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Reactive Oxygen Species Generation Is Involved in Epidermal Growth Factor Receptor Transactivation through the Transient Oxidization of SHP-2 in Endothelin-1 Signaling Pathway in Rat Cardiac Fibroblasts

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ABBREVIATIONS: ROS, reactive oxygen species; EGFR, epidermal growth factor receptor;

ET-1, endothelin-1; PTP, phosphotyrosine phosphatase; SHP-2, Src homology 2-containing

tyrosine phosphatase; DPI, diphenyleneiodonium chloride; NAC, N-acetylcysteine; IAA,

iodoacetic acid; DCF-DA, 2',7'-dichlorofluorescein diacetate; ET_A, ET-1 receptor type A; ET_B,

ET-1 receptor type B; MMP, matrix metalloproteinase.

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ABSTRACT

Endothelin-1 (ET-1) is implicated in fibroblast proliferation which results in cardiac fibrosis.

Both reactive oxygen species (ROS) generation and epidermal growth factor receptor (EGFR)

transactivation play critical roles in ET-1 signal transduction. In this study, we used rat cardiac

fibroblasts treated with ET-1 to investigate the connection between ROS generation and EGFR

transactivation. ET-1 treatment was found to stimulate the phosphorylation of EGFR and ROS

generation, which were abolished by ET_A receptor antagonist BQ485. NAD(P)H oxidase

inhibitor diphenyleneiodonium chloride (DPI), ROS scavenger N-acetylcysteine (NAC) and

p47^{phox} siRNA knockdown all inhibited the EGFR transactivation induced by ET-1. In contrast,

EGFR inhibitor AG1478 cannot inhibit intracellular ROS generation induced by ET-1. Src

homology 2-containing tyrosine phosphatase (SHP-2) was shown to be associated with EGFR

during ET-1 treatment by EGFR co-immunoprecipitation. ROS have been reported to transiently

oxidize the catalytic cysteine of phosphotyrosine phosphatases to inhibit their activity. We

examined the effect of ROS on SHP-2 in cardiac fibroblasts using a Modified Malachite Green

Phosphatase Assay. SHP-2 was transiently oxidized during ET-1 treatment, and this transient

oxidization could be repressed by DPI or NAC treatment. In SHP-2 knockdown cells,

ET-1-induced phosphorylation of EGFR was dramatically elevated and is not influenced by

NAC and DPI. However, this elevation was suppressed by GM6001 (a MMP inhibitor) and

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heparin binding (HB)-EGF neutralizing antibody. Our data suggest that ET-1-ET_A-mediated ROS generation can transiently inhibit SHP-2 activity to facilitate the MMP-dependent and HB-EGF-stimulated EGFR transactivation and mitogenic signal transduction in rat cardiac fibroblasts.

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Introduction

The progression of cardiac dysfunction, especially diastolic dysfunction, in hypertensive hearts is always accompanied by excessive cardiac fibrosis (Mann, 1999). After cardiac infarction, reactive fibrosis results in excessive scar formation as proliferating fibroblasts invade the necrotic area. This remodeling leads to an increase of the ventricular stiffness and ultimately imperils the function of the heart (Borer et al., 2002). Recent studies have shown that the expression of myocardial endothelin-1 (ET-1) is increased during cardiac fibrosis (Lapointe et al., 2002). Some have suggested that ET-1 might contribute to cardiac fibroblast proliferation, resulting in cardiac fibrosis (Ammarguella et al., 2001).

Endothelin-1 (ET-1) is a bioactive vasoconstrictor peptide formed through the specific conversion of its intermediate precursor by the endothelin-converting enzyme (ECE) (Yanagisawa and Masaki, 1989). ET-1 exerts its fibrosis stimulation effect through a heteromeric G protein-coupled receptor (GPCR) that is linked to a number of well-defined intracellular signaling pathways (Schieffer et al., 1996). Activation of phospholipase C induces the generation of diacylglycerol (DG) and inositol triphosphate (IP₃), leading to the activation of PKC and mobilization of intracellular calcium (Sugden, 1999). A variety of tyrosine kinases, such as JAK2 (Kodama et al., 1998), proline-rich tyrosine kinase 2 (Pyk2), and Src family kinases (Kovacic et al., 1998) are then stimulated. It is also found that agonist binding to ET receptors

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and other G protein-coupled receptors (GPCRs) are associated with activation of MAPK family members, including Erk (Wang et al., 1992), Jun kinase (Kodama et al., 1998), and p38 (Aquilla et al., 1996). Tyrosine kinase-dependent activation of the ras/MAPK pathway is an important step in ET-1-induced mitogenic signaling (Herman and Simonson, 1995). Intermediate signaling molecules involved in ET-1-stimulated tyrosine kinase pathways include the adaptor proteins Shc and Grb2 (Cazaubon et al., 1994).

Recently, the epidermal growth factor receptor (EGFR) transactivation has been revealed to accompany the tyrosine kinase signaling in ET-1 action (Prenzel et al., 1999). The mechanism of EGFR transactivation has been proposed whereby GPCR-mediated activation of PKC leads to metalloprotease proteolytic cleavage of the pro-heparin binding (HB)-EGF precursor to release the mature ligand, which in turn activates the EGFR (Kodama et al., 2002; Leserer et al., 2000). This event leads, through the formation of Shc/Grb-2/Sos complexes, to the activation of the ras/MAPK pathway and the transcription of early response genes (Leserer et al., 2000). Therefore, EGFR kinase activity predominantly contributes to the ability of these GPCRs to induce the activation of MAPK, Shc and ERK, and the transcription of the *c-fos* gene (Kodama et al., 2002).

Besides EGFR transactivation, it has also been shown that ET-1 stimulates membrane-bound NAD(P)H oxidase, which generates reactive oxygen species (ROS) in many

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types of cells, including cardiac fibroblasts (Cheng et al., 2003; Hua et al., 2003). The secondary messenger ROS are required for optimal activation of numerous signal transduction pathways, particularly those mediated by protein tyrosine kinases (Xu et al., 2002). The potential role of ROS in the regulation of signal transduction and gene expression in the cardiovascular system has recently been elucidated (Cheng et al., 1999; Hirotsani et al., 2002). The increase in ROS regulates various intracellular signal transduction cascades, as well as the activities of various transcription factors (Hirotsani et al., 2002). ROS modulate *c-fos* gene expression induced by ET-1, and the administration of antioxidants inhibits such expression in cardiomyocytes (Cheng et al., 1999).

Although both EGFR transactivation and ROS generation play important roles in ET-1 signaling pathway, the connection between these two events is still unclear. Recent studies have revealed a mechanism through which ROS regulate cellular processes, whereby protein tyrosine phosphatases (PTPs) are transiently inhibited through the reversible oxidization of their catalytic cysteine, which in turn suppresses protein dephosphorylation (Meng et al., 2002). Several protein tyrosine phosphatases regulate the signaling pathways of receptor tyrosine kinases, including EGFR (Markova et al., 2003; Ostman and Bohmer, 2001). This reversible oxidization mechanism may be involved in the connection between EGFR transactivation and ROS generation in the ET-1 signaling pathway. In this study, we show that ROS generation is

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involved in EGFR transactivation in the ET-1 signaling pathway in rat cardiac fibroblasts. This mechanism is associated with the transient inhibition of a PTP, SHP-2, caused by ROS.

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Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents are from Life Technologies, Inc. (NY, USA). Endothelin-1 (ET-1) and all other chemicals of reagent grade are obtained from Sigma (St. Louis, MO). To block EGFR-mediated signaling or ET-1 induced ROS generation, cells were preincubated for 30 minutes at 37°C with AG1478, N-acetyl-cysteine (NAC) or diphenyleneiodonium chloride (DPI) (Sigma) before incubation with ET-1. For blocking HB-EGF shedding or function, cells were pretreated with HB-EGF neutralizing antibody (R&D Systems, Inc., MN), or GM6001 (Calbiochem, CA) for 1 hour at 37°C before incubation with ET-1. Antibodies used in this research are purchased from BD Laboratories (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA).

