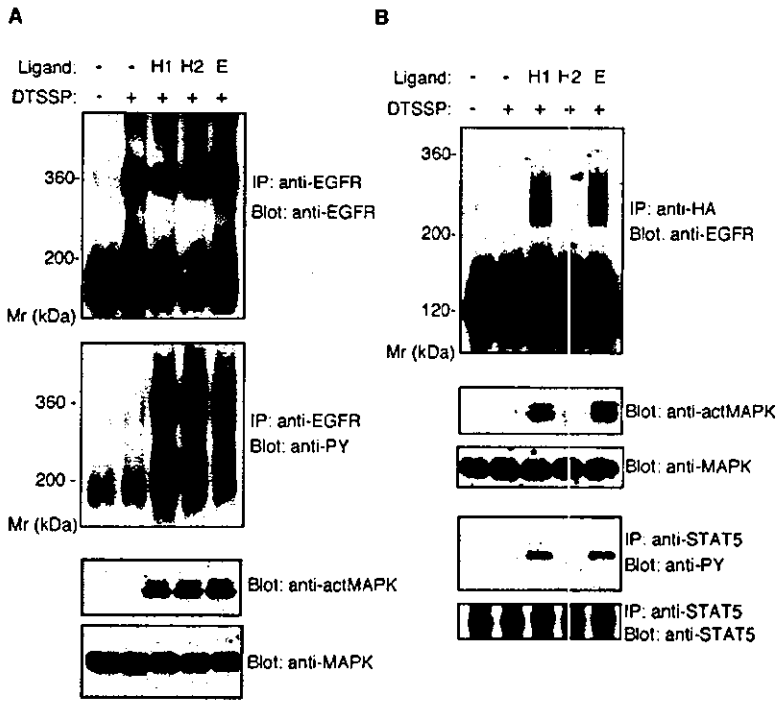

Figure 5. Dimerization and activation of EGFR and the EGFR-EpoR chimeric receptor by EGF, HB1, or HB2. (A) BE cells were treated with HB1, HB2, or EGF, followed by incubation with or without DTSSP. Cell lysates were immunoprecipitated with an anti-EGFR polyclonal antibody, and the precipitated materials were separated by 4% SDS-PAGE in the absence of a reducing agent and transferred to an Immobilon membrane. The membrane was blotted with anti-EGFR mAb or anti-phosphotyrosine mAb. Total cell lysates were used for detection of MAPK and phosphorylated MAPK by anti-MAPK or anti–activated MAPK antibody. (B) B108 cells were treated with HB1, HB2, or EGF followed by treatment with or without DTSSP. Cell lysates were immunoprecipitated with anti-HA polyclonal antibody, and the precipitated materials were separated by 4.5% SDS-PAGE in the absence of reducing agents and transferred to an Immobilon membrane. The membrane was blotted with anti-EGFR mAb. Total cell lysates were used for detection of MAPK and phosphorylated MAPK by anti-MAPK or anti–activated MAPK antibody. For the detection of STAT5, cell lysates were immunoprecipitated with anti-STAT5 antibody, and the membrane was blotted with anti-STAT 5 antibody or anti-phosphotyrosine mAb.

of EpoR (Figure 5B). However, neither chimeric receptor dimer formation nor MAPK/STAT5 activation was induced by treatment of B108 cells with HB2.

The above results, including the failure of HB2 to induce chimeric receptor dimer formation, lead to the hypothesis that HB2 activates EGFR only when it exists as a preformed dimer. To examine this possibility, we decided to induce chimeric receptor dimer formation artificially using an mAb directed to EGFR. The anti-EGFR antibody used in this experiment (EGFR.1) recognizes the extracellular domain of EGFR but neither activates EGFR nor inhibits ligand binding (Waterfield *et al.*, 1982). The ability of the antibody to induce dimers was first monitored by coimmunoprecipitation assay. Plasmids encoding EGFR and EGFR-EpoR chimeric receptor were cotransfected into COS-7 cells. Cells were treated with the anti-EGFR mAb, and the cell lysates were subjected to coprecipitation assay. As shown in Figure 6A, EGFR and EGFR-EpoR chimeras coprecipitated with each other, indicating that the anti-EGFR antibody can induce ligand-independent dimer formation between the EGFR and EGFR-EpoR chimera. Figure 6B also demonstrates antibody-mediated formation of EGFR-EpoR chimeric receptor dimers in B108 cells, as demonstrated by the cross-linking assay. B108 cells were first incubated with the EGFR.1 antibody and then treated with DTSSP. Western blotting using an anti–HA-tag antibody detected the formation of high-molecular-weight bands corresponding to or higher than the size of the chimeric receptor homodimer. Such high-molecular-weight bands were not observed in control cells that were not treated with the EGFR.1 antibody. However, analysis of the phosphorylation states of MAPK and STAT5 revealed that the antibody-mediated dimer or oligomer formation did not activate the chimeric receptor, in contrast to EGF-induced homodimer formation (Figure 6C).

Next, we compared the mitogenic activities of EGF, HB1, and HB2 with both BE and B108 cells in the presence or absence of the EGFR.1 antibody. DNA synthesis induced by EGF, HB1, and HB2 in BE cells and by EGF and HB1 only in B108 cells was not affected by the presence of the EGFR.1 antibody (Figure 7A). However, the mitogenic activity of HB2 on B108 cells was increased by ~10 times in the presence of 1 μg/ml of the EGFR.1 antibody. This effect of the EGFR.1 antibody on the mitogenic activity of HB2 was lost when only the Fab fragment of the EGFR.1 antibody was used (Figure 7B), indicating that bivalency of the antibody is required for its effect.

From these results, we concluded that the mitogenic activity of HB2 is greatly affected by the presence or absence of preformed EGFR dimers, whereas EGF and HB1 are less affected by the oligomerization state.

DISCUSSION

Crosslinking studies using the chemical cross-linker DTSSP have provided biochemical evidence for the existence of a ligand-independent preformed dimer of EGFR in BE cells. Conversely, ligand-independent dimer formation was not observed with an EGFR-EpoR chimeric receptor, excluding the possibility that the bifunctional cross-linker itself artificially forms the dimer. By cross-linking studies and by single-molecule analysis (Sako, Yu, Mekada, and Yanagida, unpublished observations), we have also observed that EGFR expressing in another cell line (CHO cells) forms a ligand-independent dimer, whereas the EGFR-EpoR chimeric receptor expressed in the same cell line does not. Thus, the present results are not specific for Ba/F3 cells. Because DTSSP cannot penetrate the cell membrane, the

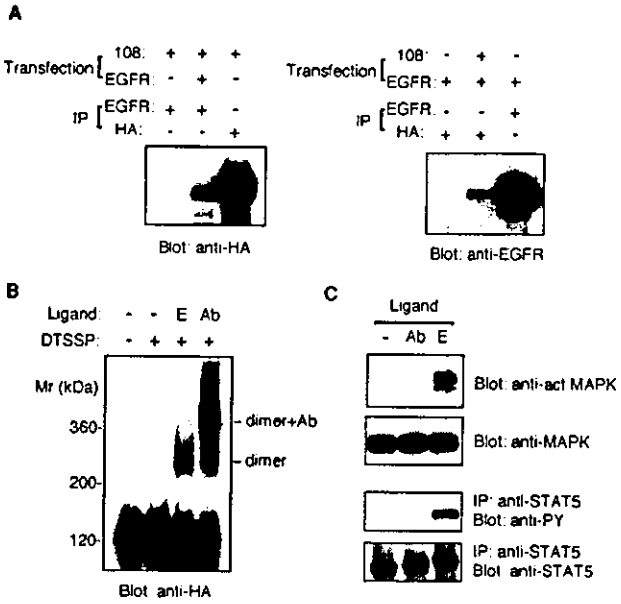

Figure 6. Antibody-mediated dimer formation of EGFR. (A) Coprecipitation assay of antibody-mediated formation of EGFR/EGFR-EpoR chimeric receptor heterodimers. COS cells were transfected with plasmids encoding EGFR and EGFR-EpoR (108). The transfected cells were incubated with anti-EGFR mAb (EGFR.1) for 2 h. Cells were lysed, and the cell lysates were subjected to the coimmunoprecipitation assay as described in MATERIALS AND METHODS and Figure 2. (B) Cross-linking assay of antibody-mediated formation of EGFR-EpoR chimeric receptor dimers. B108 cells were incubated with anti-EGFR mAb (Ab) or EGF (E) and then treated with or without DTSSP. Cells were lysed, and cell lysates were immunoprecipitated by anti-HA antibody. Precipitated materials were analyzed by SDS-PAGE and immunoblot using an anti-HA antibody. (C) The activation state of the antibody-mediated chimeric receptor dimer. B108 cells were incubated with anti-EGFR mAb (EGFR.1) (Ab) or EGF (E) and then lysed. The lysates were separated by SDS-PAGE, followed by immunoblotting with anti-phosphorylated MAPK or anti-MAPK antibody. An equal amount of cell lysate was subjected to immunoprecipitation with rabbit anti-STAT5 polyclonal antibody and then immunoblotted with anti-phosphotyrosine mAb or anti-STAT5 antibody.

preformed EGFR dimer must be on the plasma membrane. Phosphorylation analysis of EGFR and its downstream signaling molecules demonstrates that EGFR is not activated under serum-free conditions. In addition, using epitope-tagged EGFRs in coimmunoprecipitation assays, we confirmed the physical interaction between the two kinds of epitope-tagged EGFRs in COS-7 cells. Taking into account all earlier studies (Boni-Schnetzler and Pilch, 1987; Cochet *et al.*, 1988; Gadella and Jovin, 1995; Sako *et al.*, 2000; Moriki *et al.*, 2001), we conclude that EGFR forms dimers or oligomers on the cell surface in the absence of ligand stimulation.

Our work also indicates that EGFR dimer formation is not sufficient for receptor activation and downstream signaling. The amount of cross-linked EGFR dimers on BE cells was almost the same in EGF-treated or -untreated cells. However, phosphorylation assays of EGFR and its downstream molecules Cbl and MAPK indicate that EGF-untreated dimers are not activated at all. Although weak phosphory-

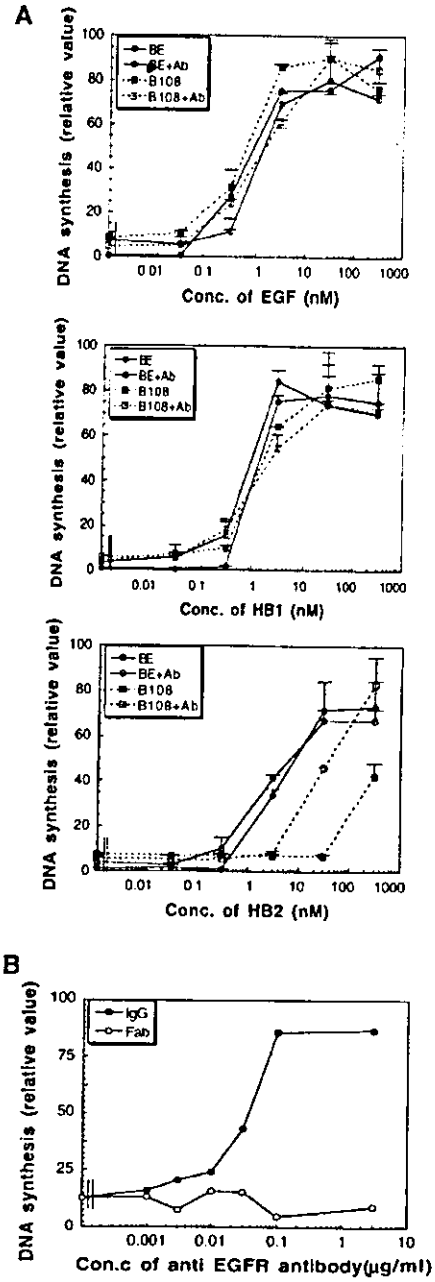

Figure 7. Effect of antibody-mediated formation of the chimeric receptor dimers on the mitogenic activities of EGF, HB1, and HB2. (A) BE and B108 cells were cultured with EGF, HB1, or HB2 in the presence or absence of anti-EGFR mAb (EGFR.1, 1 μg/ml) for 24 h, followed by incubation with [^{3}H]thymidine (37 kBq/ml) at 37°C for 4 h. Cells were harvested, and the amounts of [^{3}H]thymidine incorporated into DNA were measured. Data are shown as means ± SD obtained from three independent experiments. (B) B108 cells were incubated with HB2 (100 nM) in the presence of the indicated amounts of anti-EGFR mAb (EGFR.1) or its Fab fragment for 24 h, followed by incubation with [^{3}H]thymidine (37 kBq/ml) at 37°C for 4 h. Cells were harvested, and the amounts of [^{3}H]thymidine incorporated into DNA were measured. Conc., concentration

lation of Cbl and EGF-untreated EGFR monomers was seen in some cases, it was basal-level phosphorylation probably caused by the insufficient serum starvation. In addition, the bivalent anti-EGFR mAb used here can induce dimer formation without EGFR activation. In contrast to the ligand-independent dimerization state, binding of EGFR ligands, such as EGF and HB-EGF, to EGFRs results in strong activation of the receptor. These results clearly show that dimer formation is not enough for receptor activation. A similar phenomenon has also been demonstrated in EpoR and other receptor systems (Burke *et al.*, 1997; Constantinescu *et al.*, 2001). Therefore, the present conclusion that dimerization is not sufficient for receptor activation should be applicable to other receptor systems (Jiang and Hunter, 1999).

