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Heme oxygenase/carbon monoxide-biliverdin pathway may be involved in the antinociceptive activity of etoricoxib, a selective COX-2 inhibitor

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Abstract:
The aim of this study was to assess the interaction between the heme oxygenase-1/ biliverdin/carbon monoxide (HO-1/BVD/CO) and cyclooxygenase-2 (COX-2) pathways in the writhing test. Mice were pretreated with 0.1, 1 or 10 mg/kg, *ip* etoricoxib, a selective COX-2 inhibitor, or with one of the following HO-1/BVD/CO pathway modulators: 1, 3 or 9 mg/kg, *sc* ZnPP IX, a specific HO-1 inhibitor, 0.3, 1 or 3 mg/kg, *sc* hemin, a substrate of the HO-1/BVD/CO pathway; or 0.00025, 0.025 or 2.5 μmol/kg, *sc* DMDC, a CO donor. Mice pretreated with etoricoxib or one of the HO-1/BVD/CO pathway modulators received an injection of acetic acid (*ip*) after 30 and 60 min, respectively. Next, the number of writhes was quantified between 0 and 30 min after stimulus injection. In another series of experiments, ineffective doses of etoricoxib were co-administered with hemin or DMDC and an effective dose of etoricoxib with ZnPP IX, followed by an acetic acid injection. Four hours after the acetic acid injection, levels of bilirubin, which is a product of BVD conversion by the BVD reductase enzyme, in the peritoneal lavage were determined. Hemin or DMDC reduced ($p < 0.05$) the number of writhes, but ZnPP IX potentiated ($p < 0.05$) the effect of acetic acid by increasing ($p < 0.05$) the number of writhes. The co-administration of etoricoxib with hemin or DMDC reduced ($p < 0.05$) the number of writhes. However, the analgesic effect of etoricoxib was not observed in the presence of ZnPP IX. Pretreatment with ZnPP IX reduced bilirubin levels, but etoricoxib pretreatment significantly increased the bilirubin concentration in peritoneal exudates. The data obtained from these experiments showed that the HO-1/BVD/CO pathway was activated in the acetic acid-induced abdominal writhing model. The analgesic effect of etoricoxib was at least partially dependent on the participation of the HO-1/BVD/CO pathway.

Key words:
etoricoxib, heme oxygenase, antinociception, mice

Introduction

Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes the degradation of heme to liberate carbon monoxide (CO), biliverdin and free iron in mammalian cells [1]. To date, three isoforms of HO (HO-1, HO-2, and HO-3) have been identified. HO-1 is inducible [22, 24], whereas HO-2 and HO-3 are constitutively expressed [24]. HO-1 is induced in a variety of cells, including endothelial cells, monocytes/macrophages, neutrophils and fibroblasts, by heme, endo-

toxins, cytokines, nitric oxide and other mediators produced during inflammatory responses [1, 7, 29, 40]. HO-1 plays an important role in the antioxidant defense system, and its induction provides a negative feedback for cell activation and the production of inflammatory mediators, which could modulate, at least partially, the inflammatory pain process [1].

Nonsteroidal anti-inflammatory drugs (NSAIDs) exert their major therapeutic and adverse effects by inhibiting cyclooxygenases (COXs) [37]. COXs exist in at least two functionally distinct isoforms [18, 32]. The constitutive isoform of COX, COX-1, is a housekeeping gene that has clear physiological functions. The inducible isoform, COX-2, is induced by a variety of noxious stimuli and produces great amounts of prostaglandins (PGs) associated with inflammation and pain development [38]. This theory has led to the development of a new generation of NSAIDs with the promise of fewer adverse effects, namely selective COX-2 inhibitors (i.e., COXIBs) [21, 39].

The interplay between the HO-1 and COX-2 pathways has recently been addressed [1]. In this regard, during inflammatory processes, HO-1 overexpression could result in the inhibition of heme proteins, including cytochrome P-450 isozymes and cyclooxygenases, due to diminished heme availability [13].

