Mechanisms of Cerebral Arterial Relaxations to Hydrogen Peroxide

Yasuhiko Iida, MD; Zvonimir S. Katusic, MD, PhD

Background and Purpose—The role of hydrogen peroxide in the regulation of cerebral arterial tone is not completely understood. Previous studies have demonstrated that hydrogen peroxide causes vasodilation of small cerebral arteries. The present study was designed to determine the mechanisms responsible for relaxations of large cerebral arteries to hydrogen peroxide.

Methods—Rings of canine middle cerebral arteries without endothelium were suspended for isometric force recording in modified Krebs-Ringer bicarbonate solution bubbled with 94% O₂/6% CO₂ (37°C, pH 7.4). Radioimmunoassay technique was used to determine the levels of cAMP and cGMP.

Results—During contraction to UTP (3×10⁻⁶ or 10⁻⁵ mol/L), hydrogen peroxide (10⁻⁶ to 10⁻⁴ mol/L) caused concentration-dependent relaxations. Catalase (1200 U/mL) abolished the relaxations to hydrogen peroxide. Inhibition of cyclooxygenase by indomethacin (10⁻⁵ mol/L) significantly reduced relaxations to hydrogen peroxide. In arteries contracted by KCl (20 mmol/L), the relaxations to hydrogen peroxide were significantly reduced. In the presence of a nonselective potassium channel inhibitor, BaCl₂ (10⁻⁴ mol/L), a delayed rectifier potassium channel inhibitor, 4-aminopyridine (10⁻³ mol/L), or a calcium-activated potassium channel inhibitor, charybdoxin (3×10⁻⁸ mol/L), the relaxations to hydrogen peroxide were also significantly reduced. An ATP-sensitive potassium channel inhibitor, glyburide (5×10⁻⁶ mol/L), did not affect the relaxations to hydrogen peroxide. Hydrogen peroxide produced concentration-dependent increase in levels of cAMP. Indomethacin (10⁻⁵ mol/L) inhibited the stimulatory effect of hydrogen peroxide on cAMP production. In contrast, hydrogen peroxide did not affect the levels of cGMP.

Conclusions—These results suggest that hydrogen peroxide may cause relaxations of large cerebral arteries in part by activation of arachidonic acid metabolism via cyclooxygenase pathway with subsequent increase in cAMP levels and activation of potassium channels. (Stroke. 2000;31:2224-2230.)

Key Words: calcium ■ cyclic AMP ■ cyclooxygenase ■ potassium channels

Several previous studies demonstrated that hydrogen peroxide causes vasodilation of peripheral as well as cerebral arteries.¹⁻⁷ However, the mechanisms responsible for relaxations of vascular smooth muscle cells exposed to hydrogen peroxide are not fully understood. Studies on isolated bovine pulmonary arteries indicated that hydrogen peroxide activates guanylate cyclase and increases levels of cGMP.² In isolated rabbit aorta and small pial cerebral arteries, oxidation of sulfhydryl groups and activation of ATP-sensitive potassium channels and calcium-dependent potassium channels, respectively, have been proposed as important mechanisms of hydrogen peroxide–induced vasodilation.³⁻⁶,⁷ In cerebral circulation, the vasodilator effect of hydrogen peroxide has been studied almost exclusively on small pial arteries, with the exception of our previous study on isolated large cerebral arteries.⁸ However, the signal transduction pathways involved in mediation of hydrogen peroxide–induced relaxations of large cerebral arteries have not been characterized. Thus, the present study was designed

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to determine the mechanisms responsible for relaxations of large cerebral arteries to hydrogen peroxide.

