Nitric Oxide and S-Nitroso-L-Cysteine as Endothelium-Derived Relaxing Factors From Acetylcholine in Cerebral Vessels in Cats

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Background and Purpose: The predominant view is that the endothelium-derived relaxing factor generated by acetylcholine from blood vessels is nitric oxide. However, there is evidence suggesting that certain nitric oxide–containing compounds such as nitrosothiols resemble the endothelium-derived relaxing factor generated by acetylcholine more closely than does nitric oxide itself. Accordingly, we compared the effects of nitric oxide and S-nitroso-L-cysteine on cerebral arteriolar caliber in relation to the associated increments in nitrite concentration in the effluent.

Methods: Acetylcholine, nitric oxide, and S-nitroso-L-cysteine were administered by continuous superfusion in oxygen-free solution through the space under a cranial window in anesthetized cats. Nitrite concentration was measured in the effluent. The degree of vasodilation induced was evaluated in relation to the increment in nitrite concentration.

Results: All agents induced dose-dependent vasodilation and dose-dependent increments in nitrite concentration in the effluent. For any given degree of vasodilation, the increments in nitrite concentration were equivalent during acetylcholine or S-nitroso-L-cysteine infusion, whereas the nitrite concentrations were 10 times higher during nitric oxide infusion. After administration of nitroarginine, a competitive inhibitor of nitric oxide synthesis from arginine, there was depression in the vasodilation as well as the increment in nitrite concentration induced by acetylcholine.

Conclusions: S-Nitroso-L-cysteine resembles endothelium-derived relaxing factor from acetylcholine more closely than does nitric oxide. (Stroke. 1993;24:2010-2015.)

Key Words • acetylcholine • endothelium-derived relaxing factor • nitric oxide • cats

The endothelium-dependent vasodilation induced by acetylcholine is caused either by the release of an endothelium-derived relaxing factor (EDRF) derived from the action of nitric oxide synthase on L-arginine, which then activates vascular smooth muscle–soluble guanylate cyclase, or by the release of an unidentified endothelium-derived hyperpolarizing factor, which increases vascular smooth muscle resting transmembrane potential and causes relaxation. In the cerebral microcirculation of the cat, the vasodilator action of acetylcholine is due entirely to a nitric oxide–containing EDRF, because inhibition of nitric oxide synthase with arginine analogues at a low dose completely inhibits both the vasodilation and the production of EDRF.

It is well established that the EDRF generated by acetylcholine in large vessels in vitro as well as by bradykinin or the ionophore A23187 in cultured endothelial cells is nitric oxide or a nitric oxide–containing compound. The predominant view is that the EDRF generated under these conditions is identical to nitric oxide. However, there is evidence suggesting that nitric oxide–containing compounds, such as nitrosothiols, resemble the EDRF generated by these vasoactive agents more closely than does nitric oxide itself. For example, Myers et al compared the relaxation induced by nitric oxide or by S-nitroso-L-cysteine and by EDRF released from endothelial cells by bradykinin or by the ionophore A23187 in relation to the nitric oxide concentration generated by these agents. They found that the concentration of nitric oxide generated by bradykinin and by A23187 was inadequate to explain the relaxation and that S-nitroso-L-cysteine resembled the EDRF released by the vasoactive agents much more closely than did authentic nitric oxide. Rubanyi et al, using electron paramagnetic resonance spectroscopy of nitrosylhemoglobin, could detect nitric oxide but not EDRF or S-nitroso-L-cysteine in amounts that caused equivalent relaxation. These investigators also detected the release of radioactive sulfur from prelabeled endothelial cells under the influence of endothelium-dependent agonists, suggesting the possible release of a nitrosothiol. We found that, in cerebral arterioles of anesthetized cats, the EDRF induced by acetylcholine could be

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pharmacologically distinguished from nitric oxide.\textsuperscript{11,12} In these experiments, we found that, after exposure to oxidant stress, cerebral arterioles responded normally to the EDRF generated by acetylcholine from another source and to S-nitroso-L-cysteine but did not respond to exogenous nitric oxide.\textsuperscript{11,12} Other investigators also noted pharmacologic differences between EDRF released from cultured endothelial cells by bradykinin or by ionophore A23187 and exogenous nitric oxide.\textsuperscript{13,14}

In the present experiments, we compared the cerebral arteriolar vasodilator responses to acetylcholine, S-nitroso-L-cysteine, and nitric oxide in relation to the concentration of nitrite generated by these agents in the effluent.

