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MECHANISMS MEDIATING NITROGLYCERIN-INDUCED DELAYED-ONSET HYPERALGESIA IN THE RAT

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Abstract—Nitroglycerin (glycerol trinitrate, GTN) induces headache in migraineurs, an effect that has been used both diagnostically and in the study of the pathophysiology of this neurovascular pain syndrome. An important feature of this headache is a delay from the administration of GTN to headache onset that, because of GTN's very rapid metabolism, cannot be due to its pharmacokinetic profile. It has recently been suggested that activation of perivascular mast cells, which has been implicated in the pathophysiology of migraine, may contribute to this delay. We reported that hyperalgesia induced by intradermal GTN has a delay to onset of ~30 min in male and ~45 min in female rats. This hyperalgesia was greater in females, was prevented by pretreatment with the anti-migraine drug, sumatriptan, as well as by chronic pretreatment with the mast cell degranulator, compound 48/80. The acute administration of GTN and compound 48/80 both induced hyperalgesia that was prevented by pretreatment with octoxynol-9, which attenuates endothelial function, suggesting that GTN and mast cell-mediated hyperalgesia are endothelial cell-dependent. Furthermore, A-317491, a P2X3 antagonist, which inhibits endothelial cell-dependent hyperalgesia, also prevents GTN and mast cell-mediated hyperalgesia. We conclude that delayed-onset mechanical hyperalgesia induced by GTN is mediated by activation of mast cells, which in turn release mediators that stimulate endothelial cells to release ATP, to act on P2X3, a ligand-gated ion channel, in perivascular nociceptors. A role of the mast and endothelial cell in GTN-induced hyperalgesia suggests potential novel risk factors and targets for the treatment of migraine.

Key words: vascular pain, nitroglycerin, endothelium, mast cells, migraine, P2X3.

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Abbreviations: ANOVA, analysis of variance; GTN, glycerol trinitrate.

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INTRODUCTION

Migraine, a chronic pain disorder whose pathophysiology is still incompletely understood, affects approximately 15% of the adult population, with women about twice as likely to suffer from this condition (Blackwell et al., 2014). Neurovascular mechanisms are considered important in the pathophysiology of migraine (Gupta et al., 2011; Blackwell et al., 2014), and a role for nitric oxide (NO), a potent vasodilator, has been implicated by finding that intravenous infusion of the NO donor, glyceryl trinitrate (GTN), reliably induces an attack in migraineurs (Olesen, 2008). However, while release of NO following GTN infusion is very rapid (Persson et al., 1994) and NO can act directly on nociceptors (Holthusen and Arndt, 1995), onset of GTN-induced headache does not occur until at least 45–60 min after its infusion (Thomaides et al., 2003; Sances et al., 2004), and persists for hours (Olesen et al., 1995). This markedly delayed onset of GTN-induced migraine-like headache indicates that NO is acting indirectly to induce a migraine attack.

While perivascular nociceptor mechanisms play a role in migraine (Asghar et al., 2011), there are challenges to directly studying the role of dural nociceptors in behavioral models of migraine-like pain. Therefore, in this study we explored whether we could produce delayed-onset mechanical hyperalgesia using local administration of the classical inducer of experimental migraine, GTN, at the site of nociceptive testing, as well as by intravenous administration. We also tested the contribution of perivascular targets (mast cells, endothelium and leukocytes) in this GTN-induced delayed-onset hyperalgesia.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on adult male and female Sprague–Dawley rats, (200–250 g; Charles River, Hollister, CA, USA). Experimental animals were housed three per cage, under a 12-h light/dark cycle, in a temperature- and humidity-controlled environment. Food and water were available *ad libitum*. All behavioral nociceptive testing was performed between 10:00 AM and 4:00 PM. Experimental animals were acclimatized to the testing environment by bringing them to the room in which the experiments were to be performed in their home cages, where they were left for 15–30 min. After this acclimatization period, rats were placed in

cylindrical acrylic restrainers that have side openings that allow extension of the hind limbs for nociceptive testing.

Nociceptive threshold was defined as the mean of three readings of mechanical threshold for hind leg withdrawal, taken at 5-min intervals. All experimental protocols were approved by the University of California, San Francisco Committee on Animal Research and conformed to National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. All efforts were made to minimize the number of animals used and their suffering.

