The expression of contractile proteins in vascular smooth muscle cells is controlled by still poorly defined mechanisms. A thrombin-inducible expression of smooth muscle-specific α-actin and myosin heavy chain requires transactivation of the epidermal growth factor (EGF) receptor and a biphasic activation of ERK1/2. Here we demonstrate that the sustained second phase of ERK1/2 phosphorylation requires de novo RNA and protein synthesis. Depolymerization of the actin cytoskeleton by cytochalasin D or disruption of transit between the endoplasmic reticulum and the Golgi apparatus by brefeldin A prevented the second phase of ERK1/2 phosphorylation. We thus conclude that synthesis and trafficking of a plasma membrane-resident protein may be critical intermediates. Analysis of the expression of protease-activated receptor 1, heparin-binding EGF (HB-EGF), and the EGF receptor revealed that pro-HB-EGF is significantly up-regulated upon thrombin stimulation. The kinetic of HB-EGF expression closely matched that of the second phase of ERK1/2 phosphorylation. Because inhibition of matrix metalloproteases or of the EGF receptor strongly attenuated the late phase of ERK1/2 phosphorylation, the second phase of ERK1/2 activation is primarily relayed by shedding of EGF receptor ligands. The small interfering RNA-mediated knockdown of HB-EGF expression confirmed an important role of HB-EGF in triggering the second phase of ERK1/2 activation. Confocal imaging of a yellow fluorescent protein-tagged HB-EGF construct demonstrates the rapid plasma membrane integration of the newly synthesized protein. These data imply that the hormonal control of contractile protein expression relies on an intermediate HB-EGF expression to sustain the signaling strength within the Ras/Raf/MEK/ERK cascade.

The principal function of vascular smooth muscle (VSM) cells in a developed vascular system is the regulation of blood pressure and flow. In certain diseased states, however, VSM cells can undergo a phenotypic modulation toward a proliferative and secretory phenotype or by reverting toward the non-proliferative contractile phenotype (1). The transition from a differentiated phenotype to a fibroblast-like proliferative state is observed during the onset or progression of atherosclerosis (2–3), one of the most common diseases in developed countries (4–5). Under these conditions, the VSM cell phenotype is reminiscent of that observed during vascular development, where VSM cells play a key role in morphogenesis of the blood vessel and exhibit a high proliferative index, migrate, and produce extracellular matrix components (6). Conditions that promote the proliferative phenotype include the combined action of growth factors, proteolytic enzymes, and exposure to extracellular matrix proteins (7).

The reciprocal process has been observed upon completion of wound healing or during formation and organization of a fibrous cap. Under these conditions, VSM cells are exposed to various stimuli, including macrophage- and lymphocyte-derived cytokines and serum components that are currently being discussed as critical regulators of plaque stability.

On the molecular level, phenotypic modulation of VSM cells depends on the activation of mitogen-activated protein (MAP) kinases (8). The epidermal growth factor (EGF) receptor is transactivated after G protein-coupled receptor (GPCR) activation and recruits the guanine nucleotide exchange factor (Sos) through adaptor proteins, Shc and Grb2, thereby initiating the canonical Ras/Raf/MEK/ERK cascade (9–10). Activated MAP kinases of the extracellular signal-regulated kinase (ERK1/2) family in turn translocate to the nucleus and phosphorylate nuclear transcription factors or transcriptional coactivators (11).

Within the family of EGF receptor ligands, heparin-binding EGF (HB-EGF) has been implicated in vascular remodeling because it is a potent mitogen, acts as a chemotactic factor for VSM cells, and is abundantly expressed in vascular lesions such as atherosclerosis (12). Upon membrane insertion and signal peptide cleavage, pro-HB-EGF is synthesized as a type 1 transmembrane protein that is trafficked to the plasma membrane and proteolytically shed by matrix metalloprotease to release the biologically active soluble HB-EGF (13–14). In addition,
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shedding of pro-HB-EGF also provides an important molecular link between GPCR activation and EGF receptor transactivation (15).

