Effects in Cats of Inhibition of Nitric Oxide Synthesis on Cerebral Vasodilation and Endothelium-Derived Relaxing Factor From Acetylcholine

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Background and Purpose: We investigated the chemical identity of the endothelium-derived relaxing factor generated by acetylcholine in cerebral microvessels by studying the effects and mechanism of action of inhibitors of nitric oxide synthesis from arginine on the vasodilation and endothelium-derived relaxing factor production induced by topical application of acetylcholine in cerebral arterioles.

Methods: We determined cerebral arteriolar dilation and endothelium-derived relaxing factor production by bioassay in anesthetized cats equipped with cranial windows during superfusion of 10^{-7} M acetylcholine before and after administration of either N^ω-monomethyl l-arginine or N^ω-nitro-l-arginine, two inhibitors of nitric oxide synthesis.

Results: N^ω-Nitro-l-arginine abolished the vasodilation from acetylcholine and eliminated the production of endothelium-derived relaxing factor in the bioassay experiments. N^ω-Monomethyl l-arginine had no effect on the response to acetylcholine in the absence of pretreatment. However, after pretreatment with the detergent sodium dodecyl sulfate to increase cell membrane permeability, the inhibitor had effects identical to those of N^ω-nitro-l-arginine. l-Arginine reversed the effects of the inhibitors of nitric oxide synthesis. Neither inhibitor affected baseline vascular caliber, nor did they generate a vasoconstrictor agent in the bioassay experiments. The two inhibitors of nitric oxide synthesis did not affect the response to nitroprusside or adenosine, showing that the effect on responses to acetylcholine was specific. Also, the blockade of the response to acetylcholine induced by the inhibitors of nitric oxide synthesis was unaffected by treatment with superoxide dismutase and catalase, showing that the effect was not mediated by oxygen radicals.

Conclusion: The endothelium-derived relaxing factor generated by acetylcholine in cerebral arterioles of cats is either nitric oxide or a nitric oxide-containing substance. The effect of these inhibitors on the response to acetylcholine is mediated by inhibition of the synthesis of nitric oxide. There is no involvement of radicals, and no vasoconstrictor agent is generated. (Stroke 1992;23:1623-1629)

Keywords • acetylcholine • endothelium • vasodilation • cats

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2. Although, as noted above, other investigators found that in rodents inhibition of nitric oxide synthesis by pharmacological agents eliminated the vasodilatation from acetylcholine, the investigators also found that inhibition caused significant vasoconstriction.7,9 It is, therefore, possible that the effect of these agents may be mediated by the generation of a vasoconstrictor agent rather than by inhibition of the synthesis of nitric oxide. These possibilities can be distinguished in bioassay experiments.

3. Finally, Rosenblum et al12 reported that the blockade induced by inhibition of nitric oxide synthesis in mice by L-NMMA was prevented by either cyclooxygenase blockade or by oxygen radical scavengers, suggesting that the action of the inhibitor of nitric oxide synthesis was due to the generation of oxygen radicals from cyclooxygenase. If this could be confirmed, obviously the interpretations of the pharmacological experiments using L-NMMA or L-NOARG would be different from the classical explanation that their effect is due to inhibition of the synthesis of nitric oxide.

Materials and Methods

Experiments were carried out in cats weighing 2–3.3 kg and anesthetized with 30 mg/kg i.v. sodium pentobarbital. After tracheostomy, each cat was ventilated with a positive-pressure respirator. After all operative procedures were completed, the animals received 5 mg/kg i.v. gallamine triethiodide for skeletal muscle paralysis. Additional doses of anesthetic were given to maintain surgical anesthesia. Prior to the administration of the paralyzing agent, the administration of additional doses of anesthetic was determined by responses to corneal reflex testing or to tail pinching. After the administration of the paralyzing agent, we gave the anesthetic at regular intervals at the same rate as before the animals were paralyzed. The end-expiratory CO2 of the cats was continuously monitored with a Hewlett-Packard CO2 analyzer (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 (Glen Burnie, Md.) connected to a cannula introduced into the aorta via the femoral artery. Arterial blood samples were collected for determination of Pao2 and Paco2, pH, and hematocrit at appropriate intervals during the experiment. Blood gas tensions and pH were measured with Corning electrodes (Corning, N.Y.). Hematocrit was measured by a micromethod. Rectal temperature was monitored continuously and was kept constant with a heating blanket.

The cerebral microcirculation of the parietal cortex was visualized through acutely implanted cranial windows, as described in detail previously.13 In most cases, we used two symmetrical windows that were placed to overlap the parietal cortex of each hemisphere for comparative studies. The space under the cranial windows was filled with artificial cerebrospinal fluid (CSF) identical in composition to the CSF of cats. 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 the outlet of the window to a coiled plastic tube the free end of which 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 attached to a Wild microscope equipped with a ×6.5 dry objective. In each cat, several arterioles were observed under each window.

