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RESEARCH PAPER

Heme oxygenase/carbon monoxide-biliverdin pathway down regulates neutrophil rolling, adhesion and migration in acute inflammation

A Freitas[1,4], JC Alves-Filho[1,4], DD Secco[1,4], AF Neto[2], SH Ferreira[1], C Barja-Fidalgo[3] and FQ Cunha[1]

[1]*Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil;* [2]*Department of Pharmaceutical Science, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, São Paulo, Brazil and* [3]*Department of Pharmacology, Institute of Biology, Rio de Janeiro State University, Rio de Janeiro, Brazil*

Background and purpose: Heme oxygenase (HO) activity is known to down-regulate inflammatory events. Here, we address the role of HO and its metabolites, carbon monoxide (CO) and biliverdin (BVD), in leukocyte rolling, adhesion and neutrophil migration during inflammatory processes.
Experimental approach: Intravital microscopy was used to evaluate leukocyte rolling and adhesion in the mesenteric microcirculation of mice. TNFα and IL-1β were determined by ELISA and HO-1 protein expression by Western blot.
Key results: Intraperitoneal challenge with carrageenan enhanced HO-1 protein expression in mesentery and bilirubin concentration in peritoneal exudates. Pretreatment of mice with a non-specific inhibitor of HO (ZnDPBG) or with a HO-1 specific inhibitor (ZnPP IX) enhanced neutrophil migration, rolling and adhesion on endothelium induced by carrageenan. In contrast, HO substrate (hemin), CO donor (DMDC) or BVD reduced these parameters. The reduction of neutrophil recruitment promoted by HO metabolites was independent of the production of chemotactic cytokines. Inhibitory effects of CO, but not of BVD, were counteracted by treatment with a soluble guanylate cyclase (sGC) inhibitor, ODQ. Furthermore, inhibition of HO prevented the inhibitory effect of a nitric oxide (NO) donor (SNAP) upon neutrophil migration, while the blockade of NO synthase (NOS) activity by aminoguanidine did not affect the CO or BVD effects.
Conclusions and Implications: Metabolites of HO decreased leukocyte rolling, adhesion and neutrophil migration to the inflammatory site by a mechanism partially dependent on sGC. Moreover, inhibition by NO of neutrophil migration was dependent on HO activity.
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Keywords: neutrophil migration; heme oxygenase; carbon monoxide; biliverdin; soluble guanylate cyclase; nitric oxide

Abbreviations: AG, aminoguanidine; BVD, biliverdin; cGMP, guanosine 3′5′-cyclic monophosphate; DMDC, dimanganese decacarbonyl; eNOS, endothelial nitric oxide synthase; HO, heme oxygenase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-(1,2,4) oxadiazolo (4,3-a)quinoxalin-1-one; sGC, soluble guanylate cyclase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; ZnDPBG, zinc deuteroporphyrin 2,4-bis glycol; ZnPP IX, zinc protoporphyrin IX

Introduction

Heme oxygenase (HO) is a microsomal enzyme that catalyzes the degradation of heme into carbon monoxide (CO), biliverdin (BVD) and free iron (Abraham *et al*., 1988). BVD is subsequently converted to bilirubin by BVD reductase (Maines and Trakshel, 1993). HO-1, the inducible isoform, is expressed in a variety of cells including endothelial cells, vascular smooth cells, basophils, monocytes/macrophages, neutrophils and fibroblasts, through a diversity of conditions, such as oxidative stress, exposure to cytokines, nitric oxide (NO), endotoxin, heme and during inflammatory response (Tenhunen *et al*., 1969; Willis *et al*., 1996; Terry *et al*., 1998; Datta and Lianos, 1999; Oshiro *et al*., 1999; Alcaraz *et al*., 2003; Vicente *et al*., 2003). Moreover, a variety of pathological conditions are associated with HO-1 induction, for instance, hypertension, acute pancreatite, asthma, cancer (Ishizaka *et al*., 1997; Sato *et al*., 1997; Horvath *et al*., 1998; Doi *et al*., 1999). HO-2 and HO-3 are isoforms constitutively expressed in many tissues, such as, brain, testes, endothelium (Zakhary *et al*., 1996; Maines, 1997;

Correspondence: Professor FQ Cunha, Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Avenida Bandeirantes, 3900, Ribeirão Preto, São Paulo 14049-900, Brazil.
E-mail: fdqcunha@fmrp.usp.br
[4]These authors contributed equally to this work.
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McCoubrey *et al*., 1997). HO metabolites are involved in many biological effects. CO exhibits some properties similar to those of NO, including regulation of vascular tone, neuronal signaling and antinociception (Morita *et al*., 1995; Maines, 1997; Steiner *et al*., 2001), and BVD presents important antioxidant properties (Stocker *et al*., 1987).

Over the last few years, numerous studies have demonstrated that HO-1, its substrate, heme and its metabolites, CO and BVD, are able to modulate the inflammatory process. Heme has been reported to mediate oxidative insults and inflammation and is probably important in a wide variety of pathophysiological processes, triggering the production of reactive oxygen species and stimulating adhesion molecule expression and leukocyte infiltration in the inflammatory site (Wagener *et al*., 2003). However, a low concentration of heme can have anti-inflammatory and cytoprotective actions via the upregulation of HO-1 and stimulation of the formation of HO-1 metabolites, such as CO and BVD, which were also shown to reduce cell migration, exudation, the release of pro-inflammatory mediators and the expression of adhesion molecules (Hayashi *et al*., 1999; Vicente *et al*., 2003), suggesting that expression of HO-1 has a protective role.

An interplay between HO-1 and nitric oxide synthase (NOS) systems has recently been addressed. These systems share many common features and overlap in biological function. There is evidence that NO derived from inducible nitric oxide synthase (iNOS) induces HO-1 expression (Vicente *et al*., 2001) and potentiates HO-1 induction by ferriprotoporphyrin IX chloride (hemin) (Naughton *et al*., 2002). Conversely, HO-1 activity can lead to the inhibition of iNOS expression (Turcanu *et al*., 1998). It was shown by our group and others that NO derived from both iNOS and endothelial nitric oxide synthase (eNOS) reduces neutrophil migration to inflammatory sites, downregulating the expression of adhesion molecules and consequently decreasing leukocyte rolling and adhesion to endothelium (Hickey and Kubes, 1997; Spiecker *et al*., 1998; Secco *et al*., 2003). More recently, it was also demonstrated that the inhibition of leukocyte adherence promoted by NO is mediated by the activation of soluble guanylate cyclase (sGC), via production of the second messenger guanosine 3′5′-cyclic monophosphate (cGMP) (Ahluwalia *et al*., 2004).

