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Role of soluble guanylate cyclase activation in the gastroprotective effect of the HO-1/CO pathway against alendronate-induced gastric damage in rats ☆

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ABSTRACT

Our objective was to evaluate the role of soluble guanylate cyclase (sGC) activation in the gastroprotective effect of the HO-1/CO pathway against alendronate-induced gastric damage in rats. Rats were pretreated, once daily for 4 days, with saline, hemin (HO-1 inducer), or dimanganese decacarbonyl (DMDC, CO donor). Another group received zinc protoporphyrin IX (ZnPP IX, HO-1 antagonist) 1 h before hemin treatment or sGC inhibitor (ODQ) 30 min before hemin and DMDC treatment. After 30 min, gastric damage was induced by alendronate (30 mg/kg) by gavage. On the last day of treatment, 4 h after alendronate administration, the animals were killed. Gastric lesions were measured using a computer planimetry program, and gastric corpus pieces were assayed for malondialdehyde (MDA), glutathione (GSH), pro-inflammatory cytokines (tumor necrosis factor [TNF]-α and interleukin [IL]-1β), myeloperoxidase (MPO), or bilirubin. Another group was used to measure gastric mucus. HO-1 expression was determined after saline or alendronate administration by immunohistochemistry. Alendronate induced gastric damage, produced neutrophil accumulation, increased MDA levels and MPO activity, and reduced GSH and mucus in the gastric tissue. Alendronate also increased HO-1 immunoreactivity and the level of bilirubin in gastric mucosa. Pretreatment with hemin or DMDC reduced neutrophil infiltration and TNF-α, IL-1β, and MDA formation, and increased the levels of GSH and mucus in the gastric tissue. ODQ completely abolished the gastroprotective effect of hemin and DMDC and increased alendronate gastric damage. Our results suggest that the HO-1/CO pathway plays a protective role against alendronate-induced gastric damage through mechanisms that can be dependent on sGC activation.

1. Introduction

Bisphosphonates are a class of compounds widely used in the treatment of diseases related to bone remodeling, e.g., osteoporosis and Paget's disease (Aihara et al., 2007; Russell, 2006). However, these compounds cause serious adverse effects in patients, including inflammation, gastric ulcer, nausea, and abdominal pain in the upper gastrointestinal tract, although the mechanism underlying these reactions remains unknown (Graham, 2002; Marshall et al., 2000).

It has been demonstrated that the potential to damage the gastric mucosa is different between bisphosphonates; among these drugs, alendronate seems to have more potential for causing such effect (Lanza et al., 2000; Graham and Malaty, 1999; Graham, 2002). The use of alendronate is associated with irritant effects on the esophagus, stomach, and duodenum (Jeal et al., 1997; Lanza et al., 2000; Graham, 1998). In some cases, severe ulceration has been reported (De Groen et al., 1996). Adverse effects of alendronate in the upper gastrointestinal tract have been attributed in many cases to adherence of the drug to the mucosal surface (Jeal et al., 1997).

Heme oxygenase-1 (HO-1) is a stress-inducible protein, which catalyzes oxidative degradation of heme, thereby eliminating the potentially toxic free-heme, but releasing biliverdin, carbon monoxide (CO), and ferrous iron. Biliverdin is then converted to bilirubin by biliverdin reductase (Pae and Chung, 2009). Biliverdin

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and bilirubin also act as antioxidants (Llesuy and Tomaro, 1994). HO-1 has been identified in the gastric mucosa and participates in a number of cellular defense mechanisms (Guo et al., 2003; Morse and Choi, 2002; Becker et al., 2003). Induction of HO-1 in several animal models of diseases has been shown to protect tissues and cells against oxidative stress, inflammation, ischemia/reperfusion injury, transplant rejection, and apoptosis (Otterbein and Choi, 2000; Wagener et al., 2003).

CO is known to be an activator of soluble guanylate cyclase (sGC), which results in increased cyclic guanosine 3′5′-cyclic monophosphate (cGMP) production. The physiological function of the CO/sGC/cGMP pathway has become the subject of intensive research in recent years, and studies on the gastrointestinal tract have been an important focus of investigations (Gibbons and Farrugia, 2004). In addition, it has been suggested that CO may also have anti-inflammatory properties, including decreased free radical production and inhibition of proinflammatory cytokine expression (Gomes et al., 2010; Kirkby and Adin, 2006; Ryter et al., 2007).

Recently, studies show that CO contributes to maintaining gastric mucosal against ethanol-induced gastric damage and NSAIDs and this beneficial effect of CO seems to depend on cGMP activation (Gomes et al., 2010; Uc et al., 2012; Aburaya et al., 2006).

However, the participation of this gaseous mediator in alendronate-induced gastric damage is unknown.

Considering that there is still no fully effective therapy for gastropathy caused by alendronate and there are few studies on the mechanisms involved in its toxicity, the aim of this study was to evaluate the role of sGC activation in the gastroprotective activity of the HO-1/CO pathway against alendronate-induced gastric damage in rats.

2. Material and methods

2.1. Animals

Male Wistar rats, weighing 180–200 g, were obtained from the Department of Physiology and Pharmacology, Federal University of Ceará. The animals were housed in cages in a temperature-controlled environment under a 12 h light/12 h dark cycle. The animals had free access to drinking water and standard pellet diet (Purina chow). The animals were deprived of food for 18–24 h before the experiment, but had free access to water. All animal treatments and surgical procedures were performed in accordance with the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD) and were approved by the local ethics committee (Protocol No. 0067/10).

2.2. Drugs and solutions

ZnPP IX, Ferriprotoporphyrin IX chloride (hemin), dimanganese decacarbonyl (DMDC), 1 H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), glibenclamide, and alendronate were purchased from Sigma (St. Louis, MO). Hemin was dissolved in 1 mM NaOH, DMDC and ODQ in 0.01% DMSO. Glibenclamide was dissolved in 0.01 N NaOH containing 4% glucose. Alendronate was dissolved in saline and adjusted to pH 7.0 by adding NaOH or HCl (Kanatsu et al., 2004).

