Cerebral vasospasm is a serious complication of subarachnoid hemorrhage that frequently arises from rupture of a cerebral aneurysm, but the pathogenesis of this condition is still far from clear. A large body of evidence points to oxyhemoglobin (OxyHb) as a major causative component of blood clot responsible for vasospasm. Therefore, the present studies were conducted to explore whether the Rho/Rho kinase and PKC may be involved in a sustained vasoconstriction induced by OxyHb in cerebral arteries. OxyHb evoked sustained vasoconstriction in the endothelium-denuded rabbit basilar arteries, which was reversed by the selective inhibitors of Rho kinase, Y-27632, and HA-1077, with the IC50 values of 0.26±0.02 and 0.74±0.1 μmol/L, respectively. In quiescent cerebrovascular smooth muscle (CVSM) cells, OxyHb induced Rho translocation, as assessed by immunoblotting, with a time course, which paralleled the contractile action of OxyHb. Rho translocation was also observed in intact arteries stimulated with OxyHb for 24 hours (219%) and 48 hours (160%). The increase in Rho translocation was fully inhibited by GGTI-297, an inhibitor of Rho prenylation. OxyHb also caused significant translocation of both PKCα and PKCε (P<0.01), which was maximal at the time corresponding to maximal tension developed in response to OxyHb. Ro-32-0432, an inhibitor of PKC, attenuated vasoconstriction mediated by OxyHb in basilar artery. These results show, for the first time, that OxyHb-mediated signaling in CVSM utilizes the Rho/Rho kinase and PKC-based mechanisms.

Key Words: hemoglobin ■ vasospasm ■ smooth muscle ■ Rho kinase ■ protein kinase C
17 kDa), an inhibitor protein involved in the regulation of MLCP. Phosphorylation of CPI-17 by PKC inhibits MLCP activity and subsequently maintains the vasoconstriction by sustained phosphorylation of MLCP. Although there is considerable evidence in support of a role for PKC, it is now recognized that the monomeric G protein Rho and its downstream target Rho-kinase can participate in sustained vasoconstriction by phosphorylating and inhibiting myosin binding subunit of MLCP. 

Rho kinase has been proposed to play a role in a variety of vascular smooth muscle disorders including hypertension, coronary and cerebral vasospasm. There is recent evidence that phosphorylation of MLCP increases after experimental hemorrhage and that this increase is associated with concomitant increase in Rho kinase activity. Furthermore, HA-1077, the selective inhibitor of Rho kinase, attenuates cerebral vasospasm in experimental animals and humans. Together, these observations imply that Rho/Rho kinase pathway plays a role in the pathogenesis of cerebral vasospasm. However, to date, there is no information available regarding the mechanism by which SAH activates the Rho/Rho kinase or PKC pathways. Moreover, it remains unexplored whether Rho/Rho kinase pathway plays a role in the OxyHb-mediated cerebral vasospasm. Therefore, the goal of the present investigation was to determine the possible involvement of PKC and Rho/Rho kinase in the sustained vasoconstriction mediated by OxyHb in cerebral arteries.

Materials and Methods

Tissue Preparation

All procedures were performed according to protocols approved by the University of Alberta Animal Care Committee. New Zealand rabbits were killed by an intravenous overdose of pentobarbital, and the basilar artery was quickly removed to a dissection dish filled with oxygenated Krebs-Henseleit buffer (KHB). Each artery was cut into sections about 3 mm in length and attached to a force transducer (Grass FT.03) connected to a Grass Polygraph model 7D. Ring preparations were equilibrated for 1 hour under a resting tension of 10 mN. This value was chosen on the basis of prior experiments to set a tissue length in the optimal range for maximal force generation (Lo). Viability was determined by maximal force response to high KCl (60 mmol/L).

Cerebrovascular Smooth Muscle Cell Culture

Cells were prepared as previously published. Briefly, basilar arteries were isolated under sterile conditions and placed in a Petri dish containing Dulbecco’s modified Eagle medium (DMEM). The vessels were cut into segments and the endothelium was removed mechanically. The explants were transferred to culture flasks containing DMEM supplemented with 10% calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The confluent cerebrovascular smooth muscle (CVSM) cells were routinely subcultured at a split ratio of 1:3. The cells were tested positive for smooth muscle α actin and demonstrated the typical hill-and-valley pattern.