In some experiments, we assayed EDRF 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 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 that in this preparation acetylcholine generates a short-lived vasodilator agent that can be transferred to induce vasodilatation in the assay window. This vasodilator agent has characteristics identical to those of the EDRF induced by acetylcholine in large vessels in vitro.14 It is therefore believed to be the EDRF induced by acetylcholine from cerebral microvessels.

Acetylcholine chloride, adenosine, sodium nitroprusside, L-NOARG, sodium dodecyl sulfate (SDS), superoxide dismutase (SOD) (from bovine blood, 3,000 units/mg protein), catalase (from bovine liver; 40,000 units/mg protein), and L-arginine were obtained from Sigma Chemical Co., St. Louis, Mo. L-NMMA was obtained from Calbiochem Corp., La Jolla, Calif. Nitroglycerin was prepared from tablets as described before.10 All agents were dissolved in artificial CSF immediately prior to the experiment. The artificial CSF was equilibrated with 6% CO2, 6.5% O2, balance N2 at 37°C to give gas tensions and pH identical to that of CSF for cats. Solutions of various inhibitors were injected into the space under the window and left there for the specified periods. Vasoactive agents such as acetylcholine, adenosine, and nitroprusside were administered by constant superfusion at a rate of 1 ml/min.

Several series of experiments were performed.

1. In six cats we first determined the responses to topical superfusion with 10−7 M acetylcholine. The effect of acetylcholine was tested again after topical application of L-NMMA in doses of 10, 20, 50, and 100 μM. Each dose of L-NMMA was left in contact with the brain for 10 minutes before acetylcholine was superfused.

2. In a second series of six cats we essentially repeated the experiment described above except that the entire experiment was carried out after a 10-minute topical application of 20 μM SDS to the brain surface. This was done to increase cellular permeability and facilitate the entry of L-NMMA into vascular smooth muscle. The technique of permeabilization to facilitate the entry of various agents into the interior of cells where the agents gain access to intracellular enzymes has been used successfully in isolated hepatocytes and in cultured ovarian cells as well as very extensively in various types of muscle including vascular smooth muscle and cardiac muscle.15–18 Subsequently, we tested the responses to 10−7 M acetylcholine superfusion before
and after a 10-minute application of 20 μM L-NMMA as well as after topical application of 50, 100, 200, and 1,000 μM L-arginine. Each dose of L-arginine was left for 10 minutes before testing the responses to acetylcholine.

3. In a third series of experiments in four cats we tested the responses to 10⁻⁷ M acetylcholine superfusion as well as the presence of EDRF in the effluent by bioassay before and after a 10-minute application of L-NMMA in the donor window and after the topical application of 1 mM L-arginine for 10 minutes in the donor window. The entire experiment was done after a 10-minute application of SDS in the donor window as described above.

4. In a fourth series of experiments in six cats we tested the effects of superfusion of 10⁻⁷ M acetylcholine as well as the presence of EDRF in the effluent by bioassay before and after a 10-minute application of 20 μM L-NOARG in the donor window and after application of 1 mM L-arginine in the donor window.

5. In a fifth series of experiments in six cats equipped with double cranial windows, we tested the effect of the inhibitors of nitric oxide synthesis on the responses to nitroprusside, nitroglycerin, and adenosine. One window was treated with 20 μM L-NOARG for 10 minutes. The other window was treated first with 20 μM SDS followed by 20 μM L-NMMA for 10 minutes. Responses to topical applications of 10⁻⁶ M nitroprusside, 1.9×10⁻⁶ M nitroglycerin, and 10⁻⁴ M adenosine were tested before and after application of the nitric oxide inhibitors.

6. In a sixth series of four cats we tested the duration of inhibition of the vasodilator response to acetylcholine by the nitric oxide synthesis inhibitors as well as the dependence of their effect on oxygen radicals. We used SDS followed by L-NMMA in two cats and by L-NOARG in two other animals. L-NMMA and L-NOARG were left in contact with the brain for 10 minutes and were then washed out with fresh artificial CSF. The response to 10⁻⁷ M acetylcholine was tested before the application of the nitric oxide synthesis inhibitors and 10, 30, 60, and 90 minutes after their application and washout. At the end of the experiment we again tested the response to acetylcholine in the presence of 60 units/ml SOD plus 40 units/ml catalase.

In all protocols all vessels studied were used for all drugs tested. Statistical analyses were done by analysis of variance. If significant differences were found by this technique, differences between individual group means were evaluated by t tests modified for multiple comparisons. Data are reported as mean±SEM.

