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Original Paper

Opposite effect of oxidative stress on inducible nitric oxide synthase and haem oxygenase-1 expression in intestinal inflammation: anti-inflammatory effect of carbon monoxide

Gerard Dijkstra,[1]* Hans Blokzijl,[1] Lisette Bok,[1] Manon Homan,[1] Harry van Goor,[2] Klaas Nico Faber,[1] Peter LM Jansen[1] and Han Moshage[1]

[1]*Department of Gastroenterology and Hepatology, University Hospital Groningen, Groningen, The Netherlands*
[2]*Department of Pathology, University Hospital Groningen, Groningen, The Netherlands*

**Correspondence to:*
Dr Gerard Dijkstra, Department of Gastroenterology and Hepatology, University Hospital Groningen, PO Box 30.001, 9700 RB Groningen, The Netherlands.
E-mail: G.Dijkstra@int.azg.nl

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Abstract

Inducible nitric oxide synthase (iNOS) is expressed in intestinal epithelial cells (IEC) of patients with active inflammatory bowel disease (IBD) and in IEC of endotoxaemic rats. The induction of iNOS in IEC is an element of the NF-κB-mediated survival pathway. Haem oxygenase-1 (HO-1) is an AP-1-regulated gene that is induced by oxidative stress. The enzyme produces carbon monoxide (CO), which may attenuate the inflammatory response. The aim of this study was to investigate the regulation and interaction of iNOS and HO-1 in response to inflammation and oxidative stress. Male Wistar rats were treated with the thiol-modifying agent diethylmaleate (DEM) to induce oxidative stress and rendered endotoxaemic by LPS injection. Human colonic biopsies and the human colon carcinoma cell line DLD-1 were treated with DEM and the lipid peroxidation end-product 4-hydroxynonenal to induce oxidative stress and exposed to cytokine mix (CM) to mimic inflammation. In some experiments, cells were incubated with 250–400 ppm CO prior to and during stimulation with CM. HO-1 and iNOS expression was evaluated by RT-PCR, western blotting, and immunohistology. NF-κB activation was evaluated by EMSA. LPS induced iNOS but not HO-1 in epithelial cells of the ileum and colon. Oxidative stress strongly induced HO-1 in epithelial and inflammatory cells. Combined oxidative stress and endotoxaemia decreased iNOS expression but strongly induced HO-1 expression. Similarly, CM induced iNOS but not HO-1 in colonic biopsies and DLD-1 cells. Oxidative stress prevented iNOS induction in an NF-κB-dependent manner but increased HO-1 expression in CM-exposed DLD-1 cells. CO inhibited iNOS mRNA induction in CM-stimulated DLD-1 cells. These data demonstrate opposite regulation of iNOS and HO-1 in intestinal epithelial cells in response to cytokine exposure and oxidative stress. These findings suggest that iNOS (NF-κB driven) and HO-1 (AP-1 driven) represent mutually exclusive survival mechanisms in intestinal epithelial cells.

Keywords: haem oxygenase-1; inducible nitric oxide synthase; inflammatory bowel diseases; endotoxaemia; intestinal epithelial cells; carbon monoxide; nitric oxide; lipid peroxidation

Introduction

A monolayer of intestinal epithelial cells (IECs) forms a selective barrier in the gastrointestinal tract. During acute or chronic inflammation this barrier is compromised due to exposure to reactive oxygen species (ROS) and/or cytokines. A number of non-enzymatic (eg glutathione, flavonoids, and vitamins A, C, and E) as well as enzymatic scavengers of ROS (eg superoxide dismutases, catalase, and glutathione peroxidase) prevent the accumulation of ROS. Unfortunately, these defence mechanisms are not always adequate to counteract the toxic effects of ROS, resulting in what is termed a state of oxidative stress. The enzyme haem oxygenase-1 (HO-1), which generates carbon monoxide (CO), is induced by oxidative stress. HO-1 detoxifies the pro-oxidant haem into CO, Fe, and biliverdin [1]. The latter is subsequently converted into bilirubin, a potent antioxidant. In addition, the HO-1 product CO has been reported to have anti-inflammatory and anti-apoptotic effects [2]. The enzyme-inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO), is induced by inflammatory cytokines. NO has an important role in maintaining mucosal integrity under normal and pathological conditions. In normal conditions, small amounts of NO produced by

