British Journal of Pharmacology (2000) 130, 57–64

www.nature.com/bjp

Enhanced expression of haem oxygenase-1 by nitric oxide and antiinflammatory drugs in NIH 3T3 fibroblasts

*,[2]Maria José Alcaraz, [1]Aïda Habib, [1]Marilyne Lebret, [3]Christophe Créminon, [1]Sylviane Lévy-Toledano & [1]Jacques Maclouf

[1]Unité 348 INSERM, Institut Féderatif de Recherche Lariboisière-Circulation, 75475 Paris cedex 10, France; [2]Department of Pharmacology, University of Valencia, 46100 Burjasot, Valencia, Spain and [3]CEA, Service de Pharmacologie et d'Immunologie. Département de Recherche Médicale, 91191 Gif sur Yvette, France

1 Haem oxygenase-1 (HO-1) can exert protective effects against oxidative stress and inflammation. Fibroblasts participate in inflammatory responses where they produce high levels of prostaglandins (PGs) and nitric oxide (NO). However, little is known of the presence of HO-1 in these cells and the possible interactions among these pathways. Incubation of cells with NO donors, spermine nonoate (SPNO) and S-nitroso-N-acetylpenicillamine (SNAP), induced a dose- and time-dependent expression of HO-1 protein.

2 NO donors increased basal PGE_2 release although they reduced PGE_2 accumulated in the medium and cyclo-oxygenase (COX) activity when cells were stimulated with lipopolysaccharide (LPS). COX-2 protein was weakly induced by SPNO in basal conditions and in the presence of LPS a synergy for HO-1 and COX-2 protein expression was observed.

3 Our results indicate that reactive oxygen species participate in the inductive effect of NO donors or LPS on HO-1 expression, whereas endogenous NO production may play a role in the mechanism of the synergy exhibited by SPNO and LPS on HO-1 and COX-2 expression. In this system, zinc protoporphyrin IX did not affect nitrite levels but reduced COX activity.

4 The selective COX-2 inhibitors SC58125 and NS398 as well as the non-selective COX inhibitor, indomethacin, strongly reduced PGE_2 synthesis and showed a synergy with NO donors in HO-1 and COX-2 induction. Addition of PGE_2 had no effect, suggesting a mechanism independent of PGs formation.

5 In inflammatory conditions a number of factors could cooperate to induce HO-1 and COX-2, with a positive regulation by COX inhibitors.

British Journal of Pharmacology (2000) **130,** 57–64

Keywords: Haem oxygenase; nitric oxide; NIH 3T3 fibroblasts; cyclo-oxygenase-2; non-steroidal antiinflammatory drugs; lipopolysaccharide; reactive oxygen intermediates

Abbreviations: CO, carbon monoxide; COX, cyclo-oxygenase; DF, deferoxamine; ECL, enhanced chemiluminescence; HO, haem oxygenase; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; NAC, N-acetylcysteine; NSAIDs, non-steroidal antiinflammatory drugs; PBS, phosphate buffered saline; PG, prostaglandin; SNAP, S-nitroso-N-acetylpenicillamine; SOD, superoxide dismutase; SPNO, spermine nonoate; ZnPPIX, zinc protoporphyrin IX

Introduction

Haem oxygenase-1 (HO-1) is an inducible stress protein that catabolizes haem to form biliverdin, which is then reduced to bilirubin, carbon monoxide (CO) and free iron. The constitutive isoform HO-2 is present in tissues such as brain and testis (Abraham *et al*., 1988) and recently, another closely related constitutive isoform (HO-3) has been described (McCoubrey *et al*., 1997). Recent evidence has placed HO-1 as a key event in defence mechanisms against oxidative injury by different agents such as haemoglobin, hypoxia, glutathione depletion, cytokines... (Camhi *et al*., 1995; Shibahara *et al*., 1987; Terry *et al*., 1998). Mice lacking functional HO-1 show an altered iron metabolism, with anaemia, iron accumulation in liver and kidneys, as well as chronic inflammation (Poss & Tonegawa, 1997a). Increased mortality and hepatic necrosis after lipopolysaccharide (LPS) challenge is also observed (Poss & Tonegawa, 1997b). Interestingly, induction of HO-1 can ameliorate some inflammatory conditions such as nephrotoxic glomerulonephritis (Mosley *et al*., 1998) or carrageenan pleuritis in rats (Willis *et al*., 1996), as well as ocular inflammation in rabbit corneas produced by contact lens (Laniado-Schwartzman *et al*., 1997). In addition, overexpression of HO-1 in the lung by gene transfer before exposure to hyperoxia, results in a marked reduction in lung injury and inflammation in rats (Otterbein *et al*., 1999).

The antiinflammatory properties of HO-1 can be related to the generation of biliverdin and bilirubin, possessing antioxidant (Frei *et al*., 1988; Stocker & Peterhans, 1989) and anticomplement properties (Nakagami *et al*., 1993), besides the upregulation of ferritin, which sequesters free iron and the generation of CO. In addition, overexpression of HO-1 offers cellular protection against haem/haemoglobin oxidative injury and haem-mediated expression of adhesion molecules (Wagener *et al*., 1997).

Nitric oxide (NO) is an important regulatory and effector mediator produced constitutively by endothelial (eNOS) or neuronal NO synthases (nNOS), or in high concentrations by the activity of inducible NO synthase (iNOS) (Knowles & Moncada, 1994; Moncada & Higgs, 1993). At the vascular level, there is a cooperation between NO and HO-1 in the

*Author for correspondence.

control of the vascular tone. The induction of HO-1 by NO has been described in vascular smooth muscle cells (Durante *et al*., 1997) and endothelial cells (Foresti *et al*., 1997) resulting in the generation of CO, which could act in a manner similar to NO and control blood pressure under stress conditions (Motterlini *et al*., 1998). In endothelial cells, HO-1 overinduction may also participate in cell proliferation and angiogenesis, as an adaptive mechanism against cell damage in inflammatory sites of haemorrhage, thrombosis and hypoxia (Deramaudt *et al*., 1998). Interestingly, rapid expression of HO-1 in endothelial and smooth muscle cells in the heart, can be determinant for long-term survival of cardiac xenografts in rodents (Soares *et al*., 1998).

