kinase inhibitor, PP2, attenuated Ang II-induced ERK1/2 activation in C9 cells but not in HEK 293 cells (Fig. 9, A and C). Similarly, overexpression of the negative regulatory Src kinase (Csk) significantly inhibited ERK1/2 phosphorylation by Ang II in C9 cells but not in HEK 293 cells (Fig. 9, B and D). These results show that Src kinase is required for Ang II-induced ERK1/2 activation in C9 cells and acts upstream of the EGF-R. However, agonist-activation of the AT_1-R in HEK 293 cells does not activate Src and thus lacks MMP induction and consequent EGF-R transactivation.

The signaling pathways activated by ectopic GPCRs, including the AT_1-R in certain cell types, have shown significant differences compared with those expressed in native target cells (11, 19). To determine whether such differential signaling in native and transfected cells is a general feature of the AT_1-R, we used COS-7 cells that lack AT_1-R but express EGF-R. In COS-7 cells transfected with AT_1-R, Ang II caused tyrosine phosphorylation of the EGF-R as demonstrated by immunoprecipitation of the EGF-R and immunoblotting with phosphotyrosine antibody (PY20). Tyrosine phosphorylation of the EGF-R was attenuated by inhibition of MMP and

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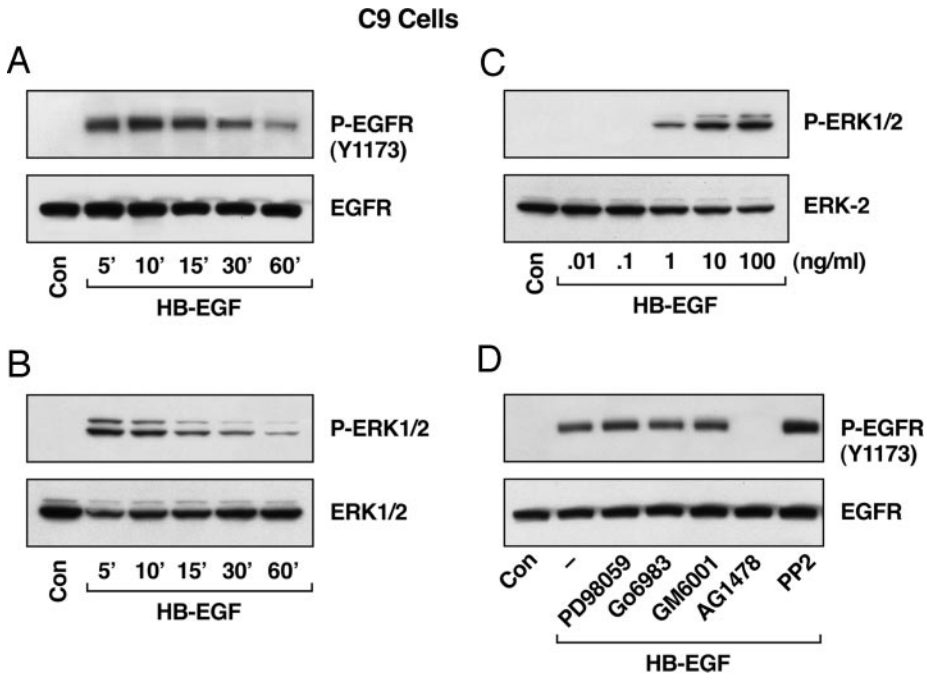


Fig. 6. HB-EGF Causes ERK1/2 Activation through Phosphorylation of the EGF-R in C9 Cells

A and B, Time course of the effects of HB-EGF (20 ng/ml) on phosphorylation of EGF-R (Tyr1173) and ERK1/2 in C9 cells. C, Concentration-dependent effects of HB-EGF on ERK1/2 phosphorylation. D, Effects of MEK1/2 inhibitor, PD98059 (10 μM), PKC inhibitor, Go6983 (1 μM), MMP inhibitor, GM6001 (20 μM), EGF-R antagonist, AG1478 (200 nM) and Src inhibitor, PP2 (5 μM) on phosphorylation of the EGF-R (Y1173) by HB-EGF. C9 cells were treated with the inhibitors for 15 min and stimulated with HB-EGF (20 ng/ml) for 5 min.

