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The nitroxyl donor, Angeli's salt, inhibits inflammatory hyperalgesia in rats

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ABSTRACT

Nitric oxide modulates pain development. However, there is no evidence on the effect of nitroxyl (HNO/NO^-) in nociception. Therefore, we addressed whether nitroxyl inhibits inflammatory hyperalgesia and its mechanism using the nitroxyl donor Angeli's salt (AS; $Na_2N_2O_3$). Mechanical hyperalgesia was evaluated using a modified Randall and Selitto method in rats, cytokine production by ELISA and nitroxyl was determined by confocal microscopy in DAF (a cell permeable reagent that is converted into a fluorescent molecule by nitrogen oxides)-treated dorsal root ganglia neurons in culture. Local pre-treatment with AS (17–450 μg/paw, 30 min) inhibited the carrageenin-induced mechanical hyperalgesia in a dose- and time-dependent manner with maximum inhibition of 97%. AS also inhibited carrageenin-induced cytokine production. AS inhibited the hyperalgesia induced by other inflammatory stimuli including lipopolysaccharide, tumor necrosis factor-α, interleukin-1β and prostaglandin E_2. Furthermore, the analgesic effect of AS was prevented by treatment with ODQ (a soluble guanylate cyclase inhibitor), KT5823 (a protein kinase G [PKG] inhibitor) or glybenclamide (an ATP-sensitive K^+ channel blocker), but not with naloxone (an opioid receptor antagonist). AS induced concentration-dependent increase in fluorescence intensity of DAF-treated neurons in a L-cysteine (nitroxyl scavenger) sensitive manner. L-cysteine did not affect the NO^+ donor S-Nitroso-N-acetyl-DL- penicillamine (SNAP)-induced anti-hyperalgesia or fluorescence of DAF-treated neurons. This is the first study to demonstrate that nitroxyl inhibits inflammatory hyperalgesia by reducing cytokine production and activating the cGMP/PKG/ATP-sensitive K^+ channel signaling pathway *in vivo*.

1. Introduction

Sensitization of primary sensory neurons is an essential process of inflammatory pain. In humans, this nociceptor sensitization usually leads to clinical conditions known as hyperalgesia (an increased response to a stimulus that is normally painful) or allodynia (pain due to a stimulus that does not normally provoke pain) (Verri et al., 2006). The mechanisms involved in the sensitization of primary sensory neurons may be divided into two processes. The first process includes non-neuronal events in which resident and recruited immune cells produce a sequence of hyperalgesic inflammatory mediators initiated by tumor necrosis factor α (TNFα). This triggers the release of interleukin-1β (IL-1β) and chemokines that in turn stimulate release of directly acting hyperalgesic mediators (Verri et al., 2006; Valério et al., 2009). The most well known directly acting hyperalgesic mediators are prostaglandins, which activate their specific receptors present on the nociceptive neuron membrane. The second process includes neuronal events involving activation of receptors on primary nociceptive neurons that trigger intracellular signaling pathways, such as cAMP, protein kinase A, and protein kinase C (Aley and Levine, 1999; Khasar et al., 1999). These signaling pathways result in subsequent phosphorylation of voltage-dependent sodium channels (Gold et al., 1998) and

Abbreviations: AS, Angeli's salt; HNO/NO^-, Nitroxyl; PGE_2, Prostaglandin E_2; TNFα, Tumor necrosis factor alpha; LPS, Lipopolysaccharide; IL-1β, Interleukin-1 beta; sGC, Soluble guanylate cyclase; PKG, Protein kinase G.

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inhibition of voltage-dependent potassium channels (Evans et al., 1999). Consequently, the nociceptor threshold is lowered and ultimately leads to an enhancement of neuron excitability.