Immunoblotting

PKC isozyme expression and translocation were measured by immunoblot analysis with isofrom-specific antibodies using rat brain (PKCα, β, γ, δ, ε, ζ) and Jurkat cell (PKCθ) lysates as positive controls for antibody specificity (BD Transduction Laboratories). Rho expression was examined using a mouse monoclonal anti-Rho antibody (Santa Cruz Biotechnology). Total cellular proteins were obtained by homogenization of basilar arteries or cultured cells in sample buffer containing the following: (in mmol/L) Tris-HCl (pH 7.5) 50, EDTA 5, EGTA 5, sodium orthovanadate 1; and (in μg/mL) phenylmethylsulphonyl fluoride 50, leupeptin 10, aprotinin 10, and pepstatin 10. Protein-matched samples were electrophoresed by SDS-PAGE, transferred to nitrocellulose membranes, and blocked with nonfat milk. Equal lane loading was confirmed by inspection of the membranes after reversible Ponceau staining. Blots were then incubated with a primary antibody followed by HRP-labeled goat anti-mouse secondary antibody. The bands were detected on film using an Epson perfection 636 scanner with Epson Twain scanning software. The density of bands was measured using Sigma-Gel software (Jandel Corp).

In the experiments in which Rho and PKC isozyme translocation was measured, the cytosolic and particulate fractions of total cellular contents were separated by centrifugation as described previously.

Solutions and Materials

KHB contained (in mmol/L) 120 NaCl, 5.9 KCl, 2.5 CaCl2, 1.2 MgCl2, 25 NaHCO3, 1.2 NaH2PO4, and 11 dextrose at pH 7.4 when bubbled with 95% O2/5%CO2.

The following drugs were used: hemoglobin (Sigma), Ro-32-0432, HA-1077, and GGTI-297 (Calbiochem). Hemoglobin obtained commercially was converted before use to OxyHb (about 96% of OxyHb) as previously described. Y-27632 was generously provided by the Wellside Corporation (Osaka, Japan). All other reagents were of analytical grade.

Statistics

All values are expressed as mean ± SE. The results were analyzed using ANOVA. Differences were considered significant when values were P < 0.05.

Results

Inhibition of Rho Kinase Activity Reverses Cerebral Vasoconstriction Induced by OxyHb

The concentration of OxyHb in the blood clot after subarachnoid hemorrhage is in the high micromolar range, thus, a concentration of 10 μmol/L OxyHb was used in our experiments. This treatment caused a slowly developing sustained contraction of the endothelium-denuded ring preparations of rabbit basilar arteries, which reached a plateau at about 20 minutes and was maintained for several hours. To assess the involvement of Rho kinase in the sustained vasoconstriction induced by OxyHb, we examined the effects of two selective inhibitors of Rho kinase, Y-27632 and HA-1077. To mimic the situation in vasospasm, in which therapeutic agents are usually administered after vasospasm had developed, cumulative concentrations of Y-27632 and HA-1077 were administered to preparations, in which a tonic response to OxyHb had developed. Both Y-27632 and HA-1077, potently inhibited OxyHb-induced sustained contraction, causing a concentration-dependent relaxation to the basal force with the IC50 values of 0.26 ± 0.02 and 0.74 ± 0.135 μmol/L, respectively (Figures 1A through 1D). Y-27632 administered at a single concentration of 1 μmol/L rapidly reversed contraction, thus demonstrating that contractile activity of OxyHb could be abolished by inhibition of Rho kinase (Figure 1B).

OxyHb Stimulates Membrane Targeting of Rho

Recruitment of Rho to the plasma membrane is crucial for activation of Rho GTP-ase activity and subsequent activation of Rho kinase. We investigated, therefore, in quiescent CVSM cells, the distribution of Rho mediated by OxyHb (10 μmol/L). In these experiments, cultured CVSM cells were...
exposed to OxyHb for 10, 30, 60 minutes, and 3 hours, and then subjected to SDS-PAGE followed by Western blotting using the anti-Rho monoclonal antibody. The expression of Rho was determined in both cytosolic and membrane fractions. The increase in Rho level in the membrane fraction (M) peaked after 30 minutes of stimulation (241% of control; $P<0.001$), it remained significantly elevated after 60 minutes (196% of control; $P<0.01$), and declined after 3 hours (147% of control; $P<0.01$) (Figure 2). The increases were associated with concomitant decreases of Rho in the cytosolic fractions (86%, 86%, and 119% after 30, 60, and 3 hours of stimulation, respectively). The time course of the changes in translocation of Rho in cultured CVSM cells, therefore, closely corresponds to the development of sustained contraction induced by OxyHb in cerebral artery preparations.