Results

Mean arterial blood pressure in all the cats averaged 119±2.0 mm Hg; PaCO₂ was 28±0.5 mm Hg, and PaO₂ was 113±1.7 mm Hg. None of the interventions used had any influence on mean arterial blood pressure or blood gases. Figure 1 shows that, in the absence of pretreatment, L-NMMA did not affect baseline cerebral arteriolar caliber and did not modify the vasodilator response to topical superfusion of acetylcholine. Figure 2 shows that responsiveness to topical applications of acetylcholine, nitroprusside, nitroglycerin, and adenosine were not altered by prior treatment with the detergent SDS. The doses of acetylcholine, nitroprusside, nitroglycerin, and adenosine used gave responses on the ascending limb of the dose–response curve for each agent and none causes supermaximal vasodilation. Similarly, SDS did not alter baseline arteriolar diameters. Following SDS application to increase cellular permeability, topical application of L-NMMA eliminated the vasodilator response to acetylcholine (Figure 3). Subsequent topical application of L-arginine restored the vasodilator response to acetylcholine in a dose-dependent fashion (Figure 3). L-NMMA after SDS with or without L-arginine did not alter baseline vessel caliber. At 100–1,000 μM L-arginine by itself was not vasoactive (data not shown).

Figure 4 shows that, after SDS, L-NMMA eliminated the vasodilator response to acetylcholine and caused...
disappearance of the EDRF induced by acetylcholine from the effluent as indicated by the absence of vasodilation in the assay window during superfusion with acetylcholine. L-Arginine restored both normal vasodilator responsiveness to acetylcholine in the donor window as well as the presence of EDRF in the effluent. Figure 5 shows that L-NOARG completely blocked the vasodilator response to acetylcholine and caused the disappearance of EDRF from the effluent in the donor window as indicated by the absence of vasodilation in the assay window during superfusion with acetylcholine. Subsequent application of L-arginine restored normal vasodilator responsiveness to acetylcholine in the donor window as well as the presence of EDRF in the effluent at normal concentrations.

Figure 6 shows that L-NMMA, given after pretreatment with SDS, and L-NOARG did not affect responses to nitroprusside and adenosine, indicating that their effect on the response to acetylcholine is specific. Figure 7 shows the time course of the effect of inhibition of synthesis of nitric oxide by L-NMMA or L-NOARG on the vasodilator response to acetylcholine. The data with L-NMMA and L-NOARG were combined since the effects of the two compounds were essentially identical. It is seen that the blockade lasted without abatement for at least 90 minutes. Topical application of SOD plus catalase did not affect the blockade of the response to acetylcholine.

Discussion

The short-lived vasodilator material generated by acetylcholine in these experiments is very likely the EDRF induced by this agent in cerebral vessels for four reasons. 1) Various agents that eliminate the vasodila-
plus catalase were ineffective in reversing effect of inhibition of nitric oxide synthesis on vasodilator response to topical application of nitroprusside and adenosine. Results are from 26 vessels with L-NOARG and 25 vessels with L-NMMA in six cats. Mean ± SEM baseline diameter from which percent change was derived is shown in μm above each column. Note that neither L-NOARG nor L-NMMA had any significant effect on baseline diameter and that they did not affect responses to nitroprusside or adenosine.

An important finding of these experiments was that L-NMMA was effective in blocking the vasodilator action of acetylcholine and the generation of EDRF induced by acetylcholine only after cellular permeability was increased with the topical application of a detergent. This suggests that unlike L-NOARG, L-NMMA does not readily penetrate the cell membrane of vascular smooth muscle in cerebral arterioles of cats. The possibility that low cellular membrane permeability to the nitric oxide synthase inhibitors may explain negative results obtained with the use of these agents must be considered. Clearly, such negative experiments do not warrant the conclusion that an EDRF different from nitric oxide or a nitric oxide–containing compound is involved, unless the presence of a permeability barrier preventing access of these inhibitors to the interior of vascular smooth muscle is excluded.

In our preparation, administration of the nitric oxide synthesis inhibitors did not alter basal arteriolar diameter. This is consistent with the view that there is no basal secretion of EDRF in this preparation. This also agrees with earlier findings in which we found that in bioassay experiments no detectable basal secretion of EDRF occurred in the absence of an agonist\textsuperscript{14} and no change in vascular caliber occurred when the vascular bed was treated with agents that destroyed the EDRF. It is likely that acetylcholine is prevented from entering the brain substance by a high concentration of acetylcholinesterase in the glia limitans.

