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Nitric oxide synthase inhibitors can antagonize neurogenic and calcitonin gene-related peptide induced dilation of dural meningeal vessels

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1 The detailed pathophysiology of migraine is beginning to be understood and is likely to involve activation of trigeminovascular afferents.

2 Clinically effective anti-migraine compounds are believed to have actions that include peripheral inhibition of calcitonin gene-related peptide (CGRP) release from trigeminal neurones, or preventing dural vessel dilation, or both. CGRP antagonists can block both neurogenic and CGRP-induced dural vessel dilation.

3 Nitric oxide (NO) can induce headache in migraine patients and often triggers a delayed migraine. The initial headache is thought to be caused *via* a direct action of the NO–cGMP pathway that causes vasodilation by vascular smooth muscle relaxation, while the delayed headache is likely to be a result of triggering trigeminovascular activation. Nitric oxide synthase (NOS) inhibitors are effective in the treatment of acute migraine.

4 The present studies used intravital microscopy to examine the effects of specific NOS inhibitors on neurogenic dural vasodilation (NDV) and CGRP-induced dilation.

5 The non-specific and neuronal NOS (nNOS) inhibitors were able to partially inhibit NDV, while the non-specific and endothelial NOS (eNOS) inhibitors were able to partially inhibit the CGRP induced dilation.

6 There was no effect of the inducible NOS (iNOS) inhibitor.

7 The data suggest that the delayed headache response triggered by NO donors in humans may be due, in part, to increased nNOS activity in the trigeminal system that causes CGRP release and dural vessel dilation.

8 Further, eNOS activity in the endothelium causes NO production and smooth muscle relaxation by direct activation of the NO–cGMP pathway, and may be involved in the initial headache response.

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Abbreviations: cGMP, cyclic guanosine monophosphate; CGRP, calcitonin gene-related peptide; GTN, glyceryl trinitrate; DPI, diphenyleneiodonium; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NAME, L-nitroarginine methylester; L-NIO, N^5-(Iminoethyl)-L-ornithine; L-NMMA, N^G-methyl-L-arginine; NDV, neurogenic dural vasodilatation; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; SMT, s-methylisothiourea; SMTC, s-methyl-l-thiocitrulline; TNC, trigeminal nucleus caudalis

Introduction

The detailed pathophysiology of migraine is beginning to be understood and is likely to be related to activation of trigeminovascular afferents (Goadsby, 2001). There are sensory fibres within the trigeminal nerve that innervate the cranial blood vessels and contain the vasodilator neuropeptides calcitonin gene-related peptide (CGRP) and substance P (Edvinsson *et al*., 1987; Jansen *et al*., 1991). These trigeminal sensory fibres become active during the migraine and their activation results in the release of CGRP (Gallai *et al*., 1995; Goadsby *et al*., 1990). The increased level of CGRP is reversed with sumatriptan (Goadsby & Edvinsson, 1993), which is an extremely effective treatment for acute migraine (Ferrari *et al*., 2001). The most reliable human experimental model for migraine induction (Edvinsson, 1999) is achieved with administration of nitric oxide (NO) donors to migraineurs (Thomsen *et al*., 1994). The mechanism of this response and its interaction with CGRP-induced changes in the dural circulation are, therefore, of considerable interest.

The successful clinical action of acute specific anti-migraine drugs, ergot derivatives and triptans-5-$HT_{1B/1D}$ receptor agonists, is likely to relate their ability to inhibit release of neuropeptides from trigeminal sensory nerve fibres (Goadsby, 2000). When the dura mater is electrically stimulated in rats it causes the dilation of dural blood vessels (Williamson *et al*., 1997b). This is likely to be caused by CGRP release from trigeminal sensory nerves that innervate the cranial blood vessels since the effect is abolished by the CGRP receptor

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antagonist $hCGRP_{(8-37)}$ (Williamson *et al*., 1997a). Significant attenuation of the neurogenic meningeal vasodilator response is similarly seen with triptans, such as sumatriptan (Williamson *et al*., 1997b) and rizatriptan (Williamson *et al*., 1997c). Intravenous administration of CGRP also causes dural blood vessel dilation that is similarly abolished by the CGRP antagonist $hCGRP_{(8-37)}$, although it was not abolished by sumatriptan, indicating that it is likely the triptans act prejunctionally to prevent CGRP release (Williamson *et al*., 1997a, b).