2.3. Effect of CO on alendronate-induced gastric damage

The animals were initially treated with hemin (HO-1 inducer: 1, 3, and 10 mg/kg, *i.p.*), DMDC (CO donor: 9, 27, and 81 μmol/kg, *i.p.*), or an equivalent volume of their respective vehicles. After 30 min, the rats received alendronate (30 mg/kg, pH 7.0) by gavage. All drugs were administered once daily for 4 days (adapted from Sener et al., 2004). On the last day of treatment, 4 h after alendronate administration, the animals were killed and their stomachs removed. Gastric damage was measured using a computer planimetry program (Image J). A sample of the stomach was fixed in 10% formalin immediately after its removal for subsequent histopathological assessment and immunohistochemistry for HO-1. Other samples were then weighed, frozen, and stored at −70 °C until assayed for glutathione (GSH) (Sedlak and Lindsay, 1968), malondialdehyde (MDA) (Mihara and Uchiyama, 1978), myeloperoxidase activity (Bradley et al., 1982), and cytokine concentrations (Cunha et al., 1993).

2.4. Role cGMP in the gastroprotective effect of CO

To investigate the role of cGMP in the gastroprotective effect of CO, rats were pretreated with ODQ (guanylate cyclase inhibitor: 10 mg/kg, *i.p.*). One hour before, the rats received hemin (HO-1 inducer: 10 mg/kg, *i.p.*) or DMDC (CO donor: 81 μmol/kg, *i.p.*). After 30 min, the animals received alendronate (30 mg/kg, pH 7.0) by gavage. The control group received only saline or dimethyl sulfoxide (DMSO, 0.01%) plus alendronato or ODQ plus alendronate. All drugs were administered once daily for 4 days. On the 4th day of treatment, 4 h after alendronate administration, the animals were killed, their stomachs removed, and gastric damage was determined as described above. In other groups, the stomachs were removed for measurement of the amount of mucus adhered to the gastric wall (Corne et al., 1974).

2.5. Role of HO-1 on the gastroprotective effect of hemin

To study the role of HO-1 on the gastroprotective effect of hemin, rats were pretreated with HO-1 antagonist (ZnPP IX: 1 mg/kg, *i.p.*). One hour after, rats were treated with hemin (10 mg/kg, *p.o.*). After 30 min, the animals received alendronate (30 mg/kg, pH 7.0) by gavage. The control group received only saline or ZnPP IX plus alendronate. All drugs were administered once daily for 4 days. On the 4th day of treatment, 4 h after alendronate administration, the animals were killed, their stomachs removed, and gastric damage was determined as described above.

2.6. Histological evaluation of gastric lesions

For histological evaluation, stomach samples were fixed in 10% formalin solution, where they remained for 24 h. After this procedure, the samples were transferred to a solution of alcohol 70%. Then, the samples were embedded in paraffin and sectioned. Four-micrometer-thick sections were deparaffinized, stained with hematoxylin and eosin, and then examined under a microscope. The specimens were assessed according to the criteria of Laine and Weinstein (1988), who assigned scores according to the following parameters: epithelial cell loss (a score of 0–3), edema in the upper mucosa (a score of 0–4), hemorrhagic damage (a score of 0–4), and presence of inflammatory cells (a score of 0–3), yielding a maximum total score of 14. Afterward, the sections were assessed by an experienced pathologist who was blinded to the treatment.

2.7. Immunohistochemistry for HO-1

The samples from the stomach of rats undergoing alendronate-induced gastric damage were assessed for the expression of HO-1 by employing an immunohistochemical technique. The slides mounted from the paraffin blocks were deparaffinized and then hydrated. Endogenous peroxidase activity was blocked with 1% H_2O_2 diluted in methanol. Then, the slides were washed in phosphate-buffered saline (PBS). Next, the slides were incubated with primary antibody (1:400, Santa Cruz Biotechnology, Santa

Cruz, CA) overnight at 4 °C. After washing, the slides were incubated with biotinylated secondary antibody, diluted in PBS plus bovine serum albumin (PBS-BSA). Negative control sections were processed simultaneously as described above, but without adding an antibody. Finally, the tissue was stained for antigen-antibody complexes using a peroxidase detection system and then viewed under a microscope.

2.8. Glutathione analysis

The reduced glutathione (GSH) content of stomach tissues was estimated according to the method described by Sedlak and Lindsay (1968). Briefly, 50–100 mg of frozen gastric tissue was homogenized in 1 ml of 0.02 M EDTA for each 100 mg of tissue. Aliquots (400 μl) of the homogenate were mixed with 320 μl of distilled water and 80 μl of 50% (w/v) trichloroacetic acid to precipitate proteins. The tubes were centrifuged at 3000 rpm for 15 min at 4 °C. Supernatants (400 μl) were mixed with 800 μl of Tris buffer (0.4 M, pH 8.9) and 20 μl of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 0.01 M). The mixture was then stirred for 3 min and the absorbance was read at 412 nm using a spectrophotometer. The results were expressed as micrograms of GSH per gram of tissue.

2.9. Malondialdehyde analysis

The level of malondialdehyde (MDA) in the homogenates from each group was measured using the method of Mihara and Uchiyama (1978), which is based on the reaction with thiobarbituric acid. Fragments of gastric mucosa weighing between 100 and 150 mg were homogenized with cold KCl (1.15%) to prepare a 10% solution of homogenate. In brief, 250 μl of this homogenate was added to 1.5 ml of 1% H_3PO_4 and 0.5 ml of 0.6% thiobarbituric acid (aqueous solution). Then, the mixture was stirred and heated in a boiling water bath for 45 min. Next, the reaction mixture was cooled immediately in an ice water bath, followed by addition of 4 ml of n-butanol. This mixture was shaken for 1 min, and the butanol layer was separated by centrifugation at 1200*g* for 10 min. Optical density was determined at 535 and 520 nm, and the optical density difference between the 2 determinations was calculated and considered as thiobarbituric acid value. MDA concentrations were expressed as nanomoles per gram of tissue.