Because the activities of both the HO-1 and COX-2 enzymes are induced by the same pro-inflammatory mediators, the present study was designed to investigate the effect of hemin (a HO-1 inducer), DMDC (a CO-releasing molecule) or ZnPP IX (a specific HO-1 inhibitor) on the antinociceptive effect of etoricoxib, a COXIB that has been approved for the treatment of inflammatory and painful conditions [25]. We also investigated whether the analgesic effect of etoricoxib in the writhing test was dependent on the integrity of the HO-1/BVD/CO pathway.

Material and Methods

Animals

Male Swiss mice (25–30 g) were housed at 25 ± 1°C under a 12/12 h light/dark cycle, and food and water were supplied *ad libitum*. All efforts were made to minimize animal suffering and the number of animals used. The study protocol was approved by the local Committee of Animal Use and Care in accordance with the "Guide for the Care and Use of Laboratory Animals" from the Brazilian College of Animal Experimentation (COBEA).

Measurement of antinociceptive activity

Nociception was assessed using the writhing test [3, 20]. Briefly, acetic acid, (0.1 ml of a 0.6% v/v solution per 10 g of body weight) was injected intraperitoneally (*ip*) in mice. These animals were placed in a large glass cylinder, and the intensity of nociception was quantified by counting the total number of writhes that occurred between 0 and 30 min after stimulus injection. The writhing response is characterized by a wave of contractions of the abdominal musculature followed by an extension of the hind limbs.

Drugs

Zinc protoporphyrin IX (ZnPP IX; a specific HO-1 inhibitor), hemin (an HO-1 substrate), dimanganese decacarbonyl (DMDC; a CO donor), and indomethacin were purchased from Sigma (St. Louis, MO, USA). Acetic acid was purchased from Reagen Quimibrás Ind. Química (Rio de Janeiro, RJ, Brazil). Etoricoxib (Merck Sharp and Dohme, Whitehouse Station, NJ, USA) was diluted in 0.9% sterile saline solution. Indomethacin was diluted in a 5% $NaHCO_3$ solution, and the pH was adjusted to 8.0 using 0.1 M HCl. ZnPP IX was dissolved in 50 mM Na_2CO_3. Hemin was dissolved in 1 mM NaOH, and DMDC was dissolved in Tween 80. All drugs were protected from light, except DMDC, which was exposed to cold light before administration to mice [15].

Study design

To assess the effects of the test drugs, animals received (*ip*) the selective COX-2 inhibitor, etoricoxib (0.1, 1 or 10 mg/kg), or an equivalent volume of its respective vehicle 30 min before an acetic acid (0.6%) injection. The specific HO-1 inhibitor ZnPP IX (1, 3 or 9 mg/kg), the HO-1 substrate hemin (0.3, 1 or 3 mg/kg), the CO-releasing molecule DMDC (0.00025, 0.025 or 2.5 μmol/kg), or an equivalent volume of their respective vehicles were administered (*sc*) 1 h before acetic acid (0.6%) injection, except for those mice pretreated with ZnPP IX, which received a lower dose of acetic acid (0.3% *ip*). This dose of 0.3% acetic acid per cav-

ity was used in mice pretreated with the specific HO-1 inhibitor to promote a submaximal writhing response and to allow a recording of a possible enhancement of the number of writhes by treatment with the specific HO-1 inhibitor [10].

To validate the data, a positive control group of mice was pretreated (*sc*) with indomethacin (5 mg/kg) 1 h before stimulus injection. Untreated groups (NT) consisted of mice that received only acetic acid (*ip*) followed by 0.9% sterile saline (*ip*). The etoricoxib dose selection was based on a previous study [5]. The doses of HO-1 pathway agents were selected in accordance with the literature [10, 35]. It is important to mention that 1 mol of DMDC (Mn_2CO_{10}) in experimental conditions releases 4 moles of CO [8].

