Mechanism of RhoA/Rho kinase activation in endothelin-1-induced contraction in rabbit basilar artery

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Miao, Liyan, Yun Dai, and John Zhang. Mechanism of RhoA/Rho kinase activation in endothelin-1-induced contraction in rabbit basilar artery. Am J Physiol Heart Circ Physiol 283: H983–H989, 2002. First published May 9, 2002; 10.1152/ajpheart.00141.2002.—This study was undertaken to demonstrate the role of the RhoA/Rho kinase pathway in endothelin-1 (ET-1)-induced contraction of the rabbit basilar artery. Isometric tension and Western blot were used to examine ET-1-induced contraction and RhoA activation. The upstream effect on ET-1-induced RhoA activity was determined by using ET_a and ET_b receptor antagonists, protein kinase C (PKC), tyrosine kinase, and phosphorylidyinositol-3 kinase inhibitors. The downstream effect of ET-1-induced contraction and RhoA activity was studied in the presence of the Rho kinase inhibitor Y-27632. The effect of Rho kinase inhibitor on ET-1-induced myosin light chain (MLC) phosphorylation was investigated by using urea-glycerol-PAGE immunoblotting. We found 1) ET-1 increased RhoA activity (membrane binding RhoA) in a concentration-dependent manner; 2) ET_a, but not ET_b, receptor antagonist abolished the effect of ET-1 on RhoA activation; 3) phosphodylinositol-3 kinase inhibitor, but not PKC and tyrosine kinase inhibitors, reduced ET-1-induced RhoA activation; 4) Rho kinase inhibitor Y-27632 (10 μM) inhibited ET-1-induced contraction; and 5) ET-1 increased the level of MLC phosphorylation. Rho kinase inhibitor Y-27632 reduced the effect of ET-1 on MLC phosphorylation. This study demonstrated that RhoA/Rho kinase activation is involved in ET-1-induced contraction in the rabbit basilar artery. Phosphodylinositol-3 kinase and MLC might be the upstream and downstream factors of RhoA activation.

cerebral vasospasm; rabbit basilar artery

CEREBRAL VASOSPASM is a leading cause and a frequent complication of the morbidity and mortality in patients with subarachnoid hemorrhage (SAH). Cerebral vasospasm is characterized by a delayed and prolonged contraction of the major cerebral arteries (13, 39). The cause of vasospasm might be vasoactive substances released into the subarachnoid space by the dissolution of the resultant blood clot (14, 32) or by vasoactive agents released from the vessel walls. There is evidence that endothelin-1 (ET-1) is a major cause of cerebral vasospasm after SAH (40). The levels of ET-1 in bloody cerebrospinal fluid are elevated in patients with SAH. ET-1 causes long-lasting vasoconstriction. ET-1 receptor antagonists, endothelin-converting enzyme inhibitors, and ET-1 antisense oligonucleotide prevent cerebral vasospasm in animal models (40).

It is commonly accepted that increased intracellular calcium is a determinant for contraction in vascular smooth muscle in response to agonists (14). Recent studies have shown that most of these agonists are able to modulate contraction by altering myofilament calcium sensitivity or through calcium independent pathways. The involvement of protein kinase C (PKC) (2, 6, 11), tyrosine kinase (18), phosphatidylinositol (PI)-3 kinase (41), and also RhoA/Rho kinase in calcium sensitization has been reported in vascular smooth muscle (5, 20). In addition, cross talk between these different kinase pathways may be the key signaling event of calcium sensitization of the contractile activation of vascular smooth muscle (25). RhoA/Rho kinase is an important pathway to mediate myofilament Ca^{2+} sensitization of smooth muscle cells (16), and RhoA/Rho kinase has been suggested to be involved in cerebral vasospasm (15, 26, 30, 35). However, the possible role of the RhoA pathway in ET-1-induced contraction is not clear. In this study, we investigated the effect of ET-1 on RhoA activation and the types of ET receptors involved, and the implication of PKC, tyrosine kinase, and PI-3 kinase in the mechanisms controlling ET-1-induced contraction was also studied.

MATERIALS AND METHODS

Materials. ET-1 was purchased from Alexis. Staurosporine (PKC inhibitor), genistein (tyrosine kinase inhibitor), and LY-294002 (PI-3 kinase inhibitor) were purchased from BioMol. Y-27632 (Rho kinase inhibitor) was purchased from TOCRIS. Anti-RhoA (26C4), ROKI, and ROKII antibodies were purchased from Santa Cruz Biotechnology. BQ-610, BQ-788, monoclonal anti-myosin (light chain 20K) antibody, and other chemicals were purchased from Sigma Chemical.