2.10. Myeloperoxidase activity

Myeloperoxidase (MPO) is an enzyme found primarily in neutrophil azurophilic granules. It has been used extensively as a biochemical marker for granulocyte infiltration into various tissues, including the gastrointestinal tract. The extent of neutrophil accumulation in the gastric mucosa was measured by MPO activity evaluation as previously described (Bradley et al., 1982). Briefly, 50–100 mg of tissue was homogenized in 1 ml of potassium phosphate buffer (50 mM, pH 6.0) with 0.5% of hexadecyltrimethylammonium bromide (HTAB) for each 50 mg of tissue. Then, homogenates were centrifuged at 40,000*g* for 7 min at 4 °C. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using *o*-dianisidine dihydrochloride and 1% hydrogen peroxide. The results were reported as MPO units/mg of tissue. A unit of MPO activity was defined as that converting 1 μmol of H_2O_2 to water in 1 min at 22 °C.

2.11. Bilirubin determination in tissue

Briefly, 100 mg of frozen gastric tissue were homogenized in 1 ml of saline. Aliquots (500 μl) of homogenates were added to 250 mg of $BaCl_2$ and vortex-mixed thoroughly, as described by Foresti et al. (2003) with some modifications. Then, 0.75 ml of benzene was added to the mixtures, and the tubes were vigorously vortex-mixed again. The benzene phase containing extracted bilirubin was separated from the aqueous phase by centrifugation at 13,000*g* for 30 min. A standard bilirubin curve was obtained using commercial bilirubin (Labtest, Brazil). Bilirubin was measured spectrophotometrically, as absorbance difference between 450 and 600 nm and expressed as mg/dl (Foresti et al., 2003).

2.12. Measurement of the amount of mucus adhered to the gastric wall

The mucus adhered to the gastric wall in the alendronate-induced damage model was determined according to the method of Corne et al. (1974). Glandular segments from stomachs were collected and weighed. Each segment was transferred to 1% Alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8). Excess dye was removed by washing the segments with 0.25 M sucrose solution. The mucus dye complex was extracted by placing the segments in 0.5 M $MgCl_2$ for 2 h. The dye extract was mixed with diethyl ether, centrifuged at 1400*g* for 10 min, and absorbance of the supernatants was measured at 598 nm. The quantity of extracted Alcian blue (μg/g of glandular tissue) was then calculated using a standard curve of Alcian blue.

2.13. Cytokine measurements

The animals had a sample of their stomach removed on day 4 for analysis of cytokines. The specimens were stored at −70 °C until required for assay. The collected tissues were homogenized and processed as described by SaWeh-Garabedian et al. (1995). The concentration of tumor necrosis factor (TNF)-α and interleukin (IL)-1β was determined by using an enzyme-linked immunosorbent assay (ELISA), as described previously (Cunha et al., 1993). Briefly, microtiter plates were coated overnight at 4 °C with an antibody against rat TNF-α or IL-1β (4 μg ml^{-1}, DuoSet ELISA Development kit R&D Systems; Catalog DY501 or DY510, respectively). After blocking the plates, the samples and standards were added at various dilutions in duplicate and incubated at 4 °C for 24 h. The plates were washed 3 times with buffer. After washing the plates, biotinylated sheep polyclonal anti-TNF-α or anti-IL-1β (diluted 1:1,000 with assay buffer containing 1% bovine serum albumin [BSA]) was added to the wells. After further incubation at room temperature for 1 h, the plates were washed and 50 μl of avidin-conjugated horseradish peroxidase diluted 1:5,000 was added to the wells. The color reagent *o*-phenylenediamine (OPD; 50 μl) was added 15 min later and the plates were incubated in the dark at 37 °C for 15–20 min. The enzyme reaction was stopped with H_2SO_4 and absorbance was measured at 490 nm. Values are expressed as pictograms of cytokines per milliliter (pg/ml).

2.14. Statistical analysis

All values are expressed as mean ± S.E.M. ANOVA and Student–Newman–Keuls test were used to determine statistical significance of differences between groups. For histological assessment, the Kruskal–Wallis nonparametric test was used, followed by Dunn's test for multiple comparisons. Differences were considered to be significant when $P < 0.05$.

3. Results

The present study confirmed that alendronate damaged both the corpus and the antral mucosa of the stomach and this damage

was mostly surface epithelial injury. The direct exposure of the rat gastric mucosa to alendronate for 4 days produced widespread damage to the epithelium, and the luminal surface was covered with cellular debris (data not shown). Chronic oral administration of alendronate caused hemorrhagic lesions in the mucosa of the glandular stomach, indicating true ulcer formation and this was also supported by the histopathological findings (Table 1).

In or study, we demonstrated that hemin (HO-1 inducer) and DMDC (CO-donor) treatment prevented alendronate-induced macroscopic and microscopic gastric damage (Fig. 1A and B, and Table 1). From the microscopic analysis, alendronate increased hemorrhagic damage, edema, epithelial cell loss, and infiltration of inflammatory cells, when compared to the control group. Hemin and DMDC significantly decreased infiltration of inflammatory cells, formation of edema, and epithelial cells loss induced by alendronate, but other parameters did not change (Table 1). HO-1 inhibition by ZnPP IX significantly reversed the hemin-mediated protection in both macroscopic and microscopic assessments (Fig. 1A and Table 1). Rats treated with ZnPP IX plus alendronate showed an increase of gastric damage as in those treated with alendronate alone (Fig. 1A); however, ZnPP IX alone had no effect on the gastric damage parameters. Because hemin at a dose of 10 mg/kg and DMDC at a dose of 81 μmol/kg afforded the most protection against gastric lesions induced by alendronate, these doses were selected for the study of possible mechanisms of action involved in CO-mediated gastroprotective effects.