Materials and Methods

Organ-Chamber Experiments

The experiments were performed on 4-mm middle cerebral artery rings taken from mongrel dogs (15 to 20 kg) of either sex, anesthetized with 30 mg/kg IV sodium pentobarbital. All procedures were conducted in accordance with institutional guidelines. Rings were studied in modified Krebs-Ringer bicarbonate solution (control solution) of the following composition (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 all rings the endothelium was removed mechanically by gentle rubbing of the intimal surface with a stainless steel wire. Each ring was connected to an isometric force transducer (model FT03, Grass Instrument Co) and suspended in an organ chamber filled with 25 mL control solution (37°C, pH 7.4) bubbled with 94% O₂/6% CO₂ gas mixture. Isometric force was recorded continuously. Each ring was then gradually stretched to the

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optimal points of its length-tension curve as determined by the contraction to UTP (10^{-5} \text{ mol/L}). In most of the studied arteries, optimal tension was achieved at approximately 3 g of force. The endothelial removal was confirmed by the absence of relaxation to bradykinin (10^{-6} \text{ mol/L}).

Concentration-response curves to hydrogen peroxide were obtained in a cumulative fashion. Responses to hydrogen peroxide were obtained during submaximal contractions to UTP (3 \times 10^{-5} \text{ or } 10^{-5} \text{ mol/L}). Because 4-aminopyridine, BaCl_{2}, and charybdotoxin increased resting tension, care was taken to match the contractions induced by UTP in control and treated rings. Responses were expressed as a percentage of the maximal relaxations to papaverine (3 \times 10^{-4} \text{ mol/L}). The incubation periods were 30 minutes for indomethacin and 4H[1,2,4]oxadiazolo[4,3-c]quinoxalin-1-one (ODQ); 15 minutes for 4-aminopyridine, BaCl_{2}, charybdotoxin, deferoxoxime, and glyburide; and 5 minutes for catalase and superoxide dismutase (SOD).

Radioimmunoassay of cAMP and cGMP
Radioimmunoassay techniques were used to determine the levels of cAMP and cGMP. Rings without endothelium were immersed in control solution bubbled with a 94% O_{2}/6% CO_{2} gas mixture and kept at 37°C, pH 7.4. After 1 hour, rings were incubated for another 30 minutes in a solution containing 3-isobutyl-1-methylxanthine (IBMX; 10^{-3} \text{ mol/L}) to inhibit the degradation of cyclic nucleotides by phosphodiesterases. In some rings, indomethacin (10^{-5} \text{ mol/L}) or ODQ (3 \times 10^{-6} \text{ mol/L}) was added with IBMX. Hydrogen peroxide (10^{-3} \text{ to } 10^{-4} \text{ mol/L}) was added during the last 1 minute of incubation. All rings were then removed from the solution and frozen in liquid nitrogen. cAMP and cGMP radioimmunoassay kits (Amersham International, Amersham) were used to perform the measurements. Protein assay was conducted by DC Protein Assay Kit (Bio-Rad).

Drugs
The following pharmacological agents were used: BaCl_{2}, catalase (from bovine liver; 40 000 U per milligram protein), charybdotoxin, deferoxoxime mesylate, dimethyl sulfoxide (DMSO), hydrogen peroxide, indomethacin, IBMX, papaverine hydrochloride, SOD (from dog erythrocyte; 3000 U per milligram protein), UTP, all from Sigma; glyburide, ODQ (BIOMOL Research Laboratories, Inc); 4-aminopyridine (Research Biochemicals International); and KCl (EM SCIENCE). Drugs were dissolved in distilled water such that volumes of <0.2 mL were added to the organ chambers. Stock solutions of IBMX (10^{-3} \text{ mol/L}), charybdotoxin (10^{-3} \text{ mol/L}), glyburide (5 \times 10^{-6} \text{ mol/L}), and ODQ (10^{-3} \text{ mol/L}) were prepared in DMSO. Stock solutions of indomethacin (10^{-3} \text{ mol/L}) were prepared in equal molar concentrations of Na_{2}CO_{3}. The concentrations of drugs are expressed as final molar bath concentration.

Statistical Analysis
The data are expressed as mean±SEM; n refers to the number of animals studied. Statistical analysis was performed by using repeated-measures ANOVA, followed by Bonferroni/Dunn test for changes in tension, and 1-way ANOVA, followed by Fisher’s test for changes in levels of cAMP and cGMP.