endothelial NOS (eNOS) maintain the blood supply to the gastrointestinal mucosa. In inflammatory conditions, large amounts (micromolar) of NO are produced by iNOS. It has been demonstrated that intestinal epithelial cells express iNOS during inflammatory conditions such as coeliac disease [3], inflammatory bowel disease (IBD) [4–7], diverticulitis [5], and endotoxaemia [8]. The exact role of iNOS-derived NO in these conditions is unknown. In endotoxaemia, overproduction of NO may contribute to epithelial dysfunction. Selective inhibition of iNOS reduces hyperpermeability and bacterial translocation in endotoxaemic rats [9]. The HO-1 gene is predominantly regulated by the oxidative stress responsive transcription factor AP-1, whereas iNOS is predominantly regulated by the inflammation-activated transcription factor NF-κB. Cross-talk in the regulation of these enzymes exists, eg NO is a potent inducer of HO-1 and CO can decrease NO production [10]. In inflammatory conditions, inflammation and oxidative stress usually occur together. In the present study we have investigated the regulation of HO-1 and iNOS genes and their products in LPS-induced endotoxaemia and in cytokine-activated human colon carcinoma cells, both in the presence and absence of additional oxidative stress. We demonstrate opposite regulation of iNOS and HO-1 in inflammation and oxidative stress. These findings suggest that iNOS (NF-κB driven) and HO-1 (AP-1 driven) represent mutually exclusive survival mechanisms in intestinal epithelial cells.

Material and methods

In vivo endotoxaemia model

Specific pathogen-free male Wistar rats (200–250 g; purchased from Harlan-CPB, Zeist, The Netherlands) were kept at the Central Animal Laboratory of the University of Groningen under routine conditions and had free access to drinking water and standard chow. The local committee for care and use of laboratory animals approved the study. Rats were injected intraperitoneally at $t = 0$ h with 5 mg/kg endotoxin (LPS: *E coli* serotype 0127:B8; Sigma Chemical Co, St Louis, MO, USA) with ($n = 6$) or without ($n = 6$) intraperitoneal administration of 4 mmol/kg diethylmaleate (DEM) dissolved in olive oil (1 : 1 vol/vol; Sigma Chemical Co) at $t = -0.5$ h and $t = 3$ h. DEM is an oxidative stress-inducing alkylating agent which rapidly depletes reduced glutathione [11]. Two additional groups of rats received only PBS ($n = 6$) or DEM ($n = 6$) at 4 mmol/kg. Six hours after LPS injection rats were anaesthetized with pentobarbital (60 mg/kg, ip) and sacrificed. Blood was collected by cardiac puncture and colon and ileum were removed. For routine histology, tissue specimens were fixed by immersion in 4% buffered paraformaldehyde and embedded in paraffin wax. For immunohistochemistry, tissue specimens were snap frozen in isopentane. For western blotting and RNA isolation, tissue specimens were snap frozen in liquid nitrogen and stored at −80 °C.

Cell culture experiments

In vitro experiments were performed with fresh biopsy specimens obtained from healthy humans and with the human colon carcinoma cell line DLD-1. Control subjects comprised one man and two women aged 49, 72, and 51 years respectively. These patients underwent colonoscopy because of abdominal pain or polyps. The protocol was approved by the local medical ethics committee and informed consent for research use was obtained from all patients. Biopsies were taken from macro- and microscopically normal mucosa from the transverse colon. Biopsies and cells were cultured in a humidified incubator at 37 °C in an atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with Glutamax, 10% v/v fetal calf serum (Gibco BRL, Breda, The Netherlands), penicillin (50 U/ml), streptomycin (50 μg/ml), and fungizone (5 μg/ml) (Bio Whittaker, Verviers, Belgium). To mimic the pathophysiological situation of inflammation *in vitro*, DLD-1 cells were stimulated for 6–12 h with a cytokine mix (CM) composed of 10 ng/ml human interleukin-1β (IL-1β), 10 ng/ml human interferon-γ (IFN-γ) and 10 ng/ml human tumour necrosis factor-α (TNF-α). DEM at 1 mmol/L was added 30 min before and 3.5 h after stimulation with CM. DLD-1 cells were also exposed to the lipid peroxidation end product 4-hydroxy-nonenal (4-HNE). NF-κB activation was blocked using the proteasome inhibitor MG-132 at 52 nmol/L added at 30 min before and 3.5 h after stimulation with CM. In some experiments, DLD-1 cells were incubated in the presence of 250–400 ppm CO at 37 °C in a humidified incubator containing 5% CO_2.

Immunohistochemistry

For immunohistochemistry, 4 μm cryostat sections were cut, dried, and fixed in acetone for 10 min at room temperature. For iNOS detection, a rabbit polyclonal antibody developed in our laboratory was used [11]. For HO-1 detection, a monoclonal antibody (OSA-111) from Stressgen (Victoria, BC, Canada) was used. Slides were incubated with the polyclonal iNOS antibody (1 : 300) or monoclonal HO-1 antibody (1 : 500) in PBS containing 1% BSA for 60 min at room temperature. Subsequently, endogenous peroxidase activity was blocked by incubating for 30 min in PBS containing 0.075% H_2O_2. For iNOS detection, peroxidase-conjugated goat anti-rabbit Ig (1 : 50) and peroxidase-conjugated rabbit anti-goat Ig (1 : 50) and for HO-1 detection peroxidase-conjugated rabbit anti-mouse Ig (1 : 50) and peroxidase-conjugated rabbit anti-mouse Ig (1 : 50), all from Dako (Glostrup, Denmark), were used as secondary and tertiary antibodies. Colour was developed using 3-amino-9-ethylcarbazole

(10 mg/2.5 ml dimethylformamide in 50 ml 0.1 mol/L acetate buffer pH 5.0) containing 0.03% H_2O_2 for 10 min at room temperature. Counterstaining was performed with haematoxylin, and the slides were covered with Kaiser's glycerin–gelatin.

