Role of Tyrosine Kinase in Serotonin-Induced Constriction of the Basilar Artery In Vivo

Takanari Kitazono, MD; Setsuro Ibayashi, MD; Tetsuhiko Nagao, MD; Tomoko Kagiyama, MD; Jiro Kitayama, MD; Masatoshi Fujishima, MD

Background and Purpose—Serotonin is one of the most potent constrictors of cerebral blood vessels and is implicated in several pathological conditions, including migraine and cerebral ischemia. Recent evidence has suggested that tyrosine kinase is involved in vasoconstrictive responses. The objective of this study was to test the hypothesis that activation of tyrosine kinase contributes to serotonin-induced constriction of the basilar artery in vivo.

Methods—Using a cranial window in anesthetized Sprague-Dawley rats, we examined effects of inhibitors of tyrosine kinase and tyrosine phosphatase on constrictor responses of the basilar artery to serotonin in vivo.

Results—Serotonin \(10^{-8}, 10^{-7}, \) and \(10^{-6}\) mol/L produced constriction of the basilar artery by 12±2%, 27±2%, and 37±3%, respectively. Genistein \(3\times10^{-6}\) mol/L, an inhibitor of tyrosine kinase, did not affect baseline diameter of the basilar artery but attenuated serotonin-induced vasoconstriction \(\left(P<.05\right)\) versus control responses. Daidzein, an inactive analogue of genistein, did not affect serotonin-induced constriction of the basilar artery. Tyrphostin 47 \(10^{-7}\) mol/L, another inhibitor of tyrosine kinase, also attenuated serotonin-induced vasoconstriction, and tyrphostin 63, an inactive analogue of tyrphostin 47, did not affect the vasoconstriction. Sodium orthovanadate \(10^{-3}\) mol/L, an inhibitor of tyrosine phosphatase, enhanced serotonin-induced vasoconstriction. Phorbol 12,13-dibutyrate, a direct activator of protein kinase C, also caused constriction of the basilar artery, which was not affected by genistein or sodium orthovanadate.

Conclusions—These results suggest that serotonin-induced constriction of the basilar artery is mediated, at least in part, by activation of tyrosine kinase in vivo. (Stroke. 1998;29:494-498.)

Key Words: cerebral arteries || genistein || protein kinase C || tyrphostin

Cerebral blood vessels are richly innervated by serotonergic nerve fibers. Although serotonin causes dilatation of small cerebral arterioles, it has potent constrictive actions on large cerebral arteries such as basilar artery. It is also suggested that serotonin has an important role in several pathological conditions including migraine, cerebral vasospasm, and cerebral ischemia. Murray et al have found a role of calcium and protein kinase C in serotonin-induced constriction of the basilar artery. However, the precise mechanism by which serotonin produces constriction of the basilar artery is not fully understood.

Activity of tyrosine kinase appears to be an important determinant of cell growth and oncogenesis. Recent evidence has suggested that the activity of tyrosine kinase has a major influence on the contractility of vascular smooth muscle in vitro. There are data, however, regarding the role of tyrosine kinase in constrictor responses of cerebral arteries in vivo. Because vascular responses in vivo may not be same as those in vitro, it is valuable to study the mechanisms of vascular responses in vivo. Thus, the goal of the present study was to response to serotonin is mediated by activation of tyrosine kinase in vivo. For this purpose, we tested the effects of test the hypothesis that constriction of the basilar artery in

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inhibitors of tyrosine kinase, genistein and tyrphostin 47, on serotonin-induced vasoconstriction using a cranial window technique.