To analyze the effect of HO-1 pathway agents on etoricoxib-induced antinociception, another series of experiments was performed. Animals were pretreated (*sc*) with the lower doses of the HO-1 pathway agents, hemin (0.3 mg/kg) or DMDC (0.00025 μmol/kg), and 30 min later, the animals received (*ip*) the lower dose of etoricoxib (0.1 mg/kg). After 30 min, acetic acid (0.6%) was injected, and the total number of writhes was counted as described above. Furthermore, to analyze the effect of ZnPP IX on the analgesic efficacy of etoricoxib, the animals were pretreated (*sc*) with ZnPP IX (3 mg/kg), followed by an injection (*ip*) of an effective dose of etoricoxib (1 mg/kg) 30 min later. After 30 min, acetic acid (0.6%) was injected (*ip*), and the total number of writhes was counted for the next 30 min.

Determination of bilirubin

Four hours after acetic acid injection, the animals were sacrificed, and the peritoneal exudate was collected for the determination of the amount of bilirubin. Bilirubin was measured in peritoneal exudate using a commercial kit (Labtest®, Lagoa Santa, MG, Brazil) following the manufacturer's protocol. Samples were read at 540 nm, and the results were expressed as mg bilirubin per ml of exudate [10].

Data analysis and statistics

The results are presented as the mean ± SEM of measurements made for six animals in each group. Differences between means were compared using a one-way ANOVA followed by Tukey's test. In these tests, the criterion for statistical significance was $p < 0.05$.

Results

Effect of pretreatment with etoricoxib on the writhing response to acetic acid

The injection (*ip*) of a 0.6% (v/v) solution of acetic acid (0.1 ml/10 g) in mice induced a significant writhing response between 0 and 30 min, which was significantly ($p < 0.05$) inhibited by pretreatment (*sc*) with indomethacin (5 mg/kg). Etoricoxib (1 or 10 mg/kg), injected (*ip*) 30 min prior to the stimulus injection, significantly inhibited ($p < 0.05$) the nociceptive response by 52.54% and 93.02%, respectively, compared to the NT group (Fig. 1). Although the lower dose of etoricoxib (0.1 mg/kg) tended to reduce the number of writhes, it failed to exhibit a significant ($p > 0.05$) antinociceptive effect.

Effect of pretreatment with ZnPP IX, hemin, or DMDC on the writhing response to acetic acid

The pretreatment of animals with ZnPP IX (3 mg/kg) potentiated ($p < 0.05$) the number of writhes ($p > 0.05$) (Fig. 2). Indeed, pretreatment of the animals with hemin (3 mg/kg) or DMDC (2.5 μmol/kg) significantly inhibited ($p < 0.05$) the nociceptive response by 70.38% and 60.44%, respectively, compared to the NT group

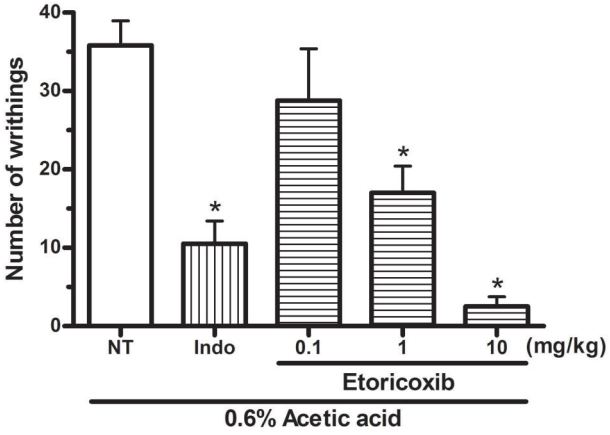

Fig. 1. Effect of the systemic administration of etoricoxib on the writhing response induced by acetic acid in mice. The number of writhes was determined between 0 and 30 min after injection (*ip*) of acetic acid (0.6% (v/v), 0.1 ml/10 g of animal mass). A positive control was pretreated (*sc*) with indomethacin (Indo) (5 mg/kg). Etoricoxib (0.1, 1 or 10 mg/kg, *ip*) was given 30 min before injection of acetic acid. Data are expressed as the mean ± SEM of 6 mice for each group. * $p < 0.05$ indicates a significant difference from the untreated (NT) group (ANOVA; Tukey's test)