In this study, HO-1-immunoreactive cells were observed in the gastric mucosa after hemin administration (Fig. 2B, black arrows), alendronate administration (Fig. 2C, black arrows), or hemin plus alendronate administration (Fig. 2D, black arrows) when compared with the control group (Fig. 2A) and alendronate treatment resulted in bilirubin accumulation in gastric tissues (Fig. 2E). Rats treated with hemin plus alendronate showed an increase in HO-1-immunoreactive cells as in those treated with alendronate alone (Fig. 2E). This result demonstrated that hemin alone or alendronate alone increased HO-1 expression in gastric mucosa when compared to the control group and hemin plus alendronate causes a greater induction than alendronate alone (Fig. 2).

In the present study, we investigated whether cGMP participated in the gastroprotective effects of hemin and DMDC against alendronate-induced gastric damage. Pretreatment with ODQ, a sGC inhibitor, significantly reversed hemin and DMDC-induced gastroprotection in alendronate-induced macroscopic and microscopic gastropathy (Fig. 3), but ODQ by itself had no effect on the

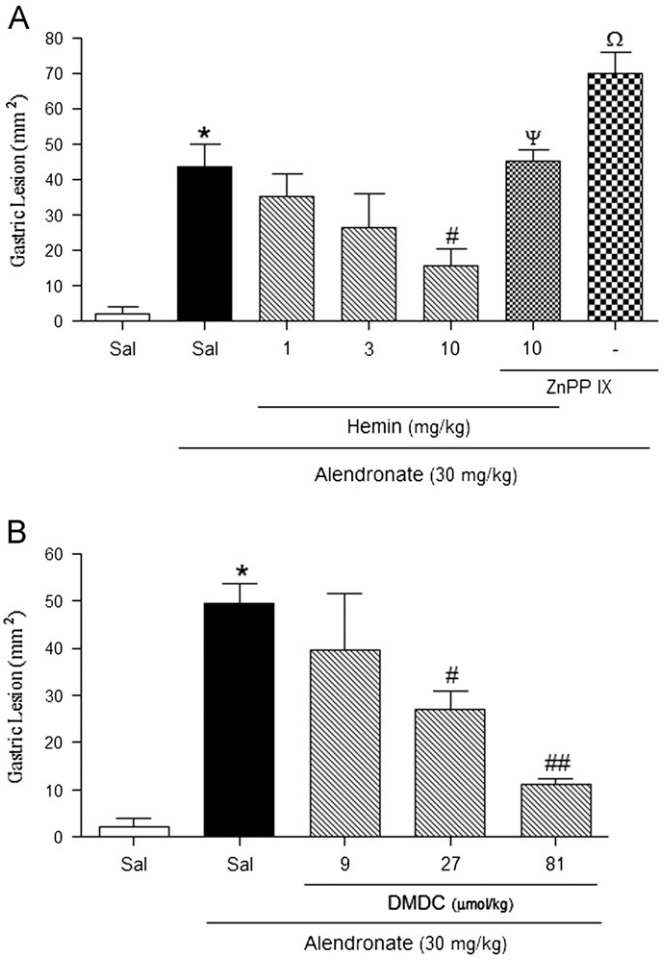

Fig. 1. Hemin (A) and dimanganese decacarbonyl (DMDC) (B) prevents alendronate-induced gastric damage in a dose-dependent manner. Rats were treated by gavage with hemin or DMDC 30 min before alendronate (30 mg/kg) administration. Another group received zinc protoporphyrin IX (ZnPP IX, HO-1 antagonist) 1 h before hemin treatment (panel A). The control group was treated with saline only. All drugs were administered once daily for 4 days. The total area of macroscopic gastric lesions was determined after 4 days. Results are expressed as mean ± S.E.M. of at least 5 rats per group. (*) $P < 0.05$, when compared to control. (#) $P < 0.05$, when compared to the alendronate group; ($^{\Psi}$) $P < 0.05$, when compared to the hemin plus alendronate group; ANOVA and Newman–Keuls test were used for evaluation.

Table 1
Effect of ODQ (10 mg/kg) and ZnPP (1 mg/kg) pretreatment on protective effect of hemin (10 mg/kg) or DMDC (81 μmol/kg) in alendronate-induced microscopic gastric damage.

Experimental group	Hemorrhagic damage (score 0–4)	Edema (score 0–4)	Epithelial cell loss (score 0–3)	Inflammatory cells (score 0–3)	Total (score 0–14)
Saline	0 (0–1)	0	0 (0–1)	0	1 (0–2)
Alendronate	3 (1–3)[a]	3 (2–3)[a]	3 (2–3)[a]	2 (2–3)[a]	9 (7–12)[a]
Hemin+alendronate	2 (1–2)	1 (0–1)	0 (0–2)[b]	0 (0–1)[b]	2 (2–5)[b]
ZnPP+hemin+alendronate	3 (1–3)	3 (1–3)	3 (2–3)[c]	3 (2–3)[c]	10 (6–12)[e]
ZnPP+alendronate	3 (2–3)	3 (1–3)	3 (2–3)	3 (2–3)	8 (7–12)
ODQ+hemin+alendronate	3 (1–3)	2 (2–3)[c]	3 (2–3)[c]	2 (2–3)[c]	10 (7–12)[c]
DMDC+alendronate	2 (0–2)	0 (0–1)[b]	0 (0–1)[b]	0 (0–1)[b]	2 (2–4)[b]
ODQ+DMDC+alendronate	3 (1–4)	3 (2–3)[d]	3 (2–3)[d]	3 (2–3)[d]	11 (7–13)[d]
ODQ+alendronate	4 (1–4)	3 (2–3)	3 (2–3)	3 (2–3)	10 (7–13)

Data shown are medians with minimal and maximal scores shown in parentheses. Kruskal–Wallis nonparametric test, followed by Dunn's test was used for multiple comparisons for histological assessment.

