Calcitonin Gene-Related Peptide Mediates Nitroglycerin and Sodium Nitroprusside-Induced Vasodilation in Feline Cerebral Arterioles

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The cerebral vasodilator response induced by topical nitroglycerin and nitroprusside was examined in cats equipped with cranial windows for the observation of the cerebral microcirculation. In cats subjected to chronic unilateral ganglionectionomy, the vasodilator responses to nitroprusside and nitroglycerin were markedly depressed on the denervated side. Application of a selective calcitonin gene-related peptide (CGRP) antagonist [CGRP(8-37)] on the innervated side reduced the response to nitroprusside to the same extent as seen on the denervated side. The vasodilator response to acetylcholine was unaffected by trigeminal ganglionectomy. CGRP(8-37) almost abolished the vasodilator response to nitroglycerin and sodium nitroprusside and to CGRP, but did not affect the response to adenosine or to adenosine diphosphate. Pretreatment with LY83583, a drug that lowers cyclic GMP levels, diminished the vasodilation to CGRP and to nitroprusside but not to adenosine. We conclude that the nitrovasodilators activate sensory fibers to release CGRP, which in turn relaxes cerebral vascular smooth muscle by activating guanylate cyclase. Hence, nitrovasodilators possess a novel mechanism of action within the cerebral circulation which may explain both the occurrence of vasodilation and headache. (Circulation Research 1992;70:1313–1319)

**Key Words** • calcitonin gene-related peptide • nitroglycerin • sodium nitroprusside • vasodilation • trigeminovascular system

The frequent occurrence of pain (headache) following nitrovasodilator administration suggests that nitrates activate trigeminovascular fibers. Sensory nerve fibers innervate large and small pial blood vessels and project from nerve cells within the ipsilateral trigeminal ganglion. Destruction of this ganglion causes degeneration of neuropeptide-containing sensory fibers within the adventitial layer of cephhalic blood vessels, and blockade of the hyperemia that accompanies reperfusion following global ischemia, severe acute hypertension, or seizures. Trigeminovascular fibers store and release calcitonin gene-related peptide, a 37 amino acid–containing peptide and one of the most potent vasodilating substances, as well as substance P and neurokinin A. Superfusion with potassium, or the pungent chemical capsaicin, causes neuropeptide release from perivascular pial fibers by calcium-dependent mechanisms and CGRP release from the Langendorff heart preparation. Electrical stimulation of sensory nerves innervating the meninges also releases CGRP into the venous effluent. Receptors for CGRP reside on vascular smooth muscle and mediate relaxation by cyclase-dependent mechanisms. The competitive antagonist CGRP(8-37) blocks CGRP-induced relaxation selectively and inhibits the increases in blood flow caused by electrical stimulation of splanchnic nerve fibers. An important role for CGRP as a mediator of neurogenic vasodilation has thus been established.

In this study we examined the possibility that the nitrovasodilators nitroglycerin and/or sodium nitroprusside activate trigeminovascular fibers and by so doing, promote neuropeptide release and vasodilation within the pial vasculature. We also examined whether LY83583, a drug that lowers cyclic GMP, blocks the relaxation mediated by the topical application of the released neuropeptide CGRP or by sodium nitroprusside. We now report that nitrovasodilators activate perivascular sensory fibers directly, and by so doing, relax vascular smooth muscle via CGRP and cyclic GMP–dependent mechanisms within the cranial vasculature.

**Materials and Methods**

**Preparation of Animals**

Experiments were carried out in mongrel cats (2-4 kg) anesthetized with sodium pentobarbital (30 mg/kg i.v.). After tracheostomy, each cat was ventilated with a positive-pressure respirator. After all operative procedures were completed, the animals received gallamine triethiodide (5 mg/kg i.v.) for skeletal muscle paralysis.
End-expiratory CO₂ was continuously monitored with a Hewlett-Packard CO₂ analyzer and was maintained at a constant level of about 30 mm Hg. Arterial blood pressure was measured with a Statham pressure transducer 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. Hematocrit was measured with a micromethod. Rectal temperature was monitored continuously and was kept constant with a heating blanket.

Cranial Window Preparation

The cerebral microcirculation of the parietal cortex was visualized through acutely implanted 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 cerebrospinal fluid (CSF) identical in composition to that 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 another outlet of the window to a coiled plastic tube whose free end was placed at the appropriate height to give the desired pressure. Two ports of the cranial window were used as inlet and outlet, allowing topical application of various solutions by superfusion. Pial arteriolar diameter was measured with a Vickers image-splitting device attached to a Wild microscope equipped with a ×6.5 dry objective. In each cat, several arterioles were observed under each window. Responses of small and large arterioles (small or larger than 100 μm) were analyzed separately to identify potential size dependence. A minimum of four and a maximum of nine vessels were measured per window.

