Endothelin-1 modulates hemoglobin-mediated signaling in cerebrovascular smooth muscle via RhoA/Rho kinase and protein kinase C

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Lan, Christopher, Debarsi Das, Andrew Włoskowicz, and Bozena Vollrath. Endothelin-1 modulates hemoglobin-mediated signaling in cerebrovascular smooth muscle via RhoA/Rho kinase and protein kinase C. *Am J Physiol Heart Circ Physiol* 286: H165–H173, 2004. First published September 18, 2003; 10.1152/ajpheart.00664.2003.—Endothelin-1 (ET-1) and oxyhemoglobin (OxyHb) have been implicated in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage. However, the contribution of ET-1 to this condition has not been definitively established. In this study, we investigated whether threshold concentration of ET-1 enhances cerebrovascular smooth muscle (CVSM) contraction to OxyHb by activating the RhoA/Rho kinase and protein kinase C (PKC) pathways. CVSM contraction was measured in endothelium-denuded rabbit basilar arteries. Cytosolic and particulate fractions of CVSM cells were examined for RhoA and PKC reactivity with specific antibodies using immunoblotting procedures. ET-1 (0.1 nM) alone did not produce any significant contraction, but it markedly potentiated the magnitude (223% of control) and rate (149% of control) of contraction in response to OxyHb, which was attenuated by the inhibitors of Rho kinase Y-27632 and HA-1077. ET-1-mediated potentiation of the contraction was also inhibited by inhibitors of PKC, Ro-32-0432, and GF-109203X. BQ-123 prevented potentiation of vasoinhibition mediated by ET-1, indicating that the action of ET-1 was mediated by the endothelium type A receptor. Pretreatment with ET-1 significantly enhanced OxyHb-mediated RhoA translocation in CVSM cells and intact basilar arteries. ET-1 also caused potentiation of PKC-ε expression in membranes of CVSM cells exposed to OxyHb for 10 and 60 min but did not markedly change the distribution of PKC-α. Thus, in CVSM, threshold concentration of ET-1 potentiates contraction induced by OxyHb via RhoA/Rho kinase- and PKC-ε-dependent mechanisms. This process may contribute to the pathological contraction of cerebral arteries observed after subarachnoid hemorrhage.

**A POTENT VASOACTIVE PEPTIDE** generated in vascular endothelium and smooth muscle cells, endothelin-1 (ET-1), has been implicated in the pathogenesis of a number of cerebrovascular disorders, including stroke, ischemia, and, in particular, cerebral vasospasm, which develops after aneurysmal subarachnoid hemorrhage (SAH) (1, 2, 29, 46). The following findings favor a role for ET-1 in the pathogenesis of cerebral vasospasm: 1) intracranial injections of this peptide induce vasospasm in experimental animals (10), 2) the levels of ET-1 are elevated in the cerebrospinal fluid of patients after SAH (29, 32, 36, 46), and 3) selective antagonists of ET type A (ET(A)) receptors and inhibitors of ET-1 synthesis attenuate vasospasm in animal models (1, 13, 45, 46). Furthermore, evidence has been provided that synthesis of ET-1 is stimulated by a number of vasoactive agents, including oxyhemoglobin (OxyHb) and thrombin, which are liberated during posthemorrhagic clot lysis (9, 21). Although there is considerable evidence supporting the concept of a causative role for ET-1 in the development of cerebral vasospasm, the importance of this peptide in the pathogenesis of vasospasm has been questioned on the basis of clinical findings that the plasma and cerebrospinal fluid levels of ET-1 detected in patients with angiographic vasospasm are outside a range of concentrations that can directly induce cerebral vasospasm and that these levels do not correlate well with the temporal course and severity of vasospasm (4, 23, 46). Furthermore, not all studies have shown that ET-1 receptor antagonists have beneficial effects in cerebral vasospasm or delayed ischemic deficit associated with this condition (1, 2, 46). Therefore, it has been argued that ET-1 alone cannot induce cerebral vasospasm. It is conceivable, however, that low concentrations of ET-1 could augment the effects of other agents liberated from the blood clot after SAH and, thus, substantially contribute to the pathological contraction and cerebral ischemia observed in clinical vasospasm. Indeed, a potentiation of the effects of vasoconstrictors by threshold concentrations of ET-1 has been reported for serotonin, norepinephrine, angiotensin II, and eicosanoids (18, 34, 39, 44). However, most evidence indicates that these agents play little, if any, role in the pathogenesis of cerebral vasospasm, and it is unclear whether a low concentration of ET-1 could enhance the effects of the blood clot components, such as OxyHb, a major causative factor in cerebral vasospasm (15). Also, the molecular mechanisms of the augmenting action of ET-1 have not been fully explored. There is evidence that a monomeric G protein (RhoA) and protein kinase C (PKC) participate in the mechanism for Ca\(^{2+}\) sensitization of smooth muscle contraction (6, 17, 35), which is activated by a variety of agonists acting via G\(_{q}\)- and G\(_{12}/G_{13}\)-coupled receptors, including the ET\(_{A}\) receptor. This mechanism plays an important role in the maintenance of enhanced vascular tone under pathological conditions, including coronary and cerebral vasospasm (16, 26). Furthermore, our recent study indicated that the RhoA/Rho kinase and PKC signaling pathways play a role in the contractile effects of OxyHb (42). Together, these observations suggest that RhoA/Rho kinase and PKC could play a role in the potential interactions of the agents implicated in the pathogenesis of cerebral vasospasm. Therefore, the present study was designed to examine whether threshold concentrations of ET-1 augment the contractile effects of OxyHb in cerebral arteries and, if so, whether the RhoA/Rho kinase pathway and PKC are involved in these interactions.