Results
During contractions to UTP (3 \times 10^{-6} \text{ or } 10^{-7} \text{ mol/L}), hydrogen peroxide (10^{-6} \text{ to } 10^{-4} \text{ mol/L}) caused concentration-dependent relaxations. The effect of hydrogen peroxide was reproducible 30 minutes after the first concentration-response curve was obtained (n=8; data not shown). Catalase (1200 U/mL) abolished the relaxations to hydrogen peroxide (Figure 1), whereas SOD (150 U/mL) and deferoxoxime (10^{-4} \text{ mol/L}) had no effect (Table 1). In arteries contracted by KCl (20 mmol/L), relaxations to hydrogen peroxide were strongly reduced (Figure 2). A nonselective potassium channel inhibitor, BaCl_{2} (10^{-4} \text{ mol/L}; Ca^{2+}-activated potassium channel inhibitor, charybdotoxin (3 \times 10^{-5} \text{ mol/L}); and voltage-dependent potassium channel inhibitor, 4-aminopyridine (10^{-3} \text{ mol/L}), significantly reduced the relaxations induced by hydrogen peroxide (Figures 3, 4, and 5), whereas an ATP-sensitive potassium channel inhibitor, glyburide (5 \times 10^{-6} \text{ mol/L}), had no effect.

| Table 1. Effect of SOD and Deferoxoxime on Relaxations to Hydrogen Peroxide in Canine Middle Cerebral Arteries Without Endothelium |
|---|---|---|---|---|---|
| H_{2}O_{2}, −log mmol/L | 6 | 5.5 | 5 | 4.5 | 4 |
| Control | 0 | 1.9±1.9 | 9.7±2.3 | 27.2±4.9 | 86.6±3.75 | 6 |
| SOD (150 U/mL) | 0 | 1.3±1.3 | 9.1±4.8 | 31.0±7.1 | 84.1±3.1 | 6 |
| Deferoxoxime (10^{-4} \text{ mol/L}) | 0 | 3.2±2.0 | 19.0±10.9 | 46.2±8.9 | 92.3±3.2 | 5 |

Values are mean±SEM; n=number of dogs. Relaxations are expressed as percentage of maximal relaxation induced by papaverine (3 \times 10^{-4} \text{ mmol/L}; 100%=2.8±0.4 \text{ g}, 100%=3.2±0.4 \text{ g}, and 100%=2.2±0.6 \text{ g} for control rings, rings treated with SOD, and rings treated with deferoxoxime, respectively).
Inhibition of cyclooxygenase by indomethacin (10⁻²⁵ mol/L) significantly reduced relaxations to hydrogen peroxide (Figure 6). In contrast, a selective guanylate cyclase inhibitor, ODQ (3×10⁻¹⁶ mol/L), did not affect hydrogen peroxide–induced decrease in vascular tone (Table 3).

Hydrogen peroxide produced concentration-dependent increase in levels of cAMP (Figure 7, top). This effect of hydrogen peroxide was inhibited by indomethacin (10⁻³ mol/L) (Figure 7, bottom). In contrast, hydrogen peroxide (10⁻³ to 10⁻¹ mol/L) had no effect on formation of cGMP (n=7; data not shown).

