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Independence of Angiotensin II-Induced MAP Kinase Activation from Angiotensin Type 1 Receptor Internalization in Clone 9 Hepatocytes

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The agonist-induced internalization of several G protein-coupled receptors is an obligatory requirement for their activation of MAPKs. Studies on the relationship between endocytosis of the angiotensin II (Ang II) type 1 receptor (AT_1-R) and Ang II-induced ERK1/2 activation were performed in clone 9 (C9) rat hepatic cells treated with inhibitors of endocytosis [sucrose, phenylarsine oxide (PAO), and concanavalin A]. Although Ang II-induced endocytosis of the AT_1-R was prevented by sucrose and PAO, and was partially inhibited by concanavalin A, there was no impairment of Ang II-induced ERK activation. However, the specific epidermal growth factor receptor (EGF-R) kinase inhibitor, AG1478, abolished Ang II-induced activation of ERK1/2. Sucrose and PAO also inhibited EGF-induced internalization of the EGF-R in C9 cells, and the inability of these agents to impair EGF-induced ERK activation suggested that the latter is also independent of receptor endocytosis. In COS-7 cells transiently expressing the rat AT_{1A}-R, Ang II also caused ERK activation through EGF-R transactivation. Furthermore, a mutant AT_{1A}-R with truncated carboxyl terminus and impaired internalization retained full ability to activate ERK1/2 in response to Ang II stimulation. These findings demonstrate that Ang II-induced ERK1/2 activation in C9 hepatocytes is independent of both AT_1-R and EGF-R endocytosis and is mediated by transactivation of the EGF-R. (*Molecular Endocrinology* 16: 610–620, 2002)

THE EXTRACELLULARLY regulated MAP kinases, ERK-1 and ERK-2, are phosphorylated and activated by protein kinase cascades that are initiated by agonist binding to G proteincoupled receptors (GPCRs) and tyrosine kinase growth factor receptors (1, 2). Many agonist-activated GPCRs stimulate ERKs phosphorylation both directly through the Ras/Raf pathway and/or indirectly via transactivation of epidermal growth factor (EGF) or platelet-derived growth factor receptor tyrosine kinases (3–6). Exposure of GPCRs to their agonists is often followed by rapid attenuation, or desensitization, of their G protein-mediated signaling responses. This process appears to be initiated by receptor phosphorylation by GPCR kinases (GRKs) and second messenger-activated kinases (7). The consequent binding of β-arrestin to GRK-phosphorylated receptors terminates agonist-induced signaling by uncoupling the receptors from their cognate G protein(s), leading to attenuation of the target cell response (8). Desensitized GPCRs are rapidly internalized into endosomes via clathrin-coated vesicles by β-arrestin and dynamin-dependent (9–11) or -independent pathways (12, 13). The phosphorylated arrestin-bound receptors are subsequently dephosphorylated and then recycled to the plasma membrane or targeted to lysosomes for degradation (8, 14).

Three distinct pathways of GPCR sequestration have been defined by Claing *et al.* (12), based on their sensitivity to overexpression of GRK-interactor-1 (GIT1), a recently identified GRK-interacting factor that inhibits signaling and internalization of the β_2-adrenergic receptor. These are: 1) internalization through a GIT1, β-arrestin, and dynamin-sensitive pathway (β_2-AR, β_1-AR, μ-opioid receptors, M_1 muscarinic-R); 2) internalization through a dynamin-sensitive pathway that is insensitive to GIT1 or β-arrestin (VIP and endothelin B receptors); and 3) internalization through a pathway that is insensitive to GIT1, β-arrestin, and dynamin [Ang II type 1 receptor (AT_{1A}-R), M_2 muscarinic-R]. Based on the mechanisms involved, the above pathways were designated as 1) clathrin-coated pit, 2) caveolae-mediated, and 3) dynamin-independent, respectively. However, other studies have indicated that AT_1-R internalization is dynamin- and β-arrestin-dependent (11, 15) and occurs primarily via clathrin-coated vesicles and to a lesser extent through noncoated pits, possibly caveolae (15–17).

Several recent reports have implicated events associated with the termination of receptor signaling, including receptor phosphorylation, recruitment of β-arrestin, and clathrin-mediated endocytosis, in signaling to the MAPK cascade (8, 18, 19). Such GPCR internalization-dependent ERK activation was observed for α- and β-adren-

Abbreviations: Ang II, Angiotensin II; AT_1-R, angiotensin type 1 receptor; C9 cells, clone 9 rat liver cells; Con A, concanavalin A; EGF, epidermal growth factor; EGF-R, EGF receptor; GIT1, GRK interactor 1; GPCR, G protein-coupled receptor; GRK, GPCR kinase; HA, hemagglutinin; InsP, inositol phosphates; LPA, lysophosphatidic acid; MDC, monodansylcadaverine C; PAO, phenylarsine oxide; PKB, protein kinase B; WT, wild type.

ergic receptors in transfected COS-7 cells (20), muscarinic m1, m2, and m4 receptors in transfected HEK 293 cells (21), endogenous lysophosphatidic acid (LPA), thrombin, and bombesin receptors in Rat-1 fibroblasts (22), and μ-, σ-, and κ-opioid receptors in transfected COS-7 cells (23, 24). An analysis of the mechanism of ERK activation via internalized β_2-adrenergic receptors by Luttrell *et al.* (3) showed that β-arrestin binding to activated receptors recruits c-Src and initiates a second wave of signal transduction. In this process, the desensitized receptor appears to function as a critical structural component of the mitogenic signaling complex (25). However, in some cases, data obtained through morphological and biochemical approaches have shown that agonist-stimulated ERK activation is independent of GPCR endocytosis. This has been observed in α_{2A}-, α_{2B}-, and α_{2C}-adrenergic receptors (26, 27), κ-, μ-, and δ-opioid receptors (28, 29), the m3 muscarinic receptor (30), and the B2 bradykinin receptor (31).