Materials and Methods

Animal Preparation

Experiments were performed on male Sprague-Dawley rats (mean±SEM weight, 354±29 g; mean±SEM age, 3.7±0.1 month; n=36) anesthetized with amobarbital (50 mg/kg IP). Anesthesia was supplemented intravenously at 20 to 25 mg/kg per hour. The trachea was cannulated, and the animals were mechanically ventilated with room air and supplemental oxygen. Skeletal muscle paralysis was produced with \(-tubocurarine chloride (2 mg/kg). 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. 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 CaCl\(_2\), 0.65 MgCl\(_2\), 24.6 NaHCO\(_3\), 3.69 d-glucose) that

Received July 22, 1997; final revision received October 14, 1997; accepted November 20, 1997.

From the Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan.
Correspondence to Takanari Kitazono, MD, Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan. E-mail kitazono@intmed2.med.kyushu-u.ac.jp
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was bubbled continuously with 5% CO₂ and 95% N₂. Cerebrospinal fluid sampled from the cranial window had a pH of 7.42±0.01, a PCO₂ of 36±1 mm Hg, and a PO₂ of 109±3 mm Hg. The diameter of the blood vessel was measured with a microscope equipped with a television camera coupled to an autowidth analyzer (C3161, Hamamatsu Photonics K.K.). After a craniotomy was prepared, pH, PCO₂, and PO₂ of arterial blood were adjusted by changing rate and volume of the respirator and the oxygen content of inspiratory air. Arterial blood gas monitored during the experiments had a pH of 7.44±0.01, a PCO₂ of 37±1 mm Hg, and a PO₂ of 119±6 mm Hg.

Experimental Protocol
We examined responses of the basilar artery to topical application of serotonin (10⁻⁸ to 10⁻⁶ mol/L). Serotonin was mixed in artificial cerebrospinal fluid and suffused over the cranioptmy for 5 minutes. Diameters of the basilar artery were measured immediately before and during the last minute of application of the agonist. We used two different inhibitors of tyrosine kinase – genistein, tyrphostin 47. To show specificity of these inhibitors, we used daidzein, an inactive analogue of genistein, and tyrphostin 63, an inactive analogue of tyrphostin 47. We also used sodium orthovanadate, an inhibitor of tyrosine phosphatase. Genistein, daidzein, and tyrphostins were dissolved in dimethyl sulfoxide (DMSO). The maximum final concentration of DMSO was 0.05%, and the concentration of DMSO did not cause any significant changes in diameter of the basilar artery (data not shown). Sodium orthovanadate was dissolved in saline. Inhibitors were suffused starting from 15 minutes before and during application of the agonist. Topical application of these agents did not cause any changes in systemic arterial pressure (data not shown).

We next examined responses of the basilar artery to phorbol 12,13-dibutyrate (PDBu) (10⁻⁸ to 10⁻⁷ mol/L), a direct activator of protein kinase C. PDBu was mixed in DMSO and suffused over the cranioptmy for 15 minutes. Because phorbol esters are metabolized slowly, they persist in the cell membrane for long periods of time. For this reason, PDBu was applied to the basilar artery only once during an experiment, either in the absence or the presence of inhibitors. The maximum final concentration of DMSO was 0.1% in these experiments. The concentration of DMSO did not cause any significant changes in diameter of the basilar artery (data not shown).

Statistical Analysis
All values were expressed as mean±SEM. One-way repeated-measures ANOVA was used to compare concentration-dependent responses to vasoconstrictors. 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 for responses to serotonin and Mann-Whitney’s U test for responses to PDBu. A value of P<.05 was considered significant.

Results

Effects of Genistein on Serotonin-Induced Vasocostriction
Under control conditions, the diameter of the basilar artery was 255±5 μm (n=36). Topical application of serotonin (10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/L) produced constriction of the basilar artery by 12±2%, 27±2%, and 37±3%, respectively (Fig 1). Serotonin-induced vasoconstriction was reproducible, since there was no significant attenuation of the response during repeated application of serotonin (n=6, data not shown). Genistein (3×10⁻⁷ mol/L), an inhibitor of tyrosine kinase, had no effect on baseline diameter of the basilar artery but attenuated serotonin-induced vasoconstriction (Fig 1). In the presence of 3×10⁻⁷ mol/L genistein, serotonin (10⁻⁸ and 10⁻⁷ mol/L) produced constriction of the artery by 3±1% and 15±2%, respectively (P<.05 versus control responses, Fig 1).

