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Involvement of nitric oxide in the modulation of dural arterial blood flow in the rat

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1 Nitric oxide (NO) has been proposed to be a key molecule in the pathogenesis of migraine pain and other headaches that are linked to vascular disorders. Several lines of evidence indicate that the meningeal vascularization is crucially involved in the generation of these headaches. In an experimental model in the rat a dominating role of calcitonin gene-related peptide (CGRP) in causing neurogenic vasodilatation and increased blood flow has been shown. The aim of the present study was to clarify the role of NO in this model with regard to the meningeal blood flow.

2 The blood flow in and around the medial meningeal artery (dural arterial flow) was recorded in the exposed parietal dura mater encephali of barbiturate anaesthetized rats using laser Doppler flowmetry. Local electrical stimulation of the dura mater (pulses of 0.5 ms delivered at 7.5–17.5 V and 5 or 10 Hz for 30 s) caused temporary increases in dural arterial flow for about 1 min that reached peaks of 1.6–2.6 times the basal flow. The effects of NO synthase (NOS) inhibitors on the basal flow and the electrically evoked increases in flow were examined.

3 Systemic (i.v.) administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME) at cumulative doses of 10 and 50 mg kg^{-1} lowered the basal flow to 87 and 72%, respectively, of the control and reduced the evoked increases in blood flow to 82 and 44% on an average. Both these effects could partly be reversed by 300 mg kg^{-1} L-arginine. The systemic arterial pressure was increased by L-NAME at both doses. Injection of the stereoisomer D-NAME at same doses did not change basal flow and evoked increases in flow.

4 Topical application of L-NAME (10^{-4}–10^{-2} M) was effective only at the highest concentration, which caused lowering of the basal blood flow to 78% of the control; the evoked increases in flow were not changed. Topical application of 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), a specific inhibitor of the inducible NOS, at concentrations of 10^{-4}–10^{-2} M lowered the basal flow to 89, 87.5 and 85%, respectively, but did not significantly change the evoked flow increases. Same concentrations of 7-nitroindazole monosodium salt (7-NINA), a specific inhibitor of the neuronal NOS, had no significant effects on basal flow and evoked increases in flow.

5 It is concluded that NO is involved in the maintenance of the basal level of dural arterial blood flow as well as in the electrically evoked flow increases, which have been shown to be mainly mediated by CGRP released from dural afferent fibres. The most important source of NO is probably the endothelium of dural arterial vessels. The synergistic effect of NO and CGRP on the stimulated blood flow may be in part due to a NO mediated facilitation of the CGRP release.
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Abbreviations: AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; ANOVA analysis of variance; BP, blood pressure; CGRP, calcitonin gene-related peptide; $CGRP_{8-37}$, CGRP receptor antagonist; D-NAME, N^{ω}-nitro-D-arginine methyl ester; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LSD test, least significance difference test; MMA, medial meningeal artery; 7-NINA, 7-nitroindazole monosodium salt; NO, nitric oxide; NOS, NO synthetase; SNP, sodium nitroprusside; VIP, vasoactive intestinal polypeptide

Introduction

Nitric oxide (NO) is an important messenger in the cerebrovascular regulation by mediating vasodilatation and participating in the maintenance of cerebral blood flow (Buchanan & Phillis, 1993; Faraci & Heistad, 1998; Iadecola *et al*., 1994). NO, originally described as an endothelium derived relaxing factor (Moncada *et al*., 1988), can be released from endothelial cells of arterial vessels by mechanical stimuli (shear stress) and by several chemical mediators (Wahl & Schilling, 1993). Other possible sources of NO in meningeal tissues are perivascular nerve fibres (Berger *et al*., 1994) and immunocompetent cells, in which NO production is induced during inflammation (Korytko & Boje, 1996).

NO is also suggested to be a key molecule in the cascade of nociceptive processes that lead to migraine pain and other vascular headaches (Olesen *et al*., 1994), which are believed to originate in the trigeminovascular system (Goadsby & Edvinsson, 1993; Moskowitz *et al*., 1989). Exogenous NO derived from nitroglycerin has been shown to decrease the blood flow velocity in the middle cerebral artery indicative for vasodilatation (Thomsen *et al*., 1993) and to cause headache (Iversen *et al*., 1989). These reactions are pronounced in migraine patients, who seem to be supersensitive to nitrovasodilators and presumably to endogenous NO release (Olesen *et al*., 1993).

