H₂O₂ and Endothelium-Dependent Cerebral Arteriolar Dilation
Implications for the Identity of Endothelium-Derived Relaxing Factor Generated By Acetylcholine

Enoch P. Wei and Hermes A. Kontos

We studied the mechanism of the vasodilator effect of H₂O₂ on cerebral arterioles and its effect on endothelium-dependent responses to acetylcholine. Topical application of H₂O₂ (0.1–1 μM) on the brain surface of anesthetized cats equipped with cranial windows induced dose-dependent arteriolar dilation, which was markedly inhibited by topical deferoxamine, showing that it was probably mediated by generation of hydroxyl radical. Higher concentrations of H₂O₂ (3 μM) also induced dilation, which was unaffected by deferoxamine, indicating the participation of other mechanisms. After topical application of H₂O₂, endothelium-dependent responses to acetylcholine were eliminated or converted to vasoconstriction, and in bioassay experiments, acetylcholine-mediated endothelium-derived relaxing factor (EDRF) was absent. Superoxide dismutase plus catalase restored the appearance of transferable EDRF after 1 μM H₂O₂ but not after 3 μM H₂O₂. Application of H₂O₂ in the assay window eliminated the responses to nitroprusside and nitric oxide but did not affect responses to adenosine, to EDRF from the donor window, or responses to S-nitroso-L-cysteine. The inhibiting effect of H₂O₂ on the response to nitroprusside was partially eliminated after topical application of N-acetyl-L-cysteine. The results show that H₂O₂ inhibits the vasodilator action of nitroprusside and nitric oxide probably because it oxidizes thiols in vascular smooth muscle and prevents the formation of a nitrosothiol. EDRF from acetylcholine and S-nitroso-L-cysteine still produce dilation in the presence of the blockade induced by H₂O₂. The findings suggest strongly that the EDRF from acetylcholine in cerebral vessels is a nitrosothiol like S-nitroso-L-cysteine. (Hypertension 1990;16:162–169)

In earlier studies, we found that topical application of 29.4 μM H₂O₂ on the brain surface of anesthetized cats for 30 minutes induced pronounced cerebral arteriolar dilation that outlasted the washout of H₂O₂ for at least 30 minutes and was accompanied by evidences of endothelial and vascular smooth muscle damage. The dilated arterioles in these experiments showed discrete morphological lesions of the endothelium consisting of blebs or craters. These are similar in appearance to the blebs described in cultured cells in response to a variety of influences that induce oxidant stress. In these cultured preparations, the lesions were ascribed to oxidation of thiols with consequent alterations in the cytoskeleton of the cells. Vascular smooth muscle in cerebral arterioles exposed to H₂O₂ also showed evidences of damage consisting of vacuolization or the appearance of electron-dense inclusion bodies. The dilated arterioles displayed reduced responsiveness to the vasoconstrictor effect of arterial hypocapnia. This abnormality was manifested only in the smaller arterioles (<100 μm in diameter) but not in the larger ones.

In the present experiments, we further explored the effects of H₂O₂ on cerebral arterioles. We specifically addressed the following issues: 1) Whether H₂O₂ had significant effects on cerebral arterioles in concentrations lower than those used earlier, which are more likely to be achieved under naturally occurring pathological conditions in vivo. 2) We investigated the possibility that hydroxyl radical might mediate the effects of H₂O₂. This possibility was suggested by earlier findings that the effect of H₂O₂ on cerebral arterioles was similar to that seen from a combination of H₂O₂ and ferrous iron, which produces hydroxyl radical, and by the fact that indige-
nous cerebrospinal fluid (CSF) contains enough catalytic iron to catalyze the Haber-Weiss reaction.\(^8\)\(^9\)\(^3\) We explored the functional aspects of the vascular damage induced by H\(_2\)O\(_2\) by studying various aspects of endothelium-dependent vasodilation in response to acetylcholine after exposure to H\(_2\)O\(_2\). The findings of these experiments have important implications about the identity of the endothelium-derived relaxing factor (EDRF) induced by acetylcholine in cerebral arterioles.