Trigeminal Ganglion Sectioning

Unilateral trigeminal ganglionectomy was performed as follows: Cats were anesthetized by intraperitoneal injection of 25–30 mg/kg ketamine hydrochloride and 30 mg/kg sodium pentobarbital. The trigeminal ganglia were removed under sterile conditions using an operating microscope and microsurgical techniques. A subtemporal craniectomy was performed to expose the trigeminal ganglion after retracting the temporal lobe. After an injection of lidocaine into the trigeminal ganglion, it was cut at the apex of the petrous bone. The three divisions were cut anteriorly at the point where they leave Meckel's cave. The ganglion was then removed. An ipsilateral tarsorrhaphy was performed to prevent exposure keratitis. The animals were anesthetic to pin prick in the cutaneous trigeminal dermatomes but otherwise had no neurological deficits.

Materials

Acetylcholine chloride, sodium nitroprusside, adenosine, and adenosine diphosphates were obtained from Sigma; CGRP was obtained from Peninsula Labs; CGRP(8-37) was from Bachem. LY83583 was generously supplied by Eli Lilly Co; nitroglycerin was prepared from tablets. All drugs were dissolved in artificial CSF immediately prior to use. Dissolved drugs were applied topically by filling the space under the cranial window with the appropriate solution. The solution was then left in place until a steady state response was obtained. This usually occurred 2–4 minutes after application. CGRP(8-37) (1 μM) and LY83583 (10 μM) were applied for 15 minutes before responses to vasoactive agents were tested.

Experimental Procedures

We carried out the following experiments in a total of 27 cats: 1) In five cats with unilateral trigeminal ganglionectomy, we obtained dose–effect curves to nitroglycerin (0.4, 1.2, and 1.9 μM) and nitroprusside (0.3, 0.5, and 0.8 μM) in the denervated hemispheres. Subsequently, we retested the responses to nitroglycerin and nitroprusside following topical application of CGRP(8-37) (1 μM) in the innervated hemisphere. 2) After chronic unilateral trigeminal ganglionectomy, we tested responses to acetylcholine (10⁻⁵M) in the innervated and denervated hemispheres in six cats, four of whom were studied in protocol 1. 3) In five cats, we tested the responses to topical CGRP before and after application of CGRP(8-37) to verify the effectiveness of the antagonism induced by the latter compounds. 4) In five cats, we tested responses to nitroglycerin and to adenosine before and after application of CGRP(8-37). 5) In five cats, we tested responses to nitroprusside and to adenosine diphasphate before and after application of CGRP(8-37). 6) In five cats, we tested the responses to CGRP, nitroprusside, and adenosine before and after application of LY83583 for 15 minutes.

Statistical Analysis

The results were analyzed 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.

Results

Figure 1 summarizes the responses of normal and denervated pial arterioles to the topical application of nitroglycerin, sodium nitroprusside, and acetylcholine. Sodium nitroprusside and nitroglycerin dilated small (Figure 1A) and large (Figure 1B) arterioles on the innervated side in a dose-dependent manner. With dervation, dilation was significantly less at each of the three tested doses. Small and large pial arterioles responded similarly. Acetylcholine (0.1 μM) relaxed vessels on the innervated and denervated hemispheres equally.

Figure 2 demonstrates that the CGRP antagonist (1 μM) completely blocked nitroglycerin-induced vasodilation and markedly attenuated the response to sodium nitroprusside. Both small (Figure 1A) and large (Figure 1B) arterioles responded similarly. CGRP(8-37) (10 nM to 1 μM) alone did not change the diameter of large or small arterioles. Figure 2 also shows that CGRP-induced dilatation (0.01, 0.1 μM) was completely blocked by the CGRP antagonist but that the responses to ADP and adenosine were not (Figure 2).

Figure 3 shows that LY83583 significantly decreased the response to CGRP and nitroprusside at each of the tested doses. Nitroglycerin was not tested. By contrast, the response to adenosine (100 μM) was unaffected by the guanylate cyclase inhibitor.
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A

Figure 1. Chronic trigeminal denervation significantly decreases the responsiveness of denervated small (A) and large (B) arterioles to the application of nitroglycerin (NTG; 0.4 μM, 1.2 μM, and 1.9 μM; n=5 cats) or sodium nitroprusside (SNP; 0.3 μM, 0.5 μM, and 0.8 μM; n=5 cats) but not to topical acetylcholine (Ach; 0.1 μM, n=6 cats). Drugs were applied through symmetrically placed closed cranial windows 10–14 days after nerve sectioning. The response of intact (filled symbols) and denervated (open symbols) arterioles are shown. Baseline diameter (μm) for large and small vessels were 154±7.5 and 66±3.1, Ach; 148±7.3 and 62±2.7, SNP; 151±8.0 and 62±3.3, NTG. Data are expressed as mean±SEM. *p<0.01 as compared with the response on the unoperated side.