Nitric oxide (NO) is a potent endogenous vasodilator with an impressive array of biological actions (Moncada *et al*., 1991). NO causes an immediate headache in migraine sufferers and less often in control subjects, and in migraineurs triggers a delayed headache several hours after a NO infusion has ceased that fulfils the International Headache Society criteria (Headache Classification Committee of The International Headache Society, 1988) for migraine (Iversen *et al*., 1989; Olesen *et al*., 1993; 1994). This is also seen when NO is given exogenously (Krabbe & Olesen, 1980; Lassen *et al*., 1995), and is likely to be related to endothelial activation (Jansen Olesen *et al*., 1997). In experimental animals NO is also able to cause meningeal vessel dilation when given intravenously (Akerman *et al*., 2001). Although the immediate headache caused by NO can be attributed to a direct action on blood vessels, and a possible role in the NO–cyclic guanosine monophosphate (NO–cGMP) pathway, a direct action is unlikely to explain the delayed headache response (Olesen *et al*., 1995).

It seems possible that NO might act on the trigeminovascular system, including trigeminal neurons, to trigger the delayed headache response. Glyceryl trinitrate (GTN) when infused through the intra-carotid artery was able to sensitize and increase the discharge rate of trigeminovascular neurons which have received inputs from the superior sagittal sinus immediately and also beyond the time of infusion of GTN (Lambert *et al*., 2000). Fos expression in the trigeminal nucleus caudalis is affected by NO (Hoskin *et al*., 1999; Jones *et al*., 2001; Tassorelli *et al*., 1997; 2000; Tassorelli & Joseph, 1995). It would seem likely that the activation of the trigeminovascular system by NO might involve an interaction with calcitonin gene-related peptide (CGRP) to cause the dural blood vessel dilation during migraine. There is evidence that a relationship between NO and CGRP does exist. Indeed CGRP and NOS seem to coexist in trigeminal ganglion cells, although it was found that L-nitroarginine methylester (L-NAME), a nitric oxide synthase inhibitor (NOS) had no effect on cortical blood flow following nasociliary nerve stimulation while the $hCGRP_{8-37}$ reduced the blood flow (Edvinsson *et al*., 1998).

The effectiveness of NOS inhibitors as a potential migraine treatment has been examined in a single small study. The authors demonstrated, using a non-specific NOS N^G-methyl-L-arginine (L-NMMA), that it was possible to provide relief to patients experiencing headache (Lassen *et al*., 1997). A side effect of using a non-specific NOS inhibitor were the changes in blood pressure and heart rate over time that would limit the usefulness of this compound in the clinic. It is remarkable that the same NOS inhibitor could reduce pain in patients with Chronic Tension-Type Headache (Ashina *et al*., 1999).

In this series of experiments we sought first, to determine if a non-specific NOS inhibitor was able to interact with CGRP in the trigeminovascular system by observing its effects on both the neurogenically mediated dural blood vessel dilation and also on CGRP-induced dilation. Secondly, we administered specific inhibitors to endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) to determine which is likely to be involved in trigeminovascular transmission, and therefore be a potential therapeutic target.

Methods

Surgical preparation

All experiments were conducted under UK Home Office Animals (Scientific Procedures) Act (1986). Male Sprague-Dawley rats (300–400 g) were anaesthetized throughout the experiments with sodium pentobarbitone (60 mg kg^{-1} i.p. and then 18 mg kg^{-1} hr-i.v. infusion). The left femoral artery and vein were cannulated for blood pressure recording and intravenous infusion of anaesthetic, respectively. Temperature was maintained throughout using a homeothermic blanket system. The rats were placed in a stereotaxic frame, the skull exposed and the right parietal bone thinned by drilling with a saline-cooled drill until the blood vessels of the dura were clearly visible through the intact skull.

Intravital microscopy

The cranial window was covered with mineral oil (37°C) and a branch of the middle meningeal artery viewed using an intravital microscope (Microvision MV2100, U.K.) and the image displayed on a television monitor (Williamson *et al*., 1997b). Dural blood vessel diameter was continuously measured using a video dimension analyser (Living Systems Instrumentation, U.S.A.) and displayed with blood pressure on a chart recorder and a data analysis system (MI^2, Modular Instruments, U.K. and Cambridge Electronic Design, spike 2 software).

