Consequences of Epidermal Growth Factor Receptor (ErbB1) Loss for Vascular Smooth Muscle Cells From Mice With Targeted Deletion of ErbB1

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Objective—Pathophysiological effects of the epidermal growth factor receptor (EGFR or ErbB1) include vascular remodeling. EGFR transactivation is proposed to contribute significantly to heterologous signaling and remodeling in vascular smooth muscle cells (VSMC).

Methods and Results—We investigated the importance of EGFR in primary VSMC from aorta of mice with targeted deletion of the EGFR (EGFRΔVSMC→VSMCEGFR−/− and EGFRVSMC→VSMCEGFR+/+) and the respective littermate controls (EGFR+/+ VSMC→VSMCEGFR+/+) with respect to survival, pentose phosphate pathway activity, matrix homeostasis, extracellular signal–regulated kinase 1/2 (ERK1/2) phosphorylation, and Ca2+ homeostasis. In VSMCEGFR−/−, epidermal growth factor–induced signaling was abolished; VSMCEGFR+/− showed an intermediate phenotype. EGFR deletion enhanced spontaneous cell death, reduced pentose phosphate pathway activity, disturbed cellular matrix homeostasis (collagen III and fibronectin), and abolished epidermal growth factor sensitivity. In VSMCEGFR−/−, endothelin-1- or α1-adrenoceptor-induced ERK1/2 phosphorylation and the fraction of Ca2+ responders were significantly reduced, whereas responsive cells showed a significantly stronger Ca2+ signal. Oxidative stress (H2O2) induced ERK1/2 activation in VSMCEGFR+/+ and VSMCEGFR−/− but not in VSMCEGFR+/−. The Ca2+ signal was enhanced in VSMCEGFR−/−, similar to purinergic stimulation by ATP.

Conclusion—in conclusion, EGFR was found to be important for basal VSMC homeostasis and ERK1/2 activation with respect to the fraction of responders and magnitude of the signal. Thus, EGFR seems to be Janus-faced for VSMC biology. (Arterioscler Thromb Vasc Biol. 2011;31:1643-1652.)

Key Words: growth factors ■ receptors ■ signal transduction ■ vascular biology ■ vascular muscle

The epidermal growth factor receptor (EGFR) family consists of 4 related tyrosine kinase receptors: EGFR (ErbB1), HER2 (ErbB2, with no ligand described), HER3 (ErbB3, without kinase function), and HER4 (ErbB4).1,2 There are more than 10 ligands for these receptors with different specificities.3 On ligand binding, the receptors undergo dimerization, leading to Tyr phosphorylation of the cytosolic domain and subsequent activation of various signaling pathways, including mitogen-activated protein kinases (extracellular signal–regulated kinase 1/2 [ERK1/2], p38, c-Jun N-terminal kinase), protein kinase B, c-Src kinase, and phospholipase Cγ, which affect extra- and intracellular events.4 Activation includes formation of homo- and heterodimers, with the EGFR possessing the ability to interact with all 3 family members. Activated ErbB receptors regulate various aspects of cell fate, such as proliferation, survival, differentiation, migration, and matrix homeostasis.5

EGFR (ErbB1) dimers can be activated by epidermal growth factor (EGF), transforming growth factor-α, amphi- regulin, heparin-binding (HB)-EGF, β-cellulin, and epiregu- lin. ErbB1/ErbB4-dimers can be activated by neuregulins.6 The ligands may derive from neighboring cells (paracrine) or from the same cell (autocrine), as in the case of shedding of membrane-bound pro-HB-EGF.1 As pro-HB-EGF shedding can also be induced by activation of other receptors (eg, G-protein–coupled receptors) or radical stress, Ca2+ signaling was modulated by EGFR differentially with respect to the fraction of responders and magnitude of the signal. Thus, EGFR seems to be Janus-faced for VSMC biology. (Arterioscler Thromb Vasc Biol. 2011;31:1643-1652.)
a variety of hormones also in vascular smooth muscle cells (VSMC).6,9
Pathophysiological effects of EGFR include cell transformation and tumorigenesis, as well as parainflammatory dysregulation of tissue homeostasis, leading, for example, to vascular dysfunction and fibrosis.6 There are several reports suggesting that EGFR transactivation is responsible for G-protein–coupled receptor–mediated ERK1/2 phosphorylation (eg, induced by angiotensin II [ang II],10,11 α₁ and β-adrenergic agonists,12,13 thrombin,14 endothelin-1 [ET-1],15–17 and purinergic receptor ligands18) and the resulting pathophysiological effects on VSMC and vascular function, including atherosclerosis.6 However, other hormones also, such as steroids19 or prostaglandins20 as well as reactive oxygen species21 and cardiotonic steroids,22 (mis)use the EGFR as signal transducer.
EGFR supports fibrosis in cardiovascular and renal tissue.15,23,24 The importance of EGFR in mediating pathophysiological effects of heterologous signaling systems is supported by its role in endothelin-induced fibrogenic effects.15 Endothelin-induced phosphorylation of the mitogen-activated protein kinase and endothelin-induced increase in collagen I gene activity were completely prevented by an inhibitor of the EGFR kinase. Ang II also “uses” the EGFR to elicit certain effect in cardiovascular cells. For example, Bokemeyer et al reported that ang II–induced growth of VSMC requires activation of EGFR,25 and Mazak et al showed that ang II– or aldosterone-induced stimulation of ERK1/2 in VSMC depends on EGFR.19 In cardiomyocytes, ang II receptor type 1A promotes hypertrophy via transactivation of the EGFR,26,27 thus proving that EGFR serves as a heterologous transducer for pathological vascular signals such as ET-1, ang II, and catecholamines, all of which have been linked to vascular remodeling.1
EGFR affects not only protein kinases and transcription factors but also influences cellular Ca²⁺ signaling via phospholipase Cγ.28,29 Phospholipase Cγ can induce inositoltrisphosphate-mediated Ca²⁺ release and also Ca²⁺ influx across the cell membrane.30 This effect is elicited by, eg, CAM, arachidonic acid metabolites, or internal store depletion.31,32 Additionally, EGFR kinase activity may also contribute to or support the Ca²⁺ response to other mediators after transactivation. For ang II and ET-1, a small contribution of EGFR to Ca²⁺ signaling in VSMC has been suggested on the basis of pharmacological data.17,33 Thus, EGFR is not only relevant for mitogen-activated protein kinase but also for Ca²⁺ signaling.
Most of the studies that have been performed relied on pharmacological or immunologic tools to unveil the contribution of EGFR to the aforementioned deleterious effects on vascular tissue homeostasis. Our work, presented here, aims at testing the hypothesis of EGFR as a heterologous signal transducer in VSMC using the genetic model of targeted EGFR deletion. We generated mice with deletion of EGFR in VSMC (EGFRΔ/Δ VSMC) and compared the cells in primary culture with cells derived from wild-type littermates (EGFR⁺/+ VSMC), with respect to ERK1/2 phosphorylation, Ca²⁺ homeostasis, and cell survival.