The Ras/Raf/MEK/ERK cascade is involved in the control of both cell proliferation and differentiation (16), and different kinetic patterns have been shown to control the outcome (17). In thrombin-stimulated VSM cells, the protease-activated receptor 1 (PAR1) induces a biphasic and long-lasting activation of ERK1/2 and, subsequently, promotes the expression of contractile proteins and differentiation markers such as smooth muscle-specific α-actin and smooth muscle-specific myosin heavy chain (18). Although an EGF receptor transactivation and subsequent signal propagation through the Ras/Raf signaling module are required for the second and long lived phase of ERK1/2 phosphorylation in thrombin-stimulated VSM cells (19), the molecular basis of this differentiation-promoting signal cessation and reappearance has been unknown. In this study, we tested the hypothesis that an intermediate gene expression may underlie the delayed appearance of a second wave of ERK1/2 activation, and provide evidence for a functionally relevant de novo expression of HB-EGF that contributes to the second phase of ERK1/2 activation and subsequent SM-α-actin expression in newborn rat aortic VSM cells.

EXPERIMENTAL PROCEDURES

Reagents—The modulators brefeldin A, cytochalasin D, heparin, cycloheximide, and AG1478 were obtained from Sigma. Thrombin and GM6001 were from Calbiochem and Biomol, respectively. The thrombin receptor agonist peptide was from Cell Signaling. The HB-EGF receptor were purchased from Cell Signaling Technology. AllStars negative control siRNA and a rat HB-EGF-specific siRNA (sense 5′-r(UAGCUCAUCAAAUCC-1000). Intensities were integrated, corrected for background and antisense 5′-r(CGCUGGAUUUGAU-1000) Intensities were integrated, corrected for background

Electrophoresis and Immunoblotting—Cells were lysed into a buffer containing 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 10 μg/ml aprotinin. The lysates were passed six times through a 26-gauge needle and centrifuged (12,000 M phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 μg/ml aprotinin. The lysates were passed six times through a 26-gauge needle and centrifuged (12,000 × g) for 20 min at 4 °C. The pellet was resuspended in Laemmli buffer.

Confocal Microscopy—VSM cells were nucleofected with 7 μg of an expression plasmid encoding HB-EGF-YFP. One hour later, the cells were treated with 7 μM brefeldin A or 2 μM cytochalasin D, incubated for another 4 h, and visualized with an inverted confocal laser scanning microscope (LSM 510 Meta; Carl Zeiss) using an α-Plan-Fluar 100×/1.45 objective. YFP was excited at 488 nm and detected through a 505-nm long pass filter. All experiments were performed at room temperature in HBS buffer (10 mM HEPES, 128 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5.5 mM glucose, and 0.2% bovine serum albumin, pH 7.4).

Cloning of HB-EGF cDNA—Total RNA of VSM cells was reversely transcribed using oligo(dT) primers and Superscript

base, 0.2 M glycine, and 20% ethanol. Blots were probed with respective primary and horseradish peroxidase-conjugated secondary antibodies. For densitometry, signal intensity was detected by a “cooled CCD camera.” Integrated band intensities were background-corrected and evaluated in a TINA 2.09 software (Raytest). Statistical significance was calculated using a Student’s unpaired t test (comparison of band intensities with and without modulators) or a paired t test (comparison of stimulated samples to basal signal intensities). Significance was accepted at p < 0.05 (*) or p < 0.01 (**).

RNA Extraction and Multiplex Reverse Transcription-PCR—Total RNA was extracted using the TRizol reagent (Invitrogen) and reversely transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and a (dT)₁₈ primer. The PCR primers were selected based on published gene sequences for Rattus norvegicus HB-EGF (GenBank accession number NM_012945), PAR1 (GenBank accession number NM_012950), and GAPDH (GenBank accession number NM_017008). A 605-nucleotide (nt) fragment of the HB-EGF transcript was amplified using a sense 5′-GCT GTC GGT GGT GCT GAA-3′ and antisense 5′-GCG ATG CCC AAC TCT TTC TTT T-3′ primer pair. A 354-nt PAR1 fragment was amplified using a sense 5′-ATG AGA CAG CCA GAA-3′ and antisense 5′-GTT GCC ACC CGC AGG TAC T-3′ primer pair. A 439-nt EGF receptor fragment was amplified using a sense 5′-GGG AGC TGC CGT GTC AAA GA-3′ and antisense 5′-GCT GCC ACC CCAG AGG TAC T-3′ primer pair. A 454-nucleotide (nt) fragment of GAPDH was coamplified as an internal control, using a sense 5′-TTA GCC CCC CTG GCC AAG G-3′ and antisense 5′-CTT ACT CCT TGG AGG CCA TG-3′ primer pair. The PCR amplicons were analyzed and stained on 1.5% agarose gel containing 0.002% ethidium bromide, and the quantitative analysis of signals was performed with a fluorescence imaging system (Fujifilm LAS-1000). Intensities were integrated, corrected for background signals, and evaluated with TINA 2.09 software (Raytest).