Discussion

The mechanism underlying relaxation of vascular smooth muscle by the nitrodilators nitroglycerin and sodium nitroprusside has been studied for the most part in large peripheral vessels in vitro such as the aorta, pulmonary artery, and vein (see Reference 21). The classical view about their main mechanism of action is that they act directly on vascular smooth muscle to generate nitric oxide, either spontaneously
FIGURE 2. Application of CGRP(8-37) (1 μM) decreases the responsiveness of normal small (A) and large (B) vessels to the topical application of nitroglycerin (NTG; 0.4, 1.2, and 1.9 μM; five cats), nitroprusside (SNP; 0.3, 0.5, and 0.8 μM; five cats) or CGRP (0.01, 0.1 μM; five cats) but not to topical adenosine (1, 10, 100 μM; five cats) or adenosine diphosphate (ADP; 0.1, 1, 10 μM; five cats). Filled symbols correspond to untreated and open symbols to CGRP(8-37) treated vessels. The antagonist was applied to the cranial windows as described in methods. Baseline diameter (μm) for large and small vessel were 139±7.4 and 57±2.8, NTG; 147±11.4 and 62±3, SNP; 152±6.7 and 65±3.0, CGRP; 142±7.7 and 60±3.1, adenosine; 149±10.8 and 61±2.9, ADP. CGRP(8-37) (0.01–1 μM) was not vasoactive when applied to large (n=11) and small (n=11) arterioles (three cats) (data not shown). Data are expressed as mean±SEM. *p<0.01 as compared with the response in the absence of CGRP(8-37).

or through interaction with tissue components (see Reference 21). This agent then activates soluble guanylate cyclase either directly or through the formation of nitrosothiol intermediates. The result is an increase in cyclic GMP and cyclic GMP-dependent protein kinase with resultant smooth muscle relaxation.
CGRP Release by Nitrodilators Relaxes Cerebral Vessels

Figure 3. Application of LY83583 (1 μM) significantly decreases the dilation of normal small (A) and large (B) pial vessels to topically applied sodium nitroprusside (SNP; 0.5 μM), and CGRP (0.001, 0.01, 0.1 μM) but not to adenosine (100 μM). Filled symbols correspond to untreated and open symbols to LY83583 treated vessels. Baseline diameters (μm) for large and small vessel were 147 ± 6 and 60 ± 4, SNP; 150 ± 6.6 and 60 ± 3, CGRP; 153 ± 7.2 and 61 ± 3.3, adenosine. Data are expressed as mean ± SEM. *p<0.01 as compared with the response without LY83583.

The studies reported above show that feline cerebral arterioles possess a hitherto undescribed indirect mechanism underlying nitrovasodilator-induced vasodilation. The nitrovasodilators act on sensory fibers to release CGRP, which then diffuses to the vascular smooth muscle where it activates soluble guanylate cyclase to cause vasodilation. This appears to be their main mechanism of action on feline cerebral arterioles. The possibility that other vessels may utilize this mechanism merits further investigation especially in view of the likelihood that release mechanisms are difficult to demonstrate in sensory fibers using isolated blood vessel preparations. Our data also suggest that a secondary mechanism of relaxation is apparent, particularly at
high doses of nitrovasodilators. CGRP(8-37) did not block a substantial portion of the vasodilation induced by high-dose nitroprusside while it blocked completely the vasodilator action of CGRP in doses that induced vasodilation comparable with that caused by nitroprusside. The residual dilation may represent the direct action of the nitrovasodilators on vascular smooth muscle, although the finding that LY83583 had a much greater inhibitory effect on responses to CGRP than SNP (Figure 3) suggests a mechanism other than one dependent on guanylate cyclase activation.

