Age-Related Changes in Release of Endothelium-Derived Relaxing Factor From the Carotid Artery

Roberto Paternò, MD; Frank M. Faraci, PhD; Donald D. Heistad, MD

Background and Purpose The goal of this study was to determine whether release of endothelium-derived relaxing factor (EDRF) from carotid artery in response to acetylcholine is altered by aging.

Methods Responses were examined in arteries from Wistar rats 6 to 8 months old (young rats), 24 to 26 months old (old rats), and 30 to 32 months old (very old rats). We used a bioassay technique to measure release of EDRF from the carotid artery (donor vessel) and, in the same experiment, measured the diameter of pressurized donor arteries.

Results Responses of the donor arteries and the detector vessels (aortas) to sodium nitroprusside were not altered in old (24 to 26 months) and very old (30 to 32 months) rats compared with responses in the young rats. Dilator responses in the carotid artery to acetylcholine tended to be lower in the old rats and were significantly lower in very old rats compared with the young rats. Relaxation of the detector vessel during administration of acetylcholine to the donor artery was not significantly different in young, old, and very old rats.

Conclusions Endothelium-dependent dilatation in carotid arteries of old rats is impaired, but release of EDRF and responsiveness of vascular muscle to nitroprusside are normal. Thus, impairment of endothelium-dependent relaxation in the carotid artery during aging is not due to impaired release of EDRF.

See Editorial Comment, page 2461

Materials and Methods

Animals

Experiments were performed using common carotid arteries (donor vessels) from Wistar rats of three different ages: young (6 to 8 months, n=18), old (24 to 26 months, n=13), and very old (30 to 32 months, n=6). Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, IP) and exsanguinated, and both common carotid arteries were removed and used as donor vessels. The aortas were used as the detector vessels in the bioassay.

Bioassay Detector

The descending thoracic aorta was removed and placed in room-temperature modified Krebs’ solution that was bubbled with a gas mixture of 95% O₂ and 5% CO₂. The vessel was cleaned of adherent loose connective tissue. Two rings were cut, and the endothelium was removed by gently rubbing the luminal surface with fine forceps on a moist towel.

Two stainless-steel stirrups were passed through the lumen of each ring, and one stirrup was connected to an isometric force transducer to measure tension in the vessels. The bioassay rings were suspended directly below the effluent from the donor vessels. The transit time of the effluent from the donor vessel to the detector vessel was 4 seconds. When the transit time was increased to 2 minutes, relaxation of the detector in response to acetylcholine was abolished.

Donor Artery

After their removal, the carotid arteries were aerated with modified Krebs’ solution ([mmol/L] NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ • 7H₂O 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, and dextrose...
11) at room temperature. Blood in the lumen of the vessels was washed out with Krebs' solution. Adherent loose connective tissue was removed from the carotid arteries. Each arterial segment (1.5 cm) was mounted between two plastic cannulas and placed in an organ chamber. The vessels were perfused intraluminally with oxygenated modified Krebs' solution at a constant flow (0.3 mL/min) using a peristaltic pump. Arteries in the organ chamber were superfused with aerated Krebs' solution maintained at a constant temperature of 37°C.

In one carotid artery from each animal, intravascular pressure was maintained at 100 mm Hg throughout the experimental protocol by use of a screw clamp downstream from the donor vessel. In preliminary experiments, we found that to enable detection of changes in carotid artery diameter during administration of drugs, it was important to increase pressure in the donor vessel. Perfusion pressure was measured upstream from the donor vessel. Between the pump and the pressure transducer, an air-filled 10-cc syringe was attached as a compliance chamber to maintain pulse pressure close to 0 mm Hg. The donor artery was observed with a microscope (magnification, 15 x) and the image was displayed on a video screen using a video camera. The outer diameter of the vessel was measured using a computer-assisted edge detection system and digital analysis of the video image. We have used this method previously.12

**Experimental Protocol**

Bioassay rings were superfused with aerated modified Krebs' solution passed through plastic cannulas for 60 minutes. During this time, the aortic rings were stretched in small steps approximately every 5 minutes, until the basal tension reached 2.5 g. Successful removal of endothelium was verified for all rings, which were contracted with phenylephrine, by absence of relaxation to 10^{-6} mol/L acetylcholine.

