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Antinociception synergy between the peripheral and spinal sites of the heme oxygenase-carbon monoxide pathway

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We have shown that the peripheral and spinal cord heme oxygenase (HO)-carbon monoxide (CO)-soluble guanylate cyclase-cGMP pathways play an important role in antinociception in the rat experimental formalin model. Our objective was to determine if there is synergism between peripheral (paw) and spinal HO-CO pathways in nociception. Rats were handled and adapted to the experimental environment for a few days before the formalin test, in which 50 µL of a 1% formalin was injected subcutaneously into the dorsal surface of the right hind paw. The animals were then observed for 1 h and the frequency of flinching behavior was taken to represent the nociceptive response. Thirty minutes before the test, rats were pretreated with intrathecal injections of the HO inhibitor, zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) or heme-lysinate, which is a substrate of the HO pathway. The paw treatments took place 20 min before the test. Low doses of ZnDPBG did not increase nociception, while a low heme-lysinate dose did not change flinching behavior after paw or spinal injections. Combined subactive spinal (50 nmol) and peripheral (40 nmol) low doses of ZnDPBG induced hypernociception (increase of 80% in the first and 25% in the second phase flinching), whereas combined spinal-peripheral heme-lysinate (50 and 30 nmol) led to second phase antinociception (40% reduction in flinching). These findings suggest a synergy between the peripheral and spinal HO-CO pathways. Local activation of the HO system probably regulates the nociception initiation in peripheral tissue and participates in buffering the emerging nociceptive signals at the peripheral and spinal sites of action. In short, an antinociceptive synergy exists between peripheral and spinal HO pathways, which may reduce the doses required and side effects.

Key words: Nociception; Carbon monoxide; Formalin; Hypernociception; Antinociception

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Introduction

Recently, carbon monoxide (CO) has been recognized to act as a neurotransmitter or neuromodulator in the nervous system (1,2). The synthesis and consequently the levels of CO in cells and tissues depend on heme oxygenase (HO) activity. Endogenous CO arises from the cleavage of the heme molecule by HO, producing biliverdin, free iron and the most active substrate in the nervous system, CO (3). There are three HO isoforms, of which isoforms one (HO-1) and two (HO-2) are the best known (3). Both HO-1 (inducible) and HO-2 (constitutive) have been detected in various tissues, including endothelial cells, smooth muscle and neural tissue (1,3-5). There is evidence that CO stimulates soluble guanylate cyclase activity and consequently increases the cellular levels of cyclic GMP (cGMP) (3,4,6). The HO-CO pathway is involved in many physiological processes, acting as a vasoactive substance and

a neurotransmitter or neuromodulator in the nervous system (4).

CO plays a key role in the central nervous system by controlling core body temperature (fever and hypothermia) and nociception. CO has also been reported to participate in both stress- and endotoxin-induced fever (7,8), in hypoxia-induced hypothermia (9) and to have an anti-hyperalgesic effect in inflamed paws, probably by increasing intracellular levels of cGMP in primary afferent neurons. These data have been obtained using both the hypernociception induced by carrageenan (10), and the inflammatory formalin model (11). Moreover, the spinal HO-CO pathway may also have an anti-hyperalgesic effect (12). Since both peripheral and spinal events are involved in the development of the hypernociceptive response, the possible synergy between the heme oxygenase pathway in spinal and peripheral nociceptor becomes a relevant subject of study. In the present study, we investigated the synergy between spinal and peripheral functions of the HO-CO pathway in nociception. We evaluated the effect of intrathecal and paw co-administration of pharmacological modulators of the HO pathway on the nociceptive response of rats during the formalin test.

Material and Methods

Animals

Adult male Wistar rats weighing 220-260 g were housed at a room temperature of 24-26°C, with free access to water and food and on a 12-h light/dark cycle. All procedures were approved by the Animal Use and Ethics Committee of Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, and followed the ethical guidelines for investigations of experimental pain in conscious animals of the International Association for the Study of Pain (13).

Drugs

Formalin was diluted in sterile saline. The HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), 50 and 200 nmol, was dissolved in 50 mM Na_2CO_3. Heme-lysinate was prepared as described by Linden (14), and L-lysine solution was used as the control vehicle. Both ZnDPBG and hemin were purchased from Porphyrin Products, USA.