Western blot analysis

Ileum and colon samples were homogenized with a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland) in a homogenization buffer containing 20 mmol/L Tris HCl (pH 7.2, 4 °C), 0.2 mmol/L phenylmethylsulphonyl fluoride, 1 mmol/L ethylenediaminetetraacetic acid and 1 mmol/L dithiothreitol. DLD-1 cells were harvested in the same homogenization buffer. After centrifugation (30 min, 11 600 × *g*), the protein concentration in the supernatant (crude lysates) was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard. The crude lysates were fractionated on a 10% sodium dodecylsulphate polyacrylamide gel and transferred to nitrocellulose (Amersham International plc, Buckinghamshire, UK), using a semi-dry blotting system according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Pre-stained molecular weight standards (Bio-Rad) were used as marker proteins. For iNOS detection on rat samples, a polyclonal antibody was used [11] and for detection of iNOS on human samples (DLD-1 cells) a monoclonal iNOS antibody (cat. no. N39120, Transduction Laboratories, Lexington, KY, USA) was used. For HO-1 detection, a polyclonal antibody (SPA-896) from Stressgen (Victoria, BC, Canada) was used. Antibodies were diluted in PBS containing 4% skimmed milk powder and 0.1% Tween-20, subsequently incubated with the appropriate peroxidase-labelled Ig (dilution 1 : 2000) and finally developed using the ECL Chemiluminiscence detection reagents (Amersham).

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from tissue specimens and cell layers using Trizol reagent (Life Technologies Ltd) according to the manufacturer's instructions. Reverse transcription was performed on 5 μg of total RNA using random primers in a final volume of 75 μl (Reverse Transcription System, Promega, Madison, WI, USA). Polymerase chain reaction (PCR) on cDNA was performed with Taq polymerase (Eurogentec, Seraing, Belgium). The primer set for human iNOS was sense: 5′-CTA TGC TGG CTA CCA GAT GC-3′, antisense: 5′-CCA TGA TGG TCA CAT TCT GC-3′; and for rat iNOS, sense: 5′-CGA GGA GGC TGC CCT GCA GAC TGG-3′, antisense: 5′-CTG GGA GGA GCT GAT GGA GTA GTA-3′; resulting in amplified products of 505 bp and 1383 bp respectively. The primer set for human HO-1 was sense: 5′-ACA TCT ATG TGG CCC TGG AG-3′, antisense: 5′-TGT TGG GGA AGG TGA AGA AG-3′; and for rat HO-1, sense: 5′-CAC GCA TAT ACC CGC TAC CT-3′, antisense: 5′-AAG GCG GTC TTA GCC TCT TC-3′; resulting in amplified products of 348 bp and 227 bp respectively. Primers specific for glyceraldehyde-3-phosphate dehydrogenase[28] (GAPDH, sense: 5′-CCA TCA CCA TCT TCC AGG AG-3′, antisense 5′-CCT GCT TCA CCA CCT TCT TG-3′), resulting in an amplified product of 576 bp, were used as a control for the RT-PCR procedure. The tubes were incubated in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT, USA) at 95 °C for 5 min to denature the primers and cDNA. The cycling programme was 95 °C for 40 s (iNOS) or 30 s (HO-1 and GAPDH), 60 °C for 40 s (iNOS) or 30 s (HO-1 and GAPDH), 72 °C for 40 s (iNOS) or 30 s (HO-1 and GAPDH), and for 5 min in the last cycle, and comprised 30 cycles for both iNOS primer sets, 28 cycles for the human HO-1 primer set, 26 cycles for the rat HO-1 primer set, and 22 cycles for the GAPDH primer set.

Quantitative real-time PCR

RNA was isolated from DLD-1 cell layers using Trizol reagent (Life Technologies Ltd) according to the manufacturer's instructions. Reverse transcription was performed on 2.5 μg of total RNA using random primers in a final volume of 50 μl (Reverse Transcription System, Promega, Madison, WI, USA).