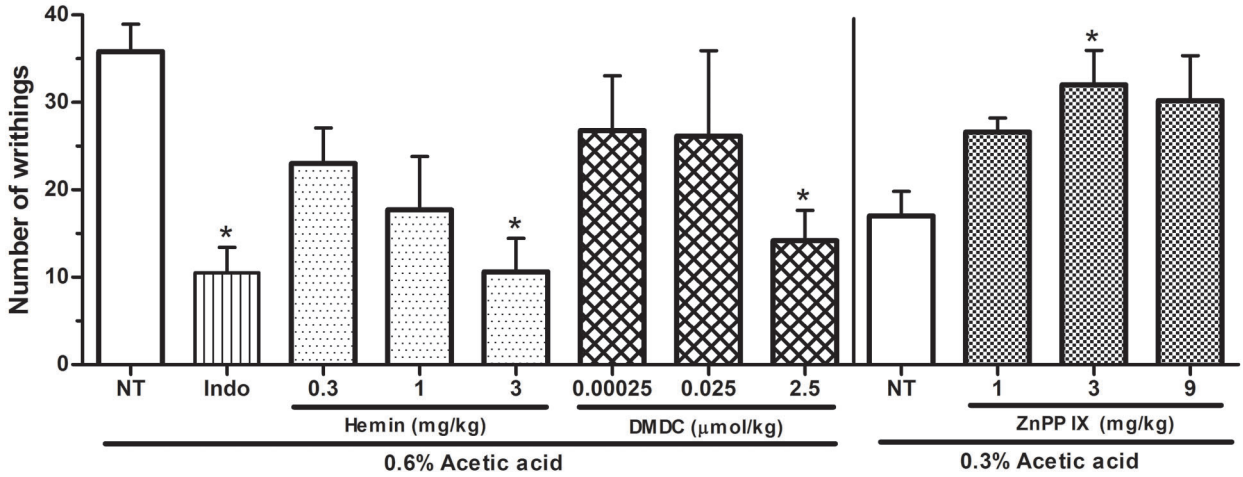

Fig. 2. Effect of the systemic administration of HO-1 pathway agents on the writhing response induced by acetic acid in mice. The number of writhes was determined between 0 and 30 min after an *ip* injection of acetic acid (0.6% (v/v), 0.1 ml/10 g of animal mass). A positive control was pretreated (*sc*) with indomethacin (Indo) (5 mg/kg). Hemin (0.3, 1 or 3 mg/kg) or DMDC (0.00025, 0.025 or 2.5 μmol/kg) was given *sc* 1 h before acetic acid (0.6%). ZnPP IX (1, 3 or 9 mg/kg) was given *sc* 1 h before acetic acid (0.3%). Data are expressed as the mean ± SEM of 6 mice for each group. * $p < 0.05$ indicates a significant difference from the untreated (NT) group (ANOVA; Tukey's test)

(Fig. 1). Although the lower doses of hemin (0.3 or 1 mg/kg) or DMDC (0.00025 or 0.025 μmol/kg) tended to reduce the number of writhes, both failed to exhibit significant ($p > 0.05$) effects.

Effect of ZnPP IX, hemin or DMDC on etoricoxib-induced antinociception in the writhing response to acetic acid

To investigate the role of HO-1 activity in the antinociceptive effect of etoricoxib, animals were pretreated (*sc*) with ZnPP IX (3 mg/kg). After 30 min, the animals received (*ip*) an effective dose of etoricoxib (1 mg/kg), followed by an acetic acid (0.6%) injection (*ip*) 30 min later. The total number of writhes was counted for the next 30 min. An analgesic effect of etoricoxib (1 mg/kg) on the acetic acid-induced writhing test in mice was not observed in the presence of ZnPP IX (3 mg/kg), a specific HO-1 inhibitor (Fig. 3). Because we observed that the inhibition of HO-1 activity reduced the analgesic effect of etoricoxib in the writhing response to acetic acid, we next investigated whether its substrate, hemin, or its metabolite, CO, could also interfere with the analgesic effect of etoricoxib in this animal model. The combination of ineffective doses of etoricoxib (0.1 mg/kg) with hemin (0.3 mg/kg) or DMDC (0.00025 μmol/kg) significantly ($p < 0.05$) inhibited the nociceptive responses by 92.70% and 60.60%, respectively, compared to the NT group (Fig. 3).