During the acute inflammation induced by intraperitoneal injection of carrageenan, neutrophil migration to the peritoneal cavity is mainly mediated by cytokines (tumor necrosis factor-α (TNFα), interleukin (IL)-6, interleukin 1 beta (IL-1β)) and arachidonic acid metabolites (Dozen *et al*., 1989; Utsunomiya *et al*., 1991). A downregulation of inflammatory events, induced by NOS and HO activity, has been proposed by several groups (Willis *et al*., 1996; Hickey and Kubes, 1997; Secco *et al*., 2003). Thus, in the present study, we attempted to clarify the interrelationship between the inhibitory roles of HO and NOS systems on neutrophil migration during an acute inflammatory response, and investigated whether sGC activation was involved in the inhibitory effect of HO metabolites on carrageenan-induced neutrophil migration in mice. We show that sGC activation is essential for the inhibitory effects of CO, but not for those of BVD, on carrageenan-induced neutrophil rolling, adhesion and migration. We also demonstrate that HO activity is probably a key step in mediating the inhibitory effect of NO on neutrophil migration.

Methods

Animals

Adult Balb/c mice weighing 18–20 g were used in this study. The animals were housed in temperature-controlled rooms (22–25°C), with access to water and food *ad libitum*, until use in the Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo. All experiments were conducted in accordance with the ethical guidelines of the School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil.

Determination of neutrophil migration into peritoneal cavity

Mice were anesthetized intraperitoneally (i.p.) with tribromoethanol (250 mg kg^{-1}) and were killed in a CO_2 chamber, 4 h after carrageenan administration and the peritoneal cavity cells were harvested by washing the cavity with 3 ml of phosphate-buffered saline containing ethylenediaminetetraacetic acid (37.2 mg 100 ml^{-1}). Total counts were performed on a cell counter (COULTER A ^{C}T, Miami, FL, USA) and differential cell counts (100 cells total) were carried out on a cytocentrifuge (Cytospin 3; Shandon Lipshaw, Pittsburgh, PA, USA) and slides were stained by the May-Grünwald-Giemsa (Rosenfeld, Laborclin, Pinhais, PR, Brazil) method. The results are presented as the number of neutrophils per cavity.

Determination of leukocyte rolling and adhesion to the mesenteric microcirculation by intravital microscopy

The leukocyte rolling and adhesion were examined as described previously (Fortes *et al*., 1991). Briefly, mice were anesthetized i.p. with tribromoethanol (250 mg kg^{-1}) and the mesenteric tissue was exposed for microscopic examination. The animals were maintained on a special board thermostatically controlled at 37°C. Images were recorded on a video recorder using a long-distance objective lens ($\times$40) with a 0.65 numerical aperture. Vessels selected for study were third-order venules, defined according to their branch-order location within the microvascular network. These vessels corresponded to postcapillary venules, with a diameter of 10–18 μm. Rolling leukocytes were defined as the white blood cells that moved at a lower velocity than erythrocytes in the same stream and were determined at 10-min intervals 2 h after challenge with carrageenan. Adherent leukocytes were considered to be the white blood cells that remained stationary on the venular endothelium at the end of the observation period and were determined 4 h after challenge with carrageenan. The venular area in which the adhesion process was determined varied from 350 to 450 μm^2 and the results were expressed as the number of adherent leukocyte per 100 μm^2 of venule (Alves-Filho *et al*., 2006). The time points selected to determine rolling (2 h) and adhesion (4 h) processes were based on a previous studies,

which was observed that these process peak at these times after inflammatory stimuli injection (Secco *et al*., 2003).

*Enzyme-linked immunosorbent assay for TNFα and IL-1*β

TNFα and IL-1β levels in the peritoneal exudate of mice were detected by enzyme-linked immunosorbent assay. Briefly, microtiter plates were coated overnight at 4°C with an immunoaffinity-purified polyclonal sheep antibody against TNFα (2 μg ml^{-1}) or IL-1β (2 μg ml^{-1}). After blocking the plates, recombinant murine TNFα or IL-1β standards at various dilutions and the samples were added. Rabbit biotinylated immunoaffinity-purified polyclonal antibody anti-TNFα (1:1000) or IL-1β (1:1000) was added, followed by incubation at room temperature for 1 h. Finally, 50 μl of avidin-horseradish peroxidase (HRP) (1:5000 dilution; DAKO A/S, Glostrup, Denmark) was added to each well, and after 30 min the plates were washed and the color reagent *O*-phenylenediamine (200 μg per well; Sigma, St Louis, MO, USA) was added. After 15 min, the reaction was interrupted with 1 M H_2SO_4 and the optical density measured at 490 nm. The results were expressed as pg TNFα or IL-1β per ml of exudate.

Western blot

Protein expression was determined in extracts of mesentery by Bradford's method. Tissue were lysed in 400 μl of buffer (1% Triton X-100, 1 M NaF, 100 mM NaPPi, 1 M Na_3VO_4, 1 mg ml^{-1} aprotinin, 1 mg ml^{-1} leupeptin, 1 mg ml^{-1} phenylmethylsulphonyl fluoride) and centrifuged at 4°C for 20 min at 12 300 *g*. Equal amounts of protein (90 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel etetrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Rainbow markers (Bio-Rad Laboratories, Hercules, CA, USA) were run in parallel to estimate molecular weight. Membranes were blocked in Tris-buffered saline-T (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) containing 2% bovine serum albumin. After blocking, membranes were incubated with a goat polyclonal antibody anti-HO-1 (1:500 dilution; Santa Cruz, CA, USA), followed by incubation at 4°C overnight. Membranes were then incubated with anti-goat immunoglobulin G conjugated with biotin (1:2000; Santa Cruz, CA, USA) at room temperature for 1 h. Finally, avidin-HRP (1:1000 dilution; DAKO A/S, Denmark) was added, and after 1 h the membrane was washed and immunoreactive proteins were visualized by 3,3′-diaminobenzidine (Sigma, St Louis, MO, USA) staining. A computer-based imaging system (Gel-Pro Analyzer) was used to measure the intensity of optical density of 32 kDa bands that represent the molecular weight of HO-1.