Consistent with our previous observations, the present study demonstrates that hydrogen peroxide causes relaxation in the isolated canine middle cerebral artery, and this effect is not dependent on the presence of intact endothelial cells. Catalase inhibited these relaxations, whereas SOD and deferoxamine had no effect, demonstrating that the relaxations are due to the effect of hydrogen peroxide rather than formation of hydroxyl radical. Our control experiments indicated that the responses to hydrogen peroxide were reproducible without apparent tachyphylaxis. Furthermore, in arteries contracted with UTP and exposed to hydrogen peroxide (10⁻⁴ mol/L), reduction of vascular tone was recovered to control level after hydrogen peroxide was removed from Krebs-Ringer solution. These findings indicate that hydrogen peroxide was used in concentrations that do not cause nonspecific damage of contractile proteins in smooth muscle cells. Although the exact concentration of hydrogen peroxide in normal or diseased cerebral arterial wall is unknown, the results of our study are consistent with a number of previous reports demonstrating that in very high concentrations (that may be outside the physiological range) from 10⁻⁶ to 10⁻⁴ mol/L, hydrogen peroxide is a potent vasodilator. Relaxations to hydrogen peroxide were reduced in arteries contracted by increasing concentration of extracellular potassium, suggesting that potassium channels could be involved in mediation of the hydrogen peroxide–induced relaxations. This conclusion was reinforced by the fact that potassium channel inhibitors BaCl₂, charybdotoxin, and 4-aminopyridine significantly reduced relaxations to hydrogen peroxide. Our results also suggest that the effects of hydrogen peroxide are mediated in part by calcium-activated and delayed rectifier potassium channels. The results of the present study are in agreement with the previously reported ability of hydrogen peroxide to produce vasodilatation of rat

**Discussion**

Figure 2. Concentration-response curve to hydrogen peroxide in the absence and presence of KCl (20 mmol/L) obtained in canine middle cerebral arteries without endothelium. Data are shown as mean±SEM and expressed as percentage of maximal relaxation induced by papaverine (3×10⁻⁴ mol/L; 100%=3.1±0.2 g [n=7] and 2.4±0.5 g [n=7] for control rings and rings treated with KCl, respectively). Difference between control rings and rings treated with KCl is statistically significant (P<0.05).

Figure 3. Concentration-response curve to hydrogen peroxide in the absence and presence of BaCl₂ (10⁻⁴ mol/L) obtained in canine middle cerebral arteries without endothelium. Data are shown as mean±SEM and expressed as percentage of maximal relaxation induced by papaverine (3×10⁻⁴ mol/L; 100%=3.2±0.5 g [n=6] and 2.9±0.7 g [n=6] for control rings and rings treated with BaCl₂, respectively). Difference between control rings and rings treated with BaCl₂ is statistically significant (P<0.05).

Figure 4. Concentration-response curve to hydrogen peroxide in the absence and presence of charybdotoxin (CTX; 3×10⁻⁸ mol/L) obtained in canine middle cerebral arteries without endothelium. Data are shown as mean±SEM and expressed as percentage of maximal relaxation induced by papaverine (3×10⁻⁴ mol/L; 100%=3.5±0.5 g [n=6] and 2.9±0.7 g [n=6] for control rings and rings treated with charybdotoxin, respectively). Difference between control rings and rings treated with charybdotoxin is statistically significant (P<0.05).
cerebral arterioles by activation of calcium-dependent potassium channels.\textsuperscript{7} Our results differ from the results of a study performed on cat cerebral arterioles, demonstrating the activation of ATP-sensitive potassium channels by hydrogen peroxide.\textsuperscript{6} Differential effects of hydrogen peroxide on potassium channel subtypes between canine and cat cerebral arteries may be due to species differences, different size and regional localization of studied arteries, and different experimental conditions (in vivo versus in vitro).

The relaxation to hydrogen peroxide was inhibited in the presence of a cyclooxygenase inhibitor, indomethacin. This finding is consistent with the results of several previous reports. Hydrogen peroxide has been shown to stimulate arachidonic acid release from vascular smooth muscle cells by activation of phospholipase A\textsubscript{2}.\textsuperscript{19} In newborn piglet cerebral arterioles, topical application of hydrogen peroxide causes an increase in formation of 6-ketoprostaglandin F\textsubscript{1α}, thromboxane B\textsubscript{2}, and prostaglandin E\textsubscript{2}.\textsuperscript{20} In our previous study we demonstrated that in arteries without endothelium, free radicals generated by xanthine plus xanthine oxidase stimulate the production of 6-ketoprostaglandin F\textsubscript{1α}.\textsuperscript{8} Taken together, these findings strongly suggest that hydrogen peroxide may release prostacyclin from vascular smooth muscle.

