Heparin-binding EGF-like growth factor mediates oxyhemoglobin-induced suppression of voltage-dependent potassium channels in rabbit cerebral artery myocytes

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Koide M, Penar PL, Tranmer BI, Wellman GC. Heparin-binding EGF-like growth factor mediates oxyhemoglobin-induced suppression of voltage-dependent potassium channels in rabbit cerebral artery myocytes. Am J Physiol Heart Circ Physiol 293: H1750–H1759, 2007. First published June 8, 2007; doi:10.1152/ajpheart.00443.2007.—Oxyhemoglobin (OxyHb) can suppress voltage-dependent K+ channel (Kv) currents through protein tyrosine kinase activation, which may contribute to cerebral vasospasm following subarachnoid hemorrhage. Here we have tested the hypothesis that shedding of heparin-binding EGF-like growth factor (HB-EGF) and the resulting activation of the tyrosine kinase EGFR receptor (EGFR) underlie OxyHb-induced Kv channel suppression in the cerebral vasculature. With the use of the conventional whole cell patch-clamp technique, two EGFR ligands, EGF and HB-EGF, were found to mimic OxyHb-induced Kv suppression in rabbit cerebral artery myocytes. Kv current suppression by OxyHb or EGF ligands was eliminated by a specific EGFR inhibitor, AG-1478, but was unaffected by PKC inhibition. Compounds (heparin and CRM-197) was eliminated by a specific EGFR inhibitor, AG-1478, but was unaffected by PKC inhibition. Compounds (heparin and CRM-197) that specifically interfere with HB-EGF signaling eliminated OxyHb-induced Kv suppression, suggesting that HB-EGF is the EGF ligand involved in this pathway. HB-EGF exists as a precursor protein that, when cleaved by matrix metalloproteases (MMPs), causes EGFR activation. MMP activation was detected in OxyHb-treated arteries by gelatin zymography. Furthermore, the MMP inhibitor (GM-6001) abolished OxyHb-induced Kv current suppression. We also observed Kv current suppression due to EGFR activation in human cerebral artery myocytes. In conclusion, these data demonstrate that OxyHb-induced MMP activation, causing HB-EGF shedding and enhanced EGFR activity, ultimately leads to Kv channel suppression. We propose that EGFR-mediated Kv suppression contributes to vascular pathologies, such as cerebral vasospasm, and may play a more widespread role in the regulation of regional blood flow and peripheral resistance.

vascular smooth muscle; growth factors; subarachnoid hemorrhage; tyrosine kinase

VOLTAGE-DEPENDENT DELAYED rectifier K+ (Kv) channels play an important role in the maintenance of vascular smooth muscle membrane potential and the regulation of arterial diameter (13, 35). A reduction in Kv channel activity and/or a decrease in Kv channel expression in vascular smooth muscle would promote membrane potential depolarization, increased Ca2+ influx via voltage-dependent Ca2+ channels, and vasoconstriction (27). Decreased Kv currents have been linked to a number of endogenous and exogenous vasoconstrictors, and Kv current suppression has been implicated in systemic and pulmonary hypertension, as well as cerebral vasospasm following subarachnoid hemorrhage (SAH) (22, 24, 42, 49). A large body of evidence suggests that the blood component oxyhemoglobin (OxyHb) contributes to SAH-induced vasoconstriction (3), and our laboratory has recently reported that OxyHb suppresses Kv currents in cerebral artery myocytes via a mechanism involving enhanced protein tyrosine kinase activity and Kv channel endocytosis (22).

Protein tyrosine kinases represent an abundant and diverse group of proteins that encompass both cytosolic (second messenger activated) and membrane-spanning (receptor mediated) kinases (11). EGFR receptor (EGFR) is one receptor-mediated tyrosine kinase, originally identified as a cancer-promoter protein (33), involved in a variety of cellular responses including vascular smooth muscle proliferation, migration, and contraction (19, 46). Previous studies have demonstrated that vasoconstrictors, such as adrenergic agonists, angiotensin II, and endothelin, acting through G protein-coupled receptors, lead to EGFR activation. Furthermore, two EGFR ligands, EGF and heparin-binding EGF-like growth factor (HB-EGF), cause constriction of systemic arteries (4, 7, 16, 31). EGF and HB-EGF exist on the cell surface as precursor proteins (pro-EGF and pro-HB-EGF) that are proteolytically processed by a matrix metalloprotease (MMP) and/or a disintegrin and metalloprotease (ADAM) into active EGF ligands. In arteries, MMP-7 (16) and MMP-9 (31) have been reported to cleave pro-HB-EGF, leading to EGFR activation and vasoconstriction.