Experimental protocols

Defining electrical stimulation parameters In the preparations where electrical stimulation was used to evoke dilation of the dural blood vessels a bipolar stimulating electrode (NE 200X, Clark Electromedical) was placed on the surface of the cranial window approximately 200 μm from the vessel of interest. The surface of the cranial window was stimulated at 5 Hz, 1 ms for 10 s (Grass Stimulator S88, Grass Instruments) with increasing voltage until maximal dilation was observed. Subsequent electrically induced responses in the same animal were then evoked using that voltage.

Control group

In a separate set of experiments we tested the reproducibility of the neurogenic ($n=7$) and CGRP ($n=6$) vasodilator responses to four consecutive stimuli in order to test whether there was any systematic effect over time.

CGRP induced dilation In the preparations where CGRP was used to dilate dural blood vessels, CGRP was given as an

intravenous bolus of 1 μg kg^{-1}. This has been shown to produce a maximal dilation (Williamson *et al*., 1997a).

Effects of nitric oxide synthase inhibitors on electrically and CGRP induced dilation The effects of the non-selective NOS inhibitor, (L-NAME, 40 mg kg^{-1} over the time-course of 15 min) was studied. L-NAME was administered intravenously 10 min after the control response to electrical stimulation or bolus CGRP, the electrical stimulation or CGRP bolus was then repeated after a further 15 min.

This protocol was also used for the eNOS inhibitor, N^5-(Iminoethyl)-L-ornithine (L-NIO, 5 mg kg^{-1} given as a bolus). The effects of the eNOS inhibitor diphenyleneiodonium chloride (DPI) were similarly studied. DPI (0.1 mg kg^{-1}) was administered intravenously 10 min after control response to electrical simulation or bolus CGRP; this was followed 15 min later by a repeat electrical stimulation or CGRP bolus and then another 10 min later by an increased dosage of DPI (0.3 mg kg^{-1}) followed by another electrical stimulation or CGRP bolus after a further 15 min. Finally DPI (1.0 mg kg^{-1}) was given after another 10 min and then electrical stimulation or bolus CGRP repeated after 15 min.

This protocol was also used for the nNOS inhibitor s-methyl-l-thiocitrulline (SMTC; 1, 3 and 10 mg kg^{-1}) and the iNOS inhibitor s-methylisothiourea hemisulphate (SMT; 3 and 10 mg kg^{-1}). Up to three doses were used in the SMTC protocol in one animal for a cumulative dose-response curve; SMT always had the two doses given.

Data analysis Dural vessel diameter was measured in arbitrary units, since calibration varies with vessel selected and the magnification used for each experiment that seeks to optimise the image–analysis device. The effects of electrical stimulation and CGRP on dural vessel diameter were calculated as a percentage increase from the pre-stimulation baseline diameter. Data are presented as mean$\pm$s.e.mean. The dose response relationship was determined for each compound. An ANOVA with repeated measures (SPSS v10.0) followed by paired *T*-test was used to examine the effect of test compounds at the various doses over time, with a factor for the control and treated animals as part of the design. Single dose control versus post-dose effects were tested with a paired *T*-test. Significance assessed at the $P<0.05$ level.

Drugs

L-nitroarginine methyl ester, s-methylisothiorea (both Sigma-Aldrich, U.K.), s-methyl-l-thiocitrulline (Bachem, U.K.) and N^5-(Iminoethyl)-L-ornithine (Tocris Cookson, U.K.) were all dissolved in 0.9% saline. Diphenyleneiodonium chloride (Tocris Cookson, U.K.) was dissolved in 5% glucose solution. Calcitonin gene related peptide (Sigma-Aldrich, U.K.) was dissolved in deoxygenated water, aliquotted and frozen until required.

Results

Control group

In control animals consecutive stimuli were carried out with intervals comparable to those used in the pharmacological studies. The mean dilations were 136$\pm$9%, 136$\pm$9%, 130$\pm$11% and 137$\pm$8%, respectively, after electrical stimulation ($n=7$; $F_{3,18}=0.61$, $P=0.62$). The mean dilations after CGRP were 111$\pm$15%, 108$\pm$38%, 106$\pm$38%, and 99$\pm$30%, respectively ($n=6$; $F_{3,15}=2.4$, $P=0.11$). Over the time course of the study there was no effect of time on the responses to either electrical stimulation or CGRP.

