British Journal of Pharmacology (2001) 132, 1673–1682

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Role of the haeme oxygenase/carbon monoxide pathway in mechanical nociceptor hypersensitivity

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1 The cleavage of haeme by haeme oxygenase (HO) yields carbon monoxide (CO), a biologically active molecule which exerts most of its effects *via* activation of soluble guanylate cyclase (sGC). In the present study, we tested the hypothesis that endogenous CO could modulate inflammatory hyperalgesia. The intensity of hyperalgesia was investigated in a model of mechanical nociceptor hypersensitivity in rats.

2 The intra-plantar (i.pl.) administration of the HO inhibitor, ZnDPBG (Zinc deuteroporphyrin 2,4-bis glycol), potentiated in a dose-dependent manner the mechanical nociceptor hypersensitivity evoked by i.pl. administration of carrageenan.

3 The mechanical hypersensitivity evoked by i.pl. injection of interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), but not interleukin-8 (IL-8), prostaglandin E_2 (PGE_2) or dopamine, was also enhanced by ZnDPBG.

4 Moreover, the haeme (HO substrate) injection in the paws reduced the hypersensitivity evoked by IL-1β, but not PGE_2. Furthermore, i.pl. administration of the gas CO reduced the hypersensitivity elicited by PGE_2.

5 The inhibitory effect of haeme and CO upon mechanical nociceptor hypersensitivity were counteracted by a soluble guanylate cyclase (sGC) inhibitor, ODQ (1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one), suggesting that this effect of CO is mediated *via* cyclic GMP.

6 Finally, the inhibitory effect of CO upon mechanical nociceptor hypersensitivity was prevented by the NO synthase blocker, L-NMMA (N^G-monomethyl L-arginine), suggesting that the impairment of mechanical hypersensitivity elicited by CO depends on the integrity of the NO pathway.

7 In conclusion, the results presented in this paper imply that endogenously CO produced by HO plays an anti-hyperalgesic role in inflamed paws, probably by increasing the intracellular levels of cyclic GMP in the primary afferent neurone.

British Journal of Pharmacology (2001) **132,** 1673–1682

Keywords: Inflammatory hyperalgesia; cyclic GMP; carbon monoxide; cytokines; nitric oxide; soluble guanylate cyclase; carrageenan; Interleukin-1β (IL-1β); tumour necrosis factor-α (TNF-α)

Abbreviations: CO, carbon monoxide; HO, Haeme oxygenase; IL-1β, interleukin-1 beta; IL-8, interleukin-8; L-NMMA, N^G-monomethyl L-arginine; NO, nitric oxide; ODQ, 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one; PGE_2, prostaglandin E_2; TNF-α, tumour necrosis factor alpha; sGC, soluble guanylate cyclase; ZnDPBG, zinc deuteroporphyrin 2,4-bis glycol

Introduction

It has been long recognized that the gaseous compound carbon monoxide (CO) is noxious and harmful. However, the notion that CO is only a poison has been challenged since 1949 when Sjostrand (1949) reported that CO could be endogenously synthesized, but it was not until 1991 that Marks *et al.* (1991) assumed that CO could have a physiological function. Currently, CO is recognized as an important signalling molecule in cardiovascular system, being a vasoactive substance (Dawson & Snyder, 1994; Johnson *et al.*, 1999) and reducing the expression of endothelin-1 in endothelial cells (Morita & Kourembanas, 1995). CO has also been recognized to act as a neurotransmitter or neuromodulator in the nervous system. Accordingly, it has been reported that CO inhibits the release of immunoreactive IL-1β from the rat hypothalamus *in vitro* (Mancuso *et al.*, 1998), as well as the release of oxytocin (Kostoglou-Athanassiou *et al.*, 1996) and vasopressin (Mancuso *et al.*, 1997; Kostoglou-Athanassiou *et al.*, 1998), although its effect on CRH appears to vary according to the precise experimental condition (Parkes *et al.*, 1994; Pozzoli *et al.*, 1994; Kostoglou-Athanassiou *et al.*, 1998). Furthermore, CO appears to be a mediator of endotoxin fever in the central nervous system (Steiner *et al.*, 1999; Steiner & Branco, 2000).

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There is evidence that CO stimulates soluble guanylate cyclase (sGC) activity and increases the cellular levels of cyclic GMP (Maines, 1993; 1997; Morita *et al*., 1995).

Endogenous CO arises mainly from the cleavage of the haeme molecule producing biliverdin, free iron and CO, a process catalyzed by the enzyme haeme oxygenase (HO) (Maines, 1997). Three HO isoforms have been identified to date: HO-1, HO-2, and HO-3, among which the isoforms 1 and 2 are the best known (Ewing *et al*., 1992; Maines, 1997). Both isoenzymes, one of them inducible (HO-1) and the other constitutive (HO-2), have been found in various tissues, including endothelial cells, smooth muscle and neural tissue (Dawson & Snyder, 1994; Maines, 1997; Johnson *et al*., 1999). The expression of HO-1 is increased in the site of experimental inflammation or inflammatory diseases such as arthritis and sepsis (Willis, 1999). Sensitization of the primary afferent neurone is the common denominator of all types of 'inflammatory pain'. Following such sensitization, previously ineffective stimuli cause 'overt pain' in humans, or a characteristic animal behaviour useful as an end point in nociceptive tests (Handwerker, 1976; Perl, 1976). The hyperalgesia measured by an increase in the mechanical nociceptor sensitivity is thought to result from the release of a cascade of cytokines (Ferreira *et al*., 1988; Cunha *et al*., 1991) and consequent production and release of the hyperalgesic mediators, the eicosanoids, i.e., prostaglandins (PGE_2, PGI_2) and sympathomimetic mediators, which in turn act directly in the nociceptor to produce the response (Cunha *et al*., 1992).