AT_1-R stimulation causes ERK activation via multiple signaling pathways that include PKC/Ras/Raf, Pyk-2, and growth factor receptor (*e.g.* EGF and platelet-derived growth factor) tyrosine kinases (4–6, 32–34). Because EGF-induced ERK activation is dependent on clathrin-mediated endocytosis (35), it was suggested that ERK activation by GPCRs acting via transactivation of EGFR would be sensitive to inhibitors of endocytosis, regardless of whether the GPCR itself underwent agonist-induced internalization (19, 20, 36). However, there are conflicting reports regarding the mechanism(s) involved in AT_1-R internalization. For example, there is no consensus on the role of various components of the endocytic machinery, including β-arrestins, dynamin, clathrin, and caveolae, in AT_1 receptor internalization (9, 13, 15, 17, 35–38). In this study, the relationship between AT_1-R internalization and ERK activation was analyzed by determining the effects of several inhibitors of endocytosis in clone 9 (C9) rat liver cells that express endogenous AT_1-Rs. We found no correlation between AT_1-R endocytosis and angiotensin II (Ang II)-mediated ERK activation in wild-type (WT) C9 cells and also observed that an internalization-deficient mutant AT_1-R was as effective as the WT receptor in mediating ERK activation when expressed in COS-7 cells.

RESULTS

In Ang II-treated C9 cells, AT_1 receptors underwent rapid internalization with loss of about 50% of the cell surface receptor population within 10 min. When the effects of several inhibitors of receptor internalization (15, 22, 39–41) on agonist-induced AT_1-R endocytosis were examined in C9 cells, AT_1-R internalization was completely inhibited by hyperosmolar sucrose (0.45 M) and phenylarsine oxide (PAO) (2.5 μM), partially inhibited (40%) by concavalin A (Con A; 250 μg/ml), and unaffected by monodansylcadaverine [MDC; 300 μM (Fig. 1)]. The absence of an inhibitory effect of MDC on AT_1-R internalization contrasts with that observed on β_2-adrenergic receptor internalization (18–20).

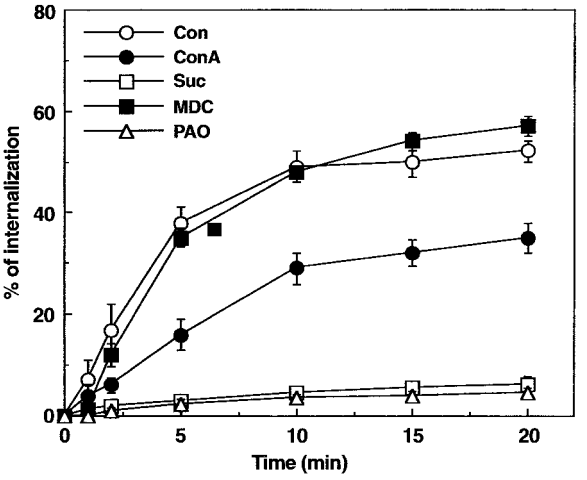

Fig. 1. Effects of Inhibitors of Endocytosis on AT_1-R Internalization in C9 Cells

After treatment with Con A (250 μg/ml), MDC (300 μM) and PAO (5 μM) for 30 min, and sucrose (Suc) (0.45 M) for 5 min, cells were incubated with ^{125}I-Ang II at 37 C for the indicated times. Con indicates untreated control. Acid-resistant and acid-sensitive binding (cpm) were determined, and the specific internalized (acid-resistant) binding is expressed as a percentage of the total binding at each time point. The data in each panel represent mean ($\pm$SEM) values from four independent experiments.

Recent studies have shown that, at least in certain cell types, receptor internalization is required for activation of downstream mitogenic signaling cascades including those leading to ERK phosphorylation (20–24). To examine the relationship between AT_1-R internalization and ERK activation, we measured the effects of endocytosis inhibitors on Ang II-induced ERK activation by immunoblotting the phosphorylated ERK1/2 using phospho-specific ERK1/2 antibodies. Ang II caused a rapid and marked increase in ERK activation, reaching a peak within 5 min and then declining to the basal level within 15–30 min (Fig. 2A). Agonist-induced ERK activation was almost completely abolished by the AT_1-R antagonist, losartan (DuP753), but was unaffected by the AT_2-R antagonist, PD123177 (Fig. 2B). Pretreatment of cells with several internalization inhibitors had differential effects on Ang II-induced ERK activation. Hypertonic sucrose and PAO completely, and Con A partially, inhibited AT_1-R internalization, but none of these inhibitors had any effect on Ang II-induced ERK activation (Fig. 2C). AT_1-R internalization was blocked by as little as 2.5 μM PAO, but at higher concentrations ($>$5 μM) this compound inhibited Ang II-induced ERK activation. This effect could result from inhibition of inositol phosphate (InsP) production (42) or nonspecific toxic effects of the compound (43). MDC, which has been reported to block β_2-adrenergic receptor internalization, and thus