Culture of cardiac fibroblasts. The investigation was conducted in accordance with the Guide for the care and use of laboratory animals published by the National Institutes of Health (NIH Publication No 85–23, revised 1996) and approved by the institutional animal care and use committee of Institute of Biomedical Sciences, Academia Sinica. Primary cultures of neonatal rat cardiac fibroblasts were prepared as previously described (Mervaala et al., 2001). Cardiac fibroblasts grown in either 60 or 100 mm culture dishes from the second to fourth passage were used for the experiments. The purity of the fibroblasts was > 95% as determined by morphological characterization and by immunostaining with antibodies to von Willebrand factor

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VIII. Cardiac fibroblasts were grown in DMEM without phenol red containing antibiotics and 10% fetal calf serum until 24 hours before experimentation, when cells were placed in a defined serum-free medium containing insulin (0.5 mM) and transferrin (5 mg/ml) for all experiments.

Assay of Intracellular ROS. Intracellular ROS production was measured with the ACAS interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI) using the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Eugene, OR) as described previously (Cheng et al., 1999). A 10 mM stock solution of DCF-DA was prepared in ethanol on a daily basis and diluted to a final concentration of 10 μ M just before the experiments. Cardiac fibroblasts were preincubated with 10 μ M DCF-DA in DMEM for 30 min at 37°C before treatment. After exposure to the dye, the cells were rinsed with Tyrode's solution. The cells were maintained in Tyrode's solution and examined by using the laser cytometer at 37°C. Excitation of dichlorofluorescein (DCF) was achieved by using the 488-nm line of a 20-mW argon-ion laser. The emission above 515 nm was quantitated from two-dimensional scans generated by using a 1- μ m laser beam and an X-Y scanning stage to obtain a fluorescence value from a single cell. To provide a valid comparison, the same acquisition parameters were used for all observations. Quantification of the levels of DCF fluorescence was assessed on a relative scale from 0 to 4000 units. Baseline values from unstimulated cells were used as control values in the comparison with ET-1-stimulated cells. Values represent mean \pm S.E.M. of DCF fluorescence from 20

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randomly selected cells for each experiment in the five investigations.

Western Blot Analysis. Rabbit polyclonal anti-phospho-specific ERK antibodies were purchased from New England Biolabs (Beverly, MA). Anti-ERK, anti-EGFR, anti-PTP1B, anti-SHP2 and pY20 antibodies were purchased from Santa Cruz Biotechnology Inc. Whole-cell extracts were obtained in a RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1% Deoxycholate, 5 mM EDTA, 1mM sodium orthovanadate, 1mM PMSF, and protease inhibitor cocktail (Complete, Roche Diagnostics GmbH, Germany)). Extracts or proteins were separated by SDS-PAGE followed by electrotransfer to PVDF membranes and probed with antisera followed by horseradish peroxidase-conjugated secondary antibodies. The proteins were visualized by chemiluminescence according to the manufacturer's instructions (Pierce Biotechnology Inc., IL).

DNA Synthesis. To measure the synthesis of new DNA, the cells (1×10^5 per well) were seeded in six-well (35-mm) dishes 24 h before the experiments were performed. Cardiac fibroblasts were incubated with [^3H]thymidine (5 $\mu\text{Ci/ml}$) for 24 h before harvest. The cells were harvested by incubation with trichloroacetic acid (5%) at 4°C, followed by solubilization in 0.1 N NaOH. Radioactivity was determined by scintillation counting. Data are presented as the mean \pm S.E.M. for 9 to 12 determinations in three to four cell preparations and normalized with data from the untreated sample and multiplied by 100 (i.e., as a percentage of control).

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Immunoprecipitation. Cardiac fibroblasts were starved overnight in serum-free culture medium and stimulated with or without 10nM ET-1 at 37°C. The cells were lysed at 4°C in lysis buffer (50mM Tris, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150mM NaCl, protease inhibitors). Particular proteins were collected by using immunoprecipitation kit (Roche Molecular Biochemicals, Germany) with specific antibodies and protein-G-agarose, following the manufacturer's instructions.

Modified Malachite Green-Protein Tyrosine Phosphatase Assay. Cardiac fibroblasts were starved overnight in serum-free culture medium and stimulated with 10nM ET-1 at 37°C. The cells were lysed at room temperature in the dark for 20 min in lysis buffer (50mM Tris, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150mM NaCl, protease inhibitors) with 100 mM iodoacetic acid (IAA). Before lysis, the buffer was degassed for at least 20 min. Those PTPs that had not encountered ROS in the cell became irreversibly inactivated by alkylation of their active site Cys by IAA. In contrast, any PTPs in which the active site Cys had been oxidized in response to the stimulus were resistant to alkylation. PTP1B and SHP-2 were collected by immunoprecipitation with specific antibodies and protein-G-agarose beads. The beads were washed three times in lysis buffer, incubated with 10 mM DTT for 10 min on ice, washed three times and stored in distilled water. To assess specific phosphatase activity, the same quantity of protein tyrosine phosphatases coupled with protein G-agarose beads was incubated with a

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phosphotyrosine peptide (RRLIEDAEpYAARG) at 37°C for two hours. Liberated phosphate was detected by malachite green using a Tyrosine Phosphatase Assay Kit 1 (Upstate Biotechnology, NY), following the manufacturer's instructions. Liberated phosphate was measured in picomoles from a standard curve. The data are representative of two experiments, each performed in duplicate.

siRNA-mediated gene knockdown. SHP-2 siRNA (sc-36489), p47^{phox} siRNA (sc-36157) and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology Inc, and used for SHP-2 knockdown, p47^{phox} knockdown and mock control respectively. Transfection of siRNA in cardiac fibroblasts was performed using siRNA transfection reagent according to the manufacturer's instruction (Santa Cruz Biotechnology Inc). Transfected cells were applied to advanced assays and Western Blotting analysis.

Statistical Analysis. Results are expressed as mean \pm S.E.M. of at least three experiments. The statistical significance of differences between groups was estimated by one-way analysis of variance. For the comparisons, *p* values of less than 0.05 were considered to indicate statistically significant differences.

Results

The Patterns of EGFR Transactivation Induced by ET-1. To determine whether EGFR transactivation can be induced by ET-1 in rat cardiac fibroblasts, EGFR was immunoprecipitated

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using goat anti-EGFR antibody. The phosphorylation of EGFR was consequently detected by pY20 antibody against phosphorylated tyrosine residues. As shown in Fig. 1A, different concentrations of ET-1 induced significant phosphorylation of EGFR. Among these experimental concentrations, 10 nM of ET-1 caused a maximal effect on the induction of EGFR phosphorylation. With 10nM of ET-1 treatment, the stimulation of EGFR phosphorylation occurred within 30 seconds (reaching a maximum at 1 min) and sustained through 5 min of treatment (Fig. 1B). Total EGFR detected by anti-EGFR antibody did not change over the 30-min exposure period. Extracellular signal-regulated kinases (ERK) are important signal molecules in the ET-1 signaling pathway. The phosphorylation of ERK also increased within 30 seconds of ET-1 exposure, peaked at 1 min (Fig. 1C). We therefore conclude that EGFR transactivation induced by ET-1 exists in rat cardiac fibroblasts, and is associated with the ET-1 signal transduction.

To determine the ET-1 receptor subtype responsible for EGFR transactivation in rat cardiac fibroblasts, cells were pretreated with ET-1 receptor type A (ET_A) antagonist (BQ485) or type B (ET_B) antagonist (BQ788) before the ET-1 exposure. BQ485 is a potent and selective ET_A receptor antagonist, whereas BQ788 selectively blocks ET_B receptors (Cheng et al., 2003). As shown in Fig. 1D, in contrast to BQ788 treatment, BQ485 significantly inhibited the ET-1-induced EGFR transactivation. This result indicates that induction of EGFR transactivation

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upon ET-1 treatment is mediated via the ET_A receptor.

ROS Generation Is Necessary for EGFR Transactivation in ET-1 Signaling Pathway. It

has been shown that ET-1 stimulates membrane-bound NAD(P)H oxidase to generate reactive oxygen species (ROS) in cardiac fibroblasts (Cheng et al., 2003). To determine the influence of ROS generation on EGFR transactivation in the ET-1 signaling pathway, the phosphorylation of EGFR induced by ET-1 was monitored with the treatment of NAD(P)H oxidase inhibitor diphenyleneiodonium chloride (DPI), or the ROS scavenger acetylcysteine (NAC). As shown in Fig. 2A, DPI, NAC and the EGFR inhibitor AG1478 were able to reduce the ET-1-induced phosphorylation of EGFR. The phosphorylation of ERK showed a similar pattern to that of EGFR when treated with NAC, DPI and AG1478. ET-1-induced [³H]thymidine uptake was also significantly suppressed by the pretreatment of NAC, DPI and AG1478 (Fig. 2B). These data suggest that both ROS generation and EGFR transactivation are involved in the mitogenic signaling pathway of ET-1 in rat cardiac fibroblasts, and ROS generation is also necessary for EGFR transactivation.