In this study, we used two kinds of recombinant HB-EGF. HB1, which contains the EGF-like domain of human HB-EGF, showed mitogenic activity similar to that of EGF. HB2, which covers the EGF-like domain of human HB-EGF but has three basic amino acids, which are thought to contribute in part to the heparin-binding property of HB-EGF, was replaced with the corresponding amino acids from EGF. The initial purpose of making these substitutions was to inactivate the heparin-binding region of HB-EGF and to examine instead its diphtheria toxin-binding activity, because interaction of the heparin-binding domain with heparin or heparin-like molecules is important for diphtheria toxin binding (Shishido *et al.*, 1995). However, we noticed in this study that HB2 differed from HB1 in its mitogenic activity; EGF and HB1 showed similar mitogenic activity in BE, BE2, and B108 cells, whereas HB2 was quite a weak mitogen for B108 cells compared with EGF and HB1. Cross-linking experiments indicated that HB2 is defective in its induction of EGFR-EpoR chimeric receptor dimer formation and therefore fails to activate MAPK and STAT5; in contrast, EGF and HB1 are able to induce chimeric receptor dimer formation under the same conditions. Although HB2 is defective in dimer formation, it seems to retain its ability to induce activation and phosphorylation of preformed EGFR dimers. The finding that pretreatment of B108 cells with a bivalent, but not monovalent, anti-EGFR mAb increases the mitogenic activity of HB2 indicates that the reduced mitogenic activity of HB2 on B108 cells is a result of its deficiency in inducing dimer formation. These results suggest that native EGFR ligands, including EGF and HB-EGF, have both receptor dimerization and receptor activation activities, whereas HB2 has little or no capacity to induce receptor dimerization.

What are the physiological implications of preformed EGFR dimers? As mentioned above, native EGFR ligands are generally able to induce EGFR dimer formation. However, without predimers, dimerization would take longer, because receptor molecules need to move around the cell surface looking for their partner molecules. We have recently observed that EGFR induced tyrosine phosphorylation within 1 minute after the addition of EGF, whereas the EGFR-EpoR chimeric receptor required much longer to induce significant tyrosine phosphorylation (Sako *et al.*, unpublished observations). Therefore, it is likely that predimers are responsible for the accelerated signal transduction of EGFR. The amounts of EGFR on the in vivo cell surfaces are much smaller than those of the cell lines we used in experiments, so ligand-induced dimer formation would not be efficient. Preformed dimers may also circumvent such disadvantages as the low surface EGFR concentration. As shown in Figure 3, BE2 cells, which express much smaller amounts of EGFR than BE cells, respond to EGF in the same way as BE cells. It has been reported that in tissue cells, EGFR can still respond to EGF stimulation even when receptor numbers go down to 2000 per cell (Carpenter, 1987), perhaps suggesting the existence of ligand-independent dimers in vivo. Although cross-linking studies would be ineffective for detecting preformed dimers in cells expressing such low amounts of EGFR, HB2 may be a useful tool to monitor the dimerization states of EGFR.

The present study also provides information regarding the domain of EGFR required for predimer formation. The EGFR-EpoR chimeric receptor does not form a predimer. This chimeric molecule possesses the extracellular and transmembrane domains of EGFR, but the cytoplasmic domain of EGFR is substituted with the cytoplasmic domain of EpoR. Thus, the results indicate that the cytoplasmic domain of EGFR is necessary for ligand-independent dimer formation. The present results are supported by an earlier report that only the extracellular domain of EGFR does not form a dimer without a ligand (Lax *et al.*, 1991). The extracellular domain of EGFR has a role in the prevention of EGFR autoactivation (Adelsman *et al.*, 1996), as shown in the platelet-derived growth factor receptor (Urer *et al.*, 1997). Further studies on truncated EGFR mutants indicate that the region from ^{835}Ala and ^{918}Asp of EGFR is required for EGFR predimer formation. This region is included in the kinase domain of EGFR, located close to the ATP binding site, and may regulate the kinase domain orientation (Groenen *et al.*, 1997). Thus, it suggests the intimate relationship between EGFR predimer formation and its activation. The dual function of this region may also support the "twist model" of EGFR activation, i.e., EGF induces rotation of each molecule of the EGFR predimer, rather than one of simply dimerization (Gadella and Jovin, 1995; Burke and Stern, 1998; Bell *et al.*, 2000).

The mechanism by which dimers form in a ligand-independent manner remains to be elucidated. It would be possible that another molecule is involved in ligand-independent predimer formation. Although there is no direct evidence identifying such a molecule, proteins such as ZPR1 (Galcheva-Gargova *et al.*, 1996), which has two identical domains that bind to the cytoplasmic domain of EGFR, may contribute to predimer formation.

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Distinct Mechanisms of Receptor and Nonreceptor Tyrosine Kinase Activation by Reactive Oxygen Species in Vascular Smooth Muscle Cells: Role of Metalloprotease and Protein Kinase C-δ

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Reactive oxygen species (ROS) are implicated in cardiovascular diseases. ROS, such as H_2O_2, act as second messengers to activate diverse signaling pathways. Although H_2O_2 activates several tyrosine kinases, including the epidermal growth factor (EGF) receptor, JAK2, and PYK2, in vascular smooth muscle cells (VSMCs), the intracellular mechanism by which ROS activate these tyrosine kinases remains unclear. Here, we identified two distinct signaling pathways required for receptor and nonreceptor tyrosine kinase activation by H_2O_2 involving a metalloprotease-dependent generation of heparin-binding EGF-like growth factor (HB-EGF) and protein kinase C (PKC)-δ activation, respectively. H_2O_2-induced EGF receptor tyrosine phosphorylation was inhibited by a metalloprotease inhibitor, whereas the inhibitor had no effect on H_2O_2-induced JAK2 tyrosine phosphorylation. HB-EGF neutralizing antibody inhibited H_2O_2-induced EGF receptor phosphorylation. In COS-7 cells expressing an HB-EGF construct tagged with alkaline phosphatase, H_2O_2 stimulates HB-EGF production through metalloprotease activation. By contrast, dominant negative PKC-δ transfection inhibited H_2O_2-induced JAK2 phosphorylation but not EGF receptor phosphorylation. Dominant negative PYK2 inhibited H_2O_2-induced JAK2 activation but not EGF receptor activation, whereas dominant negative PKC-δ inhibited PYK2 activation by H_2O_2. These data demonstrate the presence of distinct tyrosine kinase activation pathways (PKC-δ/PYK2/JAK2 and metalloprotease/HB-EGF/EGF receptor) utilized by H_2O_2 in VSMCs, thus providing unique therapeutic targets for cardiovascular diseases.

Reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide (H_2O_2), are known to act as second messengers (21, 41). ROS activate a wide variety of serine-threonine and tyrosine kinases, which are key regulatory proteins of signal transduction pathways important in mediating cellular growth, apoptosis, survival, migration, and aging (22, 35). An increasing body of evidence suggests that ROS and tyrosine kinase play prominent roles in the development and progression of the cardiovascular remodeling associated with hypertension, atherosclerosis, and restenosis after balloon angioplasty (2, 29).

ROS activate nonreceptor tyrosine kinases JAK2 (1, 59), PYK2/CAKβ (25), and Src (11, 67) and receptor tyrosine kinases epidermal growth factor (EGF) receptor (23, 53, 67) and platelet-derived growth factor receptor (34) in vascular smooth muscle cells (VSMCs) as well as other cell lines. A few studies have shown an inhibition of ligand-stimulated receptor tyrosine kinase induced by ROS (33), suggesting a dominant role for ROS as a tyrosine kinase activator. Among tyrosine kinases activated by ROS, the EGF receptor and JAK2 are of particular interest in VSMCs. A G-protein-coupled receptor (GPCR) agonist, angiotensin II (AngII), has been shown to utilize ROS to activate the EGF receptor in VSMCs (23, 66). The activation of the EGF receptor by AngII or thrombin appears to be required for extracellular signal-regulated kinase (ERK) activation and the subsequent growth of VSMCs (15, 17, 19, 38). By contrast, ROS-dependent JAK2 activation is required for AngII-induced cytokine induction (56) and thrombin-induced heat shock protein induction (47) in VSMCs. Thus, ROS-dependent activation of the EGF receptor and JAK2 could mediate two distinct functions in VSMCs, such as growth and inflammatory responses, respectively. However, whether ROS activate the EGF receptor and JAK2 through distinct mechanisms remains unknown.

Recently, it has become apparent that the EGF receptor is also a part of the signaling networks activated by stimuli that do not directly interact with this receptor. These stimuli include agonists that specifically bind to other membrane receptors and environmental stressors (6). Collectively, EGF receptor transactivation by these factors is employed in a wide array of biological signaling responses (32, 45), which may participate in several disease processes (4, 42, 50). In this regard, EGF receptor transactivation is a current topic of signal transduction research.

ROS have been proposed to exert their effects through targeting the cysteine regions of the active sites of tyrosine phosphatases, which in turn activates tyrosine kinases (21). In fact,

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H_2O_2 has been shown to inhibit the dephosphorylation of the EGF receptor through the inhibition of a tyrosine phosphatase (39). Protein kinase C-δ (PKC-δ) is also implicated in ROS-dependent activation of tyrosine kinases, such as c-Abl and Src. H_2O_2 stimulates binding between PKC-δ and c-Abl where the activation of c-Abl is dependent on PKC-δ activation (61). Interestingly, H_2O_2-induced activation of PKC-δ is reported to be independent from tyrosine phosphatase inhibition (68). Alternatively, ROS may activate a tyrosine kinase by generating growth factors, such as heparin-binding EGF-like growth factor (HB-EGF), through metalloprotease cleavage. ROS production and metalloprotease-dependent HB-EGF generation are implicated in EGF receptor transactivation initiated through several GPCRs (9, 52). Our group has shown that both mechanisms are indispensable for EGF receptor transactivation induced by AngII in VSMCs (14, 23). In addition, a metalloprotease, ADAM17 (TACE), was reported to require PKC-δ activation to generate HB-EGF (37).

In this study, we examined the hypothesis that the activation of receptor and nonreceptor protein tyrosine kinases by ROS utilizes distinct signal transduction mechanisms involving a metalloprotease or PKC-δ. We found that a metalloprotease-dependent shedding of HB-EGF is required for H_2O_2-induced EGF receptor transactivation but not for JAK2 activation. By contrast, PKC-δ is required for H_2O_2-induced JAK2 activation but not for EGF receptor transactivation. The activation of JAK2 but not of the EGF receptor also requires PYK2 activation. Taken together, our findings provide a unique example of two distinct signaling pathways that mediate ROS-dependent tyrosine kinase activation in vascular cells.