Determination of bilirubin

Bilirubin was measured in peritoneal exudate using a commercial kit (Labtest, Lagoa Santa, MG, Brazil). The manufacturer's protocol was followed, with the modification that the reaction volume was scaled down to 0.5 ml. Samples were read at 540 nm and results expressed as μg bilirubin per ml of exudate.

Experimental protocols

Protocol 1. Mice were injected subcutaneously (s.c.) with zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), a nonspecific HO inhibitor (1.5, 4.5 or 15 μmol kg^{-1}), or zinc protoporphyrin IX (ZnPP IX), a specific HO-1 inhibitor (0.45, 1.5 or 4.5 μmol kg^{-1}), with hemin (0.45, 1.5 or 4.5 μmol kg^{-1}), with dimanganese decacarbonyl (DMDC), a CO donor (2.5, 7.5 or 25 μmol kg^{-1}), with BVD (1.5, 4.5 or 15 μmol kg^{-1}), with DMDC + BVD (7.5 and 15 μmol kg^{-1}, respectively) or with an equivalent volume of their respective vehicles. Fifteen minutes later carrageenan (500 μg per cavity) was injected into the peritoneal cavity of all animal groups, except those pretreated with HO inhibitors or their vehicles, which received a lower dose of carrageenan (250 μg per cavity). This dose of 250 μg per cavity of carrageenan was used in mice pretreated with HO inhibitors to promote a submaximal neutrophil migration (Secco *et al*., 2003) and to allow a possible enhancement of the neutrophil migration by treatment with the HO inhibitors. After 2 h of carrageenan injection, leukocyte rolling and cytokines concentration (TNFα and IL-1β) were evaluated, and after 4 h leukocyte adhesion and neutrophil migration were determined. In another set of experiments, mice were injected i.p. with saline or carrageenan (500 μg per cavity) and 4 h later mesentery was collected for Western blot analyses. It is important to mention that 1 mol of DMDC (Mn_2CO_{10}) in experimental conditions releases 4 mol of CO (Dearden *et al*., 1989).

Protocol 2. Mice were injected s.c. with ZnDPBG (4.5 μmol kg^{-1}) or *S*-nitroso-*N*-acetylpenicillamine (SNAP) (3 mg kg^{-1}), 15 min later carrageenan (500 μg per cavity) was injected i.p. and after 2 h, bilirubin was measured in the peritoneal exudate. In another set of experiments, mice were injected s.c. with ZnDPBG (4.5 μmol kg^{-1}), ZnPP IX (4.5 μmol kg^{-1}), hemin (1.5 μmol kg^{-1}), DMDC (7.5 μmol kg^{-1}) or BVD (15 μmol kg^{-1}), after 2 h hemodynamic parameters and blood leukocyte counts were determined.

Protocol 3. Mice were pretreated i.p. with 1H-(1,2,4) oxadiazolo (4,3-a)quinoxalin-1-one (ODQ) (5 μmol kg^{-1}) or with its respective vehicle. After 15 min, they were injected s.c. with DMDC (7.5 μmol kg^{-1}) or BVD (15 μmol kg^{-1}). Fifteen minutes later, the animals received an i.p. injection of carrageenan (500 μg per cavity) and leukocyte rolling, adhesion and neutrophil migration were determined after 2, 4 and 4 h, respectively. In order to avoid its systemic inactivation by hemoglobin (Zhao *et al*., 2000), ODQ was injected *in situ*, into the peritoneal cavity of mice. Other investigators have used ODQ *in vivo* to evaluate local responses of sGC (Steiner *et al*., 2001; Nabah *et al*., 2005).

Protocol 4. Mice were pretreated s.c. with aminoguanidine (AG), a selective inhibitor of iNOS (50 mg kg^{-1}) or ZnDPBG

(4.5 mol kg^{-1}). After 15 min, the animals pretreated with vehicle or AG were injected s.c. with DMDC (7.5 μmol kg^{-1}), BVD (15 μmol kg^{-1}) or their respective vehicles. Animals treated s.c. with ZnDPBG (4.5 μmol kg^{-1}) or its vehicle were injected s.c. with SNAP (3 mg kg^{-1}) or vehicle. Fifteen minutes later carrageenan (500 μg per cavity) was injected i.p. in all groups, and the neutrophil migration was determined 4 h later.

Statistical analysis

Each experiment was repeated twice with five animals per group and the results are presented as means ± s.e.m. of the total number of mice per group (10 animals). The differences between the experimental groups were compared by analysis of variance (ANOVA) and, in the case of statistical significance; individual comparisons were subsequently made with Bonferroni's *t* test for unpaired values. The statistical analysis of the Western blot was evaluated using Student's *t*-test. The level of significance was set at $P<0.05$.

Drugs

Carrageenan, AG and SNAP were purchased from Sigma (St Louis, MO, USA), ZnDPBG, ZnPP IX, hemin and BVD were purchased from Porphyrin Products (Logan, UT, USA), ODQ was purchased from Tocris Cookson (St Louis, MO, USA) and DMDC was a gift from Professor Dr Alberto Federman Neto. ZnDPBG and ZnPP IX were dissolved in 50 mM Na_2CO_3, hemin and BVD in 1 mM NaOH, DMDC and ODQ in DMSO, and carrageenan, AG and SNAP were dissolved in saline. All drugs were protected from light, except DMDC, which was exposed to cold light before administration to mice (Johnson *et al*., 2003).

Results

The inhibition of HO enhances neutrophil migration, rolling and adhesion induced by carrageenan

To investigate the role of HO activity in neutrophil migration induced by carrageenan, a nonspecific HO inhibitor (ZnDPBG) and a specific HO-1 inhibitor (ZnPP IX) were administered to mice, at different doses, before the inflammatory challenge. Carrageenan was given at a dose (250 μg per cavity) that induces a submaximal neutrophil migration into the peritoneal cavity (Figure 1). The pretreatment of the animals with ZnDPBG or ZnPP IX potentiates the neutrophil migration induced by carrageenan, in a dose-dependent manner (Figure 1). To clarify the mechanisms by which the inhibition of HO activity modulated neutrophil migration to the inflammatory site, we also investigated the effect of ZnDPBG and ZnPP IX on leukocyte–endothelium interactions (rolling and adhesion) in mesenteric postcapillary venules. The effect of carrageenan, increasing the rolling (Figure 2a) and adhesion (Figure 2b) of leukocytes to endothelium, was significantly enhanced by ZnDPBG and ZnPP IX. These results suggest that HO activity downregulates neutrophil–endothelium interactions and consequently neutrophil migration, during the inflammatory process. Subsequently, we determined the expression of HO-1 protein in mesentery by Western blot and bilirubin concentration in the peritoneal exudates, as indexes of HO-1 expression and activity, in our experimental conditions, respectively. As shown in Figure 3 and Table 1, i.p. challenge with carrageenan promoted an increase in HO-1 protein expression in mesentery and in bilirubin levels in peritoneal exudate, indicating that the HO-1 expression and activity is enhanced in carrageenan-induced peritoneal inflammation. Moreover, we observed that bilirubin levels

