Resistence of endothelium-dependent relaxation to elevation of \( \text{O}_2 \) levels in rabbit carotid artery


Resistance of endothelium-dependent relaxation to elevation of \( \text{O}_2 \) levels in rabbit carotid artery. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2109–H2114, 1999.—Endogenous superoxide anion \( (\text{O}_2^-) \) interferes with the bioactivity of nitric oxide \( (\text{NO}) \) in endothelium-dependent arterial relaxation (EDR). Using the lucigenin chemiluminescence assay, we measured \( \text{O}_2^- \) in the thoracic and abdominal aortas and the carotid artery of rabbits to determine whether ambient \( \text{O}_2^- \) varies among the three arteries and differentially diminishes the effect of \( \text{NO} \). Basal levels of \( \text{O}_2^- \) were significantly higher in carotid arteries than in the thoracic aorta \( (23 \pm 1.1 \text{ vs. } 3.9 \pm 0.7 \text{ chemiluminescence units (CU)}; \text{P} < 0.05) \), whereas EDR in response to \( \text{ACh} \) \((10^{-5} - 10^{-7} \text{ M}) \) was not significantly different on ANOVA.

After treatment with the superoxide dismutase (SOD) inhibitor diethyldithiocarbamate (DDC; 10 mM), \( \text{O}_2^- \) levels were significantly elevated, becoming greater in the carotid artery and abdominal aorta than in the thoracic aorta \( (185 \pm 31.2 \text{ and } 202 \pm 40.3 \text{ vs. } 89 \pm 18 \text{ CU}; \text{P} < 0.05) \). DCC significantly reversed EDR in the thoracic aorta but not in the carotid artery; at \( 10^{-6} \text{ M} \text{ACh} \), the decrease seen with DDC was \( 48 \pm 6.2 \text{ vs. } 6.8 \pm 8.0\% \text{ of maximal relaxation in the thoracic aorta and carotid artery, respectively. In the thoracic aorta, exogenous SOD reversed the inhibition of EDR caused by DCC. Moreover, DDC/O}_2^-\text{-resistant EDR in the carotid artery was ablated by the addition of nitro-L-arginine methyl ester (300 \text{ mM; } \text{P} < 0.05), an NO synthase inhibitor, consistent with peroxynitrite or an \( \text{O}_2^-\text{-resistant NO donor being involved in carotid relaxation. Indeed, exogenous peroxynitrite caused similar relaxation of the carotid artery and thoracic aorta, which was unaffected by DDC. Our studies show a greater production of nitrite and \( \text{O}_2^- \) per unit area by the carotid artery, suggesting a greater amount of their product peroxynitrite. These findings support the hypothesis that peroxynitrite is the relaxing agent that resists high \( \text{O}_2^- \) in the carotid artery.}


daorta; reactive oxygen species; superoxide anion

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fied Krebs-HEPES buffer of the following composition (in mM): 119 NaCl, 20 HEPES, 4.6 KCl, 1.0 MgSO₄, 0.15 Na₂HPO₄, 0.4 KH₂PO₄, 5 NaHCO₃, 1.2 CaCl₂, and 5.5 glucose (pH 7.4). HEPES buffer was used to maintain pH at 7.4 in the absence of O₂-CO₂ bubbling. Rings were placed in 1 ml of HEPES buffer in a 1.6-ml, 8 x 50-mm polypropylene tube (Evergreen, Los Angeles, CA) containing lucigenin (250 µM), which was then equilibrated in the dark for 10 min at 37°C. The tubes were placed in a Turner 20e luminometer (Mountain View, CA) with the light chamber maintained at 37°C. Our methods of detecting acute changes in O₂ levels over an extended period of time and at physiological temperature, along with calibration and data collection, have been described previously in detail (25, 26). Units of chemiluminescence were converted to nanomoles of O₂ by standardization with the xanthine oxidase-cytochrome c assay.

Measurements of NO. NO can react with molecular O₂ and water to form nitrite (NO₂⁻), which is more stable than NO. A purge vessel of a chemiluminescent NO analyzer (Sievers no. 207B) at room temperature containing glacial acetic acid and 1% potassium iodide as reducing agents was used to convert NO₂⁻ back to detectable NO. The analyzer can detect picomole levels on the basis of the reaction between NO and ozone, which liberates a photon. A photomultiplier tube detects the photon and feeds an electrical signal into a recorder/ integrator (Fig. 1).