Similar studies were performed in intact basilar artery preparations exposed to OxyHb for 10, 30, 60 minutes, and 3 hours, and then subjected to SDS-PAGE followed by Western blotting using the anti-Rho monoclonal antibody. The expression of Rho was determined in both cytosolic and membrane fractions. The increase in Rho level in the membrane fraction (M) peaked after 30 minutes of stimulation (241% of control; $P<0.001$), it remained significantly elevated after 60 minutes (196% of control; $P<0.01$), and declined after 3 hours (147% of control; $P<0.01$) (Figure 2). The increases were associated with concomitant decreases of Rho in the cytosolic fractions (86%, 86%, and 119% after 30, 60, and 3 hours of stimulation, respectively). The time course of the changes in translocation of Rho in cultured CVSM cells, therefore, closely corresponds to the development of sustained contraction induced by OxyHb in cerebral artery preparations.

Similar studies were performed in intact basilar artery preparations exposed to OxyHb. The rings were incubated in serum-free DMEM at 37°C in the presence or absence of OxyHb for 24 and 48 hours, and the Rho translocation was examined as described above. As shown in Figure 3, OxyHb significantly ($P<0.01$) increased Rho translocation after 24 (219%) and 48 hours (160%) of exposure.

Rho proteins are major substrates for posttranslational modification by isoprenylation, which is necessary for translocation of inactive Rho to the plasma membrane. Therefore, to assess the role of isoprenylation in the OxyHb-mediated Rho translocation, CVSM cells were exposed to a selective,
Contractile Action of OxyHb Involves PKC Activation

To determine if PKC may play a role in sustained vasoconstriction induced by OxyHb, we examined the effect of a bisindoylmaleimide inhibitor of PKC, Ro-32-0432, which exhibits several fold greater selectivity for the classical PKC isoforms over PKCe. As shown in Figure 5, Ro-32-0432 administered at the concentrations twice as high as the IC50 values for the PKC (18 and 56 nmol/L for PKCα, PKCβ, and PKCe, respectively), produced a concentration-dependent attenuation of sustained contraction evoked by 10 μmol/L OxyHb, which was 85±5%, 57±13% (P<0.05), and 10±6% (P<0.01) of control, respectively. Ro-32-0432 at 360 nmol/L was found to significantly block contractions evoked by OxyHb, thus indicating that PKCe may play a major role in the OxyHb-mediated sustained vasoconstriction. Representative experiments are shown in Figure 5A, and the results from multiple experiments are shown in Figure 5C. To further examine whether PKC is involved in the sustained phase of contraction, we assessed the effect of Ro-32-0432 on contraction induced by phorbol myristate acetate (PMA) (160 nmol/L), a potent PKC activator. Ro-32-0432 (360 nmol/L) produced significant (P<0.01) relaxation of the PMA-induced contraction, suggesting that PKCe was the predominant isoform involved in the effects of PMA (Figure 5B). The contractile action of PMA was unaltered by either Y-27632 or HA-1077, applied at the range of concentrations that markedly reduced contraction to OxyHb, indicating that PKC was not activated by Rho kinase (results not shown).

PKC Isoform Expression in Cerebrovascular Smooth Muscle in the Absence of Stimuli

Because PKC represents a large family of related isoenzymes, we performed analysis of the PKC isoform expression in basilar artery homogenates using Western blotting techniques with specific antibodies against individual PKC isoforms. Positive controls for all the antibodies were performed with either rat brain lysate (PKCs α, β, γ, δ, ε, and λ) or Jurkat cell lysate (PKCθ). These studies have demonstrated that rabbit basilar artery expresses both the Ca2+-dependent PKC isoforms (PKCα and PKCβ) as well as the Ca2+-independent PKC enzymes (PKCe, PKCθ, and PKCa) (Figure 6). In these studies, PKCα and PKCe, previously shown to be universally
expressed in tissues, were indeed expressed in basilar artery walls. In contrast, PKCγ and PKCδ were undetectable.