Western blot. Thirty male New Zealand White rabbits were anesthetized by an injection of thiopental (20 mg/kg iv) and euthanatized by exsanguination. The basilar arteries were removed from the base of the brain and incubated with ET-1 with different concentrations and for different time periods in the

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Krebs-Henseleit buffer. Some arteries were treated with specific antagonists for 30 min before being treated with ET-1. After treatment, the arteries were immediately frozen in liquid nitrogen. Western blot analysis was performed as previously described (28). Strips of basilar arteries were homogenized in lysis buffer. After the sample was centrifuged at 15,000 g for 1 h to generate membrane and cytosolic fractions, equal amounts of protein from the membrane fractions were loaded into each lane of SDS-12% polyacrylamide gels. The gels were then electrophoresed, transferred to a nitrocellulose membrane, and finally analyzed by Western blotting by using mouse monoclonal anti-RhoA antibody (Santa Cruz, CA). As an internal control, β-actin was blotted in the same membrane.

The Animal Care and Use Committee approved all the procedures using rabbit tissues.

Isometric tension. The basilar arteries were removed and cut into 2-mm rings in a dissecting chamber filled with modified Krebs-Henseleit bicarbonate solution containing (in mM) 120 NaCl, 4.5 KCl, 1 MgSO4, 27 NaHCO3, 1.2 KH2PO4, 2.5 CaCl2, and 10 dextrose and bubbled with 95% O2-5% CO2. We removed endothelial cells by gently rubbing the rings with a steel hook.

Rings were suspended at the resting tension of 400 mg (Radnoti transducer, Radnoti Glass) between stainless steel hooks in 10 water-jacketed tissue baths (Radnoti Glass) in modified Krebs-Henseleit bicarbonate buffer with 95% O2-5% CO2 at 37°C. Rings were incubated for 90 min until a stable rest tension was achieved, and the solution was changed every 20 min to remove metabolites. The tissues were challenged with 60 mM KCl twice at 30-min intervals before the experiment. Isometric force transducers were connected to arterial rings, and contraction was recorded with an eight-channel MacLab 8E and stored on a Power Macintosh computer.

Measurements of myosin light chain phosphorylation. The extent of myosin light chain (MLC) phosphorylation in the rabbit basilar artery strips was measured by glycerol-PAGE followed by electrophoretic transfer of the proteins to a nitrocellulose membrane. The amounts of the phosphorylation form were quantified by immunoblot procedures, as described by Persechini et al. (19).

ET-1- and antagonist-treated rings were frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) cooled with dry ice. Frozen tissues were washed twice with acetone containing 10 mM DTT to remove the TCA and then dried. The dried ring was cut into small pieces and exposed to the urea sample buffer (20 mM Tris, 22 mM glycine, 10 mM DTT, 8.3 M urea, and 0.1% bromophenol blue) for purposes of extraction, and then the proteins were processed for urea-glycerol-PAGE and immu-

Fig. 1. Time- and concentration-dependent effect of endothelin (ET)-1 on the membrane RhoA activity. Left, representative immunoblot band is shown as well as a corresponding β-actin band. Right, graph displaying protein band density (% of control). A: concentration-dependent effect of ET-1 at 1, 10, and 100 nM on the RhoA band density for 10 min. B: time-dependent effect of ET-1 (10 nM) at 5, 10, and 30 min on RhoA band density. Values are means ± SE. * and **P < 0.05 and 0.01, respectively (vs. control, n = 4).

A

<table>
<thead>
<tr>
<th>Control</th>
<th>ET-1 (1nM, 10min)</th>
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B

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<th>Control</th>
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[Graphs showing protein band density (% of control) for different concentrations and times]
moblotting with anti-MLC monoclonal antibody and horse-
radish peroxidase-conjugated second antibody. Quantitative
evaluation of phosphorylated MLC was performed by densi-
tometric analysis using Quantity One software (Bio-Rad).
The percentage of the phosphorylated form in total MLC was
calculated to indicate the extent of MLC phosphorylation.

Statistical analysis. The data were expressed as means ±
SE. Statistical analysis was performed using one-way
ANOVA. Differences were considered to be significant when
the P value was <0.05.

RESULTS

ET-1 increased RhoA activity in the rabbit basilar
artery. Membrane binding RhoA protein levels were
measured in the basilar arteries treated with different
concentrations of ET-1 and at different time periods
(Fig. 1). The amount of the membrane binding RhoA
fraction was markedly enhanced in the concentration-
dependent manner. ET-1 at 10–100 nM significantly
increased RhoA activity (P < 0.01). When the rings
were treated with 10 nM ET-1 for different time peri-
ods, RhoA activity was increased but not in a time-
dependent fashion.

ET_A and ET_B receptor antagonist on ET-1-induced
RhoA activation. Figure 2 shows that ET_A receptor
antagonist BQ-610 (1 μM), but not ET_B antagonist
BQ-788 (1 μM), completely abolished ET-1-induced
RhoA activation (P < 0.01).

