

Acute stress-induced antinociception is cGMP-dependent but heme oxygenase-independent

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Abstract

Endogenous carbon monoxide (CO), which is produced by the enzyme heme oxygenase (HO), participates as a neuromodulator in physiological processes such as thermoregulation and nociception by stimulating the formation of 3',5'-cyclic guanosine monophosphate (cGMP). In particular, the acute physical restraint-induced fever of rats can be blocked by inhibiting the enzyme HO. A previous study reported that the HO-CO-cGMP pathway plays a key phasic antinociceptive role in modulating noninflammatory acute pain. Thus, this study evaluated the involvement of the HO-CO-cGMP pathway in antinociception induced by acute stress in male Wistar rats (250-300 g; n = 8/group) using the analgesia index (AI) in the tail flick test. The results showed that antinociception induced by acute stress was not dependent on the HO-CO-cGMP pathway, as neither treatment with the HO inhibitor ZnDBPG nor heme-lysinate altered the AI. However, antinociception was dependent on cGMP activity because pretreatment with the guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ) blocked the increase in the AI induced by acute stress.

Key words: Heme oxygenase; Carbon monoxide; cGMP; Lateral cerebral ventricle; Acute stress; Antinociception

Introduction

The exposure of animals to threatening situations of either an innate or learned nature results in species-specific defensive behaviors, autonomic alterations and pain inhibition. These reactions are crucial for the survival of the species (1). In this context, an important component of the response to emergency situations is the decreased capacity to perceive pain. Indeed, a number of studies have shown that the antinociceptive system can be activated by a variety of external or environmental noxious stimuli and plays an important role in control of defensive affective behaviors (2). In this context, fear induced by conditioned or unconditioned aversive stimuli is known to inhibit behavioral responses to pain (3). The onset of analgesia can be explained by multiple mechanisms that may be activated, depending on the nature of the noxious stimulation, its intensity, and its duration (4). In fact, it has been demonstrated that acute stress can trigger an increase in pain threshold and promote antinociception. In contrast, chronic stress may not interfere

with nociception because an adaptation to stressful stimuli may take place or may even promote hypernociception (5).

Endogenously formed CO arises from heme degradation in a reaction catalyzed by the enzyme heme oxygenase (HO) and has been reported to participate as a neuromodulator in a number of physiological processes including thermoregulation and nociception through the formation of 3',5'-cyclic guanosine monophosphate (cGMP) (6-8). According to this notion, Steiner et al. (8) demonstrated that the restraint-induced fever of rats may be prevented with an intracerebroventricular (*icv*) microinjection of zinc-deuteroporphyrin-2,4-bis glycol (ZnDPBG), a nonselective inhibitor of HO. It has also been reported that the HO-CO-cGMP pathway plays a key phasic antinociceptive role in modulating noninflammatory acute pain (6). However, the involvement of this pathway in the modulation of stress-induced antinociception has not been assessed. Thus, in the present study, we evaluated the involvement of

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the HO-CO-cGMP pathway in antinociception induced by acute stress, using the analgesia index (AI) in the tail flick test in rats.

Material and Methods

Experimental procedure

Experiments were performed on male Wistar rats weighing 250-300 g obtained from the animal facility of the Universidade de São Paulo, Ribeirão Preto, SP, Brazil. The animals were housed in a room with controlled temperature ($24 \pm 1^\circ\text{C}$) and a 12-h light/dark cycle (lights on at 6:00 am), with food and water *ad libitum*. The Animal Use and Ethics Committee of the Universidade de São Paulo, Ribeirão Preto approved all experimental procedures (protocol no. 07.1.997.53.4).

