Modulation of Dural Nociceptor Mechano-sensitivity by the Nitric Oxide-Cyclic GMP Signaling Cascade

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INTRODUCTION

Nitric oxide (NO) is a gaseous signaling molecule that exerts modulatory effects on a wide range of neural functions, acting in part through the activation of soluble guanylyl cyclase (GC) and the subsequent synthesis of cyclic guanosine monophosphate (cGMP) (Ahern et al. 2002). Numerous studies have found evidence that NO plays a critical role in the modulation of nociception and the development of states of pain hypersensitivity, partly through actions on the peripheral nervous system (see following text). However, these studies suggest a complex role for NO in that both pro- and anti-nociceptive effects have been found.

A pro-nociceptive role for NO in the periphery is supported by a number of findings. For example, in humans, NO elevates pain when administered into paravascular tissue or isolated cutaneous veins (Holthusen and Arndt 1995), and the NO-cGMP cascade mediates the inflammatory pain induced by bradykinin (Holthusen 1997). Likewise, in animals local NO elaboration plays a role in the development and maintenance of neuropathic pain (Levy et al. 2000, 2001) and the NO-cGMP cascade contributes to pain hypersensitivity induced by local administration of the inflammatory mediator prostaglandin E2 (PGE2) (Aley et al. 1998).

In contrast, other studies provide evidence that the NO-cGMP cascade exerts peripheral anti-nociceptive effects. These include inhibition of the inflammatory pain induced by PGE2 (Durate et al. 1990) and mediation of the anti-nociceptive actions of a number of peripherally acting analgesic agents such as morphine and nonsteroidal anti-inflammatory drugs (NSAIDs) (Ferreira et al. 1991; Tonussi and Ferreira 1994). Further adding to this inconsistency are some additional studies suggesting that NO can exert either pro- or anti-nociceptive effects in the same experimental model depending on its local concentration (Kawabata et al. 1994; Prado et al. 2002).

This discrepancy in the peripheral effects of the NO-cGMP cascade raises the question of what actions it has on the primary afferent nociceptive neurons, which presumably mediate the behavioral nociceptive effects. A small number of electrophysiological studies have also yielded mixed results. These studies showed that inhibition of the NO producing enzyme, nitric oxide synthase, can either oppose PGE2-induced sensitization of cutaneous C fibers (Chen and Levine 1999) or enhance C-fiber responsiveness to bradykinin (Kelly et al. 2001) and that cGMP itself is incapable of modulating responses of cutaneous C-fibers (Kress et al. 1996).

To further address the role of the NO-cGMP signaling cascade in peripheral nociception, we examined its direct effect on the spontaneous activity and mechano-sensitivity of both C- and A-delta nociceptive neurons that innervate the intracranial dura. Activation and sensitization of these nociceptors has been suggested to play a key role in mediating the head pain of migraine and other clinically occurring vascular headaches (Strassman et al. 1996). Current theories of migraine have proposed a critical role for the NO-cGMP cascade, based in part on the headache-generating properties of agents that produce NO or increase cGMP (Iversen et al. 1989; Kruuse et al. 2003). Although NO has been suggested to play a role in promoting headache by directly acting on meningeal nociceptors (Ashina et al. 2000; Knyihar-Csillik et al. 2001; Kruuse et al. 2003), its effect on the activity and mechano-sensitivity of these neurons has not been examined.
METHODS

Surgery and electrophysiological recording

Experiments were carried out on adult Sprague-Dawley male rats (300–400 g). The experimental protocol was approved by the institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Rats were anesthetized with urethani (2.0 g/kg ip) and were maintained under anesthesia throughout the experiment with supplemental injections as needed to suppress blink and hind paw nociceptive reflexes. At the end of the experiments, the animals were killed with an intravenous bolus injection of 1 M KCl.

The method for recording activity of dural nociceptors has been described previously (Levy and Strassman 2002a). Briefly, anesthetized rats were placed in a stereotaxic head-holder, and a craniotomy was used to expose the left transverse sinus. The exposed dura was bathed with a modified synthetic interstitial fluid (SIF, pH 7.2) containing (in mM) 135 NaCl, 5 KCl, 1 MgCl2, 5 CaCl2, 10 glucose, and 10 HEPES. A tungsten microelectrode was advanced through a second craniotomy into the left trigeminal ganglion. Dural nociceptors in the ganglion were identified by their constant latency response to single-shock stimulation of the dura overlying the ipsilateral transverse sinus of 12.5 mm. Neurons were classified as described previously (Strassman and Raymond 1999), and were maintained under anesthesia throughout the experiment with 1%–1.5% isoflurane (0.03–0.05 mls, 50 mA, 0.5 Hz). The shortest latency site was described previously (Strassman and Raymond 1999), and the response latency at this site was used to calculate conduction velocity. After the experiment, the animals were killed with an intravenous bolus injection of 1 M KCl.

