Mechanism of Endothelin-1–Induced Contraction in Rabbit Basilar Artery

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Background and Purpose—Endothelin-1 (ET-1) is suggested to be a major cause of cerebral vasospasm after subarachnoid hemorrhage. However, the mechanism of ET-1–induced contraction in cerebral arteries remains unclear. This study was undertaken to demonstrate the possible role of protein tyrosine kinase (PTK), mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) in ET-1–induced contraction.

Methods—PD-98059, damnacanthal, wortmannin, AG-490, genistein, calphostin C, and staurosporine were used to inhibit, or relax, the ET-1–induced contraction of basilar artery, studied with an isometric tension system. Immunoprecipitation of MAPK in ET-1–stimulated rings of basilar artery without or with the above inhibitors was studied with Western blot.

Results—(1) ET-1 produced concentration-dependent contraction and MAPK immunoprecipitation in rabbit basilar artery by activation of ET_a but not ET_b receptors. (2) MAPK inhibitors PD-98059 and U-0126 produced dose-dependent inhibition of ET-1–induced contraction. (3) The Src tyrosine kinase inhibitor damnacanthal, the phosphatidylinositol-3 kinase inhibitor wortmannin, and the Janus tyrosine kinase_2 inhibitor AG-490 abolished ET-1–induced contraction. (4) The PKC inhibitor staurosporine but not calphostin C abolished ET-1–induced contraction, and the PTK inhibitor genistein partially reduced ET-1–induced contraction. (5) In arteries precontracted by ET-1, PD-98059, U-0126, wortmannin, AG-490, genistein, and staurosporine produced concentration-dependent relaxation. (6) ET-1 induced a biphasic and time-dependent MAPK immunoprecipitation. (7) PD-98059, U-0126, genistein, AG-490, and damnacanthal, but not staurosporine or wortmannin, abolished the effect of ET-1 on MAPK immunoactivity.

Conclusions—This study demonstrated that MAPK may be involved in ET-1–induced contraction in rabbit basilar artery. MAPK is downstream of PTK, Src, and Janus tyrosine kinase pathways but may not be downstream of phosphatidylinositol-3 kinase pathways. The possible involvement of PKC in ET-1–induced contraction requires further investigation. Inhibition of these pathways may offer alternative treatment for ET-1–induced contraction and cerebral vasospasm. (Stroke. 2000;31:526-533.)

Key Words: cerebral vasospasm ■ endothelins ■ protein kinases ■ rabbits

Endothelin-1 (ET-1) is a major cause of cerebral vasospasm after subarachnoid hemorrhage (SAH).^1^ The level of ET-1 in bloody cerebrospinal fluid is elevated in patients with SAH. ET-1 produces a potent and long-lasting vasoconstriction of cerebral arteries. In animals, intracisternal injection of ET-1 induces cerebral vasospasm that resembles the vasospasm in humans. Endothelin receptor antagonists, endothelin-converting enzyme inhibitors, and ET-1 antisense oligonucleotide prevent cerebral vasospasm in animal models.

However, the mechanism of ET-1–induced contraction of cerebral arteries remains unclear. In peripheral arteries, ET-1 has been reported to act as an agonist for the G-protein–coupled receptors and the growth factor receptors. Thus, signals from both pathways may be involved in ET-1–induced contraction. We examined the effect of ET-1 in rabbit basilar artery using different inhibitors of protein tyrosine kinase (PTK), mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) as well as MAPK antibody in this study.

Materials and Methods

Materials

PD-98059, damnacanthal, wortmannin, AG-490, genistein, calphostin C, and staurosporine were purchased from BIOMOL. Endothelin-1, BQ-610, and BQ-788 were purchased from Calbiochem-Novabiochem Corp. U-0126 was purchased from Promega. Anti-MAPK (ERK1/ERK2) antibodies were purchased from Zymed Laboratories. Other chemicals were purchased from Sigma.