EGFR Transactivation Does Not Influence ROS Generation in ET-1 Signaling Pathway.

Using 2',7'-dichlorofluorescein diacetate (DCF-DA) staining, we also monitored the influence of EGFR transactivation on ROS generation caused by ET-1. ROS generation was found in ET-1-treated cardiac fibroblasts, and was reduced by DPI, NAC and ET_A antagonist

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BQ485 (Fig. 3A). AG1478 and ET_B antagonist BQ788 treatment, however, has no influence on ROS generation induced by ET-1. Additionally, DPI is an inhibitor of many flavoprotein enzymes, but not specific to NADPH oxidase. To confirm the role of NADPH oxidase in ET-1-induced ROS generation, NADPH oxidase subunit p47^{phox} siRNA were used for NADPH oxidase knockdown cells. As shown in Fig. 3B, the expression level of p47^{phox} was significantly reduced in p47^{phox} siRNA transfection cells compared with the mock controls. The ET-1-induced ROS generation was also decreased apparently in siRNA transfection cells (Fig. 3C). These results reveal that ET-1 induces ROS generation via ET_A receptor and NADPH oxidase in rat cardiac fibroblasts, and EGFR transactivation is not necessary for ROS generation in ET-1 signaling pathway.

The Active Site Cys Residue of SHP-2 Is Transiently Oxidized in ET-1 Signaling Pathway. It is possible that the mechanism by which ROS regulates EGFR transactivation is the transient inhibition of phosphotyrosine phosphatases (PTPs) through the reversible oxidization of their catalytic cysteine, which suppresses protein dephosphorylation (Meng et al., 2002). PTPs, SHP-2 and PTP-1B are often found to be associated with the signaling pathways of receptor tyrosine kinases (Agazie and Hayman, 2003; Markova et al., 2003). We therefore wished to test whether SHP-2 and PTP-1B were involved in EGFR transactivation stimulated by ROS in the ET-1 signaling pathway. To pursue this line of investigation, we developed a modified malachite

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green-PTP activity assay to monitor the catalytic Cys oxidization of PTPs. As described in methods, the PTPs registered as active in this assay would be those originally protected from post-lysis alkylation by a stimulus-dependent modification at the active site Cys, which can be reversed by DTT, consistent with oxidation of the Cys (Fig. 4A). In contrast, the active site Cys without oxidation modification will be blocked by iodoacetic acid (IAA) and lose its catalytic function, which can not be reversed by DTT. The data shown in Fig. 4B illustrate that the catalytic Cys oxidization of SHP-2 significantly increased during the initial stage (within approximately 3 minutes) of ET-1 treatment in Modified Malachite Green-PTP Assay. There was, however, no significant change in the catalytic Cys oxidization of PTP-1B during ET-1 treatment. In other words, the active site Cys residue of SHP-2 was oxidized to inhibit the dephosphorylation activity during the initial stage of ET-1 treatment, and then recovered quickly.

The Transient Oxidation of SHP-2 Is Highly Associated with ROS Generation Induced by ET-1. The influence of ROS on the catalytic Cys oxidization of SHP-2 was determined in rat cardiac fibroblasts treated with H₂O₂ and ET-1 combined with AG1478, NAC or DPI. SHP-2 in each sample was immunoprecipitated with anti-SHP-2 antibody, and applied in the Modified Malachite Green-PTP Activity Assay. As shown in Fig. 5A, H₂O₂ and ET-1 induced the catalytic Cys oxidization in SHP-2, which was reversed by the addition of NAC or DPI, but not by treatment of AG1478. Additionally, the SHP-2 oxidization was also reduced in p47^{phox}

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knockdown cardiac fibroblasts (Fig. 5B). It is apparent that ET-1 regulates the activity of SHP-2 via NADPH oxidase-mediated ROS generation but not via EGFR transactivation.

SHP-2 Can Interact with EGFR during ET-1 Treatment. The transient inhibition of SHP-2 implies that SHP-2 plays an important role in the regulation of EGFR transactivation in ET-1 signaling pathway. To further explore this implication, we also investigated the interaction between SHP-2 and EGFR by co-immunoprecipitation. The EGFR in ET-1-treated cardiac fibroblasts was immunoprecipitated with anti-EGFR antibody. As shown in Fig. 6, SHP-2 was co-immunoprecipitated significantly within one minute, and was sustained through the ET-1 treatment. On the other hand, there was no co-immunoprecipitated PTP-1B detected (data not shown). This result reveals that SHP-2 can interact with EGFR in cardiac fibroblasts and may play a crucial role in the regulation of EGFR transactivation in the ET-1 signaling pathway.

SHP-2 is involved in ET-1-induced EGFR Transactivation. To further determine the regulatory role of SHP-2 in ET-1-induced EGFR transactivation, SHP-2 siRNA was used for SHP-2 knockdown in rat cardiac fibroblasts. Compared to the mock controls, SHP-2 siRNA significantly reduced SHP-2 expression in rat cardiac fibroblasts (Fig. 7). The basal phosphorylation levels of EGFR in SHP-2 knockdown cells were slightly higher than those in mock controls. ET-1-induced phosphorylation of EGFR was dramatically elevated in SHP-2 knockdown cardiac fibroblasts. It has been shown that ET-1-induced phosphorylation of EGFR

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was suppressed by NAC and DPI (Fig. 2). However, NAC and DPI do not reduce the prominent increases in EGFR phosphorylation in the absence of SHP-2 from the siRNA-transfected cells although they do cause the expected small decreases in EGFR phosphorylation in the mock controls (Fig. 7A). This result supports that ROS-mediated transient inhibition of SHP-2 is necessary for ET-1-induced EGFR transactivation. In addition, the mechanism of EGFR transactivation has been proposed to involve matrix metalloproteinase (MMP) proteolytic cleavage of the pro-heparin binding (HB)-EGF precursor to yield the release of mature ligand, which in turn activates the EGFR (Kodama et al., 2002; Leserer et al., 2000; Prenzel et al., 1999). To understand the role of SHP-2 in this mechanism, the effects of HB-EGF neutralization and inhibition of ectodomain shedding on EGFR phosphorylation were assessed in SHP-2 knockdown cells (Fig. 7B). Addition of HB-EGF neutralizing antibody or GM6001 (a MMP inhibitor) blocked ET-1-induced EGFR phosphorylation in both mock controls and SHP-2 knockdown cells. The same result was also found in ERK phosphorylation detection. It is supposed that SHP-2 is a regulatory component downstream of the release of mature HB-EGF in the signaling pathway of EGFR transactivation.

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Discussion

ET-1, a vasoactive peptide, is an important factor for cardiac fibroblast proliferation and cardiac fibrosis. Both EGFR transactivation and ROS generation are two important early events to trigger mitogenic signal molecules, such as ERK, in the ET-1 signaling pathway. The connection between these two events, however, is not completely understood. In this study, we demonstrate that both ROS generation and EGFR transactivation are mediated via ET_A receptors in ET-1 signaling pathway in cardiac fibroblasts. Moreover, ROS generation is necessary for EGFR transactivation in ET-1-ET_A-mediated pathway. It is possible that the connection mechanism between ROS and EGFR transactivation is the transient inhibition of PTPs through the reversible oxidization of their catalytic cysteine, which suppresses protein dephosphorylation (Meng et al., 2002). Using a modified Malachite Green-PTP Activity Assay, we found that the catalytic cysteine of SHP-2, a PTP, was significantly oxidized at an early period of ET-1 treatment. The interaction between SHP-2 and EGFR was also found by co-immunoprecipitation. ET-1-induced EGFR phosphorylation was dramatically elevated in SHP-2 knockdown cells, which was not suppressed by either NAC or DPI. These results reveal that SHP-2 is a potent mediator in the stimulation of EGFR transactivation in the ET-1 signaling pathway. In contrast, another oxidation-sensitive PTP, PTP-1B, has no interaction with EGFR and no transient oxidization in ET-1 system although the transient oxidization of PTP-1B plays a crucial role in

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insulin signaling pathway (Meng et al., 2004). Therefore, SHP-2 plays a specific role in the connection between EGFR transactivation and ROS generation in ET-1 signaling pathway.

