Effect of Aging on Endothelium-Dependent Vascular Relaxation of Isolated Human Basilar Artery to Thrombin and Bradykinin

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Using strips of human basilar arteries mounted in organ chambers to record isometric tension, we investigated vascular reactivity to thrombin and bradykinin. Both agents produced endothelium-dependent relaxation of basilar artery strips precontracted with phenylephrine but had no effect on resting tension in strips with or without endothelium. The relaxations caused by thrombin were abolished by antithrombin III/heparin, hirudin, and MD805. Thrombin but not bradykinin caused complete tachyphylaxis toward a second exposure. Indomethacin did not inhibit the relaxations induced by thrombin or bradykinin, whereas bromophenacyl bromide and methylene blue did. Aging decreased the relaxation induced by thrombin but did not affect the concentration needed to reach 50% maximal relaxation, nor did it affect the maximal relaxation in response to bradykinin, calcium ionophore A23187, and sodium nitroprusside. Our results suggest that thrombin and bradykinin produce endothelium-dependent relaxations mediated by an endothelium-derived relaxing substance and that the relaxation caused by thrombin is mediated by a proteolytic action on endothelial cells. The decrease in relaxations in response to thrombin with increasing age might be due to a decrease in the number or sensitivity of thrombin receptors on endothelial cells. (Stroke 1990;21:1039-1043)

An endothelium-derived relaxing substance, discovered by Furchgott and Zawadski and usually called endothelium-derived relaxing factor (EDRF), mediates endothelium-dependent relaxation in response to a number of vasoactive substances. The relaxation decreases in pathophysiological states, such as hypertension and atherosclerosis, and with increasing age, as found in isolated preparations from dogs and rats. We do not know whether the results in experimental animals are applicable to isolated human arteries since there are few studies on this subject.

In isolated human basilar arteries, thrombin and bradykinin have recently been shown to induce endothelium-dependent relaxation. Because both vasodilators are known to be generated during blood coagulation, it is important to consider whether vascular responsiveness to them changes with age. The aim of our study was therefore to determine whether relaxations in human basilar arteries in response to thrombin and bradykinin change with increasing age.

Materials and Methods

Basilar arteries were removed from 94 human cadavers (77 males aged 15–91 years and 17 females aged 15–90 years) during autopsy 2–20 hours after death not due to cerebrovascular disease or tumors affecting the central nervous system. The arteries were placed in a Petri dish containing fresh Krebs-Ringer solution and carefully dissected free of arachnoid tissue. The arteries were then cut into spiral strips approximately 2 mm wide and 15 mm long. Two strips were obtained from each body. The strips were fixed vertically between hooks in a 10-ml tissue bath containing Krebs-Ringer solution maintained at 37°C and aerated with a gas mixture of 95% O2 and 5% CO2, which gave a pH of 7.4. The Krebs-Ringer solution had a millimolar composition of 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4 • 7H2O, 10 glucose, and 25 NaHCO3.

Isometric tension was monitored with a force-displacement transducer (Nihon Kohden Kogyo Co., Tokyo, Japan) to which the upper end of a strip was connected, as previously reported, and was recorded with a pen recorder (Nihon Kohden Kogyo Co.). The

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strips were equilibrated for approximately 2 hours, during which time the organ bath medium was replaced every 15 minutes, and the hooks were then adjusted to give a resting tension of 1.5 g.

In the first experiment, the endothelium was removed from 24 strips by rubbing the intimal surface with filter paper.1 Denudation of the endothelium was confirmed by the loss of relaxation induced by 10^{-6} M bradykinin, an endothelium-dependent vasodilator.8 The response of endothelium-denuded strips to 0.5 unit/ml thrombin (n=6), 10^{-5} M bradykinin (n=6), 10^{-6} M calcium ionophore A23187 (n=6), or 10^{-9}—10^{-6} M sodium nitroprusside (n=6) was compared with that for endothelium-intact strips to the same four agonists (n=6 for each). Cumulative concentrations of bradykinin (10^{-10}—10^{-5} M, n=90), A23187 (10^{-8}—10^{-6} M, n=40) and sodium nitroprusside (10^{-9}—10^{-6} M, n=83) were used in endothelium-intact strips to obtain dose-response curves. The agonists were added to the organ bath medium at the peak of submaximal contractions elicited by 10^{-6} M phenylephrine (baseline).

