Role of Superoxide Anions in the Mediation of Endothelium-Dependent Contractions

Francesco Cosentino, J. Christopher Sill, Zvonimir S. Katušić

Abstract We designed experiments to characterize the role of superoxide anions in isolated canine basilar arteries. Rings with and without endothelium were suspended for isometric tension recording in Krebs-Ringer bicarbonate solution bubbled with 94% O₂-6% CO₂ (37°C, pH 7.4). Radioimmunoassay was used to determine the levels of cyclic GMP and cyclic AMP. Calcium ionophore A23187 (10⁻⁶ to 10⁻⁵ mol/L) caused concentration-dependent contractions. The removal of endothelium abolished the effect of A23187. Contractions to A23187 were reversed into relaxations in the presence of superoxide dismutase (150 U/mL) or the prostaglandin H₂/thromboxane A₂ receptor antagonist SQ29548 (10⁻⁶ mol/L). Nitric oxide and inhibitors of prostacyclin synthesis. 16-19 we provided evidence that translocation of calcium into endothelial cells of this artery causes activation of arachidonic acid metabolism with subsequent release of cyclooxygenase products and contraction of smooth muscle cells. Prostaglandin H₂ (PGH₂), thromboxane A₂ (TXA₂), and superoxide anions have been proposed as mediators of these contractions.5,6,11-15 Superoxide anions are potent chemical inactivators of nitric oxide and inhibitors of prostacyclin synthesis.16-19 In contrast, they do not inhibit production of contractile prostanoids.12 Thus, increased production of superoxide anions may impair the balance between relaxing and contracting factors released from endothelium and favor contraction of underlying smooth muscle cells. In the present study we examined the mechanisms of endothelium-dependent contractions to more precisely characterize the role of superoxide anions.

Methods
The experiments were performed on rings (4 mm in length) of basilar arteries taken from dogs (15 to 20 kg) anesthetized with pentobarbital sodium (30 mg/kg IV). All procedures followed were in accordance with institutional guidelines. The arteries were placed in modified Krebs-Ringer bicarbonate solution (control solution) containing (mmol/L) NaCl, 118.3; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; calcium EDTA, 0.026; and glucose, 11.1. In certain rings, the endothelium was removed mechanically. Each ring was connected to an isometric force transducer (Gould 3000) suspended in an organ chamber filled with 25 mL of control solution (37°C, pH 7.4), and gassed with 94% O₂-6% CO₂. Isometric tension was recorded continuously.

The arteries were allowed to stabilize at a resting tension of 200 to 400 mg for 1 hour. Each ring was then gradually stretched to the optimal point of its length-tension curve (2.5 g) as determined by the contraction to uridine 5'-triphosphate (UTP, 10⁻⁴ mol/L). The functional integrity of endothelium was tested by the presence of relaxations to vasoressin (10⁻⁴ mol/L).20

Radioimmunoassay of Cyclic Nucleotides
Radioimmunoassay techniques were used to determine the levels of cyclic GMP (cGMP) and cyclic AMP (cAMP). Rings with endothelium were initially incubated in control solution bubbled with a 94% O₂-6% CO₂ gas mixture and kept at 37°C. After 1 hour, rings were incubated for an additional 30 minutes in a fresh solution containing 3-isobutyl-1-methylxanthine (IBMX, 10⁻⁴ mol/L) to inhibit the degradation of cyclic nucleotides by phosphodiesterases. When N⁵-nitro-L-arginine methyl ester (L-NAME, 3×10⁻⁴ mol/L) and indomethacin augmented A23187-induced production of cyclic GMP. Selective augmentation of cyclic GMP production by indomethacin appears to be due to protection of nitric oxide or a closely related molecule released following translocation of calcium into endothelial cells. Our findings suggest that (1) an increased concentration of calcium in endothelial cells may activate both cyclooxygenase and the L-arginine/nitric oxide pathway, (2) arachidonic acid metabolism via cyclooxyge

