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Nitroxyl inhibits overt pain-like behavior in mice: role of cGMP/PKG/ATP-sensitive potassium channel signaling pathway

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Abstract

Background—Several lines of evidence have indicated that nitric oxide (NO) plays complex and diverse roles in modulation of pain/analgesia. However, the roles of charged and uncharged congeners of NO are less well understood. In the present study, the antinociceptive effect of the nitroxyl (HNO) donor, Angeli's salt (Na₂N₂O₃; AS) was investigated in models of overt pain-like behavior. Moreover, whether the antinociceptive effect of nitroxyl was dependent on the activation of cGMP (cyclic guanosine monophosphate)/PKG (protein kinase G)/ATP-sensitive potassium channels was addressed.

Methods—The antinociceptive effect of AS was evaluated on phenyl-p-benzoquinone (PBQ)- and acetic acid-induced writhings and via the formalin test. In addition, pharmacological treatments targeting guanylate cyclase (ODQ), PKG (KT5923) and ATP-sensitive potassium channel (glybenclamide) were used.

Results—PBQ and acetic acid induced significant writhing responses over 20 min. The nociceptive response in these models were significantly reduced in a dose-dependent manner by

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Conflict of interest

There is no conflict of interest to declare.

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subcutaneous pre-treatment with AS. Furthermore, AS also inhibited both phases of the formalin test. Subsequently, the inhibitory effect of AS in writhing and flinching responses were prevented by ODQ, KT5823 and glybenclamide, although these inhibitors alone did not alter the writhing score. Furthermore, pretreatment with L-cysteine, an HNO scavenger, confirmed that the antinociceptive effect of AS depends on HNO.

Conclusion—The present study demonstrates the efficacy of a nitroxyl donor and its analgesic mechanisms in overt pain-like behavior by activating the cGMP/PKG/ATP-sensitive potassium channel (K^+) signaling pathway.

Keywords

pain; Angeli's salt; nitroxyl; nociception; formalin

Introduction

Nitric oxide (NO) is a gaseous substance that acts primarily as the endothelium-derived relaxing factor (EDRF) [1]. Nevertheless, NO also acts as a neuronal messenger in the central and peripheral nervous systems and is involved in various biological events such as synaptic plasticity and neurotransmission [2,3], vascular homeostasis, platelet aggregation, inflammation, angiogenesis, fibrinolysis [4], pain and antinociception [5]. NO is formed from L-arginine by action of three primary isoforms of NO synthase: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) [5, 6, 7, 8]. The effects of NO are categorized as dependent or independent on activation of soluble guanylate cyclase (sGC), which subsequently induces the increase of the second messenger cyclic guanosine monophosphate (cGMP) signaling pathway [4].

Several lines of evidence have shown a dual role of NO in the nociceptive system. In fact, NO can either induce or reduce pain depending on the nociceptive system level (central or periphery) and the amount of NO [9, 10]. Concerning the antinociceptive effect, use of NO donors has shown that NO induces analgesia [11, 12]. NO also mediates the peripheral and central antinociceptive effect of analgesic compounds, such as opioids, some non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac [13, 14], dipyron [15], natural products [16], statins [17], cannabinoids, the antiepileptic gabapentin, adenosine, PPAR-c agonists, the α_2 -adrenoceptor agonist xylazine, hormones such as estradiol and melatonin, bovine lactoferrin, the anesthetic gas nitrous oxide isosorbide dinitrate spray, hydrogen sulfide releasing drugs and phosphodiesterase inhibitors [5]. Importantly, these analgesic drugs are capable of blocking ongoing hyperalgesia. These data indicate that NO is likely the common denominator for the mode of action of peripheral analgesic drugs that directly block ongoing nociceptor sensitization. Therefore, the relevance of the antinociceptive effect of NO is already established. Furthermore, the antinociceptive effect of NO depends on formation of guanylate cyclase-derived cGMP [10], leading to the activation of cGMP-dependent protein kinase (PKG), which phosphorylates ATP-sensitive potassium channels, thereby diminishing nociceptive information transmission [9, 15, 18]

