Endothelin-1 activates mesangial cell ERK1/2 via EGF-receptor transactivation and caveolin-1 interaction

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Hua, Hong, Snezana Munk, and Catharine I. Whiteside. Endothelin-1 activates mesangial cell ERK1/2 via EGF-receptor transactivation and caveolin-1 interaction. Am J Physiol Renal Physiol 284: F303–F312, 2003. First published September 17, 2002; 10.1152/ajprenal.00127.2002.—Endothelin-1 (ET-1) stimulates glomerular mesangial cell proliferation and extracellular matrix protein transcription through an ERK1/2-dependent pathway. In this study, we determined whether ET-1 activation of mesangial cell ERK1/2 is mediated through EGF receptor (EGF-R) transactivation and whether intact caveolae are required. We showed that ET-1 stimulated tyrosine phosphorylation of the EGF-R in primary cultured, growth-arrested rat mesangial cells. In response to ET-1, ERK1/2 phosphorylation was increased by 27 ± 1-fold and attenuated by AG-1478, a specific EGF-R inhibitor, to 9 ± 1-fold. Moreover, filipin III and β-cyclodextrin, two cholesterol-depleting drugs known to disrupt caveolae, significantly reduced ET-1-induced phosphorylation of ERK1/2. In addition, preincubation of mesangial cells with a myristoylated peptide that binds to the caveolin-1 scaffolding domain diminished ET-1 activation of ERK1/2. ET-1 caused interaction of caveolin-1 with phosphorylated ERK1/2 identified by coimmunoprecipitation. Activation of ERK1/2 and its interaction with caveolin-1 were reduced by AG-1478, β-cyclodextrin, or inhibition of PKC. Phosphorylated ERK1/2 localized at focal adhesion complexes along with phospho-caveolin-1, suggesting specific sites of compartmentalization of these signaling molecules. Hence, ET-1 activates mesangial cell ERK1/2 predominantly through a pathway involving EGF-R transactivation, leading to a mechanism involving attachment to caveolin-1, presumably in caveolae.

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cantly reduced ET-1 stimulation of phosphorylation of 
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ET-1 activation of ERK1/2. Phosphorylated ERK1/2 
interaction with caveolin-1, identified through co-
munoprecipitation, appears to be dependent on EGF-R 
transactivation, in part PKC dependent, and requiring 
intact caveolae. Finally, phospho-ERK1/2 localized at 
attachment complexes along with phospho-caveolin-1, 
suggesting specific sites of compartmentalization of 
these signaling molecules.

MATERIALS AND METHODS

Materials

The following materials were used: DMEM (GIBCO Life 
Sciences), fetal bovine serum (Winsent), MatrigEL (BD Bio-
sciences, Bedford, MA), ET-1, EGF, PMA, filipin III (Sigma, 
St. Louis, MO), calphostin C, AG-1478, β-cyclodextrin (Cal-
biochem, San Diego, CA), enhanced chemiluminescence (KPL, 
Gaithersburg, MD), and immmobilon polyvinylidine 
fluoride membranes (Millipore, Bedford, MA).

Antibodies

The following antibodies were utilized for immunoblotting, 
immunoprecipitation, and immunofluorescence imaging: 
phosphorylated and total ERK1/2, phosphorylated EGF-R
(Cell Signaling Technology, Beverly, MA), caveolin-1, phos-
phorylated caveolin-1 (BD Transduction Labs), A/G-agarose-
conjugated caveolin-1 (Santa Cruz, Santa Cruz, CA), vinculin 
(Serotec, Oxford, UK), horseradish peroxidase-labeled goat 
anti-rabbit IgG (Bio-Rad, Hercules, CA), horseradish pero-
oxidase-labeled goat anti-mouse IgG, and FITC-conjugated 
anti-rabbit IgG (Jackson ImmunoResearch).

Cell Culture

Primary rat mesangial cells were isolated from Sprague-
Dawley rat kidney glomeruli as previously described (23). 
Passages 10–15 were used for all studies. Mesangial cells 
were grown on thin layer MatrigEL in DMEM containing 
20% fetal bovine serum (FBS) to confluence and then grown 
invitro in 0.5% FBS for 72 h.