\textbf{Materials and Methods}

Experiments were carried out in adult cats of either sex anesthetized with sodium pentobarbital (30 mg/kg IV). Additional doses of anesthetic were given subsequently to maintain adequate surgical anesthesia, as indicated by testing of corneal reflexes and the motor responses to tail pinch. The animals were subjected to tracheostomy and were ventilated artificially using a positive-pressure respirator. The rate and volume of the respirator were adjusted to maintain arterial blood PC\textsubscript{O\textsubscript{2}} at ≈30 mm Hg. Arterial blood gases and pH were measured periodically during the experiment using oxygen and CO\textsubscript{2} electrodes and a pH meter. Arterial blood pressure was monitored continuously using a pressure transducer connected to a catheter placed into the aorta via the femoral artery.

The pial microcirculation of the parietal cortex was directly visualized via an acutely implanted cranial window as described in detail previously.\textsuperscript{15} The window was equipped with outlets for superfusion of solutions and for measuring intracranial pressure. One outlet of the window was connected to a coiled tube whose distal end was set at a predetermined height to maintain an intracranial pressure of 5 mm Hg. The space under the window was initially filled with artificial cerebrospinal fluid (CSF) with the same composition as endogenous CSF of cats. Vessel diameter was measured with a Vickers image-splitting device attached to a microscope as described previously. The diameter of several vessels was measured in each animal.

The concentration of free nitric oxide in in vitro solutions was measured using an Iso-NO meter (World Precision Instruments, Inc), which determines polarographically the concentration of nitric oxide gas in aqueous solution. The electrode gave stable and repeatable readings and was linear in the entire range of concentrations of nitric oxide solutions used in these experiments.

The experimental design consisted of superfusing at a constant rate of 1 mL/min various vasoactive solutions through the space under the cranial window. The administration of each solution was maintained for 5 minutes. This allowed us to obtain measurements over a period of several minutes under steady-state conditions. Baseline arteriolar caliber measurements were obtained during superfusion of deoxygenated CSF without vasoactive agents. The effluent from the window was collected and used to measure nitrite concentration. CSF effluents from the window were centrifuged at 1500g for 15 minutes, and supernatants were saved. Nitrite levels in the supernatants were measured by the Greiss reaction.\textsuperscript{16} To 1-mL CSF samples we added 33-μL sulfanilic acid (6 mg/mL) and 33 μL of 12N HCl; the samples were vortexed and placed on ice for 10 minutes. Thirty-three microliters of N-1-naphthyl-1-ethylenediamine hydrochloride (3 mg/mL in distilled water) was added to the samples and vortexed. The samples were read at 548 nm against the standard curve of nitrite covering the concentration range of interest.

We tested solutions of acetylcholine at 10⁻⁷ and 10⁻⁵ mol/L, solutions of nitric oxide at 0.5 to 10 μmol/L, and solutions of S-nitroso-L-cysteine at 0.1 to 2 μmol/L. We also tested acetylcholine after a 10-minute application of a 10 μmol/L solution of N⁵-nitro-L-arginine, an agent that inhibits the synthesis of nitric oxide from arginine.\textsuperscript{17} All reagents were obtained from Sigma Chemical Co, St Louis, Mo. All solutions of vasoactive agents were prepared in artificial CSF that was gassed with 95% N\textsubscript{2}–5% CO\textsubscript{2} for 2 hours to eliminate oxygen to the extent possible. In all solutions the Po\textsubscript{2} was <7 mm Hg. The pH of the solution was adjusted to 7.35. In preliminary experiments we found that the response to superfusion with acetylcholine in CSF prepared in this fashion was less pronounced than in CSF with a Po\textsubscript{2} of 50 mm Hg. In 61 vessels in 10 animals, 10⁻⁷ mol/L acetylcholine induced a 13±0.8% increase in diameter in CSF at 50 mm Hg Po\textsubscript{2} and only an 8±1.0% increase in vascular diameter at Po\textsubscript{2} of <7 mm Hg, from a baseline of 101±5.8 μm. Also, in the same animals acetylcholine (10⁻⁷ mol/L) induced a 27±1.7% increase in vascular diameter at Po\textsubscript{2} of 50 mm Hg versus a 21±1.6% increase at Po\textsubscript{2} of <7 mm Hg, from a baseline of 104±5.6 μm.

