Role of the haem oxygenase/carbon monoxide pathway in *Clostridium difficile* toxin A-induced enteritis in mice

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Clostridium difficile is the major cause of antibiotic-associated colitis, a disease with significant morbidity and mortality. This study investigated the role of the haem oxygenase-1 (HO-1)/carbon monoxide (CO) pathway in *C. difficile* toxin A-induced enteritis in mice. The HO substrate haemin, zinc protoporphyrin IX (ZnPP IX), a specific HO-1 inhibitor, dimanganese decacarbonyl (DMDC), a CO donor, or an equivalent volume of their respective vehicles were injected subcutaneously 30 min prior to local challenge with toxin A (25 or 50 µg per ileal loop) or PBS. Intestinal ileal loop weight/length ratios were calculated 3 h later. Ileal tissues were collected for histological analysis and measurement of myeloperoxidase (MPO) activity, tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) production by ELISA and immunohistochemistry for HO-1. Treatment of mice subjected to *C. difficile* toxin A (TcdA) with haemin or DMDC prevented oedema, mucosal disruption and neutrophil infiltration observed in histological analysis. It also decreased TcdA-induced MPO activity and TNF-α or IL-1β production. In contrast, the specific HO-1 inhibitor (ZnPP IX) exacerbated all these evaluated parameters. TcdA increased HO-1 expression as seen by immunohistochemistry. These results suggest that the HO-1/CO pathway exerts a protective role in TcdA-induced enteritis and that its pharmacological modulation might be important for the management of *C. difficile*-associated disease.

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INTRODUCTION

Clostridium difficile is the major causative agent of antibiotic-associated diarrhoea. The main *C. difficile* virulence factors are two large exotoxins, toxin A (TcdA) and toxin B (TcdB), which show substantial sequence similarity and share common structural elements (Pothoulakis *et al.*, 1986). A recent report demonstrated that, instead of TcdA, TcdB is actually the essential virulence factor for the development of *C. difficile*-associated disease (Lyras *et al.*, 2009). However, by using a gene knockout system to inactivate the toxin genes permanently, Kuehne *et al.* (2010) found that *C. difficile* producing either one or both toxins showed cytotoxic activity *in vitro* that translated directly into virulence *in*

vivo, re-establishing the importance of both toxins in the pathogenesis of *C. difficile*-associated disease. The mechanism of TcdA-induced enteritis involves toxin binding to enterocyte receptors, which leads to infiltration of the mucosa by neutrophils, degranulation of mast cells and activation of sensory and enteric nerves, resulting in enhanced intestinal secretion and motility (Brito *et al.*, 2002b; Kelly *et al.*, 1994a; Pothoulakis *et al.*, 1998).

Haem oxygenase (HO; EC 1.14.99.3) catalyses haem degradation to iron, carbon monoxide (CO) and biliverdin (BVD) (Abraham *et al.*, 1988). Three isozymes have been identified: HO-2 and HO-3, which are constitutive forms (Maines *et al.*, 1986; McCoubrey *et al.*, 1997), and HO-1, which is induced by various stimuli such as haem, haemin, cytokines, mitogens, metals, reactive oxygen species, heat shock, UV radiation, hypoxia or hyperoxia (Maines *et al.*, 1986; Otterbein & Choi, 2000). Over the past years, numerous studies have demonstrated that HO-1, its substrate, haem, and its metabolites, CO and BVD, are able to modulate the inflammatory process (Willis *et al.*,

Abbreviations: BVD, biliverdin; CO, carbon monoxide; DMDC, dimanganese decacarbonyl; HO, haem oxygenase; IL-1β, interleukin-1 beta; MPO, myeloperoxidase; s.c., subcutaneous/subcutaneously; TcdA, *C. difficile* toxin A; TNF-α, tumour necrosis factor alpha; ZnPP IX, zinc protoporphyrin IX.

1996; Paine *et al.*, 2010; Vijayan *et al.*, 2010). In the gastrointestinal tract, the protective role of HO-1 has been demonstrated indirectly in trinitrobenzene sulphonic acid-induced colitis in rats (Wang *et al.*, 2001). However, the relevance of HO-1 in the pathophysiology of *C. difficile*-associated disease has never been assessed before to our knowledge.

The present study evaluates the role of the HO-1/BVD/CO pathway in TcdA-induced enteritis in mice.