Western Blot Analysis

Mesangial cells were grown arrested on six-well plates and 
stimulated with ET-1 (100 nM for 10 min), EGF (50–200 
ng/ml for 10 min), and PMA (100 nM for 10 min) with or 
without indicated inhibitors. Cellular protein was extracted 
with 2× Laemmli sample buffer (0.13 mol/l Tris-base, pH 6.8, 
20% glycerol, and 4% SDS). Aliquots were taken for protein 
assay with Bradford Protein Assay (Bio-Rad). The remaining 
cell extracts were denatured in 4× sample buffer (0.13 mol/l 
Tris, 40% glycerol, 8% SDS, 4% β-mercaptoethanol, and 
0.02% bromophenol blue). Equal amounts of protein were 
separated by SDS-PAGE at 120 V for 1–2 h. The protein was 
transferred to Immobilon polyvinylidine fluoride membranes 
(Millipore) overnight at 4°C in transfer buffer (25 mol/l 
Tris-base, 192 mol/l glycine, pH 8.3, and 20% methanol). 
Membranes were blocked in 5% skim milk powder in Tris 
buffer (pH 8) containing 0.05% Tween-20 or 5% BSA and 
probed with the indicated antibody.

Immunoprecipitation

Mesangial cells were grown arrested in 10-cm plates and 
stimulated with ET-1, EGF, or PMA with or without inhibi-
tors and lysed with ice-cold radioimmunoprecipitation assay 
(RIPA) buffer containing 50 mM HEPES, 5 mM EDTA, 50 
mM NaCl, pH 7.4, 1% Triton X-100, 10 μg/ml aprotinin, 1 
mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 
vanadate, 1 mM benzamide, and 50 mM sodium fluoride. 
Lysates were centrifuged at 14,000 rpm for 10 min, and 300 
μg of the supernatant were immunoprecipitated with 10 μg of 
the indicated A/G-agarose-conjugated antibody overnight 
at 4°C. Immune complexes were washed with cold RIPA 
buffer and denatured in Laemmli sample buffer. Immunopre-
cipitated proteins were resolved by protein electrophore-
sis on 10–12% SDS-PAGE, transferred to polyvinylidine flu-
oride membrane, and probed with the indicated antibody.

Cellular Fractions

To obtain membrane fractions, mesangial cells grown on 
10-cm plates were lysed in ice-cold buffer A containing 50 
mol/l Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 1 
mol/l benzamide, 1 mM NaF, vanadate, 1 mM phe-
nethylmethylsulfonyl fluoride, and 25 μg/ml leupeptin. 
Cells were disrupted by passage through a 26-gauge needle and 
centrifuged at 100,000 g for 30 min at 4°C (TL-100, Bechman 
Instruments Canada, Mississauga, ON). The pellet was re-
suspended in buffer A plus 1% Triton X-100 and centrifuged 
at 100,000 g for 30 min. The supernatant was collected as the 
plasma membrane-enriched fraction.

Confocal Immunofluorescence

Mesangial cells were grown arrested on a glass coverslip 
coated with a thin-layer MatrigEL and fixed with 3.7% 
formaldehyde. Cells were permeabilized with 0.1% Triton 
X-100, blocked in goat serum containing 0.1% BSA, and 
incubated with the indicated antibodies. FITC-conjugated 
anti-mouse IgG or rhodamine-conjugated goat anti-rabbit 
IgG were used as secondary antibodies. Cells were imaged 
with a Zeiss confocal laser-scanning microscope (LSM 410, 
Dusseldorf, Germany), with excitation and emission wave-
lengths of 488 and 520 nm.