The molecular events associated with mechanical nociceptor hypersensitivity triggered by the hyperalgesic mediators (i.e., prostanoids and sympathomimetic amines) are dependent on cyclic nucleotides. Our research group and others have provided experimental evidence to suggest that up- or down-functional regulation of the primary sensory neuron sensitivity to mechanical stimulation may result from a neuronal balance of Ca^{2+}/cyclic AMP and cyclic GMP concentrations, respectively. Accordingly, cyclic AMP has been shown to mediate mechanical nociceptor hypersensitivity in response to mechanical stimulation (Ferreira & Nakamura, 1979; Taiwo *et al*., 1989; Taiwo & Levine, 1991), whereas cyclic GMP seems to counteract the effects of cyclic AMP, producing antinociception (Taiwo *et al*., 1989; Follenfant *et al*., 1990; Ferreira *et al*., 1991; Ferreira & Lorenzetti, 1994; Wang *et al*., 1996). Therefore, we hypothesized that an increase in cyclic GMP levels in the primary sensory neuron by NO could block the onset of mechanical nociceptor hypersensitivity.

Taking into account that the HO-1/CO pathway is upregulated in the inflammatory site (Willis, 1995; 1999; Willis *et al*., 1996) and that CO activates sGC and promotes an increase in cyclic GMP (Maines, 1993; 1997; Morita *et al*., 1995), we tested the hypothesis that endogenously formed CO could down-regulate mechanical nociceptor hypersensitivity. In addition, in view of the suggested coordinated interaction between the CO and the NO pathway (Kitamura *et al*., 1998a,b; Matsuoka *et al*., 1994; Sato *et al*., 1998; Ingi *et al*., 1996; Throup *et al*., 1999), we also investigated whether the possible role of the CO pathway in mechanical nociceptor hypersensitivity depends on the integrity of the NO pathway or vice-versa.

Methods

Animals

Male Wistar rats weighing 180–200 g were housed in temperature-controlled rooms (22–25°C) with water and food *ad libitum* until use.

Nociceptive tests

Mechanical nociceptor hypersensitivity Mechanical nociceptor hypersensitivity was tested in rats (Ferreira *et al*., 1978). A constant pressure of 20 mmHg (measured using a sphygmomanometer) was applied (*via* a syringe piston moved by compressed air) to an area of 15 mm^2 of the dorsal surface of the hind paws of rats and discontinued when they presented a typical 'freezing reaction'. The freezing reaction was signalized by a brief apnoea, concomitant with a retraction of the head and forepaws and a reduction in the escape movements that animals frequently make to escape from the position imposed by the experimental situation. Usually, the apnoea was associated with successive waves of muscular tremor. For each animal, the latency to the onset of the freezing reaction (from the time of first application of the pressure) was measured before administration (zero time) and at different times after administration of the hyperalgesic agents. The intensity of mechanical hypersensitivity was quantified as the reduction in reaction time, calculated by subtracting the value of the second measurement from the first (Ferreira *et al*., 1978). Reaction times were typically 32–34 s (with standard errors of the mean [s.e.m.] of 0.5–1.0 s) before injection of the hyperalgesic agents. Multiple paw treatments with control solutions did not alter basal reaction times. Different individuals prepared the solutions to be injected, made the injections, and measured the reaction times.

Experimental protocols

Effect of pre-treatment of the animals with the HO inhibitor, ZnDPBG, on the mechanical nociceptor hypersensitivity induced by sub-maximal doses of carrageenan Mechanical hypersensitivity was measured 1, 2, 3 and 4 h after the injection of a sub maximal dose of carrageenan (Cg, 30 $\mu g\ paw^{-1}$), injected in a final volume of 100 μL, into the hind paws (intraplantar, i.pl.) of rats. It was used as a sub maximal dose of carrageenan to allow measurements of a possible potentiating effect of ZnDPBG (Cunha *et al*., 1999a). Zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG at 50, 100, 200 and 400 nmoles) was diluted in 100 μl of sodium carbonate buffer (50 mM) and injected i.pl. 5 min before carrageenan administration. The control animals (C) were i.pl. injected with sodium carbonate buffer 5 min before the hyperalgesic stimulus or with ZnDPBG (200 nmoles in 100 μl) 5 min before the administration of saline (100 μl).

Effect of pre-treatment of the animals with the HO inhibitor, ZnDPBG, on the mechanical nociceptor hypersensitivity induced by TNF-α, IL-1β, IL-8, PGE_2 and dopamine Mechanical hypersensitivity was measured 3 h after the injection of sub maximal doses of tumour necrosis factor-α (TNF-α, 0.8 pg paw^{-1}), interleukin-1β (IL-1β, 0.15 pg paw^{-1}), inter-

leukin-8 (IL-8, 0.03 ng paw^{-1}), prostaglandin E_2 (PGE_2, 30 ng paw^{-1}), or dopamine (30 μg paw^{-1}), each injected in a final volume of 100 μl, into the hind paws (intraplantar, i.pl.) of rats. It was used as a sub maximal dose of the hyperalgesic substances to allow measurements of a possible potentiating effect of ZnDPBG (Cunha *et al*., 1999a; 2000). ZnDPBG at the dose of 200 nmoles paw^{-1} was i.pl. administrated 5 min before TNF-α, IL-1β, IL-8, PGE_2 or dopamine. The control animals (C) were i.pl. injected with sodium carbonate buffer 5 min before the hyperalgesic stimuli.