It is generally accepted that vasodilation produced by prostacyclin is mediated by formation of cAMP.\textsuperscript{21} In cerebral arteries, hydrogen peroxide–induced relaxation appears to be mediated by the formation of prostacyclin and subsequent increase in cAMP levels. This conclusion is supported by our findings that hydrogen peroxide stimulates formation of cAMP, and this effect was inhibited by indomethacin.

Elevation in cAMP concentration may relax vascular smooth muscle by several different mechanisms. Agonist-dependent increase in cAMP may enhance Ca\textsuperscript{2+} extrusion and sequestration and therefore decrease [Ca\textsuperscript{2+}].\textsuperscript{22–24} Alternatively, increase in cAMP or cGMP induces relaxation by activating potassium channels, inducing hyperpolarization, and decreasing Ca\textsuperscript{2+} influx.\textsuperscript{25,26} Previous studies demonstrated that calcium-activated potassium channels in smooth muscle from rat aorta and porcine coronary arteries are activated by cAMP-dependent protein kinase.\textsuperscript{27,28} Furthermore, the dilation of cerebral arterioles induced by forskolin, a direct activator of adenylate cyclase, is inhibited by charybdotoxin and iberiotoxin.\textsuperscript{29} These findings suggest that adenylate cyclase–mediated activation of calcium-activated potassium channels may play an important role in cerebral vasodilation. In the present study, although we did not directly evaluate the effects of cAMP on potassium channels,
it is possible that relaxations to cAMP may be mediated in part by calcium-dependent potassium channels.

In bovine pulmonary arteries, hydrogen peroxide causes relaxation by activation of soluble guanylate cyclase. However, in the present study a selective guanylate cyclase inhibitor, ODQ, did not affect the vasodilator action of hydrogen peroxide. Furthermore, hydrogen peroxide had no effect on the levels of cGMP. These findings are consistent with the results obtained in cat cerebral arterioles demonstrating that soluble guanylate cyclase inhibitor, LY-83583, does not affect the vasodilator action of hydrogen peroxide. Thus, it appears that in the cerebral circulation, activation of guanylate cyclase does not play a role in mediation of the vasodilator effect of hydrogen peroxide.

Reduction of relaxations to hydrogen peroxide in arteries contracted by a depolarizing solution of potassium chloride suggests that hydrogen peroxide may cause relaxation by hyperpolarization of smooth muscle cells. This finding is in agreement with the results of a previous study using large coronary arteries, indicating that relaxations to hydrogen peroxide are mediated by hyperpolarization of smooth muscle cell. Hyperpolarization of cell membrane closes voltage-dependent calcium channels, leading to a decreased influx of extracellular calcium.

Hydrogen peroxide is released from activated phagocytic cells (e.g., neutrophils and monocytes) during tissue injury or inflammation. Furthermore, ischemia/reperfusion is also associated with increased formation of free radicals, including hydrogen peroxide. Under these conditions, vasodilator effects of hydrogen peroxide may play an important role in the control of vascular tone and regulation of local blood flow in the brain. Our results suggest that in canine middle cerebral arteries, effects of hydrogen peroxide are in part due to activation of arachidonic acid metabolism via the cyclooxygenase pathway. Increased formation of prostanoids apparently stimulates adenylate cyclase and biosynthesis of cAMP. This, in turn, may activate potassium channels, producing hyperpolarization and relaxations of smooth muscle cells.