Effect of a non-selective NOS inhibitor on electrical and CGRP induced dilation

In rats treated with L-NAME (40 mg kg^{-1} over 15 min, $n=6$) the response to electrical stimulation of the cranial window was significantly inhibited from 138.0$\pm$18% to 85.6$\pm$20% ($P<0.05$). Increases in dural blood vessel diameter evoked by rat CGRP (1 μg kg^{-1}, i.v.) were significantly inhibited from 114.2$\pm$9.6% to 75.2$\pm$9% ($n=7$, $P<0.05$) with pre-treatment with L-NAME (Figure 1). L-NAME caused an increase in blood pressure of 43.4$\pm$4 mm Hg that was accompanied by a drop in vessel diameter of 31.33$\pm$4% which returned to its pre-injection level before the electrical stimulation of CGRP injection was repeated.

Effect of eNOS inhibitors on electrical and CGRP induced dilation

In rats treated with DPI (0.1, 0.3 and 1.0 mg kg^{-1}, $n=6$) and L-NIO (5 mg kg^{-1}, $n=3$) there was no change in diameter from 110.7$\pm$17% to 108.4$\pm$15%, 123.5$\pm$21% and 120.1$\pm$21% (DPI, 0.1, 0.3 and 1.0 mg kg^{-1}, respectively, $F_{3,15}=0.92$, $P>0.05$) and 114.7$\pm$9% to 128.3$\pm$11% (L-NIO,

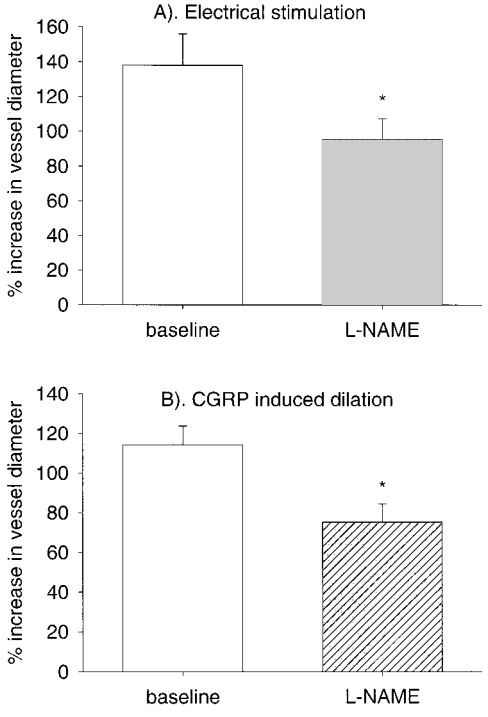

Figure 1 Effects of L-NAME on (A) electrical stimulation and (B) CGRP induced dilation. Following control responses rats were injected with L-NAME and then electrical stimulation (50–300 μA) or CGRP (1 μg kg^{-1}) injection repeated. *$P<0.05$.

$P>0.05$) with electrical stimulation of the cranial window. Increases in dural blood vessel diameter evoked by rat CGRP (1 μg kg^{-1}, i.v.) were inhibited from 90.0±7% to 84.0±7%, 85.7±9% and 66.5±7% (DPI, 0.1, 0.3 and 1.0 mg kg^{-1}, respectively, $n=6$, $F_{3,15}=3.7$, $P<0.05$) and 124.7±24 to 85.9±20% (L-NIO, 5 mg kg^{-1}, $n=6$). The 1.0 mg kg^{-1} dosage of DPI and L-NIO dosage represent significant reductions ($P<0.05$; Figure 2). DPI had no effect on arterial blood pressure until the highest dose where it caused an increase of 34.5±6.8 mm Hg, this increase led to a 24.4±7% drop in vessel diameter that was restored to pre-injection level by the time electrical stimulation or CGRP injection was repeated. L-NIO caused a 4.5±10 mm Hg increase in blood pressure and a 10.7±5% drop in vessel diameter that were restored to pre-injection levels by the time CGRP or electrical stimulation was repeated.