Until recently, most of the biological effects of NO have been attributed directly to NO itself. However, NO can be both oxidized (NO^+) and reduced (NO^-). Under physiological

conditions, the species one-electron reduced from NO is protonated (HNO; nitroxyl) [19]. Unlike NO[•], HNO has yet to be shown to be endogenously biosynthesized. However, numerous chemical reactions have been described to suggest pathways for production of HNO under biological conditions [20]. For example, biochemical studies show that nitroxyl can be formed directly from NO synthase [21], particularly in the absence of tetrahydrobiopterin [21] or via oxidation of the NOS intermediates N^G-hydroxy-L-arginine [21,22] or of hydroxylamine [23]. On the other hand, nitroxyl can also be formed via reduction of NO[•] by mitochondrial cytochrome c, xanthine oxidase, ubiquinol, haemoglobin or manganese superoxide dismutase (SOD) [19,21].

Nitroxyl donors displays vasorelaxant and cardioprotective effects in experimental models [19, 24], which are mediated predominantly by activation of sGC, production of intracellular cGMP and consequently leading to interaction with voltage-gated potassium channels [25]. Importantly, *in vitro* organ bath and contractility studies demonstrated that nitroxyl has a prominent role as an EDRF in mesenteric arteries. Additionally, nitroxyl functions as a hyperpolarizing agent in resistance arteries [26]. Together, this suggests the importance of nitroxyl as a signaling molecule and HNO donors as pharmacological tools. The best known and well-studied nitroxyl donor is trioxodinitrate (Na₂N₂O₃) or Angeli's salt (AS) [27], which has been pivotal in the analysis of the biology/pharmacology of nitroxyl.

Recently, our laboratory demonstrated that AS inhibits mechanical hyperalgesia in rats via activation of sGC/cGMP/PKG/ATP-sensitive potassium channel signaling pathway and inhibition of cytokine production [28]. Distinct from mechanical hyperalgesia or thermal external stimuli, models of inflammatory overt pain are characterized by an almost immediate behavior such as abdominal contortions (writhing) and paw flinch and/or licking. These behaviors occur through direct or indirect (via release of inflammatory mediators) activation of the primary nociceptive neurons by chemical (phenyl-p-benzoquinone - PBQ; acetic acid; formalin) and biological (zymosan; CFA) stimuli [29, 30, 31].

In the present study, we investigated whether the Angeli's salt reduces the overt pain-like behavior induced by PBQ, acetic acid or formalin and if its antinociceptive mechanism depends on the activation of the cGMP/PKG/ATP-sensitive potassium channels signaling pathway.

Material and Methods

Chemicals

The following materials were obtained from the sources indicated: acetic acid from Mallinckrodt Baker, S.A (Mexico City, Mexico); glybenclamide, KT5823 (2,3,9,10,11,12-hexahydro-10R-methoxy-2,9-dimethyl-1-oxo-9S,12R-epoxy-1H diindolo [1,2,3-fg:3',2', 1'-kl] pyrrol [3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester) and L-cysteine were obtained from Sigma-Aldrich (St. Louis, MO, USA). ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was obtained from Calbiochem (San Diego, CA, USA). Angeli's salt (Na₂N₂O₃, sodium trioxodinitrate) was synthesized and utilized as previously described [32]. 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) [33]. The selected doses of drugs were chosen based on pilot studies and previous data of our laboratory. We detected that the doses of drugs used do not alter the nociceptive response *per se*, and inhibit the respective standard stimulus [16, 18, 34, 35,36, 37]

Animals

Male Swiss mice (25–30 g) from the Universidade Estadual de Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and kept at 21 °C. Different investigators prepared the solutions, treated the mice, injected the stimulus and quantified the nociceptive behaviors. All behavioral testing was performed between 9 am and 5 pm in a temperature controlled room. Animal care and handling procedures were in accordance with the International Association for the Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Universidade Estadual de Londrina, which follows the National Council of Science of Animal Research. All efforts were made to minimize the number of animals used and their suffering.