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**References**


**TABLE 3. Effect of ODQ on Relaxations to Hydrogen Peroxide in Canine Middle Cerebral Arteries Without Endothelium**

<table>
<thead>
<tr>
<th>H₂O₂, −log mmol/L</th>
<th>6</th>
<th>5.5</th>
<th>5</th>
<th>4.5</th>
<th>4</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1.6±1.6</td>
<td>12.2±3.3</td>
<td>37.3±7.8</td>
<td>85.2±5.1</td>
<td>7</td>
</tr>
<tr>
<td>ODQ (3×10⁻⁶ mmol/L)</td>
<td>0</td>
<td>0</td>
<td>4.7±1.5</td>
<td>23.6±3.7</td>
<td>86.4±2.9</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=number of dogs. Relaxations are expressed as percentage of maximal relaxation induced by papaverine (3×10⁻⁴ mmol/L; 100%=3.4±0.5 g and 100%=3.6±0.6 g for control rings and rings treated with ODQ, respectively).

**Figure 7.** Top, Effect of hydrogen peroxide on cAMP production in canine middle cerebral arteries without endothelium. Values are expressed as mean±SEM. *Significantly different from control level (P<0.05). Bottom, Effect of hydrogen peroxide on cAMP production in canine middle cerebral arteries without endothelium in the absence or presence of indomethacin (10⁻⁵ mol/L). Values are expressed as mean±SEM. *Significantly different from control level (P<0.05).
In most studies, H$_2$O$_2$ relaxes and dilates arteries. In the cerebral circulation, H$_2$O$_2$ is a potent dilator of small cerebral arteries.$^{1,2}$ In contrast, in the isolated canine basilar artery, H$_2$O$_2$ causes vasoconstriction.$^{3}$ In one study this was dependent on activation of cyclooxygenase and release of vasoconstrictor prostanoids,$^3$ whereas in another it resulted from endogenous vasoconstrictor mechanisms without the release of a mediator.$^4$

A variety of mechanisms have been implicated in the vasodilator action of H$_2$O$_2$. These include increased production of cyclic GMP, oxidation of sulfhydryl groups, generation of hydroxyl radical with consequent activation of ATP-sensitive potassium channels, and direct activation of calcium-activated potassium channels. In the eloquent study above, Iida and Katusic provide additional needed information about the action of H$_2$O$_2$ on large cerebral arteries. They found that in isolated endothelium-denuded canine middle cerebral arteries, H$_2$O$_2$ activated cyclooxygenase with increased production of prostacyclin, which in turn activated adenylate cyclase and resulted in the opening of calcium-activated potassium channels. This mechanism is consistent with what has been found previously in rat cerebral arteries. Iida and Katusic also provided chemical and pharmacological evidence that H$_2$O$_2$ in this preparation did not activate potassium channels.$^5$ Because of the absence of catalase in the extracellular environment, the extracellular concentration of H$_2$O$_2$ can occur under abnormal conditions, such as in the presence of inflammation, where phagocytic cells may secrete oxygen radicals that would give rise to H$_2$O$_2$ in the extracellular space. In terms of the potential role of H$_2$O$_2$ in the physio-
logical regulation of vascular tone, in which $H_2O_2$ would be generated in normal parenchymal or vascular cells, the situation is quite different. Because of the presence of catalase intracellularly, the concentration of $H_2O_2$ that can be achieved under physiological conditions is low. It has been estimated that, even in the absence of catalase, it cannot exceed $10 \mu M$. Accordingly, the concentration of $H_2O_2$ in the extracellular space that can be achieved under these conditions is likely to be considerably less. Only one study was performed at very low concentrations of $10^{-6}$ to $10^{-8} M$. In this study it was found that in cat cerebral arterioles, $H_2O_2$ generated hydroxyl radical and caused dilation due to opening of ATP-sensitive potassium channels. It should be noted that at these concentrations, the isolated middle cerebral artery in the experiments of Iida and Katusic was unresponsive. Hence, the concentrations used were considerably higher, and one may question whether such concentrations are achievable under physiological conditions. Future experiments should also address whether there are differences in the mechanism of action of $H_2O_2$ in large conduit-type vessels, such as the middle cerebral artery or the basilar artery, versus the smaller arterioles. Finally, it is rare for investigators to study with the same techniques and in the same setting more than one species. In fact, none of the studies have been repeated by the same investigators in different species. Hence, the potential exists that the recorded differences in the literature result from species differences.

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