Methods

Generation of EGFRΔ/Δ VSMC Mice
C57BL/6 mice containing floxed EGFR alleles (EGFRfloxed) after removal of the neo cassettes were obtained from Dr M. Sibilia (University of Vienna) and used for further breeding.34 EGFR was inactivated tissue specifically in smooth muscle cells by using Sm22-cre transgenic mice, in which the cyclization recombination recombinase is under the control of the smooth muscle cell–specific Sm22 promoter.35 Genotyping of the mice was performed on tail biopsies by polymerase chain reaction (PCR) against the floxed EGFR allele as well as cyclization recombination. Mice were kept in the facilities of the University of Halle-Wittenberg in accordance with institutional policies and federal guidelines.

Cell Culture
Primary culture of VSMC from 4- to 5-month-old mice (EGFRfloxed VSMC, EGFRΔ/Δ VSMC, EGFR⁺/+ VSMC) was performed as described by Ray et al.36 Cells from passages 2 to 6 were cultured in DMEM with 10% fetal bovine serum. Cell number and cell size were determined with a CASY cell counter system (CASY, Reutlingen, Germany).

Western Blot
Cells were lysed with Cell Signaling Technology lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris [Base], pH 7.4, 1% Triton X-100, protease inhibitor cocktail I from Calbiochem, 1 mmol/L EDTA, and 184 mg/L sodium orthovanadate), and proteins were separated by 12% SDS-PAGE for ERK/phospho-ERK. Proteins were transferred to nitrocellulose and incubated overnight with anti-phospho-ERK or anti-ERK (1:1000, Cell Signaling Technology) respectively with anti-EGFR or anti-heat shock protein 90 (1:1000, Cell Signaling Technology) according to the manufacturer’s protocol. Protein bands were detected by horseradish peroxidase–conjugated secondary antibody (anti-rabbit IgG, Biotrend, Cologne, Germany).

Determination of Cytosolic Ca²⁺
Cytosolic free calcium was determined using the Ca²⁺ sensitive dye fura-2 (Molecular Probes, Leiden, the Netherlands) as described previously37 with an inverted Zeiss 100 TV microscope (magnification X400, oil immersion, Zeiss, Oberkochen, Germany) and an automatic filter change device (Hamamatsu, Herrsching, Germany). Cells were cultivated on glass coverslips, and serum was removed 24 hours before the measurements. Subsequently, cells were incubated for 15 minutes with 5 μmol/L fura-2-AM. Finally, coverslips were transferred to the stage of the microscope. The fluorescence signal was monitored at 510 nm, with excitation wavelength alternating between 334 and 380 nm, using a 100-W xenon lamp (fura-2 ratio). The sampling rate was 1 ratio every 2 s. At the end of each experiment thapsigargin was applied as a positive control and only cells responding to thapsigargin were included for further analysis. We did not observe significant differences with respect to the thapsigargin response for the 3 genotypes. Stimulus-induced changes in Ca²⁺ were accepted only if the induced change in the fura-2 ratio exceeded signal standard deviation of 10 preceding measurements by more than 4-fold. Thus, a cell was categorized as a responder if the Ca²⁺ signal increased by more than 4 times the SD of the baseline signal before the addition of the stimulus. The percentage of responders was determined from the sum of all measurements under a certain experimental condition (number responding/number measured). The response behavior of the cells during the different measurements for a given condition was very similar, with a variance of 10% or less.
Real-Time Quantitative Polymerase Chain Reaction
RNA was isolated using the RNeasy Mini Kit from Qiagen (Hilden, Germany) according to the manufacturer’s instructions. Subsequently, remaining DNA was digested with DNase I. Reverse transcription was performed with 1 μg of RNA and random primers using qScript from Invitrogen according to the manufacturer’s instructions. As a control, each sample was also analyzed without reverse transcription. The signals obtained without reverse transcription were negligible (<1%). Finally, real-time amplification was performed with the Stratagene Mx 3000P using the SYBR Green kit (Invitrogen) according to the manufacturer’s instructions. Quantitative PCR (qPCR) efficiency was >90%. The relative expression of mRNA was calculated according to the 2ΔΔCt method, using the 18S signal for normalization. Each sample was analyzed in triplicate, as follows: EGFR sense primer, GCCATCCTACCTGCGAGTGG; EGFR antisense primer, GCAATGAGGGCTCTAAGACC; 18S sense primer, GTGTGAGGAGCCTTGCTGTG; 18S antisense primer, AGGGAGGGACCTAATCACC. Annealing temperatures were 63°C and 64°C for EGFR and 18S, respectively.

Lactate Dehydrogenase Release
Lactate dehydrogenase (LDH) activity in media and in cell lysates was measured using a standard protocol adapted to a lower scale (200 μL) in a multiwell-multilabel reader (Infinite, Tecxan, Berlin).