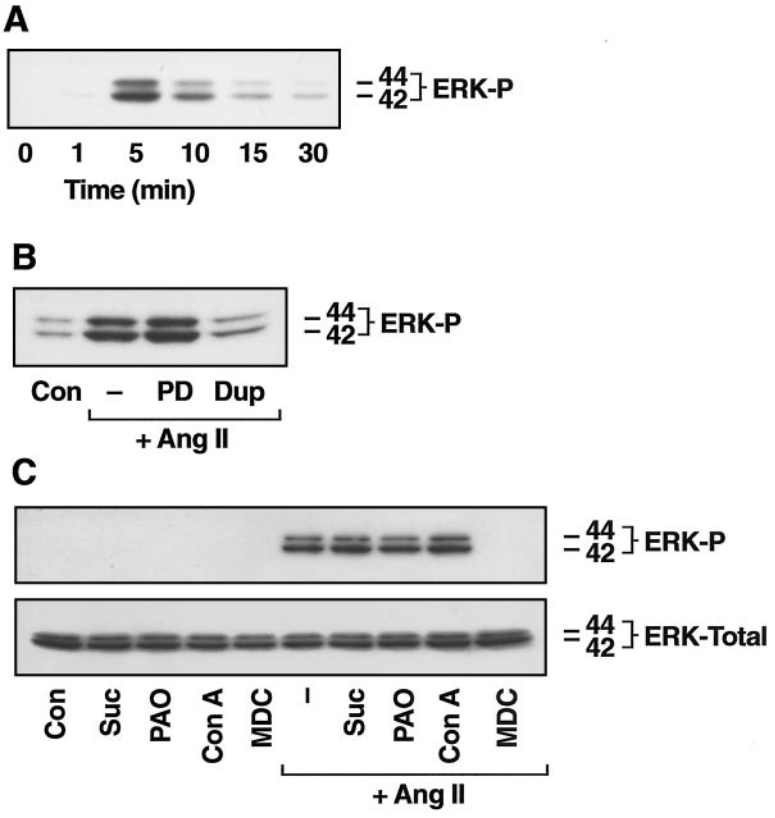

Fig. 2. Effects of Endocytosis Inhibitors in AngII-Induced ERK Phosphorylation

A, Time course of Ang II-induced ERK1/2 activation in C9 cells. Serum-starved cells were treated with Ang II (100 nM) for the indicated times, washed twice with ice-cold PBS, and collected in Laemmli sample buffer for analysis and immunoblotting with anti-phospho-ERK (ERK-P) anitbody. B, Ang II-stimulated ERK activation is mediated by the AT_1-R. Specific AT_1-R and AT_2-R antagonists, DuP753 (2-n-butyl-4-chloro-5-hydroxymethyl-l-[(2′-(1H-tetrazole-5-yl)biphenyl-4-yl)-methyl]imidazole; 10 μM) and PD123177 (1-[(4-amino-3-methylphenyl)methyl]-5-(dephenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazol[4,5-*C*]pyridine-6-carboxylic acid; 10 μM), were added 20 min before exposure to Ang II (100 nM) for 5 min. C, Effects of sucrose (0.45 M), PAO (2.5 μM), Con A, (250 μg/ml), and MDC (300 μM) on Ang II-induced ERK1/2 activation. Cells were incubated with inhibitors for the same times as in Fig. 1, followed by stimulation with Ang II for 5 min. The data shown are representative of three independent experiments.

ERK activation, by disrupting clathrin cages (19) had no effect on AT_1-R internalization but reduced Ang II-induced ERK activation.

Several recent studies have shown that Ang II-induced ERK activation is mediated through transactivation of the EGF-R in certain cell types (5–6, 41, 44). In C9 cells, pretreatment with AG1478, an EGF-R kinase inhibitor, completely abolished ERK activation induced by Ang II, as well as by EGF (Fig. 3A). This finding indicates that transactivation of the endogenous EGF-R is the major pathway for Ang II-induced ERK stimulation in hepatic C9 cells. Recently, Con A was shown to inhibit AT_1-R internalization as well as Ang II-induced ERK activation in vascular smooth muscle cells, effects that were attributed to degradation of the EGF-R (41). Similarly, PAO was shown to cause the cleavage of Erb4B, a transmembrane tyrosine kinase receptor (45). Evaluation of the effects of internalization inhibitors on EGF-R receptor integrity revealed that none of the compounds, either alone or in the presence of Ang II, caused degradation/proteolysis of the EGF-R (Fig. 3B).

Treatment of C9 cells with EGF caused marked activation of ERK that reached a peak at 5 min and declined thereafter (Fig. 4A). EGF treatment also led to a marked reduction of EGF-R immunoreactivity, suggesting rapid internalization and degradation of the EGF-R. However, whereas Ang II caused ERK activation through transactivation of the EGF-R, it did not affect the immunoreactive EGF-R level (Fig. 4B). Time course studies revealed that 50% of EGF-R internalization, as measured by radioligand binding with ^{125}I-EGF, occurred within 5 min and reached a maximum of 75% within 10 min (Fig. 5A). Although PAO has been extensively used for blocking clathrin-mediated receptor internalization, at higher concentrations it exerts toxic actions and causes a wide variety of unrelated effects on other signaling molecules (42, 43). For this reason, we determined the minimum effective concentration of this compound that inhibited receptor endocytosis. An analysis of the concentration-dependent effects of PAO showed that as little as 2.5 μM PAO is effective in blocking AT_1-R as well as EGF-R endocytosis (Fig. 5B).

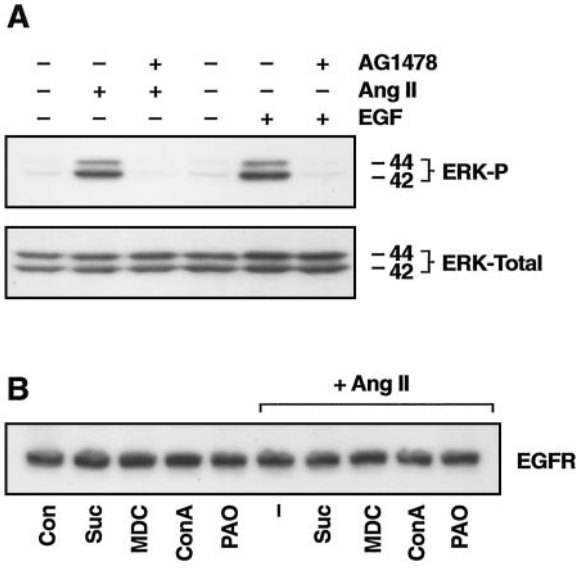

Fig. 3. Dependence of Ang II-Induced ERK Phosphorylation on EGF-R Transactivation

A, Inhibition of Ang II- and EGF-induced activation of ERK1/2 by the EGF-R kinase inhibitor, AG1478 (100 nM). B, Effects of inhibitors and Ang II on EGF-R degradation. C9 cells were treated with inhibitors and Ang II, and then processed as described in Fig. 2 and immunoblotted with phospho-ERK (ERK-P) or EGFR antibody. Similar results were obtained in two other experiments. Con, Control; Suc, sucrose.