Here we have examined whether EGFR activation is involved in OxyHb-induced suppression of Kv currents in cerebral artery myocytes. We have found that EGFR ligands mimicked the action of OxyHb to suppress Kv currents. In addition, inhibitors of EGFR, MMPs, and HB-EGF abolished OxyHb-induced Kv suppression. Furthermore, we observed that OxyHb increased MMP activity. These data suggest that OxyHb through MMP activation causes cleavage of pro-HB-EGF, leading to stimulation of EGFR and decreased Kv current. This novel pathway of Kv current suppression may contribute to enhanced cerebral artery constriction following SAH.

MATERIALS AND METHODS

Tissue preparation. Posterior cerebral and cerebellar arteries were obtained from healthy New Zealand White rabbits (males, 3.0–3.5 kg) as described previously (23). All protocols were conducted in accor-
dance with the Guidelines for the Care and Use of Laboratory Animals [National Institutes of Health (NIH) Publication No. 85-23, Revised 1996] and followed protocols approved by the Institutional Animal Care and Use Committee of the University of Vermont. Human cerebral arteries, removed as a necessary part of a required procedure, were obtained from three consenting surgical patients. The University of Vermont has an approved assurance of compliance on file with the Department of Health and Human Services covering this activity (Assurance identification No. FWA723; and Institutional Review Board identification No. 0485).

Measurement of $K^+$ current. Vascular smooth muscle cells were enzymatically isolated from cerebral arteries (50), and $K^+$ currents were measured using the conventional whole cell configuration of the patch-clamp technique (22). The bath solution contained (in mM) 134 NaCl, 6 KCl, 1 MgCl$_2$, 0.1 CaCl$_2$, 10 Glucose, and 10 HEPES (pH 7.4). Patch pipettes (3–5 MΩ) were filled with an internal solution that contained (in mM) 87 $K^+$ aspartate, 20 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 EGTA, and 25 KOH (pH 7.2). Outward $K^+$ currents were elicited by a series of 10-mV depolarizing steps to +50 mV from a holding potential of −70 mV. Measurements were obtained from cells before and after 10 min of exposure to purified OxyHb A$_0$ (OxyHb), EGF, or HB-EGF. Inhibitors were applied for 10 min before incubation with OxyHb, EGF, or HB-EGF and were present throughout the remainder of the experimental protocol. Activation time constants ($\tau_{act}$) were determined from an exponential fit of individual voltage-evoked current traces. Steady-state activation and steady-state inactivation curves were obtained using double-pulse voltage-step protocols (1, 44). Steady-state activation curves were obtained from tail currents at −40 mV following 500-ms test depolarizations from −40 to +50 mV from a holding potential of −70 mV. Steady-state inactivation curves were generated from currents obtained at +50 mV following 10-s voltage steps from −90 to +30 mV. The holding potential for these cells were −70 mV, and voltage steps were applied at 10-s intervals. The voltage for half-maximal activation ($V_{0.5, act}$) and half-maximal inactivation ($V_{0.5, inact}$) were obtained from Boltzman fit of steady-state activation and inactivation curves.

Gelatin zymography. Cerebral arteries were incubated with or without OxyHb for 10 min and then minced in 2× gel loading buffer containing 100 mM Tris, 2% sodium dodecyl sulfate (SDS), and 20% glycerol. The protein concentration of lysate was measured by a modified Bradford assay (Coomassie Plus, Pierce, Rockford, IL) using serum albumin as a standard, and lysate (15 µg of protein) was then applied onto a 10% SDS-polyacrilamide gel copolymerized with the MMP substrate gelatin (1 mg/ml). Following electrophoresis, the gel was rinsed with incubation buffer (50 mM Tris and 5 mM CaCl$_2$, pH 7.5) containing 0.25% Triton X-100 overnight to remove SDS and then placed in incubation buffer for 20 h at 37°C to allow gelatinolytic activity. The gel was then stained with Coomassie Brilliant blue G-250, and MMP activity was detected as unstained bands against the background of blue-stained gelatin. The unstained bands depicting MMP activities were densitometrically analyzed using ImageJ software (NIH).