Cyclo-oxygenase (COX), the key enzyme in the synthesis of prostanoids, is present in mammalian tissues in two isoforms, the constitutive COX-1 and the inducible COX-2, which would be responsible for the high levels of prostaglandins (PGs) detected in inflammatory responses (Fu *et al*., 1990; Hla & Neilson, 1992; Kujubu *et al*., 1993). Conventional non-steroidal antiinflammatory drugs (NSAIDs) inhibit PG synthesis without a clear selectivity for any of the two isoforms, although selective inhibitors have been extensively searched for. One of these new drugs is SC58125, a tricyclic methyl sulphone derivative exhibiting antiinflammatory and analgesic efficacy comparable to dual COX-1/COX-2 inhibitors. Nevertheless, this drug does not modify gastric PG synthesis nor exert ulcerogenic effects in animals (Seibert *et al*., 1994).

Fibroblasts participate in inflammatory responses by the release of soluble mediators and through cell-to-cell interactions with other inflammatory cells such as macrophages, which results in the exacerbation of chronic inflammatory states (Steinhauser *et al*., 1998).

Evidence for HO-1 induction by oxidative stress in human fibroblasts is provided by activation of HO-1 gene expression after glutathione depletion, through extracellular-signal regulated kinases and c-Fos protein (Numazawa *et al*., 1997). These data have been consolidated by observations showing that HO-1(−/−) embryonic fibroblasts exhibited an increased generation of reactive oxygen species and cytotoxicity after treatment with different stimuli (Poss & Tonegawa, 1997b) and that HO-1 mediates cytoprotection to oxygen in the hamster fibroblast cell line O2R95 (Dennery *et al*., 1997).

On the other hand, possible interactions between NO and CO have been suggested in different systems, especially at the vascular level (Durante *et al*., 1997; Hartsfield *et al*., 1997) or in rat brain homogenates (Willis *et al*., 1995). Since the expression of some primary response genes such as iNOS and COX-2 is induced by cytokines, growth factors or LPS in murine fibroblasts (Herschman *et al*., 1997), these pathways may coexist with HO-1 during inflammatory responses. However, little is known about the mechanisms involved in the regulation of HO-1 expression in fibroblasts and the possibility of interactions with NO and PGs. In the current study, we have examined the regulation of HO-1 and COX-2 expression by NO at the protein level in a murine cell line of fibroblasts (NIH 3T3), as well as its modulation by NSAIDs.

Methods

Cell culture

NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA, U.S.A.) were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin. When cells reached confluence, the culture medium was replaced with serum-free DMEM to perform the experiments. Cells in serum-free DMEM medium were incubated with drugs for various times at concentrations given in the Results section. LPS was added at a final concentration of 1 μg ml^{-1}. After incubation, supernatants were collected to measure PGE_2 and nitrite levels. To evaluate COX activity, cells were washed with Hanks buffer, pH 7.4, containing 1 mg ml^{-1} of bovine serum albumin (BSA), and incubated at 37°C in the same buffer with 10 μM arachidonic acid. After 30 min of incubation, supernatants were collected to measure PGE_3. Cytotoxicity was evaluated by neutral red incorporation in viable cells according to Zhang *et al*. (1990). The presence of endotoxin was determined by the Limulus assay using a commercial kit (Sigma Chemical Co., St Louis, MO, U.S.A.). Neutral red incorporation assays indicated absence of cell toxicity by drug treatments (NO donors, L-NMMA or NSAIDs) and the possibility of contamination of drug solutions with endotoxin was excluded after performing the Limulus test (data not shown).

COX-2 activity in immunoprecipitates

NIH 3T3 cells were incubated in the presence of LPS (1 μg ml^{-1}) for 18 h. COX-2 was immunoprecipitated in cell lysates using COX-2 specific antibodies and enzyme activity was assayed according to Habib *et al*. (1997). The buffer for reaction consisted of 50 mM Tris, pH 8.0, 1 mM phenol, 0.1% BSA and 1 μM hematin. When mentioned, increasing concentrations of zinc protoporphyrin IX (ZnPPIX) were added. After 10 min incubation at 37°C in the presence of 20 μM arachidonic acid, the reaction was stopped by dilution in cold buffer and PGE_2 was determined.

Measurement of PGE_2 and nitrite levels

PGE_2 was measured by enzyme immunoassay with acetylcholinesterase-labelled PGE_2 as tracer (Pradelles *et al*., 1985). Aliquots (100 μl) of supernatants were used for nitrite determination with the Griess reagent (Green *et al*., 1982).