METHODS

Animals. We used 104 male C57BL/6 mice, 25–30 g body weight, from the animal colony of the Federal University of Ceará. The animals received both sterilized water and food *ad libitum*. All experimental protocols were approved by the local Animal Care and Use Committee.

Drugs and toxins. Purified TcdA from *C. difficile* (strain #10463; molecular mass 308 kDa) was kindly provided by Dr David Lyerly (TechLab) and used diluted in PBS (pH 7.4); haemin and dimanganese decacarbonyl (DMDC) were purchased from Sigma Aldrich Products and zinc protoporphyrin IX (ZnPP IX) was purchased from Porphyrin Products. Haemin was dissolved in 1 mM NaOH, ZnPP IX in 50 mM Na₂CO₃ and DMDC in DMSO. All drugs were protected from light, except DMDC, which was exposed to cold light before administration to mice (Johnson *et al.*, 2003).

Induction of intestinal inflammation. The TcdA-induced enteritis mouse model was used as described previously (Castagliuolo *et al.*, 1998). Briefly, mice were fasted overnight but with free access to water. Prior to surgery, mice were anaesthetized with ketamine and xylazine (100 mg kg⁻¹ and 10 mg kg⁻¹ intramuscularly, respectively). Through a midline laparotomy, one 4 cm ileal loop was ligated and injected with either 0.1 ml PBS (pH 7.4; control) or buffer containing TcdA (25 or 50 µg). The abdomen was closed, and the animals were allowed to regain consciousness. Three hours after administration of TcdA or PBS, mice were sacrificed. Intestinal loops were removed and the loop length and weight were recorded. A portion of the loop was frozen at -70 °C for determination of myeloperoxidase (MPO) activity, and for TNF-α and IL-1β assay by ELISA; the remaining tissue was fixed in 10% formalin and processed for histology and immunohistochemistry.

Experimental protocol. Mice were injected subcutaneously (s.c.) with ZnPP IX, a specific HO-1 inhibitor (4.5 µmol kg⁻¹), with haemin, HO substrate (4.5 µmol kg⁻¹), with DMDC, a CO donor (25 µmol kg⁻¹), or with an equivalent volume of their respective vehicles. Thirty minutes later, TcdA (50 µg) was injected in the ileal loop of all animal groups, except those pretreated with the HO inhibitor, which received a lower dose of TcdA (25 µg per ileal loop), or their vehicles, which received PBS in the ileal loop (100 µl). This dose of 25 µg TcdA per ileal loop was used in mice pretreated with HO inhibitor to induce a submaximal inflammatory response (Dal Secco *et al.*, 2003) thus allowing a possible enhancement of the inflammatory response by treatment with the HO inhibitors. It is important to mention that 1 mol DMDC (Mn₂CO₁₀) under experimental conditions releases 4 mol CO (Dearden *et al.*, 1989).

Histology. Ileal tissues were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin. The severity of inflammation was scored in coded slides by a pathologist on a scale of 0 (absence of alterations), 1 (mild), 2 (moderate) to 3 (severe) for

epithelial damage, congestion/oedema, neutrophil infiltration and haemorrhage as previously described (Kirkwood *et al.*, 2001; Cavalcante *et al.*, 2006).

Fig. 1. Effect of haemin, ZnPP IX or DMDC on weight of ileal loops injected with *C. difficile* TcdA in mice. The mice were pretreated s.c. with haemin (a), ZnPP IX (b) or DMDC (c) and with their respective vehicles. After 30 min, 50 µg *C. difficile* TcdA was injected in ligated ileal loops for animals pretreated with either haemin or DMDC and 25 µg was injected in ligated ileal loops for animals pretreated with ZnPP IX. Bars represent the mean value ± SEM of the weight/ileal loop length (mg cm⁻¹) of at least five mice per group. **P* < 0.05 represents statistical differences compared to PBS mice; #*P* < 0.05 represents statistical differences compared to mice subjected to *C. difficile* TcdA and injected with NaOH or DMSO. Data were analysed by using analysis of variance (ANOVA) and Bonferroni's test.

Table 1. Microscopic analysis of ileal tissues

Mice ileal loops were treated with either PBS (100 µl) or TcdA (25 or 50 µg per loop). Mice challenged with 50 µg TcdA in ileal loops received s.c. injection of either 4.5 µmol haemin kg⁻¹ or 25 µmol DMDC kg⁻¹, and mice challenged with 25 µg TcdA in ileal loops received 4.5 µmol ZnPP IX kg⁻¹ s.c. Control groups consisted of animals that received s.c. injection of either NaOH or DMSO plus 50 µg TcdA per loop or PBS or Na₂CO₃ plus 25 µg TcdA per loop or PBS. Data represent the median values (and range) of microscopic scores in six mice per group. Data were analysed using Kruskal–Wallis and Dunn's tests.