Formalin test

All rats were adapted to the test chamber and experimental room for 30 min each day for 3 days. The experimental room had little human activity and a controlled temperature of 25 ± 1°C. The rats were hand held and formalin was injected subcutaneously into the dorsal surface of the right hind paw (50 µL of a 1% solution). After formalin injection the rats' behavior was observed in the experimental chamber (30 x 30 x 30 cm). The amount of flinching behavior was recorded and grouped into 5-min intervals for 1 h. The data collected between 0 and 5 min post-formalin injection represented phase 1 and the data collected between 20 and 60 min post-formalin injection represented phase 2. At the end of the experiment, each animal was sacrificed with an overdose of anesthetic.

Intrathecal drug injection

The drugs and the control solutions, 20 µL, were injected intrathecally between the L5-L6 lumbar spinal segments (15,16) 30 min before the formalin test, by direct transcutaneous injection. One-milliliter syringes and 30 G1/2 (0.30 x 13) needles were used for all drug administrations. The heme oxygenase inhibitor, ZnDPBG, and the enzyme substrate, heme-lysinate, were used at different doses. For the control group, the rats received only the drug vehicle, Na_2CO_3 for ZnDPBG, and L-lysine solution for the heme-lysinate treatment. To establish a control response regarding the injection volume, the saline group received 0.9% saline 30 min before the formalin injection.

Hind paw drug injection

The drugs and the control solutions were injected subcutaneously in a 40-µL volume 20 min before formalin into the dorsal surface of the right hind paw. One-milliliter syringes and 30 G1/2 (0.30 x 13) needles were used for all drug administrations. ZnDPBG and the enzyme substrate, heme-lysinate, were used at different doses. Control rats received only vehicles, Na_2CO_3 for ZnDPBG, and L-lysine solution for the heme-lysinate treatment. To establish a control response regarding the injection volume, the saline group received 0.9% saline 20 min before the formalin injection.

Statistical analysis

The time course of the experiment is shown at 5-min intervals and starts at the formalin injection into the hind paw. All behavioral results are reported as means ± SEM. Data were analyzed by analysis of variance (ANOVA), followed by the Tukey test for *post hoc* analysis. The differences between groups were determined at each established time during the course of the experiment. The level of significance was set at $P < 0.05$.

Results

ZnDPBG and the nociceptive response

The intrathecal administration of ZnDPBG produced a dose-dependent increase in flinching nociceptive behavior

during both phases of the formalin test (Figure 1A). The 200 nmol dose had a strong statistically significant hypernociceptive effect, while the 50 nmol dose failed to induce any significant change in nociception. The biphasic standard response was maintained but the quiescent period disappeared, being replaced by some nociception (Figure 1A).

As observed with the spinal injection (Figure 1A), the hind paw administration of ZnDPBG increased the frequency of flinching nociceptive behavior in the formalin test (Figure 1B). The higher dose of 400 nmol had a strong statistically significant hypernociceptive effect ($P < 0.05$), while 100 and 40 nmol doses did not significantly change nociception. The biphasic standard response was maintained, but the values during the first and second phases were higher than the vehicle control (Figure 1B).

Heme-lysinate and nociceptive response

The HO substrate (heme-lysinate) was administered by intrathecal injection using an L-lysine solution as the control vehicle. Solutions were injected 30 min prior to the formalin test and heme-lysinate produced a dose-related antinociception. The rats injected with 600 and 200 nmol heme-lysinate, but not 50 nmol, showed a significant decrease of the flinching nociceptive response, especially during the second phase of the formalin test (Figure 2A). Only the 600 nmol dose induced antinociception during the first non-inflammatory phase.

The hind paw injections of 100 and 300 nmol heme-lysinate had an antinociceptive effect, while the lower dose of 30 nmol induced no changes in nociception when compared to control animals injected with the heme-lysinate vehicle (L-lysine control solution; Figure 2B). The drugs were injected 20 min before the beginning of the formalin test.

Spinal-podal synergy of subactive doses of ZnDPBG and heme-lysinate

The co-administration of subactive low doses, when given alone, of both spinal (50 nmol) and peripheral (40 nmol) ZnDPBG induced hypernociception in both phases of the formalin test compared to vehicle control and single site injections (Figure 3A).

The co-administration of subactive low doses (again, when given alone) of both spinal (50 nmol) and peripheral (30 nmol) heme-lysinate induced antinociception during the second phase of the formalin test compared to control and single site injections (Figure 3B).