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Previous examinations have shown that another important mediator of vasodilatation and increased blood flow, the neuropeptide calcitonin gene-related peptide (CGRP), may be implicated in the pathogenesis of headaches. During attacks of migraine and cluster headache, increased levels of CGRP have been found in the venous outflow from the head suggesting its release in the trigeminovascular system (Goadsby & Edvinsson, 1994; Goadsby *et al*., 1990). Experimentally, CGRP can be released from trigeminal afferents upon electrical stimulation of the trigeminal ganglion (Goadsby *et al*., 1988) or of meningeal structures in rat and cat (Ebersberger *et al*., 1999; Zagami *et al*., 1990). Local electrical stimulation of the rat dura mater causes arterial vasodilatation (Williamson *et al*., 1997) and increased meningeal blood flow, which can be suppressed by application of the CGRP antagonist, $CGRP_{8-37}$ (Kurosawa *et al*., 1995). Wei *et al*. (1992) have shown that the vasodilatory effect of the NO donors nitroglycerin and sodium nitroprusside on cerebral arterioles of the cat is reduced after chronic trigeminal denervation (trigeminal ganglionectomy) and after application of $CGRP_{8-37}$. Thus CGRP released from trigeminal afferents seems to act synergistically to NO on the intracerebral circulation.

Clinical and experimental data suggest, however, that the trigeminovascular system of large blood vessels in the dura mater encephali, rather than blood vessels of the pia mater or intracerebral vessels, are involved in meningeal nociception and the pathogenesis of headaches (Davis & Dostrovsky, 1988; Ray & Wolff, 1940; Schepelmann *et al*., 1999; Strassman *et al*., 1996; Wirth & Van Buren, 1971). Little is known about the role of NO in the regulation of the blood flow in the dura mater but it seems likely that similar mechanisms as described by Wei *et al*. (1992) for the cerebral arterial vessels (see above) also apply to the vascularization of the dura mater. In the present study we have used the NO synthetase (NOS) inhibitor L-NAME (N^{ω}-nitro-L-arginine methyl ester) to examine the role of NO in the control of dural arterial blood flow in the rat. Besides, an inhibitor of the neuronal NOS and an inhibitor of the inducible NOS were tested to clarify which cells are likely to produce endogenous NO.

Methods

Anaesthesia and general preparation

Male Wistar rats (240–420 g, Charles River, Germany) were used for the study. The experiments were performed in accordance with the ethical issues for animal care and treatment and received institutional approval by a committee of the local district government. The animals were anaesthetized by an initial dose of 140–150 mg kg^{-1} thiopentone (Trapanal, Byk Gulden, Konstanz, Germany) i.p., followed by supplemental doses of 25–30 mg kg^{-1} thiopentone i.p. when required. Depth of anaethesia was routinely assessed and held at a level in which noxious stimuli (pinching of earlobes and feet) failed to elicit motor reflexes or changes of the systemic arterial pressure. A catheter was inserted into the right femoral vein for the infusion of solutions. Arterial blood pressure was continuously recorded with a pressure transducer connected to catheter in the right femoral artery and maintained stable above 90 mmHg (mean) by i.v. infusion of 4% Ficoll 70 (Serva, Heidelberg, Germany) and 5% glucose solutions, if required. Some animals were tracheotomized, paralyzed with pancuronium bromide (Pancuronium Organon, Organon Teknika, Eppelheim, Germany) and artificially ventilated. In this case, the end-expiratory CO_2 was held at 4%, and invariance of the blood pressure under noxious stimulation served as the control for the depth of anaesthesia. The body temperature of the animals was maintained at 37.0–37.5°C with a thermostatically regulated heating plate. The experiments were terminated by an i.v. overdose of thiopentone.

Preparation for stimulation and recording

The head of the animals was fixed in a stereotaxic frame, the scalp was incised and the skin pulled aside. Using an electric drill, the parietal bone at one side was trepanized while being cooled with Tyrode solution (4°C) to avoid thermal lesions. Two cranial windows, one of about 2 × 6 mm (for stimulation) and one of 4 × 6 mm (for recording), were drilled into the skull to expose the dura mater (see Figure 1 in Kurosawa *et al*., 1995). In the small opening parallel to the sagittal suture, a pair of stimulating electrodes (platin wires with a diameter of 0.2 mm and a length of about 4 mm, separation 1 mm) was lowered on the dural surface, the cathode placed laterally. This opening was filled with paraffin oil. In the recording window, at a distance of 2-3 mm from the lateral stimulation electrode, the probe (needle type, tip diameter 0.8 mm) of a laser Doppler flowmeter (Moore Instruments) was fixed pointing with its tip onto a branch of the medial meningeal artery (MMA). Care was taken to choose recording sites that were distant to pial arteries. The flow signal at these sites was usually much higher than at sites between dural arterial vessels, provided that no pial artery was underneath the dural recording field. In previous experiments, optical insulation of the dura from the underlying cortex had not significantly changed the dural arterial flow (Kurosawa *et al*., 1995). The dura mater around the probe in the recording window was covered with pieces of gauze soaked with Tyrode solution (pH 7.4) to prevent drying and to maintain a fluid medium between probe and dura.