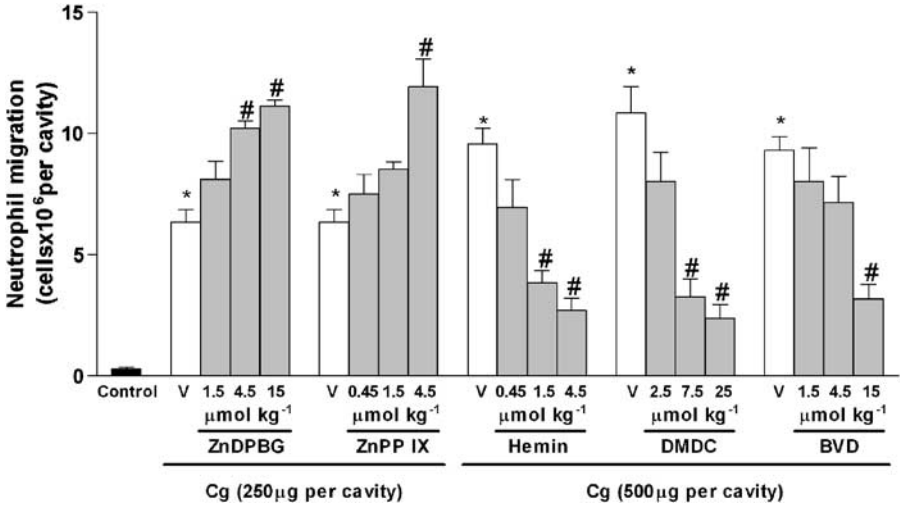

Figure 1 Role of HO in neutrophil migration induced by carrageenan into mouse peritoneal cavity. The mice were pretreated s.c. with ZnDPBG, ZnPP IX, hemin, DMDC, BVD or with their respective vehicles, as shown. After 15 min, carrageenan (Cg) was injected i.p.: 250 μg per cavity for animals pretreated with ZnDPBG or ZnPP IX and 500 μg per cavity for other groups (see Methods). The neutrophil migration was determined 4 h after. The first bar represents the neutrophil migration induced by saline injected i.p. (Control). The results are expressed as the mean ± s.e.m. of 10 animals per group. *$P<0.05$ compared with control; $^{\#}P<0.05$ compared with mice pretreated with vehicle and injected with carrageenan (ANOVA, followed by Bonferroni's test).

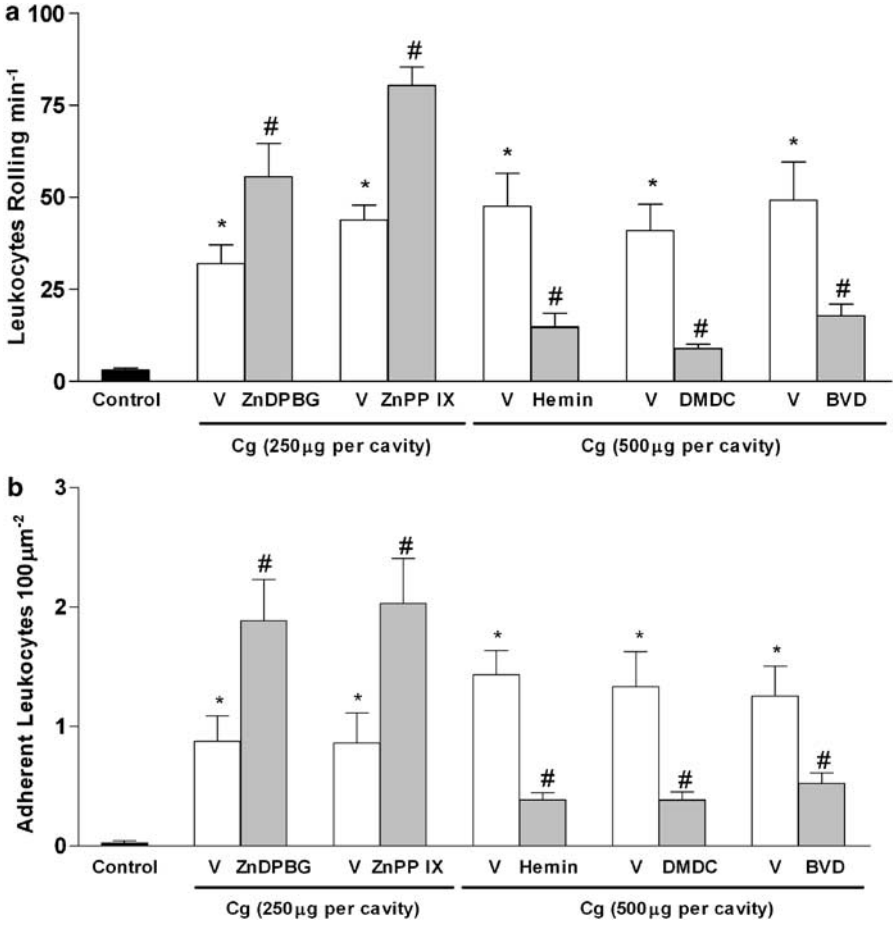

Figure 2 Role of HO in leukocyte rolling and adhesion induced by carrageenan. The mice were pretreated s.c. with ZnDPBG (4.5 μmol kg^{-1}), ZnPP IX (4.5 μmol kg^{-1}), hemin (1.5 μmol kg^{-1}), DMDC (7.5 μmol kg^{-1}), BVD (15 μmol kg^{-1}) or with their respective vehicles. After 15 min, carrageenan (Cg) was injected i.p.: 250 μg per cavity for animals pretreated with ZnDPBG or ZnPP IX and 500 μg per cavity for the other groups (see Methods). The leukocyte rolling (**a**) and adhesion (**b**) were evaluated by intravital microscopy in the mesentery 2 and 4 h later, respectively. The first bar in each panel represents the leukocyte rolling (**a**) and adhesion (**b**) induced by saline injected i.p. (Control). The results are expressed as the mean $\pm$ s.e.m. of 10 animals per group. *$P<0.05$ compared with control; $^{\#}P<0.05$ compared with mice pretreated with vehicle and injected with carrageenan (ANOVA, followed by Bonferroni's test).

in peritoneal exudate were reduced by pretreatment with ZnDPBG.