Src activities after stimulation with Ang II, but not EGF (Fig. 10, A and B). Recent studies have suggested that the C-terminal last five tyrosines (residues 292, 302, 312, 319, and 339) of the AT_1-R are important in G protein coupling and generation of inositol trisphosphate and Ca^{2+} (30). Recently, Seta and Sadoshima (18) reported that Tyr319 has a key role in Ang II-induced EGF-R transactivation because mutation of Tyr319 to phenylalanine (Y319F) abolished this effect. However, when we determined whether Y319F AT_1-R has impaired transactivation potency during Ang II action, agonist stimulation of the mutant receptor caused ERK1/2 activation that was comparable to the response mediated by the wild-type AT_1-R in COS-7 cells. Moreover, there was no difference between the wild-type and mutant receptor signaling in terms of the dependence of ERK1/2 phosphorylation on MMP, Src, and EGF-R activation (Fig. 10, C and D). Similar to its effects in C9 cells, as shown above, inhibition of MMP did not change EGF-induced ERK1/2 activation. However, blockade of Src by PP2 had minor inhibitory effects on EGF responses (Fig. 10E). Taken together, these data suggest that activation of Src and MMPs causes transactivation of the EGF-R in COS-7 and C9 cells and also argue against an essential role of phosphorylation of Tyr319 of the AT_1-R during this process.

DISCUSSION

Ang II-induced ERK1/2 activation occurs through a variety of signaling pathways that can be dependent on or independent of RTK transactivation. The cell types exhibiting Ang II signaling through EGF-R activation include VSMCs (9, 31), cardiac myocytes (32, 33), and fibroblasts (9), COS-7 cells (17, 18), C9 hepatic cells (7), glomerular mesengial cells (34, 35), prostate stromal cells (36), anterior pituitary cells (37), and breast cancer cells (38). However, this cross-communication is not a universal process (39, 40) and has a minor or no role in HEK 293 cells and preglomerular smooth muscle cells (11, 41). Although the reasons for these differential effects are obscure, the present findings indicate that selective induction of MMPs and generation of HB-EGF in C9 and COS-7 cells, but not in HEK 293 cells, is the major determinant of the EGF-R transactivation cascade.

EGF-related growth factors such as amphregulin, TGFα, betacellulin, HB-EGF, and epiregulin are synthesized as membrane spanning pro-growth factors, and are cleaved by matrix MMPs and MMP-disintegrin proteins (42). MMPs are well known to contribute to both normal and pathological tissue remodeling by regulating the processing of matrix proteins, cytokines, growth factors, and adhesion molecules (25, 33, 42). The expression of HB-EGF and EGF receptors is

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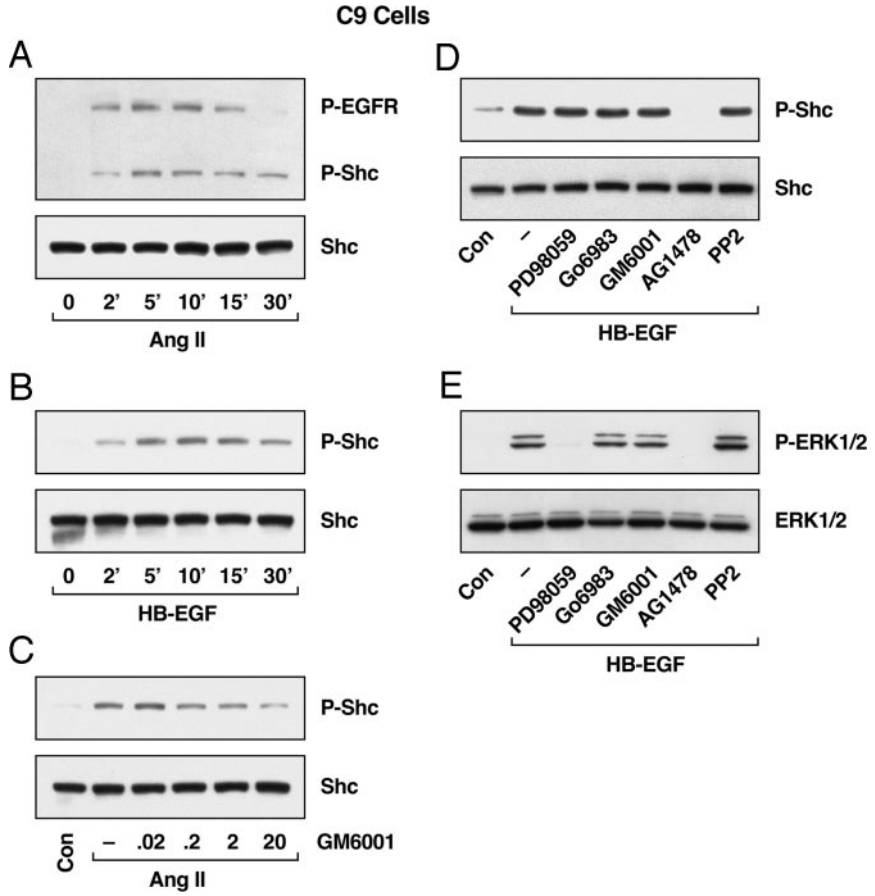