Experimental group	Epithelial damage	Neutrophilic infiltrate	Congestion/oedema	Haemorrhage
PBS	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
TcdA 50 µg (NaOH or DMSO)	2 (2–3)*	2.5 (2–3)*	3 (2–3)*	2.5 (1–3)*
Haemin 4.5 µmol (TcdA 50 µg)	1 (0–1)†	1 (0–1)†	1 (1–1)†	0 (0–1)†
Haemin 4.5 µmol (PBS)	0.5 (0–1)	0 (0–0)	0.5 (0–1)	0 (0–1)
DMDC 25 µmol (TcdA 50 µg)	1 (0–1)†	1 (0–1)†	1 (1–1)†	0 (0–1)†
DMDC 25 µmol (PBS)	0.5 (0–1)	0 (0–0)	0.5 (0–1)	0 (0–1)
TcdA 25 µg (Na ₂ CO ₃)	1 (0–1)*	1 (0–1)*	0 (0–2)	0 (0–1)
ZnPP IX 4.5 µmol (TcdA 25 µg)	2 (1–3)†	2 (1–2)†	1 (1–2)†	1.5 (0–2)†
ZnPP IX 4.5 µmol (PBS)	0 (0–0)	1 (0–1)*	0.5 (0–1)	0 (0–0)

* $P < 0.05$ compared to PBS mice.

† $P < 0.05$ compared to mice subjected to TcdA and NaOH, Na₂CO₃ or DMSO.

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Fig. 2. Effect of haemin, DMDC or ZnPP IX on *C. difficile* TcdA-induced histological alterations. (a) Histopathology in ligated ileal loops treated with PBS only. (b) Mucosal disruption in ligated ileal loops injected with TcdA (50 µg per loop). (c, d) Substantial prevention of TcdA (50 µg)-induced mucosal disruption in the presence of haemin (4.5 µmol kg⁻¹ s.c.) or DMDC (25 µmol kg⁻¹), respectively. (e) Mucosal oedema and inflammatory cell infiltration in ligated ileal loops injected with TcdA (25 µg per loop). (f) Substantial amplification of the mucosal damage induced by ZnPP IX (4.5 µmol kg⁻¹ s.c.) in TcdA (25 µg per loop)-treated tissues. Haematoxylin and eosin staining, original magnification ×100.

Determination of MPO activity. The extent of neutrophil accumulation in ileal tissue was estimated by measuring MPO activity as previously described (Bradley *et al.*, 1982). Briefly, ileal

Fig. 3. Effect of haemin, ZnPP IX or DMDC on MPO activity in the ileal mucosa of mice subjected to *C. difficile* TcdA. The mice were pretreated s.c. with haemin (a), ZnPP IX (b) or DMDC (c) or with their respective vehicles. After 30 min, 50 µg TcdA was injected in ligated ileal loops for animals pretreated with haemin or DMDC and 25 µg TcdA in ligated ileal loops for animals pretreated with ZnPP IX. Bars represent the mean value $\pm$ SEM of MPO units (mg ileal mucosa)⁻¹ of at least five mice per group. * $P < 0.05$ represents statistical differences compared to PBS mice; # $P < 0.05$ represents statistical differences compared to mice subjected to TcdA and injected with NaOH, Na₂CO₃ or DMSO. Data were analysed using analysis of variance (ANOVA) and Bonferroni's test.

tissue was homogenized in 1 ml hexadecyltrimethylammonium bromide buffer for each 50 mg tissue. The homogenate was then centrifuged at 4000 *g* for 7 min at 4 °C. MPO activity in the resuspended pellet was assayed by measuring the change in A_{450} using *o*-dianisidine dihydrochloride and 1 % hydrogen peroxide. The results were reported as MPO units (mg tissue)⁻¹. A unit of MPO activity was defined as the amount of enzyme that converts 1 µmol hydrogen peroxide to water in 1 min at 22 °C.

Quantification of TNF- α and IL-1 β by ELISA. Ileal tissue was harvested from animals and TNF- α and IL-1 β concentrations were measured by ELISA as described previously (Safieh-Garabedian *et al.*, 1995).