Experimental protocols

Mice were treated with Angeli's salt (0.03–10 mg/kg/*sc*, diluted in 10 mM NaOH) 40 min before *ip* injection of PBQ, acetic acid or formalin. In another sets of experiments, the mice were pre-treated with the inhibitors ODQ (0.1–1 mg/kg/*ip*, diluted in saline, 30 min), KT5823 (0.17 or 0.5 µg/mouse/*ip*, diluted in 5% DMSO in saline, 5 min), glybenclamide (0.03–1 mg/kg/*po*, diluted in 20% Tween 80 in saline, 45 min) before treatment with Angeli's salt (3 mg/kg/*sc*, diluted in 10 mM NaOH) with the exception that only one dose of ODQ (1 mg/kg), KT5823 (0.5 µg/mouse) or glybenclamide (1 mg/kg) was tested in the formalin model. After an additional 40 min, mice received *ip* injection of PBQ, acetic acid or formalin. As control groups, mice received ODQ (1 mg/kg), KT5823 (0.5 µg/mouse) or glybenclamide (1 mg/kg) before PBQ, acetic acid and formalin injection. In another set of experiments, mice were pre-treated with the HNO scavenger (L-cysteine, 0.3–1 mg/kg, *ip*, diluted in saline, 30 min), before treatment with Angeli's salt (3 mg/kg/*sc*, diluted in 10 mM NaOH) with the exception that only one dose of L-cysteine (1 mg/kg) was tested in the acetic acid or formalin models. After an additional 40 min mice received injection of PBQ, acetic acid or formalin stimulus.

Writhing response tests

The PBQ [38] or acetic acid [29]-induced writhing model was performed as previously described [34, 35]. PBQ diluted in 2% DMSO/saline (1890 µg/kg), acetic acid (0.8% v/v, saline, 10 ml/kg) or vehicle was injected into the mouse peritoneal cavity. After PBQ or acetic acid *ip* injection, each mouse was placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. The writhing response consists of a contraction of the abdominal muscle together with a stretching of hind limbs. The intensity of the writhing response was expressed as the cumulative writhing score over 20 min.

Formalin Test

The number of paw flinches were determined between 0 and 30 min after intraplantar (*ipl*) injection of 25 μ L of 2,5% formalin, as previously [30]. The period was divided in intervals of 5 min and clearly demonstrated the presence of the first and second phases, which are characteristic of the method [16, 30, 39]. Results were obtained for both the first (0–5 min) and second (10–30 min) phases.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 4.0 (La Jolla, 5 CA). Results are presented as means $\pm$ SEM of two independent experiments. The *n* in the legends refers to the number of mice per group in each experiment. The differences between the experimental groups were compared by one-way ANOVA and individual comparisons were subsequently made by Tukey's post hoc test. The level of significance was set at $P < 0.05$ and are represented by symbols (*, **, # or *f*; please see legends) in case of significance, otherwise a non-significant statistical difference was indicated.

Results

Angeli's salt inhibits the phenyl-p-benzoquinone or acetic acid-induced writhing response

Mice were treated with AS (0.03–10 mg/kg or 1–3 mg/kg, *sc*), or vehicle (19.35 μ L of 10 mM NaOH plus saline to complete 150 μ L) 40 min before PBQ or acetic acid *i.p.* injection, respectively. The writhing response was determined over 20 min after stimulus injection (Fig. 1). AS inhibited the PBQ-(Fig. 1A) or acetic acid- (Fig. 1B) induced writhing response. In the PBQ test, a U shaped curve was observed with a larger effect observed 3 mg/kg AS, but without difference with the lower doses of AS. The doses between 0.03 and 1 mg/kg and 10 mg/kg of AS did not significantly reduce the PBQ-induced writhing response (Fig. 1A). In the acetic acid test, the dose of AS of 1 mg/kg did not reduce the nociception, and significant antinociception was observed only with a 3 mg/kg dose of AS (Fig. 1B). Thus, a dose of AS of 3 mg/kg was selected for the next experiments.

Pre-treatment with ODQ, an inhibitor of soluble guanylate cyclase, reverses the antinociceptive effect of AS in PBQ- and acetic acid-induced writhing response in mice

Mice were pre-treated with ODQ (0.1–1 mg/kg, *ip* - an inhibitor of soluble guanylate cyclase) or vehicle (saline) 30 min before administration of AS (3 mg/kg, *sc*) and after additional 40 min mice received *ip* stimulus with PBQ (Fig. 2A) or acetic acid (Fig. 2B). ODQ inhibited in a dose-dependent manner the antinociceptive effect of AS in the PBQ-induced writhing response (Fig. 2A). The dose of ODQ of 0.1 mg/kg was ineffective while the doses of 0.3 and 1 mg/kg prevented the antinociceptive effect of AS. In the acetic acid model, the dose of ODQ of 0.1 mg/kg present significant inhibition of the antinociceptive effect of AS, and the inhibition by ODQ was even increased with the dose of 0.3 mg/kg (Fig. 2B). ODQ (1 mg/kg, *ip*) alone did not affect the writhing response induced by PBQ or acetic acid (Fig. 2A and 2B). These results show that the antinociceptive effect of nitrotyl depends on activation of soluble guanylate cyclase.