Effect of an nNOS inhibitor on electrical and CGRP induced dilation

In rats treated with SMTC (1 mg kg^{-1}, $n=6$, 3 mg kg^{-1}, $n=7$ and 10 mg kg^{-1}, $n=5$) the response to electrical stimulation of the cranial window was reduced from 130.8±13% to 112.9±23% (1 mg kg^{-1}), 125.9±13% to 74.0±20% (3 mg kg^{-1}) and 120.2±12% to 60.1±7% (10 mg kg^{-1}, $P<0.05$). These values represent a dose dependent reduction from the baseline response with the 10 mg kg^{-1} dose being significant. Increases in dural blood vessel diameter evoked by rat CGRP (1 μg kg^{-1}, i.v.) were unchanged from 84.4±5% to 87.5±5%, 75.0±13% and 63.3±10% (1, 3 and 10 mg kg^{-1}, $n=7$, respectively). Although there is a dose dependent reduction in vessel diameter this is not significant (Figure 3). SMTC caused increases in blood pressure of 20.7±2% (1 mg kg^{-1}), 36.3±5% (3 mg kg^{-1}) and 35.2±4% (10 mg kg^{-1}). This blood pressure increase caused a drop in blood vessel diameter of 26.3±5 mm Hg (1 mg kg^{-1}), 23.8±4% (10 mg kg^{-1}) and 26.9±6% (3 mg kg^{-1}) that was restored to pre-injection levels by the time electrical stimulation or CGRP injection was repeated.

Effect of an iNOS inhibitor on electrical and CGRP induced dilation

In rats treated with SMT (3 and 10 mg kg^{-1}, $n=6$) the response to electrical stimulation of the cranial window showed no changes from baseline 106.8±10% to 113.4±7% and 114.1±12% (3 and 10 mg kg^{-1}, respectively). Increases in dural blood vessel diameter evoked by rat CGRP (1 μg kg^{-1}, i.v.) also produced no change from baseline 112.2±15% to 103.3±12% and 100.2±20% (3 and 10 mg kg^{-1}, respectively, $n=8$; Figure 4). SMT caused an increase in arterial blood pressure of 25.9±2 mm Hg which resulted in a drop in vessel diameter of 23.2±4%, that was restored to pre-injection level by the time electrical stimulation or CGRP injection was repeated.

Discussion

Nitric oxide synthase is a potential target for migraine treatment. The present series of experiments demonstrate that

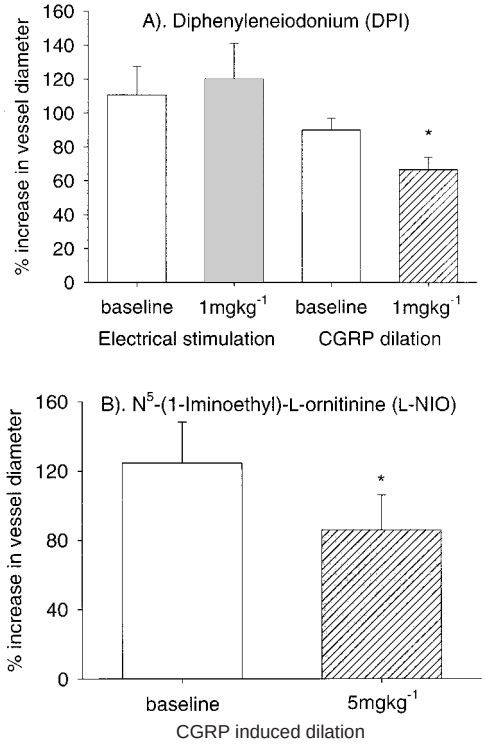

Figure 2 Effects of (A) DPI on electrical stimulation and CGRP induced dilation and (B) L-NIO on CGRP induced dilation. Following control responses rats were injected with DPI or L-NIO and then electrical stimulation (50–300 μA) or CGRP (1 μg kg^{-1}) injection repeated. *$P<0.05$.