**Drugs.** OxyHb A$_0$ was provided as a gift by Hemosol (Toronto, Canada), Iberiotoxin (Alomone, Jerusalem, Israel), heparin (Abbott, North Chicago, IL), and CRM-197 and GM-6001 (EMD Biosciences; San Diego, CA) were commercially obtained. All other compounds were purchased from Sigma (St. Louis, MO).

**Statistics.** Data are expressed as means ± SE and analyzed by paired Student’s t-test between two groups of data and Tukey means comparison after an ANOVA for multiple groups. Statistical significance was considered at the level of $P < 0.05$.

**RESULTS**

**Inhibition of EGFR abolishes OxyHb-induced $K_V$ current suppression.** Our previous work demonstrated that OxyHb decreases $K_V$ currents through activation of an unidentified tyrosine kinase leading to channel internalization (22). Here initial studies were designed to examine whether activation of EGFR, a receptor-mediated tyrosine kinase, contributes to OxyHb-induced suppression of $K_V$ currents. Outward voltage-dependent $K^+$ currents were observed in smooth muscle cells freshly isolated from rabbit cerebral arteries using the conventional whole cell configuration of the patch-clamp technique. OxyHb (10 µM) suppressed outward $K^+$ current density by about 32% (current density at +50 mV: 31.4 ± 1.4 pA/pF and 21.3 ± 0.8 pA/pF, in the absence and presence of OxyHb, respectively, $n = 7$ (Fig. 1A). A specific inhibitor of EGFR, tyrphostin AG-1478 (3 µM) (29), significantly decreased OxyHb-induced suppression of $K^+$ currents (Fig. 1B). The residual decrease in currents observed in the combined presence of OxyHb and tyrphostin AG-1478 (3.1 ± 1.0 pA/pF at +50 mV, $n = 4$) was similar to the decrease in currents observed in time controls (3.7 ± 0.7 pA/pF at +50 mV, $n = 7$) or tyrphostin AG-1478 alone (3.0 ± 1.2 pA/pF at +50 mV, $n = 5$). These data indicate the involvement of EGFR activation in OxyHb-induced $K^+$ current suppression.

![Fig. 1.](http://ajpheart.physiology.org/.../AJP-Heart-Circ-Physiol-VOL-293-SEPTEMBER-2007-WWW.AJPHEART.ORG)
EGF mimics OxyHb-induced Kv current suppression. We next examined whether ligand-mediated activation of EGFR could mimic the effect of OxyHb to decrease Kv currents. EGF, an endogenous EGFR ligand, caused a concentration-dependent decrease in voltage-dependent K⁺ current amplitude, with an IC₅₀ of 10.9 ± 3.1 ng/ml (Fig. 2A). We have previously shown that OxyHb suppresses Kv but not large-conductance Ca²⁺-activated K⁺ channel (BKCa) currents in rabbit cerebral artery myocytes (22). To clarify the effect of EGF on Kv currents, EGF-induced K⁺ current suppression was examined in the presence of the Kv channel blocker 4-aminopyridine (4-AP, 10 mM) or iberiotoxin (IBTX, 100 nM), a specific blocker of BKCa channels. 4-AP, but not IBTX, abolished EGF-induced suppression of outward K⁺ currents (Fig. 2B). In the presence of 4-AP alone, current density at +50 mV was 21.5 ± 1.1 pA/pF and was not significantly different from current density obtained from the same five cells in the combined presence of 4-AP and EGF (19.3 ± 1.7 pA/pF). However, EGF significantly decreased K⁺ currents in the presence of IBTX (100 nM), from 27.5 ± 1.4 to 20.6 ± 0.6 pA/pF at +50 mV (n = 4), consistent with EGF suppression of Kv but not BKCa currents in rabbit cerebral artery myocytes (Fig. 2B). Since IBTX was without effect on either OxyHb- or EGF-induced K⁺ current suppression, subsequent studies were performed in the absence of IBTX. We also observed that Kv suppression caused by EGF was abolished by EGFR inhibition in a manner similar to OxyHb. As illustrated in Fig. 2, C and D, EGF (100 ng/ml) caused a decrease in currents of ~40% at +50 mV that was abolished by tryphostin AG-1478 (3 μM, n = 5). We also examined whether EGFR activation by EGF could mimic OxyHb-induced K⁺ current suppression in the human cerebral vasculature. Smooth muscle cells were enzymatically isolated from small diameter cerebral arteries obtained from two consenting surgical patients. With the use of the same voltage-step protocol as for rabbit myocytes, EGF decreased K⁺ channel current density by ~25% (n = 5 cells...
from two individuals, Fig. 2, E and F). These findings indicate that EGFR activation by the ligand EGF can mimic OxyHb-induced $K_V$ suppression in rabbit and human cerebral artery myocytes.