Caveolin-1 Scaffold Domain Peptide

The caveolin-1 scaffolding domain peptide corresponding 
to amino acids DGIVKASFTTFTVTKYWFYR (41, 42, 50, 
54, 59, 73) with an additional myristoylated sequence was 
synthesized by Biotechnology Service Centre (The Hospital 
for Sick Children Peptide Synthesis Laboratory, Toronto, 
ON). The purity of the peptide was >95% as determined by 
high-pressure liquid chromatography and mass spectroscopy. 
The cells were incubated with the peptide for 30 min before 
stimulation with ET-1 and lysed for immunoblotting or im-
munoprecipitation.

Statistical Analysis

All results are expressed as means ± SE. Statistical analysis 
was performed with InStat 2.01 statistics software 
(GraphPad, Sacramento, CA). The means of three or more 
groups were compared by one-way ANOVA. If significance 
of P < 0.05 was obtained in the ANOVA, the Tukey multiple 
comparison posttest was applied.
ET-1 ACTIVATES ERK VIA EGF-R AND CAVEOLIN-1

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**RESULTS**

**ET-1 Activation of ERK1/2**

EGF-R transactivation and function of caveolae. To determine whether ET-1 causes transactivation of EGF-R, primary rat mesangial cells were growth arrested (0.5% FBS for 72 h) and stimulated with ET-1 (100 nM for 10 min), and cell membrane isolates were blotted with an antibody directed against an EGF-R-specific Src kinase phosphorylation site required for subsequent EGF-R activation. As a control, mesangial cells were also stimulated with EGF (100 ng/ml). Figure 1 shows acute ET-1-activated EGF-R phosphorylation (by 3.1 ± 0.2-fold) but not to the same extent as EGF (12.2 ± 0.2-fold). Pretreatment with a specific EGF-R inhibitor, AG-1478 (0.2 μM for 20 min), reduced ET-1-induced phosphorylation of EGF-R to 0.6 ± 0.3-fold. When mesangial cells were preincubated with filipin III or β-cyclodextrin, cholesterol-depleting agents that disrupt caveolae formation, ET-1 activation of EGF-R was also significantly reduced to 2 ± 0.2 and 1.6 ± 0.05-fold, respectively (Fig. 1). Furthermore, inhibition of PKC with calphostin C (1 μM for 1 h) also inhibited EGF-R transactivation to 0.9 ± 0.3-fold (Fig. 1).

To ascertain the role of EGF-R transactivation in ET-1 activation of ERK1/2, mesangial cells were stimulated with ET-1 (100 nM for 10 min) in the absence or presence of AG-1478. Figure 2 illustrates that acute ET-1 stimulated ERK1/2 phosphorylation by 27 ± 1-fold. AG-1478 at 0.2 μM (20 min) significantly decreased ET-1 activation of ERK1/2 to 9 ± 1-fold. The effect of AG-1478 at 2 μM was not significantly different from 0.2 μM; therefore, the lower concentration was used throughout all the remaining experiments. As a control, mesangial cells were stimulated with EGF and total cell lysates were probed for phospho-ERK1/2. EGF (50–200 ng/ml) stimulated ERK1/2 phosphorylation by 20 ± 3-fold in mesangial cells. This response was completely abolished by AG-1478 (Fig. 2B).
To determine the role of intact caveolae in ET-1 activation of ERK1/2, mesangial cells were pretreated with β-cyclodextrin (10 mM for 1 h) or filipin III (5 μg/ml for 1 h) and immunoblotted with phosphospecific and total ERK1/2 antibodies. Densitometric analysis of at least 3 separate experiments was calculated. Values are fold increase over control, means ± SE. *P < 0.01.

Fig. 3. Effect of cholesterol depletion on ET-1 activation of phosphorylated ERK1/2. Mesangial cells were stimulated with ET-1 (100 nM for 10 min) with or without β-cyclodextrin (10 mM for 1 h) or filipin III (5 μg/ml for 1 h) and immunoblotted with phosphospecific and total ERK1/2 antibodies. Densitometric analysis of at least 3 separate experiments was calculated. Values are fold increase over control, means ± SE. *P < 0.01.