Four to six arterial rings were placed in a test tube in 1 ml of buffer gassed with 95% O₂-5% CO₂ and kept at 37°C. The buffer was changed every 0.5 h. After 90 min of incubation, the rings were placed in 1 ml of buffer for 30 min, after which the buffer was collected and 250 µl were injected into the purge vessels of the analyzer. The rings were then placed in an additional 1 ml of buffer and challenged with ACh (10⁻⁶, 3 x 10⁻⁶ M) over 30 min, after which the buffer was collected and the accumulated content of nitrite analyzed. The rings in each group were challenged with an ACh concentration response only once. NO₃⁻ was integrated and normalized to the total intimal area of the rings. Picomoles of NO generated by the arteries were calculated from the standard curves for nitrite resulting from standard injections of authentic NO. The ACh-stimulated release of NO was determined by subtracting the amount of nitrite contained in a collection of supernatant produced in the 30 min immediately preceding the stimulation with ACh. Our group has previously shown that the release of nitrite stimulated by ACh did not occur if the endothelium was removed by mechanical rubbing or if the vessel was treated with atropine (5).

Measurement of SOD levels. Rings of the thoracic aorta and carotid artery were frozen on dry ice under control conditions or after treatment with DDC and were kept at -70°C. They were shipped on dry ice to the laboratory of S. Marklund, where measurements of Cu/Zn SOD, Mn SOD, and extracellular Cu/Zn SOD were separated (28) and analyzed (17, 18) as described previously.

Synthesis and application of peroxynitrite. Peroxynitrite was produced according to the methods of Beckman et al. (3). Briefly, all solutions were cooled on ice in Erlenmeyer flasks. After synthesis, peroxynitrite (~170–180 mM) was diluted 1:10 or 1:100 in 1.2 M NaOH (4°C) and then applied directly to the organ chambers. Controls were carried out using the vehicle. No relaxation was caused by the addition of equivalent amounts of 1.2 mM NaOH to organ chambers.

Drugs. ACh, DDC, and L-phenylephrine (Sigma, St. Louis, MO) were dissolved in distilled water. Indomethacin (Sigma) was dissolved in 50 mM Na₂CO₃ and buffered to pH 7.4, and SOD (Fluka, Ronkonkoma, NY) was dissolved in bicarbonate buffer. All values represent final molar concentrations in the organ chambers. All drugs were either prepared the day of the experiment or taken from frozen aliquots.

Statistical analysis. Relaxation responses to all agonists are reported as the maximum relaxation following the addition of each concentration of drug and were calculated as a percentage of the contraction induced by phenylephrine. Data are expressed as means ± SE; geometric means of drug concentrations were analyzed. Statistical evaluation of the cGMP levels and time-course responses were performed using Student’s t-test for paired or unpaired comparisons. The relaxation responses were analyzed using ANOVA for repeated measures. Differences between groups at individual concentrations were tested with the Student’s t-test for paired comparisons. P < 0.05 was considered statistically significant. For all data, n is the number of animals from which rings were taken.

RESULTS

Comparison of Tiron-inhibitable lucigenin-enhanced chemiluminescence showed a two- to fourfold higher basal O₂ level in carotid arterial rings compared with levels in rings from the abdominal and thoracic aortas, respectively (carotid vs. thoracic aorta, P < 0.05; Fig. 1A). However, Table 1 shows that the activities of the three isoforms of SOD in blood vessels were not different between the thoracic aorta and carotid artery. DDC (10 mM, a concentration shown to maximally inhibit Cu/Zn SOD levels (25)) caused significantly higher elevation of O₂ in the abdominal aorta and carotid artery than in the thoracic aorta (P < 0.05; Fig. 1B), resulting in levels that were twofold greater in the abdominal aorta and carotid artery. Furthermore, DDC was equally effective in inhibiting total SOD in the thoracic artery and thoracic aorta (98.1 ± 0.52% and 98.0 ± 0.39%, n = 3 and 4, respectively; Table 1). DNA was 689 ± 63.6 in the thoracic aorta compared with 553 ± 15.7 µg/wet wt tissue in the carotid artery (n = 8 and 7, respectively), indicating a larger ratio of cell number to mass in the thoracic aorta.

Comparison of NO levels as measured by nitrite in basal and ACh-stimulated rings showed that significantly more NO was released from the carotid artery than from the thoracic aorta when expressed per unit volume of tissue (Fig. 2). ACh-induced relaxation of phenylephrine-precontracted thoracic aorta reached a maximum of 74% compared with 41% for tissue treated with DDC (P < 0.01, ANOVA; Fig. 3). Treating DDC-pretreated rings with exogenous SOD (150 U/ml) caused a partial but not statistically significant shift of the relaxation response curve back to the left. Abdominal aortic rings relaxed more in response to ACh (P < 0.01 vs. thoracic aorta, ANOVA), reaching a maximum of 95%; DDC significantly inhibited this response, with a maximal relaxation of 67% (P < 0.01, ANOVA; Fig. 4). Interestingly, applying exogenous SOD to DDC-pretreated abdominal aortic rings caused a statistically significant reversal of inhibition (P < 0.05, ANOVA). Carotid arterial rings reached a maximum relaxation at 94% despite higher basal levels of O₂, and this was not significantly affected by pretreatment with DDC (Fig. 5). Exogenous SOD also had no effect on carotid arterial...
relaxation, consistent with the lack of effect of elevated O$_2^-$. Moreover, SOD administration had a slight tendency to enhance basal and ACh-induced relaxations in non-DDC-treated blood vessels (data not shown). However, those relaxations were not statistically different from control (n = 10–17).