Four microlitres of 20-fold diluted complementary DNA was used for each PCR reaction in a final volume of 20 μl, containing 900 nmol/L sense and antisense primers, 200 nmol/L of fluorogenic probe, 5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosphate mix, real-time buffer (10×), and 0.5 U Hot Goldstar DNA polymerase (Eurogentec, Seraing, Belgium). The primer set and probe for human iNOS were sense: 5′-GGC TCA AAT CTC GGC AGA ATC-3′, antisense: 5′-GGC CAT CCT CAC AGG AGA GTT-3′, probe: 5′ FAM-TCC GAC ATC CAG CCG TGC CAC-TAMRA 3′. The primer set and probe for 18S were sense: 5′-CGG CTA CCA CAT CCA AGG A-3′, antisense: 5′-CCA ATT ACA GGG CCT CGA AA-3′; probe: 5′ FAM-CGC GCA AAT TAC CCA CTC CCG A-TAMRA 3′. Real-time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems) initialized by 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was analysed in duplicate. 18S mRNA levels were used as an endogenous control.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of cultured DLD-1 cells were prepared using a final concentration of Nonidet P-40 of 0.25%. EMSA for NF-κB was performed as described previously [12]. Equal amounts of nuclear extract protein were used for EMSA. As controls, competition experiments were performed with excess non-labelled NF-κB probe and excess non-labelled non-NF-κB

probe as well as super-shift assays using a specific antibody against the NF-κB p65 subunit (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

All data are expressed as the mean $\pm$ SD. Statistical significance was determined by the Mann–Whitney U-test; $p < 0.05$ was considered statistically significant.

Results

Expression of iNOS and HO-1 in endotoxaemic rats

In the normal ileum (Figure 1A) and colon (data not shown), iNOS is only expressed in some inflammatory cells scattered throughout the lamina propria (Figure 1A). Treatment with DEM did not change this expression pattern (Figure 1B). Six hours after LPS administration, rat ileum displayed diffuse and intense epithelial iNOS expression (Figure 1C) whereas rat colon displayed focal epithelial iNOS expression (data not shown). Epithelial iNOS expression in the ileum was both in the crypts, along the crypt axis, and abundant in the villus, whereas in the colon iNOS expression was confined to crypt epithelial cells. The expression of iNOS in inflammatory cells was not altered in the endotoxaemic gut. Epithelial iNOS expression was almost completely abolished in endotoxaemic rats treated with the oxidative stress-inducing agent DEM (Figure 1D), with only low-level expression in some crypt cells remaining (Figure 1D, arrow). DEM treatment did not change iNOS expression in inflammatory cells of endotoxaemic rats. RT-PCR analysis supports the immunohistochemistry results (Figure 2).

In the normal ileum (Figure 1E) and colon (data not shown) HO-1 is only expressed in some inflammatory cells scattered throughout the lamina propria (Figure 1E). HO-1 expression was strongly induced in both the ileum (Figure 1F) and colon (data not shown) of normal non-endotoxaemic rats treated with the oxidative stress-inducing agent DEM. HO-1 expression was present in the epithelial cells in the intermediate part of the crypt–villus axis and in inflammatory cells. Six hours after LPS administration, rat ileum displayed only focal HO-1 expression in leucocyte