Isometric Tension

New Zealand White rabbits (n=45), of either sex and weighing 5 to 6 pounds, were anesthetized with an injection of thiopental (20 mg/kg IV) and euthanatized by exsanguination. The basilar arteries were removed and cut into 3-mm rings in a dissecting chamber filled...
with modified Krebs-Henseleit bicarbonate solution, bubbled with 95% O2/5% CO2. No attempt was made to remove endothelial cells. The modified Krebs-Henseleit solution contained the following (mmol/L): NaCl 120, KCl 4.5, MgSO4 1, NaHCO3 27, KH2PO4 1, CaCl2 2.5, and dextrose 10. All procedures were approved by the Animal Care and Use Committee at the University of Mississippi Medical Center.

The rings were suspended at 500 mg resting tension (Radnoti transducer, Radnoti Glass) between stainless steel hooks in 10-mL water-jacketed tissue baths (Radnoti Glass). The tissue bath was filled with modified Krebs-Henseleit buffer and bubbled with 95% O2/5% CO2 at 37°C. Rings were equilibrated for 90 minutes, and the bath solution was changed every 20 minutes. After equilibration, tissues were challenged with KCl (90 mmol/L) 2 times at 30-minute intervals to obtain stable contractions. Only data with recovery of 90% to 110% of the initial contraction by KCl (90 mmol/L) were included. Tension was recorded continuously with a force-displacement transducer, as described previously.2

**Western Blot**

The basilar arteries were removed from the base of the brain and incubated with ET-1 (1 μmol/L) for 1, 3, 5, 10, 30, 60, and 120 minutes in Krebs-Henseleit buffer. Some arteries were treated with specific antagonists for 30 minutes (see Materials) before being treated with ET-1. After treatment, the arteries were immediately frozen in liquid nitrogen. The arteries were homogenized for 20 minutes at 4°C in the following (mmol/L): Tris-HCl (pH 7.5) 50, NaCl 100, EDTA 5, phenylmethylsulfonyl fluoride 1, and IGEPAL CA-630 100 μL. The insoluble materials were removed by centrifugation (13,000g, 10 minutes, 4°C). The samples (30 μg protein) were applied to 12.5% SDS-PAGE. After electrophoretic transfer of the separated polypeptides to nitrocellulose membrane, the membranes were blotted with 8% nonfat milk in Tris-buffered PBS for 1 hour. The membranes were washed with Tris-buffered PBS and incubated at 4°C overnight in a 1:5000 dilution of mouse anti-MAPK antibodies (ERK1/ERK2, monoclonal mouse antibody, Zymed Laboratories). These antibodies recognize both phosphorylated and nonphosphorylated MAPK. The nitrocellulose membranes were later washed with Tris-buffered PBS and incubated with a 1:5000 dilution of goat anti-mouse IgG antibody, linked with horseradish peroxidase. The enhanced chemiluminescence system (Amersham) was used for visualization of protein bands. The results were quantified by SigmaGel software (SPSS Inc).

**Data Analysis**

Data are expressed as mean±SEM. Statistical differences between the control and other groups were compared by 1-way ANOVA and then Tukey’s multiple comparison procedure (95% CI) if significant variance was found. A value of P<0.05 was considered statistically significant.

**Results**

**Effect of ET-1 on Contraction and MAPK Immunoprecipitation**

The initial contraction was observed with 10−9 mmol/L ET-1, and the maximum contraction was not obtained even at 10−6 mmol/L of ET-1 in rabbit basilar artery (Figure 1). Since the maximum contraction to ET-1 was not obtained, we could not calculate the pA2 values.

Preincubation of the arteries with a selective ETα receptor antagonist BQ-610 (10−6 mmol/L) significantly inhibited ET-1–induced contraction (Figure 1A). The selective ETα receptor antagonist BQ-788 (10−6 mmol/L) was used in the same fashion as BQ-610 but failed to reduce the effect of ET-1 (Figure 1B). None of the inhibitors changed the resting tension alone.

Five minutes of treatment with ET-1 (10−6 mmol/L) produced a significant elevation of MAPK immunoprecipitation in the rabbit basilar artery samples (Figure 2). Pretreatment of tissues (30 minutes) with BQ-610 (10−6 mmol/L) but not BQ-788 (10−6 mmol/L) prevented the enhancement of MAPK immunoprecipitation induced by ET-1 (Figure 2).