We further show that ET-1 activation of ERK is most likely through the ET-A receptor. BQ-123, the ET-A receptor blocker, reduced ET-1-induced ERK1/2 activation by 65% (0.1 μM) (Fig. 5). Conversely, the ET-B receptor blocker BQ-378 had insignificant effects on the phosphorylation of ERK by ET-1.

Direct binding of ERK1/2 with caveolin-1. Several studies have implicated caveolin-1 as a scaffolding protein that enables compartmentalization of specific signaling molecules. To test whether ET-1-induced activation of ERK1/2 involved direct binding of ERK1/2 to caveolin-1, we immunoprecipitated caveolin-1 and blotted for phospho-ERK1/2. Figure 6 demonstrates that in the absence of ET-1 treatment, there was little binding of phospho-ERK1/2 to caveolin-1. After acute exposure to ET-1, phospho-ERK1/2 communoprecipitated with caveolin-1 (32 ± 3-fold). Both AG-1478 and β-cyclodextrin reduced this association to 6 ± 2 and 13 ± 3-fold, respectively. Furthermore, inhibition of
PKC with calphostin C also attenuated phospho-ERK1/2 binding to caveolin-1 to 13 ± 3-fold in response to ET-1. In the same manner, PMA stimulated coimmunoprecipitation of phospho-ERK1/2 and caveolin-1. EGF (50 ng/ml) stimulated the association of phospho-ERK1/2 with caveolin-1 by 51 ± 2-fold, which was markedly attenuated by AG-1478 to 5 ± 2-fold. As a further control, after ET-1 activation, cell lysates were immunoprecipitated with an anti-phospho-ERK1/2 antibody, and caveolin-1 was found to colocalize with phospho-ERK1/2 on the immunoblot (data not shown).

To test whether the EGF-R localized with caveolin-1, cell lysates were immunoprecipitated with a caveolin-1 antibody and blotted with an antibody to phosphorylated EGF-R. Although the EGF-R communoprecipitated with caveolin-1, the amount did not change with ET-1 transactivation of the EGF-R (Fig. 7).

### Caveolin-1 Interactions

To study the functional consequences of ERK1/2 binding to caveolin-1, we used a myristoylated synthetic peptide that specifically binds to the caveolin-1 scaffolding domain, designated as myristoylated caveolin-1 scaffolding domain (mCSD) peptide. This domain has been determined to be responsible for caveolin-1 interaction with signaling molecules (41, 42, 52, 54, 59, 73). Mesangial cells were treated with different concentrations of mCSD peptide for 30 min and stimulated with ET-1. Figure 8 illustrates that mCSD peptide attenuated ET-1 activation of ERK1/2 in a concentration-dependent manner (ET-1 alone, 28 ± 2-fold; ET-1 + mCSD, 5 μM, 22 ± 2-fold; 10 μM, 15 ± 3-fold; and 20 μM, 9 ± 1-fold). Furthermore, mCSD peptide also prevented the communoprecipitation of phospho-ERK1/2 with caveolin-1 in the presence of ET-1 (ET-1, 25 ± 4-fold; ET-1 + mCSD, 10 μM, 8 ± 2-fold; and ET-1 + mCSD, 20 μM, 6 ± 1-fold) (Fig. 9).

### Immunolocalization of Caveolin-1 and Phospho-ERK1/2

Several reports have indicated that caveolin-1 is involved in integrin-mediated signaling (7, 69, 71). We performed dual-channel confocal imaging to determine whether mesangial cell caveolin-1 is localized within focal adhesion sites. Figure 10 illustrates that caveolin-1, immunostained with a polyclonal primary antibody, is found dispersed throughout the cell, with some localization within focal adhesion sites. However, we found that the intensity of immunofluorescence was significantly reduced when cells were treated with ET-1 (data not shown).
not altered in response to ET-1. In contrast, a monoclonal antibody to the phosphospecific caveolin-1 showed an immunoreactivity pattern similar to the monoclonal antibody to vinculin (Fig. 11). Moreover, phospho-ERK1/2 immunoreactivity was found in the cell nucleus as well as in regions that colocalized with vinculin (Fig. 11).