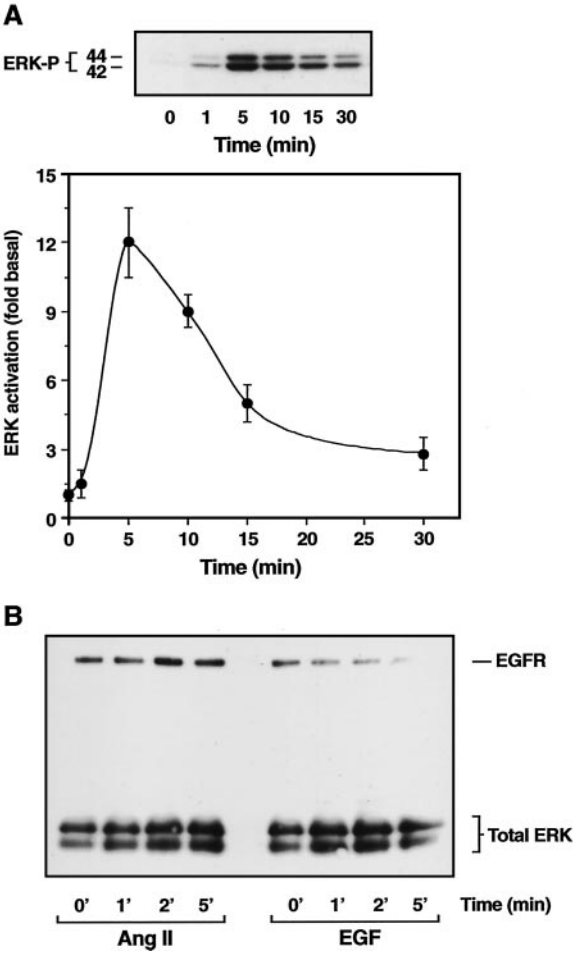

Fig. 4. Effects of EGF on ERK Activation and EGF-R Levels

A, Time course of EGF-induced ERK1/2 activation in C9 cells. Serum-starved cells were treated with EGF (50 ng/ml) for indicated times, and then analyzed and immunoblotted for phospho-ERK (ERK-P) (n = 3). B, Time-course effects of Ang II and EGF on EGF-R immunoreactivity in C9 cells. Cells were collected and lysates were immunoblotted for EGF-R or ERK. The data shown are representative of two similar experiments.

To examine the role of EGF-R endocytosis in agonist-induced MAPK activation, we measured the effects of endocytosis inhibitors on EGF-R internalization and EGF-induced ERK activation. Although EGF-R internalization was completely blocked by sucrose and PAO and partially by Con A (Fig. 6A), neither compound impaired EGF-induced ERK activation (Fig. 6B). These effects of endocytosis inhibitors are similar to those observed for the AT_1-R, as shown above, and argue against a role of receptor endocytosis in ERK activation.

Agonist-induced endocytosis of the EGF-R has been shown to be dependent on the tyrosine kinase activity of the receptor (46). To investigate the differential effects of Ang II and EGF on EGF-R levels, we measured the tyrosine phosphorylation of the EGF-R. As shown in Fig. 7A, EGF caused marked stimulation of EGF-R phosphorylation compared with Ang II. To further verify the limited or selective stimulation of signaling pathways by Ang II downstream of EGF-R, we measured the phosphorylation of protein kinase B (PKB/Akt). Interestingly, Ang II-induced phosphorylation of PKB/Akt was mediated through EGF-R transactivation but was much less than that induced by EGF (Fig. 7B). These data suggest that ERK activation by Ang II does not require full activation of the EGF-R and also emphasize the prominent effects of EGF-R activation on its downstream signaling pathways. Because agonist stimulation of AT_1-R leads to activation of $G_{q/11}$ proteins, and thus stimulation of PLC, we measured the effects of internalization inhibitors on InsP production as an early index of AT_1-R activation. Although Ang II caused 4-fold stimulation of InsP production, none of the inhibitors significantly affected this increase in C9 cells (data not shown).

Ang II stimulation causes rapid phosphorylation and internalization of the AT_1-R expressed in adrenal glomerulosa cells and transfected cells (47–48). Truncation of the carboxy terminus of the AT_1-R markedly impairs its ability to undergo internalization (41, 46). To complement the above studies on endogenous AT_1-Rs expressed in C9 cells, COS-7 cells transfected with WT and truncated mutant AT_1-Rs were used to examine the relationship between AT_1-R internalization and Ang II-induced ERK activation. WT AT_1-R showed rapid and marked internalization, whereas that of the mutant AT_1-R was substantially reduced (Fig. 8A). However, Ang II-induced ERK activation was similar in WT and mutant AT_1-R in COS-7 cells (Fig. 8B). Moreover, ERK activation was

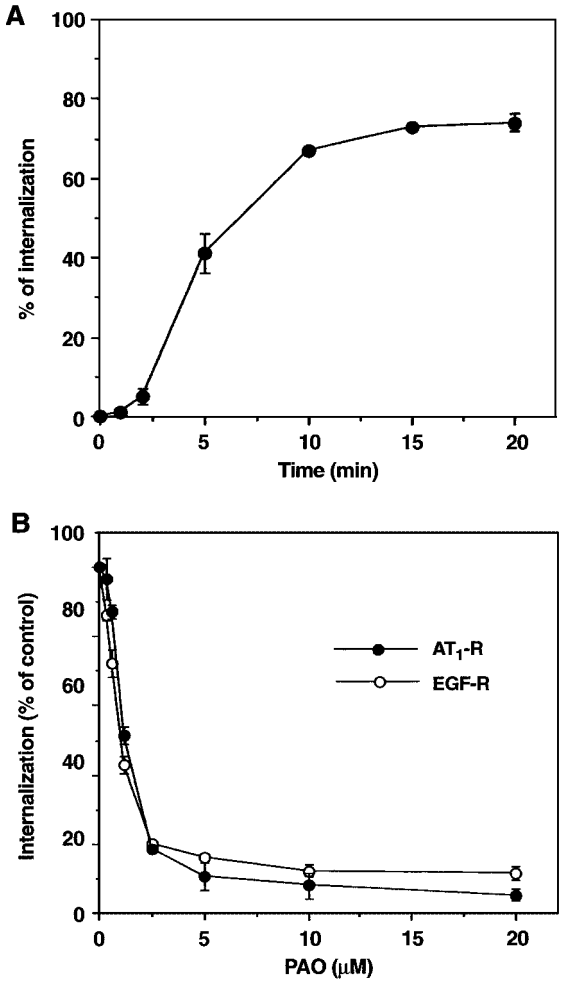

Fig. 5. Internalization Kinetics of EGF-R in C9 Cells

A, Internalization kinetics of EGF-R as indicated by the endocytosis of ^{125}I-EGF during incubation at 37 C for the indicated times. Acid-resistant and acid-sensitive binding (cpm) were determined, and the specific internalized (acid-resistant) binding was expressed as a percentage of the total binding at each time point. B, Concentration dependence of the inhibitory effects of PAO on AT_1-R and EGF-R internalization in C9 cells. The data in each panel represent mean (±SEM) values from three independentexperiments.

sensitive to the EGF-R kinase inhibitor, AG1478, as observed in C9 cells (Fig. 8C). These data indicate that Ang II-induced ERK activation occurs through transactivation of the EGF-R and is independent of AT_1-R receptor internalization.