Nitric oxide was obtained from Matheson Gas Products, Secaucus, NJ, and purified by the method of Kon.\textsuperscript{18} Nitric oxide solutions were prepared by injecting the appropriate amounts of nitric oxide gas into deoxygenated CSF in a glass gastight syringe. S-Nitroso-L-cysteine was synthesized from cysteine and nitrogen dioxide and was kept in methyl alcohol as a 0.1 mol/L solution at −20°C until used.\textsuperscript{19} The appropriate dilutions of the S-nitroso-L-cysteine were prepared just before the experiment in deoxygenated CSF. Under these conditions, the vasoactivity of the solutions was stable for a period that exceeded the duration of the experiment. Methyl alcohol in concentrations equivalent to those present in the solutions of S-nitroso-L-cysteine did not have detectable effects on cerebral arterioles.

Statistical analysis of the results was carried out using analysis of variance followed by t tests modified for multiple comparisons.

\textbf{Results}

We measured nitrite concentration in the effluent under conditions of continuous superfusion of deoxygenated CSF through the cranial window space in 29 cats. The average concentration of nitrite was 0.22±0.07 μmol/L with a range from 0 to 1.87 μmol/L. Twelve animals had an undetectable concentration of nitrite in the effluent.

Fig 1 shows that acetylcholine induced dose-dependent vasodilatation of cerebral arterioles as well as a dose-dependent increment in nitrite concentration in the effluent.

The concentration of free nitric oxide was determined in the airtight syringes used in the superfusion experi-
Fig 1. Graph shows effect of acetylcholine on arteriolar diameter (○) and on nitrite concentration (▲) in the effluent. The data are from 47 vessels in seven cats. Values are mean±SEM. The baseline diameters and the baseline nitrite concentration in the effluent from which the changes were derived are shown at the upper left portion of the figure. Note the dose-dependent increase in diameter and the dose-dependent increase in nitrite concentration in the effluent.

Fig 2. Graph shows effects of superfusion of nitric oxide (NO)−containing solutions on arteriolar diameter (○) and on nitrite concentration (▲) in the effluent. Values are from 59 vessels in eight animals. The baseline vessel diameter and the baseline nitrite concentration from which the changes are derived are shown in the left upper portion of the figure. Note the dose-dependent increment in the vessel diameter and the dose-dependent increment in nitrite concentration. Also note that the increment in nitrite concentration is approximately equivalent to the concentration of NO in the perfusate, showing that NO was quantitatively recovered as nitrite in the effluent.

Fig 3. Graph shows effect of superfusion with S-nitroso-L-cysteine on arteriolar diameter (○) and on nitrite (▲) concentration in the effluent. Values are from 47 vessels in seven animals. Baseline diameters and baseline nitrite concentration in the effluent are given in the left upper portion of the figure. Values are mean±SEM. Note the dose-dependent dilation and the dose-dependent increment in nitrite concentration induced by S-nitroso-L-cysteine. Also note that the increment in nitrite concentration in the effluent is equivalent to the concentration of the compound in the effluent, showing that the nitric oxide portion of the nitrosothiol is quantitatively recovered as nitrite in the effluent.

The increment in concentration in nitrite induced by each nitric oxide solution was equivalent to the concentration of nitric oxide infused, showing that the nitric oxide infused was quantitatively recovered in the effluent as nitrite. For the same degree of vasodilation, the increment in nitrite concentration induced by the nitric oxide solution was ~10 times higher than that induced by acetylcholine. There was no difference in the vasodilation induced by solutions of nitric oxide used immediately after preparation from that induced by the same solution 1.5 hours later, indicating that nitric oxide was stable over this period of time.