Endothelium-dependent contractions, mediated by the activation of arachidonic acid metabolism via the cyclooxygenase pathway, have been described in arteries and veins.1 The role of these contractions in the regulation of the cardiovascular system is unclear. However, selective impairment of endothelium-dependent relaxations associated with augmentation of endothelium-dependent contractions has been described in arteries removed from animals with high blood pressure or diabetes or after ischemia-reperfusion or aging.2-10

The canine basilar artery has been used as a model to study the mechanisms of endothelium-dependent contractions. In previous studies11,12 we provided evidence that translocation of calcium into endothelial cells of this artery causes activation of arachidonic acid metabolism with subsequent release of cyclooxygenase products and contraction of smooth muscle cells. Prostaglandin H₂ (PGH₂), thromboxane A₂ (TXA₂), and superoxide anions have been proposed as mediators of these contractions.5,6,11-15

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Chloride (Sigma), pentobarbital sodium (Fort Dodge Laboratories), endothelium obtained during contractions induced by uridine 5'-triphosphate (UTP) (10⁻⁵ mol/L). Note that removal of endothelium abolished contractions to A23187. At the end of the experiment, papaverine (PPV) (3×10⁻⁴ mol/L) caused complete relaxation of preparations.

**Materials and Methods**

**Drugs**

The following pharmacologic agents were used: calcium ionophore A23187 (Sigma), 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin F₂₀ (U46619, Cayman Chemical Co), indomethacin (Sigma), L-NAME (Sigma), papaverine hydrochloride (Sigma), pentobarbital sodium (Fort Dodge Laboratories), superoxide dismutase (SOD) (from dog blood, 3000 U/mg protein, Sigma), [1S-[1α,2β(52),3β,4α]β-7-[3-[2-[(phenylamino)carbonyl]-hydroxymethyl]-7-oxabicyclo[2.1.2]hept-2-yl]-5-heptenoic acid (SQ29548, Squibb & Sons), and UTP (Sigma). Stock solutions of the drugs were freshly prepared every day. Drugs were dissolved in distilled water such that volumes of less than 0.2 mL were added to the organ chambers. A stock solution of 10⁻³ mol/L indomethacin was prepared in equal molar concentrations of Na₂CO₃. A stock solution of 10⁻⁴ mol/L A23187 was prepared in 1.5×10⁻⁴ mol/L dimethyl sulfoxide. All concentrations are expressed as final molar (mol/L) concentration in the bath solution.

Concentration-response curves were obtained in a cumulative fashion. Several rings cut from the same artery were studied in parallel; only one concentration-response curve was made per preparation. Responses to the calcium ionophore A23187 were obtained during submaximal contractions to UTP (10⁻⁴ mol/L). Responses to U46619 and PGH₂ were obtained in quiescent rings. Because L-NAME increased resting tension, care was taken to match the contractions induced by UTP in control and treated rings. The incubation time was 30 minutes for indomethacin, 15 minutes for L-NAME and SQ29548, and 5 minutes for SOD. The contractions were expressed as a percentage of maximal contractions induced by papaverine (3×10⁻⁴ mol/L).

**Statistical Analysis**

Results are expressed as mean±SEM; in each set of experiments n equals the number of animals studied. Statistical evaluation of the data was performed by Student's t test for paired observations. A value of P<.05 was considered statistically significant.

**Results**

**Endothelium-Dependent Contractions to A23187**

In rings of canine basilar arteries contracted with UTP (10⁻⁵ mol/L), the calcium ionophore A23187 (10⁻⁴ to 10⁻⁶ mol/L) caused concentration-dependent contractions. The removal of endothelium abolished the effect of A23187 (Fig 1). In rings with endothelium, indomethacin (10⁻⁵ mol/L), SOD (150 U/mL), and the PGH₂/TXA₂ receptor antagonist SQ29548 (10⁻⁶ mol/L) each converted contractions to A23187 to relaxations (Figs 2 and 3). In the presence of SQ29548, relaxations to A23187 were significantly bigger than relaxations obtained in the presence of SOD (Table 1). In contrast, the nitric oxide synthase inhibitor L-NAME (3×10⁻⁴ mol/L) significantly augmented endothelium-dependent contractions to A23187 (area under the curve, 583±56 and 793±77, n=8 for control and L-NAME–treated rings, respectively; P<.05). Furthermore, SOD did not affect A23187-induced contractions in the presence of L-NAME (Fig 4).