[a] $P < 0.05$, when compared with control group.
[b] $P < 0.05$, when compared with alendronate group.
[c] $P < 0.05$, when compared with Heminplus alendronate group.
[d] $P < 0.05$, when compared with DMDC plus alendronate group.

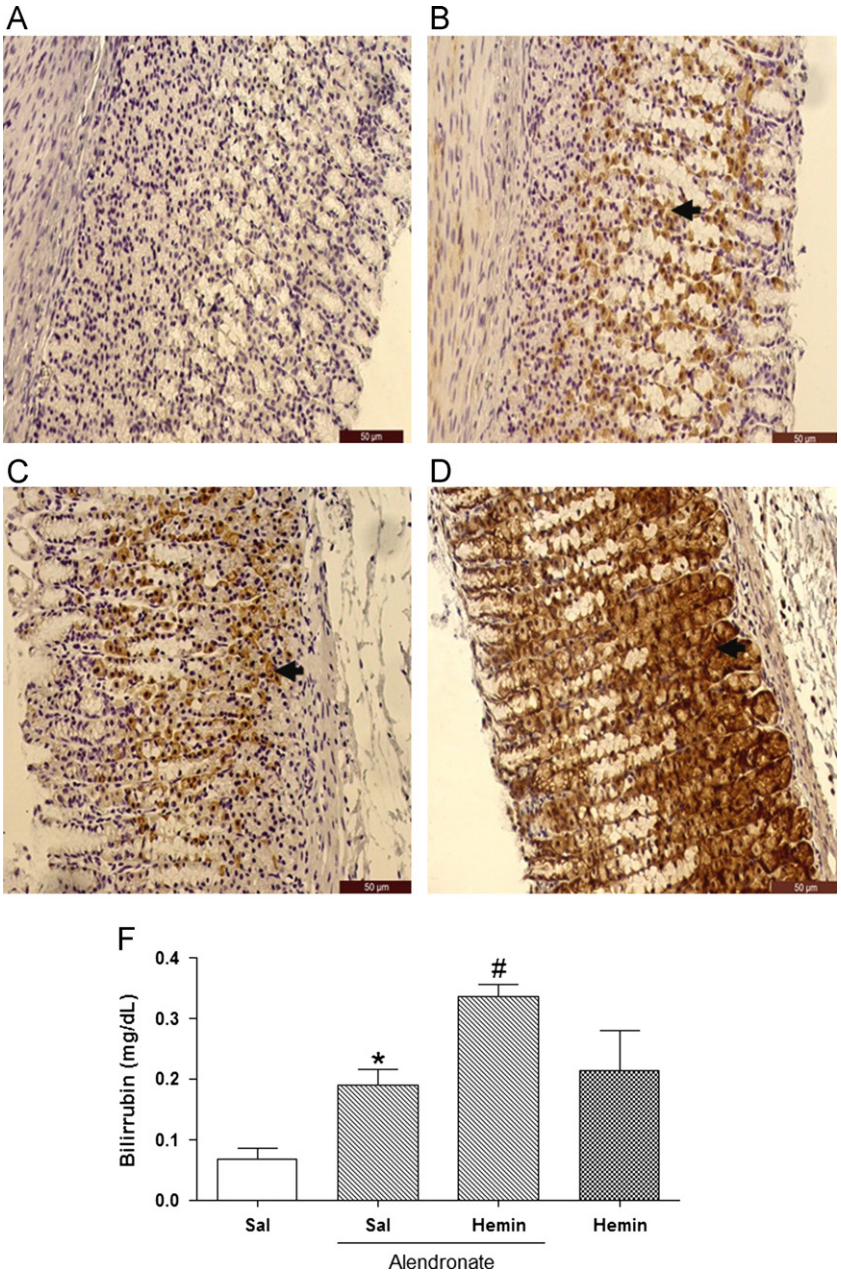

Fig. 2. Photomicrographs of gastric mucosa (magnifications, ×100) showing HO-1 immunoreactivity was absent in normal gastric tissue (panel A), but HO-1 immunoreactivity was detected in the gastric mucosa tissue after alendronate administration once daily for 4 days (panel B) and hemin (10 mg/kg) administration once daily for 4 days (panel C). Other group was treated with hemin (10 mg/kg) plus alendronate (panel D) and the HO-1 immunoreactivity was greater than with alendronate alone. Panel E: Bilirubin levels in rats treatment with saline, hemin, alendronate or hemin + alendronate administered once daily for 4 days. (*) $P < 0.05$, when compared to control (saline). (#) $P < 0.05$, when compared to the alendronate group. ANOVA and Newman–Keuls test were used for evaluation.

gastric damage parameters. Furthermore, ODQ plus alendronate treatment significantly increased macroscopic gastric damage when compared to alendronate alone (Fig. 3). These results suggest that endogenous HO-1/cGMP pathway is involved on alendronate induced gastric damage.

Administration of alendronate resulted in reduced GSH levels, increased MDA concentration and MPO activity (Table 2), and increased levels of pro-inflammatory cytokines (TNF-α and IL-1β, Fig. 4A and B, respectively) when compared with the control group. Hemin and DMDC pretreatments reversed the effect of alendronate on these biochemical parameters. Hemin and DMDC also reduced the levels of TNF-α and IL-1β (Fig. 4A and B, respectively). The only treatment with ODQ did not alter the alendronate-induced changes in cytokines levels and MPO activity (Table 2 and Fig. 4), but amplified the effect of alendronate on GSH and MDA levels (Table 2).

Alendronate decreased significantly the amount of gastric adherent mucus when compared to the control group (saline). DMDC treatment prevented this effect of alendronate. However, ODQ pretreatment reversed the gastroprotective effect of DMDC. Mucus amount in these animals was decreased to levels similar to those in animals of the alendronate group (Fig. 5).