To characterize DDC-resistant carotid relaxation, L-NAME, an NO synthase (NOS) inhibitor, was used to determine whether NO is a component of this response. Indeed, L-NAME (300 µM) was completely effective at inhibiting relaxation in both control and DDC-pre-treated carotid artery rings (Fig. 6). Application of peroxynitrite to phenylephrine-precontracted rings from the thoracic aorta and carotid artery caused similar concentration responses even when the rings were pretreated with DDC (Fig. 7).

**DISCUSSION**

Our data support the existence of O$_2^-$-sensitive NO as the major relaxing factor in the thoracic aorta. In contrast, EDR appears to be resistant to O$_2^-$ in the carotid artery but less so in the abdominal aorta. To our knowledge, these results also represent the first report of systematic comparison of the various SOD isozymes in various rabbit blood vessels and demonstrate the effectiveness of DDC at inhibiting SOD activity. Furthermore, the fact that peroxynitrite is a product of the reaction of NO and O$_2^-$, and that levels of these precursors are higher in the carotid artery, suggests that peroxynitrite is more abundant there as well.

**Table 1. Comparisons of SOD activity between homogenates of thoracic aorta and carotid artery**

<table>
<thead>
<tr>
<th>Vessel Type</th>
<th>n</th>
<th>Cu/Zn SOD, U/g wet wt</th>
<th>Mn SOD, U/g wet wt</th>
<th>Extracellular SOD, U/g wet wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoracic aorta</td>
<td>4</td>
<td>3,834 ± 542.1</td>
<td>59 ± 10</td>
<td>2,544 ± 709</td>
</tr>
<tr>
<td>Carotid artery</td>
<td>3</td>
<td>3,627 ± 199.4</td>
<td>70 ± 5.7</td>
<td>2,318 ± 417</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of rabbits from which rings were taken. SOD, superoxide dismutase.

**Fig. 1.** Superoxide anion (O$_2^-$) levels in rings from rabbit thoracic and abdominal aortas and carotid arteries. Units of lucigenin chemiluminescence were averaged over 5 min and expressed as mean of each 30-s interval per milligram of tissue wet weight; this was repeated in presence of Tiron. The difference between these two values (O$_2^-$ avg. interval units/mg) multiplied by 10$^4$ is expressed on y-axis. Shown are basal O$_2^-$ levels (A) and levels in 10 mM diethyl-dithiocarbamate (DDC)-treated [superoxide dismutase (SOD) inhibited] rings (B). *P < 0.05 compared with thoracic aorta.

**Fig. 2.** Nitric oxide levels in rings from rabbit thoracic aortas and carotid arteries. Arterial rings (4–6) were equilibrated at 37°C. Rings were placed in 1 ml of buffer for 30 min, after which buffer was injected into analyzer. Rings were then placed in an additional 1 ml of buffer and challenged over 30 min with a cumulative concentration response to ACh (10$^{-8}$–3 × 10$^{-6}$ M), after which buffer was collected and its nitrite content analyzed. Data are expressed as picomoles of NO generated by arteries per unit intimal surface area. *P < 0.05 compared with basal levels.

**Fig. 3.** Attenuation of ACh-induced relaxation of thoracic aorta by elevated levels of O$_2^-$. Aortic rings were equilibrated in buffer and stretched in a stepwise fashion to a tension of 6 g. Rings were first contracted by addition of half- to full-logarithmic increases of phenylephrine (10$^{-8}$–10$^{-5}$ M) to ~40% of maximal contraction. They were then relaxed by addition of half-logarithmic increases of ACh (10$^{-8}$–10$^{-5}$ M). Relaxation was observed in control (○, n = 17), DDC-treated (□, n = 14), and DDC-treated rings administered exogenous 150 U/ml SOD (▲, n = 8). Data are expressed as means ± SE of %relaxation. **P < 0.01 by ANOVA.
capacity of the carotid artery to relax in response to peroxynitrite without being affected by DDC and the ability of l-NAME to inhibit ACh-induced carotid relaxation support a role for peroxynitrite in this relaxation.