Caspase-3 Activity
Caspase-3 activity was measured as described previously. Briefly, cells were washed once with PBS buffer (4°C) and incubated with 100 μL of cell lysis buffer (10 mM Triton, 100 mM NaCl, 1 mM EDTA, 0.01% Triton X-100, pH 7.5) for 10 minutes on ice, harvested, and centrifuged at 16 000 g for 10 minutes at 4°C. Sixty microliters of the supernatant was incubated with 65 μL of reaction buffer (20 mM L-piperazine-N,N′-bis(ethanesulfonic acid), 4 mM EDTA, 0.2% CHAPS, 10 mM dithiothreitol, pH 7.4) containing 42 μmol/L DEVD-7-amino-4-trifluoromethylcoumarin (AFC) (end concentration) at 37°C, and fluorescence of the cleaved product, AFC, was measured at 400 nm excitation and 505 nm emission wavelength using a multiwell-multilabel counter (Infinite). Cleaved AFC was quantified by a calibration curve using known AFC concentrations. Protein content was determined with bicinchoninic acid assay (Interchim, Montluçon, France) using bovine serum albumin as a standard.

Glucose-6-Phosphate Dehydrogenase Activity
Glucose-6-phosphate dehydrogenase (G6PD) activity was measured as described. Briefly, cells were washed with phosphate-buffered saline (0.9%), scraped from the plate, lysed, and centrifuged at 2000g at 4°C for 10 minutes. Enzyme activity was determined using a plate reader spectrophotometer (Sunrise, Tecan, Germany) by measuring the rate of increase of absorbance at 340 nm due to the conversion of NADP+ to NADPH by either G6PD or 6-phosphogluconate dehydrogenase (6-PGDH). To determine total dehydrogenase activity, we added 40 μL of supernatant to a well that contained 160 μL of assay buffer and substrates for both enzymes. In a second well, substrates for the enzyme 6-PGDH were added to determine the activity of this enzyme. G6PD activity was then determined by subtracting 6-PGDH activity from total dehydrogenase activity. Substrate concentrations were glucose-6-phosphate (200 μmol/L), 6-phosphogluconate (200 μmol/L), and NADP+ (100 μmol/L). Protein levels were determined for each sample, and activity results were standardized to protein concentration.

Collagen-3 and Fibronectin ELISA
Extracellular collagen III and fibronectin were determined by ELISA as described previously. Media and collagen standards (Sigma-Aldrich, Deisenhofen, Germany) were incubated for 24 hours in 96-well Nunc-Immuno Maxisorp plates (Nalge Nunc International, Naperville, IL) followed by washing and blocking with 2% bovine serum albumin. Subsequently, the wells were incubated with rabbit antibody against collagen III or fibronectin (1:1000, Biotrend, Cologne, Germany) for 1 hour at room temperature. After another 3 washes with 0.05% Tween in phosphate-buffered saline, horseradish peroxidase–conjugated secondary antibody (1:5000, Biotrend, Cologne, Germany) was applied for 1 hour at room temperature. After 3 washes with 0.05% Tween in phosphate-buffered saline, the wells were incubated with α-phenylenediamine (Sigma-Aldrich); the reaction was stopped after 15 minutes with 1 N H2SO4, and the absorbance was measured at 490 nm. Cellular protein was determined by the BCA assay from Pierce. We tested the cross-reactivity of the primary antibodies using the collagen standards and did not observe any significant cross-reactivity.

Immunofluorescence
Cells were cultivated on glass coverslips, fixed with 4% formaldehyde (15 minutes), washed 3 times with PBS, and permeabilized with 0.5% Triton X-100. Subsequently, cells were incubated for 10 minutes in 1% SDS/PBS, followed by 10 minutes of 100 mM glycine/PBS, and finally incubated for 20 minutes in 10% serum/1% BSA/PBS. After 60 minutes of incubation with primary antibody, the cells were washed 3 times with PBS and incubated for 45 minutes with anti-rabbit Alexa 568 secondary antibody or nonlabeled secondary antibody. After 3 additional washes, the cells were analyzed by fluorescence microscopy (Biozero, Keyence, Osaka, Japan) and confocal microscopy (Radiance 2000, Bio-Rad).

Materials
Unless otherwise stated, all other materials were from Sigma-Aldrich (Munich, Germany). Control Ringer solution was composed of (in mmol/L): NaCl 130.0, KCl 1.0, MgCl2 1.0, NaH2PO4 1.0, HEPES 10, and glucose 5 (pH 7.4 at 37°C), plus the respective vehicles (ethanol or DMSO ≤0.1%).

Statistics
The data are presented as mean values±SEM. Significance of difference was tested by paired or unpaired Student t test or ANOVA as applicable. Differences were considered significant if P<0.05. Cells from at least 3 mice per genotype, and 3 matching passages were used for each experimental series. Thus, all experiments were performed with cells from at least 3 different animals in 3 different passages. Cells from the 3 genotypes were treated in parallel for the various repetitions. N represents the number of cells or tissue culture dishes investigated.

Results

Basal Phenotype of EGFRΔΔ VSMC Mice
EGFRΔΔ VSMC animals showed no differences in body weight, systolic blood pressure (measured by tail-cuff or intravasal), blood glucose, or hematocrit compared with EGFR+/+ VSMC animals (Supplemental Table I, available online at http://atvb.ahajournals.org). Aortae and coronary vessels of EGFRΔΔ VSMC animals showed signs of dilatation remodeling (reduced wall/lumen ratio) and slight fibrosis (Supplemental Table II). Possible functional differences are currently under investigation (isolated aortic rings, in vivo reactivity to vasoactive substances) but were beyond the scope of the present study.