To further examine $K_V$ current suppression by OxyHb and EGF, voltage-dependent activation and inactivation properties were studied using standard double-pulse protocols (1, 44). The voltage for half-maximal activation ($V_{0.5,\text{act}}$) obtained from Boltzman fit of tail currents was similar in cells treated with OxyHb ($V_{0.5,\text{act}} = -11.0 \pm 1.2 \, \text{mV}, \, n = 5$) and EGF ($V_{0.5,\text{act}} = -11.3 \pm 1.2 \, \text{mV}, \, n = 5$), and neither compound caused a significant shift in $V_{0.5,\text{act}}$ compared with untreated cells (Fig. 3, A and B). The voltage for half-maximal inactivation ($V_{0.5,\text{inact}}$) was also not significantly altered by either OxyHb or EGF (Fig. 3, A and B). Activation time constants ($\tau_{\text{act}}$), determined from an exponential fit of individual voltage-evoked current traces, were not altered by OxyHb or EGF but were significantly increased in response to 4-AP (Fig. 3C). Deactivation time constants ($\tau_{\text{deact}}$), obtained from exponential fit of tail currents at $-40 \, \text{mV}$, were unaltered by the presence of either OxyHb or EGF (Fig. 3D). Furthermore, inactivation time constants ($\tau_{\text{inact}}$) obtained from a single exponential fit of individual 10-s voltage-step traces were not altered by either OxyHb or EGF ($n = 5$, data not shown). The lack of effect of EGF and OxyHb on $V_{0.5,\text{act}}, \, V_{0.5,\text{inact}}, \, \tau_{\text{act}}, \, \tau_{\text{inact}},$ and $\tau_{\text{deact}}$ are consistent with EGFR activation, leading to a decrease in the number of channels on the plasma membrane rather than, for example, channel block.

$K_V$ suppression by EGFR activation is independent of PKC activity. Protein kinase C (PKC) activity is required for EGFR-mediated $K^+$ channel suppression in cultured cells (47) and can directly lead to $K_V$ channel suppression in vascular smooth muscle (1). We therefore examined whether PKC activity is involved in EGFR-mediated $K_V$ suppression in freshly isolated cerebral artery myocytes. EGFR activation via EGF (100 ng/ml) and PKC activation by the diacylglycerol analog 1,2-dioctanoyl-sn-glycerol (DOG, 1 \, \mu M) caused a similar degree of $K_V$ suppression in isolated rabbit cerebral artery myocytes (Fig. 4, A–C). The PKC inhibitor chelerythrine (1 \, \mu M) was without effect on EGF-induced $K_V$ suppression, however, as anticipated, did prevent $K_V$ current suppression by DOG. Conversely, the EGFR inhibitor AG-1478 (3 \, \mu M) abolished EGF-induced $K_V$ current suppression but did not alter the ability of DOG to reduce $K_V$ currents. Furthermore, $K_V$ suppression by EGF and DOG were additive, suggesting the two compounds act via distinct pathways (Fig. 4D). Average current densities at $+50 \, \text{mV}$ were decreased from 32.9 ± 1.0 to 19.7 ± 1.7 pA/pF after EGF treatment, with a further reduction to 12.5 ± 1.6 pA/pF in the combined presence of EGF and DOG. These findings demonstrate that suppression of $K_V$ currents by EGFR activation is independent of PKC activity and support our previous observations that OxyHb-induced $K_V$ channel inhibition involves EGFR (Fig. 1) but not PKC activation (22).

Role of HB-EGF in OxyHb-induced $K_V$ channel suppression. In addition to EGF, a number of other EGFR ligands have been identified, including HB-EGF (12), which may contribute to OxyHb-induced $K_V$ current suppression. Exogenous HB-EGF suppressed outward $K^+$ currents in rabbit cerebral artery myocytes in a concentration-dependent manner ($IC_{50} = 1.9 \pm 0.8$ ng/ml, Fig. 5A). Maximal suppression (~35%) of $K_V$ currents
by HB-EGF occurred at a concentration of 30 ng/ml, decreasing K^+ current density from 31.0 ± 1.1 to 20.3 ± 0.9 pA/pF (n = 6, Fig. 5B). Furthermore, HB-EGF (100 ng/ml) reduced outward K^+ current by about 20% in freshly isolated human cerebral artery myocytes (n = 5 cells from 2 individuals, Fig. 5, C and D). Thus both EGF and HB-EGF can suppress K_V currents in cerebral artery myocytes.