Effects of Sodium Orthovanadate on Serotonin-Induced Vasocostriction
We next tested effects of sodium orthovanadate, an inhibitor of tyrosine phosphatase, on serotonin-induced constriction of the basilar artery. Under control conditions, serotonin (10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/L) produced constriction of the basilar

Serotonin (10⁻⁶ mol/L) caused constriction of the basilar artery by 30±2% in the presence of genistein, which is similar to control responses. Daidzein, an inactive analogue of genistein, did not affect serotonin-induced constriction of the basilar artery. In the presence of 3×10⁻⁷ mol/L daidzein, serotonin (10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/L) produced constriction of the basilar artery by 10±2%, 25±2%, and 37±2%, respectively.

Effects of Tyrphostin 47 on Serotonin-Induced Vasocostriction
We also tested the effects of tyrphostin 47, another inhibitor of tyrosine kinase, on serotonin-induced vasocostriction. Under control conditions, serotonin (10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/L) produced constriction of the basilar artery by 11±1%, 24±1%, and 38±3%, respectively (Fig 2). Tyrphostin 47 (10⁻⁵ mol/L) did not affect the baseline diameter of the basilar artery but inhibited serotonin-induced constriction of the basilar artery (P<.05) (Fig 2). In the presence of 10⁻⁵ mol/L tyrphostin 47, serotonin (10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/L) produced constriction of the artery by 4±2%, 11±1%, and 20±1%, respectively (P<.05 versus control responses) (Fig 2). Tyrphostin 63, an inactive analogue of tyrphostin 47, did not affect serotonin-induced constriction of the basilar artery. In the presence of 10⁻⁵ mol/L tyrphostin 63, serotonin (10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/L) produced constriction of the basilar artery by 11±1%, 25±3%, and 38±4%, respectively. Thus, constrictor responses of the basilar artery to serotonin are mediated, at least in part, by activation of tyrosine kinase in vivo.

Effects of Genistein on Serotonin-Induced Vasocostriction
Under control conditions, the diameter of the basilar artery was 36±1 mm Hg, and a P0₂ of 109±3 mm Hg. The diameter of the blood vessel was measured with a microscope equipped with a television camera coupled to an autowidth analyzer (C3161, Hamamatsu Photonics K.K.).
was not different from control responses.

3% in the presence of sodium orthovanadate, which produced vasoconstriction. Changes in diameter of the basilar artery were measured in response to serotonin (10^{-8} \text{ mol/L}) under control conditions and in the presence of sodium orthovanadate (10^{-5} \text{ mol/L}). Baseline diameters in the absence and the presence of orthovanadate (10^{-5} \text{ mol/L}) were 244\pm10 and 239\pm7 \mu m, respectively. Values are mean\pm SEM, expressed as percentages (n=6). *P<.05 vs control response.

Effects of Genistein and Sodium Orthovanadate on PDBu-Induced Vasoconstriction

**Figure 2.** Effects of tyrophostin 47 on serotonin-induced vasoconstriction. Changes in diameter of the basilar artery were measured in response to serotonin (10^{-8} to 10^{-6} \text{ mol/L}) under control conditions and in the presence of tyrophostin 47 (10^{-5} \text{ mol/L}). Baseline diameters in the absence and the presence of tyrophostin 47 were 244\pm10 and 239\pm7 \mu m, respectively. Values are mean\pm SEM, expressed as percentages (n=6). *P<.05 vs control response.