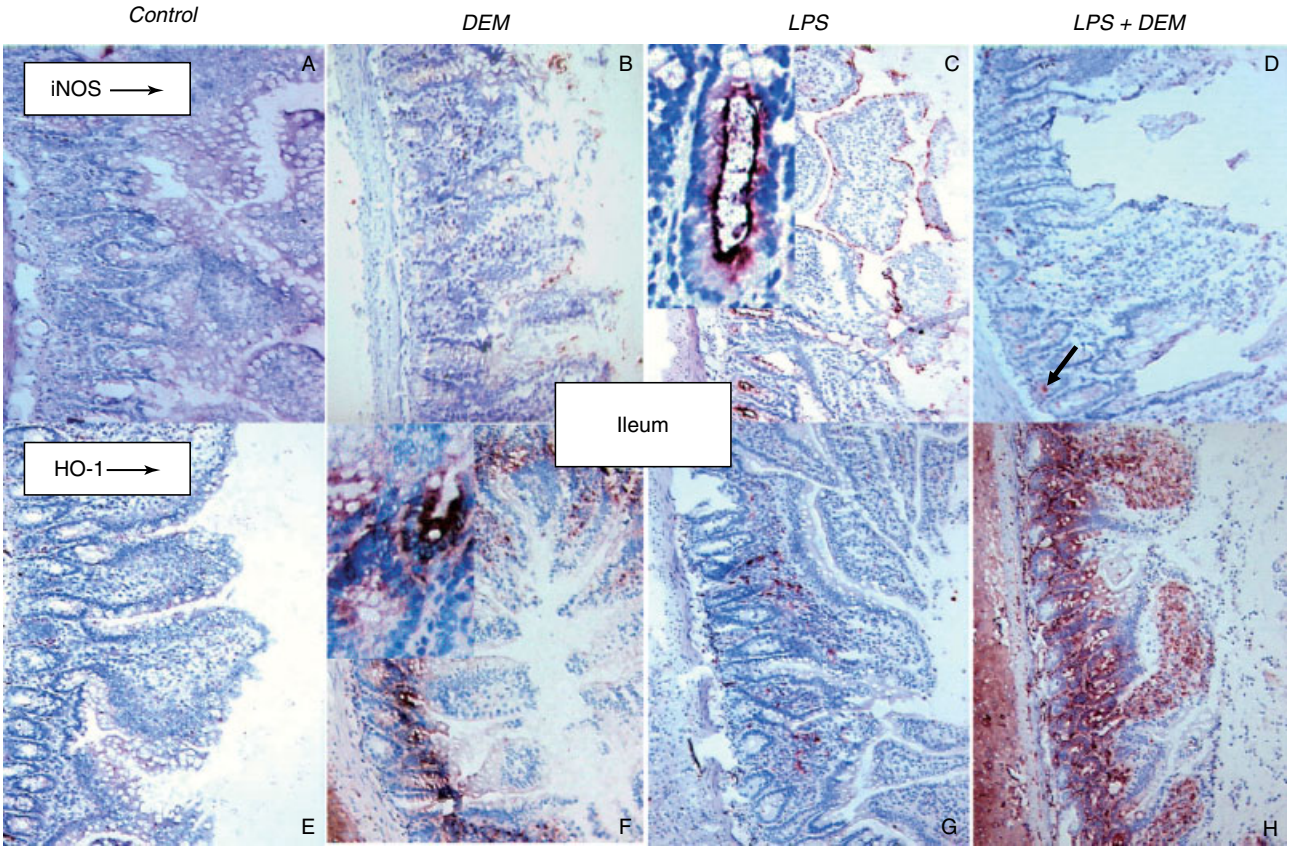

Figure 1. Staining for iNOS (A–D) and HO-1 (E–H) in rat ileum. In rats treated with PBS alone, iNOS (A) and HO-1 (E) are expressed in a few inflammatory cells scattered throughout the lamina propria. Induction of oxidative stress with diethylmaleate (DEM, 4 mmol/kg i.p. 0.5 h before and 3 h after PBS injection) did not change the iNOS staining pattern (B). In contrast, DEM induced HO-1 in epithelial cells in the intermediate part of the crypt–villus axis and in inflammatory cells (F, see inset). Six hours after LPS injection (5 mg/kg i.p.), rat ileum displayed diffuse and intense epithelial iNOS staining along the entire crypt–villus axis (C, see inset); in contrast, HO-1 was only induced in some small aggregates of inflammatory cells (G). Combined treatment with LPS and DEM almost completely abolished epithelial iNOS expression (D, arrow shows residual positivity). In contrast, HO-1 was markedly induced in epithelial cells along the crypt–villus axis and in inflammatory cells (H)

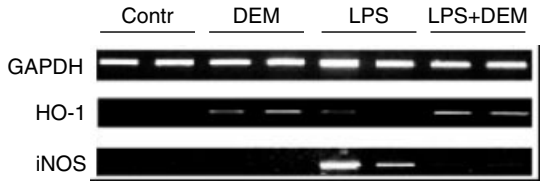

Figure 2. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of HO-1 and iNOS in whole rat ileum tissues samples using GAPDH as a control housekeeping gene (duplicate samples). In rats treated with PBS alone, HO-1 mRNA and iNOS mRNA were undetectable. Induction of oxidative stress with diethylmaleate (DEM, 4 mmol/kg i.p. 0.5 h before and 3 h after PBS injection) induced HO-1 mRNA but not iNOS mRNA. Six hours after LPS injection (5 mg/kg i.p.), HO-1 mRNA was not induced. In contrast, iNOS mRNA was markedly induced. Combined treatment with LPS and DEM markedly induced HO-1 mRNA and completely abolished iNOS mRNA induction

aggregates (Figure 1G). This focal expression of HO-1 in leucocytes was not seen in the rat colon (data not shown). No epithelial HO-1 expression was seen in the endotoxaemic gut. In endotoxaemic rats treated with DEM, the induction of HO-1 expression was even higher than in the non-endotoxaemic rats treated with DEM alone. Both in the ileum (Figure 1H) and in the colon, strong HO-1 expression was seen in epithelial cells of the crypts and the intermediate part of the crypt–villus axis together with a massive influx of HO-1 positive inflammatory cells in the lamina propria. RT-PCR analysis supports the immunohistochemistry (Figure 2).