Preparation of Membrane Fractions—VSM cells were washed with ice-cold phosphate-buffered saline buffer and lysed into a hypotonic lysis buffer containing 50 mM HEPES, 1 mM EDTA, 200 μM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 μg/ml aprotinin. The lysates were passed six times through a 26-gauge needle and centrifuged (12,000 × g) for 20 min at 4 °C. The pellet was resuspended in Laemmli buffer.
II reverse transcriptase (Invitrogen). The coding sequence of HB-EGF was amplified by PCR (Expand HF; Roche Applied Science) using appropriate primers (forward, 5'-GCCACCATGAAGCTGCTGCCG; reverse, 5'-TCTAGACAGTGGGAGCTAGCCAT) and subcloned into pcDNA3.1/V5-HIS-TOPO (Invitrogen). An expression plasmid encoding HB-EGF C-terminally tagged with yellow fluorescent protein (YFP) was generated by in-frame ligation of the HindIII/XbaI-digested HB-EGF open reading frame into the custom-made vector pcDNA3-YFP.

RESULTS

Second Phase Signaling through the Ras/Raf/MEK/ERK Pathway in Thrombin-stimulated VSM Cells Requires de Novo RNA and Protein Synthesis—Previous studies have shown that the thrombin-induced contractile protein expression in VSM cells requires a biphasic activation of members of the Ras/Raf/MEK/ERK signaling cascade (21). To analyze whether both phases of activation depend on the phosphorylation of preexisting signaling molecules or whether de novo mRNA transcription and synthesis of proteins may be required, VSM cells were pretreated with actinomycin D (4 μM) to block RNA transcription or with the corresponding solvent (control). Cells were then stimulated with thrombin (2 units/ml) for up to 180 min and lysed. Proteins were separated by 10% SDS-PAGE, electroblotted, and probed with anti-phospho-MEK or ERK1/2 antibodies. Similar results were obtained when VSM cells were pretreated with cycloheximide (10 μM, for 30 min), an inhibitor of protein translation. Shown are blots on which samples with and without the respective inhibitor were processed in parallel.

Delayed ERK1/2 Phosphorylation Is Linked to Vesicular Transport—To delineate candidate proteins that are important for the thrombin-induced second phase of ERK1/2 phosphorylation, we tested whether cytoskeletal components and trafficking processes are involved. To this end, the actin polymerization was blocked with cytochalasin D (2 μM). Again, the initial thrombin-induced ERK1/2 phosphorylation was unaffected by this pretreatment, whereas the second phase of ERK1/2 phosphorylation was almost completely abrogated (Fig. 2A). Because actin depolymerization may also affect the constitutive or agonist-triggered release of preformed secretory vesicles, possibly containing active matrix metalloproteinases that are important in the transactivation of the EGF receptor, we applied a more selective disruption of protein transport applying brefeldin A. Brefeldin A is an inhibitor of Sec7-mediated biosynthesis (see Fig. 1B). We conclude that constitutive or thrombin-induced gene expression and protein synthesis are necessary for triggering a second, long lived phosphorylation of ERK1/2, which is associated with a subsequent expression of contractile proteins.

FIGURE 1. Requirement of de novo transcription and protein synthesis for the second phase of ERK1/2 activation by thrombin. A, primary cultures of newborn rat aortic smooth muscle (VSM) cells were kept in serum-free medium for 36 h and treated with actinomycin D (4 μM) to block RNA transcription or with the corresponding solvent (control). Cells were then stimulated with thrombin (2 units/ml) for up to 180 min and lysed. Proteins were separated by 10% SDS-PAGE, electroblotted, and probed with anti-phospho-MEK or ERK1/2 antibodies. B, experiment as shown in A but with VSM cells pretreated with cycloheximide (10 μM), an inhibitor of protein translation. Shown are blots on which samples with and without the respective inhibitor were processed in parallel.

