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Role of hydrogen sulfide in the formalin-induced orofacial pain in rats

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ABSTRACT

Hydrogen sulfide (H_2S) is a gasotransmitter synthesized in peripheral tissues by the enzyme cystathionine gamma-lyase (CSE). This gas has been documented to be involved in a wide variety of processes including inflammation and nociception. The aim of the present study was to investigate the role of the peripheral H_2S pathway in nociceptive response to the orofacial formalin experimental model of pain. Orofacial pain was induced by subcutaneous injection of formalin (1.5%, 50 μl) into the upper lip of rats, and the time spent rubbing the face was measured at 3-min intervals for 45 min. Formalin induced a marked biphasic pain (first phase: 0–3 min; second phase: 15–33 min). Pretreatment with H_2S donor (Na_2S; 90 μmol/kg), CSE inhibitor (propargylglycine; 26.5 and 88.4 μmol/kg), or a preferential blocker of T-type Ca^{2+} channels (mibefradil; 0.28 and 2.81 μmol/kg) attenuated the second phase of face rubbing when injected locally as well as systemically. Pretreatment with a selective blocker of K^+_{ATP} channels (glybenclamide; 2.81 μmol/kg) suppressed the Na_2S-mediated attenuation of the formalin-induced pain second phase. Taken together these results suggest that endogenously produced H_2S plays a pronociceptive role probably via T-type Ca^{2+} channels, whereas exogenous H_2S exerts antinociceptive effects mediated by K^+_{ATP} channels.

1. Introduction

Gasotransmitters are regulatory molecules involved in the modulation of physiological and pathological functions in mammalian tissues (Wang, 2002; Boehning and Snyder, 2003). Nitric oxide (NO) was first demonstrated as an endogenous gas with a variety of biological activities, followed by carbon monoxide (CO) and, more recently, by hydrogen sulfide (H_2S). They are synthesized by enzymes, and produce an array of biological effects (Wang, 2002).

H_2S is synthesized via desulfhydration of L-cysteine, mainly by two pyridoxal 5′-phosphate-dependent enzymes: cystathionine β-synthase (CBS, mainly present in the brain) and cystathionine γ-lyase (CSE, mainly found in peripheral tissues) (Kasparek et al., 2008). The expression of CSE has been identified in several mammalian tissues, including liver, kidney, brain, ileum, and lymphocytes (Li and Moore, 2007).

One of the first identified physiological effects of H_2S was its ability to relax vascular smooth muscle (Zhao and Wang, 2002; Zhong et al., 2003). Several studies have subsequently highlighted the importance of H_2S in a number of biological processes (Wangm, 2012; Kwiatkoski et al., 2013), such as inflammation (Levine et al., 1998; Li et al., 2007, 2009; Wallace, 2007; 2010; Wallace et al., 2012), and pain (Cunha et al., 2008; Schemann and Grundy, 2009; Smith, 2009).

It has been shown that H_2S exerts pro- and antinociceptive effects (Distrutti et al., 2006a,b; Fiorucci et al., 2007; Matsunami et al., 2009; Wallace et al., 2012) which appear to result from various interconnected mechanisms dependent upon different molecular targets, being T-type Ca^{2+} channels implicated in the pronociceptive effect and K^+_{ATP} channels in antinociception (Schemann and Grundy, 2009; Smith, 2009). This interpretation is based upon pharmacological studies showing that much of the H_2S effects are dependent on K^+_{ATP} channels and/or T-type Ca^{2+} channels; these channels are known to be blocked, respectively, by glybenclamide and mibefradil (Schemann and Grundy, 2009). However, the putative role of H_2S modulating nociception in the mouth remains unknown, and there is strong evidence supporting the fact that the anatomical and physiological consequences of nerve injuries of the trigeminal system differ from those seen after peripheral nerve injury (Fried et al., 2001). It is well known that the orofacial region is densely innervated by the trigeminal nerve, which focuses on the most often acute, chronic and referred pains, accompanying the pathological states of the teeth and related structures (Raboisson and Dallel, 2004; Luccarini et al., 2006). The

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trigeminal system has a large cerebral representation and a high degree of somatotopy (Rhinn et al., 2013).

Considering that during the formalin test, a well-characterized behavioral model of tonic chemogenic pain, an inflammatory process takes place altering the peripheral neurotransmission, it seems plausible to consider that H_2S in the inflammed lip may exert a considerable impact on nociceptive response to formalin. Therefore, the present study was conducted to address the following main questions: Is H_2S endogenously produced in the lip? Does H_2S play a role in modulating nociception during the orofacial formalin test? And if so, does the effect of H_2S depends on T-type Ca^{2+} channels and/or ATP-sensitive K^+ [K^+_{ATP}] channels?

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing 220–280 g were housed at 24–26 °C, had free access to water and food, and exposed to a 12/12 h light/dark cycle. All procedures were approved by the Animal Care and Use Committee of the University of São Paulo (Protocol number 13.1.277.53.8), and followed the ethical guidelines for investigations of experimental pain in conscious animals of the International Association for the Study of Pain (Zimmermann, 1983).

2.2. Drugs

The drugs used in this study—Na_2S (H_2S donor), propargylglycine (PAG, inhibitor of CSE), mibefradil (blocker of T-type Ca^{2+} channels), and glybenclamide (blocker of K^+_{ATP} channels), were purchased from Sigma-Aldrich (St. Louis, MO), and dissolved in sterile saline (vehicle; 0.9%) in a volume of 50 μl. Glybenclamide was dissolved in 1% DMSO (vehicle) in a final volume of 50 μl.