EGFR Expression and EGF Responsiveness
EGFR mRNA level was reduced to <10% in the aorta of EGFRΔΔ VSMC animals compared with EGFR+/+ VSMC animals (Figure 1A). The same was true for isolated VSMC EGFR+/− compared with isolated VSMC EGFR+/+. In liver, EGFR was not reduced in EGFRΔΔ VSMC animals (99±22% of EGFR+/+ VSMC animals, n=9). On the protein level, EGFR
was not detectable in VSMC^{EGFR−/−}, as shown by immunofluorescence images (Figure 1B) and by Western blot (Figure 1C). Staining for α-smooth muscle actin was not affected. Loss of EGFR also reduced cell proliferation in the presence of 10% serum (ie, when non-EGF stimuli were present), emphasizing the central role of EGFR (Figure 1D). EGF responsiveness (Figure 1E and 1F, incubation with 10 μg/L EGF) with respect to ERK1/2 phosphorylation was completely absent in VSMC^{EGFR−/−}. VSMC^{EGFR−/−} showed a reduced response compared with VSMC^{EGFR+/−}. All 3 genotypes responded to phorbol-12-myristate-13-acetate (PMA) (protein kinase C [PKC] activation), which served as positive control, although the response was smaller in VSMC^{EGFR−/−} and VSMC^{EGFR+/−} compared with VSMC^{EGFR+/+} (Figure 1F), suggesting that part of the effect of PKC on ERK1/2 phosphorylation is mediated by EGFR transactivation.42 In VSMC^{EGFR+/+}, EGF elicited a slight and transient increase in cytosolic Ca^{2+}, as indicated by the increase in the fura-2 ratio (Figure 1G). The Ca^{2+} signal was similar in VSMC^{EGFR+/−} but completely absent in VSMC^{EGFR−/−} (Figure 1G to 1I). These data support the conclusion that EGFR was successfully deleted.

## Cell Survival and Metabolism

We measured parameters of cell survival and metabolism known to be relevant for VSMC pathophysiology. LDH release (normalized to total cellular LDH) was significantly higher in VSMC^{EGFR−/−} (Figure 2), indicating reduced viability. Furthermore, caspase-3 activity was elevated in VSMC^{EGFR−/−} and VSMC^{EGFR+/−} (Figure 2), indicating an enhanced basal apoptosis rate. Accordingly, protein content was reduced in VSMC^{EGFR−/−}. G6PD activity (Figure 2), rate limiting for NAPDH homeostasis and therefore reactive oxygen species defense, was significantly lower in VSMC^{EGFR−/−}. This was not the case for another enzyme of the pentose phosphate cycle, 6-PGDH.
ET-1, a ligand of the G-protein–coupled endothelin-1 receptors (ET\textsubscript{A}, ET\textsubscript{B}), induced a significant stimulation of ERK1/2 phosphorylation in VSMC\textsuperscript{EGFR\textsuperscript{+/+}} (Figure 3A, 100 nmol/L ET-1 for 5 minutes). This response was significantly reduced in VSMC\textsuperscript{EGFR\textsuperscript{+/−}} and VSMC\textsuperscript{EGFR\textsuperscript{−/−}} (Figure 3A), although ET-1 elicited a significant ERK1/2 phosphorylation in VSMC\textsuperscript{EGFR\textsuperscript{−/−}}. These data support the hypothesis that ET-1 requires EGFR (transactivation) to activate ERK1/2 signaling. The functional relevance of this transactivation is indicated by the absence of a proliferative action of ET-1 in VSMC\textsuperscript{EGFR\textsuperscript{−/−}} in contrast to VSMC\textsuperscript{EGFR\textsuperscript{+/+}} (Supplemental Table IV).

ET\textsubscript{A}/ET\textsubscript{B} also couple to cellular Ca\textsuperscript{2+} signaling and induce Ca\textsuperscript{2+} release from cellular stores, as well as Ca\textsuperscript{2+} entry via the plasma membrane.\textsuperscript{43} To test whether EGFR transactivation also has an impact on Ca\textsuperscript{2+} signaling, we compared ET-1-induced Ca\textsuperscript{2+} responses in the 3 genotypes. Figure 3B shows the typical response of VSMC\textsuperscript{EGFR\textsuperscript{+/+}} (mean±SEM of 7 cells) with a rapid peak (resulting predominantly from Ca\textsuperscript{2+} release from intracellular stores) and a subsequent plateau phase (resulting mainly from Ca\textsuperscript{2+} entry from the extracellular space). We analyzed the increase in fura-2 ratio compared with the mean of 10 control values before the addition of ET-1 during the peak phase (\textDelta\textsubscript{peak}) as well as during the plateau phase (\textDelta\textsubscript{plateau}). In addition, we determined the relative number of cells responding to the stimulus (responders). As a positive control, we used 1 \textmu mol/L thapsigargin, an inhibitor of the endoplasmic Ca\textsuperscript{2+}-ATPase,\textsuperscript{44} and only cells showing an increase in intracellular Ca\textsuperscript{2+} on thapsigargin application were included for further analysis. The percentage of total responders (ie, cells responding with either peak or plateau), as well as peak and plateau responders, was significantly higher in VSMC\textsuperscript{EGFR\textsuperscript{+/+}} compared with the 2 other phenotypes (Figure 3C). Of
note, the percentage of peak responders was very low in VSMC<sub>EGFR<sup>−/−</sup></sub>. There was no significant difference in Δpeak or Δplateau when only the responders of the 3 genotypes were compared (Figure 3C). The time to peak (Δ<sub>peak</sub>) was not different for the 3 genotypes (50±3 seconds, N=115). As shown in Figure 3D, there was no significant difference in the Ca<sup>2+</sup> response elicited by thapsigargin for the different genotypes, arguing against a general effect of EGFR knockout on basic cellular Ca<sup>2+</sup> handling.