In quiescent rings without endothelium, the PGH₂/TXA₂ receptor agonists PGH₂ (10⁻⁵ mol/L) or U46619 (10⁻¹⁰ to 10⁻⁶ mol/L) caused contractions. These con-
TABLE 1. Effect of Superoxide Dismutase and SQ29548 on Contractions to Calcium Ionophore A23187 (3×10⁻⁷ mol/L) in Canine Basilar Arteries With Endothelium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change In Tension, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>+20±26</td>
</tr>
<tr>
<td>SOD, 150 U/mL (n=8)</td>
<td>-33±16*</td>
</tr>
<tr>
<td>SQ29548, 10⁻⁶ mol/L (n=8)</td>
<td>-77±9†</td>
</tr>
</tbody>
</table>

SOD Indicates superoxide dismutase; n, number of dogs; +, contraction; and −, relaxation. Values are mean±SEM expressed as percent change of contractions to uridine 5'-triphosphate (10⁻⁵ mol/L).
*P<.05 vs control.
†P<.05 vs SOD.

Contractions were abolished by SQ29548 (10⁻⁶ mol/L, Fig 4). SOD (150 U/mL) did not affect contractions to PGH₂ (Fig 5).

Endothelium-Dependent Relaxations to A23187

The endothelium-dependent contractile effects of A23187 superimposed on contractions established with UTP were converted to relaxations by SQ29548 (Fig 3, Table 1). Furthermore, SOD (150 U/mL) in the presence of SQ29548 further augmented relaxations to A23187 (EC₅₀=6.9±0.2 [n=6] versus 7.4±0.2 [n=6] for SQ29548 and SOD plus SQ29548, respectively, P<.05). Indomethacin (10⁻⁵ mol/L) or L-NAME (3×10⁻⁴ mol/L) significantly reduced relaxations to A23187 (Figs 6 and 7). Indomethacin plus L-NAME produced significantly more inhibition of A23187-induced relaxations than indomethacin or L-NAME alone (Fig 8, Table 2). However, endothelium-dependent relaxations to A23187 were not abolished by indomethacin plus L-NAME.

In rings without endothelium contracted with UTP (10⁻⁵ mol/L), A23187 (10⁻⁶ to 10⁻⁸ mol/L), both alone and in the presence of SOD (150 U/mL) or SQ29548 (10⁻⁶ mol/L), did not cause any change in tension (Table 3). SOD (150 U/mL) and SQ29548 did not affect contractions to UTP in rings with or without endothelium (10⁻⁵ mol/L, see figure legends).

A23187-Induced Production of Cyclic AMP and Cyclic GMP

In rings with endothelium, A23187 (3×10⁻⁷ mol/L) significantly increased production of cAMP and cGMP (Fig 9). Indomethacin (10⁻⁵ mol/L) inhibited A23187-induced production of cAMP. In contrast, indomethacin augmented production of cGMP in response to A23187 (Fig 9). The stimulatory effect of A23187 on the production of cyclic nucleotides was inhibited in the presence of L-NAME (3×10⁻⁴ mol/L, Fig 9).

Discussion

The present study demonstrates that in isolated canine basilar arteries, translocation of calcium into endothelial cells simultaneously activates both arachidonic acid metabolism via cyclooxygenase and the production of nitric oxide from L-arginine. This conclusion is supported by several lines of evidence: (1) Endothelium-dependent contractions to A23187 are converted to endothelium-dependent relaxations by the PGH₂/TXA₂ receptor antagonist SQ29548; (2) A23187 significantly...
increased production of cyclic nucleotides in the arterial wall; (3) cyclooxygenase inhibition with indomethacin decreased A23187-induced production of cAMP but augmented production of cGMP; and (4) nitric oxide synthase inhibition with L-NAME significantly augmented A23187-induced contractions and inhibited the production of cyclic nucleotides. These results clearly demonstrate that in large canine cerebral arteries, endothelium-dependent relaxations to A23187 are masked by simultaneous release of contracting factors.