FIGURE 2. Effects of actin depolymerization and of disruption of vesicular transport on the second phase of ERK1/2 activation in VSM cells. A and B, cells were incubated for 36 h in serum-free medium, pretreated with 2 μM cytochalasin D (A) or with 7 μM brefeldin A (B) for 30 min, and then stimulated with 2 units/ml thrombin. Whole-cell lysates were separated on a 10% SDS-PAGE, electroblotted, and probed with anti-phospho-ERK1/2 and anti-ERK1/2. C, quantitative analysis of signals was performed with a CCD camera-based bioluminescence imaging system. Data are means and S.E. from five samples collected in three independent experiments. Statistical significance was accepted at p < 0.05 (*) or p < 0.01 (**).
guanine nucleotide exchange of Arf GTPases and thereby blocks the assembly and loading of cargo to vesicles in the exo-cytic endoplasmic reticulum-to-Golgi transport, but also a subset of Arf-dependent internalization processes. In VSM cells treated with brefeldin A (7 μM added 30 min prior to thrombin stimulation), the second phase of ERK1/2 phosphorylation, but not the early ERK1/2 phosphorylation, was suppressed, indicating that either the synthesis and trafficking of a plasma membrane-resident or secretory protein or other Sec7/Arf-dependent pathways are a critical intermediate (Fig. 2, B and C).

Expression of HB-EGF Is Up-regulated in Thrombin-stimulated VSM Cells—Candidate genes that are expressed upon thrombin stimulation in VSM cells and that are required for an EGF receptor transactivation and second phase of ERK1/2 phosphorylation include the receptor proteins PAR1 and EGF receptor as well as pro-HB-EGF. These proteins are cleaved and/or internalized and thus may require a constitutive or thrombin-induced replenishment. The gene expression of PAR1, of HB-EGF, and of EGF receptor was tested semi-quantitatively by a multiplex PCR applying nonsaturating numbers of amplification cycles and coamplification of the constitutively expressed GAPDH gene transcript as an internal control. Quantification of fluorescence intensities and normalization to the expression of GAPDH revealed that the transcription of HB-EGF was clearly induced upon thrombin stimulation in primary VSM cells (Fig. 3, A and B). The time course of HB-EGF expression showed a first significant increase in HB-EGF expression 30 min after thrombin stimulation and remained elevated for up to 3 h. Because the expression of the c-jun and c-fos immediate early genes was already induced 5–10 min after thrombin stimulation of VSM cells (data not shown), expression of HB-EGF is not part of the first wave of gene expression events. The expression of PAR1 did not change after thrombin stimulation, and expression of the EGF receptor was only slightly increased (statistically not significant). Western blot analysis of HB-EGF and EGF receptor expression in whole-cell lysates of thrombin-stimulated VSM cells confirmed these findings at the protein level (Fig. 3C).

In the context of a preliminary genome-wide analysis of thrombin-regulated genes (Affymetrix rat genome 230 2.0 chip), we could confirm an approximate 4-fold increase in HB-EGF expression 120 min after agonist application compared with unstimulated VSM cells or to VSM cells that were exposed to thrombin for only 10 min (n = 2 each). Likewise, stimulation of VSM cells with a PAR1-selective activating peptide (TRAP, 25 μM) resulted in a 3.5-fold increase in HB-EGF expression, which was not sensitive to a pretreatment with pertussis toxin, indicating a critical role of the PAR1 subtype of protease-activated receptors and downstream signaling via pertussis toxin-insensitive heterotrimeric G proteins. Thus, we conclude that a PAR1-dependent HB-EGF up-regulation can be seen on both transcriptional and protein levels.

Trafficking and Subcellular Localization of HB-EGF—To directly visualize the effects of cytochalasin D and brefeldin A on the subcellular localization of HB-EGF in living cells, VSM cells were nucleofected with a cDNA plasmid encoding a C-terminally YFP-tagged HB-EGF construct, seeded on poly-l-lysine-coated glass coverslips and treated with the respective blockers 1 h upon nucleofection. The localization of the fluorescent HB-EGF fusion protein was analyzed by confocal laser scanning microscopy. In untreated VSM cells, the HB-EGF-YFP protein was efficiently trafficked to the plasma membrane as early as 5 h after nucleofection (Fig. 4A), which was the earliest time point at which the fluorescent protein was reliably detectable (probably because of the need for an autooxidation-dependent maturation of the YFP fluorochrome). In VSM cells that were treated with cytochalasin D (2 μM), the HB-EGF protein remained in intracellular vesicular structures (Fig. 4B). Inhibition of the secretory pathway by brefeldin A (7 μM) resulted in a localization of HB-EGF-YFP in a reticular compartment, including the nuclear membrane, presumably the endoplasmic reticulum (Fig. 4C). Experiments in transiently transfected HEK293 fibroblasts essentially confirmed these results but demonstrated a less efficient integration of HB-EGF into the plasma...
Activation by TRAP Stimulation in VSM Cells