For surgical procedures, rats were anesthetized with ketamine and xylazine (75 and 10 mg/kg, respectively, administered intramuscularly). Animals designated for experiments involving *icv* injection were restrained in a stereotaxic frame, and a stainless steel guide cannula was introduced into the right lateral cerebral ventricle (coordinates: 1.0 mm posterior, 1.6 mm lateral to midline, and 3.2-3.7 mm ventral to the skull surface) (9). Displacement of the meniscus in a water manometer ensured correct positioning of the cannula in the lateral ventricle. The cannulas were attached to the bone with stainless steel screws and acrylic cement. A tight fitting stylet was kept inside the guide cannula to prevent occlusion. After surgery, rats were treated with Pentabiotic (0.3 mL/kg im; Fort Dodge, Brazil 0.3 mL/kg). The experiments were initiated 1 week after surgery.

The nonselective HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG, 50 nmol/4 μL) and hemin used in this study were obtained from Porphyrin Products (Logan, USA). ZnDPBG was dissolved in 50 mmol/L Na_2CO_3 and stored in the dark. Hemin was used to prepare 150 nmol/4 μL heme-lysinate, an HO-CO-cGMP pathway inducer, as previously described (8). Heme-free preparations were used as amino acid (L-lysine) vehicle control solutions. The vehicle of the heme-lysinate solution consisted of 14.2 $\mu\text{mol/mL}$ L-lysine, 5% ethanol, 40% propylene glycol, and 55% sterile water. The soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ, 1.3 nmol/4 μL) was purchased from Tocris Cookson (USA) and dissolved in a vehicle consisting of 1% dimethylsulfoxide (DMSO) in pyrogen-free sterile saline. The doses used were based on previous studies (6). For *icv* injections, a 10- μL Hamilton syringe connected to a dental injection needle by a PE-10 catheter was used. At the time of drug administration, the stylet was replaced with the injection needle and 4 μL of the studied drug was infused over a period of 1 min (6). To prevent reflux, the needle was kept inside the guide cannula for 40 s after completing the infusion.

Nociception was assessed with a tail flick apparatus.

Rats were restrained manually and placed on the apparatus, which measured time in seconds (latency) that the animal took to respond, that is, remove the tail away from the noxious thermal stimulus. A cut-off time of 6 s was used to prevent tissue damage. Before treatment was initiated, the response latency in seconds for each animal was determined three consecutive times at random intervals of 1 to 3 min. After measurement of basal latency, animals were divided into 8 experimental groups and were then subjected to the tail flick test after various experimental treatments at intervals of 15 min up to a maximum of 120 min.

Rats in the different experimental groups were exposed to acute stress by physical restraint for 120 min beginning 30 min after the injection. Restraint was carried out by placing the animal in a plastic tube (15 cm in length and 5 cm in diameter).

The experiments were initiated 7 days after surgery. The rats were evaluated in the tail flick test to determine the baseline, then were microinjected *icv*, and after that were divided into 8 experimental groups ($n = 8/\text{group}$). In group 1, the rats received the HO inhibitor, ZnDPBG (50 nmol/4 μL), and in group 2, the Na_2CO_3 vehicle (50 mmol/4 μL). In groups 3 and 4, the rats received heme-lysinate (150 nmol/4 μL) or the L-lysine vehicle solution (4 μL), respectively. In groups 5 and 6, the rats were microinjected with ODQ (1.3 nmol/4 μL) or with the 1% DMSO vehicle (4 μL) 30 min before *icv* administration of the L-lysine vehicle solution (4 μL). Finally, in groups 7 and 8 the rats received an *icv* injection of ODQ (1.3 nmol/4 μL) or 1% DMSO vehicle (4 μL) 30 min before *icv* administration of heme-lysinate (150 nmol/4 μL).

At the end of the experiments, rats were anesthetized with chloral hydrate (10%, 0.35 mL/100 mg body weight, *ip*) and transcardially perfused with saline (0.9% NaCl) followed by 10% formalin. The brains were then removed and fixed in 10% formalin. The material was submitted to routine histological processing and sections were observed microscopically, according to the description in the Paxinos and Watson atlas (9).