Western blot analysis

Cells were grown to confluence on 6-well plates and treated as indicated in the Results section. The culture medium was removed after activation, and cells were washed twice in phosphate buffered saline (PBS) buffer and lysed in 200 μl of ice-cold buffer (in mM: Tris-HCl (pH 7.5) 20, EDTA 1, Hecameg® 20 and benzamidine 1). Cells were scraped with a rubber policeman and centrifuged at 4°C for 10 min at 10,000 × *g*. The protein content was determined by the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.) using BSA as standard. Cell lysate (50 μg of protein) was mixed with Laemmli reagent under reducing conditions. Samples were separated by SDS–PAGE using 9% bis-acrylamide and transferred onto nitrocellulose membranes, which were saturated for 1 h at 37°C in Tris buffer saline (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, and 0.1% Tween 20) containing 5% of fat-free dry milk. Specific monoclonal antibodies were used to detect COX-2 (CX-214, 1 μg/ml) (Créminon *et al*., 1995). For HO-1, we used either the commercial monoclonal antibody for HO-1 (Stressgen, Victoria, Canada; 1/2000) or a polyclonal antibody we raised using the human peptide of HO-1: MERPQPDSMPQD. For HO-2 detection, a rabbit polyclonal antibody was raised against a specific peptide of HO-2, HSAEVETSEGVDES. These antibodies recognized

proteins with similar molecular weight as HO-1 or HO-2 (32 and 36 kDa, respectively) and the protein bands were not detected when the antibodies were saturated with the appropriate peptides. Chemiluminescence substrates (ECL, Western blotting; Amersham Pharmacia Corp.) were used to reveal positive bands visualized after 1 min exposure to Hyperfilm ECL (Amersham Pharmacia Corp.). The bands were quantitated using a laser densitometer.

Materials

LPS (*Escherichia coli* serotype 011:B4), arachidonic acid, indomethacin, BSA, hemin, hematin, flurbiprofen, N-acetylcysteine, superoxide dismutase, deferoxamine mesylate and ZnPPIX were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Dr Peter Isakson (Searle, Monsanto, St Louis, MO, U.S.A.) kindly provided SC58125. NS398, spermine nonate (SPNO) and S-nitroso-N-acetylpenicillamine (SNAP) were from Cayman Chem. (Ann Arbor, MI, U.S.A.). Enhanced chemiluminescence (ECL) substrates were from Amersham Pharmacia Corp. Donkey polyclonal anti-mouse or anti-rabbit IgGs coupled to peroxidase was from Jackson Immuno-Research Laboratories (West Grove, PA, U.S.A.). Electrophoresis reagents were from J.T. Baker (Phillipsburg, NJ, U.S.A.), and chemical reagents from Prolabo (Paris, France) and from Carlo Erba (Farmitalia, Milan, Italy). N^G-methyl-L-arginine, acetate salt (L-NMMA) was from Alexis Corporation (Coger, France). Culture reagents were from Life Technologies Inc. (Cergy Pontoise, France). Stock solutions of drugs were prepared in 0.01 M NaOH (SPNO), dimethyl sulphoxide (DMSO; Sigma Chemical Co., St Louis, MO, U.S.A.) for indomethacin, NS398, SNAP and ZnPPIX, ethanol/DMSO: 4/1, v v^{-1} (SC58125) or ethanol (flurbiprofen and PGE_2). The rest of the drugs were dissolved in the incubation medium. The maximal concentration of solvents in the final incubation media was 0.1% v v^{-1}. Appropriate controls showed that this concentration was without effect in our experiments. All other reagents were of analytical grade.

Statistical analysis

Results are shown as mean ± s.e.mean of n experiments. Data were analysed by two-way ANOVA followed by Dunnett's t-test for multiple comparisons.

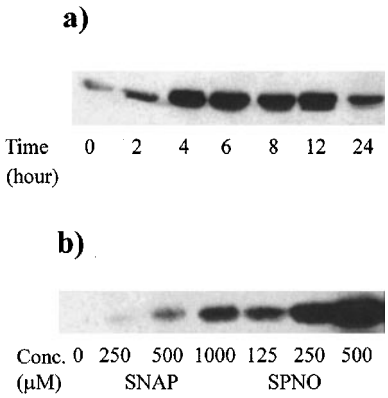

Figure 1 (a) Time course of HO-1 protein expression induced by SPNO. Cells were incubated with 250 μM SPNO. HO-1 expression was analysed by Western blotting as indicated in Methods. (b) Dose-dependent induction of HO-1 by NO donors. SNAP was added at 250, 500 and 1000 μM and SPNO at 125, 250 and 500 μM. Results are representative of three experiments.

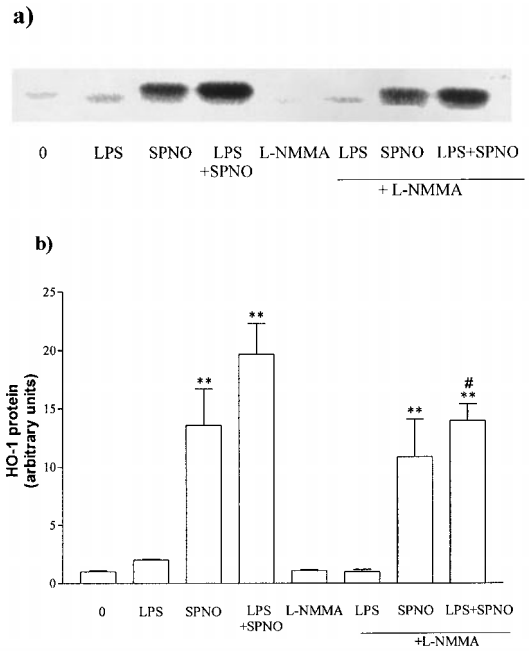

Figure 2 Induction of HO-1 by LPS (1 μg ml^{-1}) in the presence or absence of SPNO 250 μM. Influence of L-NMMA (1 mM). Coincubations were performed for 6 h. HO-1 was analysed as described in the legend to Figure 1. (a) Western blot; (b) HO-1 band intensity (fold of control based on arbitrary units, mean ± s.e.mean, $n=4$). $^{**}P<0.01$ with respect to control; $\#P<0.01$ with respect to LPS + SPNO.