EGF Receptor Transactivation Lead to the Delayed ERK1/2 phosphorylation. To test whether the PAR1-inhibition of the EGF receptor, resulting in the activation of the canonical Ras/Raf/MEK/ERK pathway. To assess the role of HB-EGF in the ERK1/2 activation by an independent approach, VSM cells were nucleofected with siRNA directed against the 3-prime noncoding region of the rat HB-EGF transcript or with a nonsilencing control siRNA. After 2.5 days, the expression of HB-EGF was almost completely abolished by the HB-EGF-specific siRNA compared with the control siRNA (Fig. 6C). The expression of amphiregulin, another member of the EGF family that is endogenously expressed in VSM cells, remained constant after nucleofection of the HB-EGF or control siRNA. Assessing the ERK1/2 phosphorylation under the same conditions, we found a strong reduction in the thrombin-induced ERK1/2 phosphorylation upon HB-EGF knockdown but not when the nonsilencing control siRNA was nucleofected (Fig. 6D).
Gene Expression in Biphasic ERK1/2 Activation

A sustained or biphasic activation pattern of ERK1/2 MAPKs is linked to cellular differentiation in several cell lines (cell biological settings), including growth factor binding to tyrosine kinase receptors such as the nerve growth factor-induced differentiation in PC-12 cells (17). Similarly, the principal mechanisms by which stimulation of a G protein-coupled receptor triggers a biphasic feed into the Ras/Raf/MEK/ERK cascade are only poorly understood. In thrombin-stimulated VSM cells, the biochemical processes that triggered the early and the delayed ERK1/2 phosphorylation seemed to be almost identical, presumably the triple membrane-spanning pathway, which has been worked out by Prenzel et al. (15). In VSM cells, both waves of thrombin- or TRAP-induced ERK1/2 phosphorylation required an MMP-mediated shedding of heparin-sensitive EGF receptor ligands, EGF receptor autophosphorylation, and MEK1 activity. Thus, without an additional cellular plasticity, it seemed difficult to explain how the ERK1/2 activation vanishes and re-appears after about 1 h of delay. Possible mechanisms for the termination and reappearance of a signaling cascade on the time scale observed may include feedback loops that employ protein inactivation or degradation, trafficking of preformed proteins to the plasma membrane, or transcriptional regulation processes. Because transcriptional activity and protein translation were required for triggering the second phase of thrombin-induced ERK1/2 phosphorylation (see Fig. 1), we concluded that de novo protein expression is a key intermediate of processes, which subsequently lead to a second and long lived ERK1/2 phosphorylation.

Because PAR1 is coupled to the \(G_{q}, G_{i},\) and \(G_{12/13}\) families of heterotrimeric G proteins (22), activation of multiple signal transduction pathways may drive numerous gene expression programs in VSM cells. Of these, immediate early genes that are activated in response to GPCR stimulation in VSM cells such as \(c-jun, c-fos, myb,\) or early growth response genes constitute the first wave of gene expression (23). The gene products encoded by these ubiquitous genes have no direct effect on the GPCR-induced EGF receptor transactivation or the subsequent signaling through the Ras/Raf/MEK/ERK pathway (24). Because in turn, both the onset and the peak of HB-EGF expression in thrombin-stimulated VSM cells are delayed compared with immediate early genes (23), HB-EGF expression may be either regulated through gene products of immediate early genes or by independent mechanisms. \(c-jun\) and \(c-Fos\) are part of the AP-1 transcription factor complex (25), which has been implicated in the mechanically activated HB-EGF expression in bladder smooth muscle cells (26). Likewise, HB-EGF up-regulation in response to hyperosmotic stress or following chemotherapy involve the AP-1 complex (27–28). These data imply that HB-EGF is part of the second wave of gene expression and may be regulated by transcriptional activity of the AP-1 complex. One should also mention that, besides transcriptional regulation, the abundance of HB-EGF mRNA may be controlled by mRNA stabilization (29). Other AP-1-independent mechanisms that regulate HB-EGF expression may include ETS (E26 transformation-specific sequence) transcription factors that bind to an overlapping AP-1/ETS site on the HB-EGF promoter (30) and are induced in VSM cells in response to various stimuli (31–38).