In each group, tail flick latency was normalized using an antinociception index (AI) derived from the tail flick test according to the following formula: tail flick test–tail flick control/cutoff time–tail flick control, where tail flick control was the average of 3 tail-flick baseline latencies. AI represents the antinociception index tail flick test; latency is the time that the animal takes to initiate a response after the experimental treatment; baseline is the latency period in the control; the noxious thermal stimulus was stopped after 6 s.

Data are reported as mean $\text{AI} \pm \text{SE}$ and were analyzed by repeated measures multivariate analysis of variance (two-way ANOVA), with the variables being time (repeated factor) and treatment (independent factor), followed by one-way ANOVA and the Newman-Keuls test. Data were considered to be statistically significant when $P < 0.05$.

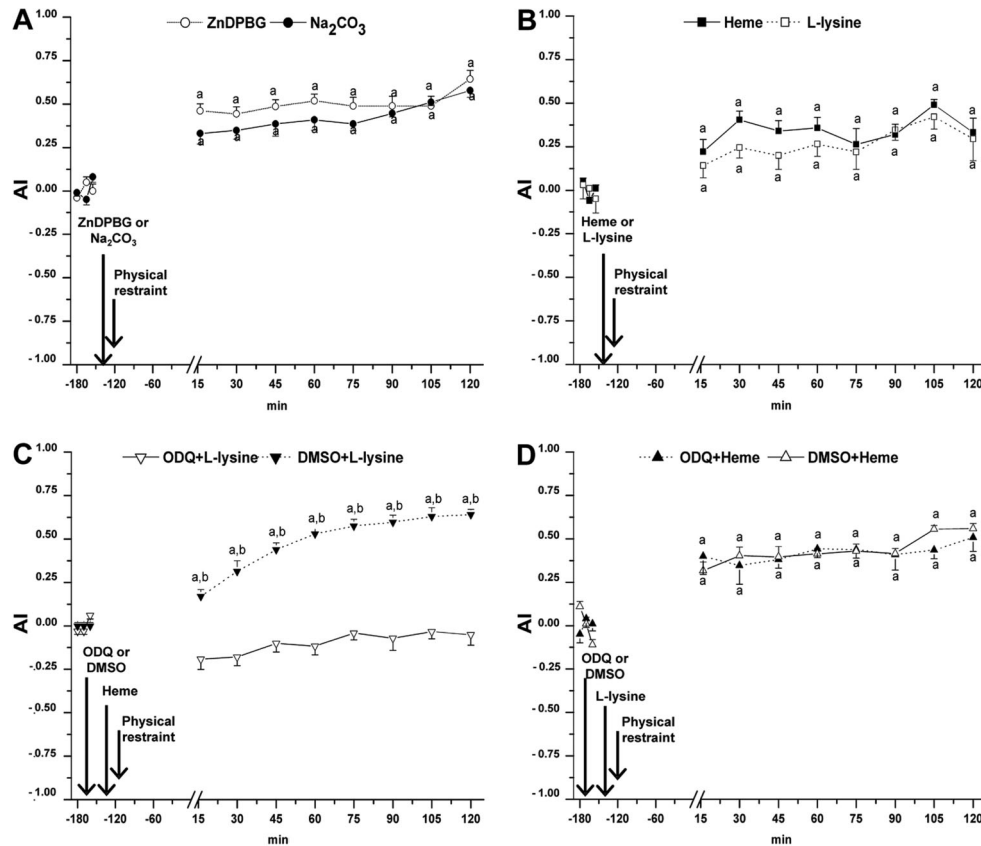


Figure 1. Antinociceptive index (AI) in the tail-flick test in rats. *A*, Administration (*icv*) of ZnDPBG (200 nmol/4 μ L, HO inhibitor) or vehicle (Na_2CO_3). *B*, Administration (*icv*) of HO substrate heme-lysinate (Heme, 150 nmol/4 μ L) or vehicle (L-lysine) on AI. *C*, Effect of *icv* injection of soluble guanylyl cyclase inhibitor (ODQ, 5.2 nmol/4 μ L) or vehicle (1% DMSO) followed 30 min later by L-lysine on AI in the tail flick test. *D*, Treatment (*icv*) of soluble guanylyl cyclase inhibitor (ODQ, 5.2 nmol/4 μ L) or vehicle (1% DMSO) followed 30 min later by heme-lysinate (150 nmol/4 μ L) on AI in the tail flick test. Data are reported as means AI (\pm SE), $n = 8$ in each group. *a*: $P < 0.05$, compared with respective control period; *b*: $P < 0.05$, ODQ + L-lysine group compared with DMSO + L-lysine group (Newman-Keuls test). The AI of the ODQ + L-lysine group did not differ over time when compared with the AI of the control period (*C*).