DISCUSSION

Studies utilizing cells transfected with native or mutant GPCRs and transducing proteins have provided useful insights into the mechanisms of receptor signaling and endocytosis. However, the characteristics of cells containing overexpressed receptors and signaling proteins may differ from those of cells expressing endogenous GPCRs and the physiological complement of transduction machinery. For example, the overexpression of multiple proteins can enhance the nonspecific interactions between GPCRs and their signaling molecules (26, 38, 49, 50). The C9 hepatic cells employed in the present study are derived from the normal rat liver and retain an epithelial phenotype. They also express endogenous AT_1-receptors that are coupled to both G_q and G_i proteins and thus represent a useful model in which to investigate AT_1-R internalization and Ang II-induced activation of the ERK1/2 signaling cascade.

Upon agonist binding, most GPCRs undergo conformational changes that lead to signal transduction and acceleration of receptor internalization. Recent studies in native and transfected cells have indicated that the internalized agonist-receptor complex also generates signals that lead to activation of MAPK (18–24). During β-adrenergic receptor stimulation, β-arrestin binding promotes the recruitment of c-Src and initiates a second wave of signal transduction in which the desensitized receptor functions as a critical structural component of a mitogenic signaling complex (3, 25). However, some reports argue against the universal role of internalized GPCRs in activation of the ERK1/2 cascade (26–31). Our results indicate that inhibitors of endocytosis exert variable effects on AT_1-R internalization, Ang II-induced InsP production, and ERK activation. Also, although the internalization of the C-terminally truncated AT_1-R (Δ335) was markedly attenuated, it was as effective as the WT AT_1-R in mediating Ang II-induced ERK activation. These findings clearly demonstrate that AT_1-R internalization in C9 hepatic cells is not required for downstream signaling, including ERK1/2 activation. The present data also support our earlier finding that G_q protein-linked signaling is independent of AT_1-R internalization, because the AT_1-R mutant (D74Y) with the most impaired internalization gave the highest InsP production in transfected COS-7 cells (47).

Endocytosis-dependent activation of ERK1/2 by GPCRs has been demonstrated for α_{2A}- and β_2-adrenergic receptors (20), m1, m2, and m4 muscarinic receptors (21), LPA, thrombin, and bombesin receptors (22), and μ-, σ-, and κ-opioid receptors (23, 24). However, internalization-independent activation of ERK1/2 has been documented for α_{2A}-, α_{2B}-, and α_{2C}-adrenergic receptors (26, 27), κ-, μ-, and δ-opioid receptors (28, 29), the m3 muscarinic receptor (30), and the B2 bradykinin receptor (31). Taken together, these reports and the present findings indicate that endocytosis-dependent signaling is not a general feature of all GPCRs and is not required for ERK1/2 activation by Ang II in C9 cells.

It has become evident that the mechanism(s) involved in agonist-induced endocytosis differ among individual receptors and cell types. Although there is abundant biochemical and morphological evidence that AT_1-R internalization occurs primarily via clathrin-

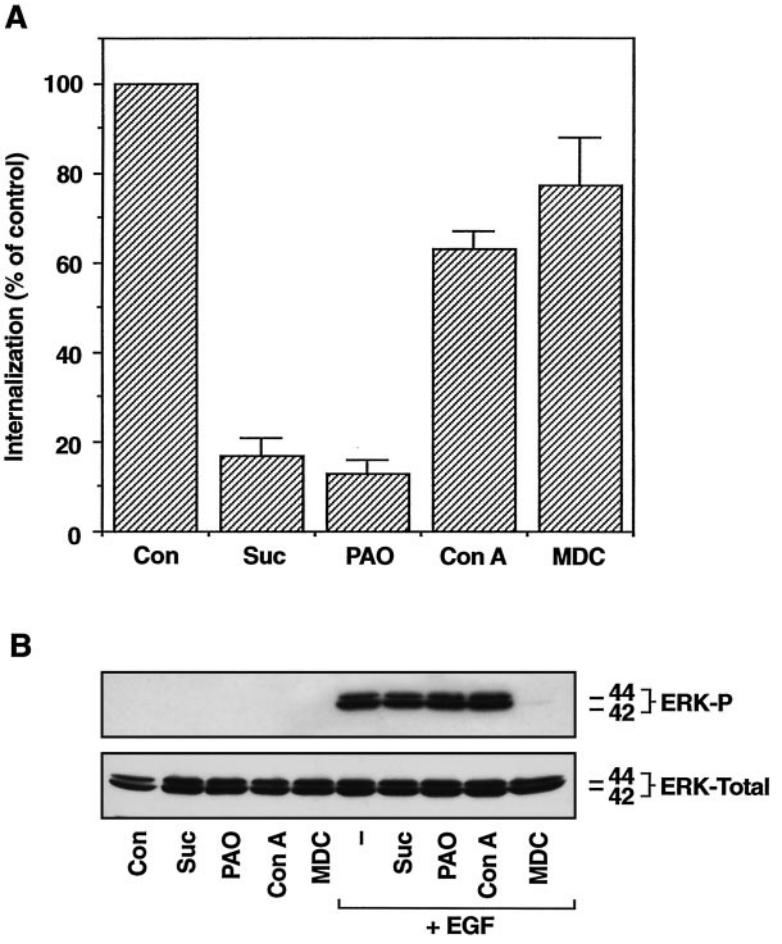

Fig. 6. Inhibition of EGF-R Endocytosis by Hypertonic Sucrose and Phenylarsine Oxide

A, Effects of internalization inhibitors on EGF-R endocytosis in C9 cells. Cells were treated with inhibitors as described in Fig. 1, and EGF-R internalization was measured as described in Fig. 5 (n = 3). B, Effects of inhibitors on EGF-induced ERK1/2 activation. Drugs were added as in Fig. 1, and cells were stimulated with EGF (50 ng/ml) for 5 min, and then analyzed and immunoblotted for phospho-ERK (ERK-P). The data shown are representative of three independent experiments. Con, Control; Suc, sucrose.

coated vesicles (9, 15), a role for caveolae has also been proposed in certain cell types (16, 17). The present study used several agents that are known to inhibit clathrin-mediated receptor endocytosis (15, 19, 39). As previously observed, both sucrose and PAO completely inhibited AT_1-R internalization (15, 47, 48, 51–53). However, the finding that neither of these compounds inhibited Ang II-induced ERK activation indicates that AT_1-R endocytosis is not required for Ang II-stimulated ERK1/2 activation in C9 cells (Figs. 1 and 2).