**Methods**

Experiments were carried out in cats (2–3.5 kg) anesthetized with sodium pentobarbital (30 mg/kg i.v.). After tracheotomy, each cat was ventilated with a positive-pressure respirator and received gallamine triethiodide (5 mg/kg i.v.) for skeletal muscle paralysis. The end-expiratory CO\(_2\) of the cats was continuously monitored with a Hewlett-Packard CO\(_2\) analyzer (Hewlett-Packard Co., Palo Alto, Calif.) and was maintained at a constant level of about 30 mm Hg. Arterial blood pressure was measured with a Statham pressure transducer (Statham Instruments, Oxnard, Calif.) connected to a cannula introduced into the aorta via the femoral artery. Arterial blood samples were collected for determination of arterial blood oxygen and CO\(_2\) partial pressures, pH, and hematocrit at appropriate intervals during the experiment. Blood gas tensions and pH were measured with Corning electrodes (Corning, Medfield, Mass.). Hematocrit was measured by a micromethod. Rectal temperature of each cat was monitored continuously, and temperature of each cat was kept constant with the aid of a heating blanket.

The cerebral microcirculation of the parietal cortex was visualized through acutely implanted cranial windows, as described in detail previously.\(^10\) In most cases, we used two symmetrical windows that were placed to overlie the parietal cortex of each hemisphere for comparative studies. The space under the cranial windows was filled with artificial CSF identical in composition to that of cats.\(^11\) Each window was equipped with three openings. One port of the window was connected to a low pressure Statham transducer for continuous monitoring of intracranial pressure. The intracranial pressure was maintained at 5 mm Hg by connecting another outlet of the window to a coiled plastic tube whose free end was placed at the appropriate height to give the desired pressure. Two ports of the cranial window were used as inlet and outlet, allowing topical application of various solutions by superfusion. Pial arteriolar diameter was measured with a Vickers image-splitting device (Vickers Instruments, York, England) attached to a Wild microscope (Wild Heezebrugg, Heezebrugg, Switzerland) equipped with a \(\times 6.5\) dry objective. In each cat, 4–10 arterioles were observed under each window. The responses of small and large (smaller or larger than 100 \(\mu\)m in diameter, respectively) arterioles were evaluated separately to assess the dependence of the responses on the size of the arterioles.

H\(_2\)O\(_2\) was obtained from J.T. Baker Inc., Phillipsburg, N.J. Acetylcholine chloride, superoxide dismutase (SOD) (3,200 units/mg protein from bovine blood), catalase (2,000 units/mg protein from bovine liver), sodium nitroprusside, N-acetyl-L-cysteine, and adenosine were obtained from Sigma Chemical Co., St. Louis, Mo. Deferoxamine was obtained from CIBA, Basle, Switzerland. Nitric oxide was purchased from Matheson Gas, East Rutherford, N.J. The nitric oxide gas was further purified by the method of Kon.\(^12\) A nitric oxide stock solution was prepared each day immediately before the experiment as follows: Artificial CSF was bubbled with 100% nitrogen in a gas-tight container for 10 minutes. Nitric oxide was then introduced at 10 psi for 1 minute. This stock solution was then anaerobically diluted with artificial CSF to the desired concentration. \(\cdot\)Nitroso-L-cysteine (cysNO) was kindly supplied by Drs. J.N. Bates and D.G. Harrison, Departments of Anesthesiology and Internal Medicine, University of Iowa, Iowa City. cysNO was synthesized from NO\(_2\) and cysteine.\(^13\) It was made available in a 0.1 M solution in methanol and kept at \(-20^\circ\) C. In this methanol solution, cysNO has a half-life of several months when kept at \(-20^\circ\) C and several hours when kept at room temperature. Changes in concentration of cysNO were monitored by measurements of absorbance at 595 nm. Solutions of cysNO were prepared immediately before the experiment by dissolving the appropriate amount of the methanol solution into artificial CSF that had previously been bubbled with 100% nitrogen as described above for nitric oxide. We detected no changes in the concentration of cysNO in the stock solution over the period covered by these experiments. Vasodilator potency of the final solutions of cysNO did not change over periods of 15–30 minutes at \(37^\circ\) C. Methanol in concentrations equivalent to those present in the final cysNO solutions did not have detectable effects on cerebral arterioles.

EDRF was assayed in vivo, as described in detail previously.\(^14\) We used cats equipped with double cranial windows. The two windows were connected in series either by a long route with a transit time of 2 minutes or by a short route with a transit time of 6 seconds. The donor window was superfused with a solution of \(10^{-7}\) M acetylcholine, and the superfusate, after passing through the donor window, was directed via either the short or the long route through the assay window. The assay window was subjected to muscarinic blockade with atropine. The absence of responsiveness to the direct effects of acetylcholine in the assay window was verified in each experiment. We showed in earlier experiments\(^14\) that, in this preparation, acetylcholine generates a short-lived vasodilator agent that can be transferred to induce vasodilation in the assay window. This vasodilator agent has characteristics identical to the EDRF induced by acetylcholine in large vessels in vitro. It is...
therefore believed to be the EDRF induced by acetylcholine from cerebral microvessels.