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MATERIALS AND METHODS

Materials. Hemoglobin and ET-1 were purchased from Sigma (St. Louis, MO); HA-1077, GF-109203X, Ro-32-0432, and GO-6976 from Calbiochem (San Diego, CA); and anti-RhoA and anti-PKC isofrom monoclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Transduction Laboratories (San Diego, CA), respectively. Y-27632 was generously provided by Welldigm (Osaka, Japan).

All other reagents were of analytic grade. Hemoglobin obtained commercially was converted before use to OxyHb, as previously described. Y-27632 was generously provided by Wellfide (Osaka, Japan).

Recording of isometric tension. New Zealand rabbits of either gender were used in these studies. All procedures were performed according to the protocols approved by the University of Alberta Animal Care and Use Committee. The animals were killed with an intravenous overdose of pentobarbitone; the brain, with cerebral arteries attached, was then removed to a dissection dish filled with oxygenated Krebs-Henseleit buffer containing (in mM) 120 NaCl, 4.5 KCl, 2.5 CaCl 2 , 1 MgSO 4 , 1.2 KH 2 PO 4 , 25 NaHCO 3 , and 10 dextrose. The endothelium was removed mechanically. Each artery was cut into ~3-mm-long sections and suspended in tissue baths containing Krebs-Henseleit buffer at 37°C, gassed with 95% O 2 -5% CO 2 , and attached to a force displacement transducer (model FT.03, Grass) connected to a polygraph (Grass). The resting tension was set at 1 g, which is optimal for inducing maximum contraction, and the preparations were allowed to equilibrate for 60 min, during which time the bathing medium was changed every 15 min (41). After the equilibration period, contractile responses of the ring preparations to 60 mM KCl were induced. Isometric tension was measured using a force displacement transducer (FT.03, Grass) and a polygraph (model 7D, Grass).

The rate of contraction of basilar artery ring preparations was determined by calculating the force generated in grams of tension per minute and expressed as a percentage of the control rate of contraction mediated by OxyHb alone. Viability of preparations was determined by maximal force response to high (60 mM) KCl. In the experiments in which the effects of Rho kinase or PKC inhibitors were measured, increasing cumulative concentrations of these agents were administered after a plateau tension in response to the vasoconstrictor agents. Preparations were preexposed for 30 min to a selective antagonist of ETA receptors, BQ-123 (1 μM) and thereafter the control rate of contraction were examined. Y-27632 and HA-1077 were preexposed for 30 min to a selective antagonist of ETA receptors, BQ-123 (1 μM).