PKC, tyrosine kinase, and PI-3 kinase inhibitors on
ET-1-induced RhoA activation. Figure 3 shows that the
PI-3 kinase inhibitor LY-294002 (50 μM), but not the
PKC and tyrosine kinase inhibitors staurosporine (30
nM) and genistein (100 μM), significantly inhibited the
ET-1-induced RhoA activation (P < 0.05).

Rho kinase inhibitor on ET-1-induced contraction
and RhoA/Rho kinase activities. The effect of Y-27632
on RhoA, ROK_I, and ROK_II activities (on the mem-
brane) was measured. Y-27632 (10 μM) significantly
inhibited ROK_I and ROK_II (P < 0.05) but not RhoA
activities (P > 0.05) (Fig. 4).

ET-1 produced a concentration-dependent contrac-
tion in the rabbit basilar arteries. Preincubation of the
rings with Rho kinase inhibitor Y-27632 (10–50 μM)
significantly inhibited ET-1-induced contraction (P <
0.01), even though the effect of Y-27632 was more
pronounced at lower ET-1 concentrations (1–10 nM)
(Fig. 5).

Rho kinase inhibitor on ET-1-induced MLC phos-
phorylation. The extent of MLC phosphorylation at the
resting level (without stimulation) was 25.3 ± 3.1%
ET-1 (10 nM, for 10 min) increased the extent of MLC phosphorylation to 43.6 ± 1.2% (n = 3, P < 0.05). Y-27632 (10 μM) abolished the effect of ET-1 on MLC phosphorylation and reduced the level to 15.9 ± 3.6%. Similarly, Y-27632 (10 μM) reduced ET-1-induced contraction from 122.3 ± 7.82% to 78.1 ± 11.2% (Fig. 6).

**DISCUSSION**

The following observations were made: first, ET-1 produced RhoA activation in the rabbit basilar artery by activation of ETA, not by ETB receptors; second, the upstream effect on ET-1-induced RhoA activation might involve PI-3 kinase but not PKC or tyrosine kinase; and finally, the downstream effect of ET-1-induced RhoA activation involves Rho kinase and MLC phosphorylation.

**ET-1 and RhoA/Rho kinase pathway in cerebral vasospasm.** Current clinical and experimental investigations support the hypothesis that ET-1 is a major cause of cerebral vasospasm after SAH. ET-1 level was found to increase significantly in the cerebrospinal fluid and plasma of patients after SAH (33). ET-1 causes long-lasting vasoconstriction, similar to cerebral vasospasm following SAH, and the constrictor effect of ET-1 was enhanced after SAH (40). Endothelin receptor antagonists, endothelin-converting enzyme inhibitors, and ET-1 antisense oligonucleotide can prevent cerebral vasospasm after SAH in animal models (40).

ET-1-induced contraction might be mediated by an elevation of intracellular Ca^{2+} and by an increased Ca^{2+} sensitivity. RhoA/Rho kinase pathway plays a very important role in the Ca^{2+} sensitivity in cerebral arteries. Ca^{2+} activates MLCK kinase to increase MLC
phosphorylation. Activated RhoA appears to inhibit MLC phosphatase activity via Rho kinase and to increase the level of MLC phosphorylation (16, 17). There are some evidences that RhoA/Rho kinases are involved in cerebral vasospasm. In a rat double hemorrhage model, RhoA and Rho kinase mRNA levels were upregulated in the rat spastic basilar artery on day 7 (15). Fasudil hydrochlorides and hydroxyfasudil, non-specific inhibitors for Rho kinase, reduced cerebral vasospasm after SAH in experimental animals and in humans (30, 35). Furthermore, the specific Rho kinase inhibitor Y-27632 reversed cerebral vasospasm in a dog double hemorrhage model (26). In the present study, we found that ET-1 activates RhoA, which leads to the activation of Rho kinase and MLC phosphorylation in the rabbit basilar artery.

RhoA/Rho kinase pathway in ET-1-induced contraction. The vasoactive function of ET-1 is mediated by three different receptor subtypes: ETA, ETB1, and ETB2. The ETA receptor subtype is located in vascular smooth muscle cells and mediates the vasocontractive effect of endothelins (1, 24). The ETB1 receptor subtype is present in vascular endothelial cells and mediates endothelium-derived relaxation (21). The ETB2 receptor subtype is located in smooth muscle cells and causes vasoconstriction (3, 4, 29, 36). The relative subtype distribution varies depending on the vessel type. In cerebral arteries, the ETA receptor subtype predominates, whereas ETB is responsible for the remaining contraction (10, 41). Our results showed that ET-1-induced RhoA activation was blocked by a ETA antagonist but not by a ETB antagonist, indicating ET-1-induced RhoA activation was mediated by ETA receptors, even though the possible contribution from an ETB1 receptor cannot be excluded.