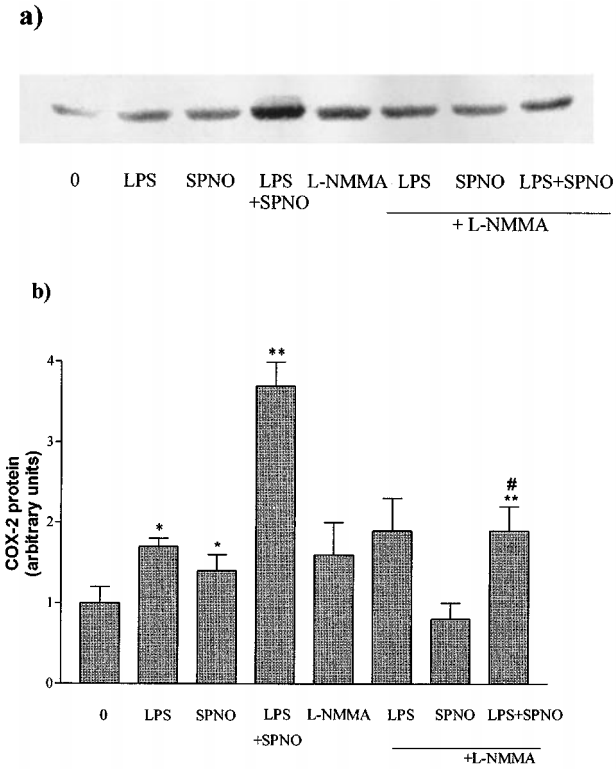

Figure 3 Induction of COX-2 by LPS (1 μg ml^{-1}) in the presence or absence of SPNO 250 μM. Influence of L-NMMA (1 mM). Coincubations were performed for 6 h. HO-1 was analysed as described in the legend to Figure 1. (a) Western blot; (b) COX-2 band intensity (fold of control based on arbitrary units, mean ± s.e.mean, $n=4$). $^{*}P<0.05$; $^{**}P<0.01$ with respect to control; $\#P<0.01$ with respect to LPS + SPNO.

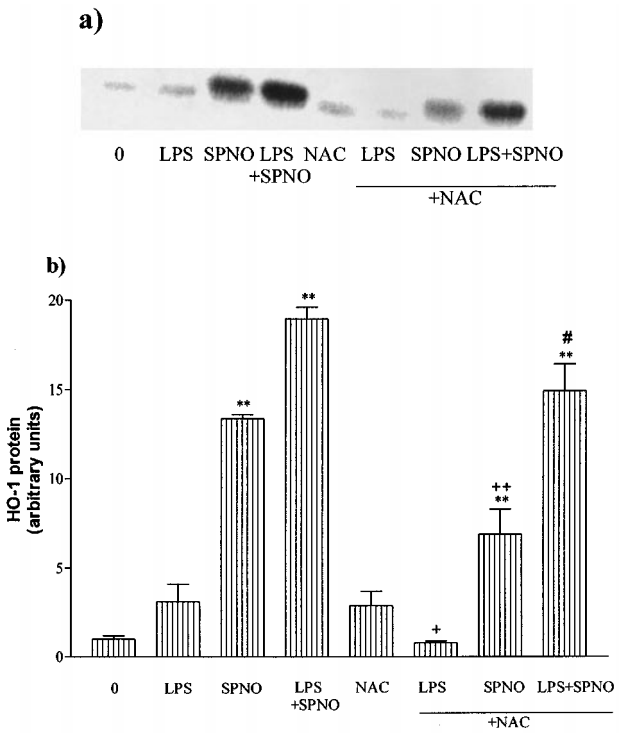

Figure 4 Effect of N-acetylcysteine (NAC) on the induction of HO-1 by SPNO, LPS or SPNO-LPS. NAC (20 mM) was incubated alone or in the presence of SPNO (250 μM), LPS (1 μg ml^{-1}) or both for 6 h. (a) Western blot; (b) HO-1 band intensity (fold of control based on arbitrary units, mean$\pm$s.e.mean, $n=4$). $^{**}P<0.01$ with respect to control; $\#P<0.01$ with respect to LPS+SPNO; $+P<0.05$ with respect to LPS; $++P<0.01$ with respect to SPNO.

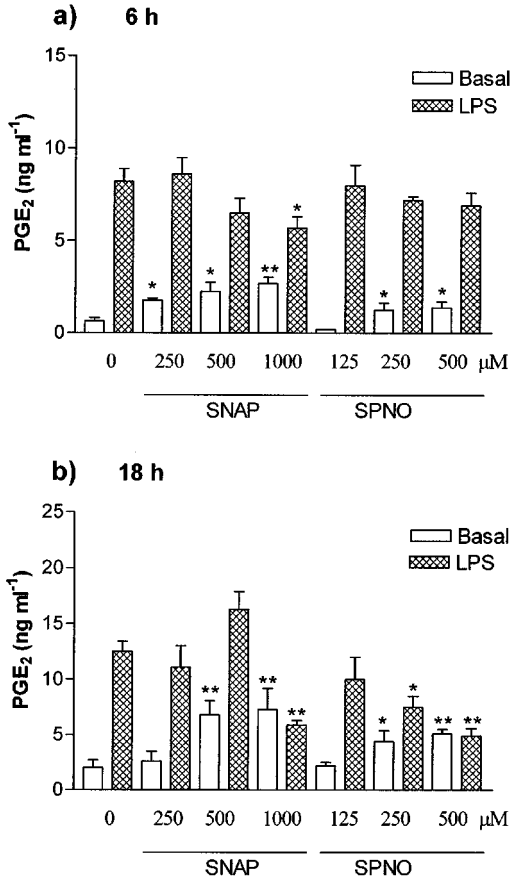

Figure 5 (a) Effect of NO donors on PGE_2 levels accumulated in the incubation medium at 6 h. SNAP or SPNO was incubated at different concentrations either with unstimulated cells (basal) or in the presence of LPS (1 μg ml^{-1}). (b) Effect of NO donors on PGE_2 levels accumulated in the incubation medium at 18 h in the same conditions. $^{*}P<0.05$; $^{**}P<0.01$. The data are the mean$\pm$s.e.mean of six experiments.