Recording of blood flow and electrical stimulation

Blood flow was recorded on-line by the DRT4 laser Doppler system (Moor Instruments) with a time constant of 1.0 s. Systemic arterial blood pressure was recorded simultaneously to the flow. To activate meningeal afferents and increase the flow, the dura was electrically stimulated with rectangular pulses of 0.5 ms length for periods of 30 s repeated at intervals of 5 min. Each period of stimulation caused transient increases in blood flow, the magnitude of which depended on the strength and the frequency of stimuli and reached its maximal size at 15–20 V. With constant stimulus parameters these increases were constant in magnitude (Kurosawa *et al*., 1995). To account for individual variations, stimulus strength and

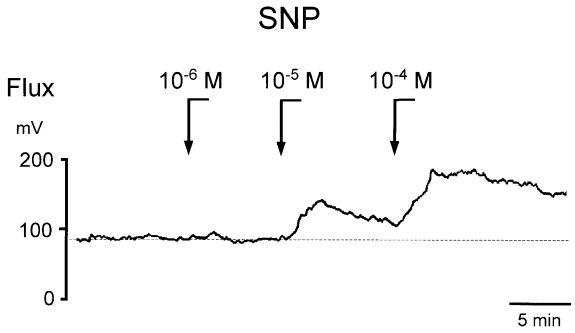

Figure 1 Effect of sodium nitroprusside, locally applied to the dura mater at three concentrations for 2 min each, on the blood flow (Flux, output reading in mV).

frequency were optimized at the beginning of each experiment to elicit stable increases in local blood flow of about 2/3 of the maximal size avoiding any changes of the systemic blood pressure. This optimal strength ranged from 7 to 12 V, in most experiments it was below 10 V. The pulse frequency was usually 10 Hz, in some experiments 5 Hz. Care was taken to use the same variation of stimulus parameters in all experimental groups to exclude any possible trends caused by different stimulus strengths and frequencies.

Measuring and evaluation of data

Blood flow values were measured by morphometry of the area under the flow curve on the printed records as previously described (Messlinger *et al*., 1997). Basal flow values were determined as the areas between the zero line and the curve of the unstimulated flow within intervals of 5 min; the intervals in which drugs were given were excluded. Evoked flow values were determined as the areas circumscribed by the curve of the flow increase above the line of the unstimulated flow. Each drug test was preceded by at least three control intervals of 5 min in which basal and evoked flow had to be stable. Measurements of basal or evoked flow, respectively, were calculated relative to the mean of the control values before the test sequence (baseline = 100%). A one-way analysis of variance (ANOVA) with extensions for repeated measurements was performed followed by a *post hoc* analysis of flow values after application of drugs. The least significance difference (LSD) test was used to test differences between means of flow values measured after L-NAME and D-NAME, respectively, at distinct time intervals. The same test was used for other sets of data to compare flow values immediately before L-NAME (control) and after L-arginine following L-NAME, and before (control) and after 7-NINA or AMT, respectively, at increasing doses. Significance was assessed at the 5% level.

Drug administration

The test substances were i.v. injected or topically applied to the exposed dural surface 2 min prior to the first test stimulation followed by four to five further stimulation periods. For topical administration of substances, the cotton swab on the dura soaked with Tyrode solution (pH 7.4; 285 mosm l^{-1}) was replaced by a swab soaked with the test solution. All drugs were dissolved in Tyrode solution. SNP (Sodium nitroprusside; Merck, Darmstadt, Germany) was topically applied at increasing concentrations of 10^{-5}–10^{-3} M for 2 min each. L-NAME (N^{ω}-nitro-L-arginine methyl ester; Sigma-Aldrich) and its stereoisomer D-NAME were i.v. administered at cumulative doses of 10 and 50 mg kg^{-1} followed by L-arginine (Sigma-Aldrich) at a dose of 300 mg kg^{-1} in a part of the experiments. L-NAME and D-NAME were also applied topically, as well as 7-NINA (7-Nitroindazole monosodium salt; Tocris Cookson, Bristol, U.K.) and AMT (2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; Tocris Cookson), at increasing concentrations of 10^{-4}–10^{-2} M.

Results

Effects of local application of sodium nitroprusside

In four experiments, the NO donor SNP applied to the dura mater at increasing concentrations caused dose-dependent increases in basal blood flow (Figure 1). SNP at 10^{-4} M increased the flow to 134% ± 14% (mean ± s.d.mean) of the baseline and SNP at 10^{-3} M to 155% ± 32% (mean ± s.d.mean) within 5–10 min post application.