Effect of treatment of the animals with the haeme (substrate) preparation, haeme-lysinate, on the mechanical nociceptor hypersensitivity induced by IL-1β and PGE_2 Mechanical hypersensitivity was measured 3 h after injection of prostaglandin E_2 (PGE_2, 100 ng paw^{-1}) or interleukin-1β (IL-1β, 0.5 pg paw^{-1}), each injected in a final volume of 100 μl into the hind paws (intraplantar, i.pl.) of rats. The doses of the hyperalgesic agents were the smallest that evoked maximum responses and they were chosen to allow measurements of a possible anti-mechanical nociceptor hypersensitivity effect of the haeme-hysinate (Ferreira *et al*., 1988; Cunha *et al*., 1991, 1992). Haeme-lysinate (30, 100 and 300 nmoles), prepared as previously described (Lindén *et al*., 1987; Tenhumen *et al*., 1987), was injected i.pl. 1 h after PGE_2 or IL-1β administration in a final volume of 100 μl. Haeme-free preparations were used as amino acid (L-lysine) vehicle control solution (C).

Effect of ODQ (1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one) on the anti-mechanical nociceptor hypersensitivity effect of haeme-lysinate Rats were injected with IL-1β (0.5 pg in 100 μl, i.pl.) into the hind paws. ODQ (8 μg in 50 μl, i.pl.) or 1% DMSO (vehicle, 50 μl, i.pl.) were injected in the same hind paws 30 min after IL-1β injection. Thirty minutes after injection of ODQ or its vehicle, haeme-lysinate (300 nmoles in 50 μl, i.pl.) was injected in the hind paws that had been injected previously with IL-1β. The mechanical hypersensitivity was measured 3 h after IL-1β injection.

Effect of treatment of the animals with CO on the mechanical nociceptor hypersensitivity induced by PGE_2 Prostaglandin E_2 (PGE_2, 100 ng) was injected in 100 μl into the hind paws (i.pl.) of rats. Two hours after PGE_2 injection, 100 μl of the carbon monoxide or nitrogen (control) gases were injected i.pl. and the mechanical hypersensitivity was determined 15, 30, 60 and 120 min after gas injections.

Effect of ODQ on the anti-mechanical nociceptor hypersensitivity effect of carbon monoxide Rats were injected with PGE_2 (100 ng in 100 μl, i.pl.) into the hind paws. ODQ (8 μg in 50 μl, i.pl.) or 1% DMSO (vehicle, 50 μl, i.pl.) were injected in the same hind paws 30 min after PGE_2 injection. Thirty minutes after injection of ODQ or its vehicle, carbon monoxide (CO, 100 μl) was injected in the hind paws that had been injected previously with PGE_2. To inject the CO or N_2 gases into the rat paw, a needle was used connected to a polyethylene tube (PE-30) which was connected to a flowmeter attached to a gas tank. The mechanical hypersensitivity was determined 15, 30, 60 and 120 min after CO injection.

Effect of the NO synthase inhibitor, L-NMMA, on the anti-mechanical nociceptor hypersensitivity effect of CO or haeme-lysinate Rats were injected with PGE_2 (100 ng in 100 μl, i.pl.) or IL-1β (0.5 pg in 100 μl, i.pl.) into the hind paws. L-NMMA (50 μg in 50 μl, i.pl.) or saline (control, C, 50 μl, i.pl.) were injected in the same hind paws 30 min after PGE_2 or IL-1β. Thirty minutes later, CO (100 μl) or haeme-lysinate (300 nmoles in 50 μl, i.pl.) were then i.pl. injected in the same hind paws, respectively. The mechanical hypersensitivity was determined 30 or 90 min after CO or haeme-lysinate injections, respectively.

Effect of ZnDPBG on the anti-mechanical nociceptor hypersensitivity effect of SNAP (S-nitroso-N-acetyl-D, L-penicillamine) Rats were injected with PGE_2 (100 ng in 100 μl, i.pl.) or IL-1β (0.5 pg in 100 μl, i.pl.) into the hind paws. ZnDPBG (300 nmoles in 50 μl, i.pl.) or its vehicle (control, 50 μl, i.pl.) were injected in the same hind paws 50 min after PGE_2 or IL-1β injection. Ten minutes later, SNAP (200 μg in 50 μl, i.pl.) was also injected in the same hind paws. The mechanical hypersensitivity was determined 3 h after the PGE_2 or IL-1β administration.

Drugs

The following drugs were used: Prostaglandin E_2 (Sigma Chemical Co., St. Louis, U.S.A.), dopamineHCl, USP grade (Research Biochemicals International-RBI), carrageenan (BHD Chemical, U.K.), 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxaline-1-one (ODQ) (Trocris Cookson, St. Louis, U.S.A.), zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) and Hemin (Porphyrin Products, Logan, Utah, U.S.A.), N^G-monomethyl-L-arginine (L-NMMA) and SNAP (BHI, St. Louis, U.S.A.), CO and nitrogen (AGA, Sao Paulo, Brazil).

Cytokines

Recombinant human IL-1β, IL-8 and TNFα (National Institute for Biological Standards and Control, NIBSC, preparations coded 86/680, 89/520, 87/650, respectively). The specific activities of these materials are IL-1β: 100,000 IU 1 μg^{-1} per ampoule; IL-8: 1000 IU 1 μg^{-1} per ampoule; TNFα: 40,000 IU 1 μg^{-1} per ampoule.

Statistical analysis

Results are presented as means$\pm$s.e.mean of at least five animals in each group. Differences between responses were evaluated by ordinary ANOVA followed by the Bonferroni *t*-test. Values with $P<0.05$ were considered to be significantly different.

Results

Effect of ZnDPBG on the mechanical nociceptor hypersensitivity response to carrageenan

The injection of carrageenan (30 μg) into the hind paw of rats evoked a small mechanical nociceptor hypersensitivity effect, measured at 1, 2, 3, and 4 h. ZnDPBG, a nonspecific HO inhibitor (50, 100, 200 and 400 nmoles paw^{-1}), injected

i.pl. into the same paw 5 min before carrageenan potentiated in a dose-dependent manner the carrageenan-evoked mechanical nociceptor hypersensitivity determined 3 h after the injection of the carrageenan (Figure 1A). The carrageenan-evoked mechanical nociceptor hypersensitivity was also potentiated by ZnDPBG (200 nmoles paw^{-1}) when the mechanical hypersensitivity was also determined 1, 2, 3 and 4 h after carrageenan injection (Figure 1B). The i.pl. administration of ZnDPBG did not change the mechanical nociceptor hypersensitivity induced by i.pl. injection of saline.