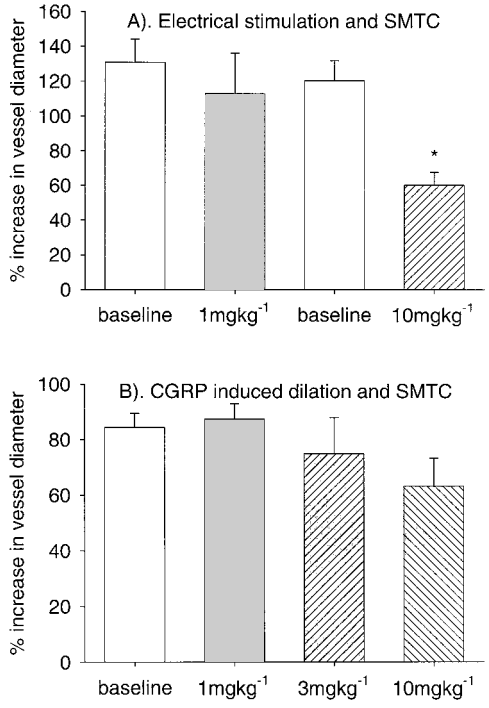

Figure 3 Effects of SMTC on (A) electrical stimulation and (B) CGRP induced dilation. Following control responses rats were injected with SMTC and then electrical stimulation (50–300 μA) or CGRP (1 μg kg^{-1}) injection repeated. *$P<0.05$.

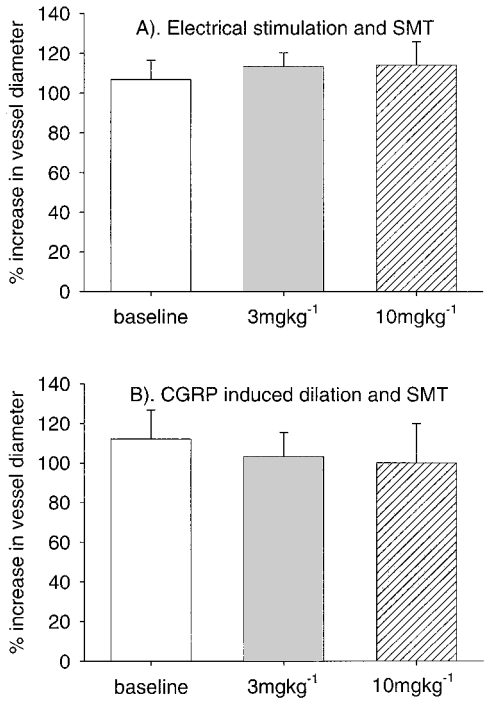

Figure 4 Effects of SMT on (A) electrical stimulation and (B) CGRP induced dilation. Following control responses rats were injected with SMTC and then electrical stimulation (50–300 μA) or CGRP (1 μg kg^{-1}) injection repeated. *$P<0.05$.

NOS inhibitors have antagonist actions within the trigeminovascular systems acting peripherally to inhibit neurogenic dural vasodilation (NDV) and also at the endothelial level to inhibit CGRP-induced dilation. Specifically, the data suggests that the nNOS and eNOS isoforms are particularly important in these dural vessel vasodilator responses. Given that none of the NOS inhibitors blocks NDV completely, it is appropriate to consider NO generation as an important, but not exclusive, mediator of the response.

The present study demonstrates that L-NAME, the non-selective NOS inhibitor, and SMTC, the more specific nNOS inhibitor, but not the eNOS and iNOS selective inhibitors antagonised NDV. This would suggest that there is NOS activity in the trigeminovascular system, including afferents up to dural sites. There is previous evidence that NO may be acting in the trigeminovascular system. It has been found that GTN induces activation of *c-fos* immunoreactivity in the trigeminal nucleus caudalis (TNC) (Tassorelli & Joseph, 1995). Furthermore, the expression of *c-fos* in the TNC of the cat, elicited by superior sagittal sinus stimulation, is reduced by L-NAME (Hoskin *et al*., 1999). The present study suggests that the effects of NO on NDV may be mediated via NO production from nNOS, as the nNOS inhibitor was able to inhibit NDV. This has been suggested previously with the nNOS inhibitor 7-nitroindazole being able to reduce *c-fos* levels in the TNC after GTN was given (Tassorelli *et al*., 1997).

Although SMTC is a compound that is highly selective at the nNOS site it does also have some affinity at the eNOS site, as does L-NAME. It seems unlikely that the actions of these compounds were at the eNOS site however, as the two highly specific eNOS inhibitors DPI and L-NIO were unable to prevent any inhibition when given as pre-treatment alone before neurogenic stimulation. Given the lack of effect of the iNOS inhibitor s-methylisothiorea hemisulphate (SMT), the iNOS site is unlikely to play a major role in the NDV response.