Another GPCR internalization inhibitor, MDC, is known to inhibit receptor trafficking at a step proximal to the formation of endocytic vesicles (54). It inhibits ERK activation induced by IGF-I (55), β-adrenergic receptors (19), and LPA (22). In COS-7 cells transfected with β_2-adrenergic receptors, MDC inhibited EGF-induced ERK activation without affecting EGF-R phosphorylation, suggesting that the MDC-sensitive step in this case is downstream of receptor activation (19). Our finding that MDC inhibited Ang II- or EGF-induced ERK activation (Fig. 2, C and B), but had no effect on AT_1-R or EGF-R internalization (Figs. 1 and 6A), suggests that its inhibitory action on ERK activation is independent of receptor internalization, or that its action is distal to membrane receptors. Alternatively, it is possible that inhibition of GPCR internalization by MDC is cell-type dependent.

As noted above, Claing *et al.* (19) have proposed that AT_1-R internalization is β-arrestin and dynamin independent and is mediated through non-clathrin-coated pits, a process that would be insensitive to MDC. However, we (15) and others (11) have shown that AT_1-R endocytosis is dependent on both β-arrestin and dynamin and is mediated through clathrin-coated pits. Similarly, AT_1-R and EGF-R internalization in C9 cells was inhibited by agents (hypertonic sucrose and PAO) that impede clathrin-mediated endocytosis by preventing the clustering of cell-surface receptors into endosomes due to loss of normal clathrin lattices

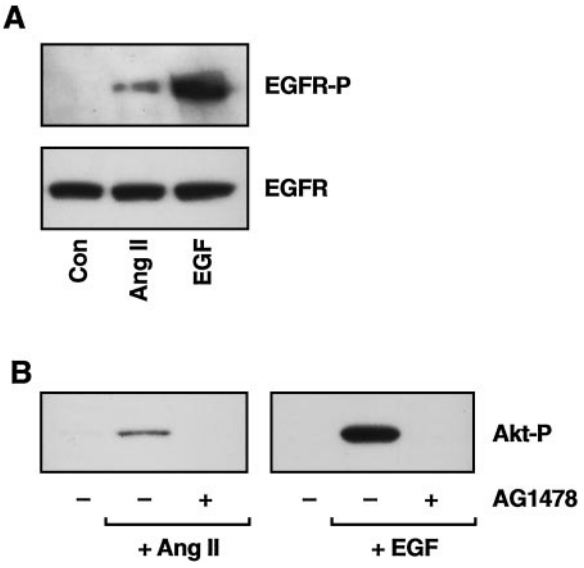

Fig. 7. Relative Effects of Ang II and EGF on Phosphorylation of EGF-R and Akt

A, Effects of Ang II and EGF on tyrosine phosphorylation of the EGF-R. Serum-starved cells treated with Ang II (100 nM) or EGF (10 ng/ml) for 1 min were washed twice with ice-cold PBS and collected in lysis buffer followed by immunoprecipitation with EGF-R antibody, SDS-PAGE analysis, and immunoblotting with antiphosphotyrosine antibody (PY20). B, Effects of Ang II and EGF on phosphorylation of PKB/Akt. Cells were pretreated with EGF-R kinase inhibitor, AG1478 (100 nM), for 20 min before stimulation with Ang II (100 nM) or EGF (50 ng/ml) for 5 min. Con, Control; EGFR-P, phospho-EGF; Akt-P, phospho-Akt.

(39, 51–52). Our data show that ERK activation by Ang II occurs through transactivation of the EGF-R, which functions as a receptor tyrosine kinase (Fig. 3A). EGF binding results in rapid endocytosis of the EGF-R through clathrin-coated pits, followed by degradation of receptor and ligand by lysosomal enzymes (35, 52, 56). Earlier studies suggested that EGF-induced signals for ERK activation originate from internalized EGF-Rs located in early endosomes (35, 53, 57). However, more recent studies in hepatocytes (Hep 2 cells) showed that internalization of the EGF-R was not required for ERK activation because K^+ depletion, which blocks clathrin-dependent EGF-R endocytosis, did not inhibit ERK activation by EGF (58). Our data support the view that EGF-induced ERK activation is independent of EGF-R endocytosis, because inhibitors that block clathrin-mediated receptor internalization did not impair ERK phosphorylation.

Agonist-activated EGF-Rs undergo rapid dimerization and phosphorylation of tyrosines within their cytoplasmic domains, followed by recruitment of other signaling proteins including PLCγ and c-Src. EGF-R activation also leads to tyrosine phosphorylation of the adaptor protein, Shc. The subsequent formation of a Shc-Grb2-Sos complex causes Ras-mediated initiation of the phosphorylation cascade leading to ERK activation (46). In many cases, the pathways of GPCR- and receptor tyrosine kinase-mediated ERK activation are convergent, largely at the point of activation of the EGF-R (19, 22, 36, 44). After tyrosine phosphorylation of the EGF-R, the signaling pathways involved in ERK activation by GPCRs and the EGFR appear to be identical, because inhibition of intrinsic EGF-R kinase activity by AG1478 blocks both EGF-R- and GPCR-mediated ERK activation (19, 41, 44). However, our results show that the degree of EGF-R endocytosis, and the magnitude of phosphorylation of PKB/Akt by Ang II and EGF, are considerably different in C9 cells (Figs. 4, B and C, and 7B). This marked divergence in signaling efficacy of the two agonists is attributable to the much lower extent of EGF-R tyrosine phosphorylation by Ang II as compared with that of EGF (Fig. 7A).