**Effect of Inhibitors on ET-1–Induced Contraction**

MAPK inhibitors PD-98059 and U-0126 (100 μmol/L), preincubated with arteries for 30 minutes, significantly reduced ET-1–induced contraction (Figure 3) (P<0.005 to P<0.001, ANOVA). At a lower concentration (30 μmol/L), U-0126 but not PD-98059 produced significant inhibition (P<0.05, ANOVA).

The Src tyrosine kinase inhibitor damnacanthal, the phosphatidylinositol-3 kinase (PI-3K) inhibitor wortmannin, and the Janus tyrosine kinase (JAK2 ) inhibitor AG-490 (30 μmol/L) were used in the same manner as MAPK inhibitions and were found to abolish the contractile effect of ET-1 (Figure 4).

The PKC inhibitors calphostin C and staurosporine and the PTK inhibitor genistein were tested. Calphostin C inhibits both phorbol ester binding and phosphotransferase activity of the PKC by binding to the regulatory domain at the diacylglycerol/phorbl ester binding site.3 Staurosporine, a microbrial alkaloid, interacts with the catalytic moiety of the PKC. Calphostin C (5×10−7 mmol/L) failed to reduce ET-1–induced contraction, while staurosporine (2×10−7 mmol/L)
completely abolished it \((P<0.001, \text{ANOVA})\) (Figure 5A and 5B). The PTK inhibitor genistein \((10^{-4} \text{ mmol/L})\), preincubated for 30 minutes, produced partial inhibition of ET-1–induced contraction \((P<0.05, \text{ANOVA})\) (Figure 5C).

In another study the rings of rabbit basilar artery were precontracted with ET-1 \((10^{-6} \text{ mmol/L})\), and once a stable contraction was obtained, cumulative concentrations of PD-98059, U-0126, AG-490, wortmannin, damnacanthal, genistein \((10^{-6} \text{ to } 10^{-4} \text{ mmol/L})\), and staurosporine \((10^{-5} \text{ to } 2 \times 10^{-7} \text{ mmol/L})\) were applied. All agents fully relaxed the sustained contraction induced by ET-1 \((10^{-6} \text{ mmol/L})\) (Figure 6). Calphostin C was not used because it failed to inhibit ET-1–induced contraction (Figure 5A).

**Effect of Inhibitors on MAPK Immunoprecipitation**

Endothelin-1 \((10^{-6} \text{ mmol/L})\) produced a time-dependent MAPK immunoprecipitation in rabbit basilar artery. The effect of ET-1 was observed at 3 minutes, peaked between 5 and 30 minutes, decayed at 60 minutes, and was reelevated at 2 to 4 hours (Figure 7).

In another study inhibitors were applied for 30 minutes before the arteries were treated with ET-1 \((10^{-6} \text{ mmol/L}, \text{5 minutes})\). PD-98059, U-0126, damnacanthal, AG-490 \((3 \times 10^{-5} \text{ mmol/L})\), and genistein \((10^{-4} \text{ mmol/L})\), but not staurosporine \((2 \times 10^{-7} \text{ mmol/L})\) or wortmannin \((3 \times 10^{-5} \text{ mmol/L})\), abolished the effect of ET-1 on MAPK immunoprecipitation (Figure 8).

**Discussion**

We have demonstrated the following: (1) ET-1 produced concentration-dependent contractions and MAPK immunoprecipitation in rabbit basilar arteries by activation of ET\(_A\) but not ET\(_B\) receptors. (2) The effects of ET-1 on contraction and MAPK immunoprecipitation were abolished by PTK-Src-JAK-MAPK inhibitors. PI-3K inhibitor abolished ET-1–induced contraction but not MAPK immunoprecipitation. (3) The contractile effect of ET-1 was probably mediated by multiple signaling pathways, ie, the PTK-Src-JAK-MAPK pathway and the PI-3K pathway, since inhibitors of all these pathways abolished the contractile effect of ET-1. (4) The possible involvement of the PKC pathway in ET-1–induced contraction requires further investigation.