Results

The results of the present study confirmed that acute stress induced antinociception (Figure 1), but neither heme-lysinate (an HO substrate, Figure 1B) nor ZnDPBG (an HO inhibitor, Figure 1A) altered the AI in rats that were evaluated using the tail flick test. However, the *icv* injection of the sGC inhibitor ODQ (Figure 1C) was able to block the increase in AI.

Statistical analysis (two-way ANOVA) showed that the AIs of group 1, which received *icv* administration of ZnDPBG, and group 2, which received the vehicle (Na_2CO_3), differed with time [$F_{(8,143)} = 26.76$, $P < 0.001$] and treatment [$F_{(1,143)} = 12.97$, $P < 0.001$], but that there was no interaction between time and treatment [$F_{(8,143)} = 0.37$, $P = 0.94$]. The *post-hoc* Newman-Keuls test showed that the AIs in the ZnDPBG and Na_2CO_3 groups were different ($P < 0.05$) at all experimental times when compared with the respective baseline values ($P < 0.05$, Newman-Keuls test, Figure 1A).

Similarly, two-way ANOVA showed that the AIs of group 3, treated with heme-lysinate (which induces the HO pathway), and group 4, which received *icv* administration of the L-lysine vehicle solution, differed with time [$F_{(8,143)} = 5.83$, $P < 0.001$], but there were no differences between the treatments [$F_{(1,143)} = 3.62$, $P = 0.06$] or in the interaction between time and treatment [$F_{(8,143)} = 0.015$, $P = 0.94$]. The *post-hoc* Newman-Keuls test showed that the AIs in the heme-lysinate and L-lysine groups were different ($P < 0.05$) throughout the experimental time when compared with the respective baseline ($P < 0.05$, Newman-Keuls test, Figure 1B).

In groups 5 and 6, which were treated with ODQ (a selective inhibitor of soluble guanylyl cyclase) or its vehicle (1% DMSO) followed by L-lysine, two-way ANOVA revealed a difference with time [$F_{(8,143)} = 713.09$, $P < 0.001$], and with treatment [$F_{(1,143)} = 515.99$, $P < 0.001$] and the interaction of time and treatment [$F_{(8,143)} = 0.19$, $P < 0.001$]. The *post hoc* Newman-Keuls test showed that the AI of the ODQ +

L-lysine group was different from that of the DMSO+L-lysine group ($P < 0.05$) at each experimental time (Figure 1C). Additionally, the AI of the DMSO+L-lysine group was different ($P < 0.05$, Newman-Keuls test) when compared with the respective baseline values. However, the ODQ+L-lysine group AI did not differ over time when compared with the baseline AI (Figure 1C).

Finally, two-way ANOVA applied to the AI of group 7, which received *icv* ODQ, or its DMSO vehicle (group 8), followed by heme-lysinate (which induces the HO pathway) showed differences with time [$F_{(8,143)} = 13.34$, $P < 0.001$] but there was no difference with treatment [$F_{(1,143)} = 0.21$, $P = 0.64$] nor was there an interaction of time and treatment [$F_{(8,143)} = 0.42$, $P = 0.91$]. The *post-hoc* Newman-Keuls test revealed that the AIs of the heme-lysinate groups pretreated with ODQ or DMSO were different ($P < 0.05$) over experimental time when compared with the respective baseline ($P < 0.05$, Newman-Keuls test, Figure 1D).