Pre-treatment with KT5823, a selective inhibitor of cGMP-dependent protein kinase (PKG), reverses the antinociceptive effect of AS in PBQ and acetic acid-induced writhing response in mice

Mice were pre-treated with KT5823 (0.17 or 0.5 µg/mouse - an inhibitor of PKG) or vehicle (5% DMSO in saline) 30 min before administration of AS (3 mg/kg, *sc*) and after additional 40 min mice received *ip* stimulus with PBQ (Fig. 3A) or acetic acid (Fig. 3B). There was a tendency of reduction of the antinociceptive effect of AS by the treatment with KT5823 at the dose of 0.17 µg/mouse and significant inhibition with the dose of 0.5 µg/mouse in the PBQ (Fig. 3A) and acetic acid (Fig. 3B) models. The treatment with KT5823 (0.5 µg/mouse, *ip*) did not affect the nociceptive response induced by PBQ and acetic acid per se (Fig. 3A and 3B, respectively). These data show that the antinociceptive effect of nitroxyl depends on activation of PKG.

Pre-treatment with glybenclamide, an inhibitor of ATP-sensitive potassium channels, prevents the antinociceptive effect of nitroxyl in the PBQ or acetic acid-induced writhing response in mice

Mice were pre-treated with glybenclamide (0.03–1.0 mg/kg, *po* - an inhibitor of ATP-sensitive potassium channels) or vehicle (20% Tween 80 in saline) 45 min before administration of AS (3 mg/kg, *sc*) and after additional 40 min mice received *ip* stimulus with PBQ (Fig. 4A) or acetic acid (Fig. 4B). The dose of glybenclamide of 0.1 mg/kg induced a tendency of reduction of the antinociceptive effect of AS, but statistically significant inhibition was observed only with doses of glybenclamide of 0.3 and 1 mg/kg in the PBQ model (Fig. 4A). In the acetic acid model, 0.1 and 0.3 mg/kg doses of glybenclamide significantly inhibited the antinociceptive effect of AS, and a dose of 0.3 mg/kg completely reversed the antinociceptive effect of AS with statistical significance compared to 0.1 mg/kg (Fig. 4B). A 0.03 mg/kg dose of glybenclamide did not inhibit the antinociceptive effect of AS in the acetic acid model. Glybenclamide per se (1 mg/kg, *po*) did not affect the writhing response induced by PBQ (Fig. 4A) or acetic acid (Fig. 4B). These results show that the antinociceptive effect of nitroxyl depends on opening of ATP-sensitive potassium channels.

Angeli's salt reduces formalin-induced overt pain-like behavior by activating the cGMP/PKG/ATP-sensitive potassium channels signaling pathway

Firstly, mice were treated with AS (0.3–3 mg/kg) or vehicle (19, 35 µl of 10 mM NaOH plus saline to complete 150 µL) 40 min before formalin (2.5%, *ipl*) administration (Fig. 5A). AS dose-dependently inhibited the first and second phases of formalin-induced overt pain-like behavior. In the first phase, a 0.3 mg/kg dose of AS had no effect while 1 mg/Kg presented a tendency of inhibition although not significant. A dose of 3 mg/kg AS significantly inhibited formalin nociception compared to vehicle group and the lower dose of AS (0.3 mg/Kg) (Fig. 5A). In the second phase of the formalin test, an AS dose of 0.3 mg/kg had no effect again, nevertheless, in this phase a dose of 1 mg/kg significantly inhibited the nociceptive response induced by formalin, and the inhibition by 3 mg/kg of AS was again significant compared to vehicle and 0.3 mg/kg AS. Therefore, a dose of 3 mg/kg AS was selected for the next experiments in which mice were treated with ODQ (1 mg/kg, *ip*, 30 min, Fig. 5B), KT5823