To clarify the identity of the EGFR ligand(s) involved in OxyHb-induced K_V suppression, we examined the action of heparin, which competitively binds to heparin-binding EGF family members including HB-EGF (43) but not to EGF. As shown in Fig. 6A, heparin (10 U/ml, equivalent to ~60–70 μg/ml) markedly reduced the ability of OxyHb to suppress outward K^+ currents. Current density at +50 mV was 27.7 ± 0.9 before and 25.7 ± 0.9 pA/pF after OxyHb-treatment in the presence of heparin (n = 6). As with OxyHb, HB-EGF-induced K_V current suppression was markedly reduced by heparin (n = 4, Fig. 6B); however, heparin did not impact EGF-induced or DOG-induced K_V current suppression (Fig. 6, C and D). CRM-197 (1 μg/ml), a nontoxic mutant of diphtheria toxin that specifically binds to and inhibits HB-EGF (34), abolished OxyHb-induced K_V current suppression in rabbit cerebral artery myocytes (n = 5, Fig. 6E). CRM-197 also abolished OxyHb-induced K_V current suppression in two cells isolated from human cerebral artery myocytes (data not shown). The ability of heparin and CRM-197 to abolish OxyHb-induced K_V current suppression implicates the involvement of HB-EGF, rather than EGF, in this phenomenon. Furthermore, the actions of OxyHb and HB-EGF were not additive; for example, in the presence of OxyHb, HB-EGF did not cause further suppression of K_V current (Fig. 6F). These data suggest that HB-EGF may be the endogenous EGFR ligand mediating OxyHb-induced suppression of K_V currents.

HB-EGF exists as a membrane-bound precursor protein, pro-HB-EGF, that is proteolytically cleaved by a MMP and/or ADAM to enable binding to EGFRs (17). We therefore hypothesized that OxyHb stimulates MMP/ADAM activity leading to HB-EGF shedding, EGFR activation, and, ultimately, K_V current suppression. We next examined the ability of GM-6001, a broad-spectrum MMP/ADAM inhibitor (30), to impact OxyHb-induced K_V current suppression. In the presence of GM-6001 (10 μM), OxyHb failed to suppress K_V currents (Fig. 7A). However, GM-6001 had no direct effect on K_V currents and did not significantly alter EGF-induced K_V suppression (Fig. 7B), demonstrating that GM-6001 does not directly interfere with EGFR or K_V channel function. Gelatin zymography was next used to examine the ability of OxyHb to increase MMP/ADAM activity. With the use of a 10% SDS-PAGE gel copolymerized with the MMP/ADAM substrate gelatin, a single band of ~65 kDa with an average arbitrary

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Fig. 4. EGFR-induced K_V suppression is independent of PKC activity. A and B: K_V currents obtained from rabbit cerebral artery myocytes incubated with either the EGFR inhibitor, AG-1478 (3 μM), or the protein kinase C (PKC) inhibitor, chelerythrine (Che, 1 μM), for 10 min before the addition of EGF (100 ng/ml; A) or PKC activator 1,2-dioctanoyl-sn-glycerol (DOG; 1 μM; B). C: summary data of K_V current suppression in the absence or presence of inhibitors. Data represent currents obtained at +50 mV. EGF (100 ng/ml; n = 8) and DOG (1 μM; n = 4) caused a similar degree of K^+ current suppression. The EGFR inhibitor AG-1478 (3 μM) significantly reduced EGF-induced (n = 5), but not DOG-induced (n = 5), K_V current suppression. Conversely, Che reduced K_V current suppression induced by DOG (n = 4) but not EGF (n = 5). **P < 0.01 vs. EGF-suppressed current in the absence of inhibitor; ††P < 0.01 vs. DOG-suppressed current in the absence of inhibitor. D: current-voltage relationship demonstrating the inhibition of K_V currents caused by EGF and DOG are additive (n = 5). Control represents currents before the addition of EGF. *P < 0.05 and **P < 0.01 vs control vs. EGF; and †P < 0.05 and ††P < 0.01 EGF vs. EGF + DOG.
Rabbit Cerebral Artery Myocytes