Experimentally, the peripheral pharmacological control of inflammatory pain is based on two main strategies. The first is the use of drugs that prevent nociceptor sensitization, such as nonsteroidal anti-inflammatory drugs that inhibit prostaglandin synthesis (Ferreira, 1972) and, therefore, prevent the development of hyperalgesia. The second strategy is direct blockade of ongoing nociceptor sensitization, which can be achieved by use of peripheral morphine (opioids), dipyrone, or diclofenac (Lorenzetti and Ferreira, 1985; Ferreira et al., 1991; Cunha et al., 2010). In fact, these drugs reverse the already established hyperalgesia induced by prostaglandin E_2 (PGE_2) in rat and mice hind paws. In addition, several studies support the hypothesis that their antinociceptive activities are due to activation of the L-arginine/NO/cGMP/protein kinase G (PKG)/ATP-sensitive K^+ channel pathway (Ferreira et al., 1991; Cunha et al., 2010).

NO is produced from the conversion of L-arginine to L-citruline by NO synthase isoforms and is involved in the regulation of several pathophysiological functions, such as vascular homeostasis, platelet aggregation, fibrinolysis, inflammation and pain (Furchgott and Zawadzki, 1980; Furlong et al., 1987; Benjamim et al., 2000; Cunha et al., 2010; Paolocci et al., 2007). Focusing on pain, NO has dual roles in that it can either induce or reduce pain depending on its concentration and locale of production or delivery (Cury et al., 2011). Concerning the antinociceptive effect of NO, evidence shows that in the periphery NO donors or substances that increase neuronal concentrations of NO directly block inflammatory hyperalgesia (Cury et al., 2011). Within nociceptive neurons, NO binds with high affinity to the ferrous heme prosthetic group of soluble guanylate cyclase (sGC), resulting in pronounced stimulation of cGMP production (Griffiths et al., 2003). In turn, cGMP activates cGMP-dependent protein kinase (PKG), which seems to be responsible for the phosphorylation of ATP-sensitive K^+ channels causing an increase in current (Cunha et al., 2010). This intracellular signaling pathway causes hyperpolarization of nociceptive neurons counteracting their enhanced excitability during the inflammatory process. This is an important peripheral mechanism of NO, which is activated, for instance, by morphine (Cunha et al., 2010).

NO• (uncharged form of NO) and oxidized nitrogen oxide congeners including peroxynitrite ($ONOO^-$), nitrite (NO_2^-), nitrate (NO_3^-), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) have attracted considerable attention in physiological and pathophysiological settings. By contrast, nitroxyl (HNO at physiological pH), the one-electron reduced and protonated sibling of NO, has been relatively overlooked (Miranda et al., 2003; Irvine et al., 2008; Wink et al., 1998). Attention to nitroxyl has increased due to its pharmacological effects, mainly in the cardiovascular system. Importantly, nitroxyl has been postulated to have a role as an endothelium-derived relaxing in mesenteric arteries and to function as a hyperpolarizing agent in resistance arteries (Andrews et al., 2009). Similarly to NO, nitroxyl also activates soluble guanylate cyclase (Wanstall et al., 2001; Irvine et al., 2003; Favaloro and Kemp-Harper, 2007; Irvine et al., 2007).

Taking into account the above information, we investigated the impact of the nitroxyl donor Angeli's salt on inflammatory hyperalgesia induced by a variety of stimuli, and the underlying analgesic mechanism of observed effect.

2. Materials and methods

2.1. Animals

A total of 520 male Wistar rats (180–220 g) were housed in temperature-controlled rooms (22–25 °C), with access to water and food *ad libitum*. Experiments were conducted in accordance with the guidelines of International Association for Study of Pain and with the approval of the Ethics Committee of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo. All effort was made to minimize suffering.

2.2. Mechanical hyperalgesia test

Mechanical hyperalgesia was analyzed as previously described (Ferreira et al., 1978). In this method, a constant pressure of 20 mmHg is applied (via a syringe piston moved by compressed air) to a 15 mm^2 area on the dorsal surface of the hindpaw, and discontinued when the rat presented a typical "freezing reaction". This reaction is comprised of brief apnoea, concomitant with retraction of the head and forepaws and reduction in the escape movements that animals normally make to free themselves from the position imposed by the experimental situation. Usually, the apnoea is associated with successive waves of muscular tremor. For each animal, the latency to the onset of the "freezing reaction" is measured before administration (zero time) and at different times after administration of the hyperalgesic agents. The intensity of mechanical hypersensitivity is quantified as the reduction in the reaction time, calculated by subtracting the value of the second measurement from the first (Ferreira et al., 1978). Reaction time was 31.5 ± 0.1 s (mean ± SEM; $n = 36$) before injection of the hyperalgesic agents.