**Phenylephrine**

Phenylephrine (PE), a ligand of the G-protein–coupled α<sub>1</sub>-adrenergic receptor, induced a significant stimulation of ERK1/2 phosphorylation in VSMC<sub>EGFR<sup>+/+</sup></sub> (Figure 4A, 1 μmol/L PE for 5 minutes). This response was significantly reduced in VSMC<sub>EGFR<sup>−/−</sup></sub> and VSMC<sub>EGFR<sup>+/−</sup></sub> (Figure 4A), although PE still elicited a significant ERK1/2 phosphorylation compared with vehicle-treated cells. These data clearly support the hypothesis<sup>12,45</sup> that the major part of α<sub>1</sub>-adrenergic receptor–induced ERK1/2 phosphorylation requires EGFR (transactivation). The functional relevance of this transactivation is indicated by the absence of a proliferative action of PE in VSMC<sub>EGFR<sup>−/−</sup></sub> in contrast to VSMC<sub>EGFR<sup>+/+</sup></sub> (Supplemental Table IV).

α<sub>1</sub>-Adrenergic receptor also couples to cellular Ca<sup>2+</sup> signaling and induces Ca<sup>2+</sup> release from cellular stores, as well as Ca<sup>2+</sup> entry via the plasma membrane.<sup>46</sup> As shown in Figure 4B, the percentage of responders was significantly reduced in VSMC<sub>EGFR<sup>−/−</sup></sub> but not in VSMC<sub>EGFR<sup>+/−</sup></sub>. Similar to the situation for endothelin, virtually none of the VSMC<sub>EGFR<sup>−/−</sup></sub> cells responded with a Ca<sup>2+</sup> peak. Surprisingly, ΔCa<sup>2+</sup> was larger in VSMC<sub>EGFR<sup>−/−</sup></sub> and VSMC<sub>EGFR<sup>+/−</sup></sub> responders compared with VSMC<sub>EGFR<sup>+/+</sup></sub> responders (Figure 4B).

These findings raised the question of whether the difference in the fraction of responsive cells is due to the acute role of EGFR in Ca<sup>2+</sup> signaling or results from altered developmental processes when EGFR is absent, possibly leading to a different expression pattern of Ca<sup>2+</sup>-handling proteins. Therefore, we performed experiments determining the fraction of responders to ET-1 or PE in wild-type VSMC with and without the EGFR-kinase inhibitor AG1478 (100 nmol/L, present during the 60 minutes of preincubation, as well as the time of hormone application, N=58 to 101 for each condition). The fraction of responders to either hormone was not reduced in the presence of AG1478 (range, 92% to 98%), indicating that the probability of a Ca<sup>2+</sup> response does not depend on acute EGFR transactivation but results from altered gene expression or adaptive processes. A more detailed investigation regarding the processes leading to these differences when EGFR is absent will be performed in future studies.

**ATP**

ATP, a ligand of the G-protein–coupled purinergic receptors, induced a significant stimulation of ERK1/2 phosphorylation in VSMC<sub>EGFR<sup>+/+</sup></sub> (Figure 5A, 10 μmol/L ATP for 5 minutes). This response was significantly reduced in...
VSMC<sup>EGFR<sup>−/−</sup></sup> but not in VSMC<sup>EGFR<sup>+/−</sup></sup> (Figure 5A), although ATP still elicited a significant ERK1/2 phosphorylation in VSMC<sup>EGFR<sup>−/−</sup></sup> compared with vehicle-treated cells. These data clearly support the hypothesis<sup>12,45</sup> that part of ATP-induced ERK1/2 phosphorylation requires EGFR (transactivation).

G-protein–coupled purinergic receptors also couple to cellular Ca<sup>2+</sup> signaling and induce Ca<sup>2+</sup> release from cellular stores, as well as Ca<sup>2+</sup> entry via the plasma membrane.<sup>47</sup> As shown in Figure 5B, the percentage of responders was not significantly different in VSMC<sup>EGFR<sup>−/−</sup></sup> compared with VSMC<sup>EGFR<sup>+/−</sup></sup> (VSMC<sup>EGFR<sup>−/−</sup></sup> were not tested). The percentage of VSMC<sup>EGFR<sup>−/−</sup></sup> cells responding with a Ca<sup>2+</sup> peak was lower, although not statistically significant. ΔCa<sup>2+</sup> of responsive cells was larger in VSMC<sup>EGFR<sup>+/−</sup></sup> compared with VSMC<sup>EGFR<sup>+/+</sup></sup> (Figure 5B). Thus, ATP-induced Ca<sup>2+</sup> signaling is modulated quantitatively by EGFR expression.

**H<sub>2</sub>O<sub>2</sub>**

Exposure to the reactive oxygen species H<sub>2</sub>O<sub>2</sub> has been proposed to induce ERK1/2 phosphorylation as part of a cell protection response against radical stress.<sup>12,48,49</sup> As shown in Figure 6A, exposure to 100 μmol/L H<sub>2</sub>O<sub>2</sub> for 30 minutes induced a significant stimulation of ERK1/2 phosphorylation in VSMC<sup>EGFR<sup>+/+</sup></sup> and VSMC<sup>EGFR<sup>+/−</sup></sup> but not in VSMC<sup>EGFR<sup>−/−</sup></sup>. In addition, H<sub>2</sub>O<sub>2</sub> may disturb cellular Ca<sup>2+</sup> homeostasis, most probably by enhanced Ca<sup>2+</sup> entry.<sup>50</sup> In VSMC<sup>EGFR<sup>+/+</sup></sup>, 100 μmol/L H<sub>2</sub>O<sub>2</sub> shifted baseline fura-2 ratio to higher values (Figure 6B). This response is typical for enhanced net Ca<sup>2+</sup> influx. In VSMC<sup>EGFR<sup>+/−</sup></sup> and VSMC<sup>EGFR<sup>−/−</sup></sup>, the percentage of responders was not significantly different from VSMC<sup>EGFR<sup>+/+</sup></sup>, but the Δplateau values were significantly higher. Thus, H<sub>2</sub>O<sub>2</sub>-induced changes of cytosolic Ca<sup>2+</sup> do not require EGFR but seem to be restricted by EGFR signaling.