The appearance of a second phase of PAR1-induced ERK1/2 phosphorylation was blocked by brefeldin A, an inhibitor of Sec7/Arf-dependent vesicular transport. Because the second...

**FIGURE 5.** The ERK activation by TRAP stimulation in VSM cells requires MMP activity and EGF receptor activation. Serum-starved cells were stimulated with 25 \(\mu\)M TRAP in the absence or the presence of 10 \(\mu\)M GM6001 (A) or 250 \(nM\) AG1478 (B) (both inhibitors added either 30 min prior to or 30 min after TRAP stimulation). Aliquots of whole-cell lysates were subjected to 10% SDS-PAGE, electroblotted, and probed with anti-phospho-ERK1/2 or anti-ERK1/2 antibodies as indicated. Shown are representative examples of three independent experiments.

**DISCUSSION**

Previous studies revealed that upon PAR1 receptor activation, vascular smooth muscle cells undergo a phenotypic modulation toward a more differentiated phenotype. However, precise signaling pathways are still incomplete.

Here we demonstrate that, upon thrombin stimulation, de novo expression of HB-EGF promotes the appearance of a long lasting second phase of ERK1/2 phosphorylation in VSM cells. An up-regulation of the HB-EGF expression can be seen on both the RNA and the protein levels, reaching a maximum at about 1–2 h following thrombin stimulation. A functional implication of HB-EGF in the sustained ERK1/2 signaling is confirmed by pharmacological and RNA interference-based approaches.

A sustained or biphasic activation pattern of ERK1/2 MAPKs is linked to cellular differentiation in several cell lines (cell biology, biochemical settings), including growth factor binding to tyrosine kinase receptors such as the nerve growth factor-induced differentiation in PC-12 cells (17). Similarly, the principal mechanisms by which stimulation of a G protein-coupled receptor triggers a biphasic feed into the Ras/Raf/MEK/ERK cascade are only poorly understood. In thrombin-stimulated VSM cells, the biochemical processes that triggered the early and the delayed ERK1/2 phosphorylation seemed to be almost identical, presumably the triple membrane-spanning pathway, which has been worked out by Prenzel et al. (15). In VSM cells, both waves of thrombin- or TRAP-induced ERK1/2 phosphorylation required an MMP-mediated shedding of heparin-sensitive EGF receptor ligands, EGF receptor autophosphorylation, and MEK1 activity. Thus, without an additional cellular plasticity, it seemed difficult to explain how the ERK1/2 activation vanishes and re-appears after about 1 h of delay. Possible mechanisms for the termination and reappearance of a signaling cascade on the time scale observed may include feedback loops that employ protein inactivation or degradation, trafficking of preformed proteins to the plasma membrane, or transcriptional regulation processes. Because transcriptional activity and protein translation were required for triggering the second phase of thrombin-induced ERK1/2 phosphorylation (see Fig. 1), we concluded that de novo protein expression is a key intermediate of processes, which subsequently lead to a second and long lived ERK1/2 phosphorylation.

Because PAR1 is coupled to the \(G_{q}, G_{i},\) and \(G_{12/13}\) families of heterotrimeric G proteins (22), activation of multiple signal transduction pathways may drive numerous gene expression programs in VSM cells. Of these, immediate early genes that are activated in response to GPCR stimulation in VSM cells such as \(c-jun, c-fos, myb,\) or early growth response genes constitute the first wave of gene expression (23). The gene products encoded by these ubiquitous genes have no direct effect on the GPCR-induced EGF receptor transactivation or the subsequent signaling through the Ras/Raf/MEK/ERK pathway (24). Because in turn, both the onset and the peak of HB-EGF expression in thrombin-stimulated VSM cells are delayed compared with immediate early genes (23), HB-EGF expression may be either regulated through gene products of immediate early genes or by independent mechanisms. \(c-jun\) and \(c-Fos\) are part of the AP-1 transcription factor complex (25), which has been implicated in the mechanically activated HB-EGF expression in bladder smooth muscle cells (26). Likewise, HB-EGF up-regulation in response to hyperosmotic stress or following chemotherapy involve the AP-1 complex (27–28). These data imply that HB-EGF is part of the second wave of gene expression and may be regulated by transcriptional activity of the AP-1 complex. One should also mention that, besides transcriptional regulation, the abundance of HB-EGF mRNA may be controlled by mRNA stabilization (29). Other AP-1-independent mechanisms that regulate HB-EGF expression may include ETS (E26 transformation-specific sequence) transcription factors that bind to an overlapping AP-1/ETS site on the HB-EGF promoter (30) and are induced in VSM cells in response to various stimuli (31–38).