Hemin, BVD and CO downregulate neutrophil migration, leukocyte rolling and adhesion induced by carrageenan

As we had observed that the inhibition of HO activity increased leukocyte recruitment during an acute inflammatory response, we next investigated whether its substrate, hemin, or its metabolites, CO and BVD, could also interfere with neutrophil migration. As shown in Figure 1, the pretreatment with hemin decreased, in a dose-dependent manner, the recruitment of neutrophils into the peritoneal cavity induced by carrageenan. This decrease in neutrophil migration was associated with a significant reduction in rolling (Figure 2a) and adhesion (Figure 2b) of leukocytes on endothelium after challenge with carrageenan. Similarly, the pretreatment of mice with BVD or with DMDC, a CO donor, reduced, in a dose-dependent manner, neutrophil migration (Figure 1), as well as the rolling and adhesion of leukocytes on endothelium (Figures 2a and b) induced by carrageenan.

HO activity does not interfere with carrageenan-induced release of the neutrophil chemotactic TNFα and IL-1β

Next, we investigated the possible involvement of HO in the release of TNFα and IL-1β, because the downregulation of neutrophil migration promoted by HO metabolites could be due to a decrease in the release of neutrophil chemotactic mediators. As shown in Table 2, mice pretreated with ZnDPBG, DMDC or BVD and challenged with carrageenan presented similar levels of TNFα and IL-1β in peritoneal exudate, when compared with mice pretreated with vehicles and i.p. injected with carrageenan.

Involvement of sGC in inhibitory effects of CO on neutrophil migration, leukocyte rolling and adhesion induced by carrageenan

On the basis of the recent evidence showing that, similar to NO, some biological effects of CO could be ascribed to the activation of sGC and the production of its second messenger, cGMP (Morita *et al*., 1995; Maines, 1997), we investigated whether cGMP could mediate the effects of CO and BVD on carrageenan-induced neutrophil migration. The

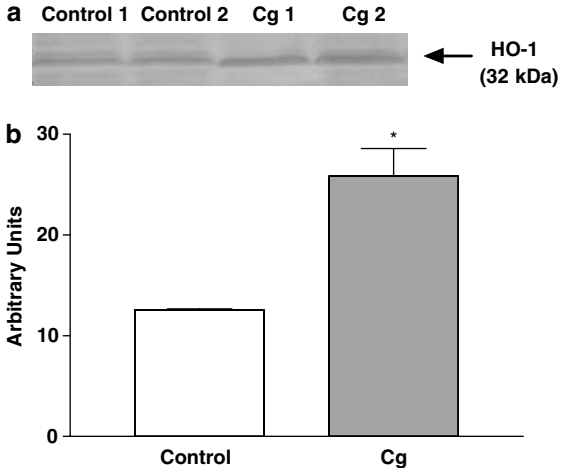

Figure 3 HO-1 protein expression in mesentery of mice after challenge with carrageenan. The mice were administrated i.p. with saline (control) or carrageenan. Mesentery was collected 4 h after, and HO-1 protein expression was analyzed by Western blot, as described in Methods. (**a**), Representative Western blot of HO-1 expression, 32 kDa. (**b**) Intensity of optical density of 32 kDa bands measured from Western blots. Protein band intensity is represented as arbitrary units. The results are expressed as the mean ± s.e.m. of four animals per group. *$P<0.05$ compared with saline injected i.p. (Control; Student's *t*-test).

Table 1 Bilirubin levels ($\mu g\,ml^{-1}$) in peritoneal exudate of mice after pretreatment with ZnDPBG or SNAP and challenge with carrageenan

	Control	*Carrageenan*		
		Vehicle	*ZnDPBG*	*SNAP*
Bilirubin	0.39 ± 0.3	5.1 ± 0.4*	2.3 ± 0.7#	8.7 ± 0.9#

Abbreviations: SNAP, *S*-nitroso-*N*-acetylpenicillamine; ZnDPBG, zinc deuteroporphyrin 2,4-bis glycol.
The mice were pretreated s.c. with ZnDPBG, SNAP or with their own vehicles (Na_2CO_3 or saline) 15 min before i.p. challenge with carrageenan. Peritoneal exudates were collected 2 h after challenge with carrageenan and bilirubin content was analyzed, as described in Methods. The results are expressed as means ($\mu g\,ml^{-1}$) ± s.e.m. of 10 animals in each group (control, ZnDPBG and SNAP). As the results obtained after treatment with the different vehicles were not statistically different, the table shows as control the bilirubin levels calculated using the results obtained with all vehicles used ($n=20$). *$P<0.05$, compared to control (saline injected i.p.); #$P<0.05$, compared with respective vehicle treatment and injected with carrageenan (ANOVA, followed by Bonferroni's test).

i.p. pretreatment of mice with ODQ, a sGC inhibitor, completely reversed the inhibition caused by the CO donor, DMDC, on neutrophil migration (Figure 4), leukocyte rolling (Figure 5a) and adhesion (Figure 5b) induced by carrageenan. In contrast, the inhibitory effect of BVD on neutrophil migration, leukocyte rolling and adhesion was not affected by the pretreatment with ODQ (Figures 4, 5a and b, respectively), suggesting that, differently from CO, the effect of BVD is not modulated by sGC activation. This difference in the mechanism of action attributed to BVD and DMDC could explain why neither of these treatments reduced the neutrophil migration to control levels (Figure 1). The combination of both treatments (DMDC + BVD) did, however, reduce the neutrophil migration to levels near to the

Table 2 TNFα and IL-1β concentrations ($pg\,ml^{-1}$) in peritoneal exudate of mice after pretreatment with ZnDPBG, DMDC or BVD and challenge with carrageenan