**Discussion**

Many studies on ErbB family functions focused on embryonic development, as well as cancer development. In recent years, however, ErbB1/EGFR has been studied more intensively with respect to its potential pathophysiological role for VSMC. It has been shown pharmacologically that several factors that are considered to be involved in vascular diseases transactivate EGFR and thereby initiate mitogen-activated protein kinase signaling.<sup>51</sup> Transactivation of EGFR was reported 15 years ago<sup>52</sup> and is now accepted as an important mechanism for signaling cross-talk that is essential to mediating critical functions of mediators and micromilieu parameters.<sup>9</sup> Thus, the mechanisms of EGFR transactivation, as well as its pathophysiological significance, are of major interest for vascular biology and medicine. EGFR transactivation seems to require intracellular second messenger activation, such as Ca<sup>2+</sup>, PKC, or the generation of reactive oxygen species. Subsequently, cytosolic nonreceptor tyrosine kinase such as Src or protein tyrosine kinase-2 may phosphorylate and transactivate EGFR. Alternatively, transactivation is accomplished by metalloprotease-dependent EGFR/ErbB ligand production from its membrane-bound proform.<sup>53</sup> HB-EGF is a ligand with strong implications in vascular diseases. HB-EGF can stimulate growth and migration of VSMCs and is expressed in pathological vascular states such as atherosclerosis and restenosis after angioplasty.<sup>51</sup> In addition to HB-EGF and ErbB1/EGFR, other combinations of ErbB ligand-receptor complexes can also be activated by A
disintegrin and metalloproteinase-dependent shedding of ErbB ligands, leading to diverse arrays of downstream signal transduction pathways with potential relevance for vascular remodeling.

These findings implicate EGFR as an element of signaling convergence by which various vascular risk factors promote vascular remodeling.8 Beyond that, the physiological significance of vascular EGFR expression and signaling is not known. Therefore, we currently do not know whether there is a yin-yang situation with respect to vascular EGFR, which might contribute to basic vascular homeostasis but could also be subject to “misuse” under certain pathophysiological conditions. In the present study, we investigated the consequence of cell-specific EGFR deletion in VSMC of mice with respect to the basal phenotype and signaling. Because general EGFR-knockout animals are not viable after birth,54 this model for the first time allows genetic testing of the importance of VSMC EGFR.

Our data show, first of all, that although VSMC EGFR had been successfully deleted (in liver cells EGFR expression was not different from wild-type animals), the animals are viable for several weeks after birth. However, aorta and coronary arteries from EGFRΔA VSMC animals showed signs of dilative remodeling (reduced wall/lumen ratio) and slight fibrosis (enhanced collagen III mRNA and Sirius red staining). The morphological data on the vascular phenotype require further experimentation to obtain mechanistic insights and draw conclusion regarding the pathomechanisms and their consequences. At present we cannot exclude the possibility that the changes in wall/lumen ratio are in part the result of different vessel contraction at the time of fixation.

Further detailed phenotyping of the animals and isolated aortae is currently in progress. Net growth of VSMCEGFR−/− was slower, as shown by the growth curve (Figure 1D), and the lower protein amount per cm² in the Petri dish after 10 days (Figure 2), despite the same starting cell number and no major differences in cell size. The reduced net growth rate is in part due to an enhanced death rate, as indicated by the higher LDH release (necrosis) and caspase-3 activity (apoptosis). Because EGF reduced LDH release (antinecrotic effect) in VSMCEGFR+/+ and VSMCEGFR−/−, we conclude that the EGFR exerts a cytoprotective action in VSMC. Surprisingly, EGF did not affect caspase-3 activity in any of the 3 genotypes. Possibly, the antiapoptotic action of EGFR depends on the activation by other ligands or on transactivation. Cytoprotection by EGFR can be explained in part by the reduced pentose phosphate pathway activity (reduced G6PDH activity in VSMCEGFR−/−). G6PD hydrogenase (H), which is known to be stimulated by EGF,55,56 is the rate limiting enzyme of the pentose phosphate pathway supplying the cell with NADPH, an important intracellular reducing equivalent to protect against oxidative injury and a cofactor for nitric oxide synthase as well as for lipogenesis. In addition, this pathway supplies the cells with ribose 5-phosphate for nucleic acid synthesis. We assume that impaired protection against oxidative injury is the most probable explanation for enhanced cell death in VSMCEGFR−/−. A nonspecific reduction in G6PDH activity can be excluded, as the activity of 6-PGDH (Figure 2) and cellular LDH (data not shown) were not significantly different from that of wild-type cells.

VSMCEGFR−/− showed an enhanced rate of extracellular collagen III and fibronectin accumulation, indicating an important role for EGFR in the control of basal matrix homeostasis. This conclusion is supported by the inhibitory effect of EGF on collagen III and fibronectin accumulation in VSMCEGFR+/+. Thus, EGFR prevents excessive matrix deposition under physiological conditions, contributing to vascular wall tissue homeostasis. In VSMCEGFR−/− collagen III and fibronectin accumulation was significantly lower compared with VSMCEGFR+/+, indicating a biphasic effect of EGFR on matrix homeostasis: low levels of EGFR are associated with a minimum of matrix deposition, which increases dramatically in the absence of EGFR but also rises when EGFR expression is elevated. This relation could explain why pathophysiological EGFR transactivation may contribute to vascular remodeling. Surprisingly, VSMCEGFR−/− responded to EGF with a partial reduction in collagen III accumulation. One explanation for this EGF responsiveness is the presence of ErbB-2/ErbB-3 heterodimers with high affinity for EGF,57,58 comparable to EGFR. By qPCR analysis, ErbB-2 and -3 were detected in all 3 genotypes at comparable levels, whereas ErbB-4 was below the detection limit (data not shown). Of note, fibronectin accumulation of VSMCEGFR−/− did not respond to EGF, suggesting the existence of different EGF signaling pathways for the regulation of matrix homeostasis. Of course, this hypothesis awaits further testing in future studies. In summary, EGFR seems to play an important role for the maintenance of physiological cell and vascular tissue homeostasis, ie, exerts a beneficial vascular effect.