HO-1 immunohistochemistry in ileal tissue. Paraffin-embedded sections were dewaxed and endogenous peroxidase activity was blocked with 3 % H₂O₂ in methanol for 30 min. After blocking in Tris buffered saline (TBS; 0.05 mol l⁻¹) containing 3 % normal horse serum and 0.3 % Triton X-100 for 30 min, sections were rinsed with TBS buffer and incubated with mouse monoclonal HO-1 antibodies diluted to 1:400 overnight at 4 °C. The tissue was then stained for antigen-antibody complexes using a peroxidase detection system (LSAB kit; DAKO).

Fig. 4. Effect of haemin or ZnPP IX on TNF- α level in the ileal mucosa of mice subjected to *C. difficile* TcdA. The mice were pretreated s.c. with haemin (a) or ZnPP IX (b) or with their respective vehicles. After 30 min, 50 µg TcdA was injected in ligated ileal loops for animals pretreated with haemin and 25 µg in ligated ileal loops for animals pretreated with ZnPP IX. Bars represent the mean value $\pm$ SEM of TNF- α level in the ileal mucosa of at least five mice per group. * $P < 0.05$ represents statistical differences compared to PBS mice; # $P < 0.05$ represents statistical differences compared to mice subjected to TcdA and injected with NaOH or Na₂CO₃. Data were analysed using analysis of variance (ANOVA) and Bonferroni's test.

Statistical analysis. Results are reported as means $\pm$ SEM or as median values and range, where appropriate. Univariate ANOVA followed by Bonferroni's test were used to compare means and the Kruskal–Wallis test followed by Dunn's test were used to compare medians. A probability value of $P < 0.05$ was considered to indicate significant differences.

RESULTS AND DISCUSSION

Haemin and DMDC prevented TcdA-induced ileal loop oedema and mucosal disruption while ZnPP IX worsened TcdA-induced epithelial damage

TcdA, when injected into animal ileal loops, elicits fluid secretion and increased mucosal permeability, and causes a marked inflammatory response (Cavalcante *et al.*, 2006; Kelly & LaMont, 1998). Pretreatment with the HO substrate, haemin ($4.5 \mu\text{mol kg}^{-1}$), significantly ($P < 0.05$) reduced the TcdA ($50 \mu\text{g}$)-induced increase in the ileal loop weight/length ratio. Haemin ($4.5 \mu\text{mol kg}^{-1}$) did not alter the ileal loop weight/length ratio in the absence of TcdA (Fig. 1a). Similarly, the pretreatment of mice with DMDC, a CO donor, reduced the increased ileal loop weight/length ratio induced by TcdA ($50 \mu\text{g}$) (Fig. 1c). However, the inhibition of HO activity with ZnPP IX ($4.5 \mu\text{mol kg}^{-1}$) did not alter the ileal loop weight/length ratio compared to the TcdA ($25 \mu\text{g}$) control group (Fig. 1b).

Histological analysis demonstrated that the lower dose of TcdA ($25 \mu\text{g}$ per ileal loop) significantly increased mucosal disruption and inflammatory cell infiltration in the ileal tissue (median injury score of 1 vs 0 in PBS loops) (Table 1). TcdA at $50 \mu\text{g}$ per loop induced intense mucosal disruption, haemorrhage, congestion/oedema and inflammatory cell infiltration compared to the PBS control ($P < 0.05$). The groups treated with either haemin or DMDC were protected from the disruptive effects of $50 \mu\text{g}$ TcdA resulting in low lesion scores ($P < 0.05$) (Table 1; Fig. 2). Pretreatment of the animals with ZnPP IX exacerbated the histopathological alterations observed in TcdA ($25 \mu\text{g}$)-treated ileal tissues ($P < 0.05$) (Table 1; Fig. 2). Supporting our results, recent data from the literature showed that tin protoporphyrin IX, an HO-1 inhibitor, aggravated toxin A-induced enteritis. The same study showed that HO-1 mediates the protective role of CX3CR1 after the induction of TcdA enteritis, suggesting that HO-1 still has anti-inflammatory properties even after the induction of the stimuli, and does not act solely as a protective factor (Inui *et al.*, 2011). Over the last few years, studies have demonstrated that upregulation of HO-1 by haemin increases BVD and CO production, and decreases redox state and inflammatory processes (Wagener *et al.*, 2003). It has also been shown that HO-1 has protective effects in colitis models (Hegazi *et al.*, 2005; Varga *et al.*, 2007). However, the role of specific HO-1 products, such as CO, in TcdA ileal mucosal damage was not elucidated. Our results show that DMDC had a protective effect in the damage and oedema induced by TcdA, suggesting that CO plays a protective role against TcdA enteritis.