Effects of i.v. administration of L- and D-NAME

In ten experiments L-NAME and in eight experiments D-NAME were i.v. administered at doses of 10 and 50 mg kg^{-1}. Figure 2A shows the effects of L-NAME on the basal blood flow, the electrically evoked flow increases, and the systemic blood pressure (BP) as original recordings in one experiment. A rapid rise of the BP by 30–50 mm Hg shortly after injection of 10 mg kg^{-1} L-NAME was accompanied by transient increases in basal and evoked flow, which turned into decreases of basal and evoked flow within 5–10 min, while the BP remained elevated. In the majority of experiments the minimum of basal flow and the maximal reduction of the evoked flow were observed 15–20 min after injection of L-NAME (Figure 2B, arrows). At this time interval the mean

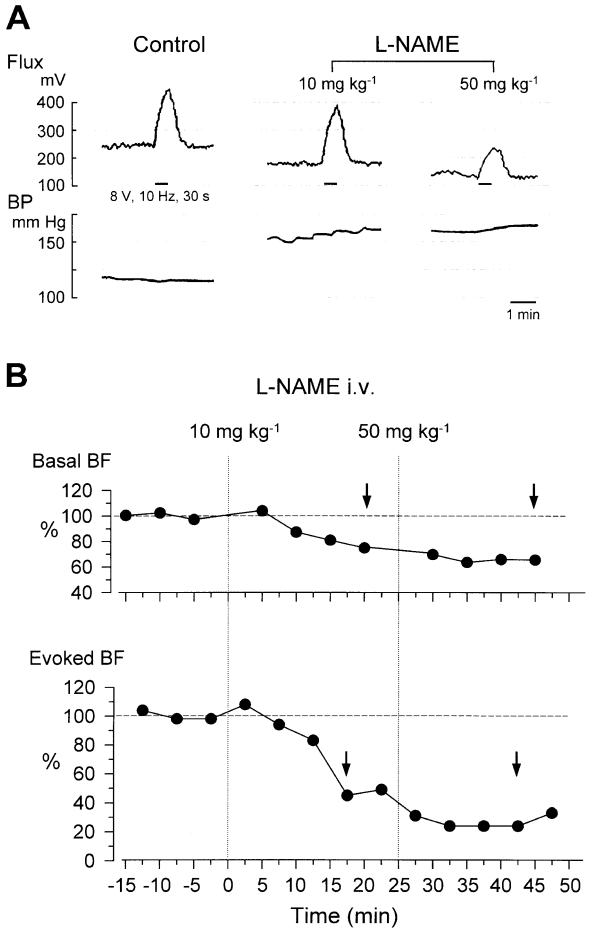

Figure 2 Effects of L-NAME, systemically administered at cumulative doses, on the dural arterial blood flow in two experiments. (A) Original recordings of blood flow (Flux) and mean arterial blood pressure (BP) in one experiment showing the last stimulation interval before L-NAME (Control) and the fourth interval after injection of L-NAME at 10 and 50 mg kg^{-1}, respectively (see time points indicated by arrows in the experiment shown in B). Bars below evoked flow increases show duration of stimulation (8 V, 10 Hz for 30 s). (B) Values of basal and evoked blood flow (BF) from a continuous recording. The mean of three control measurements is defined as 100%. Arrows point to flow values at intervals used for comparison of effects. The effect of 10 mg kg^{-1} L-NAME on the evoked (though not the basal) flow is much bigger in the experiment shown in B compared to A, indicating the variability of responses.

basal flow was lowered to 87 and 72% after 10 and 50 mg kg^{-1} L-NAME, respectively (Figure 3, upper diagram). The evoked flow was reduced to 82 and 44%, respectively, following 10 and 50 mg kg^{-1} L-NAME (Figure 3, lower diagram). In contrast, D-NAME caused neither significant changes of the BP nor changes of basal and evoked flow (Figure 3). Mean flow values after L-NAME compared to D-NAME were significantly different at both concentrations (LSD test, $p<0.05$).

In six of the experiments L-NAME at 50 mg kg^{-1} was followed by an i.v. injection of 300 mg kg^{-1} L-arginine. The lowering of the basal flow caused by L-NAME was reversed by L-arginine within 5–15 min to a mean level that was no longer different to the baseline before L-NAME (Figure 3, upper diagram, right column). There was also a clear although not complete reversal of the reduction of the evoked flow increases after the administration of L-arginine (Figure 3, lower diagram, right column). The blood pressure tended to be normalized after L-arginine but remained 5–20 mm Hg above the value registered before L-NAME.

Effects of local application of L- and D-NAME

In eight experiments L-NAME and in seven experiments D-NAME were locally administered onto the exposed dura mater in the recording opening at increasing concentrations of 10^{-4}–10^{-2} M. L-NAME caused weak but dose-dependent lowering of the basal flow with a maximal effect at 15–20 min after application. After the highest concentration of L-NAME (10^{-2} M) the mean basal flow was 78% of the control, which was significantly different to the flow after D-NAME at the same concentration (LSD test, $P<0.05$; Figure 4, upper diagram). The evoked blood flow showed some non-significant variations but was not lowered by local application of L- or D-NAME at any concentration of (Figure 4, lower diagram). The blood pressure was not affected in any of these experiments using local application.