Our data on ERK1/2 phosphorylation show that EGFR contributes substantially to the effects of ET-1, α1-adrenergic agonists (PE), ATP, reactive oxygen species (H₂O₂), and protein kinase C activation (PMA), supporting the concept of EGFR transactivation. ET-1, α₁-adrenergic agonists, and PKC activation seem to be more dependent on EGFR because their effect was also significantly reduced in VSMCEGFR−/−. This was not the case for ATP and H₂O₂, although there was a tendency for reduced ERK1/2 phosphorylation in VSMCEGFR−/−. The proliferative action of ET-1 and α₁-adrenergic agonists was completely abrogated in VSMCEGFR−/−, confirming the functional relevance of EGFR transactivation.

The effect of H₂O₂ or ATP on cytosolic Ca²⁺ was not reduced by the absence of EGFR. On the contrary, the changes in cytosolic Ca²⁺ were higher in the absence of EGFR, suggesting a negative feedback loop involving EGFR. ET-1- and PE-induced responsiveness of Ca²⁺ signaling was affected by the EGFR genotype. In the case of ET-1, EGFR expression influenced the percentage of responsive cells, which was ≈90% in VSMCEGFR+/+. Because only cells responding to thapsigargin were included in the analysis and the percentage was also reduced in VSMCEGFR−/−, we can exclude a nonspecific effect of reduced cell viability. Although the immunoblot data contain no evidence for a residual EGFR expression in knockout cells, there remains the possibility for a subthreshold amount of EGFR in few cells, because by qPCR a residual amount of mRNA (less
than 10%) was detected. At present, it is not possible to distinguish experimentally between a small “contamination” of the primary culture with non-VSMC and a small residual amount of EGFR in VSMC. However, the fraction should be very small, as we did not observe EGFR-sensitive knockout cells with respect to calcium signaling (Figure 1H). Possible EGFR-positive cells are not sufficient to explain the fraction of responders to PE or ET-1.

When the Ca2+ signals of ET-1-responsive cells were compared, no significant differences were observed. The percentage of PE-responsive cells was reduced only in VSMC/EGFR+/−. However, the Ca2+ signals of PE-responsive cells were enhanced in VSMC/EGFR+/− and VSMC/EGFR−/− compared with VSMC/EGFR+/+, also suggesting a negative feedback loop involving EGFR. At present, we have no proven explanation for the mechanisms behind this difference in Ca2+ signaling. The mechanism seems to have certain specificity, because Ca2+ handling was affected differently. An acute functional role of EGFR in Ca2+ signaling, as well as altered expression of Ca2+-handling proteins, is conceivable. As the fraction of responders was not reduced by the acute addition of the EGFR inhibitor AG1478, we favor the hypothesis that the probability of a Ca2+ response does not depend on acute EGFR transactivation but results from altered gene expression or adaptive processes. Future studies will have to address this question in more detail.

Preliminary organ bath experiments with aortic ring preparations indicate that isometric force development during α1-adrenergic stimulation is enhanced after repetitive hormone application in VSMC/EGFR−/− compared with VSMC/EGFR+/+ (data not shown). Thus, the larger Ca2+ response may result in enhanced vasoconstrictor sensitivity and finally in augmented peripheral resistance, although altered receptor handling (ie, desensitization) cannot be excluded. On the other hand, with respect to the functional vascular relevance, it might be more relevant to consider the integrated calcium signal composed of the probability of a calcium signal (Pp=number of responders) and the magnitude of the signal (∆Ca2+). The resulting integrated or average—and probably relevant for a vessel—signal is the product Pp×∆Ca2+. With respect to PE and ET-1, this average signal is lower in knockout cells compared with wild-type (compare Pp and ∆Ca2+ in Figures 3C and 4B). Thus, from the integrated perspective, the data indicate a positive role for EGFR in mediating calcium signals, as also suggested for ang II signaling in arteriolar arterioles. These hypotheses deserve more detailed investigation in the future.

In summary, our data confirm the importance of EGFR for ERK1/2 activation by a variety of unrelated stimuli. Furthermore, our data show that EGFR expression has a heterologous signaling role beyond mitogen-activated protein kinase activation and can modulate Ca2+ signaling. VSMC EGFR seems to be Janus-faced. Its absence makes cells more vulnerable and disturbs matrix homeostasis, leading to dilative remodeling. Overstimulation, on the contrary, promotes vascular fibrotic remodeling, as suggested by various reports in the literature. The model presented here offers the possibility to assess the contribution of VSMC EGFR to vascular remodeling induced by a variety of factors known to transactivate the EGFR.

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Disclosures

None.

References

Consequences of Epidermal Growth Factor Receptor (ErbB1) Loss for Vascular Smooth Muscle Cells From Mice With Targeted Deletion of ErbB1
Barbara Schreier, Maria Döhler, Sindy Rabe, Bettina Schneider, Gerald Schwerdt, Stefanie Ruhs, Maria Sibilia, Michael Gotthardt, Michael Gekle and Claudia Grossmann

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Supplement Material

Methods:

Measurement of blood pressure
Blood pressure was measured twice weekly by tail cuff using external tail pulse detection (ADI instruments, Spechbach, Germany) in awake animals. Intravasal determination of blood pressure was performed using a Millar catheter (ADI instruments, Spechbach, Germany) in anesthetised animals (isofluran). All procedures on animals were performed in accordance with the guidelines of the American Physiological Society and were approved by the local government authorities.

Harvesting of aortae
Immediately after death aortas were excised and carefully freed from adjacent tissue. Part of the aorta was immediately snap frozen in liquid nitrogen for later RNA extraction while further parts were put in 3% paraformaldehyde solution for fixation. Tissue was dehydrated by bathing in increasing concentrations of methanol or isopropanol, respectively. After embedding in paraffin, 3 \( \mu \)m sections were cut.

Determination of blood parameters
At the day of killing, blood samples were taken and glucose (Accu Check, Roche, Mannheim, Germany), hematocrit and blood cell distribution were analysed. Hematocrit was determined via differential centrifugation and is expressed in v/v %.