RESULTS

Potentiation of OxyHb-mediated cerebrovascular contraction by ET-1. In the experiments in which ability of ET-1 to augment contraction induced by OxyHb was investigated, endothelium-denuded rabbit cerebral (basilar) artery ring preparations were exposed to 10 μM OxyHb in the absence or presence of threshold concentration of ET-1 (0.1 nM). The concentration of OxyHb (10 μM) was chosen because it is within the range of perivascular concentrations of OxyHb during the development of delayed cerebral vasospasm (22). Exposure to OxyHb at a single concentration of 10 μM resulted in a slowly developing sustained contraction that peaked at a plateau at ~20–30 min and was maintained for ≥2–3 h. Administration of ET-1 markedly potentiated the magnitude (223 ± 31% of control, P < 0.001, n = 8) and rate (149 ± 17% of control, P < 0.05) of contraction induced by OxyHb (Fig. 1). ET-1 alone did not cause any significant increase in muscle tension. The representative traces are shown in Fig. 1A, and the results of multiple experiments expressed as a percentage of the maximum tension developed in response to 60 mM KCl are presented in Fig. 1B.

To elucidate the ET-1 receptor subtype responsible for the ET-1-induced potentiation of contraction to OxyHb, the ring preparations were preexposed for 30 min to a selective antagonist of ET A receptors, BQ-123 (1 μM). Exposure to BQ-123 resulted in abrogation of the potentiation mediated by ET-1 (Fig. 1).

Involvement of Rho kinase in the augmenting action of ET-1. To determine whether the RhoA/Rho kinase pathway is involved in the augmenting action of ET-1, the effects of two selective inhibitors of Rho kinase, Y-27632 and HA-1077, on the contraction were examined. Y-27632 and HA-1077 were administered in increasing cumulative concentrations to the basilar artery preparations, in which a maximal response to
A

OxyHb administered in the presence or absence of ET-1 had developed. Consistent with our previous studies (42), inhibition of Rho kinase with 0.1–1 μM Y-27632 resulted in a concentration-dependent attenuation of the contractile responses mediated by OxyHb alone (IC_{50} = 0.22 μM; Fig. 2, A and C). Y-27632 also produced a concentration-dependent relaxation of the response induced by OxyHb administered in the presence of threshold concentration of ET-1 (IC_{50} = 0.38 μM; Fig. 2, D and F). Maximal relaxation of the contraction (~90%) was observed at ~0.9 μM Y-27632. Representative traces of the contractile responses are shown in Fig. 2, A and D, and the results from multiple experiments are shown in Fig. 2, C and F.

HA-1077 (0.1–1 μM) also produced a concentration-dependent relaxation of the preparations treated with OxyHb alone (IC_{50} = 0.43 μM; Fig. 2, B and C) or in the presence of ET-1 (IC_{50} = 0.4 μM; Fig. 2, E and F), consistent with the inhibition of Rho kinase activity. The effects of both inhibitors of Rho kinase were reversible, indicating that the relaxation was not due to tissue damage.

Effects of threshold concentration of ET-1 on OxyHb-mediated RhoA translocation. To further examine the involvement of the RhoA/Rho kinase pathway in the potentiating effects of ET-1, we determined the effects of threshold concentration of ET-1 (0.1 nM) on the OxyHb-induced RhoA translocation in cultured, quiescent CVSM cells and intact basilar artery preparations. RhoA translocation from the cytosol to the plasma membrane reflects activation of this protein and is important for stimulation of Rho GTPase activity and subsequent activation of Rho kinase (6). Therefore, the expression of RhoA was determined in the cytosolic and membrane fractions of cultured quiescent CVSM cells and intact basilar artery preparations using Western immunoblot and the anti-RhoA monoclonal antibody. In these experiments, quiescent CVSM cells were exposed to 10 μM OxyHb or ET-1 + OxyHb for 10 min, 60 min, 180 min, and 24 h. Administration of ET-1 significantly potentiated the OxyHb-mediated RhoA translocation to the plasma membrane at all time points: 164 ± 10% (P < 0.01), 232 ± 23% (P < 0.01), 145 ± 13% (P < 0.01), and 185 ± 5% (P < 0.001) after 10 min, 60 min, 180 min, and 24 h, respectively (Fig. 3). ET-1 also increased the particulate-to-cytosolic RhoA expression ratio (1.95, 2.1, 1.4, and 1.5 after 10 min, 60 min, 180 min, and 24 h, respectively) compared with OxyHb alone. OxyHb (10 μM) administered alone produced a significant elevation of RhoA in the plasma membrane after 60 min and 24 h: 172 ± 18% (P < 0.01) and 147 ± 25% (P < 0.01) of control, respectively. Threshold concentration of ET-1 alone tended to increase RhoA translocation, but the difference was not statistically significant: 113 ± 16% and 99.5 ± 12% in the membrane fractions after 10 and 60 min of stimulation, respectively.