	Control	*Carrageenan*			
		Vehicle	*ZnDPBG*	*DMDC*	*BVD*
TNFα	45 ± 32	298 ± 52*	406 ± 186	230 ± 90	266 ± 29
IL-1β	12 ± 10	138 ± 21*	179 ± 94	131 ± 52	122 ± 57

Abbreviations: BVD, biliverdin; DMDC, dimanganese decacarbonyl; IL-1β, interleukin 1 beta; TNFα, tumor necrosis factor-α; ZnDPBG, zinc deuteroporphyrin 2,4-bis glycol.
The mice were pretreated s.c. ZnDPBG, DMDC, BVD or with their own vehicles (Na_2CO_3, DMSO and NaOH) 15 min before i.p. challenge with carrageenan. Peritoneal exudates were collected 2 h after challenge with carrageenan, and cytokine content was analyzed, as described in Methods. The results are expressed as means ($pg\,ml^{-1}$) ± s.e.m. of 10 animals in each group (control, ZnDPBG, DMDC and BVD). As the results obtained after treatment with different vehicles were not statistically different, the table shows as control the cytokine level calculated using the results obtained with all vehicles used ($n=30$). For statistical analysis, each drug treatment was compared to its own vehicle control and no significant differences were observed ($P>0.05$). *$P<0.05$, compared to control (saline injected i.p.; ANOVA, followed by Bonferroni's test).

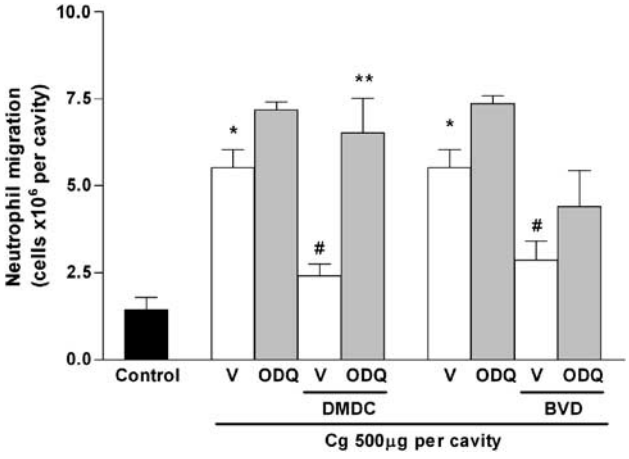

Figure 4 Role of sGC in the inhibitory effect of CO and BVD on neutrophil migration induced by carrageenan. The mice were pretreated i.p. with vehicle or ODQ ($5\,\mu mol\,kg^{-1}$) 15 min before s.c. administration of vehicle, CO donor (DMDC, $7.5\,\mu mol\,kg^{-1}$) or BVD ($15\,\mu mol\,kg^{-1}$). After 15 min, the animals received an i.p. injection of carrageenan (500 μg per cavity). The neutrophil migration was determined 4 h after. The first bar represents the neutrophil migration induced by saline injected i.p. (Control). The results are expressed as the mean ± s.e.m. of 10 animals per group. *$P<0.05$ compared to control; #$P<0.05$ compared with mice pretreated with vehicle and injected with carrageenan; **$P<0.05$ compared with mice pretreated with DMDC and injected with carrageenan (ANOVA, followed by Bonferroni's test).

control (control: $0.58\times10^6\pm0.21\times10^6$; carrageenan: $8.41\times10^6\pm0.68\times10^6$; DMDC: $2.87\times10^6\pm1.24\times10^6$; BVD: $3.12\times10^6\pm1.06\times10^6$ and DMDC + BVD: $1.00\times10^6\pm0.28\times10^6$; $n=6$–8).

HO activity modulates the inhibitory effect of NO on neutrophil migration

To investigate the involvement of NO in the inhibitory effect of CO and BVD on carrageenan-induced neutrophil migra-

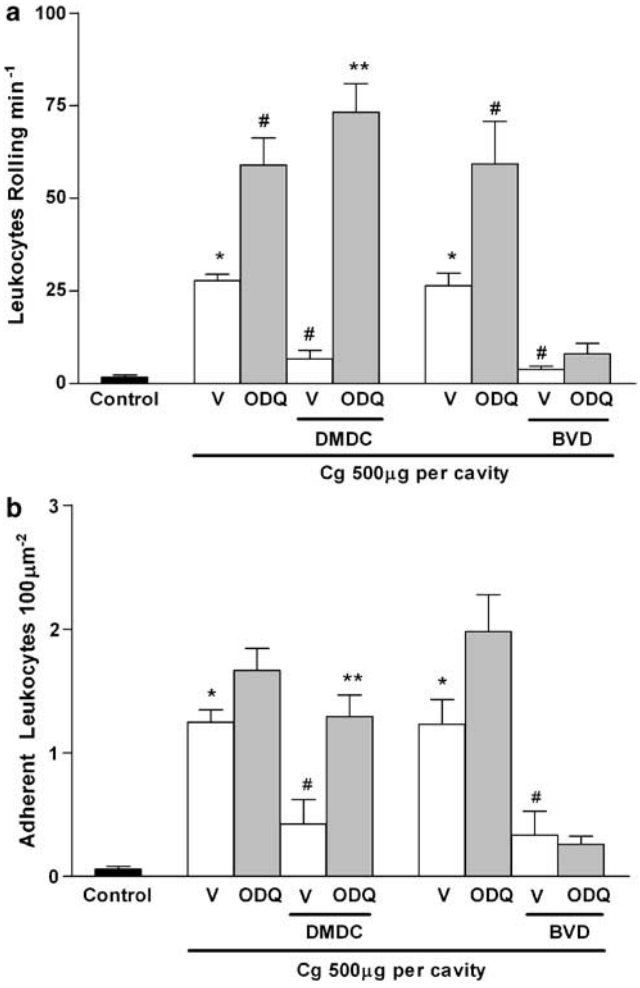

Figure 5 Role of sGC in the inhibitory effect of CO and BVD on the increase of leukocyte rolling and adhesion induced by carrageenan. The mice were pretreated i.p. with vehicle or ODQ (5 μmol kg^{-1}) 15 min before s.c. administration of vehicle, DMDC (7.5 μmol kg^{-1}) or BVD (15 μmol kg^{-1}). After 15 min, the animals received an i.p. injection of carrageenan (500 μg per cavity). The leukocyte rolling (**a**) and adhesion (**b**) were evaluated 2 and 4 h later, respectively. The first bar in each panel represents the leukocyte rolling (**a**) and adhesion (**b**) induced by saline injected i.p. (Control). The results are expressed as the mean $\pm$ s.e.m. of 10 animals per group. *$P<0.05$ compared to control; $^{\#}P<0.05$ compared with mice pretreated with vehicle and injected with carrageenan; **$P<0.05$ compared with mice pretreated with DMDC and injected with carrageenan (ANOVA, followed by Bonferroni's test).