Effect of ZnDPBG on the mechanical nociceptor hypersensitivity response to TNFα, IL-1β, IL-8, dopamine and PGE_2

Next, we investigated whether the potentiating effects of ZnDPBG occurred in response to hyperalgesic mediators. The mechanical nociceptor hypersensitivity induced by i.pl. injection of sub-maximal doses of the cytokines TNF-α (0.8 pg paw^{-1}) and IL-1β (0.15 pg paw^{-1}), but not IL-8 (0.05 ng paw^{-1}) was augmented by the pretreatment of the paw with ZnDPBG (200 nmoles paw^{-1}). Conversely, the mechanical nociceptor hypersensitivity induced by i.pl. injection of sub-maximal doses of dopamine (30 μg paw^{-1}) or PGE_2 (30 ng paw^{-1}) was not affected by i.pl. pretreatment with ZnDPBG (200 nmoles paw^{-1}) (Figure 2).

Effect of haeme-lysinate (HO substrate) on the mechanical nociceptor hypersensitivity response to PGE_2 or IL-1β

In order to investigate whether the HO/CO pathway is triggered during mechanical nociceptor hypersensitivity, we examined the effect of the HO substrate on the mechanical nociceptor hypersensitivity produced by PGE_2 and by IL-1β. The mechanical nociceptor hypersensitivity induced by i.pl. administration of PGE_2 (100 ng paw^{-1}) was not affected by i.pl. administration in the same paw with haeme-lysinate at doses of 30, 100 or 300 nmoles. On the other hand, the mechanical nociceptor hypersensitivity induced by IL-1β (0.5 pg paw^{-1}) was inhibited in a dose-dependent manner by haeme-lysinate (30, 100 and 300 nmoles paw^{-1}) administrated in the same paw 1 h after the cytokine injection. The mechanical hypersensitivity was determined 3 h after the cytokine administration. In order to investigate whether the anti-nociceptive effect of the HO/CO pathway was mediated

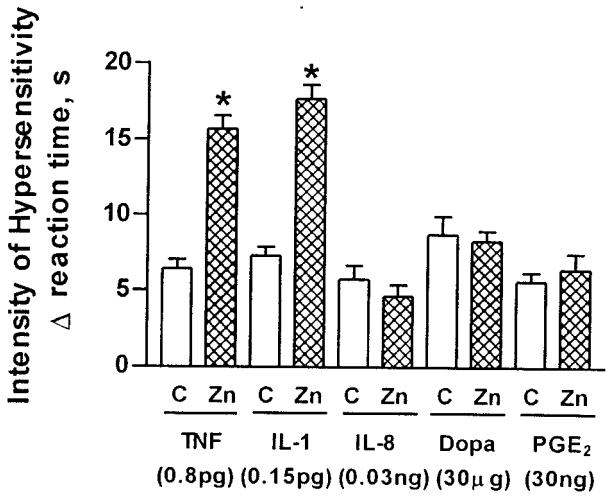

Figure 2 Effect of local administration of ZnDPBG on the mechanical nociceptor hypersensitivity responses to TNF-α, IL-1β, IL-8, dopamine and PGE_2. Mechanical hypersensitivity responses were measured 3 h after injection of sub-maximal doses of TNF-α (0.8 pg in 100 μl, i.pl.), IL-1β (0.15 pg in 100 μl, i.pl.), IL-8 (0.03 ng in 100 μl, i.pl.), dopamine (30 μg in 100 μl, i.pl.) and PGE_2 (30 ng in 100 μl, i.pl.). ZnDPBG (200 nmoles in 100 μl, i.pl.) or its vehicle (control, C, 100 μl, i.pl.) were given 5 min before the hyperalgesic mediators. Results are expressed as means±s.e.mean in groups of five rats. *$P<0.005$ compared with the respective control (ANOVA, followed by Bonferroni *t*-test).

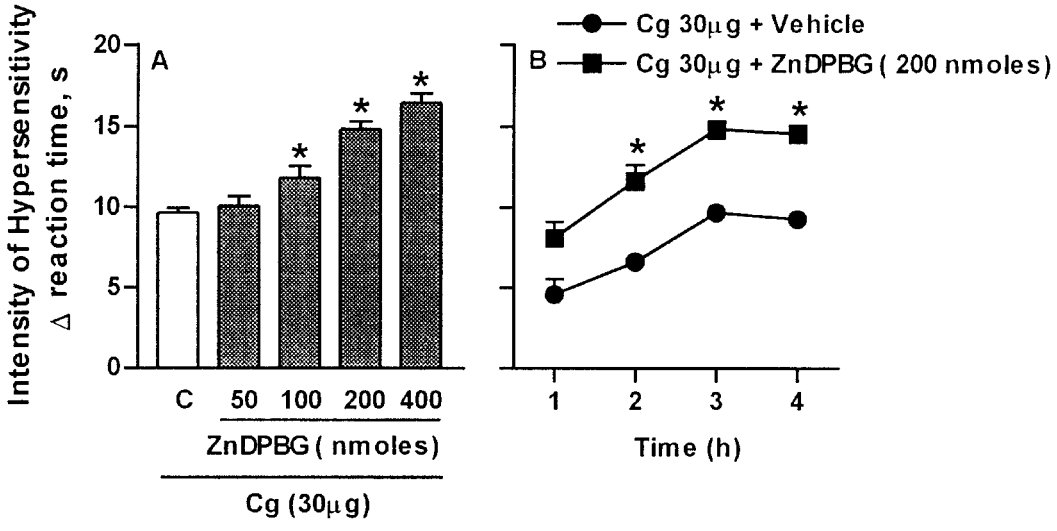

Figure 1 Effect of ZnDPBG on the mechanical nociceptor hypersensitivity response to injection (in 100 μl, i.pl.) of carrageenan (Cg, 30 μg). Vehicle (control, 100 μl, C) or ZnDPBG at doses of 50–400 nmoles, in 100 μl (A) and 200 nmoles in 100 μl (B) were injected i.pl. into paws to be injected 5 min later with carrageenan. The intensity of mechanical hypersensitivity was measured 3 h after (A), or 1, 2, 3 and 4 h after (B) injection of carrageenan. Results are expressed as means±s.e.mean in groups of five rats. *$P<0.005$ compared with the respective control (ANOVA followed by Bonferroni *t*-test).