**Role of Endothelin in Cerebral Vasospasm**

The role of ET-1 in cerebral vasospasm has been studied in the following 4 directions: (1) Elevation of ET-1 was found in bloody cerebrospinal fluid from vasospasm patients. The mean concentration of ET-1 in cerebrospinal fluid obtained from SAH patients was significantly higher than in healthy individuals. Plasma ET-1 concentrations at the onset of SAH in the patients with vasospasm were significantly higher than...
those of healthy individuals, and the high levels persisted for 3 to 14 days. 1 (2) ET-1 produced a prolonged contraction of cerebral arteries. In the control group, the arteries were contracted by exposure to ET-1 (10^{-12} to 10^{-6} mol/L) in the absence of damcanthal, wortmannin, and AG-490 in the control rings. In the treated group, damcanthal, wortmannin, and AG-490 were preincubated in separate chambers with arterial rings for 30 minutes before dose-dependent contraction with ET-1. All of these antagonists abolished ET-1–induced contraction. n indicates the number of arterial rings studied. *P<0.05, **P<0.001 (ANOVA).

Signal Transduction Pathways in ET-1–Induced Contraction

The action of endothelin is mediated by 3 different receptor subtypes: ET_{A}, ET_{B1}, and ET_{B2}. The ET_{A} receptor subtype is localized in vascular smooth muscle cells and mediates the vasoconstrictive effect of endothelins. 1 The ET_{B1} receptor subtype is present in vascular endothelial cells and mediates vasospasm after SAH. 1 Antisense oligonucleotide DNA therapy prevented vasospasm in a canine model. 9 Thus, even though some controversial reports indicate a poor correlation between cerebrospinal fluid endothelin levels and vasospasm 10 and the failure of ET_{A} receptor antagonists to reverse vasospasm, 11 the overall picture supports the indication that ET-1 is a major cause of cerebral vasospasm. 1
endothelium-derived relaxation. The ET\textsubscript{B2} receptor subtype is located in smooth muscle cells and causes vasoconstriction.\textsuperscript{1} Signal transduction pathways after ET-1 stimulation were not studied systematically in cerebral tissues.

In peripheral tissues, ET-1 stimulates pp60 c-Src and pp125 focal adhesion kinase activity, resulting in tyrosine phosphorylation of specific cellular proteins.\textsuperscript{12} Activation of c-Src by ET-1 might link G-protein–coupled receptors to the primary downstream targets of nonreceptor PTK. Simonson and Herman\textsuperscript{13} demonstrated that ET-1 caused a marked increase of the level of immunoreactive tyrosine phosphorylated proteins, suggesting that phosphorylation of PTK is a necessary step in the mitogenic response to ET-1. ET-1 activates small GTP-binding protein p21ras and increases the p21 ras-associated PI-3K activity.\textsuperscript{14} Activation of PKC, PTK, PI-3K, and Ras may lead to the activation of MAPK in a variety of cells.\textsuperscript{15}

We have demonstrated in this study that ET-1 produced contraction of rabbit basilar artery by activating ET\textsubscript{A} receptors. Activating ET\textsubscript{A} receptors (G-protein–coupled receptors) leads to the activation of phospholipase C and the generation of inositol 1,4,5-triphosphate and diacylglycerol. Diacylglycerol in turn theoretically activates PKC. Thus, it is not surprising that the PKC inhibitor staurosporine abolished the contractile effect of ET-1 in this study. Similar findings were reported in rat basilar and middle cerebral arteries in that H-7, another PKC inhibitor, significantly reduced ET-1–induced contraction, indicating that ET-1 produces contractions by activation of PKC.\textsuperscript{16} ET-1 induced a transient translocation of PKC activity from the cytosol to the membrane, and staurosporine reduced ET-1–induced contraction in bovine cerebral arteries.\textsuperscript{17} The failure of calphostin C in reducing ET-1–induced contraction in this study is actually consistent with some previous reports that staurosporine and H-7\textsuperscript{18} but not calphostin C\textsuperscript{19} attenuated cerebral vasospasm in animal models. However, because of the discrepancy between the effects of staurosporine and calphostin C in this study, the role of PKC in ET-1–induced contraction in rabbit basilar artery remains unconfirmed and requires further investigation.

