Antioxidants Inhibit ATP-Sensitive Potassium Channels in Cerebral Arterioles

Enoch P. Wei, PhD; Hermes A. Kontos, MD, PhD; Joseph S. Beckman, PhD

Background and Purpose—Hydrogen peroxide and peroxynitrite are capable of generating hydroxyl radical and are commonly suspected as sources of this radical in tissues. It would be useful to distinguish the source of hydroxyl radical in pathophysiological conditions and to clarify the mechanisms by which antioxidants modify vascular actions of oxidants.

Methods—We investigated the effect of three antioxidants—dimethylsulfoxide (DMSO), salicylate, and L-cysteine—on the cerebral arteriolar dilation caused by topical application of hydrogen peroxide and peroxynitrite in anesthetized cats equipped with cranial windows. We also tested the effect of these antioxidants on the vasodilation caused by pinacidil and cromakalim, two known openers of ATP-sensitive potassium channels.

Results—DMSO was more effective in inhibiting dilation from hydrogen peroxide, whereas salicylate and L-cysteine were more effective in inhibiting dilation from peroxynitrite. All three antioxidants inhibited dilation in concentrations that were remarkably low (<1 mmol/L). All three antioxidants inhibited vasodilation from two known potassium channel openers, pinacidil and cromakalim. Their effect was specific because they did not affect dilation from adenosine or nitroprusside.

Conclusions—The findings show that antioxidants block ATP-sensitive potassium channels in cerebral arterioles. This appears to be the mechanism by which antioxidants inhibit the dilation from hydrogen peroxide and peroxynitrite and not through scavenging of a common intermediate, ie, hydroxyl radical. The differences between effectiveness in inhibiting dilation from hydrogen peroxide and peroxynitrite by various antioxidants suggest that hydrogen peroxide and peroxynitrite act at two different sites, one in a water-soluble environment and the other in a lipid-soluble environment.

Key Words: hydrogen peroxide ■ hydroxyl radical ■ microcirculation ■ peroxynitrite ■ vascular regulation

Hydrogen peroxide and peroxynitrite cause pronounced cerebral arteriolar dilation in sufficiently low concentrations to merit consideration as mediators of cerebral vascular responses. Since hydrogen peroxide and peroxynitrite can generate hydroxyl radical, it is not surprising that both have been suggested as sources of this radical and hence have been implicated as mediators of cellular injury. We recently found that hydrogen peroxide and peroxynitrite dilate cerebral arterioles by the same mechanism, ie, by opening ATP-sensitive potassium channels.

See Editorial Comment, page 823

In the present experiments we explored the possibility that we may be able to distinguish whether vascular responses are due to hydrogen peroxide or to peroxynitrite by establishing differences in the inhibitory effects of various hydroxyl radical scavengers on their vascular actions. These experiments led to the unexpected finding that antioxidants block ATP-sensitive potassium channels.

Materials and Methods

Experiments were performed in cats anesthetized with sodium pentobarbital (30 mg/kg IV). Additional doses of anesthetic were given as required to maintain surgical anesthesia based on testing of corneal reflexes and on responses to tail pinch. The animals were subjected to tracheostomy and ventilated with a positive-pressure respirator. The end-expiratory CO₂ of the animals was continuously monitored with a CO₂ analyzer and was maintained at a constant level of approximately 30 mm Hg. Arterial blood pressure was measured with a pressure transducer connected to a cannula introduced into the aorta through the femoral artery. Arterial blood samples were collected for determination of arterial blood oxygen, CO₂ partial pressures, and pH at appropriate intervals during the experiment. Blood gas tensions and pH were measured with oxygen and CO₂ electrodes and a pH meter. The rectal temperature of the animal was monitored continuously and was kept constant with the aid of a heating blanket.

Received November 4, 1997; final revision received January 15, 1998; accepted January 21, 1998.

Reviews of this article were directed by Richard J. Traystman, PhD. To avoid possible conflict of interest, Dr Hermes Kontos was not involved in the review process.

From the Department of Medicine, Medical College of Virginia Campus of Virginia Commonwealth University, Richmond (E.P.W., H.A.K.), and Department of Anesthesiology, University of Alabama at Birmingham (J.S.B.).

Correspondence to Hermes A. Kontos, MD, PhD, Medical College of Virginia Campus of Virginia Commonwealth University, PO Box 980549, Richmond, VA 23298-0549.

E-mail hakontos@vcu.edu .