Effects of local application of 7-NINA and AMT

In eight experiments 7-NINA, an inhibitor of the neuronal NOS, and in nine experiments AMT, an inhibitor of the inducible NOS, were locally administered onto the exposed dura at concentrations of 10^{-4}–10^{-2} M. After 7-NINA at all concentrations, mean values of both basal and evoked blood flow tended to increase rather than they decreased as compared to the control values prior to application but the changes were not significant at any time (Figure 5; ANOVA followed by LSD test). Following AMT, mean values of the basal flow showed weak but significant lowering to 89, 87.5 and 85% of the control at 10^{-4}, 10^{-3} and 10^{-2} M, respectively, measured 15–20 min after the application (Figure 6, upper diagram; LSD test, $P<0.05$). The evoked flow was not significantly

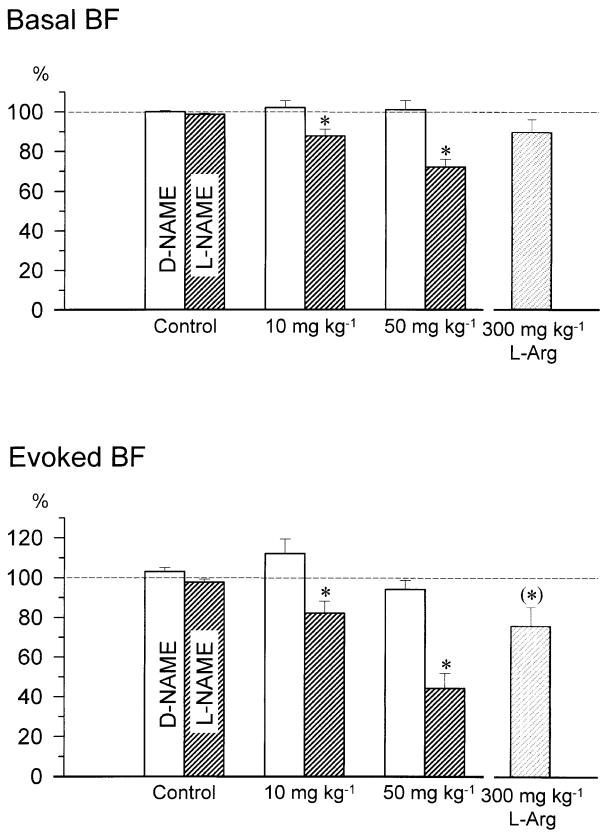

Figure 3 Comparison of mean effects of L-NAME ($n=10$) and D-NAME ($n=8$), systemically administered at two doses, and effect of L-arginine (L-Arg) on basal and evoked blood flow (BF). Control values represent last measurement before, test values fourth measurement after L-/D-NAME (see Figure 2) or second measurement after L-Arg, respectively. Error bars show s.e.mean. ANOVA followed by LSD test between L- and D-NAME (*$P<0.05$) or between L-Arg and L-NAME control (*$P<0.05$).

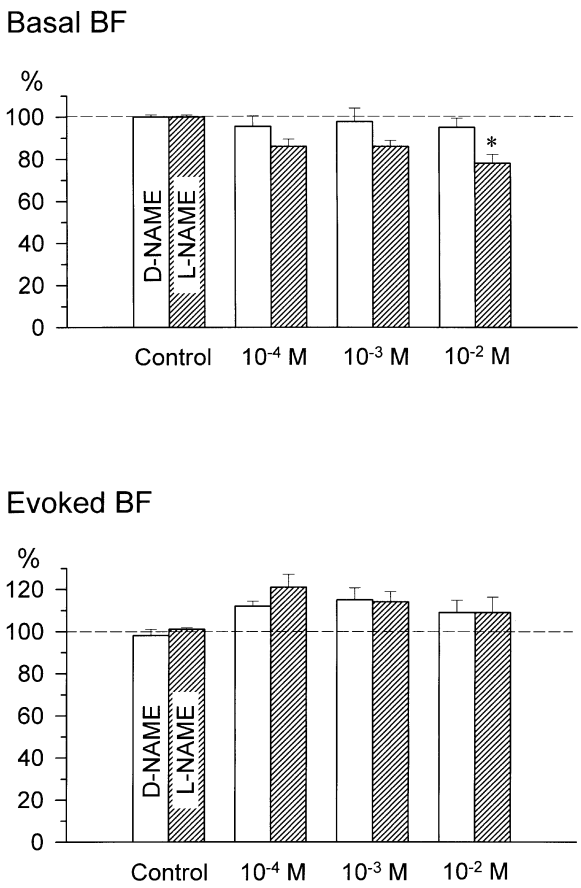

Figure 4 Comparison of mean effects of L-NAME ($n=8$) and D-NAME ($n=7$), locally applied to the dura, on basal and evoked blood flow (BF) at time points as defined in Figure 2. Error bars show s.e.mean. ANOVA followed by LSD test between L- and D-NAME (*$P<0.05$).