Fig. 7. Kinetics and Mechanism of Agonist-Induced Shc Phosphorylation (P) in C9 Cells

A and B, Time course of the effects of Ang II and HB-EGF on phosphorylation of EGF-R and Shc. C, Concentration-dependent inhibition of Shc phosphorylation by MMP inhibitor, GM6001, after stimulation with Ang II (100 nM) for 5 min. D and E, Effects of MEK1/2 inhibitor, PD98059 (10 μM), PKC inhibitor, Go6983 (1 μM), MMP inhibitor, GM6001 (20 μM), EGF-R antagonist, AG1478 (200 nM), and Src inhibitor, PP2 (5 μM) on phosphorylation of Shc and ERK1/2 by HB-EGF. C9 cells were treated with the inhibitors for 15 min and stimulated with HB-EGF (20 ng/ml) for 5 min.

enhanced in the hypertrophied left ventricle of spontaneously hypertensive rats (43) and after myocardial infarction (44). MMPs are major regulators of the extracellular matrix, and have been implicated in the genesis of tumor metastasis, myocardial infarction, left ventricular dilatation, and heart failure (6, 45, 46). Pharmacological inhibition of MMPs blocks Ang II-induced transactivation of the EGF-R and subsequent ERK1/2 activation, and growth and migration of rat VSMCs (9, 33, 47). The paradigm emerging from recent studies implicates transactivation of the EGF-R as a central point in mediating the growth-promoting effects of GPCRs, in particular those of Ang II in cardiovascular disorders including hypertension and cardiac hypertrophy (6, 9, 32, 33, 48).

Whereas Ang II signaling to ERK1/2 activation has been well characterized in the cardiovascular system, little information is available about Ang II effects in nonvascular cells. Whether Ang II-induced EGF-R transactivation occurs in such cells through MMP induction is not known. Our results show that AT_1-R activation in C9 hepatocytes causes ERK1/2 phosphorylation through MMP-dependent ectodomain shedding of the HB-EGF and subsequent transactivation of the EGF-R (Figs. 4 and 5). Four lines of evidence support our conclusion about the potential involvement of HB-EGF in this cascade. First, MMP inhibition blocked the effect of Ang II on EGF-R phosphorylation and subsequent ERK1/2 activation through recruitment of Shc/Grb/Sos. Second, neutralizing antibody to HB-EGF blocked the effects of HB-EGF, Ang II and PMA, but not that of EGF. Third, CRM, a diphtheria toxin mutant that is a selective inhibitor of HB-EGF (28), attenuated the effect of Ang II. In contrast, EGF-responses were not altered by HB-EGF antibody, CRM and MMP inhibition. Fourth, Ang II-stimulated cells showed marked increases in immunodetectable HB-EGF levels, indicating that Ang II-mediated EGF-R transactivation occurs through shedding of HB-EGF. Taken together, these data provide strong support for MMP-dependent release of HB-EGF after Ang II stimulation, and further that MMP activation occurs upstream of EGF-R during Ang II action in C9 cells.