Morphometric analysis
Morphometric analysis was performed in fixed tissue with the Keynce Biozero 8000 microscope using the integrated software (Keynce, Japan). The degree of interstitial fibrosis was determined by evaluation of Sirius Red stained area, utilizing a point counting technique.

qPCR primer
Collagen III sense primer: 5’-TGGTAGAAAGGACACACAGAGGC
Collagen III antisense primer: 5’-TCCAACTTCACCCTTAGACC
Fibronectin sense primer: 5’-TTAAGCTCACATGCCAGTG
Fibronectin antisense primer: 5’-TCGTCATAGCAGTTGCTTC
Annealing temperature was 64°C or 57°C respectively.

Cell proliferation
Cells were repeatedly counted in defined 1 mm\(^2\) areas using the Keynce Biozero 8000 microscope. Initial cell density was the same for both genotypes.
Results:

Table I. Animal phenotype. N = 10 animals for each group, except for intravasal blood pressure (N=4). Age 13-16 weeks.

<table>
<thead>
<tr>
<th></th>
<th>$EGFR^{+/+}$ VSMC</th>
<th>$EGFR^{Δ/Δ}$ VSMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>24.1 ± 1.2</td>
<td>24.4 ± 0.9</td>
</tr>
<tr>
<td>Hematocrite (%)</td>
<td>41.3 ± 1.1</td>
<td>42.3 ± 0.7</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>142 ± 5</td>
<td>143 ± 5</td>
</tr>
<tr>
<td>Systolic Blood pressure (tail cuff, mmHg)</td>
<td>132 ± 5</td>
<td>133 ± 4</td>
</tr>
<tr>
<td>Systolic Blood pressure (intravasal, mmHg)</td>
<td>75 ± 4</td>
<td>73 ± 5</td>
</tr>
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</table>

Table II. Vascular phenotype. * = p<0.05 versus $EGFR^{+/+}$ VSMC. N$_{aortae}$ = 5 animals for each group. N$_{coronary vessels}$ = 6 animals for each group.

<table>
<thead>
<tr>
<th></th>
<th>$EGFR^{+/+}$ VSMC</th>
<th>$EGFR^{Δ/Δ}$ VSMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic Col3a1/18S</td>
<td>1.0 ± 0.2</td>
<td>2.7 ± 0.7 *</td>
</tr>
<tr>
<td>Aortic FN-1/18S</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Aortic wall thickness (µm)</td>
<td>33 ± 2</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>Aortic lumen radius (µm)</td>
<td>108 ± 11</td>
<td>160 ± 38</td>
</tr>
<tr>
<td>Aortic wall crosssectional area (µm$^2$)</td>
<td>25815 ± 4355</td>
<td>36190 ± 10446</td>
</tr>
<tr>
<td>Aortic lumen crosssectional area (µm$^2$)</td>
<td>36642 ± 3732</td>
<td>80424 ± 19100</td>
</tr>
<tr>
<td>Wall/lumen area ratio</td>
<td>0.72 ± 0.07</td>
<td>0.45 ± 0.07 *</td>
</tr>
<tr>
<td>Aortic wall fractional Sirius red area</td>
<td>0.35 ± 0.01</td>
<td>0.40 ± 0.07</td>
</tr>
<tr>
<td>Aortic wall Sirius red area (µm$^2$)</td>
<td>9035 ± 1524</td>
<td>16599 ± 4178 *</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>$EGFR^{+/+}$ VSMC</th>
<th>$EGFR^{Δ/Δ}$ VSMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery wall thickness (µm)</td>
<td>7.6 ± 0.4</td>
<td>8.6 ± 0.7 *</td>
</tr>
<tr>
<td>Coronary artery lumen radius (µm)</td>
<td>20.6 ± 2.4</td>
<td>29.1 ± 3.4 *</td>
</tr>
<tr>
<td>Coronary artery crosssectional area (µm$^2$)</td>
<td>1165 ± 149</td>
<td>1805 ± 257 *</td>
</tr>
<tr>
<td>Coronary artery lumen crosssectional area (µm$^2$)</td>
<td>1333 ± 155</td>
<td>2660 ± 311 *</td>
</tr>
<tr>
<td>Wall/lumen area ratio</td>
<td>0.97 ± 0.10</td>
<td>0.68 ± 0.10 *</td>
</tr>
<tr>
<td>Coronary artery wall fractional Sirius red area</td>
<td>0.39 ± 0.04</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Coronary artery wall Sirius red area (µm$^2$)</td>
<td>454 ± 47</td>
<td>560 ± 54 *</td>
</tr>
</tbody>
</table>

Table III. mRNA levels of collagen III and fibronectin in VSMC in primary culture. N=5. * = p<0.05 versus vehicle.

<table>
<thead>
<tr>
<th></th>
<th>$EGFR^{+/+}$ VSMC</th>
<th>$EGFR^{Δ/Δ}$ VSMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic Col3a1/18S</td>
<td>1.0 ± 0.3</td>
<td>4.2 ± 1.3 *</td>
</tr>
<tr>
<td>Aortic FN-1/18S</td>
<td>1.0 ± 0.2</td>
<td>2.1 ± 0.4 *</td>
</tr>
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Table IV. Cell proliferation (% increase over 48 h in serum free media). N=30-50. * = p<0.05 versus vehicle.

<table>
<thead>
<tr>
<th></th>
<th>$EGFR^{+/+}$ VSMC</th>
<th>$EGFR^{Δ/Δ}$ VSMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>0 ± 4</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>100 µg/l EGF</td>
<td>25 ± 6 *</td>
<td>6 ± 5</td>
</tr>
<tr>
<td>100 nmol/l endothelin</td>
<td>34 ± 11 *</td>
<td>0 ± 4</td>
</tr>
<tr>
<td>1 µmol/l phenylephrine</td>
<td>22 ± 10 *</td>
<td>5 ± 3</td>
</tr>
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