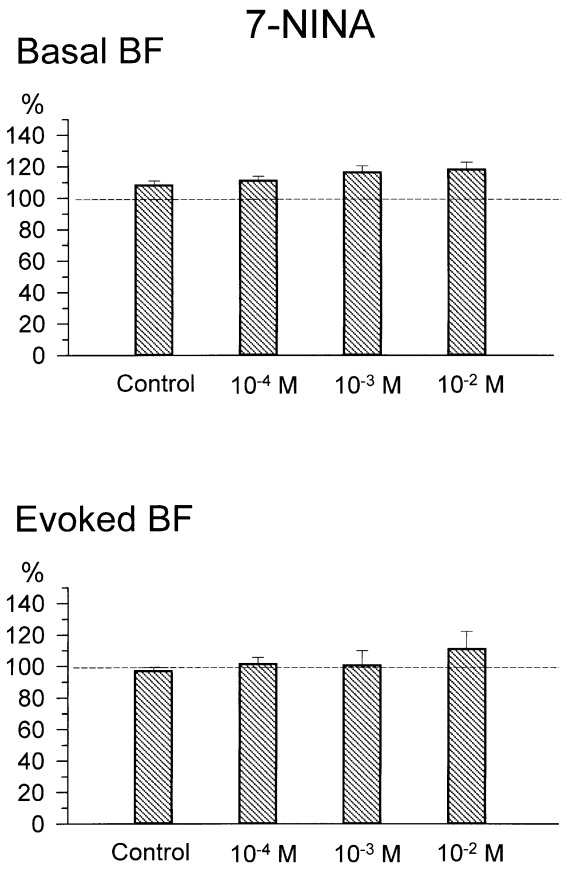

Figure 5 Mean effects of 7-NINA ($n=8$) at three doses, locally applied to the dura, on basal and evoked blood flow (BF). Error bars show s.e.mean. LSD test after ANOVA compares measurement (at time points as defined in Figure 2B) and control values.

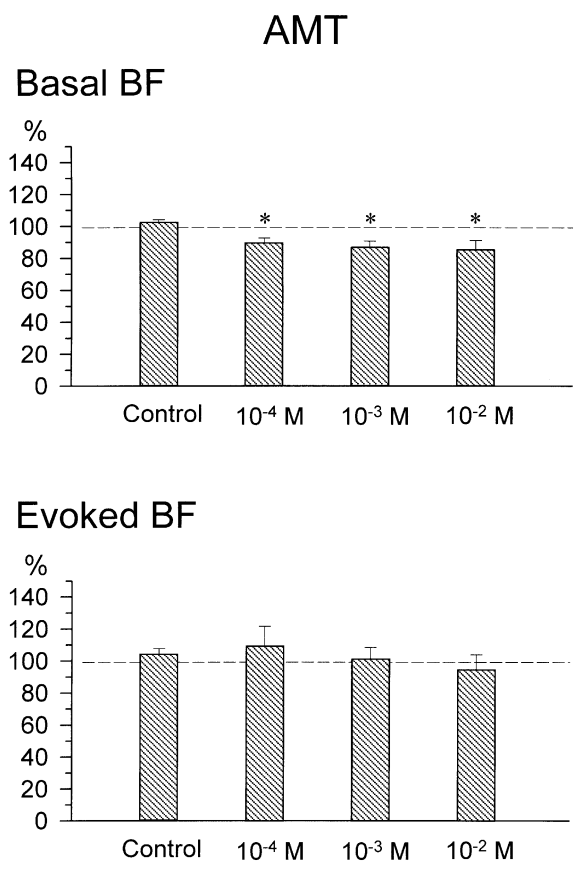

Figure 6 Mean effects of AMT ($n=9$) at three doses, locally applied to the dura, on basal and evoked blood flow (BF). Error bars show s.e.mean. LSD test ($*P<0.05$) after ANOVA comparing test measurements (at time points as defined in Figure 2B) and control values.

changed at any concentration of AMT (Figure 6, lower diagram). The blood pressure remained unchanged in all these experiments.