Discussion

The present study provides evidence that the heme oxygenase-carbon monoxide pathway plays a synergic antinociceptive role by acting on both peripheral and spinal sites. Supporting the observed data that intrathecal and hind paw injections of ZnDPBG or heme-lysinate act on the nociceptive behavior in the formalin test, the lower doses did not change the nociceptive response when the drugs were administered alone at spinal or peripheral sites, but the combination of these subactive doses resulted in synergy, i.e., hypernociception with ZnDPBG (Figure 3A) and antinociception with heme-lysinate (Figure 3B). These findings are consistent with the notion that activation of the HO pathway at the level of the spinal cord can modulate nociception originating in peripheral tissues. It is known that the association between peripheral and central sites may reduce doses and side effects. Thus, it is not surpris-

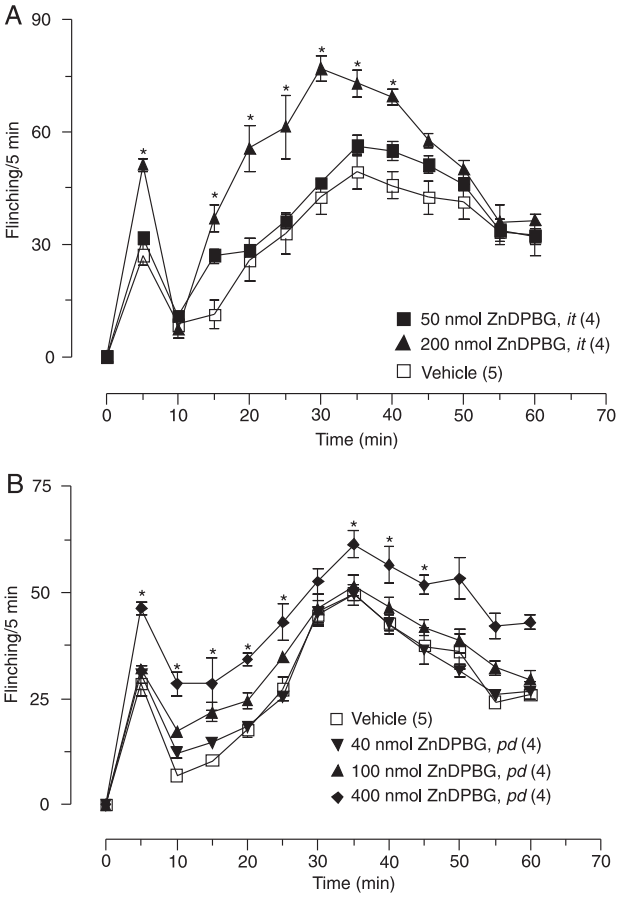

Figure 1. *A*, Hypernociceptive effect of the heme oxygenase inhibitor ZnDPBG (50 and 200 nmol) injected intrathecally (*it*) on the nociceptive response in the formalin test. *B*, Hypernociceptive effect of ZnDPBG (40, 100 and 400 nmol) in podal injections (*pd*) on the nociceptive response in the formalin test. The number of rats in each group is given within parentheses. *P < 0.05 compared to control (ANOVA followed by the Tukey *post hoc* test). ZnDPBG = zinc deuteroporphyrin 2,4-bis glycol.

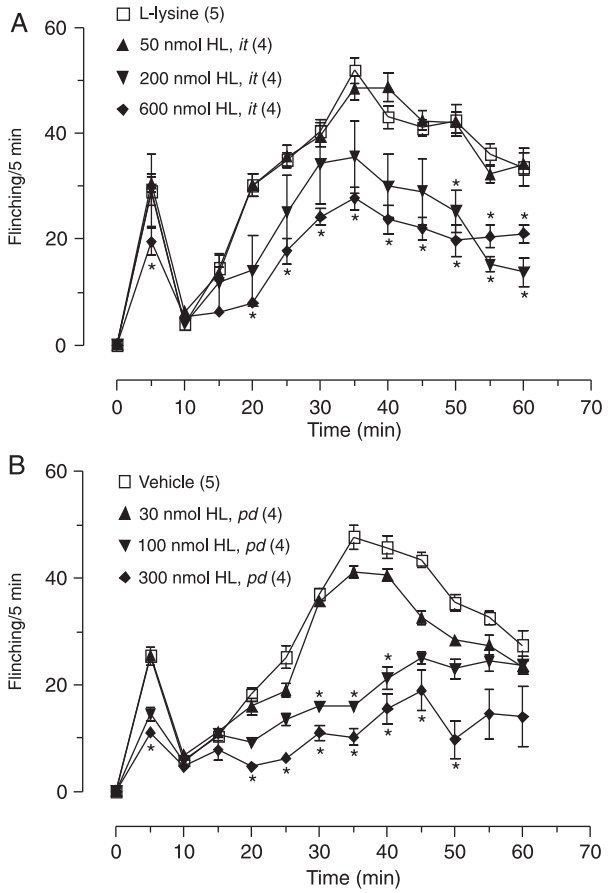

Figure 2. *A*, Antinociceptive effect of the heme oxygenase substrate heme-lysinate (HL; 50, 200 and 600 nmol) injected intrathecally (*it*) on the nociceptive response in the formalin test. *B*, Antinociceptive effect of HL (30, 100 and 300 nmol) in podal injections (*pd*) in the formalin test. The number of rats in each group is given within parentheses. *P < 0.05 compared to control (ANOVA followed by the Tukey *post hoc* test).