EGFR transactivation can be induced by many stimuli and occurs through different pathways including PKC, Ca²⁺, and Src (Carpenter, 2000; Gschwind et al., 2001). Another important mechanism of EGFR transactivation is the autocrine/paracrine release of soluble EGF ligands (Carpenter, 2000). For example, Frank et al (Frank et al., 2003) showed that H₂O₂-stimulated EGFR activation is produced by metalloprotease-dependent HB-EGF cleavage in vascular smooth muscle cells. In this study, we demonstrate that ROS can induce EGFR transactivation via transient inhibition of SHP-2 in cardiac fibroblasts. In SHP-2 knockdown cells, ET-1-induced phosphorylation of EGFR was dramatically elevated. This elevation was not suppressed by either NAC or DPI (Fig. 7A). In addition, blocking HB-EGF shedding or function suppressed ET-1-induced EGFR transactivation in SHP-2 knockdown cells (Fig. 7B). Taken together, these results suggest that SHP-2 should be a regulatory component downstream of the release of mature HB-EGF in ET-1 signaling pathway.

The mechanism of EGFR transactivation has been proposed whereby G protein-coupled receptor (GPCR)-mediated activation of PKC leads to metalloprotease proteolytic cleavage of the pro-heparin binding (HB)-EGF precursor to yield the release of mature ligand, which in turn activates the EGFR (Leserer et al., 2000). The phosphorylated EGFR will be associated with

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SHP-2 and be dephosphorylated. Mediated by ET_A receptors, ET-1 also induces ROS generation via NADPH oxidase concomitantly, which leads to the transient oxidization of catalytic Cys of SHP-2 to inhibit the dephosphorylation activity of SHP-2. This mechanism allows transactivated EGFR to transmit signals to the downstream mitogenic signaling pathway (Fig. 8). Our findings provide new insights into the molecular mechanisms by which ET-1 activates mitogenic signaling pathways in cardiac fibroblasts.

The controlled production of ROS has been reported to have beneficial effects in the regulation of cellular homeostasis, although it was initially viewed as a harmful by-product of life in an aerobic environment. The production of ROS by phagocytic leukocytes plays a critical role in the innate immune response to pathogens (Lambeth, 2004). Various stimuli lead to the assembly of a multicomponent NADPH oxidase complex that mediates a process known as the respiratory burst (DeLeo and Quinn, 1996). NADPH oxidase catalyses transfer of one electron from NADPH to molecular oxygen to generate superoxide anions, which in turn may yield hydrogen peroxide either via protonation of superoxide or through the action of superoxide dismutase (Thelen et al., 1993). The large quantities of such ROS produced in phagocytic cells have been implicated as microbicidal agents and, in certain pathological situations, can result in host cell damage (Smith and Curnutte, 1991). Many recent studies, however, have revealed that the production of ROS is tightly regulated; engendering the concept that, at lower levels than

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those generated for a microbicidal function, ROS may also function in propagating signaling responses to extracellular stimuli. For example, Choi et al. have found that the deficiency of peroxiredoxin II, a cellular peroxidase, results in increased production of H₂O₂, enhanced activation of PDGF receptor, and subsequently increased cell proliferation and migration in response to PDGF (Choi et al., 2005). Thus, in a manner analogous to reversible protein phosphorylation, the reversible oxidation of target proteins in a cell may regulate the function of those proteins in response to various agonists and thus elicit a cellular response to stimulation.

PTPs are commonly known to play either inhibitory or permissive roles in regulating the physiological response to particular ligands. For example, SHP-2 can function as either a positive or negative regulator of MAP kinase activation, depending on the specific receptor pathway stimulated (Saxton et al., 1997). The critical role of the invariant low-*pK_a* catalytic cysteine residue implies the importance of PTPs in the regulatory mechanism of tyrosine phosphorylation-dependent signaling pathways (Salmeen et al., 2003). Several studies have revealed that multiple members of the PTP family are susceptible to reversible oxidation (Chiarugi and Cirri, 2003; Salmeen and Barford, 2005). In the classical PTPs, this cysteine is oxidized to sulfenic acid and subsequently converted into a sulfenamide species (Salmeen et al., 2003). This reversible modification abrogates the nucleophilic properties of this cysteine residue, thereby inhibiting PTP activity. Conversion of the oxidized sulfenic acid to the sulfenamide form

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of the active-site cysteine induces conformational changes at the PTP active site, which both disrupt the interaction with substrate and expose the oxidized cysteine to the environment of the cell. This serves the dual purpose of preventing irreversible oxidation to higher oxidized forms of the active site cysteine (sulfinic and sulfonic acid) and facilitating the reduction of the sulfenamide to restore the active form of the PTP (Salmeen et al., 2003). This transient oxidation is supposed to be important for the regulatory role of PTPs. There is evidence that oxidation and inhibition of SHP-2 in the early phase of the response to PDGF is important for establishment of the signaling response (Meng et al., 2002). In insulin signaling pathway, the involvement of reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B has also been reported recently (Meng et al., 2004; Meng et al., 2002). It is possible that the transient inhibition of PTPs is involved in the EGFR transactivation stimulated by other G-protein coupled receptors that can trigger ROS generation.

A major obstacle in exploring the role of reversible oxidation in the regulation mechanism of PTP function is the requirement for a method by which the oxidized/inactivated PTPs could be distinguished from reduced/activated PTPs in a cellular context. Meng et al. developed a modified in-gel PTP activity assay with radioactive substrates to allow the visualization of a profile of oxidized PTPs following a particular stimulus (Meng et al., 2002). The PTPs that registered as active in this assay would be those originally protected from post-lysis alkylation by

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a stimulus-dependent modification at the active site Cys, which was reversed by DTT. The preparation of radioactive phosphorylated substrates, however, is laborious and yields a limited amount of substrate with a short half-life resulting in a low percentage of phosphorylated products. These factors limit the usefulness of radioactive phosphorylated substrates in phosphatase assays. An alternative method for the activity analysis of purified or recombinant soluble phosphatases makes use of the malachite green reagent that can detect nanomoles of phosphate released from chemically synthesized phosphorylated peptides (Stefani et al., 1993). In this study we combined IAA application with a malachite green-PTP assay to monitor the oxidation level of purified PTPs in a cellular context. Alkylation with IAA is used to subtract out the PTPs that are unaffected by the stimulus, and the remaining responsive PTPs are isolated and identified after their reduction with DTT. Restricted by the need of purified PTPs in a malachite green-PTP assay, our method is not suitable for screening unknown oxidation sensitive PTPs. Modified Malachite Green-PTP Assay is a powerful technique for the identification of specific oxidation sensitive PTPs.

In summary, the data presented here reveal that ROS generation is essential for EGFR transactivation in ET-1-ET_A-mediated signaling pathway. In rat cardiac fibroblasts, the increase of ROS specifically inhibits the SHP-2 activity within early period of ET-1 treatment to facilitate the transient increase of phosphorylation of EGFR. Following EGFR signaling pathway, ET-1

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can induce the phosphorylation of ERK to promote the proliferation of rat cardiac fibroblasts. In other words, ROS generation is involved in EGFR transactivation through the transient oxidization of SHP-2 in ET-1-triggered mitogenic signaling pathway in rat cardiac fibroblasts.

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FIGURE LEGENDS

Fig. 1. Phosphorylation patterns of EGFR and ERK induced by ET-1 in rat cardiac

fibroblasts. Rat cardiac fibroblasts were treated with different concentration of ET-1 for 2 minutes (panel A), or 10nM ET-1 for different time duration (panel B and C). Cells were also pretreated with ET_A antagonist BQ485 (100nM) or ET_B antagonist BQ788 (100nM) for 30 minutes before the ET-1 exposure of 10nM for 2 minutes (panel D). EGFR of each sample was immunoprecipitated with goat anti-EGFR antibody. EGFR and phosphorylated EGFR were detected using western blotting with anti-EGFR and pY20 antibodies respectively. Anti-ERK and anti-phosphorylated ERK antibodies were used to detect ERK and phosphorylated ERK respectively. In panel B and C, data are presented as the difference relative to the data in the 0-min groups. The results are shown as the mean \pm S.E.M. ($n = 3$). *, $p < 0.05$ versus the results at 0 min.

