Role of Tyrosine Kinase in Dilator Responses of Rat Basilar Artery In Vivo

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Abstract—We tested the hypothesis that dilator responses of the basilar artery to endothelium-dependent vasodilators are mediated by activation of tyrosine kinase in vivo. Using a cranial window in anesthetized rats, we examined responses of the basilar artery to acetylcholine and bradykinin. Topical application of acetylcholine and bradykinin increased diameter of the basilar artery in a concentration-related manner. Genistein, an inhibitor of tyrosine kinase, did not affect baseline diameter of the basilar artery but inhibited vasodilation in response to acetylcholine and bradykinin, without affecting vasodilation produced by sodium nitroprusside. Tyrphostin 47, another inhibitor of tyrosine kinase, attenuated acetylcholine-induced dilatation of the basilar artery without affecting vasodilation in response to sodium nitroprusside. Tyrphostin 63, an inactive analogue of tyrphostin 47, did not affect acetylcholine-induced vasodilation. Sodium orthovanadate, an inhibitor of tyrosine phosphatase, enhanced acetylcholine-induced dilatation of the basilar artery. These results suggest that dilator responses of the basilar artery to endothelium-dependent agonists, acetylcholine and bradykinin, are mediated in large part by activation of tyrosine kinase. Because vasodilation produced by these agonists is mediated primarily by nitric oxide, activation of tyrosine kinase may have an important role in nitric oxide production in the basilar artery in vivo. (Hypertension. 1998;31:861–865.)

Key Words: cerebral artery • acetylcholine • bradykinin • sodium nitroprusside • nitric oxide • genistein • tyrphostin • sodium orthovanadate

Endothelium-derived relaxing factor, which is considered to be NO or its related compound(s), appears to play an important role in dilator responses of cerebral blood vessels both in vitro and in vivo.1 NO synthase, an enzyme that produces NO, is activated by a calcium-dependent mechanism.2 An increase in [Ca2+]i, during activation of vascular endothelium is characterized by an initial transient peak followed by a sustained increase.3,4 An initial component reflects IP3-mediated release of Ca2+ from intracellular stores.5 The second sustained phase is considered to be transmembranous Ca2+ influx.3,4 However, the mechanisms by which agonists cause Ca2+ influx in vascular endothelium are not fully understood.

Activity of tyrosine kinase appears to be an important determinant of cell growth and oncogenesis.5 Recent evidence has suggested that Ca2+ influx during activation of cultured umbilical vein endothelial cells is dependent on the activation of tyrosine kinase.6,7 These data suggest that activation of tyrosine kinase may be involved in agonist-induced Ca2+ influx in vascular endothelium and thereby contributes to NO-dependent vasodilator responses. There are no data, however, regarding the role of tyrosine kinase in agonist-induced vasodilator responses in vitro or in vivo. The goal of the present study was to test the hypothesis that tyrosine kinase is functionally active in the basilar artery in vivo and activation of tyrosine kinase has a role in dilator responses of the artery to the endothelium-dependent agonists acetylcholine and bradykinin. For this purpose, using a cranial window, we tested effects of two different inhibitors of tyrosine kinase, genistein and tyrphostin 47,8 and an inhibitor of tyrosine phosphatase, sodium orthovanadate,9 on the vasodilation.

Methods

Animal Preparation

Experiments were performed on male Sprague-Dawley rats (400±53 g, 3.3±0.3 months old [mean±SEM]) anesthetized with amobarbital (50 mg/kg IP). Anesthesia was supplemented intravenously at 20 to 25 mg · kg−1 · h−1. The trachea was cannulated, and the animals were mechanically ventilated with room air and supplemental oxygen. Skeletal muscle paralysis was produced with d-tubocurarine chloride (2 mg · kg−1). Depth of anesthesia was evaluated by applying pressure to a paw or the tail and observing changes in heart rate or blood pressure. When such changes occurred, additional anesthetic was administered. Catheters were placed in both femoral arteries to measure systemic arterial pressure and to obtain arterial blood samples. A femoral vein was cannulated for infusion of drugs.