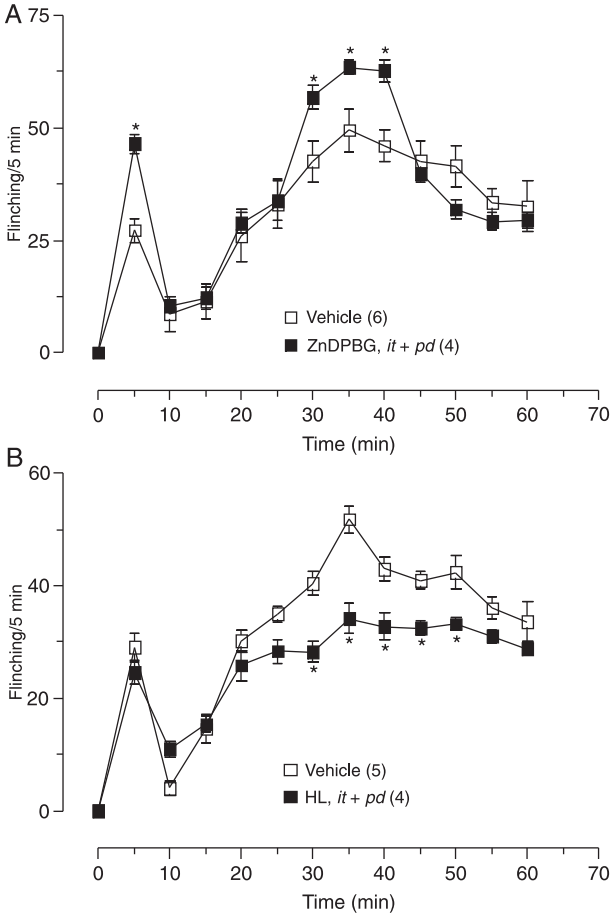

Figure 3. *A*, Synergy between spinal intrathecal (*it*) and peripheral (podal, *pd*) subactive doses (50 and 40 nmol) of ZnDPBG on the nociceptive behavioral response in the formalin test. *B*, Synergy between spinal and podal subactive doses (50 and 30 nmol) of heme-lysinate (HL) on the nociceptive behavioral response in the formalin test. Numbers within parentheses indicate the number of rats in each group. *P < 0.05 compared to control (ANOVA followed by the Tukey *post hoc* test). ZnDPBG = zinc deuteroporphyrin 2,4-bis glycol.

ing that regulation of HO activity is of great clinical interest (for a review, see Ref. 17), not only in studies related to pain but also in numerous areas of biomedical science.

Our result shows that a synergy between the two sites exists. However, there are reports in disagreement with the present data, i.e., suggesting that spinal HO inhibition reduces formalin hypernociception but not thermal nociception (18).

An acute overload of heme preparations, such as hemelysinate, has been used by ourselves (7,19) and others (2,3) to stimulate the HO pathway *in vivo*, this certainly being an important tool to investigate the physiological actions of this pathway. In agreement with our observations using a peripheral approach (11), spinal heme overload has an antinociceptive effect. HO activity leads to the production in the same molar amount of CO, biliverdine and free iron. Since CO is the only HO product that acts via soluble guanylate cyclase and the effect of heme overload could be prevented by methylene blue, it has been suggested that the HO-CO antinociceptive function is cGMP dependent, and that neither biliverdine nor iron would have a major involvement in the spinal antinociceptive role of the HO pathway (11). Most evidence supports the fact that heme overload activates the HO pathway by inducing the

transcription of HO-1 (20-23), a result that would imply that HO-1 is induced rapidly. In fact, there are data showing that HO-1 overexpression reaches maximum values within 1 h of application of the stressing stimuli (3,24). Increase in the activity of HO-2 in response to an excess of substrate, according to Michaelis-Menten kinetics, can also account for the relatively rapid development of the observed effect (3).