Fig. 2. Effects of inhibitors on ET-1-induced mitogenic signaling. Rat cardiac fibroblasts were

treated with 100nM AG1478 (A), 10mM NAC (N), or 5 μ M DPI (D) for 30 minutes. **A.** The western blotting of phospho-EGFR and phospho-ERK. The pretreated cells were then treated with 10nM ET-1 for 2 minutes. EGFR of each sample was immunoprecipitated with goat

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anti-EGFR antibody. EGFR and phosphorylated EGFR were detected using western blotting with anti-EGFR and pY20 antibodies respectively. Anti-ERK and anti-phosphorylated ERK antibodies were used to detect ERK and phosphorylated ERK respectively. The phosphorylation of EGFR and ERK induced by ET-1 was inhibited by AG1478, NAC and DPI treatment. **B.** The DNA synthesis of rat cardiac fibroblasts induced by ET-1. All experiments were performed using the incorporation of [³H]thymidine into DNA. The pretreated cells were then treated with 10nM ET-1 for 24 h, and [³H]thymidine incorporation was then assayed. Increase in the [³H]thymidine incorporation is expressed relative to the [³H] content (100%) in the control (C). All data are shown as the mean ± S.E.M. from 9 determinations in three cell preparations. *, *p* <0.05 versus the ET-1 treatment alone.

Fig. 3. ET-1-induced ROS generation in cardiac fibroblasts. A. Effects of inhibitors on ET-1-induced ROS generation. Rat cardiac fibroblasts were incubated for 30 min with culture medium containing 30μM DCF, in addition to 10mM H₂O₂ (H), 100nM AG1478 (A), 10mM NAC (N), 5μM DPI (D), 100nM BQ485 or 100nM BQ788. Except for the sample treated with H₂O₂, all samples were then treated with 10nM ET-1 for 2 min. The fluorescence intensity of cells was determined by fluorescence spectrophotometer with excitation and emission wavelengths at 475 and 525 nm, respectively. The relative fluorescence intensity of blank control

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was normalized as one fold. The results are shown as the means \pm S.E.M. of 9 determinations in three cell preparations. *, $p < 0.05$ versus the ET-1 treatment alone. **B.** Western blots of p47^{phox} in p47^{phox} knockdown cardiac fibroblasts. Control siRNA and p47^{phox} siRNA were used for mock control and p47^{phox} siRNA knockdown respectively. Proteins from fibroblasts without treatment (N) or with control siRNA (C) or p47^{phox} siRNA (R) transfection were detected by using polyclonal goat anti-p47^{phox} antibody. **C.** ET-1-induced ROS generation in p47^{phox} knockdown cells. Rat cardiac fibroblasts with control siRNA (C) or p47^{phox} siRNA (R) transfection were stained with DCF before the ET-1 exposure of 10nM for 2 min. The relative fluorescence intensity of the mock control was determined as one fold. The results are shown as the means \pm S.E.M. of 9 determinations in three cell preparations. *, $p < 0.05$ versus the ET-1 treatment alone.

Fig. 4. Detection of the oxidization levels of PTP by using Modified Malachite Green-PTP

Assay. **A.** The principle of Modified Malachite Green-PTP Assay. Cells were triggered with the appropriate stimulus and harvested in lysis buffer containing IAA. Those PTPs that had not encountered ROS in the cell became irreversibly inactivated by alkylation of their active site Cys with IAA. In contrast, any PTPs whose active site Cys had been oxidized in response to the stimulus were resistant to alkylation. Under these conditions, PTPs were collected by

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immunoprecipitation with specific antibodies, and then treated with DTT. To assess the activity of collected PTPs, the same quantity of PTPs was incubated with a phosphotyrosine substrate, and applied in a malachite green-PTP activity assay to measure liberated phosphate. The relative increase in folds of liberated phosphate represented the relative oxidization levels of PTPs in the original intracellular environment. **B.** Detection of the oxidization levels of SHP-2 and PTP-1B in ET-1-treated cardiac fibroblasts. Rat cardiac fibroblasts were treated with 10nM ET-1 for different time duration, and then treated with IAA to block catalytic residues of protein tyrosine phosphatases. SHP-2 and PTP-1B in ET-1 treated cells were immunoprecipitated with rabbit anti-SHP-2 and anti-PTP-1B antibody respectively. Purified SHP-2 and PTP-1B were then applied in Modified Malachite Green-PTP Assay. The relative oxidization levels of PTPs were recorded by measuring the relative increase in folds of liberated phosphate. The data are shown as the means \pm S.E.M. from 3 experiments performed in duplicate. *, $p < 0.05$ versus the result at 0 min.

Fig. 5. The oxidization level of SHP-2 in ET-1-treated cardiac fibroblasts. A. Effects of AG1478, NAC and DPI on the SHP-2 oxidization in ET-1 treated cells. Rat cardiac fibroblasts were pretreated with 10mM H₂O₂ (H), 100nM AG1478 (A), 10mM NAC (N), or 5 μ M DPI (D) for 30 minutes respectively. All samples were treated with 10nM ET-1 2 min except the sample

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treated with H₂O₂. Each sample was then treated with IAA. SHP-2 in rat cardiac fibroblasts was immunoprecipitated with rabbit anti-SHP-2 antibody, and then treated with DTT. The collected SHP-2 was incubated with a phosphotyrosine substrate for 2 h. The relative oxidization levels of SHP-2 were recorded by measuring the relative increase in folds of liberated phosphate. The data are shown as the means \pm S.E.M. from 3 experiments performed in duplicate. *, $p < 0.05$ versus the ET-1 treatment alone. **B.** The oxidization of SHP-2 in p47^{phox} knockdown cells. The oxidization of SHP-2 in rat cardiac fibroblasts with control siRNA (C) or p47^{phox} siRNA (R) transfection was measured using modified Malachite Green PTP Assay. The relative oxidization levels of SHP-2 were recorded by measuring the relative increase in folds of liberated phosphate. The data are shown as the means \pm S.E.M. from 3 experiments performed in duplicate. *, $p < 0.05$ versus the ET-1 treatment alone.

Fig. 6. The association of EGFR with SHP-2. Rat cardiac fibroblasts were treated with 10nM ET-1 for different time duration. EGFR of each sample was immunoprecipitated with goat anti-EGFR antibody. EGFR and phosphorylated EGFR (pEGFR) were detected using western blotting with anti-EGFR and pY20 antibodies respectively. Co-immunoprecipitated SHP-2 was detected by rabbit anti-SHP-2 antibody. The results are shown as the mean \pm S.E.M. ($n = 3$). *, $p < 0.05$ versus the result of pEGFR at 0 min. #, $p < 0.05$ versus the result of SHP-2 at 0 min.

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Fig. 7. Phosphorylation patterns of EGFR induced by ET-1 in SHP-2 knockdown

fibroblasts. Rat cardiac fibroblasts were either transfected with control siRNA as mock controls (m) or transfected with SHP-2 siRNA (r) to obtain SHP-2 knockdown cells. SHP-2 expression was determined using anti-SHP-2 antibodies. Cells were treated with 10nM ET-1 for 2 min. To block ET-1 induced ROS generation, cells were preincubated for 30 minutes at 37°C with NAC and DPI before incubation with ET-1 (panel A). For blocking HB-EGF shedding or function, cells were pretreated with HB-EGF neutralizing antibody or GM6001 for 1 hour at 37°C before incubation with ET-1 (panel B). EGFR of each sample was immunoprecipitated with anti-EGFR antibody. EGFR and phosphorylated EGFR were detected by using western blotting with anti-EGFR and pY20 antibodies respectively. Anti-ERK and anti-phosphorylated ERK antibodies were used to detect ERK and phosphorylated ERK respectively. The phosphorylation of EGFR is represented as the difference relative to the data in mock controls without ET-1 treatment. The results are shown as the mean \pm S.E.M. ($n = 3$). *, $p < 0.05$ versus the mock controls with ET-1 treatment alone. #, $p < 0.05$ versus the siRNA-transfected cells with ET-1 treatment alone.

Fig. 8. Schematic representation of the regulatory role of SHP-2 in ET-1-induced EGFR

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transactivation in rat cardiac fibroblasts. Metalloprotease (MMP) is stimulated by ET-1 treatment to cleave the pro-HB-EGF precursor to yield the release of mature ligand, which in turn transactivates the EGFR. The phosphorylated EGFR would then be associated with SHP-2 and be dephosphorylated. The ET-1 treatment, however, also induces ROS generation concomitantly via ET_A receptors and NADPH oxidases, and causes the transient oxidization of catalytic Cys of SHP-2 to inhibit the dephosphorylation activity of SHP-2. This mechanism allows transactivated EGFR to transmit signals to the downstream mitogenic signaling pathway.