**A**

![Current Density (pA/pf) vs. HB-EGF (ng/mL)](image)

**B**

![Current Density (pA/pf) vs. Membrane Potential (mV)](image)

**C**

**D**

Human Cerebral Artery Myocytes

![Current Density (pA/pf) vs. HB-EGF (100 ng/mL)](image)

Fig. 5. Heparin-binding EGF-like growth factor (HB-EGF)-induced Kv current suppression in rabbit and human cerebral artery myocytes. A: concentration-response curve of HB-EGF-induced Kv current suppression. K^{+} currents were obtained from rabbit cerebral artery myocytes (holding potential, −70 mV) using 800-ms voltage steps to +50 mV. **Currents were obtained from 4–6 cells for each concentration of HB-EGF. B: voltage-dependent K^{+} currents obtained from rabbit myocytes in the absence and presence of HB-EGF (30 ng/ml). Summarized data represent current-voltage relationships obtained from 6 cells. C: HB-EGF-induced K^{+} current suppression in freshly isolated human cerebral artery myocyte. D: summary data represent currents obtained at +50 mV in the absence and presence of HB-EGF (n = 5 cells from 2 individuals). *P < 0.05; **P < 0.01.

density unit (ADU) of 1,347 ± 130 (n = 7) was observed in the presence of cerebral artery lysate. Ten minutes of OxyHb (10 μM) treatment of cerebral arteries before lysis increased the density of the 65-kDa band (2,842 ± 238 ADU, n = 8), an effect that was abolished by GM-6001 (1,629 ± 256 ADU, n = 7, Fig. 7C). These data are consistent with a role of OxyHb-induced MMP/ADAM activity and the cleavage of pro-HB-EGF to HB-EGF in Kv current suppression.

**DISCUSSION**

Here we report a reduction in Kv currents in cerebral artery myocytes via a mechanism involving HB-EGF and activation of the tyrosine kinase EGFR. We also provide evidence that the spasmogen OxyHb, through enhanced MMP/ADAM activity, leads to HB-EGF-mediated stimulation of EGFR and Kv current suppression. The following observations are consistent with this novel pathway depicted in Fig. 8: 1) a specific EGFR inhibitor, AG-1478, abolished OxyHb-induced Kv current suppression; 2) EGFR ligands, EGF and HB-EGF, reduced Kv currents, mimicking the actions of OxyHb; 3) HB-EGF competitors, heparin and CRM-197, inhibited OxyHb-induced Kv current suppression; 4) OxyHb caused MMP/ADAM activation; and 5) the MMP/ADAM inhibitor, GM-6001, abolished OxyHb-induced Kv suppression. We also observed Kv current suppression due to EGFR activation in human cerebral artery myocytes and that the HB-EGF competitor, CRM-197, abolished OxyHb-induced Kv current suppression in this tissue.

This work suggests a mechanism linking increased activity of the tyrosine kinase EGFR to vasoconstriction. In vascular smooth muscle, EGFR-induced suppression of Kv currents would lead to membrane potential depolarization, an increase in the open-state probability of voltage-dependent Ca^{2+} channels, elevated intracellular [Ca^{2+}], and increased contraction. Several recent reports have demonstrated the involvement of HB-EGF shedding and EGFR activation in G protein-coupled receptor-dependent and -independent vasocostriction. Increased MMP activity, HB-EGF shedding, and EGFR activation have been linked to vasocostriction caused by G protein-coupled receptor agonists phenylephrine and endothelin-1 (7, 16) and increased intravascular pressure (31). In addition, exogenous HB-EGF caused constriction of rat mesenteric arteries (16), and EGF-induced contraction was greatly enhanced in aorta isolated from hypertensive rats (14, 26). Our present findings are also consistent with our previous work demonstrating that OxyHb-induced vasocostriction of small-diameter cerebral arteries involves tyrosine kinase activation leading to Kv channel internalization (22). The EC_{50} values for Kv current suppression by EGF (~11 ng/ml) and HB-EGF (~2 ng/ml) are consistent with EGFR activation in other biological systems (21, 45). The EC_{50} values that we report for EGF- and HB-EGF-mediated Kv current suppression are somewhat higher than serum concentrations of EGF (0.5–1.5 ng/ml) (6) and HB-EGF (0.05–0.15 ng/ml) (32); however, it is unclear how circulating concentrations of these peptides relate to local concentrations in tissues. Although both EGF and HB-EGF can mimic the ability of OxyHb to suppress Kv currents (Figs. 2 and 5), we provide additional data demonstrating that selective competitors of HB-EGF, heparin, and CRM-197 effectively abolished OxyHb-induced Kv current suppression (Fig. 6). These data suggest that HB-EGF, and not EGF, is the endogenous EGFR ligand leading to OxyHb-induced Kv current suppression. Future functional studies
should clarify the role of MMP activation and HB-EGF shedding in OxyHb-induced cerebral artery constriction.