Regulation of iNOS and HO-1 in DLD-1 colon carcinoma cells

Cytokine mix (CM) induced iNOS mRNA and protein expression in DLD-1 cells (Figure 3). CM-induced iNOS expression was abolished by simultaneous treatment with the oxidative stress-inducing agent DEM (Figure 3). Like DEM, 4-HNE, a lipid peroxidation end-product generated during oxidative stress, inhibited cytokine-induced iNOS expression (Figure 3). Finally, the proteasome inhibitor MG-132, used as an NF-κB inhibitor, inhibited iNOS expression (Figure 3).

The results obtained with the DLD-1 colon carcinoma cell line could be reproduced in fresh biopsies of the colon obtained from healthy humans (Figure 4). Furthermore, the HO-1 product CO reduced CM-induced iNOS mRNA induction significantly (Figure 5), indicating that the induction of HO-1 has a negative effect on iNOS induction.

The inhibition of iNOS expression by DEM, 4-HNE, and MG-132 correlated with inhibition of NF-κB activation determined by EMSA (Figure 6), showing that iNOS is an NF-κB-regulated gene and indicating that DEM and 4-HNE inhibit iNOS expression via inhibition of NF-κB activation. 4-HNE and DEM in the absence of CM did not induce iNOS mRNA or protein expression (Figure 3). HO-1 mRNA was virtually absent in control and CM-treated DLD-1 cells. HO-1 mRNA expression was strongly induced in DLD-1 cells treated with DEM, 4-HNE, and MG-132. Induction of HO-1 mRNA by DEM, 4-HNE, or MG-132 was not changed by the simultaneous treatment with CM (Figure 3A). These results confirm the *in vivo* data and demonstrate that iNOS and HO-1 are oppositely regulated by cytokines and oxidative stress.

Discussion

In this study we demonstrate that iNOS is induced in intestinal epithelial cells by cytokines both *in vivo* (endotoxaemia) and *in vitro* (cytokine mix). In contrast, oxidative stress with or without inflammation

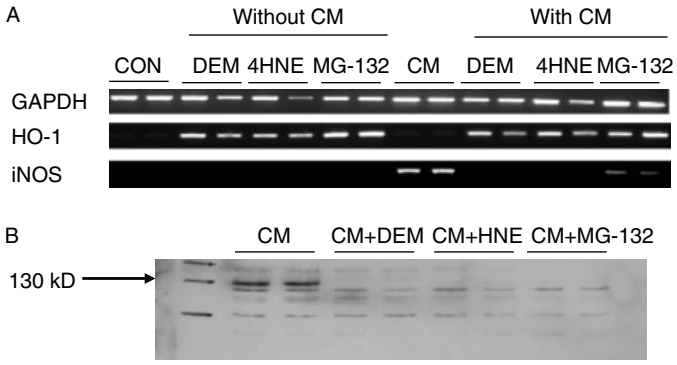

Figure 3. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis (3A) and Western blot analysis (3B) of HO-1 and iNOS of unstimulated and cytokine-stimulated human DLD-1 colon carcinoma cells (duplicate samples). Unstimulated DLD-1 cells showed a low level of HO-1 mRNA but no iNOS mRNA. Induction of oxidative stress with diethylmaleate (DEM, 1 mmol/L) or the lipid peroxidation end-product 4-hydroxy-nonenal (4-HNE, 100 μmol/L) or treatment with the NF-κB (proteasome) inhibitor MG-132 (52 nmol/L) induced HO-1 mRNA. In contrast, these agents did not induce iNOS mRNA. Treatment with a cytokine mix (CM: IL-1β 10 ng/ml, TNF-α 10 ng/ml, IFN-γ 10 ng/ml) induced iNOS mRNA (A) and protein (B) but did not change HO-1 mRNA expression (A). Combined treatment with CM and DEM, 4-HNE, or MG-132 did not change HO-1 mRNA induction compared to the unstimulated cells. In contrast, combined treatment with CM and DEM or 4-HNE completely prevented the induction of iNOS mRNA (A) and protein (B). Combined treatment with CM and MG-123 reduced iNOS mRNA induction (A) and inhibited iNOS protein production (B)

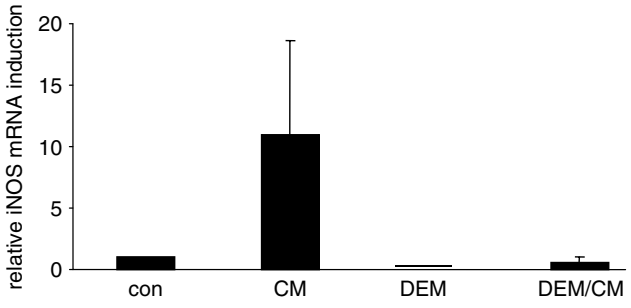

Figure 4. Quantitative real-time PCR analysis of unstimulated and cytokine-stimulated fresh colonic biopsies from healthy humans. Unstimulated colonic biopsies from healthy humans contained hardly any iNOS mRNA. Treatment with a cytokine mix (CM: IL-1β 10 ng/ml, TNF-α 10 ng/ml, IFN-γ 10 ng/ml) induced iNOS mRNA. DEM decreased the basal expression of iNOS mRNA, and combined treatment with CM and DEM prevented the induction of iNOS mRNA