© 1998 American Heart Association, Inc.
Antioxidants Inhibit $K_{\text{ATP}}$ Channels

The cerebral microcirculation of the parietal cortex was visualized through an acutely implanted cranial window, as described in detail previously. The space under the cranial window was filled with artificial cerebrospinal fluid (CSF) identical in composition to that of cats. One port of the window was connected to a pressure 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 an image-splitting device attached to a microscope. In each animal, several arterioles were observed covering a wide range of vessel caliber. The responses of small and large arterioles (smaller and larger than 100 μm in diameter, respectively) were analyzed separately to identify any size-dependent differences in responses.

Dimethylsulfoxide (DMSO), pinacidil, cromakalim, L-cysteine, sodium salicylate, adenosine, and sodium nitroprusside were obtained from Sigma Chemical Co. Peroxynitrite was synthesized as described previously. It was kept as 100 mmol/L solution in 1 mol/L sodium hydroxide at pH 14. Under these conditions, peroxynitrite is stable indefinitely. The concentration of peroxynitrite was monitored spectrophotometrically by determining absorbance at 302 nm. Because peroxynitrite when dissolved in aqueous solution decomposes very rapidly, we used the following technique for its application on the brain surface. We calculated the buffer composition of the CSF required to maintain pH at 7.3 after the addition of the appropriate amounts of peroxynitrite. After mixing the solutions rapidly, we filled the space under the window with the solution. This procedure required less than 3 seconds. To control the effects of sodium hydroxide, we added the amount of sodium hydroxide equivalent to the highest dose of peroxynitrite to the appropriate CSF volume with a buffer composition design to maintain pH at 7.3 and placed it under the window in the same fashion as used for the peroxynitrite solutions. All other solutions were prepared directly in artificial CSF and for L-cysteine 1.3 $10^{-9}$ mol/L. DMSO is lipid soluble, whereas the other two antioxidants are water soluble. In addition, L-cysteine, like other sulfhydryl-containing compounds, reacts rapidly and directly with peroxynitrite. These differences might be expected to maximize our ability to differentiate between the two sources of hydroxyl radical.

In preliminary experiments we identified the lowest dose of DMSO, salicylate, or L-cysteine that inhibited the vasodilator action of hydrogen peroxide or peroxynitrite. Systematic studies were done in the presence of 10 μmol/L DMSO, 100 μmol/L salicylate, and 250 μmol/L L-cysteine. If these did not have inhibitory effects, we retested in another series of experiments responses in the presence of 1000 μmol/L DMSO, 250 to 500 μmol/L salicylate, or 500 μmol/L L-cysteine. The space under the cranial window was filled with the appropriate solution of a hydroxyl radical scavenger and left in place for 15 minutes. The responses to hydrogen peroxide or peroxynitrite were tested before and immediately after the application of the scavenger. No attempt was made to replace the hydroxyl radical scavenger between applications of different concentrations of hydrogen peroxide and peroxynitrite.

In another series of experiments we tested the effects of DMSO, salicylate, and L-cysteine on the vasodilator responses to ATP-sensitive potassium channel openers pinacidil and cromakalim. The responses to these agents were tested before as well as after the application of the scavengers as described above. To establish the specificity of the action of antioxidant, we also tested responses to adenosine and nitroprusside before and after DMSO, salicylate, and L-cysteine.

Statistical analysis of the results was done with ANOVA followed by $t$ tests modified for multiple comparisons.

Results

None of the hydroxyl radical scavengers in any of the doses used had any significant effect on pial arteriolar caliber.

Fig 1 shows that DMSO 10 μmol/L inhibited significantly and to a pronounced extent the vasodilation from hydrogen peroxide, but it had no significant effect on the responses to peroxynitrite. DMSO 1000 μmol/L inhibited responses to peroxynitrite completely.

Fig 2 shows that salicylate 100 μmol/L inhibited to a pronounced degree the vasodilation from peroxynitrite. This dose of salicylate, on the other hand, had no significant effect on the vasodilation from hydrogen peroxide. At a concentration of 250 μmol/L, salicylate partially inhibited the re-
responses to hydrogen peroxide, and the effect was more pronounced at 500 μmol/L.

Fig 3 shows that L-cysteine 250 μmol/L completely inhibited the responses to peroxynitrite, while responses to hydrogen peroxide were not affected. L-Cysteine 500 μmol/L partially inhibited the responses to hydrogen peroxide.