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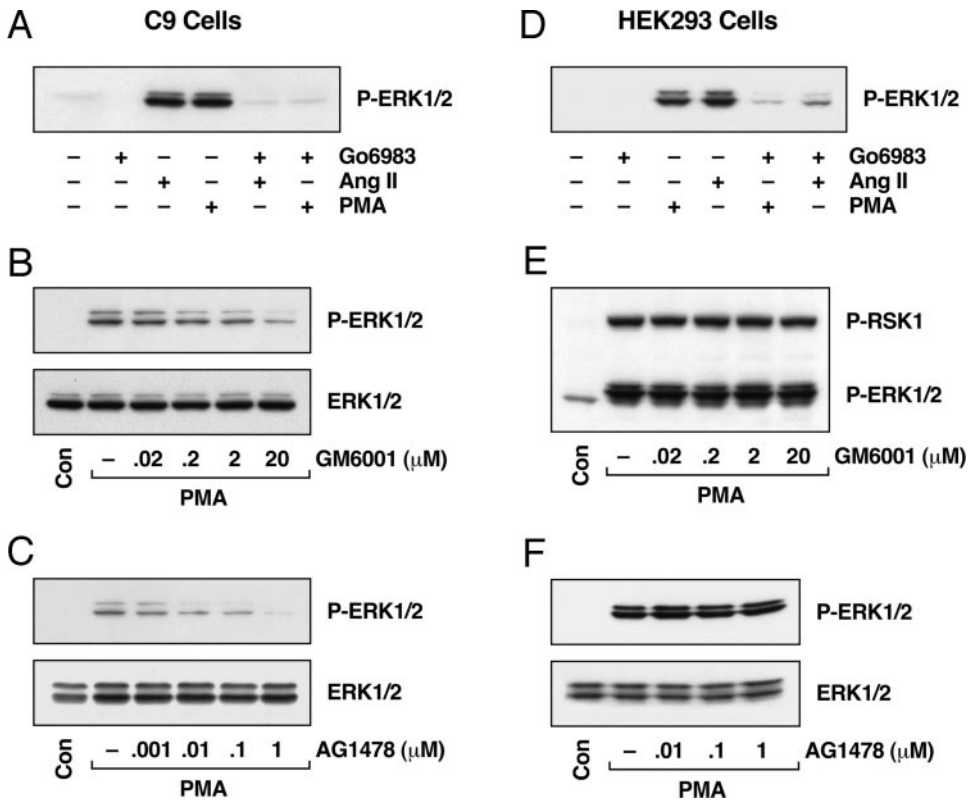


Fig. 8. PKC Acts Upstream of MMP Induction in C9 Cells

A and D, Effects of PKC inhibition on ERK1/2 activation by Ang II and PMA. C9 and HEK 293 cells were treated with PKC inhibitor, Go6983 (1 μM), and stimulated with Ang II (100 nM for 5 min) and PMA (100 nM for 10 min). B and E, Effects of MMP inhibitor, GM6001, on PMA-induced ERK1/2 activation in C9 (B) and HEK 293 cells (E). Cells were treated with increasing concentrations of GM6001 for 20 min and stimulated with PMA (100 nM) for 10 min. C and F, Concentration-dependent effects of EGF-R kinase antagonist, AG1478, on PMA-induced ERK1/2 activation in C9 (C) and HEK 293 cells (F). Cells were treated with increasing concentrations of AG1478 and stimulated with PMA (100 nM) for 10 min. P, Phosphorylation.

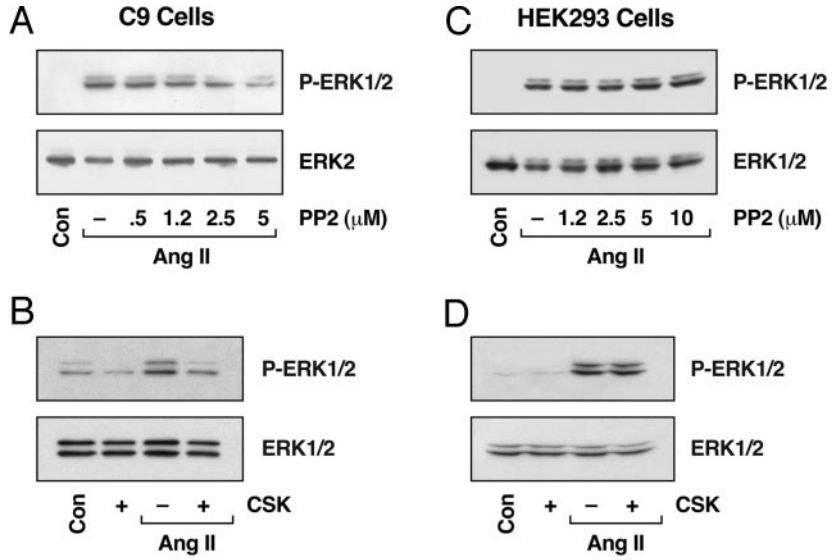


Fig. 9. Differential Effects of Src Kinase in Ang II-Induced ERK1/2 Activation in C9 and HEK 293 Cells

A and C, Serum-starved C9 and HEK 293 cells were treated with increasing concentrations of the selective Src inhibitor, PP2, and stimulated with Ang II (100 nM). B and D, Cells were transfected with Csk (1 μg) and stimulated with Ang II (100 nM) for 5 min. Agonist-induced ERK1/2 activation was measured as described (n = 2–3). P, Phosphorylation.