Subsequently, we determined the bilirubin concentration in peritoneal exudates as indices of HO-1 activity. As shown in Table 1, an *ip* challenge with acetic acid promoted an increase in bilirubin levels in peritoneal exudates, which indicated that HO-1 activity was enhanced in the writhing response to acetic acid. Accordingly, we observed that the bilirubin levels in peritoneal exudates were reduced by pretreatment with ZnPP IX. Furthermore, the pretreatment of mice with etoricoxib significantly increased the bilirubin concentration in peritoneal exudates induced by acetic acid.

Discussion

In this work, we explored the involvement of HO/BVD/CO in etoricoxib-induced antinociception and the interplay between the HO-1 and COX-2 systems and found evidence that the molecular cascade formed by COX-2-HO/CO/BVD reduced nociception during an acute inflammatory reaction.

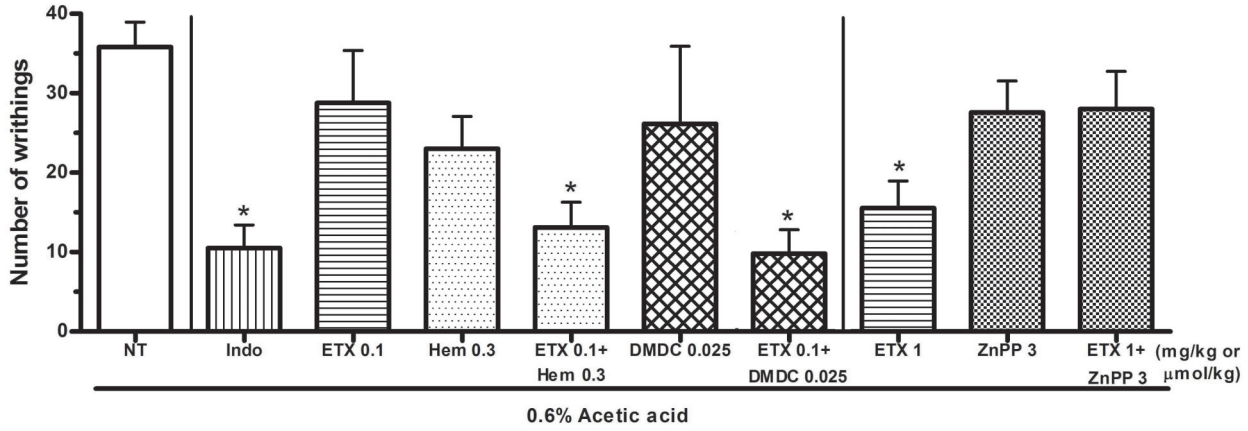

Fig. 3. Effect of the systemic administration of HO-1 pathway agents on etoricoxib-induced antinociception of the writhing response induced by acetic acid in mice. The number of writhes was determined between 0 and 30 min after an *ip* injection of acetic acid (0.6% (v/v), 0.1 ml/10 g of animal mass). A positive control was pretreated (*sc*) with indomethacin (Indo) (5 mg/kg). Hemin (0.3 mg/kg) or DMDC (0.00025 µmol/kg) was given *sc* 30 min after etoricoxib (ETX 0.1 mg/kg, *ip*) was injected. Thirty minutes later, acetic acid (0.6%) was injected, and the total number of writhes was counted. In another series of experiments, animals were pretreated (*sc*) with ZnPP IX (3 mg/kg). After 30 min, the animals received (*ip*) etoricoxib (ETX 1 mg/kg), followed by an acetic acid (0.6%) injection (*ip*) 30 min later. The total number of writhes was counted for the next 30 min. Data are expressed as the mean ± SEM of 6 mice for each group. * $p < 0.05$ indicates a significant difference from the untreated (NT) group (ANOVA; Tukey's test).