**Figure 3.** Effects of sodium orthovanadate on serotonin-induced vasoconstriction. Changes in diameter of the basilar artery were measured in response to serotonin (10^{-8} to 10^{-6} \text{ mol/L}) under control conditions and in the presence of sodium orthovanadate (1\times10^{-5} \text{ mol/L}). Baseline diameters in the absence and the presence of sodium orthovanadate were 247\pm6 and 238\pm9 \mu m, respectively. Values are mean\pm SEM (n=6). *P<.05 vs control response.

and 30\pm4\%, respectively. Neither genistein nor sodium orthovanadate affected PDBu-induced vasoconstriction (Table). Thus, constriction of the basilar artery to activation of protein kinase C is not mediated by activation of tyrosine kinase in vivo.

**Discussion**

The major new finding in the present study is that constriction of rat basilar artery in response to serotonin is mediated, at least in part, by activation of tyrosine kinase in vivo. Because PDBu-induced vasoconstriction is not affected by genistein or sodium orthovanadate, activation of tyrosine kinase may not be involved in protein kinase C-dependent constriction of the basilar artery in vivo.

The first evidence regarding the role of tyrosine kinase in vasocontractile responses was based on the observation that epidermal growth factor (EGF) produces contractile responses as well as growth of vascular muscle. It is well known that the activity of tyrosine kinase presents in EGF receptors and has a major influence on cell growth. It has also been reported that tyrosine kinase plays a role in EGF-induced vasoconstriction. Toma et al. have shown that contractile responses of rat mesenteric arteries to norepinephrine, whose receptors do not contain the activity of tyrosine kinase and are coupled to GTP-binding protein, are mediated in part by activation of tyrosine kinase in vitro. Abebe et al. have also reported that activation of tyrosine kinase is involved in norepinephrine-induced contraction of rat aorta in vitro. Thus, the activity of tyrosine kinase may be one of the major regulators of vasoconstrictive responses.

In the present study we have found, using a cranial window technique, that inhibition of tyrosine kinase markedly attenuates serotonin-induced constriction of the basilar artery and sodium orthovanadate, an inhibitor of tyrosine phosphatase, conversely enhances the vasoconstriction. Thus, activation of tyrosine kinase may also contribute to serotonin-induced constriction of the basilar artery in vivo. This is the first report thus far to show the presence of the activity of tyrosine kinase in cerebral blood vessels in vivo and to show the role of the kinase in contractile responses of the basilar artery to agonists.

A major concern regarding the findings mentioned above might be specificity of the inhibitors. In the present study both 3\times10^{-6} \text{ mol/L} genistein and 10^{-5} \text{ mol/L} tyrophostin 47 had good inhibitory effects on the vasoconstriction, and these
concentrations are very close to half-maximum concentrations for inhibition of tyrosine kinase.\textsuperscript{1,12} Moreover, daidzein and tyrophostin 63, inactive analogues of genistein\textsuperscript{14} and tyrophostin 47,\textsuperscript{11} did not affect serotonin-induced vasoconstriction. Thus, the inhibitory effects of genistein and tyrophostin 47 are likely to be specific for tyrosine kinase. The finding that genistein did not affect PDBu-induced constriction of the basilar artery may also support our interpretation that the inhibitory action of genistein and tyrophostin 47 on vasoconstriction may be specific for tyrosine kinase. The concentration of sodium orthovanadate (10\textsuperscript{−5} mol/L) is also very close to half-maximum concentration for inhibition of tyrosine phosphatase.\textsuperscript{15} The finding that the concentration of sodium orthovanadate did not affect PDBu-induced vasoconstriction may also support the interpretation that the inhibitory effects of sodium orthovanadate may not be nonspecific.