Basal O$_2$ levels varied widely among the carotid artery and the abdominal and thoracic aortas. This is likely explained by a difference in the production of O$_2$, not by differing rates of catabolism, because the levels of all three SOD isozymes did not differ among blood vessels. Whereas the carotid artery appeared to have the greatest capacity to produce O$_2$, this was not related to a higher cell number per unit mass of tissue; in fact, this ratio was slightly lower in the carotid artery. This suggests that the carotid artery has a far greater capacity to produce O$_2$ than the thoracic aorta, and differences in O$_2$ levels after DDC treatment are most likely not related to disparate O$_2$ catabolism because DDC was equally effective in reducing total SOD activity in all arteries, the carotid artery and abdominal aorta. For instance, although basal levels of O$_2$ were higher in the carotid artery than in the thoracic aorta, the degree of ACh-induced relaxation in the absence of DDC was as large or greater than that in the aorta. Consistent with the larger response, the release of NO in the carotid artery was greater than that in the aorta, suggesting the possibility of an adaptation to higher levels of basal O$_2$. Moreover, although DDC caused similar inhibition of total SOD activity in all three arteries, the carotid artery and abdominal aorta...
demonstrated higher increases in $O_2$ than the thoracic aorta. Despite the significantly higher $O_2$ levels in DDC-treated vessels, ACh-induced relaxation was greater in the abdominal than in the thoracic aorta and was highest in the carotid artery.

One possibility is that $O_2$ or one of its metabolites plays a role in the improved relaxation. Another explanation is that a variant of NO, such as nitrosoglutathione, which can act as an NO donor, is most resistant to $O_2$ in the carotid artery, less so in the abdominal aorta, and least of all in the thoracic aorta. Indeed, various biological forms of NO have been described (10, 11, 19) that would be more or less susceptible to $O_2$. As an additional example, sodium nitroprusside, which is known to be metabolized to NO intracelularly (13), causes normal vasodilator responses in arteries in which elevated $O_2$ impairs ACh-induced relaxation (22, 31). This is possibly because sodium nitroprusside releases NO$^+$ (7), which, unlike NO, does not react with $O_2$. Hence, a chemical form of endogenous NO, like that released by nitroprusside, could explain the novel results in the carotid artery. Because nitrosoglutathione or nitrosocysteine also release NO$^+$ rather than NO (7), it is possible that production of one of these nitrosodih NO donors explains the capability of ACh-induced EDR to resist elevated levels of $O_2$ in the carotid artery.

On the basis of levels of chemiluminescence, we calculated the amount of $O_2$ generated by these arteries by comparing levels to those produced by xanthine oxidase as previously described (22). $O_2$ levels in the thoracic aorta and carotid artery in the presence of DDC were 433 and 750 nM, respectively. When the concentration of NO was estimated as the amount released per square centimeter, the amounts of NO produced in response to ACh in the thoracic aorta and carotid artery were ~50 and 200 pmol/ml tissue, or 50 and 200 nM, respectively. Importantly, these data suggest that there is adequate $O_2$ in the carotid artery to react with all the NO produced. The higher production of $O_2$ and NO in the carotid artery compared with that in the thoracic aorta suggests a greater likelihood of OONO$^-$ being produced in the carotid artery. Moreover, the reaction between $O_2$ and NO in DDC-treated vessels is favored in the absence of SOD activity, with which NO must normally compete to react with $O_2$ (4). Attempts to measure OONO$^-$ levels directly using luminol-enhanced chemiluminescence were unsuccessful in the present study because of an apparent lack of sensitivity and specificity of the assay.

In vitro administration of OONO$^-$ reveals that it is a plausible relaxing factor in these arteries. Indeed, others have described peroxynitrite as a relaxing factor (14, 16). Although the exogenous amounts of OONO$^-$ that relaxed the carotid artery appear high, it is possible that substantially lower amounts of OONO$^-$ reached the smooth muscle, due in part to the bicarbonate in the buffer (16). Nonetheless, the responses of the thoracic aorta and carotid artery to OONO$^-$ were identical and equally unaffected by DDC. This lack of effect of DDC supports the potential for peroxynitrite as an $O_2$-resistant mediator. This is also supported by the ability of l-NAME to inhibit ACh-induced carotid relaxations in the presence of DDC; that is, formation of OONO$^-$ from NO and $O_2$ would be inhibited (4). OONO$^-$ has been reported to react with glutathione (GSH) to form GSNO2 (2, 33), an NO donor that would resist $O_2$. Thus the higher levels of both NO and $O_2$ suggest that OONO$^-$, or possibly a reactant of OONO$^-$ with GSH, is responsible for resisting elevated levels of $O_2$ and results in unimpaired EDR. This may also be the reason why EDR of the rabbit carotid artery resists the oxidative stress associated with hypercholesterolemia (20) or diabetes (29, 30), whereas the aorta demonstrates impaired EDR.

In summary, we propose that the carotid artery has a greater propensity to produce OONO$^-$ than the abdominal or thoracic aorta. This hypothesis is based on the higher level of the precursors for OONO$^-$ in carotid arteries as well as the resistance of its EDR response to elevated levels of $O_2$.

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REFERENCES