**Temporal Profile of PKC Isoforms Translocation Induced by OxyHb in Cerebrovascular Smooth Muscle Cells**

Because activated classical and novel PKCs translocate to the plasma membrane, a process which is a hallmark of activation, the levels of these enzymes were determined in both cytosolic and membrane fractions of CVSM cells. In these experiments, Western blot analyses were performed using CVSM cells exposed to OxyHb for 10 or 60 minutes. As can be seen in Figures 7 and 8, OxyHb induced the membrane association of both PKCa and PKCe compared with untreated controls. Densitometric analysis has shown that stimulation with OxyHb for 10 or 60 minutes resulted in 114 ± 17% and 173 ± 18% (P < 0.01) increase in the amount of membrane associated PKCa, respectively (Figure 7). Similar increases were observed in membrane-bound PKCe isoform (122 ± 9%; P < 0.05 and 141 ± 14%; P < 0.01, after 10 and 60 minutes of stimulation, respectively) (Figure 8). There were corresponding decreases in the cytosolic fractions after 60 minutes of stimulation (87 ± 4% and 86 ± 8% of control for PKCa and PKCe, respectively), thus confirming that stimulation with OxyHb resulted in spatial translocation of these PKC isoforms.

**Discussion**

Although a variety of compounds released from subarachnoid blood clot produce vasoconstriction, considerable evidence has implicated OxyHb as a major causative factor responsible for the development of cerebral vasospasm. The results presented here demonstrate, for the first time, that (1) the Rho/Rho kinase pathway is involved in OxyHb-mediated sustained cerebral vasoconstriction, (2) the selective inhibitors of Rho kinase, Y-27632 and HA-1077, reverse sustained vasoconstriction triggered by OxyHb, and (3) PKC isoenzymes, PKCa and PKCe, contribute to sustained vasoconstriction, as assessed by the vasodilatory action of a selective inhibitor of these enzymes and by the PKCs translocation.

The involvement of Rho in the contractile effects of OxyHb is documented in the present studies by the finding that the time course of Rho translocation in CVSM cells closely matches the time course of sustained vasoconstriction induced by OxyHb in cerebral arteries. Further support to this notion is provided by the ability of OxyHb to stimulate chronic translocation of Rho in intact arteries. The Rho translocation, an indicator of this protein activation, has been previously exploited in order to determine activation of Rho in response to agonists acting on G-protein–coupled receptors (GPCR) and was correlated with MLCP inhibition and Ca²⁺ sensitization of contractile force. The present study is the first to show that, although the OxyHb-mediated effects
are most likely independent of GPCRs, this compound does stimulate Rho translation and hence its activation in CVSM cells. Further evidence that Rho is involved in the effects of OxyHb is provided by the observations that Rho translocation mediated by OxyHb could be prevented by GGTI-297, a selective inhibitor of geranylgeranyl-transferase, an enzyme that catalyzes prenylation of Rho proteins. The Rho prenylation, which involves the attachment of geranylgeraniol, an isoprenoid intermediate of the cholesterol biosynthesis pathway, is a key step in the activation of Rho proteins. Inhibition of prenylation by geranylgeranyl transferase inhibitors prevents Rho translocation and hence Ca$^{2+}$ sensitization mediated by this protein. Thus, our observation that OxyHb-mediated Rho translocation is sensitive to a selective inhibitor of geranylgeranyl transferase, supports the concept that OxyHb-induced sustained vasoconstriction may involve Rho prenylation and activation, a process associated with Ca$^{2+}$ sensitization of contractile force.

It is now established that an important downstream target of Rho is Rho kinase, which phosphorylates the 130 kDa myosin binding subunit (MBS) of MLCP thereby inhibiting phosphatase activity and promoting an increase in MLCP phosphorylation and smooth muscle contraction. To assess the involvement of Rho kinase in OxyHb-mediated sustained vasoconstriction, we used two selective inhibitors of this kinase, Y-27632 and HA-1077 (fasudil). Both Y-27632 and HA-1077 have been shown to attenuate experimental vasoconstriction. To assess the involvement of Rho kinase in OxyHb-mediated sustained vasoconstriction, we used two selective inhibitors of this kinase, Y-27632 and HA-1077 (fasudil). Both Y-27632 and HA-1077 have been shown to attenuate experimental vasoconstriction.

The mechanism by which OxyHb activates the Rho/Rho kinase pathway is unclear. OxyHb was shown to activate a number of signaling processes, including free-radical generation, elevation of intracellular Ca$^{2+}$, activation of tyrosine kinases and mitogen-activated protein kinases, compatible with a prolonged vascular response. Whether free radical generation, or other actions mediated by OxyHb, may play a role in the activation of Rho/Rho-kinase pathway is presently unknown and it remains to be elucidated.