In a second experiment, we added 1 unit/ml each antithrombin III/heparin (n=6), 1 unit/ml hirudin (n=6), or 10^{-7} M of the synthetic thrombin inhibitor MD8051011 (n=6) to the organ bath medium 10 minutes before the exposure to phenylephrine. Thrombin was applied only once to each strip because tachyphylaxis occurred with a second application; since dose-response curves could not be obtained because of this tachyphylaxis, only one concentration of thrombin (0.5 unit/ml, n=75) was used.

In a third experiment, strips were exposed to 10^{-5} M indomethacin, 5×10^{-6} M bromophenacyl bromide, or 10^{-5} M methylene blue (n=8 for each inhibitor) for 60 minutes before the addition of phenylephrine. The vascular responses induced by 0.5 unit/ml thrombin, 10^{-5} M bradykinin, and 10^{-6} M A23187 in the presence of these three inhibitors were compared with those induced by each agonist using the other strip from the same body.

For all three experiments, relaxation was expressed as a percentage of the maximal relaxation induced by 10^{-4} M papaverine. The drugs were added to the organ bath medium in a volume of 50–100 μl, and their final concentrations in the medium are given.

Histologic study was carried out on paraffin-embedded sections of preselected sites from the basilar arteries. The sections were stained with van Gieson's elastica stain, and morphometric determination was performed with an image analyzer system (Quantimet 720, Cambridge Instrument, Cambridge, England) to evaluate the area of the intima and the length of the internal elastic lamina, as previously described.12,13 Indices of intimal thickening were calculated by dividing the area of the intima by the area enclosed by the internal elastic lamina in its theoretically unwrinkled state.14 Values for the intimal thickening index varied from 0.01 to 0.56. Preparations with an intimal thickening index of >0.10 were excluded from the statistical analysis. Thus, there was no significant correlation between intimal thickening index and age in the preparations used (r=0.17).

Bradykinin was purchased from Peptide Institute Inc., Osaka, Japan. Bovine thrombin (1,280 NIH units/mg) was obtained from Mochida Seiyaku Co., Ltd., Tokyo, Japan. A23187, sodium nitroprusside, L-phenylephrine hydrochloride, antithrombin III, hirudin, indomethacin, bromophenacyl bromide, methylene blue, and papaverine hydrochloride were all obtained from Sigma Chemical Co., St. Louis, Missouri. MD805 was obtained from Daiichi Seiyaku Co., Ltd., Tokyo, Japan.

The data are expressed as mean±SD and were statistically analyzed using Student's paired t test. The concentrations needed to reach 50% maximal relaxation (ED50 values) were determined graphically after linear regression of the 20–80% region of the log concentration–response curves (bradykinin, 3×10^{-8}—10^{-6} M; A23187, 2×10^{-8}—4×10^{-8} M; sodium nitroprusside, 10^{-8}—10^{-7} M). Correlations between percent relaxation and age were calculated by least-squares linear regression analysis. The level of significance was p<0.05.