Of importance, haemin or DMDC alone did not induce any histological evidence of injury compared to the PBS group. We also demonstrated that, compared to the PBS control, ZnPP IX alone induced significant inflammatory

Fig. 5. Effect of haemin, ZnPP IX or DMDC on IL-1 β level in the ileal mucosa of mice subjected to *C. difficile* TcdA. The mice were pretreated s.c. with haemin (a), ZnPP IX (b) or DMDC (c) or with their respective vehicles. After 30 min, $50 \mu\text{g}$ TcdA was injected in ligated ileal loops for animals pretreated with haemin (a) or DMDC (c) and $25 \mu\text{g}$ in ligated ileal loops for animals pretreated with ZnPP IX (b). Bars represent the mean value $\pm$ SEM of IL-1 β level in the ileal mucosa of at least five mice per group. * $P < 0.05$ represents statistical differences compared to PBS mice; # $P < 0.05$ represents statistical differences compared to mice subjected to TcdA and injected with NaOH, Na₂CO₃ or DMSO. Data were analysed using analysis of variance (ANOVA) and Bonferroni's test.

cell infiltration, but did not alter other histological parameters (epithelial damage, congestion/oedema and haemorrhage) (Table 1; Fig. 2).

Haemin and DMDC reduced neutrophil infiltration and pro-inflammatory cytokine levels and ZnPP potentiated the effect of TcdA on neutrophil infiltration and cytokine release

MPO activity was measured in the animal ileal mucosa as an indicator of neutrophil infiltration. TcdA (25 or 50 μg per loop) caused a statistically significant increase ($P < 0.05$) in MPO activity in ileal tissue compared with the PBS control group. The group treated with either haemin (4.5 $\mu\text{mol kg}^{-1}$) or DMDC (25 $\mu\text{mol kg}^{-1}$) and then challenged with 50 μg TcdA showed markedly reduced MPO activity compared with TcdA (50 μg) animals (Fig. 3a or Fig. 3c, respectively). The pretreatment of the animals with ZnPP IX potentiated MPO activity in ileal mucosa treated with 25 μg TcdA ($P < 0.05$) compared with the TcdA control (Fig. 3b). Haemin or DMDC alone did not alter MPO activity compared with PBS. ZnPP IX alone increased MPO activity compared with the PBS control ($P < 0.05$). However, this increase in MPO activity was not statistically significant in comparison with the group treated with TcdA alone.

Toxin A-induced enteritis develops with massive neutrophil infiltration, which occurs within a few hours after it is

injected into ileal loops (Pothoulakis & LaMont, 2001). The role of neutrophils in the pathogenesis of colitis caused by infection with *C. difficile* and the abundant presence of neutrophils within TcdA-induced pseudomembranes are well known (Brito *et al.*, 2002b; Kelly *et al.*, 1994b). Internalized TcdA induces mitochondrial damage and epithelial cell death through Rho inactivation (Brito *et al.*, 2002a). TcdA also causes activation of the transcription factor NF- κ B. Afterwards, its activation enhances the expression of chemokines with neutrophil chemotactic activities and increases adhesion molecule expression, thereby causing massive infiltration of neutrophils (Pothoulakis & LaMont, 2001). Here, we showed that haemin and DMDC reduced TcdA-induced MPO activity, suggesting a potent inhibitory effect of haemin and DMDC on neutrophil infiltration into the ileal tissue. The important role of neutrophils in the pathogenesis of colitis caused by infection with *C. difficile* suggests that the protective effect of haemin and DMDC on TcdA-induced mucosal disruption shown here is mediated, at least in part, by the inhibition of neutrophil recruitment.

In agreement with our data, *in vivo* and *in vitro* experiments show that haemin-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). HO metabolites decrease neutrophil migration to the peritoneal cavity induced by carrageenan, by mechanisms that can be dependent or

Fig. 6. HO-1/BVD/CO pathway and pharmacological modulation by haemin, ZnPP IX or DMDC in the inflammatory response to TcdA-induced enteritis. The induction of HO-1 by haemin and the metabolites of this pathway, such as CO and BVD, reduce the release of pro-inflammatory cytokines (IL-1 β and TNF- α) and neutrophil recruitment.

independent of activation of soluble guanylate cyclase (Freitas *et al.*, 2006). Thus, our data suggest that the HO/CO pathway plays an anti-inflammatory role in *C. difficile* TcdA-induced mouse enteritis.