MATERIALS AND METHODS

Reagents. BB2116 was kindly provided by Helen Mills (British Biotech). CGS27023, GM6001, AG1478, and rottlerin were purchased from Calbiochem. H_2O_2, AngII, *N*-acetylcysteine, and poly[Glu^{80}-Tyr^{20}] were purchased from Sigma. Antibodies were purchased from the following sources: phospho-JAK2, phospho-EGF receptor, and phospho-PYK2, BioSource International; JAK2, Upstate Biotechnology; EGF receptor, PKC-δ, PKC-α, and PKC-β1, Santa Cruz Biotechnology; PYK2, Transduction Laboratories; and neutralizing human HB-EGF, R & D Systems.

Cell culture. VSMCs were prepared from thoracic aortas of Sprague-Dawley rats (18). Subcultured cells from passages 3 to 12 were used and showed 99% positive immunostaining with smooth muscle α-actin antibody (Sigma). Human aortic VSMCs were obtained from Clonetics and subcultured according to the manufacturer's manual. For experiments, VSMCs at 80 to 90% confluency were used after serum depletion for 3 days.

Adenovirus transfection. The generation of kinase-inactive PKC-δ, PKC-α, PKC-β1, and PYK2/CAKβ mutant-encoded adenovirus constructs is described in detail elsewhere (36, 48). VSMCs were infected with adenovirus for 2 days as previously described (17).

Immunoprecipitation and immunoblotting. After stimulation, cells were lysed with ice-cold immunoprecipitation buffer (150 mM NaCl, 20 mM Tris [pH 7.5], 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 10% [vol/vol] glycerol, 10 mg of leupeptin, 10 μg of aprotinin, and 10 μg of phenylmethylsulfonyl fluoride). The lysates were centrifuged, and the supernatant was immunoprecipitated with antibody and protein A/G agarose at 4°C for 16 h (19). Cell or immunoprecipitation lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted as described previously (19).

HB-EGF shedding assay. To examine the release of soluble HB-EGF, COS-7 cells were transfected with the alkaline phosphatase (AP)-tagged HB-EGF (HB-EGF-AP) plasmid (63) by a transferrin receptor-operated transfer (8, 58) with TransFast transfection reagent (Promega). Forty-eight hours after transfection, the medium was changed to Dulbecco modified Eagle medium without phenol red and cells were stimulated with H_2O_2. HB-EGF-AP secreted into the medium was assessed by measuring AP activity (63).

PKC-δ kinase assay. Kinase activity of PKC-δ was measured by an immune complex kinase assay as described previously (26, 61). After stimulation, cells were lysed with a buffer containing 50 mM HEPES (pH 7.5), 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM NaF, 2 mM phenylmethylsulfonyl fluoride, and 10 μg each of pepstatin and leupeptin/ml. Cell lysates were centrifuged, and the supernatant was immunoprecipitated with anti-PKC-δ antibody for 2.5 h. The kinase assay was performed with a kinase assay buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 2.5 μCi of [γ-^{32}P]ATP, and a substrate, 200 μg of histone H1/ml) incubated for 15 min at 30°C.

PYK2 kinase assay. PYK2 kinase activity was measured by an immune complex kinase assay as described previously (25). In brief, the cell lysates were centrifuged and the supernatant was immunoprecipitated with anti-PYK2 antibody for 2.5 h at 4°C. After being washed, the immune complexes were incubated with or without H_2O_2 for 10 min at room temperature in the kinase buffer (100 mM sodium HEPES [pH 7.6], 60 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM Na_3VO_4, 0.2% Triton X-100). Afterwards, the lysates were incubated at room temperature in kinase buffer containing 0.25 mg of poly[Glu^{30}-Tyr^{20}] and 2.5 μCi of [γ-^{32}P]ATP for 15 min. The reaction mixture was spotted onto Whatman 3MM paper, washed, and then measured by liquid scintillation counting.

RESULTS

H_2O_2 stimulates EGF receptor activation and JAK2 activation. The activation of the EGF receptor by H_2O_2 was assessed by a phospho-specific antibody. This antibody selectively recognizes the EGF receptor only when Tyr^{1068} (a Grb2 binding site) is autophosphorylated. Also, the activation of JAK2 was assessed by a phospho-specific antibody that selectively recognizes $Tyr^{1007/1008}$ dually phosphorylated JAK2. These tyrosine residues are believed to be autophosphorylation sites, with Tyr^{1007} phosphorylation being essential for JAK2 kinase activity (57). The specificities of these antibodies have been established previously (23). As shown in Fig. 1A, H_2O_2 time-dependently stimulated the phosphorylation of the EGF receptor and JAK2, with maximal phosphorylation occurring at 10 min. As shown in Fig. 1B, H_2O_2 concentration-dependently stimulated the phosphorylation of the EGF receptor and JAK2, with maximal phosphorylation occurring at H_2O_2 concentrations of 2 to 20 μM. These data suggest that the EGF receptor and JAK2 represent ROS-sensitive tyrosine kinases in VSMCs.

Involvement of metalloprotease-dependent HB-EGF generation in H_2O_2-induced EGF receptor activation. To determine whether EGF receptor transactivation by H_2O_2 requires metalloprotease-dependent generation of an EGF receptor ligand, VSMCs were pretreated with a metalloprotease inhibitor (BB2116) and stimulated with H_2O_2. BB2116 and a structurally related compound, batimastat, have been shown to selectively inhibit the processing of several EGF receptor ligand precursors (5, 12, 13). It has previously been shown that BB2116 has no nonspecific effect on EGF receptor signals stimulated by EGF (14). As shown in Fig. 2, H_2O_2-induced EGF receptor activation was concentration-dependently inhibited by BB2116 but JAK2 activation by H_2O_2 was unaffected by BB2116. Other metalloprotease inhibitors, CGS27023 and GM6001 (10 μM each, 30-min pretreatments), also inhibited H_2O_2-induced EGF receptor activation but had no effect on JAK2 activation (data not shown).

It has previously been shown that an HB-EGF neutralizing antibody effectively antagonizes AngII-induced EGF receptor transactivation in VSMCs (14). As shown in Fig. 3A, H_2O_2-induced EGF receptor activation was markedly inhibited by

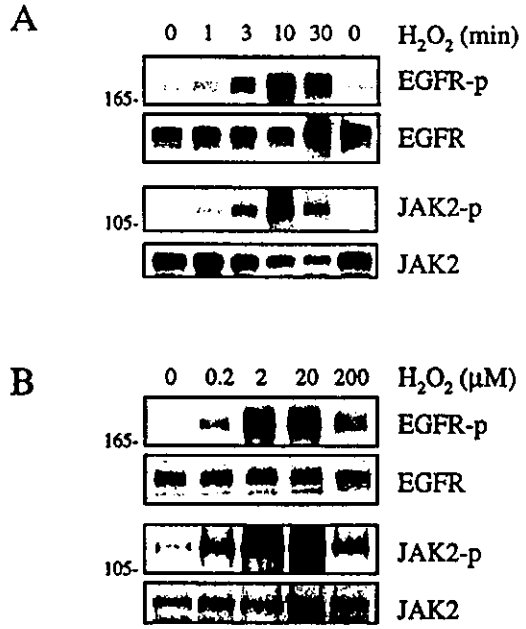

FIG. 1. Activation of EGF receptor and JAK2 by H_2O_2 in VSMCs. Cells were stimulated with 20 μM H_2O_2 for the indicated time periods (A) or with various concentrations of H_2O_2 for 10 min (B). Cell lysates were immunoblotted by antibodies toward phospho-EGF receptor (EGFR-p), EGF receptor (EGFR), phospho-JAK2 (JAK2-p), and JAK2 as indicated. Numbers on the left are molecular weights.

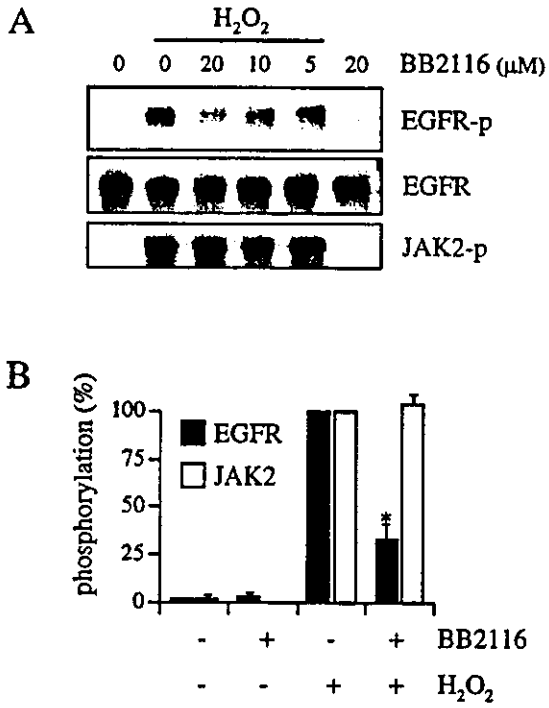

FIG. 2. Effect of metalloprotease inhibitor on H_2O_2-induced activation of EGF receptor and JAK2. (A) VSMCs were pretreated with the indicated concentrations of BB2116 for 30 min and stimulated with H_2O_2 (20 μM) for 10 min. Cell lysates were immunoblotted by antibodies toward phospho-EGF receptor (EGFR-p), EGF receptor (EGFR), and phospho-JAK2 (JAK2-p). (B) Densitometric analysis of EGF receptor and JAK2 phosphorylation. VSMCs were pretreated with or without 20 μM BB2116 for 30 min and stimulated with H_2O_2 (20 μM) for 10 min. Results are the means ± standard errors of the means (SEM) (n = 4). Asterisk, $P < 0.05$ versus H_2O_2 stimulation.

the HB-EGF neutralizing antibody. Since the detection of endogenous EGF receptor ligand generation has proven difficult (14), we utilized the HB-EGF-AP expression system, an established assay, to measure the ectodomain shedding of EGF receptor ligands. H_2O_2 time-dependently stimulated HB-EGF-AP release into the culture medium at as early as 10 min in COS-7 cells transfected with HB-EGF-AP plasmid, and BB2116 almost completely inhibited HB-EGF-AP generation in response to H_2O_2 (Fig. 3B). To confirm H_2O_2-induced EGF receptor tyrosine kinase activation, we examined the effect of AG1478, an EGF receptor kinase inhibitor (44). It has previously been shown that AG1478 specifically inhibits EGF receptor-mediated signal transduction in VSMCs (14, 15, 17, 19). AG1478 (250 nM, 30-min pretreatment) markedly inhibited H_2O_2-induced EGF receptor activation, whereas this inhibitor had no specific effect on JAK2 activation by H_2O_2 (data not shown). These data clearly implicate metalloprotease-dependent HB-EGF generation as a mechanism for H_2O_2-induced EGF receptor activation.

JAK2 activation but not EGF receptor activation requires PKC-δ. H_2O_2 has been shown to induce PKC-δ activation (40), which may in turn activate tyrosine kinases (61). PKC-δ is also implicated in the ectodomain shedding of HB-EGF (37). Given these findings, we investigated whether PKC-δ was involved in either JAK2 or EGF receptor activation by H_2O_2. VSMCs were pretreated with a PKC-δ inhibitor, rottlerin (31). The conditions required for the inhibition of PKC-δ function in VSMCs have been established previously (26). As shown in Fig. 4A, rottlerin (1 to 10 μM) concentration-dependently inhibited JAK2 phosphorylation by H_2O_2 in VSMCs. Figure 4B and C show that 10 μM rottlerin markedly inhibited H_2O_2-induced JAK2 activation but not H_2O_2-induced EGF receptor activation. A potent endogenous ROS inducer, AngII (30), produces intracellular H_2O_2 in the 10 to 100 nM range in VSMCs (64), which may mimic the exogenous addition of H_2O_2 as shown in Fig. 1A. Our group has previously reported that AngII-induced JAK2 activation requires PKC-δ (26) and is not blocked by a metalloprotease inhibitor, BB2116 (14), in VSMCs. Another study has shown that AngII-induced JAK2 activation requires ROS production in VSMCs (56). As shown in Fig. 4D, AngII-induced JAK2 activation was markedly inhibited by an antioxidant, *N*-acetylcysteine, in our VSMCs. Also, it has previously been reported that AngII-induced transactivation of the EGF receptor requires ROS (23) as well as HB-EGF production through a metalloprotease (14) in VSMCs. As shown in Fig. 4D, rottlerin had no inhibitory effect on AngII-induced EGF receptor transactivation, confirming that this pathway is independent from the JAK2 pathway activated by AngII. Taken together, these data suggest that AngII utilizes these two distinct pathways through ROS production in VSMCs, supporting the pathophysiological relevance of our findings.