A craniotomy was prepared over the ventral brain stem as previously described in detail.10–12 After a part of the dura was opened, the cranial window was suffused with artificial cerebrospinal fluid (temperature, 37°C; ionic composition [in mmol/L]: 132 NaCl, 2.95 KCl, 1.71 CaCl2, 0.65 MgCl2, 24.6 NaHCO3, 3.69 D-glucose) that was bubbled continuously with appropriate gases. Cerebrospinal fluid sampled from the cranial window had a pH of 7.40±0.01, a PaCO2 of 0.01, a PCO2 of 0.01.
Selected Abbreviations and Acronyms

ACh = acetylcholine
EDRF = endothelium-derived relaxing factor
IP$_3$ = inositol 1,4,5-trisphosphate
NO = nitric oxide
SNP = sodium nitroprusside

33±1 mm Hg, and a Po$_2$ of 110±5 mm Hg. Diameter of the blood vessel was measured using a microscope equipped with a television camera coupled to an auto-width analyzer (C3161, Hamamatsu Photonics K.K.).

After a craniotomy was prepared, pH, PCO$_2$, and Po$_2$ of arterial blood were adjusted by changing the rate and volume of the respirator and the oxygen content of inspiratory air. We also monitored arterial blood gas during the experiments and kept the values within normal limits (pH 7.43±0.01; PCO$_2$, 39±1 mm Hg; and Po$_2$, 110±4 mm Hg).

Experimental Protocol

We examined responses of the basilar artery to topical application of three vasodilators, ie, ACh (10$^{-5}$ and 10$^{-7}$ mol/L), bradykinin (10$^{-5}$ and 10$^{-7}$ mol/L), and SNP (10$^{-7}$ and 10$^{-6}$ mol/L). Agonists were mixed in artificial cerebrospinal fluid and suffused over the craniotomy for 5 minutes. Diameters of the basilar artery were measured immediately before and during the last minute of application of each agonist. After application of a specific agonist, the vessel diameter returned to baseline level within a few minutes before application of a subsequent agonist. The application sequence of agonists was randomized.

Pre-treatment of the basilar artery with N$^\gamma$-nitro-l-arginine (10$^{-5}$ mol/L) almost abolished dilator responses of the basilar artery to ACh and bradykinin (n=4, data not shown). Thus, vasodilatation produced by ACh and bradykinin appears to be mediated primarily by NO.

We used two different inhibitors of tyrosine kinase, genistein (3×10$^{-6}$ mol/L) and tyrphostin 47 (3,4-dihydroxy-a-cyanothiocinnamamide; 10$^{-5}$ mol/L). We also used tyrphostin 63 ([4-hydroxybenzyl] malononitrile; 10$^{-5}$ mol/L), an inactive analogue of tyrphostin 47, and sodium orthovanadate (10$^{-5}$ mol/L), an inhibitor of tyrosine phosphatase. Genistein, tyrphostin 47, and tyrphostin 63 were dissolved in DMSO. The maximum final concentration of DMSO was 0.1%. The concentration of DMSO did not cause any significant changes in diameter of the basilar artery. All the vasodilators and sodium orthovanadate were dissolved in water. Inhibitors were suffused starting from 15 minutes before and during application of agonists. Topical application of these agents did not cause any changes in systemic arterial pressure.

Statistical Analysis

All values are expressed as mean±SEM. One-way repeated measures ANOVA was used to compare concentration-dependent responses to vasodilators. Two-way repeated measures ANOVA was used to compare responses under control conditions and during interventions. When a significant F value was found, post hoc analysis was made with Wilcoxon’s test. A value of P<.05 was considered significant.

Results

Effects of Genistein on Vasodilatation

Under control conditions, diameter of the basilar artery was 254±7 μm (n=36). Topical application of ACh (10$^{-5}$ and 10$^{-7}$ mol/L) produced dilatation of the basilar artery in a concentration-related manner (Fig 1A). ACh (10$^{-5}$ mol/L) increased the diameter by 33±5%. ACh-induced vasodilation was reproducible because there was no significant attenuation of the response during repeated application of ACh (n=6). Genistein (3×10$^{-6}$ mol/L), a selective inhibitor of tyrosine kinase, had no effect on baseline diameter of the basilar artery. Genistein (3×10$^{-6}$ mol/L) inhibited dilatation of the basilar artery in response to ACh (10$^{-5}$ mol/L) by 74±6% (Fig 1A).

Bradykinin (10$^{-7}$ and 10$^{-6}$ mol/L) also produced dilatation of the basilar artery in a concentration-related manner (Fig 1B). Genistein (3×10$^{-6}$ mol/L) markedly attenuated bradykinin-induced vasodilatation (Fig 1B).

Application of SNP (10$^{-7}$ and 10$^{-6}$ mol/L) produced dilatation of the basilar artery (Fig 1C). Genistein (3×10$^{-6}$ mol/L) did not affect SNP-induced vasodilatation (Fig 1C).