The contractile effect of ET-1 was either abolished or attenuated by PTK, PI-3K, JAK\textsubscript{2}, Src, and MAPK inhibitors, indicating that ET-1–induced contraction in rabbit basilar arteries is mediated by multiple signaling pathways. This result differs from our previous observation of hemolysate-induced contraction in rabbit basilar arteries.\textsuperscript{2} Hemolysate-induced contraction was attenuated by PTK and MAPK inhibitors\textsuperscript{2-20} but not reduced by PI-3K, Src, or JAK\textsubscript{2} inhibitors, indicating that the signaling pathway for hemolysate is PTK-MAPK. To establish the relationship between MAPK and other signaling pathways, inhibitors for those pathways were tested on ET-1–induced MAPK phosphorylation. ET-1–induced MAPK immunoprecipitation was reduced by all but PKC and PI-3K inhibitors (Figure 8), suggesting that ET-1–induced contraction was mediated by multiple signal-
Role of PTK, MAPK, and PKC in Cerebral Vasospasm

It has been suggested that PTK regulates intracellular Ca\(^{2+}\) and contraction of cerebral smooth muscle cells.\(^{20,22}\) PTK can either increase Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores or Ca\(^{2+}\) entry from external space. PTK may potentiate the sensitivity of contractile proteins to Ca\(^{2+}\).\(^{24}\) The mechanism for MAPK-related contraction is probably mediated by phosphorylation of thin filament-associated proteins such as caldesmon.\(^{25}\) The 2 isoforms 42 and 44 kDa (ERK1/ERK2) are the most well-studied MAPK and are activated by dual phosphorylations of the threonine and tyrosine residues.\(^{15}\) Both PTK and MAPK have been suggested to be involved in cerebral vasospasm. PTK is involved in the regulation of intracellular Ca\(^{2+}\) in cultured endothelial\(^{26}\) and smooth muscle cells.\(^{23}\) PTK and MAPK are involved in the contractions of rabbit basilar arteries by hemolysate.\(^{2,20}\) MAPK activity was elevated in canine basilar arteries in a double-hemorrhage model of experimental cerebral vasospasm.\(^{27}\) This study for the first time demonstrated that PTK and MAPK are involved in ET-1–induced contraction in cerebral arteries. Since both hemolysate and ET-1 are extremely important spasmogens for cerebral vasospasm,\(^{1,2}\) and PTK and MAPK are involved in the signal transduction of both hemolysate\(^{2,20}\) and ET-1 (this study), PTK and MAPK may be important factors in the pathogenesis of cerebral vasospasm.

It has been suggested that some upstream regulators such as PI-3K, JAK, and Src may be involved in the activation of MAPK.\(^{15}\) Src family kinases play an important role in relaying signals from both \(G_{\text{q}}\) and \(G_{\text{i}}\)-coupled receptors to MAPK.\(^{28}\) It was shown that activating one of the proteins in this family, p56\(^{13}\), led to a time-dependent activation of phosphotransferase activity of the MAPK in T cells.\(^{28}\) Angiotensin II stimulation was associated with a rapid activation of c-Src and activation of MAPK in vascular smooth muscle cells.\(^{29}\) PI-3K activation may be upstream of Ras activation, and PI-3K may be involved in the function of Src, Shc, and Grb2.\(^{30}\) It has been reported that the p110 subunit of PI-3K binds to an effector domain of Ras in vitro,\(^{30}\) and the PI-3K inhibitor wortmannin reduced insulin-induced MAPK activity.\(^{31}\) The JAK family is a key mediator of mRNA expression characterized as “early growth response genes.”\(^{32}\) Recent studies have established that JAKs associate with membrane receptors and, when activated, stimulates tyrosine phosphorylation of a family of transcription factors termed signal transducers and activators of transcription (STAT).