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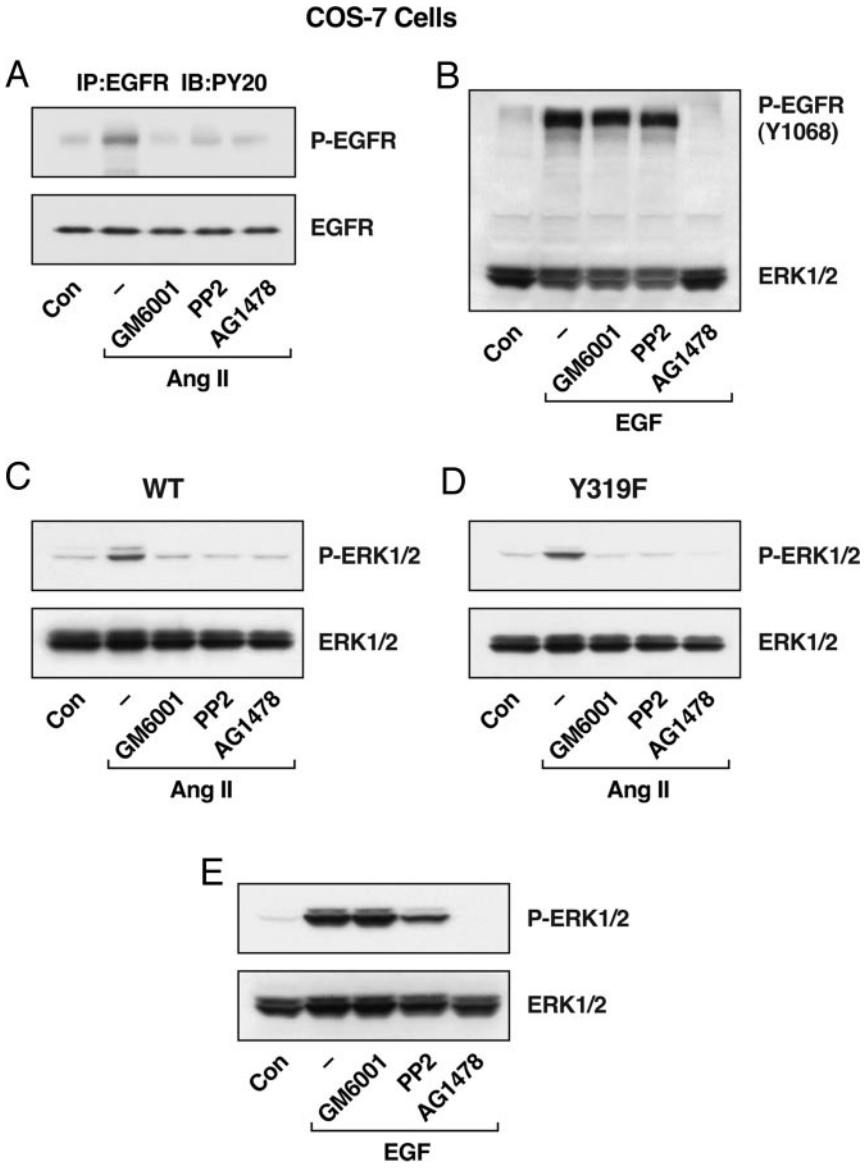


Fig. 10. Ang II-induced ERK1/2 Activation in COS-7 Cells Expressing AT_1-R Occurs through Activation of Src, MMP, and EGF-R Kinase

A, COS-7 cells transfected with wild-type AT_1-R cDNA (1 μg) were pretreated with MMP inhibitor, GM6001 (20 μM), Src inhibitor, PP2 (5 μM), and EGF-R kinase inhibitor, AG1478 (250 nM) followed by stimulation with Ang II (100 nM). Cells were collected in RIPA lysis buffer, cell lysates were immunoprecipitated with anti-EGF-R antibody and immunoblotted with PY20. B, COS-7 cells treated with GM6001 (20 μM), PP2 (5 μM) and AG1478 (250 nM) were stimulated with EGF (20 ng/ml) for 5 min. Cell lysates were collected in Laemmli lysis buffer and analyzed for phosphorylation (P) of the EGF-R at Tyr 1068. C and D, COS-7 cells transfected with wild-type (C) and mutant (Y319F) AT_1-R cDNA (1 μg) (D) were treated with GM6001 (20 μM), PP2 (5 μM) and AG1478 (250 nM) and stimulated with Ang II for 5 min. E, COS-7 cells were treated with GM6001 (20 μM), PP2 (5 μM) and AG1478 (250 nM) followed by stimulation with EGF (20 ng/ml) for 5 min. Cell lysates were collected in Laemmli lysis buffer and analyzed for ERK1/2 phosphorylation. Representative data from two to three similar experiments are shown here.