Figure 1

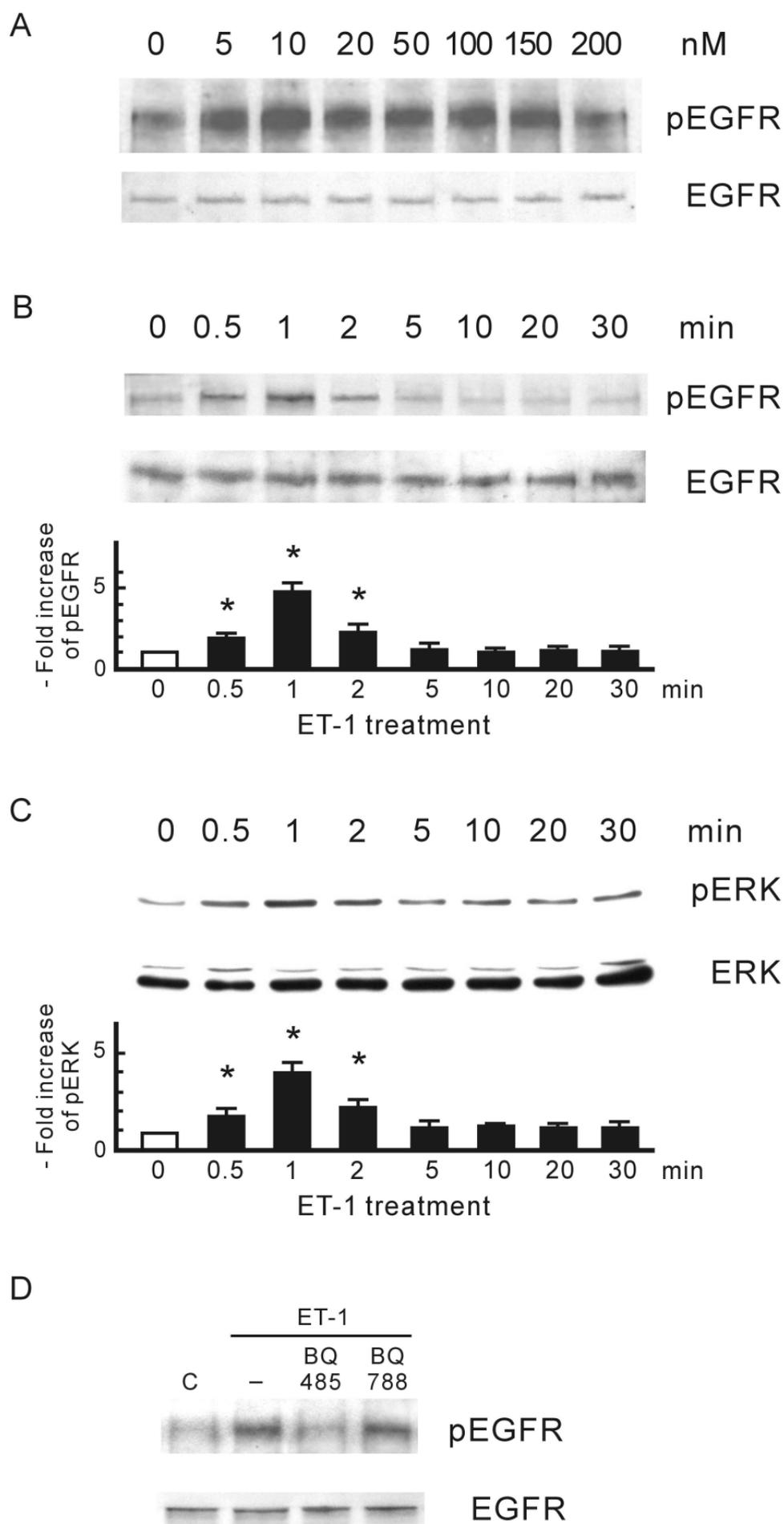
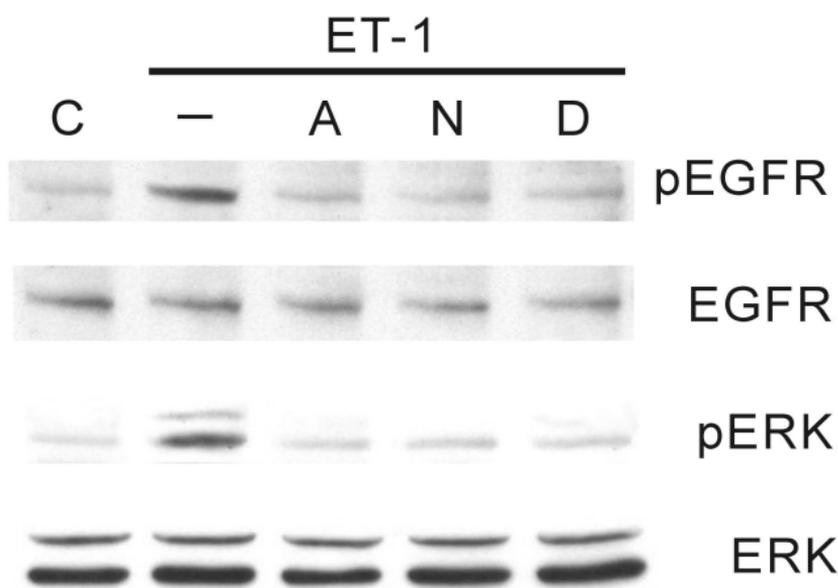


Figure 2

A



B

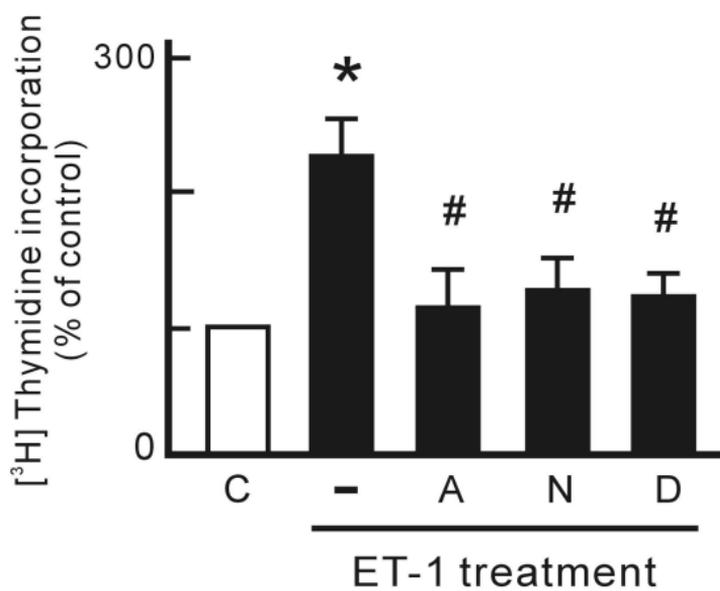
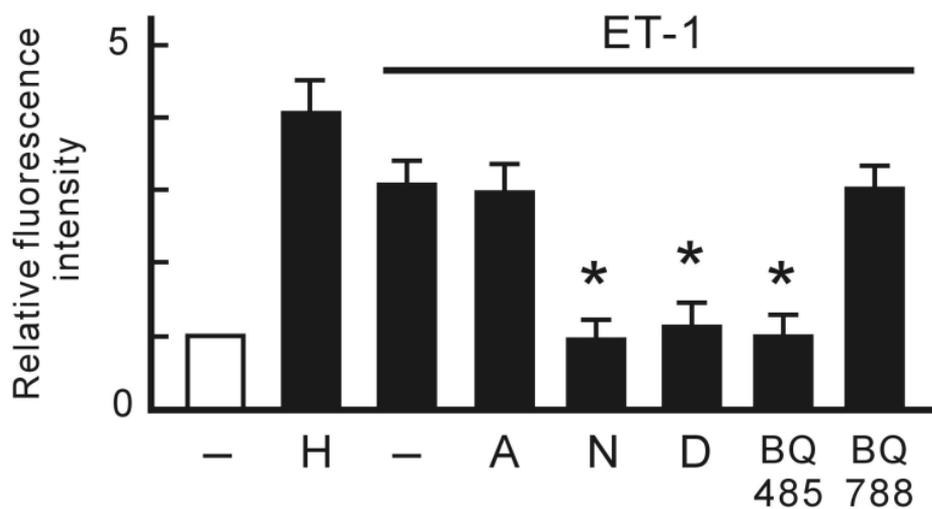
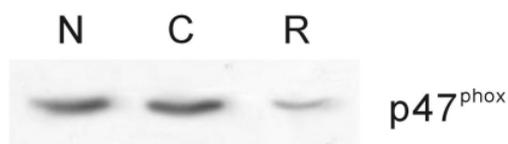