To further clarify the role of PKC-δ in ROS-dependent JAK2 activation, we transfected VSMCs with adenovirus en-

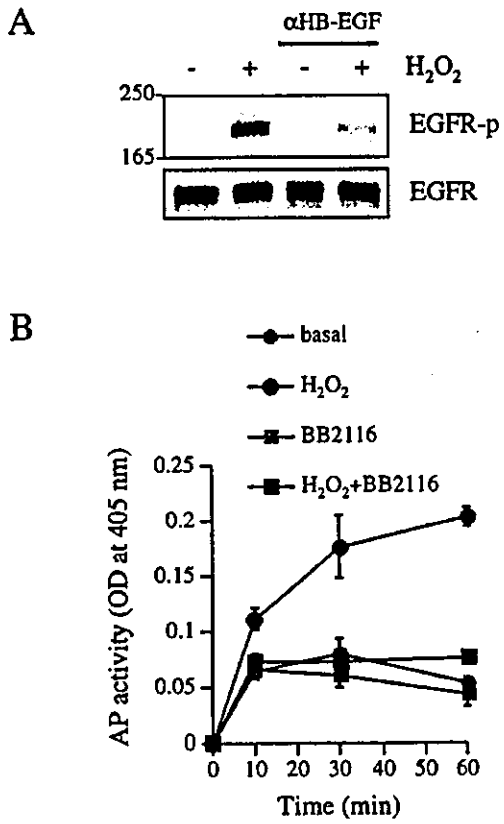

FIG. 3. Involvement of HB-EGF in H_2O_2-induced EGF receptor activation. (A) Human VSMCs were pretreated with HB-EGF neutralizing antibody (αHB-EGF; 40 μg/ml) for 1 h and stimulated with H_2O_2 (20 μM) for 10 min. Cell lysates were immunoblotted by phospho-EGF receptor antibody (EGFR-p) and EGF receptor (EGFR) antibody. Numbers on the left are molecular weights. (B) After pretreatment with or without BB2116 (20 μM) for 30 min, COS-7 cells transfected with HB-EGF-AP plasmid were stimulated with H_2O_2 (20 μM). AP activity in the medium was determined. Results are the means ± SEM (n = 3). OD, optical density; basal, control sample.

coding a kinase-deficient PKC-δ mutant that acts as a dominant negative PKC-δ (48). The specificity of this mutant has been shown previously (26). Several control studies using adenovirus encoding LacZ or vector alone also showed that the transfection of adenovirus (up to a multiplicity of infection [MOI] of 100) had no nonspecific effects in VSMCs (17, 24, 26). As shown in Fig. 5A and B, dominant negative PKC-δ transfection concentration-dependently inhibited H_2O_2-induced JAK2 activation but not H_2O_2-induced EGF receptor activation. Moreover, control studies using VSMCs transfected with kinase-inactive PKC-α and PKC-β1 revealed no inhibitory effect on H_2O_2-stimulated JAK2 phosphorylation (Fig. 5C). In addition, H_2O_2 (20 μM, 10-min stimulation) significantly stimulated PKC-δ activity in VSMCs (2.49 [±0.09]-fold increase in activity; $P \leq 0.05$; n = 4) as measured by an immune complex kinase assay. These results strongly suggest that PKC-δ is required for H_2O_2-induced JAK2 activation but not for H_2O_2-induced EGF receptor activation.

JAK2 activation but not EGF receptor activation requires PYK2. PYK2/CAKβ is a ROS-sensitive tyrosine kinase (25), and our group has recently demonstrated that this kinase is required for AngII-induced JAK2 activation in VSMCs (26). PYK2 is also implicated in EGF receptor transactivation by GPCRs (3). Therefore, we determined whether PYK2 plays a role in H_2O_2-induced activation of the EGF receptor and JAK2. VSMCs were transfected with adenovirus encoding a kinase-deficient PYK2 mutant, K457A (36). This mutant acts as a dominant negative PYK2 in VSMCs (24). As shown in Fig. 6A, kinase-deficient PYK2 mutant transfection markedly inhibited H_2O_2-induced JAK2 activation but not EGF receptor activation. Our group has previously shown that the treatment of VSMCs with H_2O_2 enhances PYK2 kinase activity (25). Thus, we further determined whether H_2O_2 directly activated PYK2 by measuring in vitro PYK2 kinase activity. However, H_2O_2 did not activate PYK2 kinase activity in vitro (data not shown), indicating that PYK2 is not a direct target of H_2O_2.

PYK2 Tyr^{402} is a major autophosphorylation site of PYK2. Our group has previously shown that H_2O_2 induces PYK2 Tyr^{402} phosphorylation by using a phospho-specific antibody (25). To determine whether PYK2 activation by H_2O_2 is regulated by PKC-δ, we examined the effect of PKC-δ inhibitors on PYK2 Tyr^{402} phosphorylation. As shown in Fig. 6B, dominant negative PKC-δ transfection markedly inhibited the phosphorylation of PYK2 induced by H_2O_2. We also observed similar inhibition by rottlerin (data not shown). These data suggest that a PKC-δ-sensitive tyrosine kinase, PYK2, is required for the activation of JAK2 by H_2O_2 but not for EGF receptor activation in VSMCs.

DISCUSSION

The significant finding reported in the present study is that ROS utilize distinct signaling mechanisms to mediate the activation of receptor and nonreceptor tyrosine kinases. Specifically, a metalloprotease-dependent cleavage of HB-EGF is required for H_2O_2-induced EGF receptor transactivation but not for JAK2 activation whereas PKC-δ is required for H_2O_2-induced PYK2/JAK2 activation and not for EGF receptor transactivation, as illustrated in Fig. 7. We believe that this is the first example showing that ROS activate tyrosine kinases through distinct mechanisms in the same cell culture system.

Here, we report that H_2O_2 stimulates EGF receptor transactivation via metalloprotease-dependent HB-EGF cleavage. This observation uncovers a previously unknown mechanism by which ROS activate a receptor tyrosine kinase. In support of this notion, our group has shown that both metalloprotease and ROS are required for EGF receptor transactivation induced by a GPCR agonist, AngII (14, 23). Although the metalloprotease responsible for HB-EGF generation induced by ROS has not been identified, both matrix metalloproteases (62, 69) and ADAM family metalloproteases (4, 37, 42) are implicated in the ectodomain shedding of HB-EGF that is stimulated by various agonists. A thiol group from a cysteine residue in the inhibitory prodomains of these metalloproteases interacts with zinc in their catalytic domains. ROS may oxidize electrophilic thiol groups and disrupt the cysteine-zinc bond, leading to the activation of the metalloproteases. In fact, H_2O_2 was shown to enhance ADAM17 activity and ADAM17-mediated ectodomain shedding (70). Therefore, it is interesting to test whether ROS activate metalloprotease directly. However, to the best of our knowledge, no reports have been published

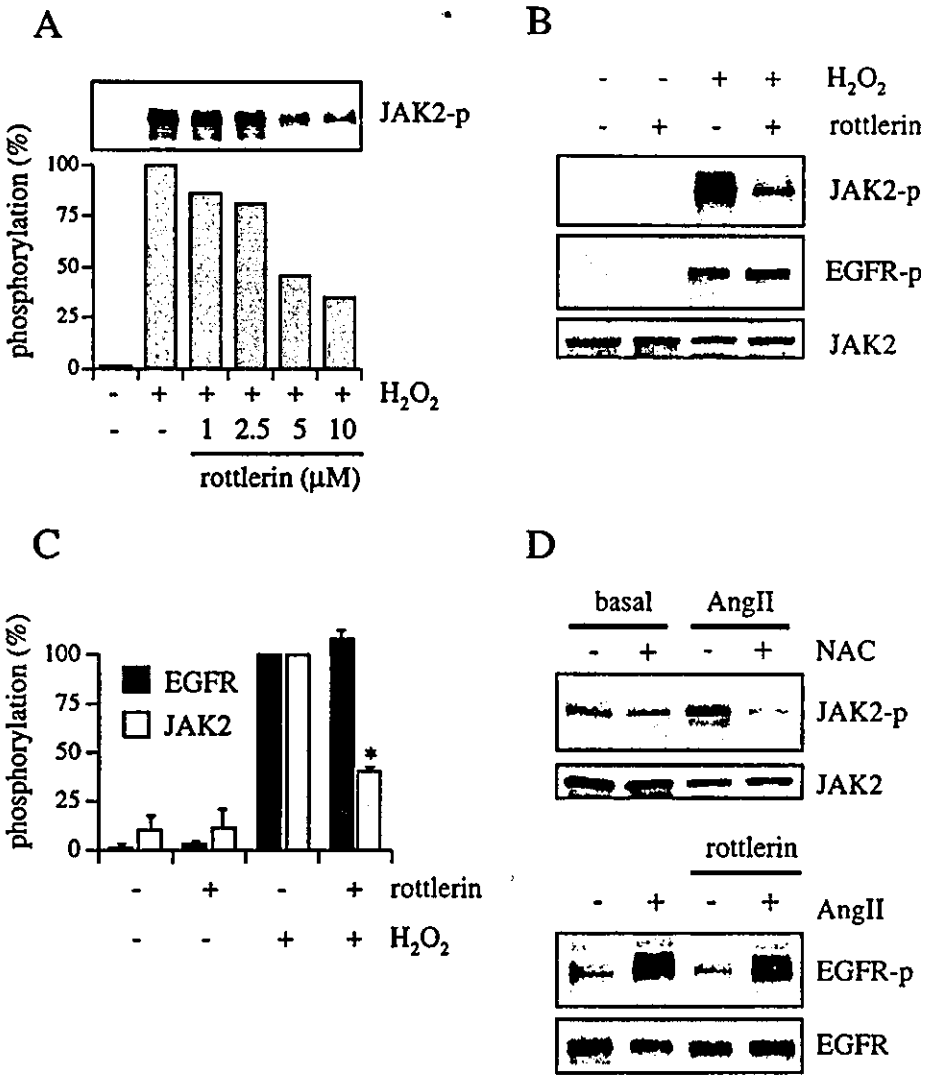

FIG. 4. Effects of rottlerin, a PKC-δ inhibitor, on H_2O_2-induced JAK2 and EGF receptor activation. (A) VSMCs were pretreated with various concentrations of rottlerin as indicated for 30 min and stimulated with H_2O_2 (20 μM) for 10 min. Cell lysates were immunoblotted by antibody toward phospho-JAK2 (JAK2-p). (B) VSMCs were pretreated with rottlerin (10 μM) for 30 min and stimulated with H_2O_2 (20 μM) for 10 min. Cell lysates were immunoblotted by antibodies toward phospho-JAK2, phospho-EGF receptor (EGFR-p), and JAK2. (C) Densitometric analysis of EGF receptor (EGFR) and JAK2 phosphorylation in the immunoblot shown in Fig. 4B. Results are the means ± SEM (n = 4). (D) VSMCs were pretreated with 20 mM *N*-acetylcysteine (NAC) for 90 min (upper panels) or 10 μM rottlerin for 30 min (lower panels) as indicated and stimulated with AngII (100 nM) for 3 min. Cell lysates were immunoblotted by antibodies toward phospho-JAK2 and JAK2 or phospho-EGF receptor and EGF receptor as indicated. Basal, control sample.

of studies that have directly measured the shedding activity toward proHB-EGF by using isolated membranes. Thus, to examine the direct activation of metalloprotease by ROS, further information is required regarding the identification of the metalloprotease responsible for HB-EGF generation and/or establishment of the assay to measure metalloprotease activity toward proHB-EGF in isolated membranes.