Results

HO-1 and COX-2 induction

The time- and dose-dependent induction of HO-1 was studied in the presence of two NO donors. Figure 1a shows the increase in HO-1 protein following the addition of 250 μM SPNO, where maximal expression occurred after 4–12 h of incubation and decreased at 24 h. A similar pattern was observed with SNAP (data not shown). Figure 1b shows the HO-1 expression in response to NO donors, which was dose-dependent with a higher potency for SPNO. This compound releases higher amounts of NO compared to SNAP at 6 h. This is reflected in the accumulation of nitrite in the culture medium, which was 118.4$\pm$8.7 and 73.0$\pm$6.3 μM (mean$\pm$s.e.mean, $n=6$) for 250 μM SPNO and 500 μM SNAP, respectively. In these cells, LPS (1 μg ml^{-1}) acted as a weak inducer of HO-1 but it increased the induction of HO-1 by SPNO (Figure 2). Western blot analysis of whole cell lysates indicated that COX-2 was weakly induced in these cells by SPNO and this effect was potentiated by LPS at 6 h (Figure 3).

Characterization of enzyme induction

The influence of endogenous NO production on the individual effects of SPNO and LPS, as well as on the synergy exhibited by coincubation of cells with both agents, was assessed by using the NOS inhibitor L-NMMA. This compound slightly attenuated the expression of HO-1 induced either by LPS or SPNO, but it reduced the synergy on HO-1 and COX-2 expression in a significant manner (Figures 2 and 3). To assess the role of oxidative stress, cells were preincubated with deferoxamine (50 μM), superoxide dismutase (50 u ml^{-1}) or N-acetylcysteine (20 mM) for 30 min and then treated with the NO donor or LPS for 6 h. Only N-acetylcysteine prevented HO-1 induction and the results with this antioxidant are shown in Figure 4. Moreover, the induction of HO-1 by coincubation with LPS+SPNO was inhibited by N-acetylcysteine in the same conditions as above and to a similar extent. In contrast, antioxidants did not exert any effect on COX-2 expression induced by SPNO, LPS or SPNO+LPS (data not shown).

PGE_2 production

In cells treated with NO donors only, PGE_2 levels accumulated in the medium were increased in a dose-dependent manner after 6 or 18 h of incubation, with a higher effect for SNAP (Figure 5). When cells were treated with LPS for 6 h or 18h, coincubation with SNAP only affected PGE_2 accumulated in the medium at a high concentration (1 mM), whereas SPNO at lower concentrations reduced the release of this eicosanoid at 18 h. For COX activity in intact cells (Figure 6), an increase in basal activity was observed in cells treated with SNAP and to a lesser extent, with SPNO. Nevertheless, only the latter compound inhibited COX activity in LPS-stimulated cells.

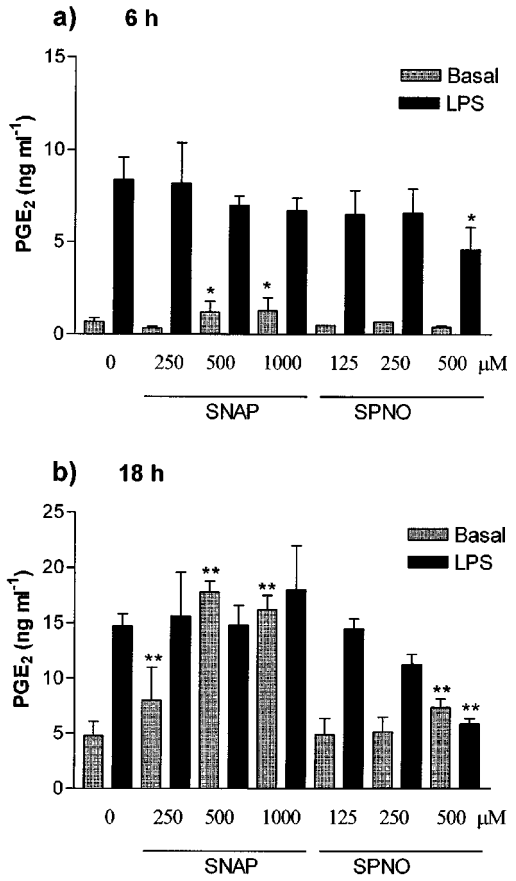

Figure 6 (a) Effect of NO donors on COX activity after 6 h incubation. SNAP or SPNO was incubated at different concentrations either with unstimulated cells (basal) or in the presence of LPS (1 μg ml^{-1}). The medium was then removed and cells were washed with Hank's buffer. After addition of arachidonic acid (10 μM), incubation proceeded for 30 min and PGE_2 was measured in supernatants. (b) Effect of NO donors on COX activity in intact cells after 18 h incubation in the same conditions. $*P<0.05$; $**P<0.01$. The data are the mean±s.e.mean of six experiments.