Inhibition of A23187-induced production of cAMP in the presence of indomethacin may be explained by decreased vasodilator prostanoit production, possibly prostacyclin. On the other hand, selective augmentation of cGMP production by indomethacin appears to be due to protection of nitric oxide from inactivation by superoxide anions. Prostaglandin H (PGH) synthase oxidizes a large number of compounds, and these oxidations are followed by chain reactions involving free radical generation. The enzyme-centered free radical intermediates generated in the PGH synthase oxidations are followed by chain reactions involving free radical generation. The enzyme-centered free radical intermediates generated in the PGH synthase oxidations are followed by chain reactions involving free radical generation. The enzyme-centered free radical intermediates generated in the PGH synthase oxidations are followed by chain reactions involving free radical generation. 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**Table 2. Comparison of Effect of Indomethacin or L-NAME With Effect of Indomethacin Plus L-NAME on Endothelium-Dependent Relaxations to Calcium Ionophore A23187 (3 x 10^-7 mol/L) in Canine Basilar Arteries**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relaxation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin, 10^-5 mol/L (n=4)</td>
<td>86±7*</td>
</tr>
<tr>
<td>L-NAME, 3 x 10^-4 mol/L (n=7)</td>
<td>58±10*</td>
</tr>
<tr>
<td>Indomethacin + L-NAME (n=7)</td>
<td>23±4</td>
</tr>
</tbody>
</table>

L-NAME indicates N^ω-nitro-L-arginine methyl ester; n, number of dogs. Values are mean±SEM expressed as percent of maximal relaxation induced by papaverine (3 x 10^-4 mol/L).

*P<.05 vs indomethacin+L-NAME.

![Fig 7. Line graph shows concentration-response curves to calcium ionophore A23187 in canine basilar arteries with endothelium in the absence and presence of N^ω-nitro-L-arginine methyl ester (L-NAME) (3 x 10^-4 mol/L). Relaxations were obtained during contractions to uridine 5'-triphosphate (10^-5 mol/L) or L-NAME (3 x 10^-4 mol/L) in rings treated with SQ29548 (10^-6 mol/L). Data are mean±SEM and expressed as percent of maximal relaxation induced by papaverine (3 x 10^-4 mol/L). 100%=3.6±0.4 [n=8] and 3.5±0.5 g [n=8] for SQ29548 and SQ29548 plus L-NAME, respectively. *Significant difference (P<.05) between the two curves.](http://hyper.ahajournals.org/)

![Fig 8. Line graph shows concentration-response curves to calcium ionophore A23187 in canine basilar arteries with endothelium in the absence and presence of indomethacin.](http://hyper.ahajournals.org/)
TABLE 3. Effect of Calcium Ionophore A23187 In Canine Basilar Arteries Without Endothelium In the Absence and Presence of Superoxide Dismutase or SQ29548

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A23187, -log M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
</tr>
<tr>
<td>SOD, 150 U/mL</td>
<td>-0.9±1.3</td>
</tr>
<tr>
<td>SQ29548, 10^-4 mol/L</td>
<td>0</td>
</tr>
</tbody>
</table>

SOD Indicates superoxide dismutase; +, contraction; and −, relaxation. Values are mean±SEM (n=8) and expressed as percent change of contractions to uridine 5'-triphosphate (10^-5 mol/L).

hypertensive rats (SHR) and diabetic rabbits and on rabbit aorta exposed to elevated concentrations of glucose. Impairment of endothelium-dependent relaxations was associated with augmented endothelium-dependent contractions. In agreement with our results, endothelium-dependent relaxations were restored by cyclooxygenase inhibition or PGH$_2$/TXA$_2$ receptor blockade.