The injection of TcdA (25 or 50 µg per loop) into mouse ligated ileal loops significantly increased the ileal tissue level of TNF- α and IL-1 β ($P<0.05$) compared to the control group challenged with PBS alone. These findings confirm previous studies showing that TcdA induces release of cytokines such as TNF- α and IL-1 β that contribute to the pathogenesis of TcdA-induced colitis (Brito *et al.*, 2002a; Flegel *et al.*, 1991).

Treatment with 4.5 µmol haemin kg⁻¹ significantly reduced TNF- α and IL-1 β levels in ileal tissue compared to loops injected only with 50 µg TcdA ($P<0.05$) (Figs 4a and 5a), and DMDC significantly reduced IL-1 β levels in ileal tissue compared to loops injected with only 50 µg TcdA ($P<0.05$) (Fig. 5c). Thus, these findings suggest that HO-1 exerts its protective effects by also preventing pro-inflammatory cytokine release, consistent with previous studies showing that HO-1 induction strikingly decreased the levels of TNF- α and IL-1 β in an experimental zymosan-injected air pouch model (Vicente *et al.*, 2003) as well as in a murine model of sepsis (Morse *et al.*, 2003).

Another mechanism not tested in the present study that is possibly associated with the protective effect of HO-1 would be an augmented anti-inflammatory cytokine release from macrophages (e.g. IL-10) (Otterbein *et al.*, 2000). Moreover, some investigators attributed the anti-inflammatory effect of HO-1 to the ability of CO to suppress the production of cytokines, whereas others have attributed this action to the potent antioxidant properties of both BVD and bilirubin (Hayashi *et al.*, 1999; Siow *et al.*, 1999; Suematsu *et al.*, 1996). Here the CO donor, DMDC, significantly reduced the TcdA-induced production of IL-1 β .

Similar to what was observed with MPO activity, ZnPP IX (4.5 µmol kg⁻¹) potentiated the increase in TNF- α and IL-1 β levels relative to that observed in 25 µg TcdA animals ($P<0.05$) (Figs 4b and 5b). Haemin alone did not affect the ileal tissue level of TNF- α and IL-1 β compared to PBS. However, even alone, ZnPP IX increased the ileal tissue levels of TNF- α and IL-1 β compared to PBS, but these increases were not statistically significant in comparison to the group that received 25 µg TcdA per loop. Hence, these findings further reinforce the anti-inflammatory effects of the HO-1/CO pathway in TcdA-induced enteritis.

The HO-1/CO pathway exerts a protective role against TcdA-induced enteritis

The present study demonstrated that treating mice with haemin (HO substrate) or DMDC (a CO donor), thereby activating the HO-1/BVD/CO pathway, strikingly reduces tissue injury and inflammation and also prevents the oedema and mucosal disruption induced by *C. difficile*

TcdA (Fig. 6). In addition, such effects were associated with diminished pro-inflammatory cytokine production and neutrophil infiltration as evident in the histological analysis and MPO activity. These findings are consistent with previous studies in which HO-1 expression along with a concurrent production of its metabolites, CO and BVD, showed anti-inflammatory properties in experimental colitis models (Wang *et al.*, 2001; Paul *et al.*, 2005; Hegazi *et al.*, 2005; Jun *et al.*, 2006) or in ethanol-induced gastric damage (Gomes *et al.*, 2010).

We also demonstrated strong HO-1 immunoreactivity in the crypt cells of the ileal tissue treated with TcdA (Fig. 7b). Therefore, we can infer that during TcdA-induced enteritis, there was an increase in HO-1 expression, probably as a response to inflammation and oxidative stress.

In summary, using a pharmacological approach, we demonstrated that the HO-1/CO pathway offers a protective effect against TcdA-induced ileal damage. This pathway might be a promising target in controlling the inflammatory response in *C. difficile*-associated diarrhoea.

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Fig. 7. HO-1 expression in TcdA-treated ileal tissue. Photomicrographs of ileal mucosa (magnifications, $\times 400$) showing absence of HO-1 immunoreactivity in crypt cells of ileal tissue treated with PBS (a) and strong HO-1 immunoreactivity detected in those treated with TcdA (b).

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