Whereas GPCR-mediated ERK1/2 activation can be either transient or sustained, EGF-induced responses are typically of short duration (7, 19, 20, 49). Accordingly, GPCRs causing ERK1/2 activation through EGF-R transactivation would be expected to mimic the signaling characteristics of EGF stimulation. Our results show that Ang II signaling has a remarkable resemblance to EGF signaling in terms of activation of Shc, ERK1/2, and RSK-1 in C9 cells (Figs. 1 and 2). HB-EGF released after Ang II stimulation also caused marked phosphorylation of the EGF-R and ERK1/2 that was blocked by the selective EGF-R kinase inhibitor, AG1478. HB-EGF stimulated signaling events downstream of the EGF-R, including phosphorylation of Shc and ERK1/2, in a manner analogous to that of Ang II and EGF stimu-

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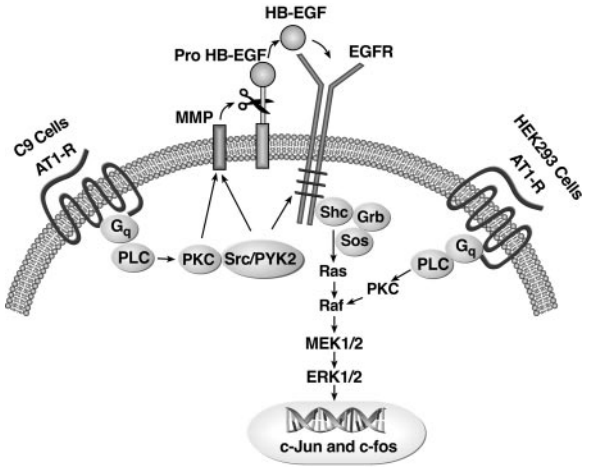


Fig. 11. Comparison of Signaling Pathways Activated by Ang II in C9 and HEK 293 Cells

In C9 cells, Ang II-induced stimulation of the AT_1-R causes transient ERK1/2 activation through activation of Src, MMP, and EGF-R in a PKC-dependent manner in C9 cells. Agonist-induced AT_1-R activation leads to sequential activation of PKC, Src, Pyk2, and MMP with subsequent release of HB-EGF. This endogenous ligand binds to and activates the EGF-R, leading to phosphorylation of ERK1/2 and its dependent protein p90 ribosomal S6 kinase 1 through recruitment of adapter molecules, Shc, Grb, Sos, and Ras/Raf/MEK. In contrast, in HEK293 cells expressing the AT_1-R, Ang II-induced ERK1/2 activation is largely dependent on PKC and is independent of Src, MMP, and EGF-R transactivation. PLC, Phospholipase C.

lation. Furthermore, the time-course of activation of these signaling events elicited by HB-EGF was closely similar to those of Ang II and EGF in C9 cells (Figs. 6 and 7). Thus, the signaling pathways activated by HB-EGF after Ang II stimulation, and those activated by EGF, are indistinguishable in these cells. In contrast, ERK1/2 activation by Ang II in HEK 293 cells was independent of MMP activity and EGF-R activation and was relatively sustained (Figs. 3 and 4).

To examine whether the differential signaling in native and transfected cells is a general feature of the AT_1-R we used COS-7 cells, which lack AT_1-R but express the EGF-R. In these cells transfected with AT_1-R, Ang II caused tyrosine phosphorylation of the EGF-R and subsequent activation of ERK1/2 that was attenuated by inhibition of MMP, Src, and EGF-R. These findings suggest a causal relationship between activation of Src and MMP, and agonist-mediated transactivation of the EGF-R. Moreover, mutation of Tyr319 to phenylalanine (Y319F) after the AT_1-R exhibited the same pattern of ERK1/2 activation as that of the wild-type receptor (Fig. 10). In this respect, our results differ from those of Seta and Sadoshima (18), who found that phosphorylation of Tyr319 is required for Ang II-induced EGF-R transactivation, and that Y319F mutation redirects Ang II signaling through Src instead of EGF-R in COS-7 cells. In the prior study, Ang II signaling was examined in COS-7 cells overexpressing the EGF-R in addition to their endogenous receptors (18). Because over- or underexpression of GPCRs, RTKs, and other signaling molecules can alter the signaling characteristics (19, 21–23), it is possible that this discrepancy is related to the relatively high expression of the EGF-R in transfected COS-7 cells.