There are several possible explanations for the mechanism by which NOS inhibitors are able to inhibit NDV. It is possible that NOS activity blocks NO production which in turn prevents the NO activation of trigeminal sensory fibres thus preventing CGRP release when they are neurogenically activated. This would indicate an indirect action of NO to cause dilation. In cats, following unilateral trigeminal ganglionectomy, the cerebral vasodilatory response to NO was depressed on the denervated side, while on the innervated side $hCGRP_{8-37}$ reduced the response to NO (Wei *et al*., 1992). This suggests an interaction between NO and CGRP in trigeminal sensory fibres that cause vasodilation. Wei *et al*. (1992) suggested that NO acts on sensory fibres to release CGRP that in turn diffuses to smooth muscle and causes vasodilation. This may in part explain the inhibition, but it is likely that this would produce a more complete inhibition of vasodilation and only partial effect was found. Another possible explanation is that both NO and CGRP are released with trigeminal fibre activation. The presence of nNOS in neurons in the trigeminal ganglion as well as those co-localised with CGRP has been demonstrated (Edvinsson *et al*., 1998). In this situation nNOS inhibition would arrest some part but not all of the vasodilation.

When CGRP was used to induce dural vessel dilation, L-NAME (pan-NOS inhibitor), DPI and L-NIO (eNOS) were all capable of inhibiting the dilation to some degree. The SMTC (nNOS) inhibition was not significant, while SMT (iNOS) was unable to inhibit the response. Taken together the data indicate that eNOS is responsible for CGRP-induced dilation, while nNOS activity is likely to take place in the trigeminovascular system, or when there is vasoneuronal activation as is seen in cortical spreading depression (Wahl *et al*., 1994). It is probable that eNOS activity results in direct action on the smooth muscle to cause dilation. It is likely that CGRP receptors present on the endothelium cause NO production by activation of eNOS, NO diffuses into vascular smooth muscle where it activates guanylate cyclase, resulting in the formation of cyclic guanosine monophosphate (cGMP). This leads to a decrease in intracellular Ca^{2+} that causes a relaxing of the smooth muscle and dilation of the blood vessels (Moncada *et al*., 1991) and may be the basis for the initial headache (Thomsen & Olesen, 1997). Indeed NOS inhibitors have been found to attenuate CGRP endothelium dependent relaxation of smooth muscle by restricting the eNOS activity in the endothelium (Gray & Marshall, 1992a, b).

One of the remarkable effects of administration of an NO donor is the delayed headache induction, which has very many features of migraine and occurs several hours later (Iversen *et al*., 1989; Olesen *et al*., 1993; 1994). An immediate direct action via the NO-cGMP pathway cannot easily explain the delayed headache response. It is possible that this takes place in the trigeminovascular system with increased activity of nNOS, NO production and consequent activation of trigeminal fibres. Fos expression has been found to be bi-phasic in its distribution in the brain when induced by NO administration (Tassorelli *et al*., 2000). It is maximally

expressed after 2 h in centres that control blood pressure while in nuclei involved in nociception, such as trigeminal nucleus caudalis, or nociceptive modulation, such as periaqueductal grey (Knight & Goadsby, 2001), it is maximally expressed after 4 h (Tassorelli *et al*., 2000). NO may then trigger a very complex series of events in the brain that leads in susceptible individuals to acute migraine.

In summary, our data suggest that NO and CGRP interact synergistically and reciprocally in different parts of the central nervous system to cause dilation of the meningeal blood vessels. The iNOS inhibitor had little effect on either form of dural vessel dilation, therefore it seems unlikely that NO production *via* the macrophage is important for either the neurogenic vasodilator response or the CGRP-mediated response. At the level of the blood vessels themselves CGRP appears to activate endothelial NO synthase to cause NO production and thus relaxation of blood vessel smooth muscle with attendant dilation. At the level of the trigeminal system nNOS appears to coordinate NO production to activate CGRP release from trigeminal fibres and trigger vasodilation. Furthermore, there may be a degree of co-localization of NO and CGRP that contributes to the vasodilation, although if this were the case one would expect that when the CGRP receptor blocker $hCGRP_{8-37}$ was given as a pre-treatment to neurogenic stimulation the response would be incompletely abolished whereas it was completely abolished (Williamson *et al*., 1997a). Therefore it seems likely that NO may activate some CGRP release from trigeminal fibres, but also that CGRP is released from trigeminal fibres by neurogenic stimulation alone.

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