EGF-R endocytosis and subsequent degradation of the receptor and its ligand are dependent on receptor tyrosine kinase activity, because a kinase-negative receptor mutant recycles to the cell surface for reutilization (46). Therefore, it is likely that the much smaller loss of immunoreactive EGF-R caused by Ang II than by EGF is related to the relative degrees of EGF-R tyrosine phosphorylation induced by these agonists. Moreover, our recent studies in hepatic C9 cells show that Ang II causes selective activation of PKCδ, which has been implicated in phosphorylation of the AT_1-R (59), as well as ERK activation (Shah, B.H., and K.J. Catt, unpublished results). Recent studies show that PKC-mediated phosphorylation of the EGF-R at threonine 654 diverts the EGF-R from degradation to recycling (60). Whether the lack of decrease in total EGF-R levels after exposure to Ang II results from a similar effect remains to be investigated. However, other recent studies in mesangial cells have shown that GPCRs acting through the Gq/PKC pathway cause ERK activation through transphosphorylation of the EGF-R but do not alter the total EGF-R levels in these cells (61).

PAO, which has been extensively used in studies on receptor endocytosis (9, 45, 47, 52), has been shown to block EGF-R endocytosis through clathrin-coated pits (52). In C9 cells, PAO concentrations above 5 μM inhibited receptor internalization and agonist-induced ERK activation (data not shown). However, lower concentrations of PAO (2.5 μM) inhibited AT_1-R and EGF-R internalization but did not reduce agonist-induced ERK activation (Figs. 2C, 5B, and 6B). PAO has been reported to inhibit EGF-dependent MAPK activation at high concentrations (52). It also affects several other signaling processes in a concentration-dependent manner, including inhibition of PI 4-kinase and calcium influx (62), calcium-dependent ATPase (63), phosphatase (64), and actin polymerization in glioma cells (65). In addition, hepatocytes are sensitive to the adverse effects of PAO on energy-dependent cellular activities (43). Taken together, these observations indicate that PAO exerts inhibitory actions on numerous signaling molecules, and that its concentration-dependent actions in individual cell types should

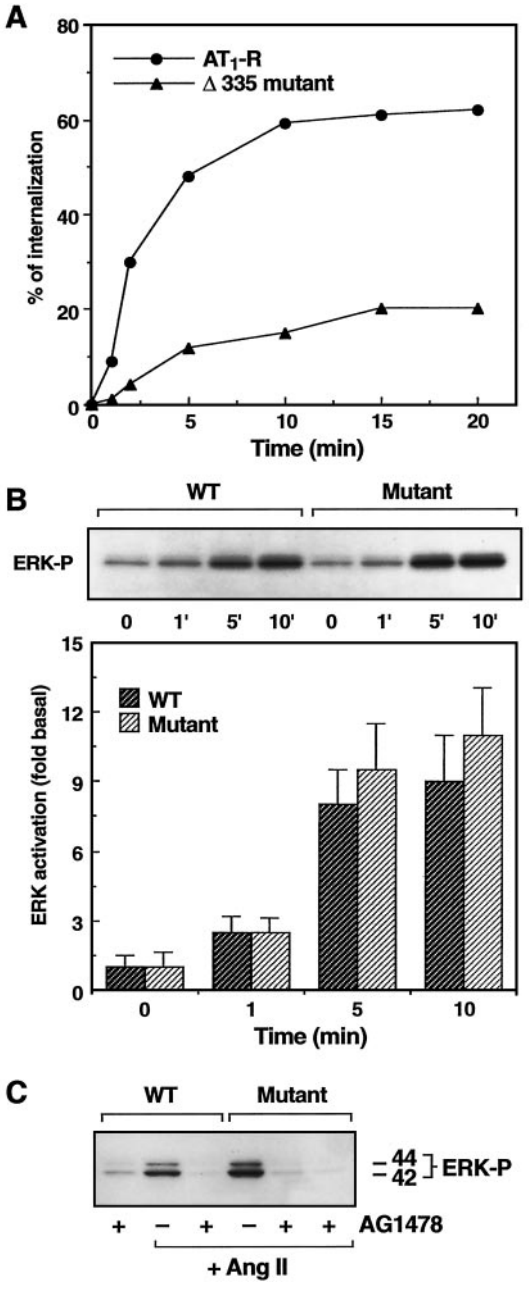

Fig. 8. A Poorly Internalized AT1-R Mutant Mediates Ang II-Induced ERK Phosphorylation in COS-7 Cells

A, Internalization kinetics of WT and carboxyl-truncated mutant AT_1-Rs (Δ335) expressed in COS-7 cells. Cells were incubated with ^{125}I-Ang II at 37 C for the indicated times, and then acid-resistant and acid-sensitive binding (cpm) were determined as described in *Materials and Methods*. The specific internalized (acid-resistant) binding was expressed as a percentage of the total binding at each time point. The data shown are derived from three independent experiments. B, Effects of Ang II on ERK activation in COS-7 cells transfected with WT and mutant AT_1-R. Serum-starved cells were stimulated with Ang II (100 nM) for indicated times, and cell lysates were analyzed and immunoblotted with phospho-ERK1/2-specific antibodies. Quantitation of data was performed on results from three independent experiments. C, Effect of the EGF-R kinase inhibitor, AG1478 (100 nM), on Ang II- and EGF-induced ERK activation in COS-7 cells transfected with WT and mutant AT_1-R. Serum-starved cells were stimulated with Ang II (100 nM) for the indicated time periods, and cell lysates were analyzed and immunoblotted with phospho-ERK (ERK-P)-specific antibodies. The data shown are representative of three similar experiments.

be taken into account when interpreting its effects on specific signaling pathways.

We observed that Con A, which inhibits GPCR endocytosis by binding to terminal mannose residues on cell surface glycoproteins and glycolipids and reducing their lateral mobility (22, 29, 41, 66), caused only partial inhibition of AT_1-R internalization. Our findings are consistent with those of Budd *et al.* (30), who did not observe Con A-mediated inhibition of ERK activation induced by stimulation of m3 muscarinic receptors in CHO cells. However, recent studies in vascular smooth muscle cells have shown that Ang II-induced AT_1-R internalization and ERK activation were inhibited by Con A and suggested that the loss of Ang II-induced ERK activation was caused by degradation of the EGF-R (41). In contrast, we found that despite its reduction of AT_1-R internalization, Con A did not exert a corresponding inhibitory effect on Ang II- or EGF-induced ERK activation (Figs. 1, 2, and 6A). Moreover, Con A and other inhibitors, either alone or in the presence of Ang II and/or EGF, did not cause proteolysis of the EGF-R in C9 cells (Fig. 3A). Thus, Con A exerts variable effects on GPCR internalization as well as ERK activation in a cell type- and receptor-dependent manner (30, 41). Taken together, these results further support the view that AT_1-R internalization is not required for ERK activation.

