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.9 2) We explored the functional aspects of the vascular damage induced by H₂O₂ by studying various aspects of endothelium-dependent vasodilation in response to acetylcholine after exposure to H₂O₂. 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 CO₂ of the cats was continuously monitored with a Hewlett-Packard CO₂ 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 CO₂ 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 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 cranial windows was connected to a low pressure Statham transducer (Statham Instruments, Oxnard, Calif.) connected to a cannula introduced into the aortal via the femoral artery. Arterial blood samples were collected for determination of arterial blood oxygen and CO₂ 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 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 cranial windows was connected to a low pressure Statham transducer (Statham Instruments, Oxnard, Calif.) connected to a cannula introduced into the aortal via the femoral artery. Arterial blood samples were collected for determination of arterial blood oxygen and CO₂ 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 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 cranial windows was connected to a low pressure Statham transducer (Statham Instruments, Oxnard, Calif.) connected to a cannula introduced into the aortal via the femoral artery. Arterial blood samples were collected for determination of arterial blood oxygen and CO₂ 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 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 cranial windows was connected to a low pressure Statham transducer (Statham Instruments, Oxnard, Calif.) connected to a cannula introduced into the aortal via the femoral artery. Arterial blood samples were collected for determination of arterial blood oxygen and CO₂ 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 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 cranial windows was connected to a low pressure Statham transducer (Statham Instruments, Oxnard, Calif.) connected to a cannula introduced into the aortal via the femoral artery. Arterial blood samples were collected for determination of arterial blood oxygen and CO₂ 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 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 cranial windows was connected to a low pressure Statham transducer (Statham Instruments, Oxnard, Calif.) connected to a cannula introduced into the aortal via the femoral artery. Arterial blood samples were collected for determination of arterial blood oxygen and CO₂ 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.
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 H₂O₂ and 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.³⁵,¹⁶ The two cranial windows were filled with a solution of H₂O₂ in CSF at concentrations of 0.1, 0.5, or 1 μM. 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 μM concentrations of H₂O₂ 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 H₂O₂ at either 1 or 3 μM 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 H₂O₂ as well as 30 minutes after its washout.

3) In another series of four cats, we tested the effects of H₂O₂ 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 adenylate cyclase. In this experiment, we tested the responses before and 30 minutes after washout of a 3-minute application of 1 μM H₂O₂.

4) In another five cats, we examined the effect of replenishment of sulphydryl (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 μM hydrogen peroxide, and for a third time after a 10-minute application of 100 μM N-acetyl-L-cysteine s.c.

5) We studied EDRF production in response to acetylcholine by bioassay before and after H₂O₂ 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-μM concentration of H₂O₂ for 3 minutes. EDRF assay was carried out 30 minutes after washout of H₂O₂. 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 μM H₂O₂ in the assay window to evaluate the effects of H₂O₂ 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 cysNO before and 30 minutes after a 3-minute application of 1 μM H₂O₂ 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 cysNO to fill the cranial window and measured changes in diameter in the second minute after application.

The results were expressed as mean±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 H₂O₂ in a concentration of 0.1 to 1 μM induced substantial cerebral arteriolar dilation, which was dose-dependent. The effects of H₂O₂ were eliminated in the presence of deferoxamine. Table 1 shows that hydrogen peroxide in a concentration of 3 μM induced pronounced cerebral arteri-
TABLE 1. Effect of Topical $H_2O_2$ (3 μM) 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>96±5.7</td>
<td>91±5.6</td>
</tr>
<tr>
<td></td>
<td>During $H_2O_2$</td>
<td>129±6.8</td>
<td>133±4.0</td>
</tr>
<tr>
<td>Large arterioles</td>
<td>Control</td>
<td>185±13.6</td>
<td>183±10.9</td>
</tr>
<tr>
<td></td>
<td>During $H_2O_2$</td>
<td>253±71.1</td>
<td>249±49.2</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.

Figure 2 shows the response to $10^{-7}$ M acetylcholine before and after 1 μM $H_2O_2$, with and without pretreatment with 1 mM deferoxamine. It is seen that $H_2O_2$ eliminated endothelium-dependent relaxation from acetylcholine, and that this effect was prevented by deferoxamine. Figure 3 shows the responses to $10^{-7}$ M acetylcholine after topical application of 3 μM $H_2O_2$. It is seen that endothelium-dependent relaxation from acetylcholine was abolished after $H_2O_2$ 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 μM $H_2O_2$, whereas the responses to adenosine were not significantly altered. Topical application of $N$-acetyl-L-cysteine after treatment with $H_2O_2$ 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
H2O2 at 1 or 3 μM, no EDRF could be detected. The effect of 1 μM H2O2 could be reversed in the presence of SOD and catalase, but the effect of 3 μM H2O2 was unaffected by SOD and catalase. Treatment of the assay window with 1 μM H2O2 did not alter the responses to EDRF from the donor window (Figure 8).

Figures 9 and 10 show that, after topical application of H2O2, 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 H2O2 is a strong dilator of cerebral arterioles. In concentrations up to 1 μM the vasodilating effect of H2O2 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 H2O2 was examined is that of Burke and Wolin.17 These investigators found that deferoxamine and scavengers of hydroxyl radical did not influence the relaxing effect of H2O2 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 H2O2 higher than 1 μM, the vasodilation is not blocked by deferoxamine. This may be due to a variety of reasons. First, it is possible that, because H2O2 is lipid-soluble and freely diffusible, it may penetrate to areas that are not accessible to deferoxamine. Alternatively, H2O2 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 Wolin17 that, in the bovine pulmonary arteries in vitro, the mechanism of the relaxation from H2O2 involved compound I of catalase as an intermediate. We did not carry out experiments to investigate this possibility.

After exposure to H2O2, 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 H2O2 (1 μM), 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 H2O2 (3 μM), 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 μM of hydrogen peroxide continued for a fairly long time after H₂O₂ had been eliminated by washout, it could not be due to a direct effect of the exogenous H₂O₂ on EDRF. Recent preliminary studies have shown that exposure of cultured endothelial
cells to H$_2$O$_2$ induces sustained generation of superoxide.$^{18}$ It is likely that an analogous mechanism was responsible for the continued absence of endothelial-dependent responses after exposure to this concentration of H$_2$O$_2$.

It is of great interest that after exposure to H$_2$O$_2$ at a concentration of 1 $\mu$M 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$^{19}$ 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$^{20,21}$ and by Ignarro et al$^{22}$ 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 H$_2$O$_2$ 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 H$_2$O$_2$ 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 $\mu$M hydrogen peroxide may be related to its SH oxidizing ability.

It has recently been suggested that the EDRF from acetylcholine is a nitrosothiol.$^{23}$ If this is correct, the effect of H$_2$O$_2$ 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$^{19}$ 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 H$_2$O$_2$ 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 arteries is a nitrosothiol like cysNO.

References


KEY WORDS  • vasodilation • endothelium • nitric oxide • oxygen radicals • vascular injury • nitrodilators
H2O2 and endothelium-dependent cerebral arteriolar dilation. Implications for the identity of endothelium-derived relaxing factor generated by acetylcholine.

E P Wei and H A Kontos

Hypertension. 1990;16:162-169
doi: 10.1161/01.HYP.16.2.162

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/16/2/162

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/