Results

Thrombin, bradykinin, and A23187 produced relaxations in the precontracted strips with the endo-

![FIGURE 1. Representative recordings of tension in human basilar artery strips show (left) endothelium-dependent relaxation in response to 0.5 unit/ml thrombin (Th), (center) tachyphylaxis to second exposure to thrombin, and (right) effect of 10^{-7} M synthetic thrombin inhibitor (MD805) on thrombin-induced vasorelaxation. Baseline tension was produced by 10^{-6} M phenylephrine (Ph). Endo (+), endothelium intact; Endo (—), endothelium removed; W, washout.](http://stroke.ahajournals.org/figs/1040F1.jpg)
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TABLE 1. Effects of Inhibitors on Endothelium-Dependent Relaxations Induced by Thrombin, Bradykinin, and A23187 in Isolated Human Basilar Arteries With Endothelium

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Indomethacin (10⁻⁵ M)</th>
<th>Bromophenacyl bromide (5×10⁻⁸ M)</th>
<th>Methylene blue (10⁻⁵ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin (0.5 unit/ml)</td>
<td>33.9±10.2</td>
<td>33.0±8.0</td>
<td>30.4±7.6</td>
</tr>
<tr>
<td>Bradykinin (10⁻³ M)</td>
<td>63.2±5.0</td>
<td>67.5±4.8</td>
<td>60.7±6.2</td>
</tr>
<tr>
<td>A23187 (10⁻⁴ M)</td>
<td>80.5±5.0</td>
<td>76.8±6.7</td>
<td>78.9±8.0</td>
</tr>
</tbody>
</table>

Values are mean±SD percent relaxation (relaxation induced by 10⁻⁴ M papaverine was taken as 100%); n=8 for each agonist.

* p<0.01 different from results in absence of inhibitor.

thelium, but the relaxations were abolished in the strips without endothelium. Representative recordings of the endothelium-dependent relaxations induced by thrombin are shown in Figure 1, left. Sodium nitroprusside produced similar relaxations in endothelium-intact and -denuded strips (ED₅₀, 3.9±0.5×10⁻⁸ M; maximal relaxation, 93.0±2.6% and ED₅₀, 3.7±0.4×10⁻⁸ M; maximal relaxation, 91.8±3.1%, respectively). Neither thrombin nor bradykinin affected resting tension.

We tested the effects of known inhibitors of EDRF-mediated relaxations to learn whether EDRF is involved in the mechanisms of relaxation in response to thrombin, bradykinin, and A23187. The relaxations induced by thrombin were not significantly affected by indomethacin, but they were abolished by bromophenacyl bromide and methylene blue (Table 1). We obtained similar results with bradykinin and A23187 (Table 1). The endothelium-dependent relaxation induced by thrombin was not produced by a second application of the enzyme (Figure 1, center). Tachyphylaxis was observed even 5 hours after the first application. However, a second application of bradykinin produced relaxation of the same magnitude as the first application (data not shown).

MD805 completely abolished the thrombin-induced relaxation (Figure 1, right). In the same manner, antithrombin III/heparin and hirudin abolished the thrombin-induced relaxations (data not shown).

Figure 2 shows the relationships between the maximal relaxations induced by thrombin or bradykinin and age. There was a significant negative correlation for thrombin (Figure 2, left) but no significant correlation for bradykinin (Figure 2, right). The maximal relaxations induced by neither sodium nitroprusside nor A23187 showed any correlation with age (Figure 3). No significant correlation between ED₅₀ and age was found for bradykinin, sodium nitroprusside, or A23187 (r=0.12, r=−0.19, and r=0.01, respectively). Also, the baseline tension induced by 10⁻⁶ M phenylephrine was not significantly correlated with age (r=−0.04).

Discussion

Since it contracts isolated canine and rabbit basilar arteries, thrombin is thought to be involved in delayed cerebral vasospasms after subarachnoid hemorrhage. However, in our study using human basilar arteries precontracted with phenylephrine, thrombin caused relaxation in endothelium-intact but not -denuded strips. When added to endothelium-intact or -denuded strips under resting tension, thrombin did not increase tension. The inability of endothelium-denuded strips to relax in response to thrombin or bradykinin is unlikely to be due to damage to the vascular smooth muscle since relaxations in response to sodium nitroprusside were unimpaired after the rubbing procedure. These results suggest that vasospasms after subarachnoid hemorrhage are not mediated by the direct action of thrombin on the blood vessel although they may be caused by the release of vasoactive substances (e.g., serotonin, thromboxane A₂) from platelets activated by thrombin.