Fig 3 shows that S-nitroso-L-cysteine caused dose-dependent dilation and a dose-dependent increment in nitrite concentration in the effluent. S-Nitroso-L-cysteine was a stronger vasodilator than nitric oxide. As was the case with nitric oxide solutions, the nitric oxide component of the nitrosothiol was quantitatively recovered in the effluent as nitrite. For the same degree of vasodilation induced by S-nitroso-L-cysteine, the increment in nitrite concentration was approximately equivalent to that caused by acetylcholine.

Fig 4 shows that after the administration of N^6-nitro-L-arginine the vasodilator response to acetylcholine was markedly reduced and the increment in nitrite concentration in the effluent was also lower than in the control experiments.

Discussion

The important findings of these experiments are that acetylcholine caused a dose-dependent increment in nitrite concentration consistent with the ability of acetylcholine to generate a nitric oxide−containing EDRF. This is the first demonstration in vivo of an increment in nitrite concentration induced by acetylcholine. It provides a basis for comparing various candidates for...
EDRF generated by acetylcholine. For the same degree of vasodilation, S-nitroso-L-cysteine caused approximately an equivalent increment in nitrate concentration in the effluent as acetylcholine, whereas nitric oxide needed a 10-fold greater increment in nitrate concentration to achieve the same vasodilation. In other words, the S-nitroso-L-cysteine resembled the EDRF from acetylcholine much more closely than nitric oxide. These results are consistent with the earlier findings of Myers et al,9 who used bovine endothelial cells in culture. Our findings of a reduction in the increment of nitrate concentration in the effluent as well as in the accompanying vasodilation from acetylcholine after nitroarginine administration are consistent with a causal relation between the release of the nitrate precursor compound and the vasodilation. The presence of a finite concentration of nitrate in the effluent under resting conditions in most animals is consistent with the release of a nitric oxide–containing compound under resting conditions. However, under resting conditions we have consistently failed to demonstrate transferable vasodilator activity in bioassay experiments.20 This suggests that the nitrate-generating compound may be released not from the surface vessels themselves but from vascular or parenchymal tissue components that are remote from the brain surface so that the nitric oxide–containing compound is oxidized fully to nonvasoactive agents by the time it reaches the effluent.

Certain important methodological aspects of these experiments need to be considered: (1) We used continuous superfusion of the various solutions because nitric oxide and related compounds are short lived. Examination of their responses during application as a bolus may be affected by minor differences in the rate of destruction of these compounds. This problem is eliminated under conditions of steady-state induced by continuous superfusion. (2) We eliminated oxygen from the solutions as much as possible. This is necessary to avoid oxidation of nitric oxide and of the nitrosothiol, which might diminish their effectiveness as vasodilators. This point merits additional elaboration. It is clear from the polarographic measurements of nitric oxide concentration in vitro that no destruction of nitric oxide took place before it reached the cranial window. This is the expected result from elimination of oxygen from the solution. We cannot, however, exclude the possibility that nitric oxide might be destroyed in transit through the window where the oxygen tension is obviously intermediate between the normally prevailing level of ~50 mm Hg and the essentially zero level used in the superfusate. Two possible alternatives exist here. The first possibility is that nitric oxide is able to reach vascular smooth muscle and that its effect is less pronounced than that of the nitrosothiol and of the EDRF generated by acetylcholine. A second possibility is that nitric oxide is destroyed more rapidly than the nitrosothiol. If this is the case, however, it constitutes a clear difference from the EDRF generated by acetylcholine, because in bioassay experiments20 the EDRF generated by acetylcholine survives transit through the donor window through an external tubing with a transit time of 6 seconds and transit through an assay window and remains essentially intact. (3) It is evident from the findings presented above that the responsiveness of the cerebral arterioles to nitric oxide and to the nitrosothiol is less than what is seen in other vessels. This factor, coupled with the fact that the amounts of nitrate generated are fairly large, allowed us to carry out the comparisons between the different compounds using simple chemical techniques for the detection of nitrite.