Tab. 1. Bilirubin levels (mg/ml) in peritoneal exudates of mice after pretreatment with zinc protoporphyrin IX (ZnPP IX) or etoricoxib and challenged with acetic acid

	Control	Acetic acid 0.6%		
		NT	ZnPP IX (9 mg/kg)	Etoricoxib (10 mg/kg)
Bilirubin	0.01 ± 0.002	0.11 ± 0.006	0.01 ± 0.001*	0.37 ± 0.030*

The mice were pretreated (*sc*) with ZnPP IX (9 mg/kg), etoricoxib (*ip*) or with vehicle 30 min before (*ip*) challenge with acetic acid. Peritoneal exudates were collected 4 h after challenge, and bilirubin content was analyzed as described in the Methods section. The results are expressed as the means ± SEM (mg/ml) of 6 animals in each group (control, vehicle, ZnPP IX and etoricoxib). * $p < 0.05$ for animals injected with acetic acid *vs.* untreated animals (NT group) (ANOVA followed by Tukey's test)

Regulatory interactions between the HO-1 and COX pathways have previously been reported [1], but there is a limited amount of data concerning these interactions in inflammatory pain modulation. During the inflammatory process, cellular heme levels affect COX expression and activity, and heme-HO has a possible regulatory role in the expression of vascular COX and the production of the vasoactive prostanoids, PGE_2 and PGI_2 [13]. Therefore, HO-1 overexpression decreases COX activity in endothelial cells, resulting in a lower production of PGI_2 and PGE_2. Heme binding to histidine residues on peroxidase binding sites of COX isoforms is required for this catalytic activity [33].

The COXIB etoricoxib is approximately three times more selective than rofecoxib or valdecoxib, and it is fifteen times more selective than celecoxib [30]. Etoricoxib has a rapid action, demonstrates good anti-inflammatory and analgesic effects in patients with osteoarthritis and rheumatoid arthritis, and has a more favorable GI safety profile than the standard nonselective NSAIDs [14, 30].

Over the last few years, numerous studies have demonstrated that HO-1 expression and the concomitant production of its metabolites, CO and BVD, have anti-inflammatory consequences [23, 34, 36]. In fact, heme-induced HO-1 results in a reduction of cell migration, exudation and pro-inflammatory mediator release in a zymosan-induced air pouch inflammation model [40]. There is evidence that CO stimulates soluble guanylate cyclase activity and increases cellular levels of cyclic GMP [8, 26, 31]. Ferreira et al. [9] have provided experimental support to suggest that elevated levels of cyclic GMP are associated with an inhibition of nociceptor hypersensitivity. In this regard, our research group recently demonstrated an increase in antinociceptive responses produced by a combination of agents that increase intracellular cy-

clic GMP concentrations [3]. Therefore, DMDC-delivered CO could reduce writhing responses by increasing cyclic GMP. Accordingly, the HO substrate hemin and the CO-releasing molecule DMDC (HO metabolite) inhibited acetic acid-induced writhes in a dose-dependent manner in the present study.

We then demonstrated that the HO/BVD/CO pathway had antinociceptive effects during acetic acid-induced nociception. In support of this hypothesis, we observed that treatment with ZnPP IX, a specific inhibitor of HO-1, enhanced the writhing response induced by a sub-maximal dose of acetic acid. Therefore, our findings corroborate other data showing that the inhibition of the HO-1 pathway is associated with a worsening of the inflammatory response [1, 2, 40]. In addition, in our experimental conditions, HO-1 activity was significantly enhanced, as increased levels of bilirubin were detected in the peritoneal exudate after an acetic acid challenge, and ZnPP IX reduced bilirubin production.