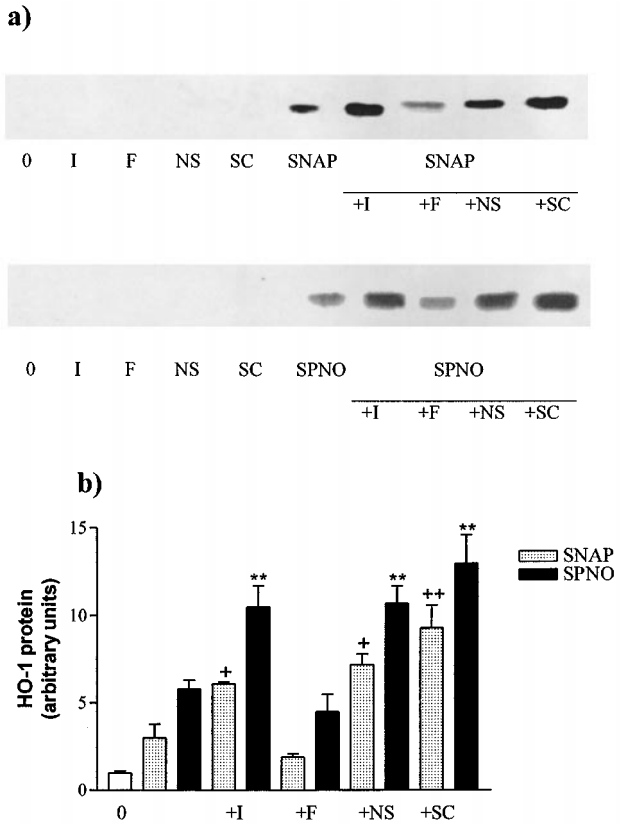

Figure 7 Effect of COX inhibitors on HO-1 expression induced by NO donors. Cells were incubated in the absence or presence of indomethacin (I, 1 μM), flurbiprofen (F, 10 μM), NS398 (NS, 10 μM) or SC58125 (SC, 25 μM) for 30 min, prior to the addition of 500 μM SNAP or 125 μM SPNO followed by 6 h incubation. (a) Western blot; (b) HO-1 band intensity (fold of control based on arbitrary units, mean±s.e.mean, $n=3$ for SNAP and $n=5$ for SPNO). $+P<0.05$, $++P<0.01$ with respect to SNAP alone; $**P<0.01$ with respect to SPNO alone.

Effect of inhibitors of HO-1 and NOS on PGE_2 release/COX-2 activity

Either control cells or cells treated with LPS for 6 h, released no detectable amounts of nitrite. Nevertheless after 18 h of incubation with LPS nitrite levels raised up to 21.6±3.8 μM (mean±s.e.mean, $n=10$). To assess the interaction among HO-1, COX-2 and iNOS activation, inhibitors of each enzyme were tested. Coincubation of cells with LPS (1 μg ml^{-1}) and L-NMMA (1 mM) for 18 h strongly reduced nitrite levels (3.5±0.2 μM; mean±s.e.mean, $n=6$; $P<0.01$) and exhibited no effect on PGE_2 levels (7.2±0.7 ng ml^{-1}, $n=6$). On the other hand, coincubation of NIH 3T3 cells with LPS and the HO inhibitor ZnPPIX (1 μM) for 18 h, did not modify nitrite levels (19.5±2.1 μM, $n=6$), but strongly reduced PGE_2 accumulated in the medium (0.6±0.2 compared to 7.7±0.6 ng ml^{-1} PGE_2, $n=6$, for LPS alone, $P<0.01$). COX activity in intact cells was also inhibited by ZnPPIX (3.6±0.6 ng ml^{-1} compared to 14.7±0.3 ng ml^{-1} PGE_2, $n=6$, for LPS alone, $P<0.01$). This effect did not depend on inhibition of COX-2 expression since this drug was unable to modify COX-2 expression induced by LPS (data not shown). To determine if ZnPPIX exerted direct inhibitory effects on COX-2 enzymatic activity, this drug was incubated with immunoprecipitated COX-2, as indicated in the Methods section. A weak inhibition of COX-2 activity was observed in the presence of 1 μM ZnPPIX (PGE_2 production in this group was 35.5±0.5 ng ml^{-1} with respect to 51.2±2.4 ng ml^{-1} in control incubations, $n=5$, $P<0.01$), likely due to competition with haem.

Effect of COX inhibitors on HO-1 expression and nitrite formation

We next examined the response of NIH 3T3 cells to NO donors in the presence of NSAIDs and found that some NSAIDs exhibited a synergy with respect to HO-1 induction. As can be seen in Figure 7, NSAIDs alone did not induce HO-1, but costimulation with SPNO or SNAP and COX inhibitors indomethacin, NS398 or SC58125 at concentrations in the micromolar range, increased by two to three times protein expression. In contrast, flurbiprofen did not show any effect. As expected, PGE_2 levels were effectively inhibited by all NSAIDs either in the presence of NO donors or LPS in the conditions indicated in Figures 5 and 6 (data not shown). Nevertheless, addition of 1 μM PGE_2 (Figure 8) or 10 nM (data not shown) did not prevent the effect of NSAIDs on HO-1 expression. A potentiation in the induction of COX-2 was observed in cells incubated with SPNO+SC58125 (Figure 9). Addition of PGE_2 did not significantly affect this synergy similarly to HO-1 expression, and PGE_2 alone induced COX-2 expression (Figure 9).