by cyclic GMP, we used the soluble guanylate cyclase (sGC) inhibitor ODQ (8 μg paw^{-1}). The anti-mechanical nociceptor hypersensitivity effect of the haeme-lysinate was completely blocked by the pre-treatment of the paw (30 min before haeme-lysinate injection) with ODQ (Figure 3).

Effect of CO on the mechanical nociceptor hypersensitivity response to PGE_2

The mechanical nociceptor hypersensitivity induced by i.pl. administration of PGE_2 (100 ng paw^{-1}) was significantly attenuated by CO (100 μl) injected in the same paw 1 h after PGE_2 injection. The anti-mechanical nociceptor hypersensitivity effect of CO was already observed 15 min after the CO administration and vanished 1 h afterwards. The i.pl. administration of the gas nitrogen (100 μl, control) did not affect the PGE_2–induced mechanical nociceptor hypersensitivity. As seen with haeme-lysinate, the anti-mechanical nociceptor hypersensitivity effect of CO was also completely inhibited by the pre-treatment of the paw (30 min before CO injection) with ODQ (8 μg paw^{-1}, Figure 4).

Effect of the NO synthase inhibitor, L-NMMA, on the anti-mechanical nociceptor hypersensitivity effects of CO and haeme-lysinate

In these experiments, mechanical nociceptor hypersensitivity was produced by i.pl. injection of PGE_2 (100 ng paw^{-1}) or IL-1β (0.5 pg paw^{-1}). CO (100 μl) or haeme-lysinate (200 nmoles) injected in the same paw 60 min after PGE_2 or IL-1β reduced the mechanical nociceptor hypersensitivity responses determined 30 and 90 min after administration of PGE_2 and IL-1β, respectively, confirming the results of the previous experiments. In order to investigate whether the anti-mechanical nociceptor hypersensitivity effects of CO and

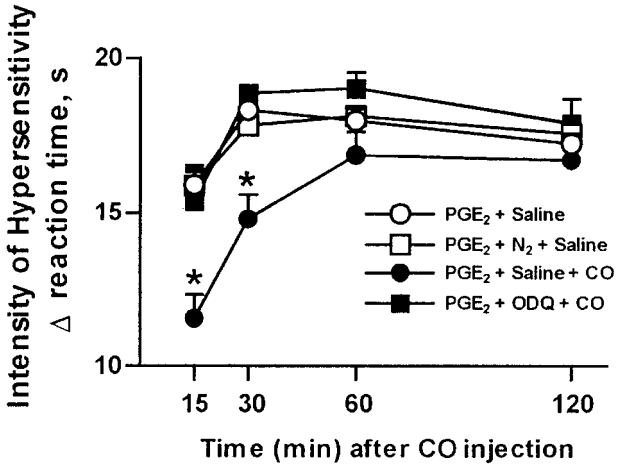

Figure 4 Effect of local administration of carbon monoxide on the mechanical nociceptor hypersensitivity responses to PGE_2. Carbon Monoxide (CO, 100 μl, i.pl.), nitrogen (control, N_2, 100 μl, i.pl., control) or saline (control, 100 μl, i.pl.) were given 1 h after the administration of PGE_2 (100 ng in 100 μl, i.pl.). The mechanical hypersensitivity responses were measured 15, 30, 60 and 120 min after CO administration. The effect of ODQ on the anti-mechanical nociceptor hypersensitivity effect of CO is also shown. ODQ (8 μg in 50 μl, i.pl.) or saline were given 30 min before CO (100 μl) administration. Results are expressed as means$\pm$s.e.mean in groups of five rats. *$P<0.005$ compared with the respective control (ANOVA, followed by Bonferroni t-test).