The following groups of experiments were done: 1) To test the effects of low concentrations of \( \text{H}_2\text{O}_2 \) and to evaluate the role of hydroxyl radical, we used six cats equipped with double cranial windows. One window was pretreated with 1 mM deferoxamine. This concentration of deferoxamine effectively scavenges the catalytic iron and prevents the generation of hydroxyl radical from the iron-catalyzed Haber-Weiss reaction, although it has insignificant direct scavenging effects on hydroxyl radical itself.\(^{15,16}\) The two cranial windows were filled with a solution of \( \text{H}_2\text{O}_2 \) at concentrations of 0.1, 0.5, or 1 \( \mu \text{M} \). Each solution was allowed to stay in contact with the brain for 4 minutes, and measurements were made after a steady state was established in the last 2 minutes of application.

2) In two additional series of experiments, each consisting of six cats, we evaluated the effects of 1 and 3 \( \mu \text{M} \) concentrations of \( \text{H}_2\text{O}_2 \) on endothelium-dependent vasodilation from acetylcholine. In these experiments, we used cats equipped with double cranial windows. One window was pretreated with deferoxamine 1 mM, and the other was left untreated. Both windows were then filled with a solution of \( \text{H}_2\text{O}_2 \) at either 1 or 3 \( \mu \text{M} \) concentration. After 3 minutes of application, the solution was washed out with fresh CSF without any additives. Responses to acetylcholine 10\(^{-7} \) M were tested before the application of \( \text{H}_2\text{O}_2 \) as well as 30 minutes after its washout.

3) In another series of four cats, we tested the effects of \( \text{H}_2\text{O}_2 \) on the responses to nitroprusside and adenosine. Nitroprusside causes vascular smooth muscle relaxation by activation of guanylate cyclase, whereas adenosine induces relaxation via activation of adenylyl cyclase. In this experiment, we tested the responses before and 30 minutes after washout of a 3-minute application of 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \).

4) In another five cats, we examined the effect of replenishment of sulfhydryl (SH) groups with \( N \)-acetyl-L-cysteine on the inhibition of the vasodilator response to nitroprusside after hydrogen peroxide application. We tested the vasodilator action of nitroprusside in the control period before application of hydrogen peroxide, 30 minutes after topical application of 1 \( \mu \text{M} \) hydrogen peroxide, and for a third time after a 10-minute application of 100 \( \mu \text{M} \) \( N \)-acetyl-L-cysteine s.c.

5) We studied EDRF production in response to acetylcholine by bioassay before and after \( \text{H}_2\text{O}_2 \) application in five cats as follows: Bioassay of EDRF in response to acetylcholine was carried out before and after topical application in the donor window of a 1- or 3-\( \mu \text{M} \) concentration of \( \text{H}_2\text{O}_2 \) for 3 minutes. EDRF assay was carried out 30 minutes after washout of \( \text{H}_2\text{O}_2 \). The assay was repeated immediately thereafter with inclusion of SOD (60 units/ml) and catalase (40 units/ml) in the solution of acetylcholine.

6) In another series of five animals, we also tested the response to EDRF from the donor window before and 30 minutes after a 3-minute application of 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) in the assay window to evaluate the effects of \( \text{H}_2\text{O}_2 \) on the responsiveness of vessels of cerebral arterioles to EDRF from another source.

7) In five cats, we tested the responses to nitric oxide and \( \text{cysNO} \) before and 30 minutes after a 3-minute application of 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) on the brain surface. Solutions of nitric oxide at various concentrations were prepared as described above and then used to fill the space under the cranial window. The changes in vessel diameter were evaluated in the second minute after application. Similarly, we used solutions of \( \text{cysNO} \) to fill the cranial window and measured changes in diameter in the second minute after application.

The results were expressed as mean\( \pm \)SEM. Statistical evaluation of the results was carried out by analysis of variance, which took into account that some vessels were from the same animal. When more than two groups of data were compared and statistically significant results were shown by analysis of variance, differences between individual means were evaluated by \( t \) tests modified for multiple comparisons.