Figure 3

A



B



C

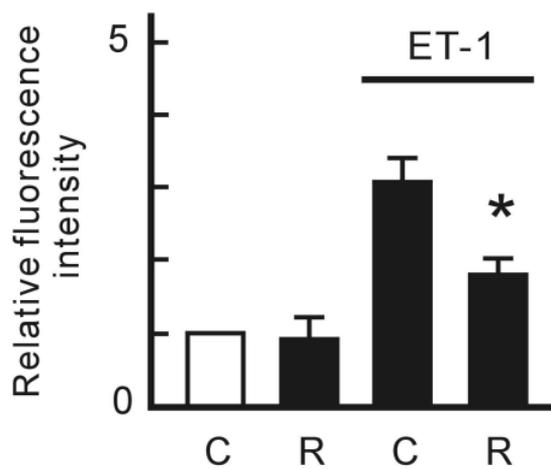
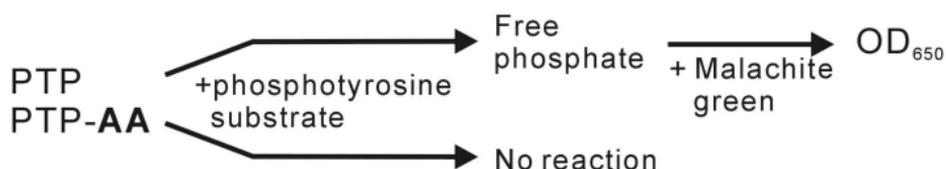


Figure 4

A



B

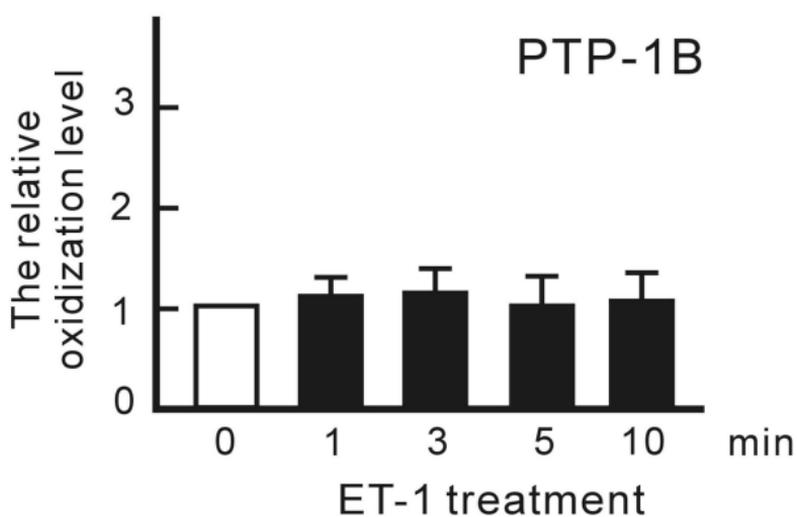
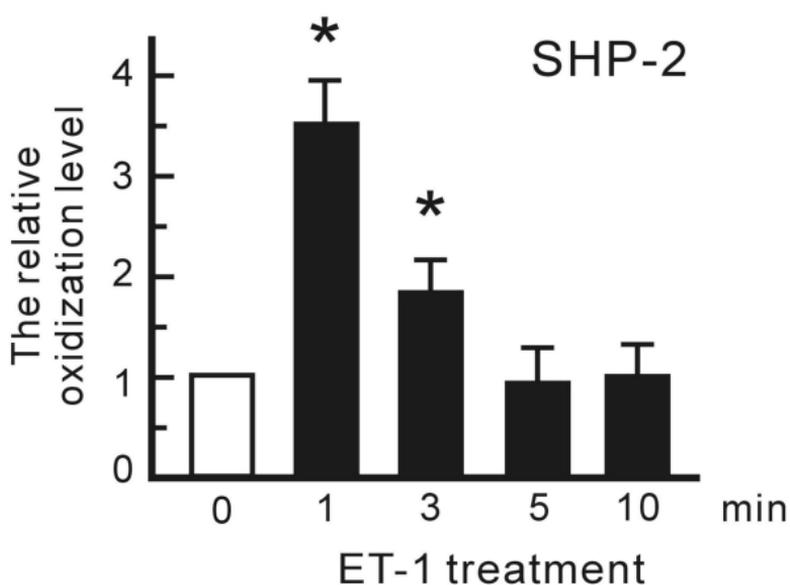
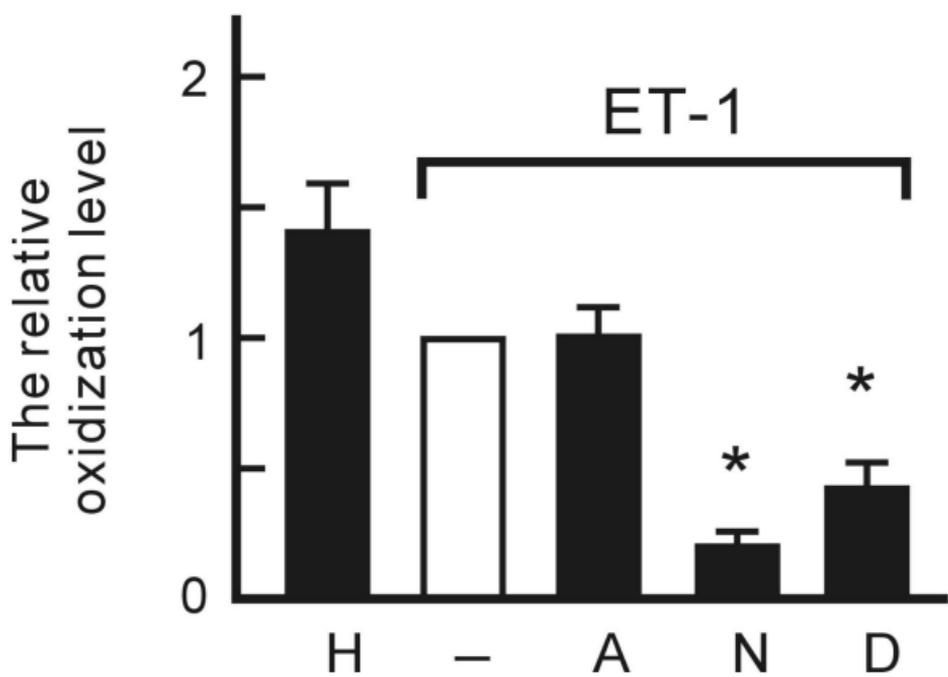


