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Abstract—A host of growth factors have been implicated in vascular pathologies; one such factor is heparin-binding epidermal growth factor–like growth factor (HB-EGF). Although HB-EGF has been shown to stimulate mitogenesis and chemotaxis of vascular smooth muscle cells (VSMC), its signaling mechanism remains undefined. In this study, we examined possible signal transduction pathways by which HB-EGF leads to mitogenesis in cultured rat VSMC. HB-EGF induced phosphorylation of the EGF receptor (EGFR) with maximum phosphorylation at 0.5 to 1 minute, whereas erbB4, the other receptor to which HB-EGF binds, was not activated on HB-EGF stimulation. HB-EGF induced a time- and concentration-dependent phosphorylation of mitogen-activated protein kinase (MAPK; p42/44 MAPK, extracellular signal-regulating kinase [ERK] 1/2). It also activated Akt and p70S6 kinase (p70S6K) but not p38 MAPK. HB-EGF–induced phosphorylation of these kinases was blocked by the EGFR kinase inhibitor AG1478. To investigate signaling molecules involved in HB-EGF–induced DNA synthesis, we pretreated VSMC with the specific ERK kinase mitogen-activated kinase (MEK) inhibitor PD98059 and the phosphatidylinositol 3-kinase inhibitor LY294002. These inhibitors significantly blocked HB-EGF–induced DNA synthesis. PD98059 inhibited HB-EGF–induced ERK activation, whereas it had no effect on Akt activation by HB-EGF. By contrast, LY294002 inhibited HB-EGF–induced Akt and p70S6K activation without effecting ERK activation by HB-EGF. These results demonstrate that HB-EGF–induced mitogenesis requires both ERK and phosphatidylinositol 3-kinase (Akt and p70S6K) pathways activated through EGFR, thereby providing a new mechanistic insight by which HB-EGF contributes to vascular remodeling. (Hypertension. 2002;39[part 2]:525-529.)

Key Words: atherosclerosis ■ epidermal growth factor ■ heparin ■ muscle, smooth, vascular ■ signal transduction

Growth and migration of vascular smooth muscle cells (VSMC) are vital factors in the pathogenesis of various cardiovascular diseases, such as atherosclerosis. In response to injury, medial VSMC proliferate and migrate to the intima of the blood vessel and form a lesion. Several mitogens have been implicated in this process, one of which is heparin-binding epidermal growth factor–like growth factor (HB-EGF). Studies have shown that HB-EGF is present in atherosclerotic plaques and that it is a potent mitogenic and chemotactic factor for VSMC. Moreover, macrophage, endothelial cells, and VSMC produce HB-EGF on stimulation, suggesting a critical role for HB-EGF in mediating vascular remodeling.

The biological actions of HB-EGF are mediated through members of the EGF receptor superfamily, EGF receptor (EGFR) also known as erbB1, and erbB4. On activation, these receptors undergo homo- or heterodimerization, followed by activation of intrinsic tyrosine kinase activity, leading to a myriad of signaling events. To this end, EGFR mediates a variety of cellular responses, such as cell proliferation, migration, and differentiation. In addition to numerous cancers, EGFR may be involved in the progression of vascular diseases. Few studies exist on the action of erbB4; however, it mediates proliferation, chemotaxis, and differentiation in some cell lines.

The mitogen-activated protein kinases (MAPKs) are a well-documented family of serine/threonine kinases that include extracellular signal-regulating kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase. The ERK cascade is the most characterized, and it mediates proliferative and chemotactic responses in various cells, including VSMC. p38 MAPK is usually associated with stress stimuli but has also been shown to lead to proliferation and migration of VSMC. Phosphatidylinositol 3-kinase (PI3K) is a serine/threonine kinase that phosphorylates phosphatidylinositol to produce PI 3,4P2 and PI 3,4,5P3, thereby activating several downstream kinases, such as Akt/protein kinase B and p70S6 kinase (p70S6K). Akt is involved in cell growth by...
eliciting cell survival/antiapoptotic effects.\textsuperscript{16,17} It has also been shown to have a role in the proliferation of VSMC.\textsuperscript{18} Activation of p70S6K contributes to cell growth by positively regulating mRNA translation and is thought to be a prerequisite for protein synthesis in various cell types, including VSMC.\textsuperscript{19–21}

EGFR stimulation by EGF results in ERK activation and growth of VSMC.\textsuperscript{22} In addition, EGFR and erbB4 are coupled to PI3K pathways in several cells.\textsuperscript{8,23} However, the signaling mechanism by which HB-EGF mediates growth of VSMC has yet to be defined. In the present study, we examined the signaling mechanisms required for HB-EGF–induced cell growth in VSMC. Here, we show that HB-EGF activates the ERK and PI3K pathways through the EGFR. We further show that these pathways are involved in HB-EGF–induced DNA synthesis in VSMC.

### Methods

#### Materials

HB-EGF was purchased from R & D Systems. PD98059 and LY294002 were purchased from Calbiochem. Antibody for Tyr\textsuperscript{1068}-phosphorylated EGFR was purchased from BioSource International. Antibodies for Tyr\textsuperscript{204}-phosphorylated ERK1/2, ERK2, p38 MAPK, EGFR, ErbB4 receptor, and p70S6K were purchased from Santa Cruz Biotechnology. Antibodies for Thr\textsuperscript{180}/Tyr\textsuperscript{182}-phosphorylated p38 MAPK, Ser\textsuperscript{411}-phosphorylated p70S6K, Ser\textsuperscript{473}-phosphorylated Akt, and Akt were purchased from Cell Signaling.