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The sustained feed into the canonical Ras/Raf/MEK/ERK pathway in newborn rat aortic VSM cells is associated with an increased expression of contractile proteins such as the smooth muscle-specific α-actin and myosin heavy chain (19) and thereby contributes to the cellular plasticity. It is tempting to speculate that the regulated expression and rapid processing of distinct membrane-bound proforms of receptor tyrosine kinases may contribute to both facets of the phenotypic modulation of VSM cells. This idea is supported by the finding that coinubation of human VSM cells with pro-atherogenic supernatants of activated macrophages induces the expression of basic fibroblast growth factor (40). The basic fibroblast growth factor-dependent FGFR-1 activation has been described to enhance the long lived ERK phosphorylation and the mitogenic effect of platelet-derived growth factor-BB stimulation in human arterial VSM cells. Taken together, the induced expression of growth factor receptor ligands is likely to modulate the long term outcome of a specific input signal by providing a sustained signaling strength via their cognate receptors. Thus, the EGF receptor transactivation and ERK1/2 phosphorylation are not only modulated by early gene expression but the rapid intracellular processing and plasma membrane targeting of growth factor receptor ligands may constitute a highly dynamic network, which contributes to the plasticity of VSM cells.

Phase of ERK1/2 phosphorylation starts at about 1 h after stimulation, the maturation and intracellular trafficking of this potential protein must thus proceed rather quickly. To investigate whether HB-EGF meets this requirement, we generated a YFP-tagged autofluorescent fusion construct and investigated the subcellular localization of HB-EGF in living VSM cells. An almost complete plasma membrane localization of the fusion protein was evident as early as 5 h after nucleofection, which contributed to its signaling properties (39). In line with the assumption that transactivation of the EGF receptor critically depends on the expression and surface localization of HB-EGF, we find that the membrane-localized HB-EGF pool is rapidly depleted upon thrombin stimulation.

In brefeldin A-treated cells, this depletion was further enhanced, and the subsequent replenishment was lacking, indicating that the induction of HB-EGF expression depends on an intact signaling via Sec7/Arf. Further work would be required to firmly establish whether the brefeldin-sensitivity is because of a block of the rapid forward trafficking of HB-EGF.

Previous studies suggest that release of HB-EGF from the plasma membrane, nuclear import, and subsequent signaling through the cytosolic C terminus of cleaved HB-EGF may contribute to its signaling properties (39). In line with the assumption that transactivation of the EGF receptor critically depends on the expression and surface localization of HB-EGF, we find that the membrane-localized HB-EGF pool is rapidly depleted upon thrombin stimulation. In brefeldin A-treated cells, this depletion was further enhanced, and the subsequent replenishment was lacking, indicating that the induction of HB-EGF expression depends on an intact signaling via Sec7/Arf. Further work would be required to firmly establish whether the brefeldin-sensitivity is because of a block of the rapid forward trafficking of HB-EGF.

From the data presented, it appears that the autooxidative maturation of the fluorescent protein allowed a gate whether HB-EGF meets this requirement, we generated a potential protein must thus proceed rather quickly. To investigate whether HB-EGF meets this requirement, we generated a YFP-tagged autofluorescent fusion construct and investigated the subcellular localization of HB-EGF in living VSM cells. An almost complete plasma membrane localization of the fusion protein was evident as early as 5 h after nucleofection, which contributed to its signaling properties (39). In line with the assumption that transactivation of the EGF receptor critically depends on the expression and surface localization of HB-EGF, we find that the membrane-localized HB-EGF pool is rapidly depleted upon thrombin stimulation.

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