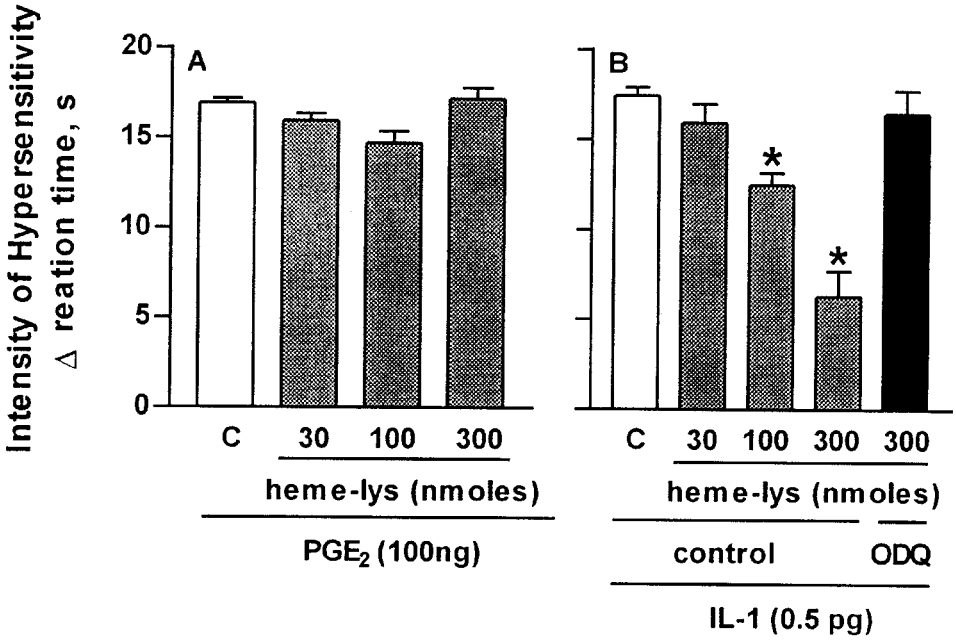

Figure 3 Effect of local administration of haeme-lysinate on the mechanical nociceptor hypersensitivity responses to PGE_2 and IL-1β. Mechanical nociceptor hypersensitivity responses were measured 3 h after PGE_2 (100 ng in 100 μl, i.pl., A) or IL-1β (0.5 pg in 100 μl, i.pl., B) injections. Haeme-lysinate (30–300 nmoles in 50 μl, i.pl.) or L-lysine, vehicle control solution (control, C, 50 μl, i.pl.) were given 1 h after the administration of the hyperalgesic mediators. B also shows the effect of ODQ on the anti-mechanical nociceptor hypersensitivity effect of haeme-lysinate. ODQ (8 μg in 50 μl, i.pl.) or saline (control) were given 30 min before the haeme-lysinate (300 nmoles) administration. Results are expressed as means$\pm$s.e.mean in groups of five rats. *$P<0.005$ compared with the respective control (ANOVA, followed by Bonferroni t-test).

haeme-lysinate depend on the integrity of the NO pathway, we used the NO synthase inhibitor L-monomethyl-L-arginine (L-NMMA, 50 μg^1 paw^{-1}). As shown in Figure 5, the anti-mechanical nociceptor hypersensitivity effects of CO and haeme-lysinate were blocked by pre-treatment of the paw (30 min before CO or haeme-lysinate) with L-NMMA. L-NMMA did not affect the mechanical nociceptor hypersensitivity effects of PGE_2 or IL-1β at the dose used.

Effect of ZnDPBG on the blockade of nociceptor hypersensitivity by the NO donor SNAP

Mechanical nociceptor hypersensitivity was evoked by i.pl. injection of PGE_2 (100 ng paw^{-1}) or IL-1β (0.5 pg paw^{-1}) and determined 3 h later. SNAP (200 μg paw^{-1}) injected in the same paw 60 min after PGE_2 or IL-1β reduced the mechanical nociceptor hypersensitivity responses. In order to investigate whether the anti-mechanical nociceptor hypersensitivity effects of SNAP were mediated by CO production, we used ZnDPBG (200 nmoles paw^{-1}). The antinociceptive effects of SNAP were not altered by pre-treatment of the paw (30 min before SNAP) with ZnDPBG (Figure 6).

Discussion

There is experimental evidence to suggest that modification of the intracellular levels of cyclic AMP or cyclic GMP in primary afferent neurones modulates hyperalgesia. Overall, the genesis of the inflammatory nociceptor sensitization is dependent on the balance between the intracellular concentrations of these cyclic nucleotides: elevation of levels of cyclic AMP are associated with enhancement of nociceptor hypersensitivity, whereas elevation of levels of cyclic GMP are associated with inhibition of nociceptor hypersensitivity (Ferreira & Nakamura, 1979; Taiwo *et al.*,, 1989; Duarte *et al.*, 1990; Ferreira *et al.*, 1991; Taiwo & Levine, 1991; Kress *et al.*, 1996; Wang *et al.*, 1996; Cunha *et al.*, 1999b).

In the present study we demonstrated that the HO/CO pathway plays an anti-mechanical nociceptor hypersensitivity role during carrageenan-evoked inflammation. Supporting this hypothesis, we observed that treatment with the nonselective inhibitor of the HO, ZnDPBG (Vreman *et al.*, 1991; Appleton *et al.*, 1999), enhanced in a dose-dependent manner the mechanical nociceptor hypersensitivity induced by a sub-maximal dose of carrageenan. In order to further investigate the role of the HO/CO pathway during inflammatory hyperalgesia, the effect of ZnDPBG on the mechanical nociceptor hypersensitivity induced by the inflammatory mediators TNF-α, IL-1β, IL-8, dopamine or PGE_2 was also investigated. Accordingly, the mechanical nociceptor hypersensitivity induced by sub-maximal doses of the cytokines TNF-α or IL-1β, but not of IL-8, were enhanced by ZnDPBG, suggesting that the HO/CO pathway is activated by TNF-α and IL-1β, but not IL-8. In fact, TNF-α and IL-1β have been reported to induce HO-1 and have been suggested to mediate the overexpression of HO-1 produced by LPS (Rizzardini *et al.*, 1993). Thus, these *in vitro* evidences suggest that HO-1 is the HO isoform involved in the inhibition of mechanical nociceptor hypersensitivity, being induced by the inflammatory mediators TNF-α and IL-1β, but not IL-8. Furthermore, the expressions of TNF-α, IL-1β and of HO-1 are enhanced in carrageenan-induced inflammation (Willis, 1995; 1999; Willis *et al.*, 1996). Corroborating with this hypothesis are our findings that ZnDPBG did not affect the mechanical nociceptor hypersensitivity induced by the