To compare the effects of the vasoconstrictors on RhoA translocation in cultured CVSM cells with those in intact basilar arteries, a separate series of experiments was conducted in which the endothelium-denuded basilar artery preparations were exposed for 10 and 60 min to 10 μM OxyHb in the absence or presence of 0.1 nM ET-1. The cytosolic and membrane fractions were separated as described above. OxyHb administered in the presence of ET-1 to the basilar artery preparations induced a significant elevation of RhoA translocation in the membrane fractions that was roughly similar to that in cultured CVSM cells: 114% (P < 0.05) and 142% (P < 0.001) vs. control after 10 and 60 min of stimulation, respectively (Fig. 4). The increases in the RhoA translocation observed in basilar artery preparations after 10 and 60 min of stimulation corresponded to the increases in the isolated basilar artery ring tension mediated by OxyHb in the presence of ET-1, indicating a temporal relation between these two processes.

Fig. 1. Effect of threshold concentration of endothelin-1 (ET-1) on contractions evoked by oxyhemoglobin (OxyHb) in rabbit basilar artery rings. A: representative traces of contractile responses to OxyHb under control conditions (left), after sequential administration of ET-1 and OxyHb (middle), and after preexposure to BQ-123 and ET-1 (right). B: contractile responses to 10 μM OxyHb administered in the absence or presence of 0.1 nM ET-1 and BQ-123. Results are expressed as percentage of maximum tension induced by 60 mM KCl. Values are means ± SE of 7 independent experiments. **P < 0.001 compared with OxyHb; ###P < 0.01 compared with ET-1 + OxyHb.
Involvement of PKC in the augmenting action of ET-1. To assess the involvement of PKC in the augmenting effects of ET-1, we examined the effects of two bisindolylmaleimide inhibitors of PKC: Ro-32-0432 and GF-109203X (Gö-6850). These inhibitors display higher selectivity for PKC than other widely used inhibitors such as staurosporine and H7. Ro-32-0432 exhibited selectivity for the classical PKCs (IC50 = 9 and 28 nM for purified PKC-α and PKC-β, respectively) over novel PKC isoforms (IC50 = 180 nM for PKC-ε) (43). GF-109203X shows high selectivity for PKC-α, -β, -γ, -δ, and -ε isoforms (12). We also used Gö-6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo-(3,4-c)-carbazole], which selectively inhibits classical PKC isoforms (IC50 = 2.3 and 6.2 for PKC-α and PKC-β, respectively) and does not affect the kinase activity of the Ca2+-independent PKCs (8). GF-109203X induced a concentration-dependent relaxation of contraction induced in the basilar artery ring preparations by OxyHb in the presence or absence of ET-1 (Fig. 5, A, C, D, and F). In contrast, Gö-6976 administered in cumulative concentrations (5–100 nM) to the preparation, in which a tonic contraction to the combined action of ET-1 and OxyHb had developed, did not produce any significant relaxation (data not shown), indicating that classical PKC isoforms may not be essential for the contraction.

Translocation of the PKC-α and PKC-ε isoforms evoked by the combined action of ET-1 and OxyHb. To test the possibility that specific isoforms of PKC are involved in the augmenting effects of ET-1 on OxyHb-mediated contraction of CVSM, we measured the PKC isoform translocation from the cytosol to the plasma membrane, a hallmark of activation of these enzymes. In these experiments, Western blot analyses were performed using specific monoclonal anti-PKC antibodies and quiescent CVSM cells exposed to 10 μM OxyHb in the presence or absence of threshold concentration of ET-1 (0.1 nM) for 10 min, 60 min, 180 min, and 24 h. The combined administration of the vasoconstrictors induced membrane association of the PKC-α and PKC-ε isoforms in the presence and absence of threshold concentration of ET-1 (0.1 nM) for 10 min, 60 min, 180 min, and 24 h. The combined administration of the vasoconstrictors induced membrane association of the PKC-α and PKC-ε isoforms compared with the controls (Figs. 6 and 7). Densitometric analysis (arbitrary