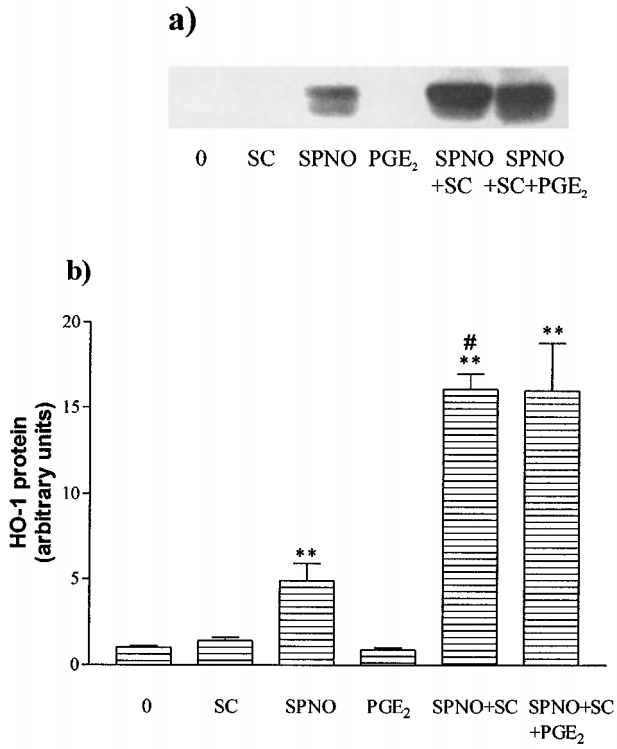

Figure 8 Effect of PGE_2 on the synergy in HO-1 expression by SPNO and SC58125. Cells were pretreated with SC58125 (SC, 25 μM) for 30 min and then incubated in the presence or absence of SPNO (250 μM) and/or PGE_2 (1 μM) for 6 h. (a) Western blot; (b) HO-1 band intensity (fold of control based on arbitrary units, mean± s.e.mean, $n=4$). $^{**}P<0.01$ with respect to control; $\#P<0.01$ with respect to SPNO.

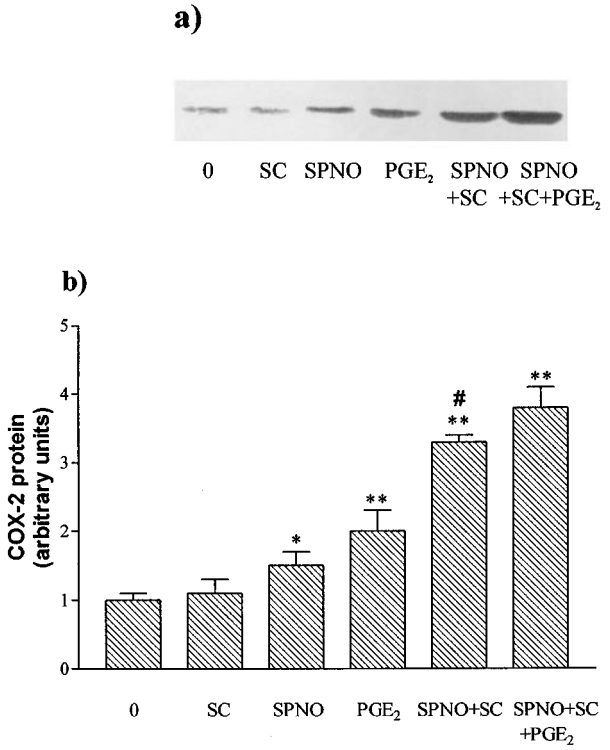

Figure 9 Effect of PGE_2 on the synergy in COX-2 expression by SPNO and SC58125. Cells were pretreated with SC58125 (SC, 25 μM) for 30 min and then incubated in the presence or absence of SPNO (250 μM) and/or PGE_2 (1 μM) for 6 h. (a) Western blot; (b) COX-2 band intensity (fold of control based on arbitrary units, mean± s.e.mean, $n=4$). $^{**}P<0.01$ with respect to control; $\#P<0.01$ with respect to SPNO.

Discussion

The present study shows that (i) exogenous or endogenous NO induces HO-1 in NIH 3T3 fibroblasts; (ii) a synergy for the inductive effects of NO donors and LPS on HO-1 and COX-2 protein expression is present and the mechanisms involved do not seem to depend on the production of oxidative stress but on endogenous NO; and (iii) selective or nonselective COX inhibitors upregulate HO-1 expression induced by NO. Expression of HO-1 by NO donors or LPS presents common features since the production of oxidative stress may be involved in the mechanism of action of both agents on HO-1, but not in COX-2, as observed in our experiments using NIH 3T3 fibroblasts. NO could interact with cellular oxidants to form more reactive species able to induce oxidative stress, which would result in the induction of HO-1 as part of a putative protective mechanism. These results extend previous observations in other systems, since participation of oxidative stress in the induction of HO-1 by NO has been described in vascular smooth muscle cells (Hartsfield *et al*., 1997), whereas in endothelial cells, NO stabilization by thiols through formation of S-nitrosothiols has been suggested as a mechanism modulating the effect of NO (Foresti *et al*., 1997). Alternatively, the release of haem by NO might play a role in the induction of HO-1 in some cells such as hepatocytes (Kim *et al*, 1995). In this regard, transcriptional activation of HO-1 gene by LPS through an oxidative pathway has been described in mouse M1 cells *via* NFκB (Kurata *et al*., 1996) and in macrophages by AP-1-dependent transcription (Camhi *et al*., 1998).

Interestingly, our data show for the first time, that some COX inhibitors upregulate HO-1 expression induced by NO. A previous report on rat mesangial cells indicate the potentiating effect of indomethacin on HO-1 mRNA expression induced by heat shock, phorbol ester or interleukin-1β, by a mechanism related to inhibition of PGE_2 production (Tetsuka *et al*., 1995). Nevertheless, we have demonstrated in NIH 3T3 fibroblasts a synergy for HO-1 induction by NO and SC58125 or NS398, which are selective COX-2 inhibitors, as well as with indomethacin, a nonselective COX-1/COX-2 inhibitor. In contrast, flurbiprofen, a nonselective COX-1/COX-2 inhibitor did not exert a significant effect although it strongly inhibited PGE_2 production in all the conditions tested. All these NSAIDs did not induce HO-1 alone and the potentiation by these drugs of NO-induced HO-1 expression was not reverted by PGE_2 addition. Thus, NSAIDs may possibly facilitate HO-1 induction of NO by means of a mechanism independent of PGE_2 production, although a possible role for other products of COX-2 activity or arachidonic acid metabolites cannot be discarded. In addition, we have shown in NIH 3T3 fibroblasts that NSAIDs and NO donors may synergize to a lesser extent, to induce COX-2 expression, also independently of PGE_2 production. One possibility is that NSAIDs act directly and independently of PGE_2 production on the transcription of HO-1 or COX-2. Recently, an example of COX-2 induction by NSAIDs was reported in human colon cancer cell lines, where some NSAIDs, fatty acids and some ligands of peroxisome proliferator-activated receptor (PPAR) induced COX-2 transcription (Meade *et al*., 1999). HO-1 transcription by PPAR ligands was also demonstrated in stimulated macrophages (Colville-Nash *et al*., 1998). Further investigations are required to know the mechanisms of action of these NSAIDs on HO-1 induction and whether this effect is of relevance *in vivo*.