Mechanical receptive fields of dural nociceptors were identified initially by stroking the dura with blunt forceps. The site of lowest mechanical threshold was determined using a calibrated set of von Frey monofilaments (0.03–6.9 g exerting 38–510 kPa, Stoelting). For quantitative determination of mechanical stimulus-response functions, graded stimuli were applied to the dura at the lowest threshold site with a servo force-controlled mechanical stimulator (Series 300B Dual Mode Servo System, Aurora Scientific, Aurora, Ontario, Canada) (Levy and Strassman 2002b). A flat-ended cylindrical plastic probe was attached to the tip of the stimulator arm. One of three probe diameters (0.5, 0.8, or 1.1 mm) was selected for each neuron, depending on the sensitivity of the neuron. The smallest probe was used unless the baseline threshold of the neuron was so low that responses were evoked even at the minimum setting of the stimulator (2 mN). In such cases, one of the larger probes was used (resulting in lower stimulus pressures). Stimulus intensity is reported in units of pressure or force per area (kPa, where 1 kPa = 1 mN/mm2). Only one probe was used for each neuron.

Experimental protocols

Stimulus trials for testing mechanically sensitive units consisted of a graded series of three square-wave stimuli (100-ns rise time, 2-s width, 10-s inter-stimulus interval) delivered in ascending order, which included a threshold and two suprathreshold stimuli. Stimuli that evoked one to two spikes (0.5–1 Hz) were considered as threshold. Suprathreshold stimuli were usually two and four times greater than threshold. Stimulus trials were delivered repeatedly at a constant interval of 5 or 10 min throughout the experiment to minimize the effect of desensitization (Peng et al. 2003). The intertrial interval was not changed once baseline testing started. A 30-s interval preceding the threshold stimulus was used for measurement of baseline spontaneaous activity. The response to each mechanical stimulus was calculated by subtracting the spontaneous firing rate from the mean firing rate during the stimulus.

In all experimental protocols, baseline measurements of spontaneous and mechanically evoked activity were obtained prior to drug administration. Only units that exhibited consistent responses at all stimulus intensities in at least three consecutive baseline trials were tested further. These trials also served as vehicle controls because the receptive field was bathed in SIF, which was the vehicle for all drugs, except 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ), which was first dissolved in DMSO and then SIF (final concentration of DMSO: 0.1%). All chemical agents were delivered topically to the dural receptive field using a small piece of cotton soaked with ~40 μl of the tested agent. In the first paradigm to examine the role of NO, sodium nitroprusside, (SNP, Sigma, 0.01–1 mM) (Ahern et al. 2000; Kim et al. 2000) was applied for 10 min followed by a wash period with SIF (see Figs. 1A and 2A). Because SNP is light-sensitive, all test solutions containing SNP were prepared immediately before use and protected from light with aluminum foil.

To examine whether cGMP can mimic the effect of SNP, we also treated dural nociceptors similarly with the stable membrane-permeable analogue of cGMP, 8-para-phenylendioxy cyclic GMP (8-pCPT-cGMP, Sigma; see Fig. 3A). To test further whether NO mediates its effects through the cGMP-protein cyclase (GC) cascade, a separate experimental protocol was used for testing the ability of either the GC inhibitor ODQ (Sigma) or the cGMP blocker Rp-8-pCPT-cGMP (RcpGMP, Sigma) to block the effect of SNP. Effective doses of 8-pCPTcGMP, ODQ, and RcpGMP were derived from previous studies (Klyachko et al. 2001; Kramer and Tibbs 1996).

Figure 4A demonstrates the sequence of drug applications in this protocol: baseline with SIF, inhibitor alone, inhibitor in combination.
with SNP, SNP alone, and wash with SIF. The alternative protocol of applying the inhibitor after the SNP was not used because there was too much variability in the time course and duration of SNP effects in different neurons.