4. Discussion

The HO enzyme represent an important anti-inflammatory pathway and has been shown to exhibit powerful protective

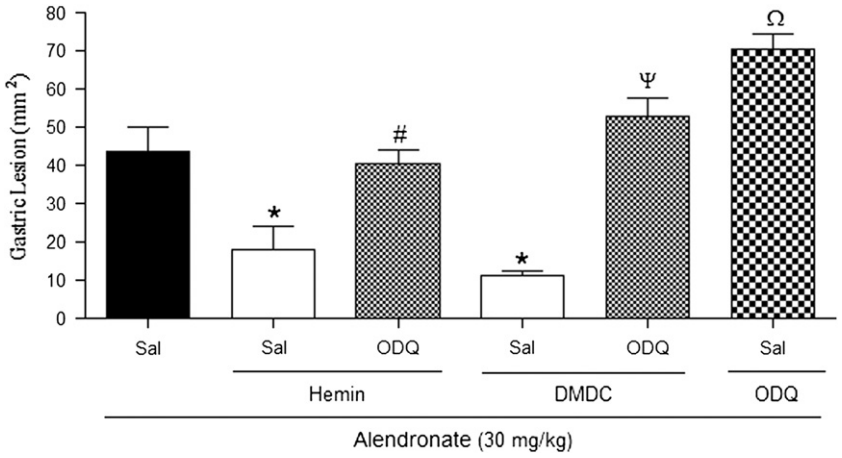

Fig. 3. Effect of pretreatment with ODQ on the protective effect of hemin and DMDC against alendronate-induced gastric damage. ODQ (10 mg/kg, *i.p.*) was injected 1 h before hemin (10 mg/kg) or DMDC (81 μmol/kg). Thirty minutes later, alendronate (30 mg/kg) was administered. The control group was treated with saline. All drugs were administered once daily for 4 days. Macroscopic gastric lesions were determined after 4 days. Results are expressed as the mean ± S.E.M. of at least 5 animals per group. (*) $P < 0.05$, when compared to the alendronate group; (#) $P < 0.05$, when compared to the hemin plus alendronate group; ($^{\Psi}$) $P < 0.05$, when compared to the DMDC plus alendronate group; ANOVA and Newman–Keuls test.

Table 2
Effect of pretreatment with hemin (10 mg/kg) or dimanganese decacarbonyl (DMDC) (81 μmol/kg) against alendronate-induced gastric damage in GSH, MDA, and MPO levels in the gastric mucosa.

Experimental group	GSH (μg/g tissue)	MDA (nmol/g tissue)	MPO (U/mg tissue)
Saline	482.5 ± 20.1	71.5 ± 2.6	7.2 ± 0.6
Alendronate	180.3 ± 21.9[a]	121.1 ± 4.3[a]	31.5 ± 3.8[a]
Hemin+alendronate	570.3 ± 21.6[b]	99.0 ± 6.3[b]	12.0 ± 2.2[b]
DMDC+alendronate	482.1 ± 73.8[b]	58.5 ± 10.5[b]	9.4 ± 0.7[b]
ODQ+hemin+alendronate	200.8 ± 42.5[c]	115.2 ± 2.3[c]	28.1 ± 3.3[c]
ODQ+DMDC+alendronate	172.7 ± 35.2[d]	102.5 ± 8.8[d]	22.2 ± 1.9[d]
ODQ+alendronate	109.2 ± 13.2[b]	196.1 ± 12.4[b]	34.2 ± 6.1

Results are mean ± S.E.M. of 5–7 rats.
[a] $P < 0.05$, when compared with control group.
[b] $P < 0.05$, when compared with alendronate group.
[c] $P < 0.05$, when compared with hemin plus alendronate group.
[d] $P < 0.05$, when compared with DMDC plus alendronate group.

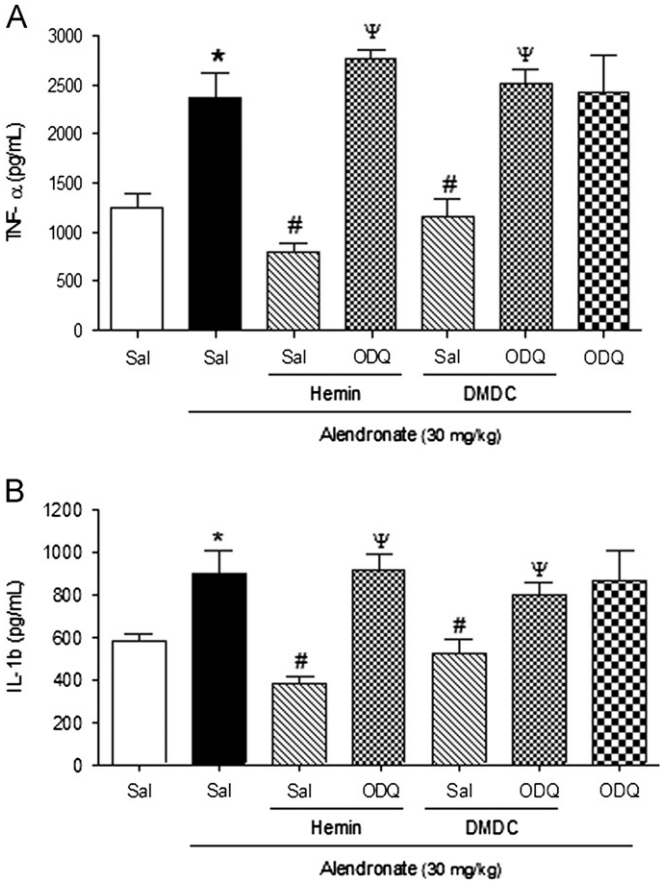

Fig. 4. Treatment with hemin (10 mg/kg) or DMDC (81 μmol/kg) decreased concentration of TNF-α and IL-1β (A and B, respectively) in alendronate-induced gastric damage. Rats were treated by gavage with hemin or DMDC 30 min before alendronate (30 mg/kg) administration. The control group was treated with saline only. All drugs were administered once daily for 4 days. Results are expressed as the mean ± S.E.M. of at least 5 rats per group. (*) $P < 0.05$, when compared to control. (#) $P < 0.05$, when compared to the alendronate group. ANOVA and Newman–Keuls test were used for evaluation.

effects against a variety of stressors in different systems (Attuwaybi et al., 2003; Giris et al., 2006; Otterbein et al., 2003). Various stressors such as oxidative stressors, ultraviolet irradiation, inflammatory cytokines, and heavy metals have been reported to induce HO-1 production (Maines, 1997).