2.3. The formalin test

All rats were previously adapted to the experimental chamber (30 × 30 × 30 cm) and room for 3 days and during 30 min each day. The experimental room had little human activity and controlled temperature (25 ± 1 °C). Rats were hand held and formalin (1.5%; 50 μl) was injected into the upper right lip. After the injection of formalin, face rubbing behavior was chronometered for 45 min at 3-min intervals. Data collected between 0 and 3 min post-formalin injection were grouped as phase 1, whereas phase 2 was composed of data collected between 15 and 33 min post-formalin injection. This protocol has been regularly used, and is well accepted by the scientific community (Tjølsen et al., 1992; Nascimento and Branco, 2007). Behavioral experiments were blinded, as the researcher who prepared drug solutions and analyzed the results was not the same one who performed the experiments.

2.3.1. Pretreatments

Before the administration of formalin to the upper lip the drugs to be tested were injected into two different (subcutaneous) locations: either the upper lip (local pretreatment) or the dorsal region of the rat (systemic pretreatment). Local pretreatment was performed (in the upper lip) with Na_2S (90 μmol/kg), PAG (26.5 and 88.4 μmol/kg), mibefradil (0.28 and 2.8 μmol/kg), or glybenclamide (3.24 μmol/kg). The same drugs, administered locally at effective doses, were also used when the systemic pretreatments were performed. Some of these doses were chosen based on previous studies in the literature and mainly on pilot experiments. All drugs (including formalin) were administered with 1-ml syringes (30 G1/2; 0.30 × 13) at a volume of 50 μl. At the end of the experiments, each animal was euthanized with an overdose of anesthetic.

2.4. Experimental groups (in vivo approach)

2.4.1. Role of K^+_{ATP} channels in the putative action of exogenous H_2S on the orofacial formalin test

To investigate whether exogenous H_2S has any effect on the orofacial formalin test, 1% DMSO (glybenclamide vehicle) was locally or systemically administered 15 min before local or systemic Na_2S injection (90 μmol/kg), and 15 min later 1.5% formalin was administered to the upper lip.

To examine the putative involvement of K^+_{ATP} channels in the antinociceptive action of exogenous H_2S, other group of animals was locally or systemically pretreated with glybenclamide (dissolved in 1% DMSO) at a dose of 3.24 μmol/kg 15 min before local or systemic administration of Na_2S (90 μmol/kg) and 15 min thereafter 1.5% formalin was administered to the upper lip.

To investigate whether the blocker (glybenclamide) of K^+_{ATP} channels has any effect on the face rubbing behavior, rats were locally or systemically pretreated with glybenclamide at 3.24 μmol/kg 15 min before local or systemic injection of 0.9% sterile saline, and 15 min thereafter 1.5% formalin was administered.

The control group received the vehicles (1% DMSO and saline): 1% DMSO was locally or systemically administrated 15 min before local or systemic injection of 0.9% sterile saline, and 15 min thereafter 1.5% formalin was administered.

2.4.2. Role of endogenous H_2S in the orofacial formalin test

To address the involvement of endogenous H2S in the genesis of nociception during the orofacial formalin test, we analyzed the effect of PAG locally (26.5 and 88.4 μmol/kg) or systemically (88.4 μmol/kg) 30 min before 1.5% formalin administration.

2.4.3. Putative involvement of T-type Ca^{2+} channels in the orofacial formalin test

The review article by Smith (2009) has suggested the involvement of a H_2S-mediated activation of T-type Ca^{2+} channels in the genesis of nociception. To provide some evidence on the putative role of T-type Ca^{2+} channels in nociception of the orofacial formalin test we injected, locally (0.28 and 2.81 μmol/kg) or systemically, mibefradil (2.81 μmol/kg) 30 min before the administration of 1.5% formalin.

2.5. H_2S measurement (ex vivo approach)

To measure the local (upper lip) levels of H_2S, we injected saline (control group) or 1.5% formalin into the upper lip, and 30 min later the animal was decapitated, upper lip skin was quickly excised, frozen by immersion in liquid nitrogen, and stored at −70 °C until assay (Lee et al., 2008; Kwiatkoski et al., 2012). The tissue was homogenized in ice-cold 100 mM potassium phosphate buffer (PBS; pH 7.4; 500 μl). The assay mixture contained the tissue homogenate, L-cysteine (5 mM; 8 μl), pyridoxal 5′-phosphate (1 mM; 8 μl) and PBS (0.01 M; 10 μl), and was incubated at 37 °C for 2 h. At the end of the incubation period, zinc acetate (1% w/v; 50 μl) was injected into a 1.5-ml plastic tube to trap generated H_2S, and then trichloroacetic acid (10% w/v; 50 μl) was also added to the tube to precipitate proteins. The tube was centrifuged thereafter (3000 rpm, 10 min, Centrifuge MiniSpin - Eppendorf, USA). Subsequently, 80 μl of the supernatant was added to a 96-well microplate to react for 15 min with *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 mM; 40 μl) dissolved in 7.2 M HCl and $FeCl_3$ (30 mM; 40 μl) dissolved in 1.2 M HCl. Absorbance was read at 670 nm using a 96-well microplate reader (PowerWave, Biotek Instruments, USA). H_2S concentration of each