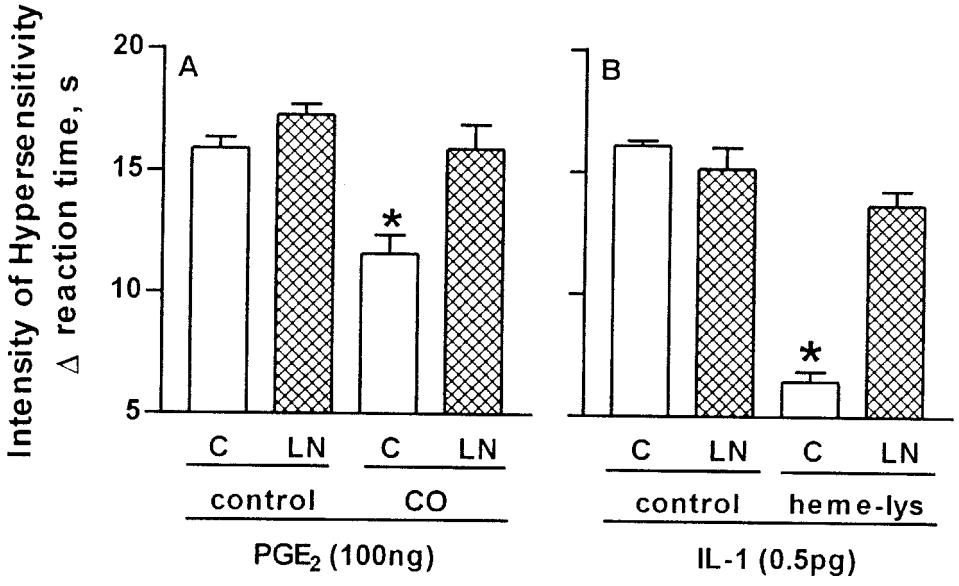

Figure 5 Effect of L-NMMA, a NO synthase inhibitor, on the anti-mechanical nociceptor hypersensitivity effects of CO and haeme-lysinate. Rats were injected with PGE_2 (100 ng in 100 μl, i.pl.) or IL-1β (0.5 pg in 100 μl, i.pl.) and 30 min later received in the same paw saline (C, 50 μl, i.pl.) or L-NMMA (LN, 50 μg/50 μl, i.pl.). After a further 30 min interval, N_2 (control) and CO (A, 100 μl, i.pl.), or L-lysine (control) and haeme-lysinate (B, 300 nmoles in 50 μl, i.pl.) were injected in the same paw previously injected with PGE_2 or IL-1β, respectively. The mechanical hypersensitivity was determined 30 and 90 min after PGE_2 or IL-1β injection, respectively. Results are expressed as means$\pm$s.e.mean in groups of five rats; $^*P<0.005$ compared with the respective control (ANOVA, followed by Bonferroni *t*-test).

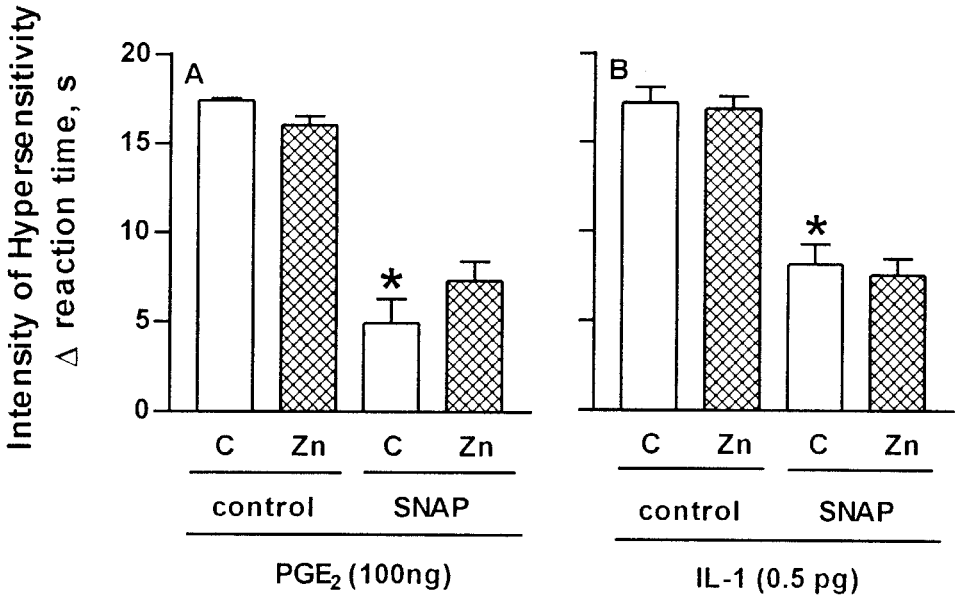

Figure 6 Effect of ZnDPBG on the anti-mechanical nociceptor hypersensitivity effect of SNAP. Rats were injected with PGE_2 (100 ng in 100 μl, i.pl.) or IL-1β (0.5 pg in 100 μl, i.pl.) and, 50 min later, received in the same paw vehicle (C, 50 μl, i.pl.) or ZnDPBG (Zn, 200 nmoles in 50 μl, i.pl.). After a further 10 min interval, saline (Control, 50 μl, i.pl.) or SNAP (200 μg in 50 μl, i.pl.) were injected in the same paw previously injected with PGE_2 or IL-1β. The mechanical hypersensitivity was determined 3 h after PGE_2 or IL-1β injection. Results are expressed as means $\pm$ s.e.mean in groups of five rats; $^*P<0.005$ compared with the respective control (ANOVA, followed by Bonferroni *t*-test).

hyperalgesic mediators, dopamine and PGE_2, that act directly in the primary sensitive neurone.

Confirming that the HO/CO pathway limits mechanical nociceptor hypersensitivity, it was demonstrated that the administration of haeme-lysinate (HO substrate) into the rat paws significantly attenuated in a dose-dependent manner the ongoing IL-1β-evoked mechanical nociceptor hypersensitivity, whereas haeme-lysinate was ineffective during PGE_2-induced mechanical nociceptor hypersensitivity, reinforcing the theory that the inhibition of the nociceptor hypersensitivity by HO/CO pathway depends on the induction of HO by cytokines. In contrast to our findings, haeme overload *per se* has been reported to induce HO-1 (Shibahara, 1988; 1994; Takahashi *et al*., 1996; Anning *et al*., 1999; Ponka, 1999; Odaka *et al*., 2000), but this seems not to be the case in the present study since haeme-lysinate counteracted the mechanical nociceptor hypersensitivity produced by IL-1β, which induces HO-1, but not by PGE_2; showing that in our experiments a previous induction of HO is necessary for haeme-lysinate effects.