Additional evidence for the lack of a relationship between AT_1-R internalization and Ang II-induced ERK activation was provided by the ability of a mutant AT_1-R with impaired internalization to transduce the Ang II-induced signals (activation of the G_q/PLC pathway) involved in ERK activation. Phosphorylation of serine/threonine residues in the cytoplasmic tail of the AT_1-R regulates its internalization in CHO and COS-7 cells (67, 68). This is attributed to their consequent binding of β-arrestins, which possess clathrin and AP2 adaptor protein-binding domains that are believed to target GPCRs to clathrin-coated pits (69). Consistent with this mechanism, deletion of the clathrin-binding regions in β-arrestin or disruption of clathrin lattices by chemical agents causes inhibition of AT_1-R internalization (15). Our finding that truncation of the carboxy-terminal tail of the AT_1-R impairs its internalization without affecting Ang II-mediated stimulation of ERK (Fig. 8), and the lack of effects of inhibitors of endocytosis thereon, clearly demonstrates that Ang II induced ERK activation does not require AT_1-R internalization in C9 hepatic cells.

MATERIALS AND METHODS

Materials

Ang II was obtained from Peninsula Laboratories, Inc. (Belmont, CA) and EGF from Life Technologies, Inc. (Gaithersburg, MD). Anti-phospho-ERK1/2 antibodies were obtained from New England Biolabs, Inc. (Beverly, MA), and secondary antibodies were from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Antibody to EGF-R was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ^{125}I-Ang II and enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). ^{125}I-EGF was obtained from NEN Life Science Products (Boston, MA). C9 cells were obtained from ATCC (Manassas, VA). MDC, Con A, and PAO were obtained from Sigma (St. Louis, MO).

Cell Culture

C9 rat liver epithelial cells were grown in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% (vol/vol) 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 as these exhibit the maximum expression of endogenous AT_1 receptors. Cells were maintained for 16 h before experiments in inositol-free medium when [^{3}H]inositol labeling was performed.

Mutagenesis and Transient Expression of Hemagglutinin (HA)-AT_{1A}-R

The influenza HA epitope (YPYDVPDYA) was inserted after the codons of the amino-terminal first two amino acids (MA) into the cDNA of the rat AT_{1A}-receptor subcloned into pcDNA3.1(+) (Invitrogen, San Diego, CA) as previously described (68). The Δ335 tail truncation mutant HA-AT_{1A}-R was created by the introduction of a stop codon at Ser335 using the Mutagene kit (Bio-Rad Laboratories, Inc., Hercules, CA), and verified by dideoxy sequencing using Thermosequenase (Amersham Pharmacia Biotech).

COS-7 cells were seeded at 8×10^5 cells per 10-cm dish in DMEM containing 10% (vol/vol) FBS, 100 μg/ml streptomycin, and 100 IU/ml penicillin (COS-7 cell medium), and maintained in a humidified atmosphere of 5% CO_2 in air at 37 C for 3 d before use. Cells were transfected using 5 ml/dish of OptiMEM containing 10 μg/ml of LipofectAMINE and the required DNA (1 μg/ml) for 6 h at 37 C. After changing to fresh COS-7 cell medium, the cells were cultured for a further 2 d before use.

Receptor Internalization

^{125}I-Labeled Ang II (0.5–1 μCi) or ^{125}I-EGF (0.5 ng/ml) was added in HEPES-buffered medium 199 to C9 cells or HA-AT_{1A}-R expressing COS-7 cells in 24-well plates, and incubations were performed at 37 C for the indicated times. Incubations were stopped by rapid washing with ice-cold Dulbecco's PBS, and the cells were treated for 10 min with 0.5 ml of an acid wash solution (150 mM NaCl-50 mM acetic acid) to remove the surface-bound radioligand. The supernatant containing the acid-released radioactivity was collected, and the cells were treated with 0.5 M NaOH and 0.05% SDS to solubilize the acid-resistant (internalized) radioactivity. Nonspecific binding was measured in the presence of an excess (10 μM) of unlabeled [Sar^1,Ile^8]Ang II. Radioactivity was measured by γ-spectrometry, and the percent internalization for each point was calculated, after deducting the respective nonspecific values, from the ratio of the acid-resistant binding to the total (acid-resistant + acid-released) binding.

InsP Measurements

[^{3}H]inositol labeling and incubation of C9 cells were performed as previously described (42). Cells were incubated [^{3}H]inositol in 0.5 ml inositol-free DMEM for 24 h, and then washed twice and incubated for 30 min at 37 C in inositol-free modified medium 199 containing 10 mM LiCl. After stimulation with 100 nM Ang II for 30 min, reactions were stopped with perchloric acid, InsPs were extracted, and radioactivity was measured by liquid scintillation γ-spectrometry.

Immunoprecipitation

After treatment with inhibitors and drugs, cells were placed on ice, washed twice with ice-cold PBS, lysed in LB buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM Na pyrophosphate, 5 mM EDTA, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin, and 1 mM 4-(2-aminoethyl)benzensulfonyl fluoride, and probe sonicated (Sonifier Cell Disruptor). Solubilized lysates were clarified by centrifugation at 14,000 rpm for 10 min, precleared with agarose, and then incubated with antibodies and protein A or G agarose. The immunoprecipitates were collected, washed four times with LB, and mixed with 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.

Immunoblot Analysis

C9 cells were grown in six-well plates in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% FCS. At 60–70% confluence, cells 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 supernatant was electrophoresed on SDS-PAGE (8–16%) gradient gels and transferred to PDVF nylon membranes. Phosphorylated ERK1 and ERK2 were detected with a 1:2,000 dilution of monoclonal mouse phospho-ERK1/2-specific antibody. Blots were probed with a 1:3,000 dilution of antimouse horseradish peroxidase-conjugated secondary antibody, and then visualized with enhanced chemiluminescence reagent; Amersham Pharmacia Biotech) and quantitated with a scanning laser densitometer. In some cases, blots were stripped and reprobed with other antibodies.

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