HO-1 degrades heme to CO, free iron, and biliverdin. Both biliverdin and CO have potent antioxidative, anti-inflammatory, and gastroprotective activity (Ponka, 1999; Maines, 1997; Gomes et al., 2010). CO mediates the activation of sGC, thereby stimulating the production of cGMP. The CO/sGC/cGMP pathway has been implicated in mediating the protective effects of CO (Ryter and Otterbein, 2004). However, the protective effects of biliverdin do not depend on activation of sGC (Gomes et al., 2010). Considering the importance of these enzymes, particularly HO-1, as therapeutic strategy for the protection against inflammatory processes and oxidative tissue damage, we investigated the role of sGC activation in the gastroprotective effect of the HO-1/CO pathway against alendronate-induced gastric damage in rats in the present study.

The mechanism by which bisphosphonates promote lesion formation in the gastric mucosa is still unknown. Our results indicate that the HO-1/CO/cGMP pathway plays a protective role against alendronate-induced gastric damage and that these preventive effects probably result from decreased free radical production and inflammation process in the gastric mucosa. As demonstrated in the literature related to ethanol-induced gastropathy (Gomes et al., 2010) and colitis models (Hegazi et al., 2005), HO-1 induction by hemin, HO-1 product, or CO donor reduced inflammatory gastropathy and colonic damage. Likewise, it was verified that a HO-1

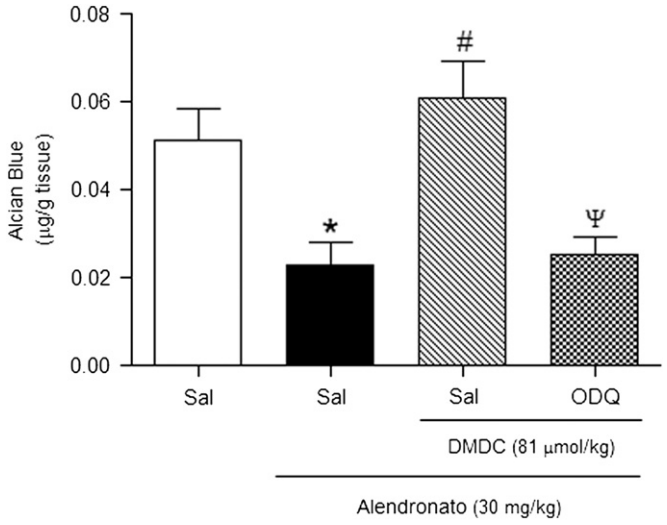

Fig. 5. Effect of pretreatment with ODQ on the protective effect of DMDC on the levels of mucus in the alendronate-induced gastric damage. ODQ (10 mg/kg) was administered by gavage 1 h before DMDC (81 µmol/kg). After another 30 min, alendronate (30 mg/kg) was administered. The control group was treated with saline only. All drugs were administered once daily for 4 days. Results are expressed as the mean ± S.E.M. of at least 5 animals per group. (*) $P < 0.05$, when compared to control; (#) $P < 0.05$, when compared to the DMDC plus alendronate group; ANOVA and Newman–Keuls test.

enzyme inhibitor, ZnPP IX, increased gastric damage induced by ethanol (Gomes et al., 2010) and exacerbated the gastric mucosa lesions induced by HCl (Ueda et al., 2008)

We confirmed the potential of alendronate to promote lesion formation in the gastric epithelium. Our results demonstrated that administration of alendronate (30 mg/kg, once daily for 4 days) promotes macroscopic and microscopic gastric mucosal damage, i.e., formation of hemorrhages and edema, migration of inflammatory neutrophils, and epithelial cell loss. We observed that both CO-donor (DMDC) and CO precursor (hemin) decreased alendronate-induced gastropathy. Therefore, we could infer that CO synthesis is essential for gastric protection against alendronate.

We also demonstrated that alendronate increased HO-1 immunoreactivity in gastric mucosal cells, which was confirmed by bilirubin accumulation, a secondary product of HO-1, in gastric tissues. Our results are in accordance with the literature. Gomes et al. (2010) demonstrated that another aggressor agent (ethanol) increased the levels of HO-1 mRNA transcripts in the gastric mucosa; they also showed HO-1 immunoreactivity in the lamina propria, submucosa, and muscle layer. Naito et al. (2011) reported that local levels of biliverdin and bilirubin are increased after HO-1 induction and this may be beneficial in protecting several types of cells from injury. Dalton et al. (1999) have reported that in order to compensate stressors agents, higher animals have evolved physiological defense mechanisms, including expression of antioxidant proteins and phase II detoxifying enzymes. Induction of phase II enzymes such as HO-1 renders cells more resistant to potential subsequent challenges of greater stress (Yeligar et al., 2010).