After 60 minutes, Krebs' solution was infused at 0.3 mL/min into the carotid artery, and pressure inside the vessel was maintained at basal level. Phenylephrine (5 x 10^{-7} mol/L) was added to the Krebs' solution that was perfusing the carotid artery and produced relaxation of the aortic ring (detector vessel). The responses of the detector vessel during infusion of acetylcholine in the carotid arteries of young, old, and very old rats were not different (see "Results"). After making these findings, which demonstrated that endothelium-dependent relaxation of the bioassay detector vessel. Thus, perfusion of the carotid artery with phenylephrine induced relaxation of the detector vessel downstream by release of EDRF from the (donor) carotid artery.

Sodium nitroprusside (10^{-9} to 10^{-7} mol/L) was also infused into the carotid artery and produced relaxation of the aortic ring (detector vessel). The responses of the detector vessel during infusion of acetylcholine in the carotid arteries of young, old, and very old rats were not different (see "Results"). After making these findings, which demonstrated that release of EDRF is normal in old rats, it was important that we confirm our previous finding that endothelium-dependent vasodilatation is impaired in old rats.13 We developed a method that allowed measurement of responses in the donor artery and simultaneous measurement of release of EDRF using a bioassay detector.

The outer diameter of one carotid artery was measured while Krebs' solution was infused at an intraluminal flow rate of 0.3 mL/min. Pressure was slowly increased until a steady-state pressure of 100 mm Hg was reached. During this time, the vessel was gradually stretched longitudinally to approximate the in situ length.

Phenylephrine was added to the Krebs' solution perfusate in the pressurized carotid artery (5 x 10^{-6} mol/L). The higher concentration of phenylephrine in the pressurized carotid artery was necessary to produce a sufficient constriction in the vessel (about 20%). When the constriction reached a steady state, acetylcholine (10^{-4} to 10^{-3} mol/L) was infused (with phenylephrine) in a cumulative fashion in the perfusate of the carotid, and the increase in diameter of the donor vessel and relaxation of the bioassay vessel were measured. After recovery, the donor carotid artery and the bioassay ring were exposed to sodium nitroprusside (10^{-7} to 10^{-4} mol/L) added to phenylephrine in Krebs' solution.

**Drugs**

Phenylephrine, acetylcholine, and sodium nitroprusside were purchased from Sigma Chemical Co. (St. Louis, Mo).

**Calculations and Statistical Analysis**

Relaxation in response to acetylcholine and sodium nitroprusside is expressed as the percentage of phenylephrine-induced force of the detector vessel and as the percentage of the phenylephrine-induced tone of the donor. Responses in the detector vessel were calculated using the formula ([isometric force during phenylephrine infusion] x 100%)/([isometric force during phenylephrine infusion]). Responses in the donor were calculated using the formula ([diameter during acetylcholine infusion] x 100%)/(isometric force during phenylephrine infusion).

Contraction of the donor to phenylephrine is expressed as percentage of the isometric force baseline of stretched bioassay detector vessel. Responses were calculated from the formula ([diameter during phenylephrine infusion] x 100%)/(isometric force during phenylephrine infusion).

Constriction of the donor artery to phenylephrine is expressed as percentage of the isometric force baseline of stretched bioassay detector vessel. Responses were calculated from the formula ([diameter during phenylephrine infusion] x 100%)/(isometric force during phenylephrine infusion).

Several experiments in which the bioassay detector did not respond to high concentrations of acetylcholine (10^{-4} mol/L) infused in the donor artery were not included in the data. We did not include data from five animals (1 young, 2 old, 2 very old). Results were expressed as mean±SEM. Comparisons between three groups were evaluated by ANOVA and Bonferroni test. Values of P<.05 for a one-tailed Student's t test were considered to be significant.

**Results**

**Nonpressurized Donor Artery**

Administration of acetylcholine to the nonpressurized donor artery produced relaxation of the bioassay vessel. In the bioassay detector vessel paired with the unpressurized carotid artery, relaxation in response to acetylcholine and sodium nitroprusside (Figs 1 and 2) and contraction in response to phenylephrine (not shown) were not significantly different in young, old, and very old rats.
Pressurized Donor Artery

In pressurized arteries preconstricted with phenylephrine, acetylcholine produced dilatation. Dilation in response to acetylcholine by the donor carotid artery tended to be lower in old rats and was significantly lower in very old rats (Table 1). Dilation in response to sodium nitroprusside was not significantly different in young, old, and very old rats (Table 1). Constriction in response to phenylephrine was not significantly different in young, old, and very old rats (not shown).