Effects of Tyrphostin 47 on Vasodilatation

We also tested effects of tyrphostin 47, another inhibitor of tyrosine kinase, on vasodilatation produced by ACh and bradykinin. Tyrphostin 47 did not affect baseline diameter of the basilar artery but inhibited ACh-induced dilatation of the basilar artery (P<.05) (Fig 2A) without affecting vasodilatation in response to SNP (Fig 2B). Tyrphostin 63, an inactive analogue of tyrphostin 47, did not affect dilatation of the basilar artery in response to ACh (Fig 2A). Bradykinin-induced dilatation of the basilar artery was inhibited by tyrphostin 47 (P<.05) but not by tyrphostin 63 (data not shown).

Effects of Sodium Orthovanadate on Vasodilatation

We tested effects of sodium orthovanadate, an inhibitor of tyrosine phosphatase, on ACh-induced dilatation of the basilar artery. Sodium orthovanadate (10$^{-7}$ mol/L) did not affect baseline diameter of the basilar artery but enhanced ACh-induced vasodilatation (Fig 3). Sodium orthovanadate did not affect vasodilatation produced by SNP (data not shown).

Discussion

The major new finding in the present study is that dilator responses of basilar artery to ACh and bradykinin are mediated at least in part by activation of tyrosine kinase in vivo. Because dilatation of the basilar artery to these two agonists is mediated primarily by NO and activation of the kinase does not contribute to vasodilatation produced by an NO donor, activation of tyrosine kinase may have an important role in agonist-induced NO production in the basilar artery in vivo. This is the first study so far to show the role of tyrosine kinase in endothelium-dependent vasodilator responses in vivo.

Role of Tyrosine Kinase in Vasodilatation

Activity of tyrosine kinase has been demonstrated in several growth-factor receptors and oncogene products and appears to be an important determinant of cell growth and oncogenesis.9 Recently, it is reported that tyrosine kinase may play an important role in rather quick responses of several cell types, including neutrophils,14 platelets,15 neurons,16 and vascular cells.17-21 In the present study, we have shown that inhibition of tyrosine kinase markedly attenuates dilatation of the basilar artery in response to ACh and bradykinin in vivo. Thus, tyrosine kinase is functional in the basilar artery in vivo and may contribute to endothelium-dependent dilator responses of the artery.

We used two different inhibitors of tyrosine kinase, ie, genistein and tyrphostin 47. Genistein is considered to compete with an ATP-binding site of tyrosine kinase, and tyrphos-
tin 47 appears to be a competitor of a substrate-binding site of the kinase. A major concern regarding the findings mentioned above might be specificity of these inhibitors. In the present study, \(1 \times 10^{-6}\) mol/L genistein had good inhibitory effects on the vasodilatation, and this concentration is very close to half maximum concentration for inhibition of tyrosine kinase. Moreover, tyrphostin 63, an inactive analogue of tyrphostin 47, did not affect vasodilatation produced by ACh and bradykinin. Thus, the inhibitory effects of these antagonists are likely to be specific for tyrosine kinase. The finding that sodium orthovanadate, an inhibitor of tyrosine phosphatase, enhanced ACh-induced dilatation of the basilar artery may also support the conclusion that activation of tyrosine kinase is involved in ACh-induced vasodilatation. Because neither genistein nor tyrphostin 47 affected dilatation of the basilar artery in response to SNP, activation of tyrosine kinase may not account for the actions of NO on vascular muscle but may be involved in NO production in vascular endothelium.

It is reported that topical application of inhibitors of NO synthase produces constriction of rat basilar artery in vivo. Thus, synthesis of NO influences the resting tone of the basilar artery in vivo. In the present study, neither genistein nor tyrphostin 47 affected baseline diameter of the basilar artery. Because vasoconstrictor responses appear to be mediated in