tion, mice were pretreated with AG, a selective inhibitor of iNOS activity. As previously demonstrated (Secco *et al*., 2003), the effect of carrageenan on neutrophil migration was enhanced in mice pretreated with AG (Figure 6). However, AG treatment was not able to reverse the effect of BVD or DMDC on the neutrophil recruitment induced by carrageenan (Figure 6), suggesting that NO is not involved in the inhibition of neutrophil migration caused by CO or BVD. In contrast, the inhibition of HO activity by the pretreatment of mice with ZnDPBG blocked the inhibitory effect of SNAP, an NO donor, on neutrophil migration triggered by carrageenan challenge (Figure 6), indicating that HO activity is involved in the inhibitory effect of NO on the recruitment of neutrophils to an inflammatory site. Accordingly, the pretreatment of mice with SNAP significantly increased the bilirubin concentration in peritoneal exudate induced by carrageenan, suggesting that NO enhances HO activity in carrageenan-induced inflammation in the peritoneal cavity (Table 1).

Discussion and conclusions

Neutrophil migration from the bloodstream into tissues during an inflammatory response results from a multistep process, including rolling, adhesion and transmigration of neutrophils on endothelial cells, which is induced by pro-inflammatory chemotactic mediators, such as TNFα, leukotriene B_4, platelet-activating factor and chemokines released by resident cells (Tessier *et al*., 1998; Szekanecz *et al*., 2003; Gaudreault *et al*., 2005). However, concomitant with the production of pro-inflammatory mediators, there is also a release of anti-inflammatory mediators, including lipoxin and NO (Papayianni *et al*., 1996; Ialenti *et al*., 2000). These mediators downregulate inflammatory events including neutrophil migration and pain (Durate *et al*., 1990; Ialenti *et al*., 2000; Secco *et al*., 2003). Over the last few years, numerous studies have demonstrated that HO-1 expression and the concomitant production of its metabolites, CO and BVD, also have anti-inflammatory consequences (Vachharajani *et al*., 2000; Soares *et al*., 2004). In this work, we explored the critical involvement of HO in neutrophil migration to the inflammatory site and the interplay between HO and NOS systems, finding evidence that a molecular cascade formed by NOS/NO/HO/CO-BVD, acting on either cGMP-dependent or -independent mechanisms, downregulates the leukocyte migration during an acute inflammatory reaction.

The inhibitory role of HO in inflammation, downregulating leukocyte recruitment to the inflammatory site, was well supported by our results. We observed that HO-1 expression and activity were significantly enhanced in our experimental conditions, as increased HO-1 protein expression in mesentery and levels of bilirubin were detected in the peritoneal exudate after carrageenan challenge, and ZnDPBG, which potently inhibits HO activity, reduced the bilirubin production. The ZnDPBG treatment enhanced the inflammatory response to carrageenan, increasing the rolling, the adhesion and the transmigration of neutrophils to the inflammatory site. Moreover, we observed that pretreatment with ZnPP IX (a specific inhibitor of HO-1) also enhanced the rolling, the adhesion and the transmigration of neutrophils to the inflammatory site, suggesting that HO-1 is the isoform involved in the process. Accordingly, the HO substrate, hemin, and also its metabolites, CO and BVD, inhibited in a dose-dependent manner carrageenan-induced neutrophil recruitment, by impairing leukocyte rolling and adhesion to the endothelium. No statistically significant differences were observed in mean arterial pressure and blood leukocyte counts after treatment with any of the drugs used (data not shown), indicating that the changes observed were not due to these vascular alterations. Our findings also corroborate with other data showing that the inhibition of HO pathway

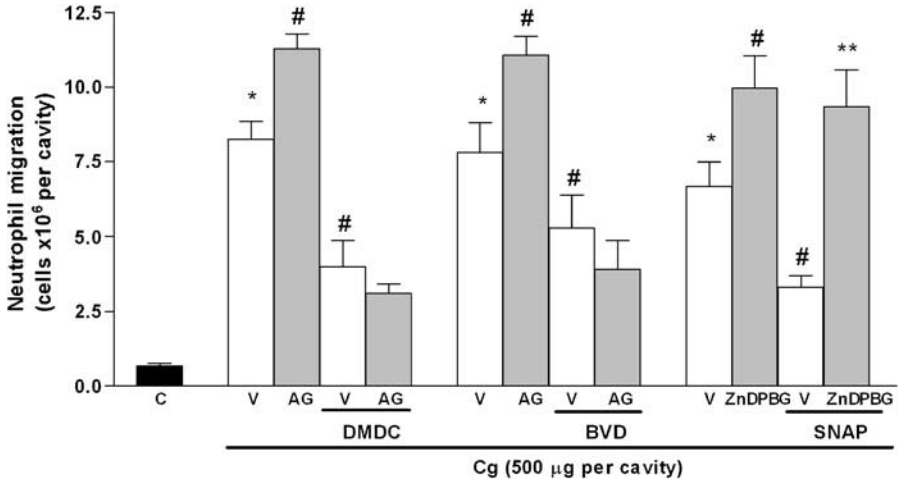

Figure 6 HO mediates the inhibitory effect of NO on neutrophil migration. The mice were pretreated s.c. with vehicle, AG (50 mg kg^{-1}) or ZnDPBG (4.5 μmol kg^{-1}). After 15 min, animals treated with AG received s.c. injection of DMDC (7.5 μmol kg^{-1}) or BVD (15 μmol kg^{-1}), whereas ZnDPBG-treated animals were injected (s.c.) with SNAP (3 mg kg^{-1}). After 15 min, carrageenan (500 μg per cavity) was injected i.p. in all groups. The neutrophil migration was determined 4 h later. The first bar represents the neutrophil migration induced by saline injected i.p. (Control). The results are expressed as the mean $\pm$ s.e.m. of 10 animals per group. $^{*}P<0.05$ compared to control; $^{\#}P<0.05$ compared with mice pretreated with vehicle and injected with carrageenan; $^{**}P<0.05$ compared with mice pretreated with SNAP and injected with carrageenan (ANOVA, followed by Bonferroni's test).

increased the leukocyte migration after an acute inflammatory challenge (Willis *et al*., 1996; Vicente *et al*., 2003).