In the bioassay detector vessel paired with the pressurized carotid artery, responses to acetylcholine in the young, old, and very old rats were not significantly different (Table 2). Relaxation of the bioassay detector in response to sodium nitroprusside (Table 2) and contraction in response to phenylephrine (not shown) were not significantly different in the three groups of rats.

Discussion

The major finding of this study is that agonist-induced release of EDRF in a cascade bioassay model is similar in young, old, and very old rats. Responsiveness of vascular smooth muscle to nitroprusside was not altered by aging. In these experiments we found, as in earlier ones, that endothelium-dependent dilatation is impaired in the donor artery by aging. The findings suggest that impairment of endothelium-dependent dilatation by aging is not due to impaired release of EDRF.

Consideration of Methods

There are no previous studies in which bioassay of endothelium-derived vasoactive substances was used to examine changes with aging. Using a perfusion-cascade bioassay model, we studied vessels in a more physiological condition than can be achieved using vascular rings. With this model, the donor vessel is exposed to flow and pressure that modulate responses of endothelial cells. The perfusion-cascade bioassay model has been used to demonstrate that flow induces release of a transferable endothelium-dependent substance that produces relaxation of denuded rings downstream. Elevation of pressure in the donor vessel reduces basal and acetylcholine-stimulated release of EDRF.

In this study, we used in one carotid artery a perfusion-cascade bioassay technique that was developed for detection of EDRF released by vasodilator agents. In the other carotid artery, in which the pressure was maintained at 100 mm Hg throughout the experimental protocol, a new method was used that allowed measurements of dilator responses in the donor artery and simultaneous measurements with a bioassay detector of the release of EDRF. The outer diameter of the carotid artery was measured with an intraluminal pressure of 100 mm Hg that was maintained throughout the experiment. In another study, a similar approach was used to

Table 1. Response of Pressurized, Perfused Carotid Arteries to Acetylcholine and Nitroprusside

<table>
<thead>
<tr>
<th>Acetylcholine</th>
<th>10^{-8} M</th>
<th>3\times10^{-8} M</th>
<th>10^{-7} M</th>
<th>3\times10^{-7} M</th>
<th>10^{-6} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (n=11)</td>
<td>8±4</td>
<td>32±10</td>
<td>60±8</td>
<td>79±5</td>
<td>87±3</td>
</tr>
<tr>
<td>Old (n=10)</td>
<td>3±1</td>
<td>18±6</td>
<td>44±7</td>
<td>67±6</td>
<td>70±8*</td>
</tr>
<tr>
<td>Very old (n=5)</td>
<td>1±1</td>
<td>11±5</td>
<td>32±8*</td>
<td>61±8*</td>
<td>74±7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitroprusside</th>
<th>10^{-8} M</th>
<th>10^{-7} M</th>
<th>10^{-6} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (n=9)</td>
<td>8±2</td>
<td>25±4</td>
<td>67±6</td>
</tr>
<tr>
<td>Old (n=7)</td>
<td>10±3</td>
<td>25±7</td>
<td>62±12</td>
</tr>
<tr>
<td>Very old (n=5)</td>
<td>2±2</td>
<td>17±4</td>
<td>54±9</td>
</tr>
</tbody>
</table>

Values are percent change in diameter (mean±SEM). *P<.05 compared with response in young rats.