Figs 4 and 5 show that DMSO 10 μmol/L, L-cysteine 250 to 500 μmol/L, and salicylate 100 to 250 μmol/L inhibited responses to either pinacidil or cromakalim.

Figs 6 through 8 show that vasodilator responses to adenosine and nitroprusside were unaffected by DMSO, L-cysteine, or salicylate.

Discussion

The results reported above show that the three hydroxyl radical scavengers we used were effective in inhibiting the vasodilation induced by hydrogen peroxide and peroxynitrite. The two water-soluble agents, salicylate and L-cysteine, were more effective in inhibiting the vasodilation from peroxynitrite than that from hydrogen peroxide, while the reverse was true for the lipid-soluble DMSO. This suggests that the sites of action of hydrogen peroxide and peroxynitrite may be different. The potency of the various inhibitors against the vasodilation of hydrogen peroxide and peroxynitrite differed sufficiently to render a distinction between the two feasible.

Our initial hypothesis was that the hydroxyl radical scavengers inhibited the vasodilation from hydrogen peroxide and peroxynitrite by scavenging hydroxyl radical. Both agents are known to generate hydroxyl radical. Hydrogen peroxide generates hydroxyl radical by the Haber-Weiss reaction via catalysis by transition metal, while peroxynitrite in aqueous solution produces peroxynitrous acid, which decomposes spontaneously to generate hydroxyl radical. In addition, we found earlier that the vasodilation in response to hydrogen peroxide...

Figure 2. Effect of pretreatment with salicylate on cerebral arteriolar responses to peroxynitrite (left panel) and to hydrogen peroxide (three right panels). Values are mean ± SE of the percent changes in diameter. Baseline diameters from which the percent changes were calculated are given in micrometers in the insets. Values were obtained from 20 small and 17 large arterioles in 5 cats in the left panel, from 20 small and 17 large arterioles in 5 cats for the 100-μmol/L salicylate panel, from 19 small and 17 large arterioles in 5 cats for the 250-μmol/L salicylate panel, and from 20 small and 17 large arterioles in 5 cats in the hydrogen peroxide data. Note that salicylate at 100 μmol/L significantly inhibited responses to peroxynitrite, but it had no effect on responses to hydrogen peroxide. Salicylate at the two higher doses induced dose-dependent, significant reductions in the responses to hydrogen peroxide.

Figure 3. Effect of pretreatment with L-cysteine on cerebral arteriolar artery responses to peroxynitrite and hydrogen peroxide. Values are mean ± SE of the percent changes in diameter. Baseline diameters from which the percent changes were calculated are given in micrometers in the insets. Values were obtained from 24 small and 15 large arterioles in 5 cats in the 250-μmol/L cysteine panel and from 16 small and 15 large arterioles in 5 cats for the 250-μmol/L cysteine panel in the peroxynitrite data, and from 24 small and 15 large arterioles in 5 cats in the 250-μmol/L cysteine panel and 16 small and 15 large arterioles in 5 cats for the 500-μmol/L cysteine panel in the hydrogen peroxide data. Note that L-cysteine at both doses significantly and completely inhibited responses to peroxynitrite, while L-cysteine at 250 μmol/L had no effect on responses to hydrogen peroxide but had a significant inhibitory effect at the higher dose.
peroxide is eliminated by scavenging iron with deferoxamine, suggesting that hydroxyl radical is the mediator of the vasodilation from hydrogen peroxide. As explained below, there are, however, strong reasons why the hypothesis that the inhibition of the vasodilation by hydrogen peroxide and peroxynitrite in the presence of antioxidants is due to their scavenging hydroxyl radical is not tenable.

Cells are well defended against the superoxide by superoxide dismutase and against hydrogen peroxide by catalase and glutathione peroxidase. These enzymes are specific and highly effective. As a result, the steady state concentrations of superoxide and hydrogen peroxide are kept at a very low range, insufficient to cause cellular damage. In contrast, there is no natural endogenous scavenger of hydroxyl radical because this radical is extremely reactive. Hydroxyl radical reacts with most common organic and biological molecules at near diffusion-limited rates. The compounds used to scavenge hydroxyl radical are useful because they can be tolerated by cells in remarkably high concentrations and not because they are more reactive with hydroxyl radical than potential biological targets. Commonly used hydroxyl radical scavengers, like the ones we used in the present experiments, must be used in 10 to 1000 mmol/L concentrations to effectively scavenge hydroxyl radical in vitro.