Data analysis

Data are presented as means ± SE. A neuron was deemed affected only if the two following criteria were fulfilled: drug-induced changes of threshold or suprathreshold responses were changed by ≥0.5 and 2.5 Hz, respectively (these values represent 2 SD of the mean response of 15 units tested for 3-4 trials while their receptive fields were bathed in SIF), and drug-induced changes in responses to mechanical stimulation showed a complete or partial recovery during the washout period. Differences between affected groups were examined using unpaired Student’s t-test. The effect of GC/cGMP blockers on NO-induced changes in mechanosensitivity was analyzed using repeated-measures ANOVA followed by a Fisher’s PLSD post hoc test. The χ² test was used to compare the proportion of neurons that were affected by different SNP concentrations and by the presence or absence of the GC/cGMP antagonist.

RESULTS

NO-induced modulation of dural nociceptor mechanosensitivity

To examine whether NO modulates their mechanical stimulus response properties, 19 mechanosensitive dural nociceptors were tested for the effect of dural application of SNP (10⁻³ M) on the neurons’ responses to graded mechanical stimulation of their dural receptive fields. The sample included 10 C units (0.3–1.45 m/s, mean: 0.83 ± 0.13 m/s), 8 slow A units (1.9–4.0 m/s, mean: 2.7 ± 0.25 m/s), and 1 fast A unit (CV: 5.2 m/s). Ten minutes after exposure to SNP (Figs. 1A and 2A), responses to mechanical stimuli were enhanced (i.e., sensitized) in 7 of 19 neurons (3 slow A units and 4 C units) and inhibited in 7 neurons (4 slow A units and 3 C units). The remaining five neurons (1 fast A unit, 3 slow A units, and 1 C unit) were not affected. SNP-induced sensitization affected threshold responses in five of seven neurons and suprathreshold responses in seven of seven neurons (Fig. 1B). SNP-induced inhibition had a similar pattern with both threshold and suprathreshold responses affected in seven of seven neurons (Fig. 2B). Both the sensitizing and inhibitory effects of SNP usually disappeared 10–20 min later during the washout period with SIF.

There was no statistically significant difference in CV between sensitized (1.7 ± 0.44 m/s) and inhibited neurons (2.0 ± 0.39 m/s). Mechanical activation thresholds (at baseline, prior to SNP administration) of sensitized neurons (27.1 ± 5.6 kPa) were, however, significantly higher (P = 0.03 unpaired Student’s t-test) than those of neurons in which SNP produced inhibition (11.6 ± 2.9 kPa). There was also a trend toward a difference in the level of baseline spontaneous activity between sensitized (0.17 ± 0.92 spikes/s) and inhibited neurons (1.4 ± 0.59 spikes/s). This difference, however, did not reach statistical significance (P = 0.06, unpaired Student’s t-test). SNP itself, however, did not change the level of spontaneous activity, regardless of its effect on the mechanosensitivity of the neurons.

Next we examined whether the effects of SNP are dose dependent by administering lower doses of SNP (10⁻⁴ M, n = 15 and 10⁻⁵ M, n = 8) and recording changes in threshold and suprathreshold responses. Because induction of mechanical hyperalgesia or analgesia has been suggested to be the result of different concentrations of NO (Kawabata et al. 1994; Prado et al. 2002), these experiments also served to examine whether SNP-induced sensitizing or inhibitory effects on mechanosensitive dural nociceptors might be related to its dose. At the lowest concentration tested (10⁻⁵ M), SNP generally failed to produce an effect on dural nociceptors; it had a sensitizing effect on the threshold response only in one of eight neurons, and no effect on suprathreshold responses. At 10⁻⁴ M, SNP induced sensitization of the threshold response in 5 of 15 neurons and inhibition in 3/15. The incidence of changes in threshold responses, and the proportion of neurons sensitized and inhibited, was not significantly different from that found with the higher dose of SNP (P > 0.3, χ², SNP 10⁻³ M vs. 10⁻⁴ M). However, the incidence of changes in suprathreshold responses induced by 10⁻⁴ M was significantly lower, as only 1 of 15 neurons was sensitized and 2 of 15 neurons were inhibited (P = 0.0019, χ², SNP 10⁻³ M vs. 10⁻⁴ M). These findings suggest that the effects of SNP are dose dependent and there is no correlation between the dose of SNP used and the induction of sensitizing versus inhibitory effects.