Translocation of RhoA from the cytosol to the membrane of the cells represents activation of RhoA. In the present study, we used Western blots to measure RhoA concentration on the membrane to represent RhoA activity. Our results (Fig. 1) showed that 1 nM concentration of ET-1 had a tendency to increase RhoA activity, which could not induce contraction (Fig. 5), whereas 10 and 100 nM ET-1 could significantly increase RhoA activity, which also could induce vascular contraction. Time response (5, 10, and 30 min) for

Fig. 5. Effect of Rho kinase inhibitor on ET-1-induced contraction. A: original trace of Rho kinase inhibitor Y-27632 on ET-1-induced concentration-dependent contraction. Solid curve is ET-1-induced contraction in the absence of Y-27632. Dotted curve is in the presence of 10 μM Y-27632 and medium dash curve is in the presence of 50 μM Y-27632. B: summary of the effect of Y-27632 on ET-1-induced contraction. Rings were incubated in Y-27632 at 10 μM or 50 μM for 30 min before application of ET-1. Values are means ± SE. * and **P < 0.05 and 0.01, respectively (vs. control, n = 5).

Fig. 6. Effect of Rho kinase inhibitor on myosin light chain (MLC) phosphorylation and force in ET-1-induced contraction. A: representative photograph of MLC immunoblot. B: summary of the MLC phosphorylation and force generation either at the resting level, induced by ET-1 (10 nM) alone for 10 min, or induced by ET-1 (10 nM) in the presence of Y-27632 (10 μM, 30 min preincubation) for 10 min. Values are means ± SE. * and **P < 0.05 and 0.01, respectively (vs. control, n = 4).
ET-1-induced RhoA activation also was shown from our results. These suggested that ET-1 increased RhoA activity of the rabbit basilar artery.

PI-3 kinase has been reported to be involved in the ET-1-induced contraction because ET-1 increased PI-3 kinase activity (38) and the PI-3 kinase inhibitor inhibited ET-1-induced contraction in the rabbit basilar artery in one of our previous publications (41). Our results showed that PI-3 kinase inhibitor LY-294002 significantly inhibited ET-1-induced RhoA activation, suggesting that PI-3 kinase is upstream of RhoA in ET-1-induced basal artery contraction, which is consistent with other reports (22). Tyrosine kinase inhibitor genistein failed to inhibit RhoA activation in the present study, which suggests that RhoA activation by ET-1 is independent of tyrosine kinase. This result is consistent with the observations of Wang and Bitar (38) and Sakurada et al. (23) that tyrosine kinase is not involved in ET-1-induced RhoA activation in the rabbit aorta and rectosigmoid smooth muscle. There are evidences that RhoA regulates Ca\(^{2+}\) sensitivity in smooth muscle via PKC-mitogen-activated protein kinase pathway or through a PKC-mediated effect on MLC phosphatase (8, 9). We tested this hypothesis in an ET-1-induced contraction and found that strausporine, a PKC inhibitor, failed to inhibit RhoA activation, suggesting that RhoA might be upstream of PKC. This view was supported by an observation that Rho inhibitors block PKC translocation/activation in endothelial or epithelial cells, suggesting a RhoA requirement for PKC activation and translocation (7).

RhoA-induced vascular contraction is mediated by its downstream Rho kinase. We found that Y-27632 markedly inhibited ET-1-induced contraction in the rabbit basilar artery. Because the inhibitory effect of Y-27632 was mainly on the contraction induced by lower concentrations of ET-1, other mechanisms could be involved in ET-1-induced contraction. Vascular contraction results from MLC phosphorylation, which is the last step of the intracellular signal transduction pathway for smooth muscle contraction. Activation of Rho kinase inhibits MLC phosphatase, thus increasing the level of phosphorylated MLC. We measured both the MLC phosphorylation and the force generated by ET-1 in the rabbit basilar artery. ET-1 increased MLC phosphorylation and force at the same time. RhoA kinase inhibitor Y-27632 inhibited ET-1-induced MLC phosphorylation and correspondingly decreased the force.

The apparent involvement of the RhoA/Rho kinase pathway in mediating ET-1-induced contraction suggests this pathway could be an important target for further drug development. There are suggestions that the Rho kinase inhibitor might be useful in the treatment of hypertension (37), coronary artery spasm (12, 31), and cerebral vasospasm following SAH (26, 34), as well as neointimal proliferation in arteries subjected to injury by balloon angioplasty (27). The combination of the relaxant and antiproliferative effect of RhoA/Rho kinase inhibitors should confer significant therapeutic benefit.