Serotonin appears to activate phospholipase C through GTP-binding protein and thereby produces inositol 1,4,5-trisphosphate and diacylglycerol.\textsuperscript{20} Inositol 1,4,5-trisphosphate and diacylglycerol activate protein kinase C,\textsuperscript{16} produced constriction of the basilar artery, which was not affected by genistein or sodium orthovanadate. Thus, PDBu-induced constriction of the basilar artery may not be mediated by activation of tyrosine kinase C in vivo.\textsuperscript{3,21} Thus, we next tested the role of tyrosine kinase in constriction of the basilar artery produced by activation of protein kinase C. PDBu, a direct activator of protein kinase C,\textsuperscript{16} produced constriction of the basilar artery, which was not affected by genistein or sodium orthovanadate. Thus, PDBu-induced constriction of the basilar artery may not be mediated by activation of tyrosine kinase in vivo. The findings are similar to the recent studies of noncerebral blood vessels.\textsuperscript{5,8} It is reported that tyrosine kinase inhibitors attenuate agonist-induced increase in the cytoplasmic Ca\textsuperscript{2+} level of vascular muscle.\textsuperscript{9,22} Thus, it may be possible that tyrosine kinase has a role in calcium signaling of the basilar arterial muscle in vivo.

Masumoto et al\textsuperscript{10} have reported that activation of tyrosine kinase is involved in pressure-induced contraction of rat cerebral artery in vitro. Thus, it may be possible that inhibition of tyrosine kinase affected the resting (myogenic) tone of the basilar artery. In the present study, however, neither genistein nor tyrophostin 47 affected the baseline diameter of the basilar artery. Activation of tyrosine kinase appears to be involved in nitrergic oxide production of vascular endothelial cells.\textsuperscript{23} Thus, inhibition of tyrosine kinase may have attenuated dilator responses as well as constrictor responses of the basilar artery and thereby masked the inhibitory effects of tyrosine kinase inhibitors on myogenic tone under control conditions. Another possibility may be that some compensatory mechanisms may have counteracted the inhibitory actions of genistein and tyrophostin 47 on myogenic tone under control conditions in vivo.

In summary, activation of tyrosine kinase may be involved in constrictor responses of rat basilar artery to serotonin in vivo. Protein kinase C−dependent constriction of the basilar artery may not be mediated by activation of tyrosine kinase.

Acknowledgments
This study was supported by a research grant for cardiovascular diseases (6A-3) from the Ministry of Health and Welfare and a grant from Sankyo Foundation of Life Sciences.

References
Although it is well known that serotonin (5-hydroxytryptamine) is a potent constrictor of large cerebral arteries, the mechanism that mediates the constriction has not been fully defined. This study provides evidence that tyrosine kinases play an important role in serotonin-induced constriction of the basilar artery.

Tyrosine kinases are thought to be a major signal transduction system in a variety of cells, including vascular muscle.\(^1,2\) For example, several effects of angiotensin II on vascular muscle, including contraction, appear to be mediated by tyrosine kinases.\(^2\) The study presented here by Kitazono et al supports this concept by providing pharmacological evidence that constriction of the basilar artery in response to serotonin in vivo is dependent on activation of tyrosine kinases. The conclusion supports previous work that implicated a role for these kinases in contraction of cerebral arteries in response to other stimuli in vitro.\(^3-5\)

Serotonin has been implicated in cerebral vascular pathophysiology, including conditions involving intravascular activation of platelets. Serotonin-induced contraction of large cerebral arteries is enhanced under pathophysiological conditions, including chronic hypertension,\(^6\) atherosclerosis,\(^7\) and subarachnoid hemorrhage.\(^8\) Because activation of tyrosine kinases appears to be an important mechanism of constriction of large cerebral arteries under normal conditions, it is tempting to speculate that enhanced activity of tyrosine kinases may contribute to augmented vasoconstrictor effects of serotonin under pathophysiological conditions. This study contributes to our understanding of signaling events in cerebral vascular muscle and may help provide insight into management of cerebral vascular disorders, including vasospasm.

Frank M. Faraci, PhD, Guest Editor
Department of Internal Medicine
Cardiovascular Division
University of Iowa College of Medicine
Iowa City, Iowa

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_Stroke_. 1998;29:494-498
doi: 10.1161/01.STR.29.2.494
_Stroke_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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