Role of cGMP in NO-induced modulation of mechanosensitivity

NO-mediated increases and decreases in mechanical pain thresholds in animal models of hyperalgesia are both thought to be produced, in part, by activation of guanylyl cyclase, result-

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ing in the production of cGMP (Aley et al. 1998; Duarte et al. 1990, 1992; Granados-Soto et al. 1997; Vivancos et al. 2003). To examine the role of cGMP in the modulation of dural nociceptor mechanosensitivity, we tested the effect of the membrane-permeable cGMP analogue, 8-pCPT-cGMP (10^{-3} M), on the threshold and suprathreshold mechanical responses of 12 dural nociceptors neurons (Fig. 3, A and B). We detected significant inhibition of threshold responses in 8 of 12 neurons and of the suprathreshold responses in 9 of 12 neurons. These responses resembled the inhibitory effect of SNP (Fig. 4, D and E). None of the neurons tested with the cGMP analogue showed sensitization.

To further examine the role of cGMP-GC cascade, using the second protocol (Fig. 4A), the GC or cGMP blocker was first applied alone, followed by the blocker in the presence of SNP, followed by SNP alone. A total of 22 neurons were tested with this protocol, using either ODQ (0.1 mM, n = 13) or RpcGMP (1 mM, n = 9). In the neurons tested with ODQ, SNP alone produced inhibition in 7 of 13 neurons and sensitization in only 2 of 13. ODQ produced partial or complete blockade of the SNP effect in five of seven neurons that showed inhibition (84.4 ± 7.3% blockade, range: 59–100%, n = 5) and in one of two neurons that showed sensitization (32% blockade). In the neurons tested withRpcGMP, SNP alone produced inhibition in five of nine neurons and had no effect in four of nine. None of the neurons was sensitized. RpcGMP produced partial or complete blockade of the SNP effect in four of five neurons that showed inhibition (73.5 ± 12.7% blockade, range: 44–100%, n = 4). Overall, inhibition of the cGMP pathway either reduced or blocked SNP-induced inhibition in 9 of 12 neurons (Fig. 4, B and C). In contrast to the results obtained in protocol 1 with SNP alone, where nearly equal numbers of neurons were inhibited or sensitized by SNP, in this paradigm, the predominant effect of SNP was inhibition. The occurrence of sensitization from SNP alone in protocol 1 (7 of 19 neurons) was significantly greater than in protocol 2 (2 of 23 neurons; P < 0.05, \( \chi^2 \)). This suggests that in protocol 2, the prior application of the GC/cGMP blockers had an effect on the subsequent responses to SNP alone, even though the blockers were no longer present.

**DISCUSSION**

An apparent controversy that has arisen in the literature is that NO can produce opposing peripheral effects on nocipe-
tion: it can promote pain, inflammatory hyperalgesia, and neuropathic pain (Aley et al. 1998; Holthusen and Arndt 1995; Levy et al. 2000) but can also produce analgesia (Durate et al. 1992). One explanation that has been proposed to account for this discrepancy is the existence of subpopulations of peripheral sensory neurons in which NO exerts opposite physiological effects (Vivancos et al. 2003). Our results in dural nociceptors show that NO can produce both sensitization and inhibition of mechanosensitivity and that these effects are likely to occur in different neurons. Our results raise the question of whether nociceptive neurons that show opposite effects from NO represent two separate subpopulations that might also be distinguished by differences in other characteristics. Although no significant difference in CV between the two groups could be found, neurons that were sensitized by NO had higher baseline (predrug) response thresholds and also exhibited a trend toward lower spontaneous activity than neurons that were inhibited. These characteristics suggest the possibility that the effect of NO might be partly related to a neuron’s baseline level of excitability, such that neurons with higher excitability are more likely to be inhibited.

Behavioral studies have found dual effects of NO on nociception at different drug concentrations (Kawabata et al. 1994; Prado et al. 2002). However, our results showed no relationship between the concentration of the NO-donating compound and the direction of the neuronal effect. Instead, our results are consistent with the idea that, acutely, NO affects different neurons differently and that an individual neuron may be sensitized, inhibited, or unaffected.

Activation of GC-cGMP has been implicated in both the hyperalgesic (Aley et al. 1998; Nakamura et al. 1996) and analgesic effects of NO (Duarte et al. 1992; Vivancos et al. 2003). Our study provides clear evidence for an involvement of cGMP in the inhibitory effects of NO on dural nociceptors because these effects could be partly or completely blocked by inhibitors of GC or cGMP. In addition, the cGMP analogue itself produced inhibition comparable to that produced by the NO donor and was never found to produce sensitization. The finding that cGMP had no effect on cutaneous nociceptors (Kress et al. 1996) raises the possibility that cGMP-mediated inhibition might be present only in nociceptors.