Several inflammatory mediators, such as TNFα and IL-1β, orchestrate the migration of leukocytes during inflammation (Tessier *et al*., 1998; Dangerfield *et al*., 2005). Our results show that peritoneal TNFα and IL-1β levels were similar among the groups pretreated with ZnDPBG, DMDC or BVD and challenged with carrageenan, suggesting that decreased production of cytokines chemotactic for neutrophils was not involved in the inhibitory effect of HO activity on neutrophil recruitment. In contrast, Vicente *et al*. (2003) demonstrated that HO-1 induction by hemin reduced the levels of TNFα and IL-1β in the zymosan-injected air pouch. A possible explanation for these divergent findings may be provided by the differences in inflammatory stimulus and experimental conditions employed in both studies: we used HO inhibitors and carrageenan as inflammatory stimulus and the above-mentioned authors used HO-1 substrate and zymosan as the inflammatory stimulus. On the other hand, the downregulation of neutrophil migration by HO metabolites was paralleled by reduced leukocyte rolling and adhesion to postcapillary venules when compared with mice challenged with carrageenan, suggesting that the decreased neutrophil recruitment could be due to reduced expression of adhesion molecules on the endothelial cell surface. Consistent with this possibility, it was previously shown that HO inhibition promotes an increase of E- and P-selectin expression induced by lipopolysacharide (LPS), whereas BVD attenuates the LPS-induced expression of endothelial selectin in different regional vascular beds (Vachharajani *et al*., 2000). Moreover, TNFα-mediated expression of VCAM-1 was significantly inhibited in endothelial cells exposed to hemin (Soares *et al*., 2004).

The inhibitory effects of hemin on neutrophil migration, rolling and adhesion found in this study are in apparent contradiction with results from other groups. Graça-Souza *et al*. (2002) demonstrated that hemin triggers a dose-dependent oxidative burst and induces IL-8 production. Moreover, hemin inhibits neutrophil spontaneous apoptosis (Arruda *et al*., 2004), suggesting a role for heme as a pro-inflammatory agent, able to induce neutrophil activation in situations of clinical relevance, that is, hemolysis or hemoglobinemia. In addition, Wagener *et al*. (2001) demonstrated that administration of heme results in a sustained increase of adhesion molecules expression. On the other hand, our results are corroborated by the report of the anti-inflammatory effects of heme, which were ascribed to its ability in inducing HO-1 expression (Wagener *et al*., 1999). Heme-induced HO-1 was reported to result in a reduction of cell migration, exudation and pro-inflammatory mediators release in a zymosan-induced air pouch inflammation model (Vicente *et al*., 2003). Moreover, *in vivo* and *in vitro* experiments show that hemin-induced HO-1 is associated with a decrease in the expression of adhesion molecules induced by inflammatory stimuli (Vachharajani *et al*., 2000; Soares *et al*., 2004). This apparent conflict can probably be accounted for by the different hemin concentrations used in those different studies. In low concentrations, as in the present study, heme acts as an anti-inflammatory and cytoprotective agent via upregulation of HO-1 and stimulation of the formation of HO-1 metabolites, CO and BVD (Hayashi *et al*., 1999; Lindenblatt *et al*., 2004). On the other hand, high concentrations of heme, such as 750 μM, are deleterious to tissue due to its pro-oxidative and pro-inflammatory function (Wagener *et al*., 2001, 2003), which can no longer be neutralized by the antioxidative and anti-inflammatory properties of the HO-1 metabolites.

Taking into account that CO was reported to activate sGC (Morita *et al*., 1995; Maines, 1997), we investigated whether sGC might be involved in the inhibitory effects of CO and BVD on neutrophil migration. We observed that, although the effects of CO in reducing leukocyte rolling, adhesion and

transmigration were impaired by ODQ, this sGC inhibitor did not affect the inhibitory effects of BVD upon these parameters. It is conceivable then, to suggest that whereas the anti-inflammatory properties of CO, similarly to NO, reside in its ability to activate sGC, BVD, another HO-1 metabolite, probably directly affects neutrophil migration due to its potent antioxidant properties (Stocker *et al*., 1987).

The ability of NO derived from both iNOS and eNOS to downregulate neutrophil migration to an inflammatory site was reported by us and other groups (Hickey and Kubes, 1997; Secco *et al*., 2003). Administration of NO donors inhibits rolling, adhesion and neutrophil migration induced by carrageenan (Ialenti *et al*., 2000; Dal Secco *et al*., 2006). Pharmacological inhibition of both eNOS and iNOS or iNOS gene deletion leads to an increase in leukocyte–endothelium interactions and most of these effects displayed by NO are mediated by cGMP, derived from sGC activation (Ialenti *et al*., 2000; Secco *et al*., 2003; Ahluwalia *et al*., 2004; Dal Secco *et al*., 2006).

The interrelationship between HO-1 and NOS systems has been the subject of numerous investigations. A given inflammatory stimuli can induce iNOS expression and also be involved in the induction of HO-1 (Camhi *et al*., 1995). It was shown that NO derived from iNOS can induce HO-1 expression (Vicente *et al*., 2001) and that CO inhibits NOS activity (Thorup *et al*., 1999). In an animal model of acute inflammation, we have demonstrated that the inhibitory effect of NO on neutrophil migratory response, *in vivo*, was blunted when HO activity was inhibited. However, the effects of CO and BVD were not altered when animals were treatment with AG. These results suggest that, at least in our experimental conditions, the downstream signaling events of the inhibitory effect of NO on neutrophil migration involve the HO pathway. Consistent with this, we observed that an NO donor (SNAP) increased the bilirubin production in peritoneal exudate induced by carrageenan, showing that NO enhances HO activity.

To summarize, the present study demonstrates, to our knowledge for the first time, that HO metabolites downregulate neutrophil migration induced by carrageenan to the peritoneal cavity, by mechanisms that can be dependent or independent of activation of sGC. Importantly, we also show that HO activity is involved in the inhibitory effect of NO on neutrophil migration to the inflammatory site. The results suggest that CO and BVD are potential tools for therapies of several clinically relevant inflammatory diseases in which the tissue lesions involve neutrophil recruitment.

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Conflict of interest

The authors state no conflict of interest.

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