In fact, alendronate has been shown to cause erosions and ulcers in rodents and human stomach and to interfere with the healing of pre-existing lesions (Elliott et al., 1998; Graham and Malaty, 1999; Wallace et al., 1999). The mechanism through which alendronate and other bisphosphonates cause mucosal injury has not been clearly identified. When bisphosphonates are administered intravenously, the occurrence of gastritis and esophagitis is uncommon. Thus, it seems that alendronate produced damage through topical irritant effects on the gastric epithelium (Geddes et al., 1994) and the changes in the gastric microcirculation do not appear to contribute significantly to the pathogenesis of epithelial injury (Wallace et al., 1999). Furthermore, it was demonstrated that neutrophil accumulation and subepithelial edema in the gastric mucosa caused by alendronate are important for the development of ulcer (Wallace et al., 1999). The latter effect is associated with increased production of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) (Yamaguchi et al., 2000; Thieâbaud et al., 1997).

In the present study, elevated accumulation of neutrophils and TNF-α and IL-1β levels along with increased MPO activity in the gastric mucosa caused by alendronate indicate the contribution of neutrophil infiltration and the impact of proinflammatory cytokines (TNF-α and IL-1β) in oxidative gastric damage. Since a CO-donor and CO precursor inhibited alendronate-induced accumulation of neutrophils in the mucosa, increased MPO activity, and abolished the TNF-α and IL-1β response concomitantly, our results suggest that neutrophil accumulation contributes to alendronate-induced gastric injury. Furthermore, the gastroprotective effect of CO may be, in part, dependent on its inhibitory effect on gastric mucosa neutrophil infiltration and neutrophil-associated TNF-α and IL-1β response.

Our results are in accordance with the literature. Recent investigations have shown that HO-1/CO inducers increase HO-1 expression in gastrointestinal mucosa and ameliorate mucosal injury as well as inflammatory cell accumulation by decreasing infiltrating neutrophils via inhibition of nuclear factor-kappa beta (NF-κB)-dependent proinflammatory cytokines, suggesting that CO can mediate the anti-inflammatory actions of HO-1. Recently, Dal-Secco et al. (2010) demonstrated that the HO-1/CO/cGMP pathway inhibits neutrophil recruitment during the inflammatory response.

Accordingly, it was demonstrated that aminobisphosphonates induce inflammatory reactions in several tissues (Yamaguchi et al., 2000). In addition to their direct damaging effects on tissues, it is well established that oxygen metabolites play a role in the recruitment of neutrophils into injured gastric mucosa (Zimmerman et al., 1990; Kettle and Winterbourn, 1997). Activated neutrophils are also a potential source of oxygen metabolites (Sullivan et al., 2000).

It has been suggested that oxygen-derived free radicals may contribute to alendronate-induced gastric mucosal lesions and reduced GSH content in stomach tissues (Sener et al., 2004, 2005). Therefore, it is possible that the effect of CO may result in a decreased redox state in alendronate-induced gastropathy. Our results showed that administration of hemin or DMDC reversed the decrease in gastric GSH levels after alendronate administration. Thus, CO may function by decreasing the redox state in alendronate-induced gastropathy. Therefore, another possibility is that an increase in GSH levels could be secondary to a decrease in free radical production. Our results demonstrated that hemin or DMDC pretreatment resulted in a significant decrease in MDA levels in alendronate-induced gastropathy. In general, it has been demonstrated that HO-1-derived CO has anti-oxidant properties against oxidative stress in vivo and in vitro (Mancuso, 2004; Ryter and Tyrrell, 2000). Thus, the mechanism through which CO exerts its gastroprotective effect seems to involve the reduction of lipid peroxidation induced by alendronate in the gastric mucosa.

Recent studies reported that CO has a number of important physiological properties that are activated through sGC and elevated intracellular cGMP levels (Gonzales and Walker, 2002; Gomes et al., 2010). Using a pharmacological approach, we demonstrated that inhibition of sGC by ODQ reversed the protective effects of a CO donor (DMDC) and a CO precursor (hemin) against alendronate-induced gastric damage and ODQ plus alendronate increase the alendronate effects. Our results are in accordance with the literature. Some works have demonstrated that CO activates sGC (Ryter and Otterbein, 2004; Morita et al., 1995) and ODQ treatment completely

abolished the protective effect of HO-1 against non-steroidal anti-inflammatory drugs and ethanol-induced gastric damage (Gomes et al., 2010; Freitas et al., 2006).

Several studies have suggested that bisphosphonates have a direct irritant effect on the gastric mucosa (Graham and Malaty, 1999; Blank et al., 1997; Elliott et al., 1998), i.e., to decrease the hydrophobic barrier (Lichtenberger et al., 2000). This damaging effect is not due to a decline in the levels of mucosal prostaglandins (Marshall et al., 2000), but through topical irritant action. The damage in the stomach is accompanied by disruption of the mucosal barrier, leading to H^+ back-diffusion into the mucosa (Kanatsu et al., 2004). Furthermore, Lichtenberger et al. (2000) showed that the topical irritant property of bisphosphonates is, in part, attributable to the ability of bisphosphonates to chemically associate with and destabilize the surface lining of phospholipids, resulting in a rapid decrease in the tissues hydrophobic barrier. In the present study, we confirmed that alendronate caused a significant reduction in gastric mucus production when compared with the negative control (saline). Pretreatment with a CO donor significantly increased amount of mucus in the gastric mucosa wall. However, when the animals were pretreated with ODQ, this gastroprotective effect was abolished. Studies linking CO with mucus secretion in the stomach are scarce. However, Brown et al. (1993) showed that other gaseous mediators, e.g., NO, also stimulate mucus secretion by rat gastric mucosal cells, and this effect appears to depend on elevation of intracellular cGMP levels.

In summary, our results indicate that CO prevents alendronate-induced gastric damage by activation of sGC, decreases direct oxidative damage, and causes inhibition of neutrophil infiltration. Although there are many mechanisms through which this effect can occur, our data support the hypothesis that activation of the HO-1/CO/cGMP pathway is of primary importance. These observations also raise the possibility that CO-releasing agents could be used to improve resistance to gastric mucosa injury.

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