![Graphs and images](https://example.com/graphs.png)
units) has shown that stimulation with OxyHb in the presence of threshold concentration of ET-1 resulted in 164 ± 7% and 151 ± 5.5% increase in PKC-α translocation after 10 and 60 min of stimulation, respectively (Fig. 5, A and B). However, these increases did not reach statistical significance. The increases in the PKC-ε translocation were 210 ± 21% (P < 0.01) and 208 ± 20% (P < 0.01) after 10 and 60 min of stimulation, respectively (Fig. 6, A and B). ET-1-mediated potentiation of the PKC-ε translocation was associated with an increase in the particulate-to-cytosolic PKC-ε expression ratio (2.7 and 2.76 after 10 and 60 min of stimulation, respectively) compared with OxyHb alone. OxyHb administered alone also produced significant increases in the amount of membrane-associated PKC-ε: 146 ± 14% (P < 0.05), 150 ± 13% (P < 0.01), 141 ± 11% (P < 0.05), and 126 ± 7.5% (P < 0.05) after 10 min, 60 min, 180 min, and 24 h, respectively. However, no significant potentiation of the PKC-ε translocation by ET-1 has been observed after 180 min and 24 h of stimulation: 121 ± 11% and 126 ± 2% of control, respectively. ET-1 (0.1 nM) alone produced small increases in the PKC-α and PKC-ε translocation to the membrane fractions (~110% of control) that were not significantly different from the controls (data not shown).

**DISCUSSION**

Although there is considerable evidence that OxyHb plays a role in the development of cerebral vasospasm, the contribution of ET-1 to this phenomenon has not been conclusively established. The findings of the present studies are as follows. First, threshold concentrations of ET-1 significantly enhanced the magnitude and rate of sustained contraction induced by OxyHb in rabbit cerebral arteries, suggesting that the concerted action of these vasoconstrictors may facilitate development of contraction observed after SAH. Second, the augmenting action of ET-1 was inhibited by BQ-123, indicating that the actions of this peptide are mediated via the ETA receptor, a receptor subtype involved in cerebral vasospasm. Third, threshold concentrations of ET-1 markedly augmented RhoA translocation induced by OxyHb in CVSM cells and intact basilar arteries, providing evidence that this protein is involved in the effects of ET-1 on the contraction. Fourth, Y-27632 and HA-1077 reversed contraction evoked by OxyHb alone, and ET-1-induced potentiation was abolished by these agents, suggesting that activation of Rho kinase, a downstream target of RhoA, plays a role in this process. Fifth, the selective inhibitors of the PKC isoforms, Ro-32-0432 and GF-109203X, attenuated sustained responses evoked by the concerted action of the vasoconstrictors, implying that PKC also plays a role in the contraction. Finally, threshold concentrations of ET-1 significantly potentiated PKC-ε translocation mediated by OxyHb, further supporting a role for this enzyme in the effects of ET-1 to this phenomenon has not been conclusively established. The findings of the present studies are as follows. 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action of the vasoconstrictors. Together, these findings support the concept that threshold concentration of ET-1 enhances the OxyHb-mediated contraction of cerebral vessels in a RhoA/Rho kinase- and PKC-dependent manner. As shown in the present study, the augmenting action of ET-1 on the contraction is mediated by the ET A receptor, which is coupled to the heterotrimeric G proteins Gq/G11 and G12/G13. The Gq/G11 protein activation leads to the initiation of

Fig. 4. RhoA translocation stimulated by OxyHb in the presence or absence of ET-1 in the rabbit basilar artery. Basilar artery preparations were stimulated with 10 μM OxyHb in the presence or absence of 0.1 nM ET-1 for 10 min (A) or 60 min (B). Cytosolic and membrane lysates were resolved by SDS-PAGE and immunoblotted with a monoclonal anti-RhoA antibody, as described in Fig. 3 legend. Representative Western blots illustrate RhoA translocation induced with OxyHb alone and in the presence of ET-1. RhoA translocation is expressed as percentage of control. Values are means ± SE for 4 experiments conducted using basilar arteries from 8 rabbits. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control; ##P < 0.01 vs. OxyHb alone.