Table 2. Response of Bioassay Detector to Effluent From Pressurized, Perfused Carotid Arteries

<table>
<thead>
<tr>
<th>Acetylcholine</th>
<th>10^{-8} M</th>
<th>3\times10^{-8} M</th>
<th>10^{-7} M</th>
<th>3\times10^{-7} M</th>
<th>10^{-6} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (n=8)</td>
<td>8±2</td>
<td>24±5</td>
<td>35±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old (n=6)</td>
<td>9±3</td>
<td>18±6</td>
<td>32±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very old (n=4)</td>
<td>3±2</td>
<td>18±8</td>
<td>31±10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitroprusside</th>
<th>10^{-8} M</th>
<th>10^{-7} M</th>
<th>10^{-6} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (n=9)</td>
<td>23±7</td>
<td>76±8</td>
<td>104±6</td>
</tr>
<tr>
<td>Old (n=9)</td>
<td>18±4</td>
<td>58±9</td>
<td>85±9</td>
</tr>
<tr>
<td>Very old (n=6)</td>
<td>19±6</td>
<td>75±8</td>
<td>100±5</td>
</tr>
</tbody>
</table>

Values are percent change in isometric force (mean±SEM). No values were significantly different in young, old, and very old rats.

Fig 2. Values are percent change (mean±SEM) in isometric force in the bioassay detector vessel during infusion of nitroprusside in the unpressurized carotid arteries of 18 young (open circles), 13 old (closed circles), and 5 very old rats (open triangles). No values were significantly different between the groups.
measured the effects of pressure in canine donor and bioassay detector vessels; responses in the perfused donor artery were measured with stainless-steel stirrups inserted into the vessel wall to measure isometric force.\textsuperscript{15} In our system, diameter, not force, was measured in the pressurized perfused vessel.

Impaired endothelium-dependent relaxation in response to acetylcholine may be due to several mechanisms, including impaired synthesis or release of EDRF, impaired responsiveness of vascular smooth muscle to EDRF, or generation of oxygen-derived free radicals that inactivate EDRF. Using the cascade bioassay model, it is possible to study release of EDRF.

Release of EDRF

Several studies suggest that agonist-induced endothelium-dependent relaxation is impaired by aging.\textsuperscript{1,4} In this study, there was no difference between young, old, and very old rats in release of EDRF, as detected with a perfusion–cascade bioassay system.

Sodium nitroprusside was administered to test responsiveness of vascular smooth muscle. Responsiveness of vascular muscle was not altered by aging. Thus, in the bioassay rings that detect release of EDRF, relaxation is not the result of impaired responsiveness of vascular muscle.

Mechanism of Impaired Vasodilatation

Responses of the donor artery in the present study confirmed previous findings that vasodilator responses to acetylcholine are impaired by aging.\textsuperscript{14} The important finding of this study is that the decrease in dilator responses in old rats is not due to a decrease in release of EDRF, indicating that other mechanisms are involved.

In a recent study, we found that L-arginine, the precursor of EDRF, partially restores dilatation of the carotid artery in response to platelets, ADP, or acetylcholine in vessels from old animals.\textsuperscript{13} Improvement in dilatation may or may not indicate that there is a reversible deficiency in L-arginine, because responses may improve with excess generation of EDRF.

Impairment of endothelium-dependent vasodilatation with aging could also be due to production of EDCF, as has been found in large arteries and cerebral arterioles in chronically hypertensive rats,\textsuperscript{17} or to the presence of oxygen-derived free radicals. Because the concentration of superoxide dismutase appears to decrease in several different tissues with aging,\textsuperscript{18,19} susceptibility to superoxide anion may increase. Oxygen radicals can influence endothelium-dependent relaxation,\textsuperscript{20} and superoxide anion and hydrogen peroxide in particular can inactivate EDRF.\textsuperscript{21} Superoxide anion may also act as a direct vasoconstrictor in vascular smooth muscle under some conditions.\textsuperscript{22}

In a previous study we found that in old rats, superoxide dismutase and indomethacin increase dilator responses of the carotid artery to platelets, ADP, and acetylcholine.\textsuperscript{12} This finding suggests that a cyclooxygenase-dependent constrictor factor, either superoxide or a prostanoid or both, may contribute to inactivation of EDRF or may produce direct vasoconstriction in vascular muscle.

In conclusion, the major findings of this study are that, although in old rats endothelium-dependent dilation is impaired in carotid arteries, intraluminal release of EDRF (as detected by bioassay) and responsiveness of vascular muscle are normal. We speculate, on the basis of findings in a previous study,\textsuperscript{17} that impairment of responses to acetylcholine may be related to release of EDCF or increased destruction of EDRF that is released abuminally.

References

Age-related changes in release of endothelium-derived relaxing factor from the carotid artery.

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