Our results indicate that a HO product plays an inhibitory action on mechanical nociceptor hypersensitivity in rats. In order to investigate whether this analgesic effect is due to the production of CO, we demonstrated that i.pl. administration of the gas, CO, was able to counteract the ongoing mechanical nociceptor hypersensitivity induced by PGE_2, implying that CO is the HO product involved in anti-mechanical nociceptor hypersensitivity. Furthermore, the fact that CO (HO product), but not haeme-lysinate (HO substrate), impaired the mechanical nociceptor hypersensitivity induced by PGE_2 reinforces that the CO involved in the down-regulation of hypersensitivity is a product of the inducible HO isoform (HO-1) since CO acts independently from HO induction, whereas haeme-lysinate requires enzyme induction.

In order to investigate whether the analgesic effect of CO is mediated by cyclic GMP, we tested the effect of ODQ, an inhibitor of soluble guanylate cyclase (sGC) (Moro *et al*., 1996), upon hypersensitivity inhibitory effect of the haeme-lysinate and CO. It was observed that the anti-hypersensitivity effects of either haeme-lysinate or CO were blocked by ODQ, suggesting that CO down-regulated the mechanical nociceptor hypersensitivity *via* action of sGC.

According to the present results, the mechanism of the anti-hypersensitivity effect of the HO/CO pathway seems to be similar to that described to the L-arginine/NOS/NO pathway. Several observations indicate that NO donors, such as SNAP or SIN-1, inhibit the ongoing mechanical nociceptor hypersensitivity induced by prostaglandin or by inflammatory stimuli such as carrageenan (Duarte *et al*., 1990; Ferreira *et al*., 1991; 1992; Lorenzetti & Ferreira, 1996; Tonussi & Ferreira, 1994; Granados-Soto *et al*., 1997). The analgesic effects of these NO donors are inhibited by ODQ or by methylene blue, a non-specific inhibitor of sGC (Duarte *et al*., 1990; Ferreira *et al*., 1991; Cunha *et al*., 1999b). Furthermore, NOS inhibitors enhance the mechanical nociceptor hypersensitivity evoked by carrageenan or cytokines, but not by PGE_2 or sympathetic amines (Duarte *et al*., 1990; 1992; Ferreira *et al*., 1991). Therefore, it was concluded that, during the inflammatory process, NO is released by an inducible NOS isoform and it limits the intensity of the inflammatory hyperalgesia. The induction of NOS is mediated by TNF-α and/or IL-1β. The hyperalgesic mediators (PGE_2 and sympathetic amines) do not participate in the NOS induction process, inasmuch as their mechanical nociceptor hypersensitivity effect was not augmented by the NOS inhibitors. On the other hand, there are also observations that indicate that the L-arginine/NO/cyclic GMP pathway has a peripheral mechanical nociceptor

hypersensitivity rather than anti-hyperalgesic effect. Thus, the intraplantar or systemic administration of N^G-nitro-L-arginine methyl ester (L-NAME, another NOS inhibitor), but not D-NAME, has been reported to produce dose-dependent antinociception in the second phase of the formalin test in rats (Haley *et al*., 1992). A nociceptive role for the L-arginine/NO/cyclic GMP pathway has also been demonstrated using other tests, such as the tail-flick and hot-plate tests, acetic acid- or phenyl-quinone-induced writhing and formalin-induced paw licking in mice (Morgan *et al*., 1992; Malmberg & Yaksh, 1993; Mustafa, 1992; Moore *et al*., 1991; Meller *et al*., 1994). The simplest explanation for these conflicting observations may be that the role and importance of this pathway varies among the groups of primary sensory neurones mobilised by different types of nociceptive stimuli.

It is interesting to point out that the same cytokines that induce the NOS are involved in the induction of HO-1. In view of these considerations, we investigated the possible interaction between the NO and CO pathways. Accordingly, the anti-hypersensitivity effects of CO or of haeme-lysinate upon the hypersensitivity induced by PGE_2 and IL-1β, respectively, were abolished by pretreatment of the paw with the NO synthase blocker, L-NMMA (Figure 5), whereas the anti-hypersensitivity effect of the NO donor SNAP was not affected by the HO inhibitor ZnDPBG, suggesting that CO-induced anti-mechanical hypersensitivity depends on the integrity of the NO pathway. On the other hand, the anti-mechanical nociceptor hypersensitivity effect of NO does not depend upon CO release. These results are not at all in line with the literature, which suggests that CO inhibits NOS activity by inhibiting all NOS isoforms (Matsuoka *et al*., 1994; Sato *et al*., 1998; Johnson *et al*., 1999; Throup *et al*., 1999). However, recent evidence has supported the theory that the interaction between the NO and CO pathways is more intricate than envisaged, being not restricted only to the NOS and HO level. In this context, Ingi *et al*. (1996) have recently demonstrated that CO modulates the activation of soluble guanylate cyclase by NO. More studies are necessary to clarify this issue.

In conclusion, the results described above provide evidence that CO, produced by HO limits the intensity of the inflammatory hyperalgesia. Moreover, the anti-nociceptive effect of CO depends on the activation of sGC, with the consequent production of cyclic GMP in the sensitive neurons, and on the integrity of the L-arginine/NOS/NO pathway.

The authors gratefully acknowledge the technical assistance of Ieda R. dos Santos. This work was supported by grants from FAPESP, Pronex and CNPq (Brazil). Alexandre A. Steiner was the recipient of a FAPESP fellowship.

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(Received November 20, 2000
Revised January 30, 2001
Accepted February 1, 2001)