**Results**

Figure 1 shows that \( \text{H}_2\text{O}_2 \) in a concentration of 0.1 to 1 \( \mu \text{M} \) induced substantial cerebral arteriolar dilation, which was dose-dependent. The effects of \( \text{H}_2\text{O}_2 \) were eliminated in the presence of deferoxamine. Table 1 shows that hydrogen peroxide in a concentration of 3 \( \mu \text{M} \) induced pronounced cerebral arteriolar...
TABLE 1. Effect of Topical \( \text{H}_2\text{O}_2 \) (3 \( \mu \text{M} \)) on Cerebral Arterioles

<table>
<thead>
<tr>
<th>Arteriolar size</th>
<th>Condition</th>
<th>Without deferoxamine</th>
<th>With deferoxamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>62±2.6</td>
<td>62±2.6</td>
</tr>
<tr>
<td>Small arterioles</td>
<td>Control</td>
<td>62±2.6</td>
<td>62±2.6</td>
</tr>
<tr>
<td></td>
<td>During</td>
<td>96±5.7</td>
<td>91±5.6</td>
</tr>
<tr>
<td>Large arterioles</td>
<td>Control</td>
<td>129±6.8</td>
<td>133±4.0</td>
</tr>
<tr>
<td></td>
<td>During</td>
<td>185±13.6</td>
<td>183±10.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM from 17 small and 15 large arterioles without deferoxamine and 22 small and 17 large arterioles with deferoxamine from six cats.

Arteriolar dilation, which was unchanged in the presence of deferoxamine.

Figure 2 shows the response to \( 10^{-7} \text{M} \) acetylcholine before and after 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), with and without pretreatment with 1 mM deferoxamine. It is seen that \( \text{H}_2\text{O}_2 \) eliminated endothelium-dependent relaxation from acetylcholine, and that this effect was prevented by deferoxamine. Figure 3 shows the responses to \( 10^{-7} \text{M} \) acetylcholine after topical application of 3 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \). It is seen that endothelium-dependent relaxation from acetylcholine was abolished after \( \text{H}_2\text{O}_2 \), in the presence of deferoxamine and converted to a significant vasoconstriction in its absence.

As seen in Figures 4 and 5, the vasodilator responses to nitroprusside were markedly reduced or eliminated after topical application of 1 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), whereas the responses to adenosine were not significantly altered. Topical application of \( N \)-acetyl-L-cysteine after treatment with \( \text{H}_2\text{O}_2 \) partially restored the vasodilator response to nitroprusside (Figure 5).

Acetylcholine induced the production of a vasodilator agent that was transferable to the assay window but was too short-lived to induce vasodilation when the transit time was increased to 2 minutes (Figures 6 and 7). After application in the donor window of
H$_2$O$_2$ at 1 or 3 $\mu$M, no EDRF could be detected. The effect of 1 $\mu$M H$_2$O$_2$ could be reversed in the presence of SOD and catalase, but the effect of 3 $\mu$M H$_2$O$_2$ was unaffected by SOD and catalase. Treatment of the assay window with 1 $\mu$M H$_2$O$_2$ did not alter the responses to EDRF from the donor window (Figure 8).

Figures 9 and 10 show that, after topical application of H$_2$O$_2$, the vasodilator response to topical application of nitric oxide was eliminated, and the response to cysNO was unchanged. cysNO was a more potent dilator of cerebral arterioles than nitric oxide. Also, the dilation caused by cysNO subsided within 4–5 minutes from application, whereas that due to nitric oxide persisted for 8–10 minutes.

**Discussion**

Our findings show that H$_2$O$_2$ is a strong dilator of cerebral arterioles. In concentrations up to 1 $\mu$M the vasodilating effect of H$_2$O$_2$ is due to the generation of hydroxyl radical, as shown by its complete inhibition by deferoxamine. The only other study in which the role of hydroxyl radical in the vasodilation from H$_2$O$_2$ was examined is that of Burke and Wolin. These investigators found that deferoxamine and scavengers of hydroxyl radical did not influence the relaxing effect of H$_2$O$_2$ on isolated precontracted pulmonary arteries. This difference may be due to the presence of catalytic iron in CSF capable of catalyzing the Haber–Weiss reaction.

Our findings show that, at concentrations of H$_2$O$_2$ higher than 1 $\mu$M, the vasodilation is not blocked by deferoxamine. This may be due to a variety of reasons. First, it is possible that, because H$_2$O$_2$ is lipid-soluble and freely diffusible, it may penetrate to areas that are not accessible to deferoxamine. Alternatively, H$_2$O$_2$ may initiate another mechanism of dilation unrelated to hydroxyl radical generation when it reaches intracellular sites. In this respect, it was found by Burke and Wolin that, in the bovine pulmonary arteries in vitro, the mechanism of the relaxation from H$_2$O$_2$ involved compound I of catalase as an intermediate. We did not carry out experiments to investigate this possibility.