MMPs represent a group of structurally similar proteases that are best characterized by their ability to degrade extracellular matrix components such as collagens, elastin, fibronectin, and laminin. MMP activity has been implicated in the process of angiogenesis, as well as in a number of vascular pathologies including abdominal and cerebral aneurysms, vascular remodeling, the formation and thinning of atherosclerotic plaques, and arterial restenosis (15, 20). A number of MMP subtypes (MMP-1, -2, -3, -7, -9, -12, -13, and -14) have been reported in vascular smooth muscle cells and macrophages (15). MMPs and the related family of ADAM proteases are also responsible for cleavage of membrane-bound proteins such as growth factors (e.g., HB-EGF and transforming growth factor-α) (15, 39). EGFR ligands, such as HB-EGF, exist on the cell surface as membrane-bound precursor proteins (pro-HB-EGF) that are cleaved by MMP/ADAMs. Enhanced MMP activity and HB-EGF shedding have been linked to vasoconstriction in small-diameter arteries. Hao et al. (16) reported that MMP-7 activation and HB-EGF shedding contribute to the sustained constriction caused by α-adrenergic stimulation in rat mesenteric
arteries. These authors also report that MMP-2 and ADAM-12 may also contribute to HB-EGF sheddase activity of arterial lysate. In mesenteric arteries of mice, an increase in intravascular pressure from 25 to 125 mmHg was associated with activation of MMP-2 and -9, HB-EGF cleavage, enhanced EGFR activity, and the development of arterial constriction (31). Consistent with the involvement of MMP/ADAM activation, we observed that OxyHb-induced KV suppression was abolished by GM-6001, a broad-spectrum MMP/ADAM inhibitor. With the use of gelatin zymography, one band demonstrating MMP/ADAM activity was detected around 65 kDa, and the intensity of this band was enhanced in OxyHb-treated arteries. The molecular mass of this band corresponding to MMP/ADAM activity in cerebral artery lysate is similar to the range (60 to 72 kDa) that has been reported for MMP-2 (16, 31). However, future studies are needed to clarify the identity and mechanistic detail of MMP/ADAM activation by OxyHb in this tissue.

Our present findings are in agreement with observations of enhanced tyrosine kinase activity leading to suppression of Kv family members in overexpression cell culture systems. Bowlby et al. (5) found that EGF caused current suppression when Kv1.3 and EGFR were coexpressed in human embryonic kidney (HEK) cells. These investigators observed that in addition to decreased peak Kv1.3 currents, EGF accelerated the slow (C type) inactivation constant (5). In the present study, using native cerebral artery myocytes, we observed a decrease in peak KV currents due to EGFR stimulation without an alteration in the voltage dependence of activation/inactivation or a change in activation/inactivation kinetics. In contrast, the KV channel blocker, 4-AP, causes a marked prolongation of the activation time constant of these currents (Fig. 3), as well as a positive shift in the voltage of half-maximal activation and inactivation (44). A decrease in current density in the absence of a change in channel activation/inactivation properties is consistent with previous reports by us (22) and others (36) of enhanced tyrosine kinase activity leading to a decrease in plasmalemmal channels via Kv channel endocytosis. We have previously shown that OxyHb caused a decrease in cell-surface staining of Kv1.5, consistent with increased channel endocytosis, that is abolished by tyrosine kinase inhibition in isolated cerebral artery myocytes (22). It has also been reported that enhanced tyrosine kinase activity causes Kv1.2 current suppression in HEK cells via enhanced dynamin-dependent channel endocytosis (36). Recently, Cogolludo et al. (10) have described serotonin-induced Kv1.5 channel suppression in rat pulmonary artery myocytes involving activation of a tyrosine kinase and caveolin-dependent channel endocytosis, and Choi et al. (8) have demonstrated the regulation of Kv1.5 channel currents by dynamin-dependent endocytosis and retrograde