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** A, Effects of genistein on ACh-induced vasodilatation. Changes in diameter of the basilar artery were measured in response to ACh (\(10^{-6}\) and \(10^{-5}\) mol/L) under control conditions and in the presence of genistein (\(10^{-6}\) mol/L). Baseline diameters under control conditions and in the presence of genistein were 254±15 and 257±11 \(\mu\)m, respectively. Values are mean±SEM (n=6). *P<.05 vs control response. B, Effects of genistein on bradykinin-induced vasodilatation. Changes in diameter of the basilar artery were measured in response to bradykinin (\(10^{-7}\) and \(10^{-6}\) mol/L) under control conditions and in the presence of genistein (\(10^{-6}\) mol/L). Baseline diameters under control conditions and in the presence of genistein were 254±15 and 253±11 \(\mu\)m, respectively. Values are mean±SEM (n=6). *P<.05 vs control response. C, Effects of genistein on SNP-induced vasodilatation. Changes in diameter of the basilar artery were measured in response to SNP (\(10^{-7}\) and \(10^{-6}\) mol/L) under control conditions and in the presence of genistein (\(10^{-6}\) mol/L). Baseline diameters under control conditions and in the presence of genistein were 258±12 and 251±11 \(\mu\)m, respectively. Values are mean±SEM (n=6).
part by activation of tyrosine kinase, inhibition of the kinase may have attenuated constrictor responses as well as dilator responses of the basilar artery and thereby masked inhibitory effects of tyrosine kinase inhibitors on vasodilator responses under control conditions. Another possibility is that some compensatory mechanisms may have counteracted the inhibitory actions of genistein and tyrphostin 47 on vasodilator responses under control conditions in vivo.

Intracellular tyrosine kinases appear to be divided into at least eight subfamilies based on the sequence similarity. It is reported that the Src family protein kinases are known to form complexes with G protein–coupled receptors in several cell types and that activation of cultured endothelial cells leads to tyrosine phosphorylation of several proteins, including paxillin and phospholipase Cγ1, which are known substrates of the Src family tyrosine kinases. Thus, the candidate tyrosine kinases that are involved in dilator responses of the basilar artery may be the Src family tyrosine kinases. However, we cannot exclude the possibility that other families of tyrosine kinases may also be involved in the responses of the basilar artery.

Mechanisms by Which Tyrosine Kinase Produces Vasodilatation

Receptors for Ach and bradykinin belong to a G protein–coupled receptor family. Activation of these receptors causes hydrolysis of inositol phospholipids by phospholipase C. It is reported that the βγ subunits of heterotrimeric G proteins activate tyrosine kinase. Thus, it is possible that G protein–coupled receptors can activate tyrosine kinase as well as phospholipase C.

Several mechanisms may account for tyrosine kinase–induced production of NO. Recently, two groups have reported that changes in [Ca2+]i of cultured vascular endothelial cells are dependent on activation of tyrosine kinase. Kruse et al have shown that herbinycin A, an inhibitor of tyrosine kinase, attenuated α-thrombin–induced Ca2+ influx but not Ca2+ release from intracellular store sites in human umbilical vein endothelial cells. Fleming et al have also shown that agonist-induced Ca2+ influx in human umbilical vein endothelial cells is attenuated by pretreatment with other tyrosine kinase inhibitors, genistein and piceatannol. These results
suggest that activation of tyrosine kinase may cause \(Ca^{2+}\) influx, thereby activating NO synthase in vascular endothelial cells. Recently, Jayaraman et al.\(^{29}\) have reported that activation of tyrosine kinase stimulates IP\(_3\) receptors and thereby causes \(Ca^{2+}\) release from intracellular stores. Thus, it may be possible that activation of tyrosine kinase is involved in \(IP_3\)-mediated release of \(Ca^{2+}\) from intracellular stores.

Other \(Ca^{2+}\)-independent mechanisms may also be involved in tyrosine kinase–activated production of NO. It is reported that NO production caused by activation of endothelin sub-type B receptors is mediated by activation of tyrosine kinase but that the responses are not dependent on a calcium/calmodulin system. Ayajiki et al.\(^{30}\) and Corson et al.\(^{31}\) have also reported that shear stress–induced NO production in cultured endothelial cells is mediated by activation of tyrosine kinase in a \(Ca^{2+}\)-independent manner. Thus, some tyrosine kinase–dependent mechanisms that are independent of calcium signaling may also be responsible for activation of NO synthase in the basilar artery.

Because inhibitors of tyrosine kinase and tyrosine phosphatase did not affect dilator responses of the basilar artery to SNP, an NO donor, tyrosine kinase may not modulate effects of NO on basal arterial tone but may mediate NO production in the basilar arterial endothelium in vivo. Because SNP is not identical to NO itself, the possibility that tyrosine kinase might also modulate effects of NO on basal arterial tone cannot be ruled out.

In summary, activation of tyrosine kinase appears to account in part for dilator responses of rat basilar artery to ACh and bradykinin. Activity of tyrosine kinase may play an important role in NO production in the basilar artery in vivo.

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