Overexpression of other GPCRs in HEK 293 cells has yielded variable results in terms of the dependence or otherwise of ERK1/2 activation on EGF-R transactivation. Studies on several GPCRs have revealed further aspects of the variable dependence of EGF-R transactivation in agonist-induced ERK1/2 signaling. For example, whereas μ-opioid receptor activation shows complete dependence of ERK1/2 activation on EGF-R transactivation (15), the receptors for LPA and thrombin (13), endothelin-1 (14), and thromboxane A2 (50) exhibit partial cross-communication with EGF-Rs, and the δ-opioid and GnRH receptors completely lack the ability to transactivate EGF-R in HEK 293 cells (12, 29). Our data show that EGF-R transactivation in HEK 293 cells has a negligible role in Ang II-induced ERK1/2 activation. A similar lack of EGF-R transactivation by AT_1-R stimulation has been documented recently in preglomerular smooth muscle cells (41). To explore the basis of this differential response, we conducted similar studies in HEK 293 cells and found that Ang II-induced ERK1/2 activation in these cells is primarily dependent on PKC but is independent of Src and MMP activation (Figs. 4, 8, and 9). However, both HB-EGF and EGF caused marked phosphorylation of EGF-R and ERK1/2 that was abolished by AG1478, consistent with HB-EGF's ability to activate the EGF-R. Thus, the lack of Src activation and/or HB-EGF formation in response to GPCR stimulation in HEK 293 cells is probably responsible for the lack of transactivation of the EGF-R. The transfected HEK 293 cells used in the present studies have higher AT_1-R expression than C9 cells. It is possible that the differential expression of AT_1-Rs alters the stochiometric relationship between AT_1-Rs and EGF-Rs, and thus modifies the signaling pathways of communication between these receptor types. Interestingly, HB-EGF generation through MMP activation is absent in native rat hepatocytes (51), and Ang II-induced ERK1/2 activation is independent of EGF-R transactivation in these cells (52), further indicating that MMP activation is a prerequisite for EGF-R transactivation after Ang II stimulation.

In summary, our findings show that Ang II-induced MAPK activation via transactivation of the EGF-R is dependent on ectodomain shedding of HB-EGF through activation of GM6001-sensitive MMPs. Furthermore, PKC and Src act upstream of MMP-dependent transactivation of the EGF-R in C9 cells, and Ang II stimulation leads to the assembly of a multiprotein complex consisting of Src, Pyk2, and the EGF-R (7). Thus, it is possible that this intracellular communication also contributes to the transactivation of the

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EGF-R. This mechanism is absent in HEK 293 cells and is attributable to the lack of Ang II-mediated induction of MMP action on EGF-like ligand precursors (Fig. 11).

MATERIALS AND METHODS

F-12K nutrient mixture (Kaighn's modification), DMEM, fetal bovine serum, and antibiotic solutions were from Invitrogen Life Technologies (Carlsbad, CA). PKC inhibitors, PP2, AG1478, CRM197, and GM6001 were purchased from Calbiochem (San Diego, CA), Ang II from Peninsula Laboratories Inc. (Belmont CA), and pertussis toxin from LIST Biologicals Laboratories, Inc. (Campbell, CA). Human recombinant EGF was from Invitrogen Life Technologies or Biosource International Antibodies (Camarillo, CA) to EGF receptor, anti-phospho-EGF receptor (Y1068 and Y1173) and Src were from Santa Cruz Biotechnology (Santa Cruz, CA). PY20 antibodies were from Transduction Laboratories (Lexington, KY). Anti-phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 antibodies were from Cell Signaling Technology Inc. (Beverly, MA). Recombinant human HB-EGF and anti-HB-EGF antibody was from R & D Systems (Minneapolis, MN). Csk was kindly provided by Dr. Zvi Naor (Department of Biochemistry, Tel Aviv University, Tel Aviv, Israel). Lipofectamine reagent was from Invitrogen Life Technologies. Secondary antibodies conjugated to horseradish peroxidase were from Kirkegaard & Perry Laboratories, Inc. (Campbell, CA), and ECL reagents were from Amersham Biosciences (Piscataway, NJ). ^{125}I-[Sar^1,Ile^8]Ang II was from PerkinElmer (Boston, MA), and clone 9 rat liver cells were obtained from ATCC (Manassas, VA).