Figure 5

A



B

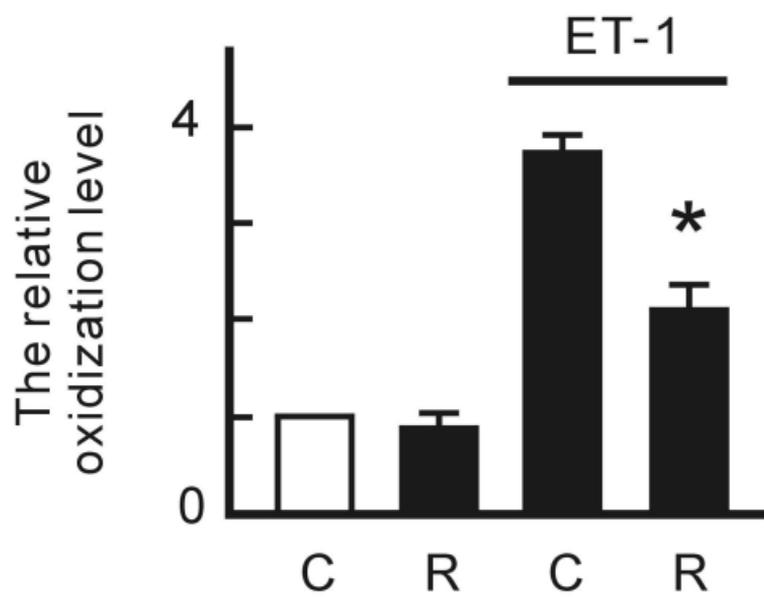


Figure 6

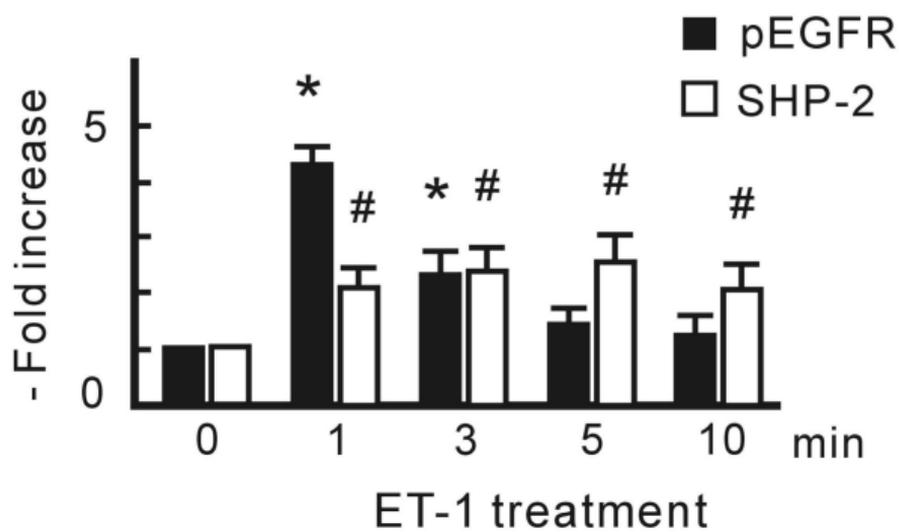
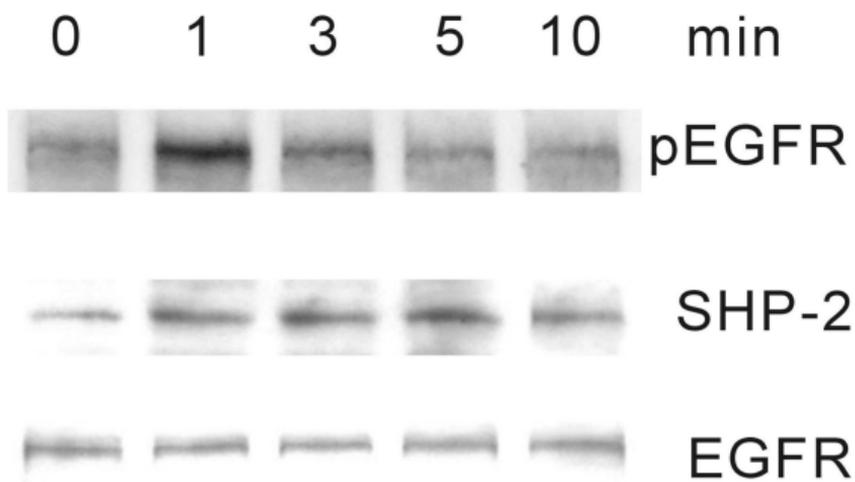
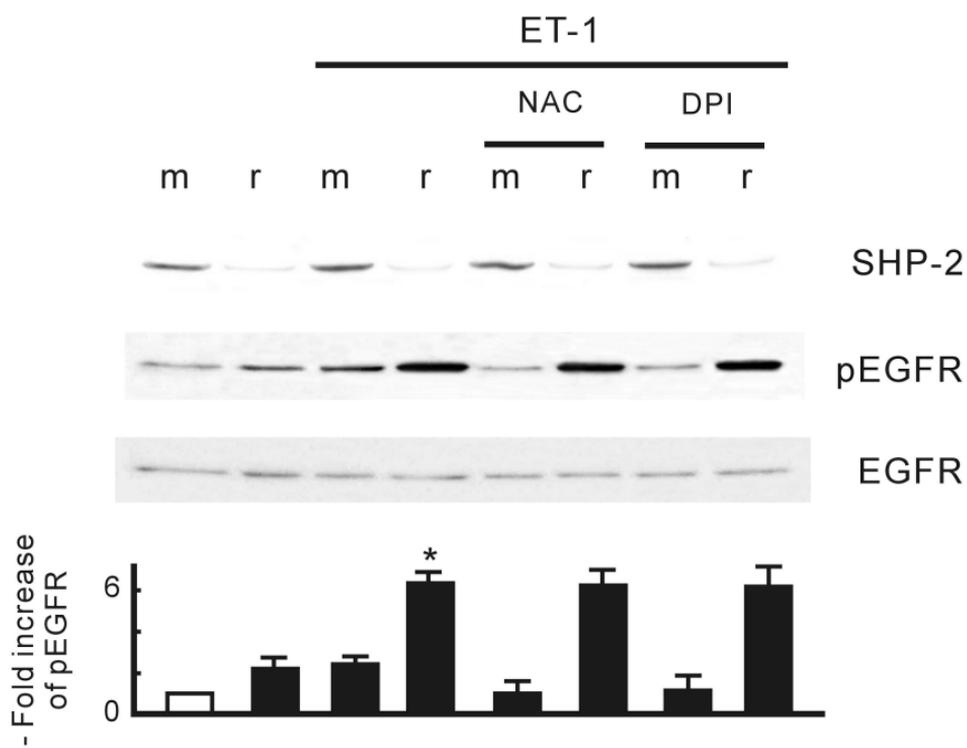


Figure 7

A



B

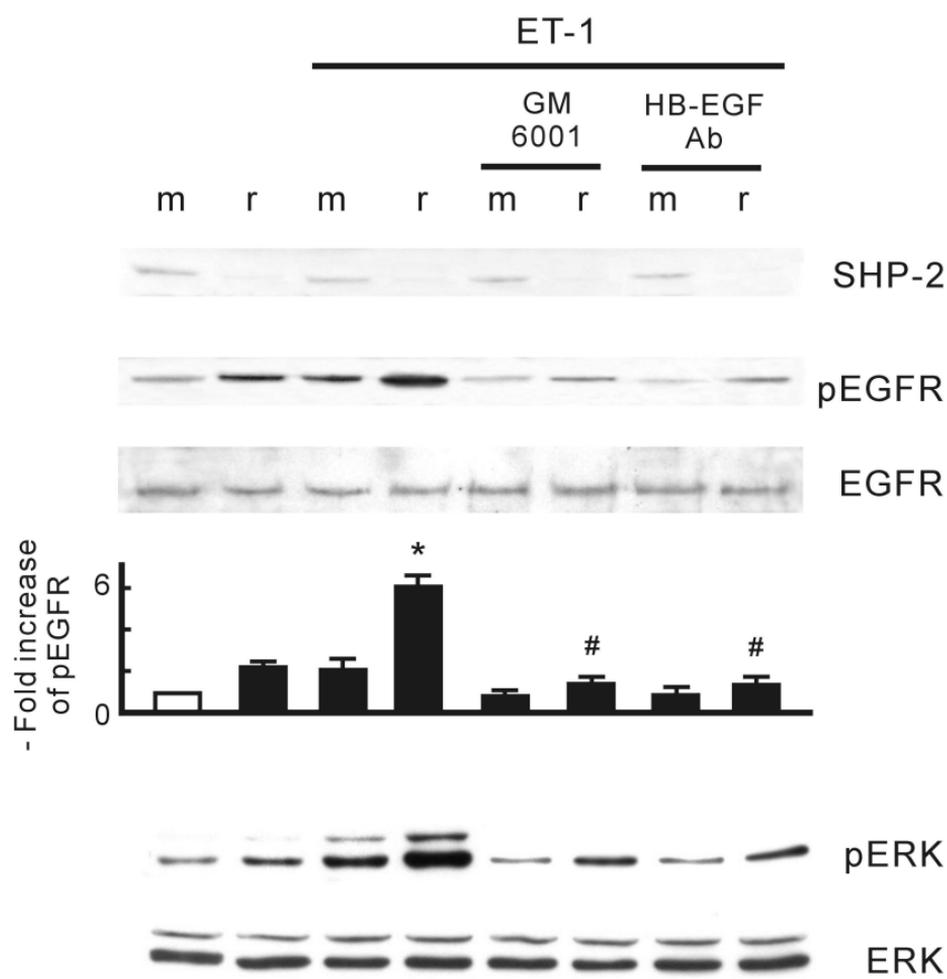


Figure 8