Discussion

NO causes vascular relaxation significantly contributing to the regular perfusion of intracranial tissues (Iadecola *et al*., 1994) and to the increased perfusion under pathological conditions such as during meningitis (Koedel *et al*., 1995). In the present study we have used laser Doppler flowmetry on dural arterial vessels to measure basal (unstimulated) arterial blood flow and increases in flow evoked by local electrical stimulation. While the basal flow represents the normal perfusion of the tissue, the evoked flow has been shown to depend at least partly on the release of calcitonin gene-related peptide (CGRP) from trigeminal afferents (Kurosawa *et al*., 1995; Messlinger *et al*., 1995). Measurements of the diameter of dural arteries by video microscopy have confirmed these findings that indicate neurogenic vasodilatation (Williamson *et al*., 1997). In the present study we have applied the unspecific inhibitor of the NOS, L-NAME, first at i.v. doses that have been shown to be effective in lowering cerebral blood flow in barbiturate anaesthetized animals (Todd *et al*., 1994) and second, topically at three different concentrations, to study the involvement of NO in basal and stimulated dural arterial flow. Furthermore, we have applied 7-NINA, an inhibitor of the neuronal NOS, and AMT, an inhibitor of the inducible NOS, to identify possible extravascular sources of NO in the dura mater.

Systemic administration of L-NAME and inhibitory effects

Systemic (i.v.) administration of L-NAME decreased both the basal and the evoked arterial blood flow. While these inhibitory effects could partly be reversed by L-arginine, the stereoisomer D-NAME had no effect on the flow. Thus these changes are very likely due to the inhibition of NOS by L-NAME.

The lowering of the basal blood flow after infusion of L-NAME suggests that there is permanent production and tonic release of NO contributing to the vasodilatory state and continuous perfusion of dural arteries. From other (unpublished) experiments we know that the basal flow depends in part on the systemic blood pressure, i.e., rapid changes of the blood pressure are accompanied by respective changes of the flow. Interestingly, in the majority of experiments, shortly after the first injection of L-NAME there was a transient increase of both the basal and the evoked flow in parallel with a rise of the systemic blood pressure. After this short phase, basal and evoked flow were lowered, while the blood pressure remained elevated. This may indicate that the vasoconstrictory effects caused by NOS inhibition were faster in other parts of the body than in dural arterial vessels resulting in rapid blood pressure elevation and the transient flow increase in the dura mater. Injection of L-arginine reversed the effects of L-NAME on the dural arterial flow and also largely normalized the blood pressure.

The electrically evoked increases in flow have been shown to be inhibited by the CGRP antagonist h-$CGRP_{8-37}$ (Kurosawa *et al*., 1995) but not by the NK_1 antagonist RP 67580 (Carmody *et al*., 1996) providing evidence for a neurogenic mechanism that is CGRP- but not substance P-mediated. The inhibitory effect on the evoked flow after i.v. administration of L-NAME suggests that NO is also involved in this neurogenic vasodilatory mechanism. CGRP, a potent vasodilator that stimulates the adenylate cyclase of vascular smooth muscles, is known to cause increased blood flow in the cerebral circulation (Edwards *et al*., 1991; McCulloch *et al*., 1986). Activation of parasympathetic fibres that release vasodilatory mediators such as vasoactive intestinal polypeptide (VIP) may additionally contribute to an increase of intracranial flow upon local electrical stimulation (Zagami *et al*., 1990) but experimental evidence in the dura mater is lacking so far.

The question is how NO can interact with the stimulation-evoked flow, and whether this effect is based on a synergistic mechanism with CGRP. Dilatation of pial arteries of the cat elicited by cortical spreading depression has been suggested to be caused by a synergistic action of CGRP and NO (Wahl *et al*., 1994). This pial arterial dilatation was reduced to 50% after topical administration of either the CGRP antagonist $CGRP_{8-37}$ or the NOS inhibitor N^{ω}-nitro-L-arginine, but by 75% after simultaneous administration of both these compounds. On the other hand, in a recent study in the cat Edvinsson *et al*. (1998) have used $CGRP_{8-37}$ and L-NAME (30 mg kg^{-1}) and found that there is a major influence of CGRP but not a significant contribution of NO production to the trigeminally mediated increase in local cortical blood flow induced by stimulation of the nasociliary nerve.

To explain the interaction of NO with the CGRP-mediated flow increase, there are two possibilities on principle. It seems clear that NO has a direct effect on the smooth muscle cells and may act synergistically to CGRP. A possible mechanism is, for example, that NO maintains a level of pre-dilatation which is necessary for the CGRP mediated vasodilatory effect. Another possibility is that NO is a mediator of the stimulation-evoked release of CGRP. Endothelial NO (see below) may diffuse into the perivascular space and facilitate the release of CGRP from trigeminal afferents. Indeed, there is considerable evidence that NO contributes to the release of CGRP in different tissues including the trigeminovascular system (Holzer *et al*., 1995; for review, Wang & Hakanson, 1995). In the feline cerebral vascularization the vasodilator response of nitroglycerin and sodium nitroprusside has been shown to be depressed on the side of trigeminal ganglionectomy and after application of $CGRP_{8-37}$, whereas the vasodilator response to acetylcholine and adenosine was unaffected (Wei *et al*., 1992). Lowering of the cyclic GMP level in the preparation of Wei *et al*. (1992) decreased both the effects of CGRP and nitroprusside indicating that these mediators are linked in a common second messenger signal pathway.