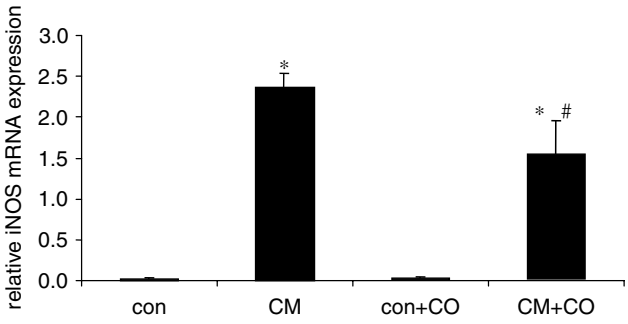

Figure 5. Cytokine-induced iNOS mRNA expression in DLD-1 is inhibited by carbon monoxide. DLD-1 cells were pre-incubated in the presence or absence of CO for 15 h. DLD-1 cells were subsequently incubated with and without CM (10 ng/µl TNF-α, 10 ng/µl IL-1β, and 10 ng/ml INF-γ) in the presence or absence of CO for 12 h. Values represent mean values ($n = 3$) $\pm$ SD. $^{*}p < 0.05$: significantly different from control values; $^{\#}p < 0.05$: significantly different from incubation without CO

or cytokines does not induce this enzyme. In fact, oxidative stress induced by DEM or 4-HNE abolishes cytokine-induced iNOS induction in intestinal epithelial cells. DEM is a non-physiological electrophilic agent that reacts with GSH via the transferase reaction and can modify thiol groups. DEM has been proposed as a therapeutic agent to prevent organ damage in inflammatory conditions [13] but its use was restricted by toxicity [14], which could be a consequence of its alkylating reactivity and/or its reactivity with cysteine residues leading to loss of protein function. It may be able to react with thiol groups in NF-κB, thus reducing its activity. 4-HNE is an important physiological lipid peroxidation-derived aldehyde generated during oxidative stress. The EMSA data show that 4-HNE prevented NF-κB activation in intestinal epithelial cells in a dose-dependent manner. Ji *et al* [15] demonstrated that 4-HNE inhibits IκB kinase activity and subsequent phosphorylation of IκBα, whereas Parola *et al* have demonstrated in hepatic stellate cells that 4-HNE directly interacts with c-Jun amino-terminal kinases (JNKs), leading to the activation of the transcription factor AP-1 [16]. Therefore, 4-HNE is probably