Our results do not provide clear evidence regarding the possible involvement of cGMP in the sensitizing effects of NO. In those experiments that examined the role of cGMP by co-application of GC or cGMP inhibitors with the NO donor, sensitization was almost never observed even after removal of the inhibitors. We have no clear explanation for the failure to observe sensitization in these experiments, but it implies that the inhibitors could have exerted a persistent inhibitory or desensitizing effect on a cellular process that is necessary for NO-induced sensitization. The fact that the cGMP analogue itself produced only inhibition, and not sensitization, suggests that cGMP produces inhibition even in those neurons that have a sensitizing response to NO (although we did not test individual neurons for the effect of both NO and cGMP). Another possibility is that there is a dose effect, such that sensitization is induced by lower concentrations of cGMP than were tested in this study and that the subgroup of neurons that show sensitization generate relatively low levels of cGMP in response to NO.

Actions of NO on ion channels have been described that could potentially underlie both the excitatory and inhibitory effects we observed in dural nociceptors. One mechanism for NO-induced excitation that has been described in some types of central neurons is the facilitation of Ca2+-activated potassium (BK) channels (Klyachko et al. 2001). This NO-induced opening of BK channels enhances K+ currents at depolarized potentials, augmenting the afterhyperpolarization and leading to facilitation of the recovery of Na+ channels from inactivation. This process may lead to an enhanced neuronal excitability.

Although NO has been reported to have both excitatory and inhibitory actions on voltage-gated Na+ channels, which could strongly modulate neuronal activity (Ahern et al. 2000; Li et al. 1998; Renganathan et al. 2002), these actions have been described as cGMP-independent and so could not account for the cGMP-dependent action of NO observed in our study in dural nociceptors. However, one cGMP-dependent mechanism of inhibition that has been described is the activation of ATP-sensitive K+–channels (Murphy and Brayden 1995), which causes hyperpolarization and inhibition of neuronal firing (Roper and Ashcroft 1995). These channels have been identified in primary sensory neurons (Sarantopoulos et al. 2003) and have been implicated in mediating the peripheral anti-nociceptive action of the NO-cGMP-PKG cascade (Sachs et al. 2004; Soares et al. 2000).

One limitation of using SNP as an NO donor is that one of its metabolites is also cyanide (CN−) (Terwel et al. 2000), which has been shown to produce discharge in arterial chemoreceptors (Brophy et al. 1999; Paton et al. 2001). Like NO, cyanide also appears to exert its biological actions partly through activation of cGMP (Garry et al. 1994). However, in our experiments, SNP had no significant effect on ongoing discharge. We took precautions to minimize SNP degradation (see METHODS), and the time course of degradation is relatively long compared with the duration of our experiments (Garcia-Pascual et al. 1999). It therefore seems unlikely that cyanide was a major contributor to the SNP effects in our experiments.

NO donors such as SNP and nitroglycerin cause an immediate headache in normal subjects (Olesen et al. 1994; Waeb er and Moskowitz 2003). Both SNP and nitroglycerin promote vasodilatation of meningeal blood vessels (Messlinger et al. 2000; Wei et al. 1992), an action that might contribute to the immediate headache by inducing activation of meningeal nociceptors. However, the available evidence suggests that local vasodilatation is not an effective stimulus for either activation or sensitization of dural nociceptors. Distension of the dural sinuses by rapid infusion of saline failed to produce neuronal effect (Strassman et al. 1996). In addition, dural application of the potent vasodilator calcitonin gene-related peptide (CGRP) failed to produce activation or sensitization of dural nociceptors (unpublished observations).

It was also proposed that the NO could promote headache by directly activating meningeal nociceptors (Ashina et al. 2000; Knyhár-Csillik et al. 2001). Based on our finding that SNP did not influence the spontaneous activity of dural nociceptors, we propose that the NO is unlikely to cause headache by directly activating dural nociceptors. The acute NO-induced mechanosensitization of dural nociceptors could, however, promote headache by lowering the mechanical activation thresholds of dural nociceptors, thus allowing previously innocuous stimuli,
such as blood vessel pulsation and changes in intracranial pressure, to evoke pain. Systemic administration of sildenafil, which acts exclusively by increasing cGMP, causes only a delayed headache in migraine patients (Kruuse et al. 2003), unlike the NO donors, which also produce an immediate headache (see preceding text). This is consistent with our finding that the cGMP analogue 8-pCPT-cGMP, unlike SNP, produced no immediate sensitization of meningeal nociceptors.

In conclusion, our results provide evidence that NO can promote both sensitization and inhibition of mechanosensitive dural nociceptors. These opposing effects could depend on the level of excitability of the nociceptor.

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