While antioxidants such as DMSO and salicylate are widely used to scavenge hydroxyl radical in brain and other organs, the minimum effective concentration for this action has rarely been investigated. The surprisingly low concentrations of DMSO and salicylate that were effective in inhibiting vasodilation from hydrogen peroxide and peroxynitrite reported above are at least 1000-fold lower than required to block the action of hydroxyl radical in vitro. This finding strongly suggests that the mechanism of action of these agents is different.

A second reason for seeking a different mechanism of action for the antioxidants was that they were not equally effective in preventing the vasodilation from hydrogen per-
oxide and peroxynitrite, suggesting that hydroxyl radical was not the common intermediate. While DMSO and salicylate do not directly affect the rate of peroxynitrite decomposition, L-cysteine reacts directly with peroxynitrite at rates that are considerably faster than with hydrogen peroxide. It is possible, therefore, that the difference in the effectiveness of L-cysteine against peroxynitrite and hydrogen peroxide may be due in part to its direct reaction with peroxynitrite.

For the reasons outlined above, we sought other mechanisms for the inhibition of the vasodilation by hydrogen peroxide and peroxynitrite. In earlier studies we found that these two agents opened ATP-sensitive potassium channels based on the finding that glyburide, a known inhibitor of these channels, abolished the vasodilation they caused in cerebral arterioles. It was therefore logical to examine whether the hydroxyl radical scavengers we used might be inactivating ATP-sensitive potassium channels rather than scavenging hydroxyl radical directly. The experiments reported above show that indeed this is the case, because all three scavengers eliminated the vasodilation from pinacidil or cromakalim, two known ATP-sensitive potassium channel openers. Therefore, the present experiments and our earlier findings suggest that in cerebral arterioles of cats, oxidants open ATP-sensitive potassium channels and antioxidants inhibit these channels.

Understanding of the structure of ATP-sensitive potassium channels has improved considerably as a result of successful attempts to clone these channels. As a result of these studies it now appears that they consist of two components: an inward rectifying potassium channel and a sulfonylurea receptor. The sulfonylurea receptor from pancreas of rats and hamsters has recently been cloned. It consists of 1582 amino acids, has two ATP-binding sites, and belongs to the ATP-binding cassette family of proteins. It appears to function by modulating the activity of the potassium pore protein. Sulfonylurea receptor–like proteins have recently been identified in extrapancreatic tissues. The sulfonylurea receptor is the binding site for sulfonylureas such as glyburide, which inhibit the ATP-sensitive potassium channels. The mechanisms by which agents like pinacidil and cromakalim open ATP-sensitive potassium channels are incompletely understood and appear to be complex. Some evidence, mainly based on the fact that these compounds reversibly and competitively antagonize the action of glyburide, suggests that they act on the same binding site as glyburide, namely on the sulfonylurea receptor. It is therefore likely that the antioxidants we used are acting on the same receptor. The findings that DMSO was more effective against hydrogen peroxide while the two water-soluble antioxidants were more effective in inhibiting the action of peroxynitrite suggest the possibility that there may be two separate sites of action, one accessible to lipid-soluble agents and the other accessible to

Figure 6. Effect of pretreatment with DMSO on cerebral arteriolar responses to nitroprusside (SNP) and to adenosine (ADO). Values are mean±SE of the percent changes in diameter. Baseline diameters from which the percent changes were calculated are given in micrometers in the insets. Values are from 12 small and 12 large arterioles in 4 cats. Note that DMSO had no significant effect on responses to either SNP or ADO.

Figure 7. Effect of pretreatment with L-cysteine on cerebral arteriolar responses to nitroprusside (SNP) or adenosine (ADO). Values are mean±SE of the percent changes in diameter. Baseline diameters from which the percent changes were calculated are given in micrometers in the insets. Values are from 12 small and 12 large arterioles in 4 cats. Note that L-cysteine had no significant effect on responses to either SNP or ADO.
Antioxidants Inhibit K\textsubscript{ATP} Channels

Water-soluble ones. The pattern of blockade of the effect of pinacidil and cromakalim by the three antioxidants is consistent with this view. Both of these agents are lipid soluble. Their effects were blocked completely by very low-dose (10 \( \mu \text{mol/L} \)) DMSO. On the other hand, the two water-soluble antioxidants, l-cysteine and salicylate, blocked the effects of pinacidil and cromakalim completely at high dose, a pattern similar to what was seen with hydrogen peroxide but unlike that seen with peroxynitrite.