Discussion

The present data showed that although cGMP is critical for the antinociceptive response induced by stress, stress analgesia is independent of the HO-CO-cGMP pathway. Additionally, our results confirmed previous studies (4,5,10) reporting that acute stress produced by physical restraint was able to promote antinociception, as evidenced by the increase in the AI, as evaluated using the tail flick test. In contrast, while the HO-CO-cGMP pathway is essential for restraint-induced fever, this system did not participate in restraint-induced analgesia.

Several previous studies have shown that different stress situations can promote antinociception in both opioid and nonopioid manners (3). Stress-induced analgesia is considered an adaptive response that occurs in both laboratory animals and humans. Thus, evidence shows that acute stress in rats (male and female) promotes increased latency of the tail flick test when compared with control animals that were not stressed, thereby demonstrating the antinociceptive effect of this manipulation (5).

Regarding the mechanisms involved in stress-induced analgesia, studies have reported both opioid-dependent and non-opioid mechanisms involving histaminergic, cholinergic, and serotonin neurotransmission, as well as the involvement of angiotensin and nitric oxide (3,11). Correlating the angiotensin system, opioid and nociceptive modulation, a previous study (11) showed that administration of angiotensin II into the lateral ventricle of rats increased the latency of responses in the tail flick test. This increase was reversed by prior administration of naloxone, suggesting that the analgesia induced by angiotensin II was mediated by endogenous opioid mechanisms. However, analgesia produced by the activation of angiotensin receptors involved the activation of guanylyl cyclases because the administration of ODQ, a selective inhibitor of the GCs in the ventrolateral periaqueductal gray matter, was able to

block angiotensin-induced antinociception in rats (12). Interestingly, evidence has consistently shown that angiotensin II is involved in the stress response (13). Additionally, the angiotensin AT₁ receptor subtype has been found in the amygdala (14), which is a limbic structure known to be involved in the emotional response to stress (15). Moreover, some findings have demonstrated that restraint stress for 120 min enhanced antinociception, as assessed by the tail flick test in rats, and that this effect was blocked by treatment with an inhibitor of nitric oxide synthase (NOS), indicating that stress restraint-induced antinociception is dependent on the release of nitric oxide (10). The NO mechanism is similar to that of CO, in that it involves an increase in the enzyme activity of GCs, thereby resulting in an increase in cGMP (16,17). In fact, some evidence indicates that the antinociceptive effect of NO occurs through the activation of cGMP (12,16,17). de Oliveira et al. (18) showed that restraint stress promotes increased expression of the neuronal enzyme NOS in the paraventricular nucleus of the hypothalamus, the medial nucleus of the amygdala and the dorsal periaqueductal gray matter. These brain areas have been closely related to nociceptive and antinociceptive modulation (19). The NO/cGMP system is involved in antinociception and in the effects produced by opioids (20) or the renin-angiotensin system (12). Taken together, these findings support the present data that the physical restraint stress promoted antinociception in the tail flick test, and this effect was blocked by pretreatment with ODQ, a GCs selective inhibitor. This evidence suggests that the antinociceptive effect was dependent on the activation of cGMP and was independent of the HO-CO-cGMP pathway, as neither a selective inhibitor of HO (ZnDPBG) nor an HO substrate (heme-lysinate) altered the increase in the AI induced by acute stress.

It is important to note that a previous report demonstrated that *icv* ZnDPBG administration did not affect the core body temperature of unrestrained rats but attenuated restraint-induced fever (8). In contrast, *icv* administration of heme-lysinate to unrestrained rats resulted in increased AI, which was blocked by pretreatment with ZnDPBG or ODQ (6); however, *icv* treatment with either heme-lysinate or ZnDPBG did not alter the AI induced by acute stress in the present study. One plausible explanation is that the antinociception induced by restraint in the present study promoted the activation of cGMP, independent of the HO-CO-cGMP pathway, and was blocked by pretreatment with ODQ (a selective inhibitor of soluble guanylyl cyclase).

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