Endothelium-dependent relaxations to A23187 were significantly reduced by indomethacin or L-NAME, suggesting that the relaxations were mediated by the production and release of nitric oxide and prostacyclin or some other vasodilator prostanoïd. Indomethacin plus L-NAME further reduced relaxations to A23187 but did not abolish the effect of A23187. Relaxation resistant to inhibition by indomethacin plus L-NAME may be mediated by a hyperpolarizing factor or some other unknown substance released from endothelium.

The present study confirmed our previous findings that SOD converts endothelium-dependent contractions evoked by A23187 to relaxations. In canine basilar arteries, superoxide anions produce a modest contraction of smooth muscle, implying that chemical inactivation of endothelial nitric oxide is a major mechanism responsible for the contractile effect of superoxide anions. In contrast, generation of superoxide anions by xanthine plus xanthine oxidase causes contractions of aorta from SHR comparable to the maximal contractions induced by a depolarizing solution of 60 mmol/L KCl. These contractions were observed in aortas without endothelium, suggesting that free radicals are very potent contractile agents in smooth muscle cells of hypertensive animals. Because superoxide anions may cross cell membrane, it is not possible to rule out the possibility that in some blood vessels, increased production of this free radical in endothelium may favor contraction of smooth muscle not only by inactivation of endothelial nitric oxide but also by direct activation of the contractile process in smooth muscle cells. On the other hand, the earlier observation of an increased production of prostacyclin in the presence of SOD plus catalase would suggest that relaxations to prostacyclin may be responsible for the inhibitory effect of SOD. However, the interpretation of this finding is complicated by the fact that prostacyclin produces both relaxation (3×10^-10 to 3×10^-3 mol/L) and contraction (10^-7 to 10^-5 mol/L) of the isolated canine basilar artery. Furthermore, the lack of an inhibitory effect of SOD in the presence of L-NAME indicates that the observed increase in the concentration of prostacyclin cannot explain the reversal of A23187-induced contractions. By contrast, the cyclic nucleotide data and the inhibitory effect of SOD alone provide strong evidence that superoxide anions modulate the endothelium-mediated responses to calcium ionophore primarily via inactivation of nitric oxide.

Endothelium-dependent contractions mediated by cyclooxygenase have been described in resistance arteries of young SHR before the development of manifest hypertension. Interestingly, these contractions were not affected by a PGH$_2$/TXA$_2$ receptor antagonist but could be abolished with an inhibitor of superoxide anion

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

**p < 0.05 as compared to control**

**p < 0.05 as compared to control and to A23187**

Fig 9. Bar graphs show stimulatory effect of A23187 on production of cyclic AMP and cyclic GMP in control canine basilar arteries with endothelium and arteries exposed to indomethacin (10^-5 mol/L) or N^6-nitro-L-arginine methyl ester (L-NAME) (3×10^-4 mol/L). Values are mean±SEM; n=number of dogs.
production. Intravenous injections of SOD reduced arterial blood pressure in SHR but not in Wistar-Kyoto normotensive rats. Furthermore, a correlation between the expression of endothelium-dependent contractions and increase in arterial blood pressure has been reported in SHR. These findings strongly support the concept that the production of superoxide anions mediated by cyclooxygenase activation may play a role in the development of high arterial tone in hypertension. Indeed, as in SHR, treatment of hypertensive patients with indomethacin improves endothelium-dependent relaxations to acetylcholine.

Our findings demonstrate the existence of superoxide anion-mediated interaction between endothelial cyclooxygenase and the L-arginine/nitric oxide pathway (Fig 10). In pathological conditions, increased production of superoxide anions could impede endothelium-dependent contractions and increase in arterial blood pressure has been reported in SHR. These findings strongly support the concept that the production of superoxide anions mediated by cyclooxygenase activation may play a role in the development of high arterial tone in hypertension. Indeed, as in SHR, treatment of hypertensive patients with indomethacin improves endothelium-dependent relaxations to acetylcholine.

Acknowledgments

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