Cell Culture

C9 rat liver epithelial cells were grown in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% (vol/vol) fetal calf serum (FCS), 100 μg/ml streptomycin, 100 IU/ml penicillin and 250 μg/ml fungizone. For all studies, C9 cells between passages 3 and 12 were used because these cells exhibit maximum expression of their endogenous AT_1 receptors. COS-7 cells were grown in DMEM containing glucose, glutamine, sodium bicarbonate, and supplemented with 10% (vol/vol) FCS. HEK 293 cells stably expressing the AT_1-R were grown in DMEM containing glucose, glutamine, sodium bicarbonate, and supplemented with G418 (200 μg/ml) and 10% (vol/vol) FCS.

Transfections and Receptor Binding Assays

HEK 293 cells were transiently transfected with the rat AT_{1A} receptor using Lipofectamine as previously described (53). The mutant AT_1-R (Y319F) was prepared by site-directed mutagenesis and the Phe replacement of the Tyr319 residue was confirmed by DNA sequencing. Transient transfections of COS-7 cells with wild-type or mutant (Y319F) AT_1-R DNA, or of HEK 293 cells with the negative regulatory Src kinase, Csk, were performed using Lipofectamine in Opti-MEM-1 (Invitrogen Life Technologies) following the manufacturer's instructions. After 5 h, cells were switched to regular serum-containing medium for 24 h followed by replacement with serum-free medium overnight. Binding of ^{125}I-[Sar^1,Ile^8]Ang II to intact cells cultured in 24-well plates was determined as described previously (54). The calculated expression levels of AT_1-R in HEK 293 and C9 cells were 1.7 $\pm$ 0.1 and 0.23 $\pm$ 0.06 pmol per sample, respectively. AT_1-R expression levels were comparable in COS-7 cells transfected with wild-type and Y319F mutant receptor.

Immunoprecipitation

After treatment with inhibitors and drugs, cells were placed on ice, washed twice with ice-cold PBS, lysed in RIPA lysis buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, 20 mM NaF, 10 mM Na-pyrophosphate, 5 mM EDTA, 1% Nonidet P-40, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstain, and 1 mM 4-(2-aminoethyl)benzensulfonyl fluoride, and probe sonicated (sonifier cell disruptor; Heat Systems-Ultrasonics, Inc., Plainview, NY). Solubilized lysates were clarified by centrifugation at 8000 $\times$ *g* for 10 min, precleared with agarose, and then incubated with specific antibodies and protein A or G agarose. The immunoprecipitates were collected, washed four times with lysis buffer, and dissolved in Laemmli buffer. After heating at 95 C for 5 min, the samples were centrifuged briefly and the supernatants were analyzed by SDS-PAGE on 8–16% gradient gels.

Measurement of MMP Activity

MMP activity was measured by incubation of cells with fluorescent MMP substrate [Dnp-PChaGCHAK(Nma)] following the manufacturer's instructions (BIOMOL, Plymouth Meeting, MA). Cells were grown in 12-well plates and stimulated with Ang II for 15 min in assay buffer [50 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35 (pH 7)] containing 10 μM fluorescent MMP substrate. Reactions were monitored in a fluorometer using excitation/emission values of 340/440 nm. In separate experiments, the amount of HB-EGF shed after Ang II stimulation was measured by immunoprecipitation and immunoblotting with HB-EGF antibody.

Immunoblot Analysis

Cells were grown in six-well plates and at 60–70% confluence were serum starved for 24 h before treatment at 37 C with selected agents. The media were then aspirated and the cells were washed twice with ice-cold PBS and lysed in 100 μl of Laemmli sample buffer. The samples were briefly sonicated, heated at 95 C for 5 min, and centrifuged for 5 min. The supernatants were electrophoresed on SDS-PAGE (8–16%) gradient gels and transferred to polyvinylidine difluoride membranes. Blots were incubated overnight at 4 C with primary antibodies and washed three times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then visualized with ECL [enhanced chemiluminescence reagent; Amersham Biosciences or Pierce (Rockford, IL)] and quantitated with a scanning laser densitometer. In some cases, blots were stripped and reprobed with other antibodies.

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Address all correspondence and requests for reprints to: Kevin J. Catt, Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, Building 49, Room 6A36, National Institutes of Health, Bethesda, Maryland 20892-4510. E-mail: catt@helix.nih.gov.

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