In a recent study Sobey et al\textsuperscript{16} found that hydrogen peroxide dilated cerebral arteries in rats by opening calcium-activated potassium channels. An obvious difference between these experiments and ours is that Sobey et al\textsuperscript{16} used 10 to 100 \( \mu \text{mol/L} \) hydrogen peroxide, ie, 100 to 1000 times the largest concentration we used. At these high concentrations hydrogen peroxide inhibits membrane and sarcocellal calcium pumps\textsuperscript{17} and would be expected to raise intracellular calcium ion concentration. Such an increase in calcium ion concentration may be responsible for activating calcium-activated potassium channels. In earlier studies\textsuperscript{18} we found that very high concentrations of hydrogen peroxide induced histologically demonstrable damage to the vascular wall associated with sustained arteriolar vasodilatation. Lower concentrations of hydrogen peroxide (1 to 3 \( \mu \text{mol/L} \)) caused reversible arteriolar dilation but also caused inhibition of release of endothelium-derived relaxing factor by acetylcholine as well as guanylate cyclase inhibition, which outlasted the vascular effects.\textsuperscript{1} The low concentrations of hydrogen peroxide and peroxynitrite we used in the present experiments had vascular effects that were readily reversible. It is evident, therefore, that large concentrations of oxidants cause tissue damage, while lower concentrations have effects that are reversible and may be involved in physiological regulation.

Acknowledgment

This study was supported by grant NS 19316.

References

4. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. \textit{Proc Natl Acad Sci U S A}. 1990;87:1620–1624.
Activity of potassium channels in smooth muscle has a major influence on vascular tone.\(^1\)\(^2\) Reactive oxygen species and peroxynitrite produce dilatation of cerebral arterioles, and recent evidence suggests that a key mechanism that mediates this response is activation of potassium channels in vascular muscle. For example, exogenously applied hydrogen peroxide produces vasodilation that can be largely blocked using inhibitors of ATP-sensitive (K\(_{ATP}\)) or calcium-activated potassium channels.\(^3\)\(^4\) Importantly, potassium channels have also been implicated as mediators of dilatation of cerebral arterioles in response to endogenously produced reactive oxygen species.\(^4\)

The K\(_{ATP}\) channel is a complex of proteins consisting of a pore-forming subunit and the sulfonylurea receptor.\(^1\) The latter component is responsible for glibenclamide sensitivity of this channel and is probably the site that confers sensitivity to K\(_{ATP}\) channel openers such as pinacidil.\(^1\)

The present study provides pharmacological evidence that antioxidants inhibit K\(_{ATP}\) channels in blood vessels. This conclusion is based on the finding that three antioxidants (dimethylsulfoxide, salicylate, and L-cysteine) inhibited cerebral vasodilatation in response to K\(_{ATP}\) channel openers (pinacidil and cromakalim) and exogenously applied hydrogen peroxide and peroxynitrite (which produce glibenclamide-sensitive vasodilatation in this model).

How would antioxidants inhibit activation of ATP-sensitive potassium channels? Glibenclamide binds to the sulfonylurea receptor and is the most commonly used inhibitor of K\(_{ATP}\) channels in studies of blood vessels.\(^1\)\(^2\) It might seem logical to assume that the site of action of other inhibitors of K\(_{ATP}\) channels would also be the sulfonylurea receptor. However, recent evidence suggests that inhibition of K\(_{ATP}\) channels by phentolamine (a nonsulfonylurea) is not mediated by effects on the sulfonylurea receptor. Rather, phentolamine inhibits channel activity by effects on the pore-forming subunit or other proteins that regulate activity of this channel.\(^5\) Thus, the site of action of antioxidants may or may not be the sulfonylurea receptor. One finding of this study was that the three antioxidants exhibited differential efficacy with respect to inhibition of responses to hydrogen peroxide and peroxynitrite. Thus, the mechanisms and sites of action of these antioxidants may differ.

**References**

Antioxidants Inhibit ATP-Sensitive Potassium Channels in Cerebral Arterioles
Enoch P. Wei, Hermes A. Kontos and Joseph S. Beckman

Stroke. 1998;29:817-823
doi: 10.1161/01.STR.29.4.817
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/29/4/817

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke 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 Stroke is online at:
http://stroke.ahajournals.org//subscriptions/