After pretreatment with ZnPP IX, an analgesic effect of etoricoxib on the writhing response was not observed, which suggests that HO-1 activity is involved in the inhibitory effect of etoricoxib in this model. Accordingly, the pretreatment of mice with etoricoxib significantly increased the bilirubin concentration in peritoneal exudates induced by acetic acid.

For many years, the analgesic and anti-inflammatory properties of NSAIDs have been ascribed to their ability to block PG synthesis by the inhibition of COX [37]. In addition to the ability to inhibit COX, NSAIDs possess free radical scavenging properties and, as a consequence, might decrease tissue damage that contributes to its anti-inflammatory and analgesic therapy [16]. In this regard, treatment with the COX-2 inhibitor rofecoxib significantly reduced oxidative stress in a rat model of aluminum-induced oxidative stress, which is used to mimic Alzheimer's disease-like conditions [28]. In fact, salicylate, the active metabolite of aspirin, exerts COX-independent anti-inflammatory effects through the induction of HO-1 [11]. Furthermore, daily doses of NSAIDs increase circulating levels of antioxidants in rheumatoid arthritis [27, 28]. Various NSAIDs activate the three families of MAP kinases, and this activation depends on the presence of reactive oxygen species [19]. Indeed, NS-398, a selective COX-2 inhibitor, increases HO-1 protein expression in vascular smooth muscle cells after inflammatory cytokine stimulation [6]. Considering all of this evidence, it would be worthwhile to clarify the role of COX-2 in oxidative stress or adaptive responses and the relationship between HO-1 and COX-2.

The discovery of two COX isoenzymes, a constitutive COX-1 serving homeostatic prostaglandin synthesis, including gastric mucosal defense and renal homeostasis, and COX-2, which synthesizes detrimental PGs that are responsible for inflammation and pain in several sites, led to the development of selective COX-2 inhibitors that promise minimal NSAID-typical toxicity with full anti-inflammatory efficacy [6, 10].

To date, the strategy of selective COX-2 inhibition has been successful. Selective COX-2 inhibitors have significantly less gastrotoxicity and no effects on platelet aggregation. However, with regard to renal adverse events, selective COX-2 inhibitors do not offer a clinically relevant advantage over non-selective inhibitors. Moreover, concerns over the cardiovascular risks of selective COX-2 inhibitors have recently been raised [12].

We also observed that an ineffective dose of etoricoxib associated with ineffective doses of HO-1 pathway agents (e.g., hemin or DMDC) significantly reduced the writhing response. Because HO-1 induction may inhibit COX-2 induction [1], this evidence could at least partially explain the increased antinociception observed when small doses of etoricoxib and hemin were administered concomitantly. Indeed, COX-2 induction is inhibited by CO [4], which provides some explanation for the increased analgesic effect observed when small doses of etoricoxib and DMDC, a CO donor, were administered concomitantly. Therefore, these associations may contribute to the therapeutic effectiveness of selective COX-2 inhibitors and might further reduce the side effects mediated by these drugs.

The local irritation provoked by acetic acid in the intraperitoneal cavity triggers a variety of mediators, such as bradykinin, substance P, PGs (especially PGI2), and some cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-8 [17]. These mediators activate chemosensitive nociceptors that contribute to the development of inflammatory pain. In the present study, etoricoxib or HO-1/BVD/CO pathway modulators (e.g., hemin or DMDC) alone or co-administered reduced writhing, which suggests that their antinociceptive effect could be related to the inhibition of the release of mediators in response to acetic acid, which reduces the activation of chemosensitive nociceptors.

To summarize, the present study provides evidence that the antinociceptive effect of etoricoxib was dependent on the integrity of the HO-1/BVD/CO pathway, which gives new insight into the mechanism of the action of COX-2 inhibitors. The combination of both HO-1 pathway agents and COXIBs may contribute to the therapeutic effectiveness of these drugs and might further reduce the side effects mediated by the blockade of the COX-2. Taking these results into account, the design of new analgesics is very encouraging. However, the pharmacological profile of these associations must be subject to further investigations.

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