The experiments reported above showed that the inhibitors of nitric oxide synthesis from arginine completely eliminated the vasodilation induced by acetylcholine in feline cerebral arterioles and caused the disappearance of EDRF from the effluent as indicated by the bioassay experiments. The effect of the inhibitors of nitric oxide synthesis occurred in the absence of baseline changes in vascular caliber, and the effect was relatively specific because the inhibitors did not affect the vasodilation from nitroprusside or adenosine. The findings, therefore, suggest strongly that the EDRF generated by acetylcholine in feline cerebral arterioles is nitric oxide or a nitric oxide–containing compound. As indicated above, earlier experiments had distinguished between the EDRF from acetylcholine and nitric oxide by pharmacological means and showed that a nitrosothiol (S-nitroso-L-cysteine) resembled the EDRF from acetylcholine much more closely than nitric oxide did.\textsuperscript{10,11}

An important finding of these experiments was that L-NMMA was effective in blocking the vasodilator response to topical application of nitroprusside as in the present experiments, does not gain access to the brain parenchyma. For example, in unanesthetized rabbits equipped with cranial windows, topical application of acetylcholine in concentrations of up to $10^{-2}$ M elicits no motor or behavioral changes. It is likely that acetylcholine was generating an EDRF-like material from sources other than vessels, this would not happen. 3) Interventions, such as short-lived acute elevations in blood pressure, that would be expected to have purely vascular effects eliminate not only the vasodilation from acetylcholine but also the appearance of the vasodilator material in the effluent (unpublished observations). 4) Acetylcholine, when applied by superfusion as in the present experiments, does not gain access to the brain parenchyma. For example, in unanesthetized rabbits equipped with cranial windows, topical application of acetylcholine in concentrations of up to $10^{-2}$ M elicits no motor or behavioral changes. It is likely that acetylcholine was generating an EDRF-like material from sources other than vessels, this would not happen. 3) Interventions, such as short-lived acute elevations in blood pressure, that would be expected to have purely vascular effects eliminate not only the vasodilation from acetylcholine but also the appearance of the vasodilator material in the effluent (unpublished observations). 4) Acetylcholine, when applied by superfusion as in the present experiments, does not gain access to the brain parenchyma. For example, in unanesthetized rabbits equipped with cranial windows, topical application of acetylcholine in concentrations of up to $10^{-2}$ M elicits no motor or behavioral changes. It is likely that acetylcholine was generating an EDRF-like material from sources other than vessels, this would not happen. 3) Interventions, such as short-lived acute elevations in blood pressure, that would be expected to have purely vascular effects eliminate not only the vasodilation from acetylcholine but also the appearance of the vasodilator material in the effluent (unpublished observations). 4) Acetylcholine, when applied by superfusion as in the present experiments, does not gain access to the brain parenchyma.
ments that acetylcholine following inhibition of nitric oxide synthesis did not generate a transferable vasoconstrictor agent.

An important finding is that acetylcholine in the presence of inhibitors of nitric oxide synthesis caused no change in vascular caliber despite the fact that acetylcholine is known to have a direct vasoconstrictor effect on vascular smooth muscle. This result is different from what is seen when the response to acetylcholine is converted to vasoconstriction in the presence of oxygen radicals. The difference between the present experiments and these earlier findings is that the vessels in the presence of radicals are dilated. Perhaps the vasoconstrictor effect of acetylcholine is expressed only when vascular smooth muscle is relaxed. Another possibility is that acetylcholine in the presence of oxygen radicals generates a vasoconstrictor agent. Finally, the vasoconstriction from acetylcholine in the presence of oxygen radicals may be due to interaction with EDRF, which eliminates the vasodilation from the radicals.

We were unable to confirm the observation that oxygen radicals participate as mediators of the effect of L-NAME on acetylcholine. The topical application of SOD and catalase, which eliminate all the reactive products of univalent reduction of oxygen including superoxide and hydrogen peroxide and prevent the formation of hydroxyl radical, did not affect the blockade of the response to acetylcholine by either L-NAME or L-NOARG. We do not know whether this represents a difference between cats and mice.

References

Editorial Comment

Since publication of the seminal paper by Furchgott and Zawadzki,1 endothelium-derived relaxing factor (EDRF) has been one of the hottest topics in vascular biology. Major questions include the following: What is the chemical nature of EDRF? What is the role of EDRF in physiology and disease states? And recently, what is the therapeutic role of augmented synthesis or inhibition of synthesis of EDRF?

This study addresses the first question. Studies by Moncada’s group (e.g., Reference 2) propose that nitric oxide (NO) is EDRF. Kontos, at a presentation several years ago, asked rhetorically whether we now know the chemical nature of EDRF. His answer, on the next slide, was NO. This ambiguous answer is still appropriate. Several investigators, including Myers et a1 and Wei and Kontos,4 have provided strong evidence that NO may not be EDRF but may be incorporated in more than one EDRF.5

This study provides evidence to support the hypothesis that the EDRF that is generated by acetylcholine in cerebral arterioles of cats is NO or an NO-containing
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