Alternatively, ROS may modulate intracellular signals such as c-Src and ERK, which may indirectly activate the metalloprotease responsible for HB-EGF generation. The contribution of the ERK cascade to HB-EGF and transforming growth factor-α generation has been reported previously (20, 27, 65). However, the ERK cascade is unlikely to mediate HB-EGF generation by H_2O_2 in VSMCs. This is because the ERK cascade exists downstream of EGF receptor transactivation in H_2O_2-stimulated VSMCs (23). c-Src is involved in EGF receptor transactivation by GPCRs (3, 46, 66), and c-Src appears to exist upstream of HB-EGF release (51). Moreover, H_2O_2-induced EGF receptor transactivation was inhibited by a selective Src inhibitor, PP2, in endothelial cells (7). The role of c-Src in mediating HB-EGF-dependent EGF receptor transactivation by H_2O_2 is under current investigation.

Our findings presented here strongly suggest the requirement of PKC-δ for JAK2 activation by H_2O_2. In the present study, we have used rottlerin as a PKC-δ inhibitor because it is commonly used as a selective inhibitor of this PKC isoform. In

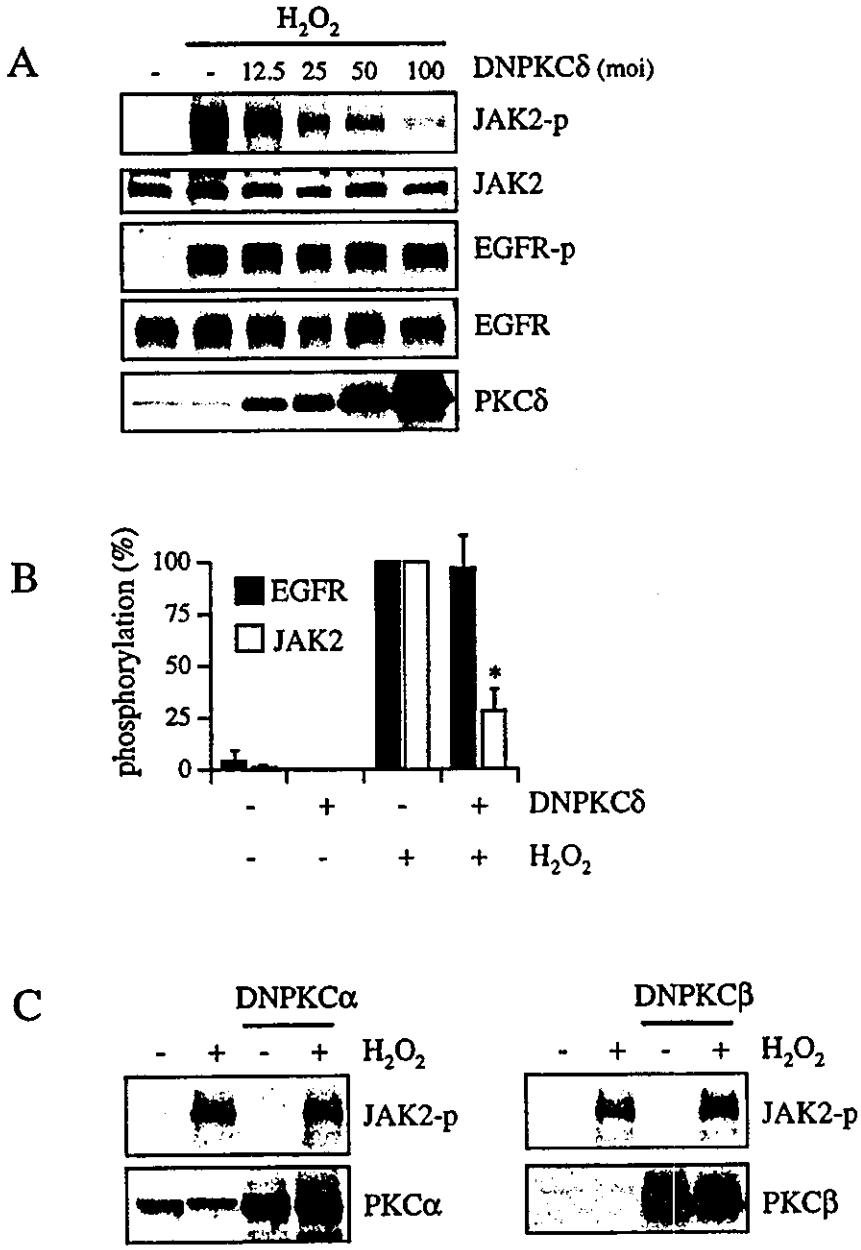

FIG. 5. Effects of dominant negative PKC-δ transfection on H_2O_2-induced JAK2 and EGF receptor activation. (A) Cells were transfected with the indicated amount of adenovirus encoding dominant negative PKC-δ (DNPKCδ) for 48 h and stimulated by H_2O_2 (20 μM) for 10 min. The cell lysates were immunoblotted by antibodies toward phospho-JAK2 (JAK2-p), JAK2, phospho-EGF receptor (EGFR-p), EGF receptor (EGFR), and PKC-δ. (B) Densitometric analysis of EGF receptor and JAK2 phosphorylation. Cells were transfected with adenovirus encoding dominant negative PKC-δ (MOI, 100) and stimulated with H_2O_2 (20 μM) for 10 min. Results are the means ± SEM (n = 3). (C) Cells were transfected with dominant negative PKC-α (DNPKCα) or dominant negative PKC-β1 (DNPKCβ) for 48 h and stimulated by H_2O_2 (20 μM) for 10 min. The cell lysates were immunoblotted by antibodies toward phospho-JAK2, PKC-α, or PKC-β1.

fact, our group has shown that this inhibitor blocks the translocation of PKC-δ toward the membrane stimulated by AngII in VSMCs and that it also inhibits the autophosphorylation of human recombinant PKC-δ in vitro (26). In contrast, two recent publications reported a failure of PKC-δ inhibition by rottlerin in a kinase assay using a synthetic substrate and one of the publications reported that rottlerin showed additional inhibitory effects besides PKC-δ inhibition (10, 60). To further evaluate the involvement of PKC-δ in JAK2 activation by H_2O_2, we utilized kinase-inactive PKC mutants and showed that only the PKC-δ mutant inhibited JAK2 activation. This is in line with the recent finding that PKC-δ is required for JAK2 activation induced by AngII (26), a well-established ROS inducer (30), in VSMCs. In addition, several reports indicate that H_2O_2 stimulates PKC-δ activity in various cell types (40, 49). In this study, we also found that H_2O_2 could stimulate

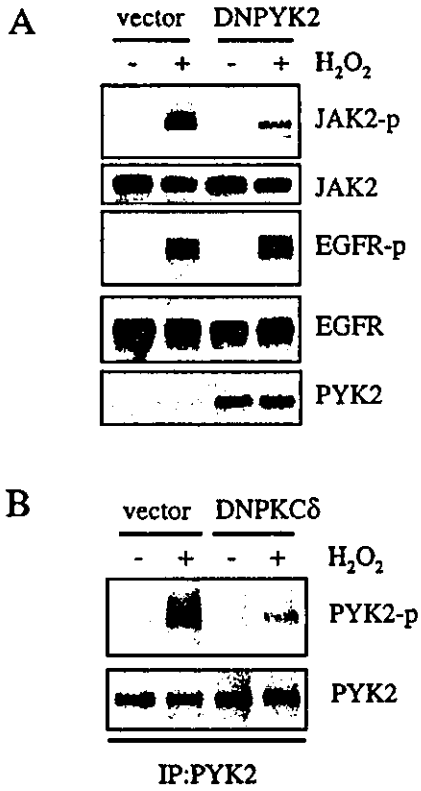

FIG. 6. Involvement of PYK2 in H_2O_2-induced JAK2 activation. (A) Cells were transfected with adenovirus (MOI, 10) encoding dominant negative PYK2 (DNPYK2) for 48 h and stimulated by H_2O_2 (20 μM) for 10 min. The cell lysates were immunoblotted by antibodies toward phospho-JAK2 (JAK2-p), JAK2, phospho-EGF receptor (EGFR-p), EGF receptor (EGFR), and PYK2. (B) Cells were transfected with adenovirus (MOI, 100) encoding dominant negative PKC-δ (DNPKCδ) for 48 h and stimulated by H_2O_2 (20 μM) for 10 min. The cell lysates were immunoprecipitated with anti-PYK2 antibody and immunoblotted by antibodies toward phospho-PYK2 (PYK2-p) and PYK2. IP, immunoprecipitate.

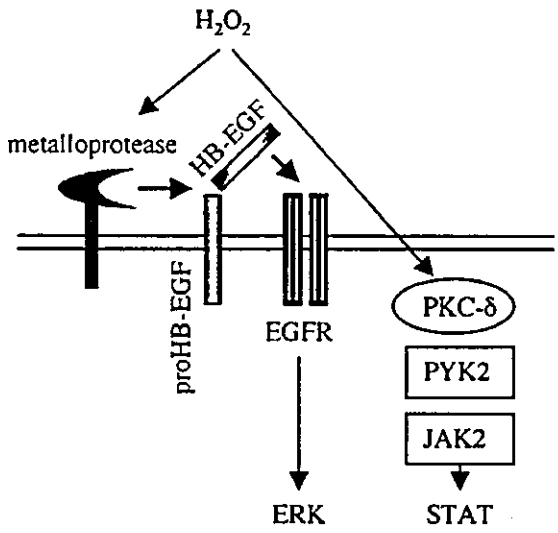

FIG. 7. Scheme illustrating proposed two distinct activation mechanisms of protein tyrosine kinases by H_2O_2 in VSMCs. EGFR, EGF receptor.

PKC-δ activity in VSMCs. In addition, PKC-δ was previously shown to be required for HB-EGF production, possibly through the activation of ADAM9 (37). However, our present findings rather eliminate the role of PKC-δ in H_2O_2-induced EGF receptor activation. This is in good agreement with previous findings by our group that PKC does not mediate EGF receptor transactivation induced by AngII (19).

VSMCs normally express PYK2 that is activated by ROS or AngII through ROS production (16, 25). It has been shown that PYK2 function is indispensable for several AngII-induced signaling pathways and subsequent hypertrophy in VSMCs (24, 54). Specifically, PYK2 is constitutively associated with JAK2 and is required for JAK2 activation by AngII (26). Although PYK2 is implicated in EGF receptor transactivation in fibroblasts (3), this may not be the case for EGF receptor transactivation in VSMCs (66). Here, we found that JAK2 activation but not EGF receptor activation by H_2O_2 requires PYK2, which appears to be downstream of PKC-δ. Interestingly, it was demonstrated that H_2O_2 stimulates PKC-δ and c-Abl association, where c-Abl is activated by a PKC-δ-dependent mechanism (61). Thus, the possibility that JAK2 or PYK2 is a substrate with which PKC-δ is capable of associating should be considered. PYK2 and its related tyrosine kinase FAK share a common structure with conserved important motifs (43, 55). Recently, FAK was shown to be involved in AngII-induced growth-promoting responses in cultured VSMCs (28). Although our present findings together with previous findings showing an interaction between PYK2 and JAK2 in VSMCs strongly suggest a critical role for PYK2 in mediating ROS-dependent JAK2 activation, it is possible that dominant negative PYK2 may interfere with the FAK function together with PYK2 function. Therefore, further studies are needed to examine the role of FAK in JAK2 activation.

In the present study, we recognized that most approaches that interfered with H_2O_2-dependent activation did not result in complete inhibition. Thus, both EGF receptor activation and JAK2 activation by ROS could involve additional pathways independent from the pathways identified in this study. In this regard, Src family kinase-dependent pathways have been proposed to mediate JAK2 activation (1) or EGF receptor activation by ROS (7, 66). Also, ROS are believed to stimulate tyrosine phosphorylation by the inhibition of tyrosine phosphatases via the cysteine residues in the active site regions of these enzymes (21). Knebel et al. (39) in fact demonstrated that H_2O_2 could inhibit the dephosphorylation of the EGF receptor through the inhibition of tyrosine phosphatases. Thus, future research should be conducted to determine whether a tyrosine phosphatase or Src kinase is involved in one or both mechanisms of tyrosine kinase activation by ROS in VSMCs.