The mechanism by which nitrovasodilators activate the release of vasodilator polypeptides from sensory fibers is not known. Several possibilities exist. For example, these agents may induce calcium-dependent neuropeptide release. Another possibility is that the nitrovasodilators generate nitric oxide, which then may interact with superoxide to form peroxynitrite. This agent then may generate hydroxyl radical. Free radicals, particularly the very reactive hydroxyl radical, are important mediators of tissue injury, and small unmyelinated C fibers are activated by real or threatened tissue injury. Of possible relevance in this respect is that nitric oxide has been proposed as a mediator of relaxation induced by non-adrenergic, noncholinergic (NANC) fibers within guinea pig trachea, an nocyclocyte muscle of the rat and mouse, rat gastric fundus strips, and canine ile-colon. Sensory fibers from the trigeminal ganglion are NANC, and the possible formation and release of nitric oxide from such fibers merits further study. Whether the two lipophilic nitrovasodilators used in our studies are taken up by sensory fibers and transformed to nitric oxide remains to be determined. Irrespective of the exact mechanism involved, the findings implicate CGRP and not nitric oxide as the major mediator of the cerebral vasodilation induced by these agents. Of course, the role of nitric oxide itself as a coexisting neurotransmitter or as a potential releasing agent deserves further consideration.

The strongest evidence implicating CGRP as the mediator of nitrodilator-induced cerebral arteriolar dilation is the finding that pretreatment with CGRP(8-37) blocked the dilation caused by these agents. CGRP(8-37) is a potent competitive antagonist that resembles the native peptide except that it lacks the cyclic loop at the amino terminus. CGRP(8-37) inhibits cAMP accumulation induced by CGRP and dose-dependently displaces [125I]-[Tyr]CGRP binding in plasma membranes prepared from rat liver. The compound appears devoid of agonist activity when tested in concentrations as high as 10 μM. Receptors sensitive to this antagonist are designated as CGRP receptors. Therefore, the receptors present on feline pial arterioles appear to be CGRP receptors. Westfall and colleagues found that CGRP(8-37) inhibited reversibly the vasodilation induced by periarterial nerve stimulation or by CGRP infusion within the mesenteric arterial bed, but not, however, by isoproterenol infusion. Donoso et al. found that the intravenous administration of CGRP(8-37) blocked the hypotensive response to CGRP in the rat. Consistent with the data reported herein, the peptide antagonist was not vasoactive.

Our findings show that CGRP relaxed vascular smooth muscle in feline cerebral arterioles by activating guanylate cyclase–dependent mechanisms. We base this on the inhibitory effects of LY83583 on the vasodilation induced by CGRP. LY83583 lowers basal levels of cGMP in guinea pig lung, heart, cerebellum but not cAMP in fragments of guinea pig lung. In rat thoracic aorta, LY83583 blocked vascular smooth muscle relaxation to nitroprusside, ATP and acetylcholine; CGRP was not tested. The importance of guanylate cyclase as a mediator of CGRP’s action in cerebral arterioles is of interest in view of recent data suggesting that cAMP levels increase in response to CGRP application. In cultured rat aortic smooth muscle cells, CGRP stimulates the formation of cAMP but not cGMP. In isolated intracerebral arterioles and feline middle cerebral arteries, the EC50 values for CGRP-induced relaxation and cAMP formation were similar and CGRP(8-37) blocked both responses. Although these results suggest an important role for cAMP in the response of vascular smooth muscle to CGRP, they do not establish that cAMP mediates the effects of CGRP, nor do they exclude an important role for cGMP. Time-dependent measurements of both cyclic nucleotides in relation to smooth muscle relaxation may help to clarify this issue as may studies utilizing inhibitors of adenylyl cyclase activation. Certainly there is precedence for peptide-induced guanylate cyclase activation as evidenced by the effects of natriuretic peptides. It is of interest that Nω-nitro-l-arginine blocked the hindquarter hyperemic vasodilator effects of human α-CGRP. This suggests a possible role for nitric oxide (and, therefore, cyclic GMP-dependent mechanisms). The relative contribution of the two cyclic nucleotides to the actions of CGRP within different vascular beds merits further study.

Nitroglycerin and sodium nitroprusside cause headaches in humans. The traditional explanation concerns drug-induced vasodilation and vasomotor effects on cerebrovascular smooth muscle. New data suggest that vasodilation is neither necessary nor sufficient to cause headache in most cases. Headache implies that trigeminovascular fibers are being depolarized. Depolarization releases neuropeptides such as CGRP; vasodilation may result. Neuropeptide release from perivascular axons, however, is not always triggered by an action potential and not always accompanied by transmission of impulses centrally. Hence pain need not always accompany vasodilation, although vasodilation will often accompany pain. As evidence, Dahl et al. determined that nitroglycerin induced headache in humans began when dilation of intracranial arteries was decreasing. The demonstration that nitrovazodilators activate sensory fibers and release neuropeptides adds a new dimension toward understanding possible mechanisms of vasodilation and pain production in cephalic blood vessels.

This work has been published previously in preliminary form.

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