(0.5 µg/mouse, *ip*, 5 min, Fig. 5C) or glybenclamide (1 mg/kg, *po*, 45 min, Fig. 5D) before AS (3 mg/kg) or vehicle (1.6125 µl of 10 mM NaOH plus saline to complete 150 µL) treatment. After an additional 40 min, mice received formalin (2.5%, 25 µL) stimulus. All inhibitors (ODQ, KT5823 and glybenclamide) reversed AS antinociception in the formalin-induced flinch response in the first and second phases (Fig. 5B–5D). These results indicate that AS presents the same antinociceptive mechanism in the PBQ or acetic acid writhing test and in the flinch response induced by formalin. The inhibitors ODQ (1 mg/kg), KT5823 (0.5 µg/mouse) and glybenclamide (1 mg/kg) did not affect the nociceptive response induced by formalin (Fig 5E) [28, 39].

Pre-treatment with L-cysteine, an HNO scavenger, prevents the antinociceptive effect of Angeli's salt in the overt pain-like behavior induced by PBQ, acetic acid or formalin

Mice were pre-treated with L-cysteine (0.3–1 mg/kg, *ip*) or vehicle (saline) 30 min before administration of AS (3 mg/kg, *sc*), and after an additional 40 min mice received PBQ (Fig. 6A), acetic acid (Fig. 6B) or formalin stimulus (Fig. 6C). There was tendency of reduction of AS antinociceptive effect in the PBQ model by treatment with L-cysteine at a dose of 0.3 mg/Kg while 1 mg/kg L-cysteine significantly inhibited the antinociceptive effect of AS over PBQ-induced writhings (Fig. 6A). Therefore, a dose of 1 mg/kg L-cysteine was selected for further experiments (Fig. 6B and 6C). L-cysteine inhibited the antinociceptive effect of AS in the acetic acid and formalin models. Furthermore, L-cysteine (1 mg/kg) did not affect PBQ, acetic acid or formalin-induced overt pain (Fig. 6A, B, and C).

Discussion

The biological roles of nitroxyl have not been explored to a similar extent as of NO. The HNO donor Angelis Salt (AS) has been pivotal in the discovery of the biology/pharmacology of HNO [40]. In the present study, we demonstrated that AS reduced the overt pain-like behavior induced by phenyl-*p*-benzoquinone, acetic acid or formalin by triggering the cGMP/PKG/ATP-sensitive potassium channel antinociceptive signaling pathway in mice. The analgesic effect of AS was amenable by treatment with the HNO scavenger L-cysteine..

The acetic acid and PBQ models have similarities in their nociceptive mechanisms since both induce the release of many endogenous inflammatory mediators as prostanoids and cytokines such as TNF-α and IL-1β [34, 35, 41]. Acetic acid or PBQ intraperitoneal injection causes a stereotypical behavior in mice, which is characterized by abdominal contractions together with a stretching of the hind limbs [34, 35, 42]. In the formalin model, the overt pain-like behavior is characterized by first (0–5 min) and second (10–30 min) phases. The first phase involves histamine and serotonin release/action on nociceptors [16, 43]. On the other hand, the second phase depends on inflammatory mediators produced in response to formalin stimulus comprising cytokines [44]. Hence, it is possible that nitroxyl inhibits mechanisms that are shared by the PBQ, acetic acid and formalin models such as cytokine production, since AS inhibited carrageenin-induced TNFα and IL-1β production [28].