In the formalin test, a small amount of diluted formalin solution is injected subcutaneously into the rat hind paw, resulting in a reproducible biphasic behavioral response (25), which can be subdivided into an early and late phase. Formalin concentration determines the nociceptive effects and behavioral expression (26). The first phase (phase 1, 0-5 min) seems to be caused by the initial tissue injury and direct activation of peripheral small afferent C fibers by formalin. The second phase (phase 2, 20-60 min) is mediated by a low level of primary afferent fiber activity secondary to the inflammatory reaction in the peripheral tissue, whose effects are then enhanced at the spinal level by central sensitization (26). It is relevant to note that while high doses of the spinal or hind paw heme-lysinate pretreatment changed both the first and second phases of the nociceptive response to subcutaneous injection of formalin, the low dose synergy modulated only the second phase. Pain behaviors in phase 1 are primarily mediated by a direct effect of injury and chemical stimulation of peripheral nociceptors, and phase 2 behaviors are generated by the ongoing stimulation of nociceptors by inflammatory mediators and/or by first phase-induced spinal cord excitability as central sensitization (27-29). Evidence exists that sustained peripheral nerve input and neurogenic tissue-mediated components are required for the expression of the second phase (30). The heme-lysinate antinociceptive effect probably results from an action of CO on the peripheral nociceptor, first synapse in the spinal cord and/or on second-order afferent neurons, increasing their intracellular cGMP levels. Thus, the action of the HO pathway may involve not only modulation of inflammatory mediator release or activity, but also changes in nociceptors, fiber excitability and spinal sensitization.

Since the beginning of the 1990's, a growing body of evidence has given support to the physiological actions of the gaseous compound CO, which has been shown to be a vasoactive substance and to act as a neurotransmitter/neuromodulator (1,4,31). In agreement, evidence has been accumulated demonstrating that the HO-CO-cGMP pathway plays a role in mechanical hypernociception (10) and in the formalin test (11). Now the knowledge has been extended to the possible functional synergy between the spinal and peripheral HO-CO pathway in the nociceptive response.

The HO enzyme inhibitor ZnDPBG prevents the cleavage of the heme molecule, and thus the production of endogenous biliverdine, free iron and carbon monoxide (3). Direct intrathecal or hind paw injection of ZnDPBG produced hypernociception, characterized by the increase of the flinching nociceptive response to the formalin test (Figure 1). The treatment with the low no-effect doses at both spinal and peripheral sites induced a hypernociceptive response to the formalin test, suggesting that a synergic effect could be involved (Figure 3).

The hypernociceptive behavioral response affected both phases of the formalin test, increasing the total number of flinches during the 5-min intervals studied. The ZnDPBG-induced hypernociception during the first phase suggests that HO inhibition may have some effect on excitability, synaptic transmission or transduction velocity of sensory neurons when stimulated by noxious chemicals. Treatment with ZnDPBG did not change the shape or the relationship between the first and the second inflammatory phase. Pretreatment with ZnDPBG vehicle, i.e., Na_2CO_3, and saline (volume control only) had no effect on flinching behavior (Figure 1).

The heme-lysinate antinociceptive synergy could only be observed with the response during the second phase of the formalin test (Figure 2), suggesting that inflammatory hypernociception is more sensitive to the activity of the heme oxygenase-carbon monoxide pathway than the first phase.

Since synergy was observed between the spinal and peripheral treatments (Figure 3), one can suggest that functional summation may occur with the different subactive low doses at the two different sites of injection. Thus, the reduced peripheral nociceptive input and the prevented central spinal sensitization could be working together, resulting in antinociception. Synergic interactions have been observed between other different drugs and systems (32,33). Cannabinoid antinociceptive synergy between topical and spinal sites has already been reported, showing that peripheral-spinal integration is not unlikely to occur (34). The synergy of one antinociceptive drug can reduce the systemic side effects by the reduction of the therapeutic doses.

There are studies showing that nociceptor activity and excitability may be modulated by intracellular cGMP (35-37). Pharmacological evidence suggests that cGMP can increase K^+ conductance by opening K_{ATP} channels directly or indirectly via protein kinase G (37,38). Since the antinociceptive potential of CO is cGMP dependent (11), we can speculate that the HO-CO pathway may act via ion channels or synaptic transmission modulation.

The present study provides evidence that functional synergy occurs between the spinal and peripheral HO pathway, modulating the nociceptive response in the formalin test, and acting as an antinociceptive mechanism. Inhibition of the HO pathway leads to hypernociception, whereas its induction results in antinociception during the nociceptive and inflammatory phases of the formalin test.

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