The involvement of PKC in the OxyHb-mediated vasoconstriction is suggested in the present study based on findings with Ro-32-0432, a selective inhibitor of PKC isoforms, at the concentration previously reported to inhibit novel PKC isoforms, nearly completely inhibited the development of cerebrovascular spasm. It has been demonstrated in a variety of blood vessels that Rho kinase inhibitors decrease the MLCP inhibition and subsequent elevation of MLC phosphorylation, mediated by receptor stimulation or the activation of G-proteins, but have no effect on the transient or sustained increase in intracellular calcium. These observations suggest that sustained elevation of calcium and MLCK activation are not sufficient to cause sustained contraction, unless MLCP is simultaneously inhibited. It has previously been shown that, although OxyHb-stimulated contraction is not mediated by G-protein-coupled receptors, this compound can trigger the sustained elevation of intracellular calcium. It therefore seems likely that prolonged elevation of intracellular calcium mediated by OxyHb in CVSM is not sufficient to produce sustained contraction, and that MLCP inhibition is also required. This contention is supported by the observations that, although the intracellular Ca$^{2+}$ level is elevated in cerebral vasospasm, the vasoconstriction is mediated through the inhibition of MLCP and the Y-27632-sensitive MLC phosphorylation.

The involvement of PKC in the OxyHb-mediated vasoconstriction is suggested in the present study based on findings with Ro-32-0432, a selective inhibitor of PKC isoforms, at the concentration previously reported to inhibit novel PKC isoforms, nearly completely inhibited the sustained contraction, indicating that this group of PKC enzymes may be involved in the late phase of the OxyHb-induced response. The same concentrations of Ro-32-0432 that reversed the sustained responses to OxyHb also reversed the PMA-dependent vasoconstriction, implying that this agent activated novel PKC isoenzymes. The concentrations of Ro-32-0432 reported to inhibit the classical PKC isoforms were less effective, suggesting that classical PKCs may not be essential for sustained contraction.
PKC activation has been associated with the translocation of PKC isoforms to the plasma membrane. This event is required for the functional PKC enzymes. The translocation of both PKCα and PKCe was observed in CVSM cells at the time corresponding to the maximal sustained contraction, pointing toward possible involvement of these isoforms in OxyHb–mediated contraction. Thus, the effects of OxyHb were quite selective, being restricted to only two isoenzymes of PKC expressed in the rabbit basilar artery. Together, these findings imply that regulation of cerebrovascular constriction by OxyHb involves selective activation of PKCe and, to a lesser extent, PKCα. How PKC may initiate vasoconstriction mediated by OxyHb is unclear, but an important step may be the phosphorylation of CPI-17, a potent repressor protein in the regulation of MLCP. It has been shown that agonists and phorbol esters evoke Ca2+ sensitization of smooth muscle contraction through the PKC-dependent phosphorylation of CPI-17, which increases MLC phosphorylation indirectly through the inhibition of MLCP. Furthermore, inhibitors of PKC reduce contraction and CPI-17 phosphorylation caused by GPCR agonists, indicating that CPI-17 functions downstream of PKC. Although there is strong evidence to support a PKC role in Ca2+ sensitization, the involvement of PKC has been questioned on the basis of findings that PKC inhibitors are not always effective in attenuation of Ca2+ sensitization caused by agonists or GTPγS. However, most of the evidence against a role for PKC is derived from permeabilized preparations from which PKC and CPI-17 may be lost.

The mechanism by which OxyHb stimulates PKC activity is unknown, but may involve the kinase oxidation mediated by the free radicals generated in the process of OxyHb oxidation to methemoglobin. This suggestion is supported by the observations indicating that PKC oxidation may be required to clarify the potential relationship between Rho and PKC activation.

Although the precise molecular events leading to activation of the Rho A/Rho kinase pathway and PKC by OxyHb and the relationship between these pathways await further elucidation, the present studies demonstrate, for the first time, that both the Rho/Rho-kinase and PKC pathways contribute to the sustained cerebral vasoconstriction mediated by OxyHb. In conclusion, results from these studies indicate that OxyHb activates the Rho/Rho kinase pathway in intact CVSM, a phenomenon that has not been previously demonstrated. We further demonstrate that the Rho kinase inhibitors, Y-27632 and HA-1077, completely reverse sustained cerebral vasoconstriction mediated by OxyHb. Our results showing that OxyHb-mediated contraction is dependent on both activation of the Rho/Rho kinase pathway and PKC provide new clues to the understanding of the functions of OxyHb and suggest that these signaling events could play a role in the development of cerebral vasospasm.

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Functional Roles of the Rho/Rho Kinase Pathway and Protein Kinase C in the Regulation of Cerebrovascular Constriction Mediated by Hemoglobin: Relevance to Subarachnoid Hemorrhage and Vasospasm
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