The antinociceptive effect of AS in overt pain-like behavior models depends on activation of the cGMP/PKG/ATP-sensitive potassium channel pathway. These results line up well with the data on mechanical hyperalgesia in which AS also activated the cGMP/PKG/ATP-sensitive potassium channel pathway [28]. Furthermore, nitroxyl is capable of activating sGC to produce cGMP [19, 45, 46, 47] and there are three possible lines of explanation for this activity. The first is that nitroxyl activates sGC via NO[•] [19]. However, although it has been demonstrated that AS decomposes to NO[•] in endothelial cells culture in the presence of superoxide dismutase [48], there is reduction of superoxide dismutase activity in inflammation [49] suggesting an inadequate environment for AS decomposition to NO[•]. Furthermore, AS decomposes to only 1–2% of NO[•] comparing to 98–99% of HNO [48]. Therefore, these data [19, 48, 49] reduce the possible relevance of this first possible mechanism of nitroxyl activation of sGC. The second possibility suggests that nitroxyl could directly activate sGC by an NO[•]-independent mechanism [19]. Nitroxyl and NO[•] present different activities in *ex vivo* pharmacological and functional studies in isolated organ baths. Further supporting that NO[•] and nitroxyl are relatively independent molecules, and that the effects of nitroxyl are not explained solely by simple conversion to NO[•], nitroxyl is known for activating voltage-dependent K⁺ (K_v) channels [25] in the same vascular bed that instead, NO[•] activates calcium-activated K⁺ channels (K_{Ca}) [50]. The third line of evidence demonstrated in anaerobic *in vitro* system that the HNO donors AS and NCTFA (1-nitrosocyclohexyl trifluoro acetate) inhibit isolated bovine lung sGC activity in the presence of NO[•] scavenging compounds by interacting with the ferrous heme of sGC [50]. On the other hand, AS also inhibited sGC possibly by modifying enzyme cysteine thiol groups. Nevertheless, it is noteworthy to mention that AS achieved maximal activation of sGC at 10 μM while inhibition of sGC was observed at the supra-physiological concentration of 100 μM [50], indicating that such high doses are not recommended for inhibition of sGC studies with nitroxyl and that nitroxyl might present opposing roles depending on its concentration. Therefore, these data corroborate that AS activates sGC by an NO[•]-independent mechanism to reduce nociception.

In addition to these three lines of evidence and the present data demonstrating that the HNO scavenger L-cysteine inhibits the antinociceptive effect of AS, we have demonstrated that AS-induced DAF-2DA fluorescence in dorsal root ganglia neurons was sensitive to L-cysteine, which further corroborates AS is delivering nitroxyl to neurons [28]. It is important to mention that similar tools (ODQ, KT5823 and glybenclamide) were used in previous studies demonstrating the anti-hyperalgesic effect and mechanism of NO [9, 15, 35, 39, 51] and those inhibitors did not affect the nociceptive behavior induced by PBQ, acetic acid or formalin *per se*.

In addition to the therapeutic potential present herein, nitroxyl seems to offer considerable advantages over traditional nitrovasodilators (e.g., the NO donor DEA/NO) since tolerance to AS was not observed in rat isolated aortae [46]. Furthermore, nitroxyl presents a similar degree of antinociceptive action to NO donors such as SNP, SNAP or SIN-1 and drugs that act by an NO-dependent mechanism including morphine [5, 9]. A possible disadvantage to be investigated is that several studies demonstrated the involvement of NO[•] in the development of opioid tolerance in models of pain [52]. It remains to be determined whether

nitroxyl is also involved in the development of morphine tolerance and further investigate the potential pharmacological applications of HNO and NO⁻.

Concluding, we demonstrated that the nitroxyl donor Angeli's salt inhibits overt pain-like behavior by triggering the antinociceptive cGMP/PKG/ATP-sensitive potassium channel signaling pathway *in vivo*.

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Figure 1. Angeli's salt inhibits the writhing response

Mice were treated with AS (0.03–10 mg/kg, *sc.*, 150 µL) or vehicle (0.20 µL of NaOH 10 mM plus saline to complete 150 µL) 40 min before phenyl-*p*-benzoquinone (PBQ, 1890 µg/kg, Panel A) or acetic acid (0.8%, 10 ml/kg, Panel B) injection. The writhing response was evaluated over 20 min. * $P < 0.05$ compared to the saline group (Panels A and B); # $P < 0.05$ compared to the PBQ or acetic acid group. One-way ANOVA followed by Tukey's test ($n = 6$).

Figure 2. Role of soluble guanylate cyclase in nitroxyl-induced antinociception

Mice were pre-treated with ODQ (0.1 – 1.0 mg/kg, *ip*, 200 µL) 30 minutes before AS (3 mg/kg, *sc*, 150 µL) or vehicle (1.075 µL of NaOH 10 mM plus saline to complete 200 µL) treatment. A group received ODQ (1 mg/kg, *ip*) without AS treatment. After additional 40 minutes all of the animals received the PBQ (Panel A) or acetic acid (Panel B) *ip* injection. The number of writhing was determined over 20 min. * $P < 0.05$ compared to saline group; # $P < 0.05$ compared to PBQ or acetic acid group; ** $P < 0.05$ compared to AS group and ^f $P < 0.05$ compared to AS group and dose of 0.1 mg/kg of ODQ (Panel B). One-way ANOVA followed by Tukey's test ($n = 6$).