Fig. 5. Effects of PKC inhibitors GF-109203X and Ro-32-0432 on contractions evoked by OxyHb in the presence or absence of threshold concentration of ET-1. GF-109203X and Ro-32-0432 were administered after maximum response to vasoconstrictors had developed (arrows). Representative traces show effects of GF-109203X and Ro-32-0432 on contraction induced by OxyHb alone (A and B, respectively) or in the presence of ET-1 (D and E, respectively). C and F: effects of GF-109203X and Ro-32-0432 on contraction induced by OxyHb in the absence (C) or presence (F) of ET-1. Results are expressed as percentage of maximum tonic response. Values are means ± SE from 23 basilar artery ring preparations from 9 animals. *P < 0.05 and **P < 0.01 vs. control.
endothelin enhances vasoconstrictor effect of hemoglobin

ET-1 is suggested in the present studies by the observations showing that ET-1 increases Ca\(^{2+}\) mobilization and myosin light chain (MLC) kinase (MLCK) activation, whereas stimulation of G\(_{12}/G_{13}\) proteins activates RhoA and its downstream target Rho kinase (5, 28). In the present study, we have shown that the major mechanism of the potentiation mediated by ET-1 is activation of the RhoA/Rho kinase pathway. This pathway has been implicated in the development of coronary and cerebral vasospasm (16, 26), and there is evidence that the Rho kinase inhibitors Y-27632 and HA-1077 attenuate experimental and clinical vasospasm (25, 26, 31). Furthermore, our previous study has shown that OxyHb, a major causative factor in vasospasm, promotes a prolonged activation of the RhoA/Rho kinase pathway in CVSM, with the time course corresponding to that for cerebral vasospasm induced by this agent (42), suggesting that this process is an important step in the signaling cascade underlying the development of cerebrovascular spasm. Rho kinase has been implicated in Ca\(^{2+}\)-sensitization of contraction mediated through the inhibition of MLC phosphatase (MLCP) (6). This action leads to a prolonged increase in the phosphorylation of MLC and subsequent smooth muscle contraction at a constant level of intracellular free Ca\(^{2+}\) (35).

The involvement of Rho kinase in the augmenting effects of ET-1 is suggested in the present studies by the observations that Y-27632 and HA-1077 reversed the contraction of isolated basilar artery rings with IC\(_{50}\) values corresponding to those for the purified enzyme (25, 38). Although both agents also inhibit PKC and MLCK activity, their affinity for Rho kinase is ~10 times higher than that for PKC-ε and ~200 and 2,000 times higher than that for Ca\(^{2+}\)-dependent PKC isoforms and MLCK, respectively (25, 38). Therefore, it is unlikely that the concentrations of the inhibitors used in the present studies affected kinase activity of PKC or MLCK. We recently showed that delayed administration of low concentrations of Y-27632 and HA-1077 was effective in attenuating the sustained cerebrovascular constriction induced by OxyHb via the RhoA/Rho kinase pathway (42). These latter studies and our present observations that ET-1-induced augmentation of the responses to OxyHb is also inhibited by Y-27632 and HA-1077 suggest that beneficial effects of Rho kinase inhibitors observed in the course of cerebral vasospasm could result, at least in part, from inhibition of contraction triggered by the concerted action of the vasoconstrictors acting via the RhoA/Rho kinase pathway. These observations also suggest that the vasoconstrictors utilize a common mechanism that involves the Rho kinase-mediated Ca\(^{2+}\)-sensitization of contraction through inhibition of MLCP in CVSM. Consistent with this suggestion are recent findings showing that ET-1 increases Ca\(^{2+}\) sensitivity of vascular contraction through inhibition of MLCP and a subsequent
increase in MLC phosphorylation at a constant level of intracellular Ca\(^{2+}\) (19, 20, 27).

Although Rho kinase effects on Ca\(^{2+}\) sensitization of smooth muscle contraction are believed to be primarily dependent on RhoA activation (6, 35), there is evidence in favor of a role for the sphingosylphosphorylcholine/Rho kinase pathway in this process (33, 37). However, evidence that this novel pathway plays a role in the augmenting action of ET-1 in CVSM is lacking, and its potential involvement in this phenomenon remains to be established.

Although the RhoA/Rho kinase pathway is an excellent candidate to be involved in the augmenting effects of ET-1, our studies suggest that PKC also may be involved. Earlier studies demonstrated that phorbol esters, potent activators of PKC, induced angiographic cerebral vasospasm in animal models (24) and that PKC inhibitors ameliorated experimental vasospasm after SAH (11, 14). Furthermore, PKC activity was shown to increase with progression of angiographic vasospasm in canine cerebral arteries, suggesting a role for this enzyme in cerebral vasospasm (11).