In conclusion, we have shown that ROS utilize distinct signal transduction mechanisms to activate nonreceptor and receptor protein tyrosine kinases in VSMCs. This important finding may lead to the selective inhibition of various distinct ROS functions that may sufficiently prevent or attenuate several cardiovascular-related diseases.

ACKNOWLEDGMENTS

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Angiopoietin-regulated recruitment of vascular smooth muscle cells by endothelial-derived heparin binding EGF-like growth factor

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ABSTRACT **Recruitment of vascular smooth muscle cells (SMC) by endothelial cells (EC) is essential for angiogenesis. Endothelial-derived heparin binding EGF-like growth factor (HB-EGF) was shown to mediate this process by signaling via ErbB1 and ErbB2 receptors in SMCs. *1)* Analysis of ErbB-ligands demonstrated that primary ECs expressed only HB-EGF and neuregulin-1. *2)* Primary SMCs expressed ErbB1 and ErbB2, but not ErbB3 or ErbB4. *3)* Consistent with their known receptor specificities, recombinant HB-EGF, but not neuregulin-1, stimulated tyrosine phosphorylation of ErbB1 and ErbB2 and migration in SMCs. *4)* Neutralization of HB-EGF or inhibition of ErbB1 or ErbB2 blocked 70–90% of the potential of ECs to stimulate SMC migration. Moreover, *5)* angiopoietin-1, an EC effector with a role in recruitment of SMC-like cells to vascular structures in vivo, enhanced EC-stimulated SMC migration by a mechanism involving up-regulation of endothelial HB-EGF. Finally, *6)* immunohistochemical analysis of developing human tissues demonstrated that HB-EGF was expressed in vivo in ECs associated with SMCs or pericytes but not in ECs of the hyaloid vessels not associated with SMCs. These results suggest an important role for HB-EGF and ErbB receptors in the recruitment of SMCs by ECs and elaborate on the mechanism by which angiopoietins exert their vascular effects.—Iivanainen, E., Nelimarkka, L., Elenius, V., Heikkinen, S.-M., Junttila, T. T., Sihombing, L., Sundvall, M., Määttä, J. A., Laine, V. J. O., Ylä-Herttuala, S., Higashiyama, S., Alitalo, K., Elenius, K. Angiopoietin-regulated recruitment of vascular smooth muscle cells by endothelial-derived heparin binding EGF-like growth factor. *FASEB J.* 17, 1609–1621 (2003)**

Key Words: angiogenesis · cancer · ErbB · HB-EGF · Herceptin

The interaction between vascular endothelial cells (EC) and smooth muscle cell (SMC) -like mural cells is essential for the formation of mature vascular structures. During angiogenesis, the newly formed endothelial-lined channels recruit pericytes (PC) or other types of vascular SMCs that give the vessel both physical and chemical support. SMCs stabilize the vessel by inhibiting cellular proliferation and migration, stimulating production of components of the extracellular matrix, and providing survival factors (1–3). If the support provided by the SMCs is inadequate or blocked, vessels become dilated and leaky or start to regress (2, 4). These phenomena lead to poorly functional blood vessels that have an impaired capacity to deliver oxygenated blood or eliminate waste products. Thus, the EC–SMC interaction is one of the targets in the quest for strategies to promote or inhibit angiogenesis (3).

Soluble growth factors are important mediators of the paracrine interactions between ECs and SMCs. Well-characterized examples include platelet-derived growth factor-B (PDGF-B) and the angiopoietins (Ang). Null mice with disrupted genes for PDGF-B, or its receptor PDGFR-β, die perinatally and indicate a

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lack of recruitment of PCs and vascular SMCs by ECs (5, 6). Mice lacking Ang-1 or its receptor Tie2 die during midgestation with cardiovascular defects explained by a failure of the ECs of the blood vessels and endocardium to signal and associate with the adjacent vascular SMCs and cardiomyocytes, respectively (7, 8). The phenotypes of Ang-1 −/− and Tie2 −/− mice resemble that of mice overexpressing another member of the Ang family, Ang-2, under an EC-specific promoter (9). Based on these in vivo findings and cell culture studies (9), it has been suggested that Ang-2 functions as a natural antagonist for Ang-1 effects on blood ECs, competing for binding to the same Tie2 receptor. Tie2 has been documented to be an EC-specific receptor not expressed by other cell types (10). This has led to the hypothesis that Ang-1 stimulates the expression or secretion of an EC-derived factor which then recruits SMCs by chemoattraction (2). The observations that the vascular defects in Ang-1 −/− or Tie2 −/− mice occur earlier in development than the vascular defects in PDGF-B −/− or PDGFR-β −/− mice suggest that this Ang-1-induced SMC chemoattractant may be distinct from PDGF-B (5).

The ErbB receptors form a subfamily of receptor tyrosine kinases that consists of four members: ErbB1 (also known as EGF-receptor or HER1), ErbB2 (c-Neu, HER2), ErbB3 (HER3), and ErbB4 (HER4) (11–15). The ErbB receptors specifically interact with approximately a dozen epidermal growth factor (EGF) -like growth factors such as EGF, transforming growth factor-α (TGF-α), amphiregulin (AR), heparin binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPR), epigen, and the neuregulins (NRG-1, NRG-2, NRG-3, and NRG-4) (16–19). Gene targeting experiments have demonstrated that the ErbB signaling system functions in a paracrine fashion in the communication between endocardial ECs and myocardial muscle cells in the heart. Homozygous mice with disrupted ErbB2, ErbB4, or NRG-1 genes all die around embryonic day 10 with defects in the formation of projections of the heart ventricles, called trabeculae (20–22). Since NRG-1 is expressed solely in the endothelial lining of the heart (endocardium), and ErbB2 and ErbB4 in heart muscle (myocardium), these findings indicate that a paracrine signaling system consisting of endocardium-derived NRG-1 signaling via ErbB2/ErbB4 heterodimer in cardiomyocytes is necessary for normal heart development.

Here we address whether ErbB receptors and their ligands also play a role in the paracrine interplay between blood vascular ECs and SMCs. Our results suggest that HB-EGF expressed by ECs can recruit SMCs by signaling via ErbB1 and ErbB2 in SMCs. Moreover, Ang-1 is capable of inducing the expression of HB-EGF in ECs as well as the potential of ECs to stimulate SMC migration, suggesting an indirect mechanism by which Ang-1 recruits SMCs. These findings provide new evidence about molecular signals that regulate EC–SMC interactions.

MATERIALS AND METHODS

Growth factors and inhibitors

Recombinant human EGF, BTC, NRG-1β1, Ang-1, and Ang-2 were purchased from R&D (Abingdon, UK). Recombinant human HB-EGF was purchased from R&D or kindly provided by Dr. J. Abraham (Scios Nova, Mountainview, CA, USA). Extracellular domain of human NRG-2/NTAK (23) was produced in SF21 cells using BAC-TO-BAC baculovirus expression system (Gibco BRL, Grand Island, NY, USA) and was purified by two successive column chromatographies on heparin-Sepharose and C4 reversed phase HPLC.

To produce recombinant Ang-1 and Ang-2 fusion proteins, expression vectors encoding Ang-1 or Ang-2 coupled to the Fc fragment of human immunoglobulin gamma (Ang-1-Fc or Ang-2-Fc, respectively) (24) were transfected to HEK293 cells using Lipofectamine reagent (Gibco BRL). Transfected and nontransfected HEK293 cells were grown to confluency, cell layers were washed with PBS, and the cultures were continued in serum-free DMEM for another 7 days. The conditioned media were centrifuged and the supernatants were concentrated 20-fold using Amicon Concentrator with YM-10 ultrafiltration membrane (Millipore, Bedford, MA, USA). Presence of Ang-1-Fc and Ang-2-Fc in the supernatant was confirmed by Western analysis using rabbit anti-human Fc antibody (Zymed, San Francisco, CA, USA) (see Fig. 6A).

For adenoviral protein expression, HUVECs were grown to 70% confluency and exposed (400 MOI) to an adenovirus encoding angiopoietin-1 (AdAng-1) (25) or β-galactosidase (AdLacZ) (26) for 2 h in RPMI containing 1% human AB serum (Finnish Red Cross). After a wash with PBS, cultures were maintained in RPMI containing 10% AB serum (for Western analysis) or in plain RPMI (to produce conditioned medium for migration assays).

Neutralizing antibodies for human HB-EGF (R&D) and ErbB2 (Herceptin; Roche, Nutley, NJ, USA) were used at final concentrations of 1 μg/mL and 10 μg/mL, respectively. CRM 197, a specific inhibitor of HB-EGF (27, 28) (Sigma, St. Louis, MO, USA), was used at the concentrations indicated in Fig. 5. The ErbB1 inhibitor PD 153035 (Compound 32; Calbiochem, San Diego, CA, USA) was used at a final concentration of 1 μM.

Cell culture

Human umbilical vein endothelial cells (HUVEC) were prepared as described (29). HUVECs were cultured on dishes coated with 2% gelatin in RPMI medium supplemented with 10% AB serum, 1% glutamine/penicillin/streptomycin (GPS) supplement (Irvine Scientific, Santa Ana, CA, USA), 1.4 IU/mL heparin (Heparin LEO, Leo Pharma, Ballerup, Denmark), and 0.02 mg/mL bovine endothelial cell growth factor (ECGF; Roche). HUVECs were passaged maximally three times. Primary bovine capillary endothelial cells (BCE) (gift from Dr. Judah Folkman, Children's Hospital, Boston, MA, USA) were cultured on gelatin-coated dishes in DMEM supplemented with 10% fetal calf serum (FCS) (Sigma or Bioclear), 1% GPS, and 3 ng/mL basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ, USA). The expression of Tie2 in ECs was confirmed by Western analysis using a rabbit polyclonal anti-Tie2 antibody (C-20; Santa Cruz, Santa Cruz, CA, USA). Human aortic smooth muscle cells (HASMC) were obtained from American Type Culture Collection (ATCC No. CRL 1999) or purchased from PromoCell (Heidelberg, Germany). HASMCs were cultured in F12K (ATCC) medium including 10% FCS and supplements as recommended by ATCC. Bovine aortic smooth muscle cells

(BASMCs) were established from tissue strips prepared from bovine thoracic aorta, as described (30). HEK293 cells (gift from Dr. Mika Scheinin, University of Turku, Finland) and BASMCs were cultured in DMEM supplemented with 10% FCS and 1% GPS.

Analysis of conditioned culture media and recombinant growth factors using Boyden chamber migration assays

To produce conditioned media for the migration assays, confluent cultures were washed with PBS and maintained for 24–48 h in plain DMEM or RPMI medium without any supplements. The media were collected, centrifuged, and diluted in DMEM or RPMI to obtain a concentration series. Recombinant HB-EGF and NRG-1 were diluted in DMEM before testing for chemoattractive potential.

The cells analyzed in the migration assays were starved overnight in serum-free medium, washed with PBS, trypsinized, and suspended in DMEM or RPMI to a final concentration of 500,000 cells/mL. Fifty microliter samples of the cell suspension were analyzed for migration in response to chemoattractants (conditioned medium, serum or recombinant proteins) using Boyden chamber apparatus, as described (31, 32). The migration assays were carried out for 5 h in 37°C.

To determine the effect of specific inhibitors on SMC migration, inhibitory reagents were either added together with the chemoattractant to the lower Boyden chamber wells (CRM 197; anti-HB-EGF antibody) or together with the cells to the upper Boyden chamber wells (ErbB1 inhibitor; anti-ErbB2 antibody).