Local administration of NOS inhibitors and source of nitric oxide

Local administration of L-NAME was not effective in lowering the evoked blood flow, and the basal flow was lowered only at a very high concentration of L-NAME (10^{-2} M). Similarly in the cat, Wahl *et al*. (1994) have found no effect of the NOS inhibitor N^{ω}-nitro-L-arginine, topically administered at a concentration of 10^{-4} M, on the resting diameter of pial arteries exposed in a cranial window. We assume therefore that the major source of NO in the dura (and possible also the pia) is the endothelium of arterial vessels. There is histological evidence for this assumption, since NADPH diaphorase activity is only visible in precapillary sections of the dural vascularization (unpublished results from our laboratories). Because blood flow changes depend on the vasomotor functions of precapillary arterioles (resistance vessels), the endothelium of small precapillary vessels is most likely the crucial target for L-NAME. As there is mainly outward filtration of fluid in precapillary vessels, the penetration of L-NAME from the perivascular tissue into the endothelial cells is probably very limited. This may explain why only the high topical concentration of L-NAME was able to lower the basal flow.

On the other hand, there was also a significant although weak effect of AMT, an inhibitor of the inducible NOS (Nakane *et al*., 1995), on the basal (but not the evoked) blood flow. We have used AMT because NO is known to play an important role in experimental meningitis (Korytko & Boje, 1996), where it may be produced by inflammatory cells or possibly by fibroblasts (Skaper *et al*., 1995). We can actually not exclude that during the preparation, though it was performed with a maximum of care to avoid lesions, and during the ongoing experiment some inflammatory reactions occurred. It seems also possible that NO can be released from dural mast cells, which have been shown to be immunoreactive to antibodies directed against NOS (Berger *et al*., 1994).

Further possible NO producing structures are perivascular nerve fibres, some of which may be immunoreactive to NOS (Berger *et al*., 1994). NOS immunoreactivity has also been found in the trigeminal ganglion of the cat, where it was colocalized with CGRP in some small neurones (Edvinsson *et al*., 1998). With regard to these reports, we have used 7-NINA, a specific inhibitor of the neuronal isoform of the NOS (Silva *et al*., 1995). This NOS inhibitor, locally administered at same concentrations as L-NAME, had no significant effect on the dural arterial flow. Therefore we conclude that perivascular nerve fibres, although they may produce NO under special circumstances, play no significant role in the basal blood flow and the flow increases induced by experimental stimulation of the dura mater.

Relevance of NO and CGRP mechanisms for the pathophysiology of headaches

Intravenous injection of NO liberating agents such as nitroglycerin have been shown to cause headaches in healthy persons (Iversen *et al*., 1989), and migraineurs report migraine-like pains that mimick their spontaneous attacks (Olesen *et al*., 1994). Therefore it has been suggested that NO release followed by meningeal vasodilatation is involved in the pathogenesis of migraine attacks and other severe headaches (Olesen *et al*., 1994). Vasodilatation and increased cerebral blood flow have long been proposed to be involved in migraine (Olesen *et al*., 1990). CGRP has been shown to be released upon experimental trigeminal stimulation and during attacks of migraine and cluster headache (Goadsby & Edvinsson, 1993, 1994; Goadsby *et al*, 1988; 1990). Interestingly, CGRP levels in the blood plasma of patients suffering from episodic cluster headache was also increased during headaches, which were experimentally provoked by nitroglycerin (Fanciullacci *et al*., 1995). Therefore it seems very likely that the NO production and neuropeptide release are functionally linked in severe headaches. Meningeal vasodilatation induced by the synergistic action of NO and CGRP may contribute to the sensitization of perivascular afferents in the dura mater and could constitute a peripheral element of nociception, which together with central processes may lead to attacks of head

pain. A recent study in the rat has shown that vasodilatation of dural vessels caused by i.v. administration of CGRP is accompanied by sensitization of neurons in the spinal trigeminal nucleus with convergent input from the dura mater and the facial skin (Cumberbatch *et al*., 1999). Synergistic mechanisms of neuropeptides and NO may also occur in the central trigeminal system. In the spinal dorsal horn, NO produced from islet cells has been suggested to trigger the release of substance P from primary afferents (Aimar *et al*., 1998). It is likely that there is also central release of CGRP upon afferent activation, and increased levels of neuropeptides may influence neurotransmission in the spinal trigeminal nucleus (Henry *et al*., 1980).

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