Testing cutaneous nociception

The nociceptive flexion reflex was quantified with an Ugo Basile Analgesymeter® (Stoelting, Wood Dale, IL, USA), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw. Nociceptive threshold was defined as the force, in grams, at which the rat withdrew its paw from this stimulus. Hyperalgesia was defined as a decrease in mechanical nociceptive threshold, here presented as percentage change from baseline nociceptive threshold. Both paws of the same rat received the same treatment, and baseline paw withdrawal threshold was defined as the mean of the three readings taken just before administration of a test agent. Each experiment was performed on separate groups of rats. Animals acted as their own controls, with a test agent injected either intradermally, on the dorsal surface of the hind paw at the site of nociceptive testing, or intravenously, before the intradermal administration of GTN. In one group of rats, GTN was administered intravenously (4 μg/kg/min, for 20 min) via a 25-ga butterfly infusion set in a lateral tail vein; mechanical nociceptive testing in this group began 30 min after start of infusion. Paw-withdrawal thresholds before and after drug treatment were compared.

Mast cell degranulation

Repeated administration of compound 48/80 causes long-lasting local mast cell depletion in the skin (Jaffery et al., 1994). We employed a protocol as previously described (Piovezan et al., 2004), wherein 3 daily escalating doses (1, 3 and 10 mg) of compound 48/80 were administered intradermally to the dorsal surface of the hind paw, at the site of nociceptor testing. On the fourth day, a 10-mg test dose of compound 48/80 was administered and nociceptive threshold was evaluated 30 min after administration. In animals receiving this protocol, compound 48/80 on the fourth day did not produce hyperalgesia, indicating mast cell degranulation, as acute administration of compound 48/80 produces robust mechanical hyperalgesia (Chatterjea et al., 2012) (and see Fig. 5).

Attenuating endothelial function

Intravenous administration of octoxynol-9 impairs the function of the endothelial cell lining of blood vessels (McLeod and Piper, 1992; Bourreau et al., 1993; Sun et al., 1997), disrupting its contribution to nociceptor sensitization (Chen et al., 2014). To evaluate the role of the endothelial cell in GTN-induced by hyperalgesia, rats received an intravenous injection, through a tail vein, of a 0.5% solution of octoxynol-9, at a volume of 1 ml/kg body weight (Joseph and Levine, 2012a), 15 min before evaluation of GTN hyperalgesia.

Drugs

Glyceryl trinitrate (GTN), compound 48/80, fucoidin, sumatriptan, octoxynol-9 and A-317491 (Sigma Chemical Co., St. Louis, MO) were dissolved in saline. Drugs administered by intradermal injection were given in a volume of 2.5 μl/paw; octoxynol-9 was administered by intravenous injections in a volume of 1 ml/kg body weight. Doses of the drugs employed in this study were based on the result of dose response studies performed during preliminary experiments for this study, or during prior studies (Piovezan et al., 2004; Joseph and Levine, 2012a).

Statistical analyses

Group data are represented as mean ± SEM. Statistical significance was determined by unpaired Student's *t*-test or by a two-way repeated-measures ANOVA, followed by Sidek's multiple comparison *post hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of GTN on nociceptive threshold

Intradermal injection of GTN (0.1 ng) produced delayed-onset mechanical hyperalgesia, peaking 30 min after administration in male and 45 min in female rats, and remaining unattenuated over the remainder of the 120-min observation period (Fig. 1A). The magnitude of GTN hyperalgesia during its plateau phase (45–120 min) is markedly greater in female rats (2-way repeated measures ANOVA, male vs. female $P = 0.039$).

Intravenous injection of GTN (4 μg/kg/min, for 20 min) in males produced a marked decrease in nociceptive threshold beginning ~60 min after starting the perfusion, plateauing from 100 to 260 min, with threshold returning to baseline by ~440 min. In females hyperalgesia began much later, ~320 min after the start of intravenous GTN and reached a plateau ~440 min (Fig. 1B); there is a significant difference between males and females (two-way repeated measures ANOVA, $P < 0.0001$).

Analysis of the neurovascular mechanism mediating this GTN-induced delayed-onset mechanical hyperalgesia was performed using the intradermal route of administration in male rats.

Effect of triptan on GTN hyperalgesia

To test whether GTN hyperalgesia can be prevented by a standard therapy used to prevent an attack of migraine, sumatriptan (1 μg 10 min prior to GTN administration) was administered at the site of GTN administration and nociceptive testing. Sumatriptan markedly attenuated mechanical hyperalgesia induced by a subsequent injection of GTN (control vs. sumatriptan, $P < 0.001$, unpaired Student's *t*-test, Fig. 2).