RT-PCR analysis of EGF-like ligands

Five micrograms of total RNA was extracted from confluent HUVEC cultures using the RNAzol B reagent (Tel-Test), and transcribed to cDNA with mouse myeloid leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI, USA), according to the manufacturer's instructions. PCR amplification was carried out using the following sense and antisense primers, respectively: 5′-TGTCCCCTGTCCCACGAT-3′ and 5′-AGCCTTGCTCTGTGCCCA-3′ for amplification of a 511 bp fragment of EGF; 5′-TGCGGGACCATGAAGCT-3′ and 5′-TCTCAGTGGGAATTAGTCA-3′ for a 638 bp fragment of HB-EGF; 5′-AAAATGGTCCCCTCGGCT-3′ and 5′-TCTGGGCTCTTCAGACCA-3′ for a 496 bp fragment of TGF-α; 5′-GCTCCCATCGCCGATGA-3′ and 5′-TTTGATGGCGCCATTCAGA-3′ for a 537 bp fragment of EPR; 5′-TGCGAAGGACCAATGAGAG-3′ and 5′-GCATGTTACTGCTTCCAGG-3′ for a 522 bp fragment of AR; 5′-TAGTGATCCTTCACTGTG-3′ and 5′-TTAAGCAATATTTGTCTCTTC-3′ for a 470 bp fragment of BTC; 5′-ATGAAAAGCCAGGAATCGG-3′ and 5′-AGTATCTCGAGGGGTTTGA-3′ for a 502 bp fragment of NRG-1; 5′-AGCCAGACGGGACAGGTG-3′ and 5′-AGGAGAGCTGGTTGATGCC-3′ for a 379 bp fragment of NRG-2; and 5′-CTACAATGAGCTGCGTGTGG-3′ and 5′-TAGCTCTTCTCCAGGGAGGA-3′ for a 450 bp fragment of human β-actin. All PCR reactions were carried out in a total volume of 50.2 μL including 5 μL of template (10% v/v of RT reaction), 31 μL sterile water, 1 μL of specific 5′ and 3′ primers (35 pmol/μl; see above), 1 μL Dynazyme DNAII polymerase (2.0 U/mL) (Finnzymes, Espoo, Finland), 5 μL 10 × Dynazyme buffer (Finnzymes), and 1.2 μL dNTP mix (10 mM; Finnzymes). The samples were denatured at 94°C for 3 min and subsequently cycled 35 times through 1 min steps of annealing at 65°C, extension at 72°C, and denaturation at 94°C. PCR products were separated in 1% agarose gels.

Western blot analysis of ErbBs, HB-EGF, and tyrosine phosphorylated proteins

Protein expression was analyzed by Western blot as described (32). The synthesis of ErbBs by SMCs was analyzed using the following primary antibodies: anti-EGFR for ErbB1, C-18 for ErbB2, C-17 for ErbB3, and C-18 for ErbB4 (all from Santa Cruz). The synthesis of HB-EGF by ECs was analyzed using a monoclonal neutralizing antibody (R&D) recognizing mature HB-EGF as well as two polyclonal antibodies (C-18 and M-18; Santa Cruz) recognizing the cytoplasmic tail of precursor HB-EGF. Peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA), goat anti-mouse IgG (Cappel, Cochranville, PA, USA), and rabbit anti-goat IgG (Chemicon International, El Segundo, CA, USA) were used as secondary antibodies.

For tyrosyl phosphorylation analysis, confluent SMC cultures were starved overnight in serum-free DMEM, stimulated with 50 ng/mL of growth factors for 10 min at 37°C, and lysed in lysis buffer containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate. Aliquots of the lysates corresponding to 75 μg of total protein were separated in 6% SDS-PAGE gels, transferred to nitrocellulose membranes, and analyzed by Western blot with anti-phosphotyrosine antibody (4G10; Upstate Biotechnology Inc., Lake Placid, NY, USA) or phospho-specific anti-ErbB1 and anti-ErbB2 antibodies [phospho-EGF receptor (Tyr1068) and phospho-HER2/ErbB2 (Tyr1248), respectively; Cell Signaling Technology, Beverly, MA, USA]. Primary antibodies were detected by peroxidase-conjugated goat anti-mouse IgG (Cappel) or goat anti-rabbit IgG (Jackson Immunoresearch Laboratories), and ECL (Amersham, Amersham, UK).

Northern blot analysis of HB-EGF

Confluent HUVEC cultures were starved for 6 h in RPMI containing 2% AB serum, washed with PBS, and treated with the test samples diluted in serum-free DMEM. Total cellular RNA was isolated using RNAzol B (Tel-Test). Ten microgram aliquots of total RNA were fractionated in 0.9% formaldehyde-agarose gels and transferred to Zetaprobe membranes (Bio-Rad, Hercules, CA, USA). The membranes were hybridized with a ^{32}P-dCTP-labeled (Rediprime II; Amersham) 665 bp ApaI-SalI fragment of the full-length human HB-EGF cDNA (33). To control loading of the RNA samples, the membranes were rehybridized with a ^{32}P-dCTP-labeled (Nick Translation; Roche Diagnostics) human 28S rRNA cDNA probe (34). After exposure to X-ray films, the hybridization signals were quantitated using MCID image analyzer (MCID M5, Imaging Research, St. Catharine's, Ontario, Canada).

Real-time RT-PCR analysis of ErbBs and HB-EGF

Quantitative real-time RT-PCR analysis (TaqMan) of ErbB mRNA expression in HASMCs was performed (T. Junttila et al., unpublished results). To analyze the stability of HB-EGF mRNA after angiopoietin treatment, test samples were applied to starved HUVEC cells as described above. Eight hours later RNA synthesis was blocked by addition of 3.3 μg/mL actinomysin D (Sigma). Total RNA was then extracted 0, 1, 2, 4, and 8 h after the application of actinomycin D, as described above. cDNA synthesis and TaqMan real-time PCR analysis were subsequently performed (T. Junttila et al., unpublished results), using the following primers and probes for HB-EGF and the reference gene β-actin: 5′-TTATCCTCCAAGCCA-

CAAGCA-3′ (HB-EGF forward primer), 5′-AGCCCCTTGCCTTTCTTCTTT-3′ (HB-EGF reverse primer), and 5′-TTCCCGTGCTCCTCCTTGTTTGGTGT-3′ (HB-EGF probe); 5′-ATCTGGCACCACACCTTCTACAAT-3′ (β-actin forward primer), 5′-CCGTCACCGGAGTCCATCA-3′ (β-actin reverse primer), and 5′-TGACCCAGATCATGTTTGAGACCTTCAACAC-3′ (β-actin probe).

Immunohistochemistry

To localize HB-EGF expression in developing vascular structures in vivo, formalin fixed paraffin sections of an aborted 10-wk-old human fetus were stained with IgG fraction of polyclonal chicken immune serum targeted against the cytoplasmic domain of human HB-EGF (1:800 dilution; gift from Drs. Rosalyn Adam and Michael Freeman, Children's Hospital, Boston, MA, USA). For the control sections, primary antibody was replaced by chicken preimmune IgG. Blood vascular ECs were localized from adjacent sections with a mouse monoclonal anti-CD34 (Becton Dickinson, Franklin Lakes, NJ, USA; 1:20 dilution) and SMCs with a mouse monoclonal anti-α-smooth muscle actin (α-SMA) (Sigma; 1:40000 dilution). Epitopes for these primary antibodies were visualized with immunoperoxidase technique using biotinylated secondary antibodies (biotinylated goat anti-chicken IgG from Vector Laboratories, Burlingame, CA, USA; biotinylated goat anti-mouse from DAKO, Carpinteria, CA, USA), Vectastain Elite ABC Kit (Vector Laboratories) or ChemMate Detection Kit (DAKO), and diaminobenzidine tetrahydrochloride (DAKO and Vector Laboratories) as a substrate.

RESULTS

ECs stimulate the migration of SMCs in vitro

To determine whether ECs and SMCs interact via paracrine signals in vitro, conditioned media were collected from EC or SMC cultures and the capacity of these media to stimulate the migration of SMCs or ECs was measured in Boyden chamber analyses. Two primary EC lines, human umbilical vein ECs (HUVEC) and bovine capillary ECs (BCE), as well as two primary SMC lines, human aortic SMCs (HASMC) and bovine aortic SMCs (BASMC), were analyzed. Conditioned medium from HUVECs stimulated the migration of HASMCs in a dose-responsive manner (**Fig. 1*A***). The maximal stimulatory effect obtained by HUVEC conditioned medium was ~60% of the maximal effect stimulated by FCS. On the contrary, conditioned medium from HASMCs did not stimulate the migration of HUVECs, although HUVECs responded to human serum by migration (Fig. 1*B*). Similar results were obtained using bovine cells. BCEs stimulated the migration of BASMC (Fig. 1*C*), but not vice versa (Fig. 1*D*), although both cell types were responsive to FCS (Fig. 1*C, D*). The maximal migratory effect obtained by BCE conditioned medium was stronger than that observed by stimulation with FCS (1.4-fold). These results suggest that both human and bovine ECs secrete soluble factors that stimulate the migration of vascular SMCs, but that SMCs do not produce factors that regulate the migration of ECs.

ECs express HB-EGF

To investigate the possibility that EGF-like ligands are EC-derived factors that stimulate the migration of SMCs, the expression of several EGF-like growth factors was analyzed by RT-PCR in HUVECs (**Fig. 2*A***). PCR products of expected sizes were detected only when

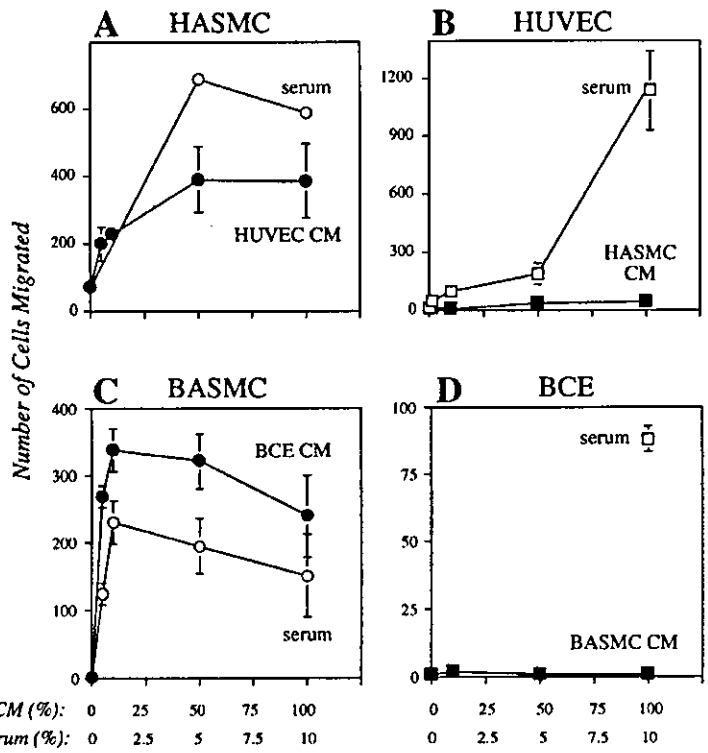

Figure 1. Regulation of SMC migration by soluble factors produced by ECs. Conditioned media collected from HUVEC (*A*), HASMC (*B*), BCE (*C*), or BASMC (*D*) cultures were analyzed at concentrations of 0–100% (v/v) for their potential to stimulate the migration of HASMCs (*A*), HUVECs (*B*), BASMCs (*C*), or BCEs (*D*). The analyses were carried out using Boyden chamber assays. Fetal calf serum (*A, C, D*) or human AB serum (*B*) at concentrations of 0–10% (v/v) were used as positive controls. All Boyden chamber assays were performed in triplicate for each concentration. The number of cells migrating through the membrane was counted under a microscope. CM, conditioned medium.

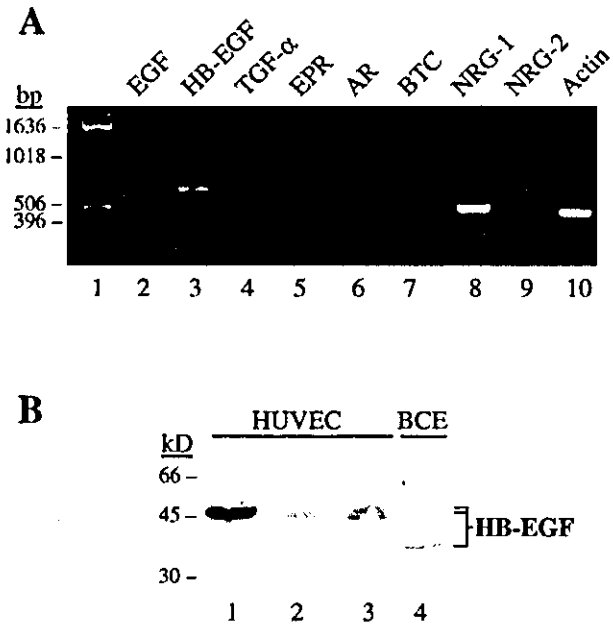

Figure 2. Expression of ErbB ligands in ECs. *A)* Total RNA was extracted from confluent HUVEC cultures (passage 3) and analyzed by RT-PCR using primers specific for EGF-like growth factors. The PCR products were separated on a 1.0% agarose gel. β-Actin-specific primers were used as a positive control (lane 10). A 1 kb DNA ladder was used as a DNA size marker (lane 1). bp, base pairs. *B)* HUVEC (lanes 1–3) and BCE (lane 4) lysates were analyzed by Western blot using three different anti-HB-EGF antibodies: a neutralizing antibody recognizing mature HB-EGF (R&D; lane 1), an antibody developed against the cytoplasmic tail of human precursor HB-EGF (C-18; Santa Cruz; lane 2), or an antibody developed against the cytoplasmic tail of mouse precursor HB-EGF (M-18; Santa Cruz; lanes 3 and 4).