**DISCUSSION**

In this study, we have demonstrated that ET-1 via ET-A receptor activation of mesangial cell ERK1/2 is mediated predominantly through a pathway requiring EGF-R transactivation and intact caveolae. ET-1-induced ERK1/2 phosphorylation was reduced significantly by AG-1478, a specific EGF-R inhibitor, as well as by two cholesterol-depleting agents (β-cyclodextrin and filipin III) known to disrupt caveolae. This finding is supported by the recent observation by Ushio-Fukai et al. (66), who observed that cholesterol-depleting agents inhibit ANG II-induced transactivation of EGF-R. We further showed that ET-1 stimulation of ERK1/2 requires the direct association of the principal protein of caveolae, caveolin-1. This study is the first to demonstrate that a myristoylated peptide to the scaffolding domain of caveolin-1 attenuates ET-1 activation of ERK1/2 in intact cells. Moreover, the sequence of EGF-R transactivation, caveolin-1 association, and ERK1/2 phosphorylation is PKC dependent. Finally, confocal imaging patterns of phospho-caveolin-1, phospho-ERK1/2, and vinculin suggest localization of these signaling molecules at least in part within focal adhesion attachment sites.

EGF-R transactivation is a novel mechanism to explain the activation of growth-mediated signaling kinases such as ERK1/2 by GPCR. Multiple GPCR-dependent agonists have been shown to act through...
EGF-R transactivation, including ET-1 (10, 28, 64, 67). Several studies have indicated that ET-1 is a mitogenic stimulus that activates ERK1/2 in mesangial cells (15, 72). Here, we show that ET-1 activation of mesangial cell ERK1/2 depends in part on EGF-R transactivation. ET-1-stimulated phosphorylation of the EGF-R as well as activation of ERK1/2, and both were attenuated by the EGF-R inhibitor AG-1478. Takemura et al. (61) have described the expression of HB-EGF in mesangial cells that was upregulated in experimental glomerulonephritis, a model wherein ERK1/2 is also activated. GPCR-induced transactivation of EGF-R is postulated to be in part mediated through a PKC-dependent mechanism, because the proteolytic cleavage of HB-EGF was shown to be activated by PKC (47). ANG II has been shown to phosphorylate EGF-R in a PKC- and metalloprotease-dependent manner in mesangial cells (65). In cardiomyocytes, ET-1-stimulated EGF-R transactivation was solely dependent on PKC (28). Similarly, we previously showed that ET-1 activation of

Fig. 10. Caveolin-1 is immunolocalized near focal adhesion sites. Mesangial cells were cultured on glass coverslips and processed for confocal immunofluorescence as described in MATERIALS AND METHODS. A–C: confocal immunofluorescence of vinculin visualized with FITC-conjugated secondary antibody and caveolin-1 visualized with rhodamine-conjugated secondary antibody in the absence of ET-1 stimulation. D–F: ET-1 (10 min) stimulation.

Fig. 11. Phospho-caveolin-1 and phospho-ERK1/2 show similar immunoreactivity to vinculin. A and B: confocal image of mesangial cells treated with and without ET-1 and labeled with phospho-caveolin-1 antibody. C: confocal image of mesangial cells in the absence of ET-1 and labeled with rhodamine-conjugated phospho-ERK1/2 antibody. D–F: confocal image of mesangial cells treated with ET-1 (10 min) and double labeled with rhodamine-conjugated phospho-ERK1/2 antibody and FITC-conjugated vinculin.
ERK1/2 was PKC dependent and, in this study, that EGF-R phosphorylation by ET-1 was abrogated by inhibition of PKC. Thus the effect of ET-1 on EGF-R transactivation in mesangial cells may be through a PKC mechanism. The attenuation by calphostin C of EGF-R phosphorylation and direct binding of activated ERK1/2 to caveolin-1 is consistent with the action of PKC principally at the EGF-R transactivation event.