![Figure 2](http://stroke.ahajournals.org/Downloaded)
Indomethacin, a cyclooxygenase inhibitor, did not inhibit the relaxations induced by thrombin, bradykinin, or A23187, suggesting that these agents do not cause relaxation by releasing prostacyclin. A likely assumption is that EDRF mediates relaxation of vascular smooth muscle via activation of soluble guanylate cyclase with production of guanosine cyclic monophosphate. Endothelium-dependent relaxations to these agents were abolished by bromophenacyl bromide (a phospholipase A2 inhibitor that depresses EDRF-mediated relaxation) and by methylene blue (a soluble guanylate cyclase inhibitor). These results are compatible with the assumption that the “relaxing substance” released in response to these agents is EDRF itself. Of course, there is likely more than one EDRF, depending on the agonist, the animal species, and the vascular bed studied. Thus, the “relaxing substance” is probably not the classical EDRF released from rabbit aorta, but this cannot be proven at present.

Antithrombin III/heparin, hirudin, and MD805 (all of which inhibit the catalytic activity of thrombin) blocked thrombin-induced relaxation. In addition, thrombin-induced relaxation led to complete tachyphylaxis to thrombin. These results confirm involvement of the catalytic activity of thrombin in its vascular effects, as previously reported. As stated by White and Robertson, the tachyphylaxis to thrombin also suggests that thrombin is not involved in vasospasms after subarachnoid hemorrhage.

Using thrombin, bradykinin, and A23187, we also investigated the effects of increasing age on the relaxations caused by these agents. Only the endothelium-dependent relaxation in response to thrombin became impaired with increasing age. As does EDRF, sodium nitroprusside relaxes vascular smooth muscle via activation of guanylate cyclase. Since the relaxation in response to sodium nitroprusside does not change with age, the age-related decrease in the relaxation in response to thrombin may occur at the level of endothelial but not smooth muscle cells.

The loss of thrombin-induced relaxation with age could be due to one or more of the following reasons. First, loss of the relaxation might be due to a decrease in the ability of the endothelium to produce and/or liberate the EDRF-like substance due to functional or structural alterations in the endothelial cells. Second, although arteries showing intimal thickening due to atherosclerosis were excluded from our study, disturbances in transport of the EDRF-like substance to smooth muscle cells and accelerated destruction of the substance may play a role. However, these two possibilities are unlikely because A23187, which causes endothelium-dependent relaxation in a manner unrelated to any receptor mechanism, did not show an attenuated response with increasing age. Further support for this view comes from the observation that the relaxation in response to bradykinin did not decrease with increasing age. Third, the degree to which a vascular strip relaxes may depend on the level of contraction before administration of the relaxing agent; as contractile tension increases, relaxation decreases. However, this is an unlikely explanation because the magnitude of the baseline tension induced by phenylephrine did not change with age. In addition, the absence of age-related changes in the responses to bradykinin, A23187, and sodium nitroprusside supports this interpretation.

The most plausible explanation is that the age-related change in thrombin-induced relaxation arises due to a decrease in the number or sensitivity of thrombin receptors on the endothelial cells with increasing age. Interestingly, since the relaxation in response to bradykinin did not change with age, this phenomenon may be specific for thrombin. Thrombin, unlike bradykinin, has a proteolytic action that may be involved in this age-related difference between thrombin- and bradykinin-induced relaxations.

How an age-related decrease in the thrombin-induced relaxation is involved in pathophysiological states is not clear. Endothelium-dependent relaxations triggered by thrombin and bradykinin during blood coagulation may flush away a developing aggregate or thrombus. Therefore, an age-related decrease in the relaxation in response to thrombin...
may be involved in the increased occurrence of cerebral infarction with aging.

References


KEY WORDS • bradykinin • endothelium-derived relaxing factor • thrombin
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