The involvement of PKC in the augmenting effects of ET-1 is suggested on the basis of findings with the selective inhibitors of PKC isoforms, Ro-32-0432 and GF-109203X. Both inhibitors reversed sustained contraction of basilar artery rings produced by OxyHb in the presence or absence of threshold concentration of ET-1, indicating that, in addition to the RhoA/Rho kinase pathway, PKC may be involved in the contraction.

Furthermore, our studies using the PKC isoform-specific antibodies and quiescent CVSM cells have shown that PKC-\(\epsilon\) is a major isoform involved in the potentiating effects of ET-1. The augmenting effects of ET-1 on PKC-\(\epsilon\) activation, as assessed by translocation of this enzyme to the membranes, were observed only during the first 60 min of stimulation, suggesting that ET-1-mediated potentiation of this process may contribute to the early phase of vasoconstriction in response to OxyHb but may not be essential for the chronic effects of this peptide. The observation that OxyHb alone produced a sustained elevation of PKC-\(\epsilon\) in the membranes of CVSM cells implies that persistence of contraction in response to this agent may be due, at least in part, to a prolonged activation of this enzyme.

Although we found that stimulation of CVSM cells with OxyHb after exposure to ET-1 did enhance PKC-\(\alpha\) translocation, much evidence pointed away from an elevation of the enzyme activity as the primary mechanism of the vasoconstriction. First, the degree of stimulation of PKC-\(\alpha\) translocation in CVSM cells was modest compared with that of PKC-\(\epsilon\). Second, Ro-32-0432 inhibited the sustained contraction at the concentrations corresponding to those for the inhibition of PKC-\(\epsilon\) activity (43). Inasmuch as the IC\(_{50}\) for the Ro-32-0432-mediated inhibition of the classical PKC isoforms is markedly lower than that for PKC-\(\epsilon\) (43), participation of these isoforms in the contraction seems unlikely. Also, G\(_{o}\)6976, a selective inhibitor of classical, but not Ca\(^{2+}\)-independent, isoforms of PKC (8, 34), had little effect on the contraction, further suggesting that classical enzymes, in particular PKC-\(\alpha\), may not be involved in the augmenting effects of ET-1.

How PKC activation may contribute to the contraction is unclear, but an important step may be the inhibition of MLCP. It has been recently shown that PKC modulates Ca\(^{2+}\) sensitivity of contraction through an indirect inhibition of MLCP mediated by CPI-17 (a 17-kDa PKC-potentiated phosphatase inhibitor protein), a smooth muscle-specific protein for the phosphatase (3, 17). Phosphorylation of CPI-17 at Thr\(^{38}\) by PKC increases the inhibitor potency of this protein and results in inhibition of MLCP activity, thereby promoting vascular contraction (17, 29). There is evidence to indicate that ET-1 increases phosphorylation of MLCP (20, 27, 30) and CPI-17 in vascular smooth muscle at a constant level of Ca\(^{2+}\) (30). In the latter study, phosphorylation of CPI-17 mediated by ET-1 was inhibited by GF-109203X and that of MLCP by the Rho kinase inhibitors, suggesting that Rho kinase and PKC play a role in the action of this peptide. As we have shown in the present studies, the augmenting effects of ET-1- and OxyHb-mediated contraction arise from activation of the RhoA/Rho kinase pathway and PKC-\(\epsilon\). It is, therefore, conceivable that these effects involve an increase in Ca\(^{2+}\) sensitivity of CVSM mediated via inhibition of MLCP that is regulated by the RhoA/Rho kinase pathway and PKC-\(\epsilon\). This would explain the effectiveness of the inhibitors of Rho kinase and PKC in attenuation of experimental cerebral vasospasm.

In conclusion, the present study provides evidence that threshold concentrations of ET-1 significantly enhance the magnitude and rate of contraction triggered by OxyHb, suggesting that the concerted action of these vasoconstrictors could facilitate the development of pathological contraction that occurs during the course of cerebral vasospasm. Enhancement of the contraction arises from augmentation of the RhoA/Rho kinase and PKC activation mediated via the ET\(_A\) receptor implicated in the development of cerebral vasospasm. Inhibition of either signaling pathway results in abrogation of the potentiation and suppression of contraction in response to OxyHb alone, suggesting that beneficial effects of the inhibitors of Rho kinase and PKC observed in experimental SAH models result from inhibition of vasoconstrictor-mediated signaling in CVSM.

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