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References


Fig 4. Graph shows effect of acetylcholine on arteriolar diameter (○, •) and on nitrate concentration (○, △) in the effluent before (○, △) and after (○, △) administration of nitroarginine. Values are mean±SEM from 23 vessels in five animals. Baseline diameters and nitrate concentrations in the effluent are given in the left upper portion of the figure. Note that after nitroarginine there is a significant reduction in the vasodilator response to acetylcholine and a comparable reduction in the increment in nitrate concentration in the effluent.
The study by Kukreja et al provides additional convincing evidence that nitric oxide does not account for all of the biologic actions of arginine-derived mediator(s) stimulated by agents such as acetylcholine. The key observations that have been made in the present study in vivo and previous studies in vitro, as discussed by Kukreja et al., are that the amount of nitric oxide metabolites detected from endogenous arginine-derived sources needed to produce smooth muscle relaxation is markedly less than the amount of exogenous nitric oxide required to produce an equivalent response. Currently, it appears that the best explanation for observations in the literature suggesting that nitric oxide does not account for all of the actions of the arginine-derived mediator(s) is that a substance(s) exists which can either generate nitric oxide in tissues or stimulate guanylate cyclase in a manner similar to nitric oxide.

In the present study and in a previous in vitro study, it has been demonstrated that the nitrosothiol, S-nitroso-L-cysteine, clearly possesses properties that could account for the actions of an arginine-derived mediator, as discussed by Kukreja et al. Nitrosothiols are thought to generate nitrosyl-heme when incubated with heme-containing guanylate cyclase, which is thought to be needed for stimulation of this enzyme. However, as recently reviewed, other identified substances may also function as endogenously generated arginine-derived mediators. Nitroxyln anion (NO⁻) and iron-nitrosyl complexes are two additional less-well-studied substances with the potential to form in tissues and to function in a nitric oxide-like regulatory manner. A particularly interesting property of nitroxyln anion is that it is converted (in a reversible manner) by superoxide dismutase (SOD) into nitric oxide. Enhancement of the actions of arginine-derived mediator(s) by SOD was initially interpreted as originating from prevention of the superoxide anion-dependent loss of nitric oxide; however, it can also be explained in terms of a role for SOD in the release of nitric oxide from nitroxyln anion. Thus, S-nitroso-L-cysteine and other nitric oxide-like biologically active substances have the potential to account for the actions of arginine-derived mediators.

Endothelial cells have recently been demonstrated to release nitric oxide at concentrations that almost reach the micromolar range at the cell surface. Since the concentrations of nitric oxide released by the endothelial cell are clearly sufficient to cause vascular relaxation, one might question why nitric oxide does not completely account for the vasodilator actions of arginine-derived mediators. A possible reason may originate from potential enzymatic or chemical interactions of nitric oxide and/or its precursors and decomposition products with substances in tissues. These interactions could result in the formation of mediators with a potency similar to that of nitric oxide but with stability properties that provide it with a greater capacity for diffusion than this unstable gas. Recent evidence for the detection in vivo of nitrosylated plasma proteins suggests consideration of this possibility.

The metabolism of arginine by nitric oxide synthase (NOS) has the potential to form active mediators, in addition to nitric oxide, like those previously discussed. For example, NOS might directly produce nitroxyln anion, or cells could potentially generate it from nitric oxide via the SOD reaction. Although there is an absence of evidence for an absolute requirement for thiols in the NOS reaction, it is possible that thiols might react with an intermediate in the metabolism of arginine by NOS, resulting in nitrosothiol formation. It is clear that nitric oxide can react with certain thiols to form nitrosothiols; however, it is not clear if this readily occurs with thiols such as cysteine at the concentrations present in tissues. Alternative potential mechanisms for the formation of S-nitroso-L-cysteine include the possibility that decomposition products of nitric oxide, such as peroxyxinitrite or nitrogen dioxide, may nitrosylate cysteine (or other thiols). In support of this concept, we should perhaps be reminded that Kukreja et al used nitrogen dioxide for the synthesis of S-nitroso-L-cysteine. It has also recently been found that exposure of vascular tissue or glutathione to peroxyxinitrite results in the time-dependent formation of nitric oxide, associated with the nitrosylation of glutathione. Nitrosylated thiols in the presence of a cysteine derivative appear to readily undergo nitrosyl transfer reactions resulting in the S-nitrosylation of cysteine. Thus, S-nitroso-L-cysteine is a very attractive candidate for a tissue-derived vasomotor mediator.

Nitrosothiols are thought to decompose releasing nitric oxide at various rates; however, their potencies as vascular relaxants are quite similar, and responses occur over the nanomolar concentration range. In addition,
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