2.3. TNFα and IL-1β production

Samples of cutaneous plantar tissue were collected 2 h after carrageenin injection and processed for ELISA assay to determine the levels of TNFα and IL-1β (Verri et al., 2010). Results are expressed as pg/100 mg of tissue.

2.4. Dorsal root ganglia (DRG) culture

Primary neuron cultures were prepared from the dorsal root ganglia (DRG) of rats as previously described (Cunha et al., 2010). Aliquots of cell suspensions were plated on Matrigel-coated coverslips (25 mm diameter) in 35 mm diameter Petri dishes at a final density of 1×10^6 cells per dish. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere for 24 h (Cunha et al., 2010). These cell cultures were analyzed by confocal microscopy as described below.

2.5. DAF-2 fluorescence measurements

After incubation of the affixed neurons with 10 μM DAF-2DA in Hanks/Hepes buffer (1 M HEPES on 1 L of Hanks, pH 7.4) for 30 min, optical microscopy was used to assess cell viability. During the experiments, cultures were maintained in the steel chamber at 37 °C. The cells were mounted onto the stage of the scanning microscope (Leica TCS SP5 water lens). Digital images of DAF-2DA fluorescence were obtained after excitation at 488 nm (argon laser) using a 515 nm long-pass emission filter. After an initial period of baseline fluorescence collection (0–120 s), the cells were treated with AS (0.1–1 mM), SNAP (0.1–1 mM) (Cunha et al., 1999), L-cysteine (3 mM) (Andrews et al., 2009) or L-cysteine for 3 min before treatment with AS (1 mM) or SNAP (1 mM). The mean fluorescence intensity was determined from a linear measurement of fluorescence of 12–15 cells in randomly chosen fields of each slide, with a total of 3 slides per group.

2.6. Image analysis

For confocal imaging, laser attenuation, pinhole diameter, photomultiplier sensitivity, and offset were kept constant for each set of experiments. Images were captured between 0 and 1200 s at 30 s intervals, and the changes in fluorescence intensities within regions of interests (circles drawn over cells) were quantitatively analyzed using the Leica SP5 software.

2.7. Drugs and stimuli

The following materials were obtained from the sources indicated. PGE_2, glybenclamide, KT5823, naloxone, L-cysteine, and SNAP (S-Nitroso-N-acetyl-DL-penicillamine) were obtained from Sigma Aldrich (St Louis, MO, USA), Dulbecco's modified Eagle medium (DMEM), Hanks, HEPES and the dye 4,5-diaminofluorescein diacetate (DAF-2DA) were obtained from Molecular Probes (St. Louis, MO, USA). Carragenin was obtained from FMC Corporation (Philadelphia, PA, USA). ODQ was purchased from Calbiochem (San Diego, CA, USA). Recombinant rat TNF-α and human IL-1β were acquired from the National Institute for Biological Standards and Control (South Mimms, Hertfordshire, UK). Angeli's salt ($Na_2N_2O_3$, sodium trioxodinitrate; AS) was synthesized and utilized as previously described (Smith and Hein, 1960). The stability of stock solutions prepared in 10 mM NaOH and stored at −20 °C, was determined from the extinction coefficients at 250 nm (ε of 8000 M^{-1} cm^{-1} for Angeli's salt) (Maragos et al., 1991). Test compounds were diluted in sterile saline, except for Angeli's salt, which was prepared at 7 mg/ml of 10 mM NaOH, PGE_2 at 500 μg/ml of ethanol, ODQ at 8 μg/50 μl of DMSO 2% in saline, KT5823 at 1.5 μg/50 μl of DMSO 2% in saline or glybenclamide at 160 μg/50 μl of