**Figure 8.** Top, Western blot demonstrating effect of PD-98059 (P), U-0126 (U), AG-490 (A), wortmannin (W, Wort.), damnacanthal (D, Damn.), genistein (G), and staurosporine (S, Staur) on MAPK immunoprecipitation after stimulation with ET-1 (10\(^{-6}\) mol/L). PD-98059, U-0126, AG-490, damnacanthal, and genistein abolished effects of ET-1 on MAPK immunoprecipitation. Wortmannin and staurosporine did not have any significant effects on MAPK immunoprecipitation. Bottom, Graph displays density of the protein band after incubation with the aforementioned antagonists. PD-98059, U-0126, AG-490, damnacanthal, and genistein abolished effects of ET-1 on MAPK immunoprecipitation. Wortmannin and staurosporine did not have any significant effects on MAPK immunoprecipitation. C indicates control; 44, p44\(^{ERK}\).
proteins translocate to the nucleus, where they stimulate transcription of early growth response genes. JAK2 PTK may represent a common component in the activation of MAPK and STAT signaling pathways, which appear to bifurcate upstream of Ras activation but converge with MAPK phosphorylation.20 The role of JAK2 in smooth muscle contraction is unclear, even though activation of 5-HT2A receptor in skeletal muscles triggers a rapid and transient tyrosine phosphorylation of JAK2 kinase. On the basis of the data obtained in this study, it is likely that PTK, JAK, and Src are involved in ET-1–induced contraction and MAPK immunoprecipitation in rabbit basilar arteries. PI3K, if involved in ET-1–induced contraction, seems to mediate the contractile action of ET-1 by other mechanisms, since wortmannin failed to reduce ET-1–induced MAPK immunoprecipitation. These results are also distinct from those observed previously with hemolyse.2 None of the proteins PI3K, JAK, and Src were involved in hemolyse-induced contraction of rabbit basilar arteries.

PKC and endothelin are 2 important factors in cerebral vasospasm.1,3,33 PKC produces prolonged contraction of cerebral arteries and may lead to contraction by activation of PTK and MAPK.32 ET-1 produces prolonged contraction, and endothelin receptor antagonists attenuate cerebral vasospasm in animal models. However, the relationship between endothelin and PKC has not been established. This study failed to confirm this relationship because of the discrepancy in the effectiveness of staurosporine and calphostin C in reducing ET-1–induced contraction. This study, on the other hand, pointed out that ET-1–induced contraction seems to be mediated by multiple signaling pathways, and ET-1–induced MAPK immunoprecipitation may be PKC independent. The effect of staurosporine, which reduced the contraction of ET-1 but failed to decrease MAPK immunoprecipitation, offered some evidence of this.

**Conclusion**

We have demonstrated that the signal transduction pathways for ET-1 in rabbit basilar arteries may involve different proteins, including Src, JAK2, PI3K, PTK, and MAPK. Among them, the Src-JAK2-PTK-MAPK pathway seems connected in contraction and activation of MAPK, and MAPK may serve as a final common pathway. PI3K may be involved in ET-1–induced contraction, although probably by other MAPK-unrelated mechanisms. The role of PKC is uncertain, but it may not be involved in ET-1–induced MAPK immunoprecipitation. The role of these proteins in ET-1–induced contraction and the possible use of these inhibitors in cerebral vasospasm require further investigation.

**Acknowledgment**

This work was supported in part by a grant-in-aid to Dr Zhang from the American Heart Association.

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Endothelin (ET), a 21-residue peptide, is the most potent vasoconstrictor currently known.1 In the human and other mammalian species there are 3 separate isopeptides termed ET-1, ET-2, and ET-3.2 ET-1 is the only isoform of the peptide produced by the endothelium.2–4 The characteristics of the ET-1–produced constrictions make it an ideal candidate as a mediator of vasospasm.5 Although not without controversy, evidence is mounting for a key role of ET in the development of vasospasm (see the Discussion section of the above article).

This article by Zubkov et al identifies protein kinases likely to be involved with ET-1 constrictions of cerebrovascular smooth muscle and raises the possibility that several pathways are involved in the response. Of significance, the study underscores the complexity of the ET-1 signaling process. This article is significant for 2 reasons. First, it provides important information regarding the signal transduction pathways associated with ET-1 in vascular smooth muscle. Second, it provides a framework around which therapeutic approaches for the clinical treatment of vasospasm can be considered.

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Stroke. 2000;31:526-533
doi: 10.1161/01.STR.31.2.526

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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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