Figure 3. Role of cGMP-dependent protein kinase (PKG) in Angeli's salt-induced-antinociception

Mice were pre-treated with KT5823 (PKG inhibitor, 0.17 or 0.5 µg/mouse, *ip*, 200 µL) 5 min before AS (3 mg/kg, *sc*, 150 µL) or vehicle (as in Figure 2) treatment. A group received KT5823 (0.5 µg/mouse, *ip*) without AS treatment. After an additional 40 minutes all of the animals received the PBQ (Panel A) or acetic acid (Panel B) *ip* injection. The number of writhing was determined over 20 min. * $P < 0.05$ compared to saline group; # $P < 0.05$ compared to PBQ group or acetic acid group; ** $P < 0.05$ compared to AS group. One-way ANOVA followed by Tukey's test ($n = 6$).

Figure 4. Role of ATP-sensitive potassium channels in Angeli's salt induced-antinociception

Mice were pre-treated with glybenclamide (0.03–1 mg/kg, *po*, 150 µL) 45 min before AS (3 mg/kg, *sc*, 150 µL) or vehicle (as in Figure 2) treatment. A group received glybenclamide (1 mg/kg, *ip*) without AS treatment. After an additional 40 minutes all of the animals received the PBQ (Panel A) or acetic acid (Panel B) *ip* injection. The number of writhing was determined over 20 min. * $P < 0.05$ compared to saline group; # $P < 0.05$ compared to PBQ or acetic acid group; ** $P < 0.05$ compared to AS group, and ^f $P < 0.05$ compared to AS group plus glybenclamide at dose of 0.1 mg/kg (Panel B). One-way ANOVA followed by Tukey's test ($n = 6$).

Figure 5. Nitroxyl inhibits formalin-induced overt pain-like behavior by activating the cGMP/PKG/ATP-sensitive potassium channel pathway

Panel A: mice were treated with AS (0.3–3 mg/kg, *sc*, 40 min) or vehicle (as in Figure 2) before intraplantar (*ipl*) stimulus with formalin 2.5% (25 μ L). Panel B, C and D (respectively): mice were treated with ODQ (1 mg/kg, 30 min), KT5823 (0.5 μ g/mouse, 5 min) or glybenclamide (1 mg/kg, 45 min) before Angeli's salt (AS; 3 mg/kg) or vehicle (as in Figure 2) treatment. Panel E: mice received ODQ (1 mg/kg, *ip*), KT5823 (0.5 μ g/mouse, *ip*), glybenclamide (1 mg/kg, *po*) or KT5823 (0.5 μ g/mouse, *ip*) without AS treatment. After, mice received formalin 2.5% (25 μ L, *ipl*) stimulus. The intensity of overt pain-like behavior was evaluated by the number of flinches within 5 min (first phase) and between 10–30 min (second phase) after formalin injection. * P <0.05 compared with the saline group, # P <0.05 compared with AS group. ** P <0.05 compared with lower AS dose (0.3 mg/kg; Panel A) or AS + vehicle treatment (Panels B–D). One-way ANOVA followed by Tukey's *t* test (n = 7).

Figure 6. L-cysteine prevents AS induced-antinociception

Mice were pre-treated with L-cysteine (0.3–1 mg/kg, *ip*, 200µL) 30 minutes before Angeli's salt (AS, 3 mg/kg, *sc*, 150 µL) or vehicle (as in Figure 2) treatment, and after an additional 40 minutes mice received PBQ (Panel A), acetic acid (Panel B) or formalin stimulus. A group received L-cysteine (1 mg/kg) without AS treatment. The number of writhing was determined over 20 min and the number of flinches was determined over 30 min. * $P < 0.05$ compared to saline group, # $P < 0.05$ compared to PBQ, acetic acid group or formalin group and ** $P < 0.05$ compared to AS group. One-way ANOVA followed by Tukey's test ($n = 6$).