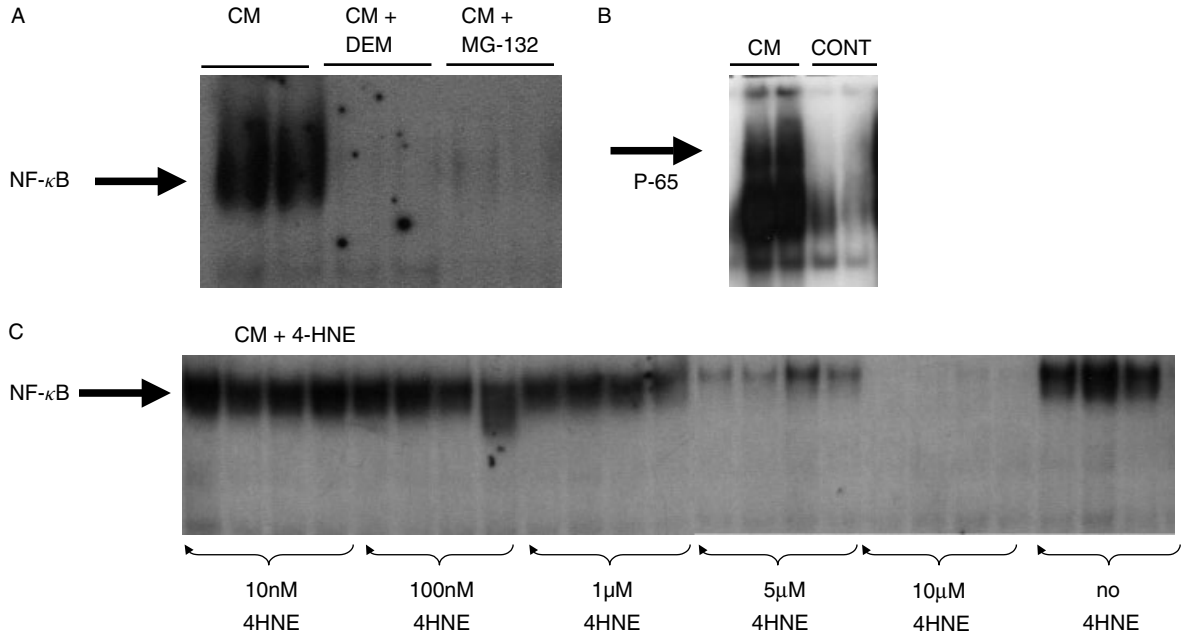

Figure 6. Electrophoretic mobility shift assay (EMSA) for NF-κB binding activity on nuclear extracts of cytokine-stimulated human DLD-1 colon carcinoma cells (duplicate samples). Nuclear extracts from cytokine mix (CM, IL-1β 10 ng/ml, TNF-α 10 ng/ml, IFN-γ 10 ng/ml)-stimulated DLD-1 cells showed NF-κB activity (A) and p65 supershift (B). This activity was completely absent in nuclear extracts from CM-stimulated cells that were exposed to oxidative stress using diethylmaleate (DEM, 1 mmol/L) or the proteasome inhibitor MG-132 (52 nmol/L) (A). Furthermore, the lipid peroxidation end-product 4-hydroxy-nonenal (4-HNE) dose-dependently inhibited CM-induced NF-κB activation in DLD-1 cells (C). Competition with excess non-labelled NF-κB probe completely abolished the specific signal, whereas competition with excess non-labelled non-specific probe did not change the specific signal

an important endogenous factor that regulates NF-κB activation during inflammatory conditions associated with oxidative stress.

In contrast, HO-1 is not induced in intestinal epithelial cells by either inflammation or cytokines. In contrast to iNOS, HO-1 is hardly expressed in the ileum and colon of LPS-treated rats. HO-1 is strongly induced by various forms of oxidative stress, including superoxide anions, hydrogen peroxide, DEM, and the lipid peroxidation end-product 4-HNE. Indeed, DEM treatment alone strongly induced HO-1 expression in ileum and colon, whilst the induction of HO-1 was maximal in rats treated with a combination of DEM and LPS. This is in accordance with HO-1 being an oxidative stress-inducible AP-1-regulated gene [17,18] that has only a weak NF-κB binding site [19,20].

We demonstrate that the HO-1 product CO directly inhibits iNOS mRNA induction, explaining our observed opposite regulation of HO-1 and iNOS. This is in agreement with Cavicchi *et al*, who showed that strong inducers of HO-1, like bismuth salts and haem, inhibited iNOS induction in intestinal epithelial cells [21]. The induction of HO-1 in intestinal epithelial cells by oxidative stress may serve a protective function. HO-1 overexpression by gene transfer protects organs against oxidative stress-induced injury [22]. Inhibition of HO-1 with tin mesoporphyrin (SnMP) showed increased damage in experimental colitis [23]. The products of the enzyme HO-1 can act as antioxidant (bilirubin) and anti-inflammatory agents (carbon monoxide). Manipulation of HO-1 activity may therefore be of interest as a new treatment option to treat inflammatory bowel disease.

In endotoxaemic rats, we observed more pronounced iNOS expression in the ileum than in the colon. Morin *et al* showed that, in the intestine, iNOS mRNA is already induced 1 h after injection of LPS [8]. Like our results, they observed more prominent induction of iNOS in the ileum than in the colon and the jejunum. The same group showed that epithelial iNOS expression was more prominent in female and older rats, indicating an age- and gender-dependent responsiveness to LPS [24]. If such massive iNOS induction and NO production in intestinal epithelial cells along the axis of the intestinal tract is also present in human endotoxaemia, it must have implications for intestinal function. Indeed, intestinal permeability is increased in human volunteers injected with LPS [25] and in septic patients [26]. Unno *et al* [9] showed that selective inhibition of iNOS reduces intestinal hyperpermeability and bacterial translocation in endotoxaemic rats. Several animal and human studies (reviewed by Kilbourn *et al* [27]) reported both detrimental and beneficial effects on blood pressure and survival after (non-selective) NOS inhibition in endotoxaemia. However, selective and local inhibition of iNOS in intestinal epithelial cells might improve intestinal dysfunction in sepsis and endotoxaemia and could be a potential new treatment option.

In conclusion, we have demonstrated a switch from an NF-κB-regulated stress response to an AP-1-regulated stress response in intestinal inflammation, which is determined by the extent of oxidative stress and may be controlled by HO-1-derived carbon monoxide. This switch is probably an adaptation to the type of stress. NF-κB-regulated protective genes include anti-apoptotic genes, eg iNOS, which protect against pro-apoptotic inflammatory cytokines like TNF-α. AP-1-regulated genes such as HO-1 and superoxide dismutases are more involved in protection against various forms of oxidative stress. These findings suggest that iNOS (NF-κB driven) and HO-1 (AP-1 driven) represent mutually exclusive survival mechanisms in intestinal epithelial cells.

Acknowledgements

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