After exposure to H$_2$O$_2$, the cerebral arterioles lost their ability to respond to acetylcholine with relaxation. The bioassay experiments show that this was due to either destruction of EDRF or inability of the endothelium to produce EDRF. At least two mechanisms seem to be involved. At low concentrations of H$_2$O$_2$ (1 $\mu$M), the elimination of endothelium-dependent relaxation from acetylcholine is due to destruction of EDRF by oxygen radicals. We base this interpretation on the rapid restoration of endothelium-dependent relaxation and reappearance of EDRF in the presence of SOD and catalase. Because these enzymes have high molecular weight and are thus unable to enter rapidly the intracellular environment of cells, this explanation seems to be the best one. On the other hand, after exposure to higher concentrations of H$_2$O$_2$ (3 $\mu$M), endothelium-
dependent responses to acetylcholine were eliminated, EDRF production was lost, and SOD and catalase were unable to restore the response to normal. We believe that this is due to injury of the endothelium and inability to produce EDRF. It is obvious that, because the loss of endothelium-dependent relaxation from acetylcholine after exposure to 1 \textmu M of hydrogen peroxide continued for a fairly long time after \textit{H}_{2}\textit{O}_{2} had been eliminated by washout, it could not be due to a direct effect of the exogenous \textit{H}_{2}\textit{O}_{2} on EDRF. Recent preliminary studies have shown that exposure of cultured endothelial
cells to H₂O₂ induces sustained generation of superoxide. It is likely that an analogous mechanism was responsible for the continued absence of endothelium-dependent responses after exposure to this concentration of H₂O₂.

It is of great interest that after exposure to H₂O₂ at a concentration of 1 μM the vasodilating effect of nitroprusside and nitric oxide, which act by activating guanylate cyclase, was eliminated, but the response to adenosine was preserved. Similarly, such vessels responded normally to EDRF from another source and to cysNO. These findings strongly suggest that interaction with SH groups is essential for nitric oxide and nitroprusside to generate an agent that is the direct stimulator of guanylate cyclase. This view is supported by the demonstration of partial restoration of the response to nitroprusside after replenishment of SH groups with N-acetyl-L-cysteine. The present findings are consistent with earlier results in which we used nitro blue tetrazolium, a dye that oxidizes SH groups, to block the vasodilating effect of nitroglycerin, nitroprusside, and nitric oxide. Nitro blue tetrazolium in these experiments did not influence the response to acetylcholine. It is clear, therefore, that EDRF from acetylcholine can bypass the essential intermediate steps through which the nitrodilators interact with vascular smooth muscle components to activate guanylate cyclase. These findings are consistent with the hypothesis advanced by Needleman and coworkers and by Ignarro et al. that the interaction of the nitrodilators with SH groups is essential for their ability to activate guanylate cyclase. The exact location of the SH groups that are destroyed by H₂O₂ is not known. They may be located in the cell membrane, in the interior of the cell, or even on the molecule of guanylate cyclase itself.

Given the known ability of H₂O₂ to oxidize SH groups, it is of interest to consider the possibility that the inability of the endothelium to produce EDRF after exposure to 3 μM hydrogen peroxide may be related to its SH oxidizing ability.

It has recently been suggested that the EDRF from acetylcholine is a nitrosothiol. If this is correct, the effect of H₂O₂ on EDRF production may be explained because the oxidation of these essential SH groups may render the endothelium unable to produce EDRF. Our findings in the present study and earlier findings are consistent with the possibility that the EDRF from acetylcholine in the cerebral vessels might be a nitrosothiol, which is capable of activating guanylate cyclase directly without the necessity for an interaction with tissue components. Our findings with cysNO support this view. The effect of cysNO contrasts with that of nitric oxide, which must interact with the SH pool and, perhaps, be converted to a nitrosothiol before it can activate guanylate cyclase. cysNO resembles the EDRF from acetylcholine much more closely than nitric oxide. Agents such as H₂O₂ or nitro blue tetrazolium, which oxidize SH groups, render the nitrodilators inactive while they leave the effect of acetylcholine unscathed. Therefore, we suggest that the EDRF induced by acetylcholine in cerebral arterioles is a nitrosothiol like cysNO.

References


KEY WORDS • vasodilation • endothelium • nitric oxide • oxygen radicals • vascular injury • nitrodilators
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