#### Cell Culture

The thoracic aorta from 12-week-old Sprague-Dawley rats were used to prepare VSMC by the explant method.\textsuperscript{24} For experiments, VSMC from passage 3 to 12 at approximately 90% confluence in culture were used after 3 days of serum depletion.

#### Immunoprecipitation

VSMC were stimulated with HB-EGF at 37°C. The cells were lysed with ice-cold immunoprecipitation buffer as previously described.\textsuperscript{25} The cell lysates were centrifuged at 14,000 g, and the supernatant was immunoprecipitated with the antibody and protein A/G-agarose at 4°C for 24 hours.

#### Immunoblotting

Cell lysate or immunoprecipitation lysate was subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane.\textsuperscript{24} The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the peroxidase-linked secondary antibody for 1 hour at room temperature, immunoreactive proteins were visualized by ECL reagent.\textsuperscript{24}

#### DNA synthesis

After pretreatment with or without inhibitors, VSMC grown on 12-well plates were incubated with HB-EGF and 1 μCi of \textsuperscript{3}H-thymidine for 24 hours. After washing with trichloroacetic acid, radioactivity was counted using a scintillation counter.\textsuperscript{26}

#### Statistical Analysis

Student’s \textit{t} test was used for the statistical analysis, and data are represented as mean±SEM, where \(n=3\).

### Results

HB-EGF can bind to 2 receptors, EGFR and erbB4.\textsuperscript{5} We determined whether these receptors were activated by HB-
EGF in cultured VSMC. As shown in Figure 1A, HB-EGF (100 ng/mL) stimulated phosphorylation of the EGFR at Tyr1068 in a time-dependent manner, with maximal phosphorylation occurring at 0.5 to 1 minute. The amount of EGFR was reduced by HB-EGF stimulation as detected by immunoblotting possibly as a result of its degradation after hyperphosphorylation and/or internalization. Also, erbB4 was not tyrosine-phosphorylated by HB-EGF in VSMC as detected by immunoprecipitation followed by immunoblotting with phosphotyrosine antibody (Figure 1B).

To determine whether members of the MAPK family, ERK and p38 MAPK, were stimulated by HB-EGF, we stimulated VSMC with HB-EGF for the indicated times and concentrations. Figure 2 shows that ERK1/2 phosphorylation was stimulated by HB-EGF in a time- (Figure 2A) and concentration-dependent (Figure 2B) manner, with maximal phosphorylation occurring at 5 to 10 minutes and 1 to 100 ng/mL HB-EGF, respectively. Although p38 MAPK is expressed in our cells, we were unable to detect its phosphorylation by HB-EGF using phospho-specific p38 MAPK antibody (Figure 2C).

To determine whether the PI3K pathway is involved in HB-EGF–induced mitogenesis, we first examined the downstream components of PI3K, Akt, and p70S6K. Figure 3A shows that Akt was stimulated in a time-dependent manner, with maximal phosphorylation occurring at 2 to 5 minutes. HB-EGF also induced phosphorylation of p70S6K with maximum phosphorylation at 10 minutes (Figure 3B).

To ensure that the ERK and PI3K pathways were actually involved in HB-EGF–induced mitogenesis, we used PD98059, the MEK inhibitor, or the PI3K inhibitor.
PI3K inhibitor, then stimulated with HB-EGF (HB) (100 ng/mL) for 24 hours. Data are presented as mean ± SEM, where n=3. *P<0.05 compared with control.

Figure 6. Effects of ERK and PI3K pathway inhibitors on HB-EGF–induced DNA synthesis. VSMC were pretreated with PD98059 (PD) or LY294002 (LY) for 24 hours. Data are presented as mean ± SEM, where n=3. *P<0.05 compared with control.

Discussion

In this study, we showed that HB-EGF activates ERK1/2, Akt, and p70S6K through the EGFR in VSMC. In addition, PD98059 and LY294002, the ERK and PI3K pathway inhibitors, respectively, blocked HB-EGF DNA synthesis. Previous studies have shown that HB-EGF can activate EGFR and erbB4. However, our data show that HB-EGF activates EGFR but not erbB4 in VSMC. We further show that an EGFR kinase inhibitor, AG1478, blocked downstream signaling of ERK, Akt, and p70S6K activated by HB-EGF, suggesting the involvement of the EGFR in HB-EGF–induced DNA synthesis.

ERK1/2 and p38 MAPK both have been shown to be positive regulators of cell growth in VSMC. As demonstrated in our present study, HB-EGF–induced activation of both Akt and p70S6K through the EGFR in VSMC. Also, HB-EGF–induced Akt and p70S6K phosphorylation was selectively inhibited by a PI3K inhibitor, LY294002, but not by PD98059, confirming the role of PI3K in mediating Akt and p70S6K activation by HB-EGF. Determining the specific involvement of the PI3K and ERK/MAPK pathways in HB-EGF–induced DNA synthesis may be difficult because both PD98059 and LY294002 inhibited control levels and HB-EGF–induced DNA synthesis. However, these data could be interpreted that both ERK and PI3K pathways may be involved in HB-EGF–induced DNA synthesis in addition to basal DNA synthesis. Recently, similar roles for both cascades were demonstrated in HB-EGF–dependent cell survival activated by tumor suppressor p53.

It should be noted that several distinct G-protein–coupled receptors “trans”–activate the EGFR through HB-EGF generation. Recently, we and others have shown that this mechanism is also involved in angiotensin II and thrombin-induced ERK activation in VSMC. Taken together, our findings will provide new mechanistic insights by which several risk factors induce vascular remodeling by activating HB-EGF/EGFR signaling pathways.

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References


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