In the present study, AG-1478 partially inhibited ET-1 activation of ERK1/2, suggesting alternative pathways of ERK1/2 stimulation by ET-1 in addition to EGF-R transactivation. Certainly, multiple cascades have been shown to stimulate ERK1/2 by ET-1 (36, 49, 57, 58).

We have further extended the understanding of EGF transactivation by a mesangial cell GPCR signal transduction by showing that intact caveolae may be necessary to bring the relevant kinases within close proximity for activation. In a number of cell types, the EGF-R is localized within caveolae (5, 31, 70) along with its downstream signaling molecules, including PLC-γ (68). Caveolae are enriched with ET receptors (43, 63). As well, the ET-A receptor and its bound ligand coimmunoprecipitated with caveolin (8). We have employed cholesterol-depleting agents to disrupt caveolae to demonstrate that an intact caveola is necessary for ET-1 transactivation of EGF-R. β-Cyclodextrin has been shown to decrease total cellular cholesterol levels, caveolin mRNA, and protein (21) as well as cause the loss of morphologically recognizable caveolae determined by electron microscopy in Madin-Darby canine kidney cells and vascular smooth muscle cells (6, 21, 53, 66). Both filipin III and β-cyclodextrin significantly attenuate ET-1-induced tyrosine phosphorylation of EGF-R, ET-1-induced communoprecipitation of phosphorylated ERK1/2 with caveolin-1, and ET-1 activation of phospho-ERK1/2.

Biochemical and morphological studies have revealed that ERK1/2, as well as its upstream kinases, localize within caveolae (34, 35, 38, 46, 74). Although caveolae disruption has been suggested to cause hyperactivation of ERK1/2 (12, 16, 18), we found that β-cyclodextrin reduced ET-1-induced stimulation of mesangial cell ERK1/2. In addition, a peptide against the scaffolding domain of caveolin-1 attenuated ERK1/2 activation and binding to caveolin-1. Binding of signaling molecules to the caveolin-1 scaffolding domain is critical for modifying signal transduction (41, 42, 52, 54, 59, 73). In vivo delivery of the caveolin-1 scaffolding domain can selectively regulate signaling in endothelial cells (4). Similarly, the caveolae-disrupting agents filipin III or cyclodextrin attenuate nerve growth factor-induced phosphorylation of ERK1/2 in PC12 cells (46), and a neutralizing antibody to caveolin-1 inhibits shear stress activation of ERK1/2 in vascular smooth muscle cells (44). In the anti-Thy-1 nephritis rat model, increased mesangial caveolin-1 protein is accompanied by mesangial cellular proliferation (62). Cholesterol depletion of caveolae causes reduced association of Ras, Grb2, and ERK1/2 but does not prevent EGF-induced hyperactivation of ERK in the caveolae (16). PDGF stimulates ERK1/2 activation in intact human fibroblasts or isolated caveolae with the kinetics of ERK activation in the cytosol being slower than in the caveolae, suggesting cytosolic ERK may originate from caveolae (34). Thus whether or not caveolae subsequently sequester ERK1/2 activity, it appears that in response to certain agonists, localization of ERK1/2 within caveolae is required for its activation. Herein, we further demonstrate a requirement for a direct association of active ERK1/2 with caveolin-1.

Caveolin has been found to be positively involved in integrin-mediated signaling (55, 69, 71). In agreement with the study by Ushio-Fukai et al. (66), we also found that phosphorylated caveolin-1 localized at focal adhesion complexes. Moreover, the downstream target of EGF transactivation, phosphorylated ERK1/2, colocalized with vinculin, suggesting a possible mechanism of caveola–regulated ERK1/2 activation involving integrin binding. Phosphorylation of caveolin-1 on tyrosine 14 by Src kinase leads to its interaction with Grb7 as well as its localization near focal adhesions (29, 30).

In summary, we showed that ET-1 activation of ERK1/2 is mediated through EGF-R transactivation, requires intact caveolae, and is regulated by caveolin-1 association through a mechanism that is PKC dependent. Phosphorylated ERK1/2 localized at attachment complexes along with phospho-caveolin-1, suggesting a potential functional link with cell adhesion.

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