HB-EGF or NRG-1 expression was analyzed (Fig. 2*A*, lanes 3 and 8, respectively). No specific products were observed for TGF-α, EPR, AR, BTC, or NRG-2 (Fig. 2, lanes 4–7 and 9). The ~400 bp band observed when EGF-specific primers were used (Fig. 2, lane 2) was a PCR artifact, as demonstrated by the unexpected size (expected size 511 bp) and sequencing of the PCR product (data not shown). RT-PCR analysis using β-actin specific primers served as a positive control for RNA quality and generated a single band of the expected size (Fig. 2*A*, lane 10). The expression of HB-EGF mRNA in HUVECs was confirmed by real-time quantitative RT-PCR. HUVECs expressed HB-EGF mRNA at levels corresponding to 36% of β-actin mRNA expression in the same sample. For comparison, HASMCs expressed HB-EGF mRNA at levels corresponding to 0.15% of β-actin mRNA expression.

The expression of HB-EGF protein in ECs was further examined by Western blot analysis of detergent-soluble EC fractions. Four different anti-HB-EGF antibodies recognized a duplet migrating at 46–48 kDa from HUVEC lysates (Fig. 2*B*). Two of the antibodies had been raised against different epitopes within the extracellular domain of human HB-EGF (Fig. 2B, lane 1; and data not shown), and two against cytoplasmic sequences corresponding to human (Fig. 2*B*, lane 2) or mouse (Fig. 2*B*, lane 3) precursor HB-EGF. The antibody against mouse HB-EGF also recognized a single 35 kDa HB-EGF band in BCE cells (Fig. 2*B*, lane 4). Mature HB-EGF was secreted to the conditioned medium of HUVECs, as demonstrated by ELISA using the two extracellular domain antibodies (data not shown). These results indicate that primary ECs express at least one ErbB-ligand, HB-EGF, at both the mRNA and protein level.

SMCs express ErbB1 and ErbB2

To determine whether vascular SMCs synthesize receptors for EGF-like ligands, the expression of the four known ErbB receptors in SMCs was analyzed by Western blot. HASMCs and BASMCs both synthesized proteins that were recognized by anti-ErbB1 or anti-ErbB2 antibodies and that migrated at the expected molecular mass for ErbB receptors, 170–180 kDa (**Fig. 3*A*, *B***, lanes 4 and 5). In contrast, no specific bands were recognized with anti-ErbB3 or anti-ErbB4 antibodies (Fig. 3*C*, *D*, lanes 4 and 5). Lysates from NIH 3T3 transfectants expressing either ErbB1, ErbB2, ErbB3, or ErbB4 (35) from T47D cells known to express all four ErbB receptors (36) and from 32D cells known to lack detectable ErbB protein expression (37) served as positive and negative controls (Fig. 3, lanes 1, 2, and 3, respectively). Consistent results were obtained when HASMC ErbB expression was quantitated using real-time RT-PCR: the ErbB mRNA expression relative to β-actin mRNA expression was 3.9%, 1.2%, 0.02%, and 0.05% for ErbB1, ErbB2, ErbB3, and ErbB4, respectively. These results demonstrate that vascular SMCs express receptors for EGF-like growth factors, i.e., ErbB1 and ErbB2, but not ErbB3 or ErbB4.

HB-EGF stimulates phosphorylation of ErbB1 and ErbB2 in SMCs

The binding of EGF-like growth factors to ErbB receptors leads to activation of the intrinsic receptor tyrosine kinases. To investigate the responsiveness of vascular SMCs to EGF-like ligands, the presence of tyrosine phosphorylated proteins in SMCs was analyzed after 10 min stimulation with 50 ng/mL of EGF-like ligands by Western blot using phosphotyrosine-specific antibodies (**Fig. 4*A***). EGF, HB-EGF, and BTC induced tyrosine phosphorylation of proteins in HASMCs and BASMCs (Fig. 4*A*, lanes 2–4 and 8–10). Consistent with these ligands activating ErbB receptors on the SMC surfaces, bands with the most prominent increase in phosphotyrosine content (Fig. 4*A*) were of the size (170–180 kDa) of ErbB receptors expressed in these cells (Fig. 3, lanes 4 and 5). In contrast, NRG-1 and NRG-2 did not stimulate tyrosine phosphorylation in SMCs (Fig. 4*A*, lanes 5, 6, and 11) over the basal level observed in unstimulated cells (Fig. 4*A*, lanes 1 and 7).

To assess ErbB phosphorylation more directly, HASMC lysates were analyzed by Western blot with

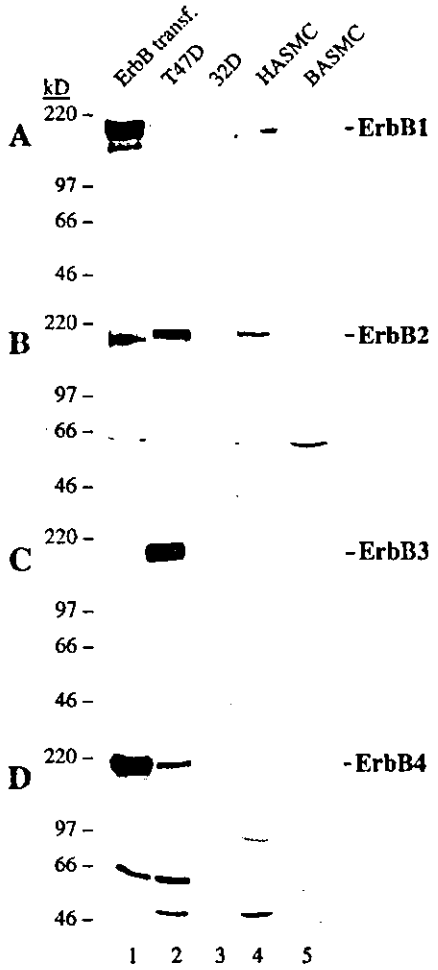

Figure 3. Western blot analysis of the ErbB receptors synthesized by SMCs. Detergent-soluble material was extrated from HASMCs (lane 4) or BASMCs (lane 5) and analyzed by Western blot using antibodies specific for ErbB1 (*A*), ErbB2 (*B*), ErbB3 (*C*), and ErbB4 (*D*). NIH 3T3 cells (lane 1) transfected with cDNAs encoding human ErbB1 (*A*), ErbB2 (*B*), ErbB3 (*C*), or ErbB4 (*D*) (35), as well as T47D cells (lane 2) that express all four ErbBs, were analyzed as positive controls. Myeloid 32D cells (lane 3) were analyzed as a negative control. The positions of the 170–180 kDa ErbB receptors are indicated.

phospho-specific anti-ErbB1 and anti-ErbB2 antibodies. As expected, HB-EGF induced phosphorylation of ErbB1 and ErbB2 (Fig. 4*B*, upper panels, lanes 2 and 5), whereas NRG-1 had no effect (Fig. 4*B*, upper panels, lanes 3 and 6). The effect of HB-EGF, but not of NRG-1, on ErbB1 was also observed in efficient down-regulation of ErbB1 protein in response to ligand stimulation (Fig. 4*B*, lower panels, lanes 2 and 3, respectively).

These data indicate that ligands capable of binding to ErbB1 (EGF, HB-EGF, and BTC) can activate receptors on SMCs, whereas ligands that need ErbB3 or ErbB4 for signaling (NRG-1, and NRG-2) cannot. No ligand is known to bind ErbB2 directly, and ErbB2 has been suggested to signal in heterodimeric complexes with other ErbBs, including ErbB1 (16). Thus, the observed specificity of the ligand responsiveness of SMCs (Fig. 4) was in accordance with the expression of ErbB1 and ErbB2 in SMCs (Fig. 3). Taken together, these findings suggest that EGF-like ligands such as HB-EGF that are capable of activating either ErbB1 homodimers or ErbB1/ErbB2 heterodimers can stimulate intracellular signaling in vascular SMCs.

HB-EGF, ErbB1 and ErbB2 are necessary for EC-stimulated migration of SMCs in vitro

Analysis of EGF-like ligands expressed by ECs (Fig. 2), the ErbB expression pattern in SMCs (Fig. 3), and the tyrosyl phosphorylation of SMCs in response to EGF-like ligands (Fig. 4) indicated that HB-EGF is the only ErbB ligand expressed by ECs that can mediate paracrine signaling from ECs to SMCs. To determine whether EC-derived HB-EGF is necessary for EC-stimu-

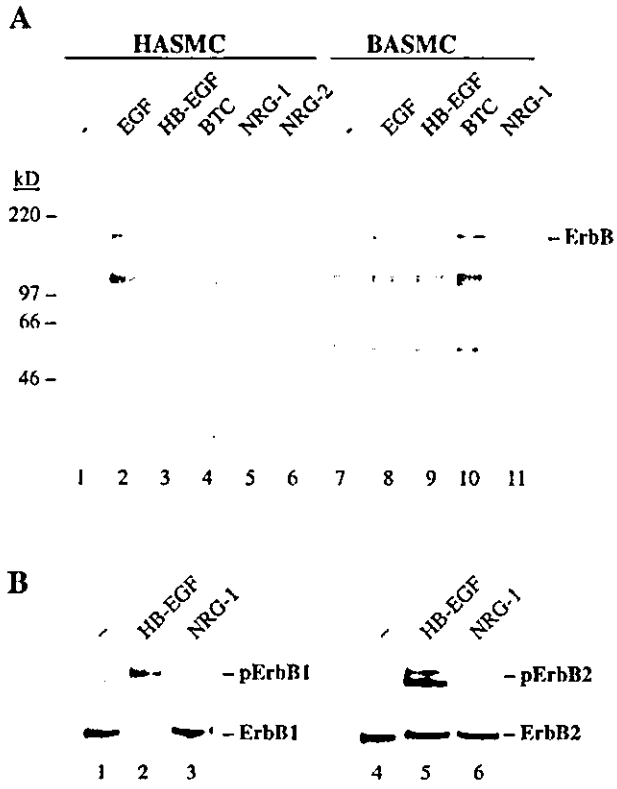

Figure 4. Tyrosine phosphorylation in SMCs in response to ErbB ligands. *A)* Confluent cultures of HASMCs (lanes 1–6) or BASMCs (lanes 7–11) were stimulated for 10 min at 37°C without (lanes 1 and 7) or with 50 ng/mL of recombinant EGF (lanes 2 and 8), HB-EGF (lanes 3 and 9), BTC (lanes 4 and 10), NRG-1 (lanes 5 and 11), or NRG-2 (lane 6). Cells were lysed and the lysates were analyzed by Western blot using an antiphosphotyrosine antibody. *B)* HASMCs were stimulated without (lanes 1 and 4) or with HB-EGF (lanes 2 and 5) or NRG-1 (lanes 3 and 6), as above, and analyzed by Western blot using antibodies specific for phospho-ErbB1 (upper panels, lanes 1–3) or phospho-ErbB2 (upper panels, lanes 4–6). After phospho-ErbB analysis, blots were reprobed with anti-ErbB1 (lower panels, lanes 1–3) or anti-ErbB2 (lower panels, lanes 4–6) antibodies to control loading. The position of the ErbB receptors is indicated.