Fig. 7. Involvement of matrix metalloprotease (MMP)/a disintegrin and metalloprotease (ADAM) activation in OxyHb-induced Kv current suppression. A and B: representative recordings and summarized data of OxyHb (n = 6; A) and EGF (100 ng/ml; n = 6; B) on Kv currents in rabbit cerebral artery myocytes in the presence of MMP/ADAM inhibitor GM-6001 (10 μM). C: gelatin zymography demonstrating MMP activity was observed around 65 kDa using an SDS-PAGE gel copolymerized with the MMP/ADAM substrate gelatin. Summary data represent MMP activity obtained from gels in arbitrary density units (ADU; n = 7 to 8). *P < 0.05, **P < 0.01 control vs. OxyHb; ††P < 0.01 OxyHb vs. OxyHb + GM-6001.

Fig. 8. Proposed signaling pathway of OxyHb-induced Kv current suppression involving HB-EGF and EGFR activation. Schematic diagram illustrates OxyHb-induced Kv current suppression via enhanced MMP activation and HB-EGF shedding. PY: phosphorylated tyrosine residue; pro-HB-EGF, precursor protein of HB-EGF.
trafficking of the channel along the microtubule cytoskeleton. Collectively, these studies support the hypothesis that OxyHb via MMP activation, HB-EGF shedding, and EGFR activation leads to enhanced Kv channel endocytosis.

It has also been reported that stimulation of m1 muscarinic receptors can decrease the amplitude of expressed Kv1.2 currents via EGF-independent activation of EGFR mediated by PKC (47). Considering that increased PKC activity can occur in cerebral artery myocytes after OxyHb exposure (51) or following SAH (37, 38), we investigated whether PKC may be involved in OxyHb-induced activation of EGFR. As have others (1, 18), we observed that activation of PKC can lead to Kv current suppression. However, our results clearly indicate that PKC and EGFR activation suppress Kv currents via independent pathways. First, the PKC inhibitor chelerythrine did not influence EGF-mediated Kv current suppression (Fig. 4). Second, although PKC-sensitive and -insensitive currents appear to exhibit similar steady-state voltage-dependent activation/inactivation curves, PKC activation does alter activation and inactivation time constants (1, 9, 18). In the present study, we have observed that EGFR ligands or OxyHb does not alter steady-state activation/inactivation or activation/inactivation time constants (Fig. 3). It is, however, unclear whether EGF/OxyHb and DOG/PKC are acting on different subpopulations of Kv channels or whether different Kv channel subtypes, with different molecular identities, exist in this cell type. Recent reports suggest that Kv currents in vascular tissue, including cerebral artery myocytes, are due to hetermultimeric channels consisting of Kv1.2 and Kv1.5 α-subunits (2, 25, 40), although mRNA of other Kv α-subunits has been observed. In the present study, we did not examine the molecular nature of Kv channels in rabbit cerebral artery myocytes. Future studies, including the actions of OxyHb and EGFR activation on cloned Kv channel α-subunits, should help to clarify whether the actions of OxyHb are limited to specific Kv channel subtypes.

Our findings suggest that OxyHb-induced Kv channel suppression may contribute to enhanced cerebral artery constriction following aneurysm rupture and SAH. OxyHb constricts cerebral arteries (3, 22, 48), and the concentration of free OxyHb in the cerebral spinal fluid in SAH patients correlates with the onset of vasospasm in these individuals (41). Furthermore, Kv channel activity is decreased (22, 42, 49) and tyrosine kinase activity is increased (28, 37) in cerebral arteries of SAH models. Our present findings provide a mechanistic link between the blood component OxyHb, enhanced tyrosine kinase activity, and Kv channel suppression. However, future studies will need to examine the role of OxyHb-induced activation of EGFR in the etiology of cerebral vasospasm in SAH animals. It is also possible that EGFR activation by additional blood components could contribute to Kv suppression associated with SAH.

In summary, here we provide evidence for a cell signaling pathway linking OxyHb to Kv current suppression via MMP/ADAM activation, HB-EGF shedding, and EGFR activation. Since decreased Kv channel activity is believed to be one mechanism contributing to a reduction in cerebral blood flow and the development of neurological deficits in SAH patients, our work provides insight into potential novel therapeutic targets for this devastating phenomenon. Furthermore, Kv suppression due to activation of EGFR may represent a more widespread mechanism of vasoconstriction involved in the regulation of arterial diameter, regional blood flow, and peripheral resistance.

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