We have shown a dual effect of exogenous NO on PGE_2 accumulation and COX activity in NIH 3T3 fibroblasts. In cells not treated with LPS there is a low level of COX-1 (Reddy & Herschman, 1994) and after incubation with NO donors, basal levels of PGE_2 were increased accompanied by a weak induction of COX-2 protein. Since all NSAIDs were able to inhibit COX activity at the same level reached by the COX-2 inhibitors SC58125 or NS398, it is very likely that COX-2 is responsible for the major PGE_2 production induced by NO donors, in our experimental conditions. In addition, an increase in PGH_2/PGE_2 isomerase activity by exogenous NO is possible and it has been reported earlier in this cell line (Kelner & Uglik, 1994). Nevertheless, in the presence of LPS, where COX-2 was strongly induced and PGE_2 levels are produced by COX-2 activity (Reddy & Herschman, 1994), there is a potentiation in protein expression by NO donors and LPS, without an increase in PGE_2 levels, indicating that NO treatment may lead to partial decrease in enzyme activity. It is known that NO can interact with haem groups, Fe-S clusters etc. NO or most likely products of reaction with oxygen or iron, form complexes such as the iron-nitrosyl complex that can modify thiol groups of enzymes. S-nitrosylation or nitration of proteins can result in modification of signalling pathways or COX activity (Kröncke *et al*., 1997).

Our finding that an inhibitor of HO-1 activity strongly reduces PGE_2 production would indicate a possible relationship between both enzymes in this cell line. Although this drug weakly inhibits COX-2 activity, this represents only a small contribution to the strong inhibitory effect of ZnPPIX on PGE_2 production in intact cells, thus suggesting that HO-1 activity would generate some mediator increasing COX activity. Our results indicated an effect of this HO-1 inhibitor independent of protein expression, in contrast to the increased COX-2 expression observed after treatment of endothelial cells with a different inhibitor of HO-1 activity (Haider *et al*., 1999).

In comparison with the effect of NO donors, the endogenous release of NO by LPS-stimulated fibroblasts is low, as well as the induction of HO-1. In inflammatory conditions, apart from their endogenous NO synthesis, fibroblasts can be exposed to high fluxes of NO by adjacent activated cells such as macrophages, neutrophils or endothelial cells which also secrete reactive oxygen species able to react with NO yielding other reactive species. It is thus likely the cooperation of factors to induce HO-1 and our results indicate a positive regulation by NSAIDs. Definition of signalling pathways governing HO-1 synthesis may indicate novel pharmacological interventions for the treatment of inflammatory states.

This work was supported by grants from INSERM, the Association de Recherche pour le Cancer ARC (J. M. Grant #2076), and CICYT SAF97-0249. The authors are grateful to Dr P. Isakson (Searle, Monsanto, St Louis, MO, U.S.A.). for providing the COX-2 inhibitor SC58125. This paper is dedicated to the late Jacques Maclouf who initiated and supported this study. He will always be remembered as a great mentor, colleague and friend.

References

ABRAHAM, N.G., LIN, J.H., SCHWARTZMAN, M.L., LEVERE, R.D. & SHIBAHARA, S. (1988). The physiological significance of heme oxygenase. *Int. J. Biochem.,* **20,** 543 – 558.

CAMHI, S.L., ALAM, J., OTTERBEIN, L., SYLVESTER, S.L. & CHOI, A.M. (1995). Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation. *Am. J. Respir. Cell Mol. Biol.,* **13,** 387 – 398.

CAMHI, S.L., ALAM, J., WIEGAND, G.W., CHIN, B.Y. & CHOI, A.M. (1998). Transcriptional activation of the HO-1 gene by lipopolysaccharide is mediated by 5′ distal enhancers: role of reactive oxygen intermediates and AP-1. *Am. J. Respir. Cell Mol. Biol.,* **18,** 226 – 234.

COLVILLE-NASH, P.R., QURESHI, S.S., WILLIS, D. & WILLOUGHBY, D.A. (1998). Inhibition of inducible nitric oxide synthase by peroxisome proliferator-activated receptor agonists: correlation with induction of heme oxygenase 1. *J. Immunol.,* **161,** 978 – 984.

CRÉMINON, C., HABIB, A., MACLOUF, J., PRADELLES, P., GRASSI, J. & FROBERT, Y. (1995). Differential measurement of constitutive (Cox-1) and inducible (Cox-2) cyclooxygenase expression in human umbilical vein endothelial cells using specific immunometric enzyme immunoassays. *Biochim. Biophys. Acta,* **1254,** 341 – 348.

DENNERY, P.A., SRIDHAR, K.J., LEE, C.S., WONG, H.E., SHOKOOHI, V., RODGERS, P.A. & SPITZ, D.R. (1997). Heme oxygenase-mediated resistance to oxygen toxicity in hamster fibroblasts. *J. Biol. Chem.,* **272,** 14937 – 14942.

DERAMAUDT, B.M., BRAUNSTEIN, S., REMY, P. & ABRAHAM, N.G. (1998). Gene transfer of human heme oxygenase into coronary endothelial cells potentially promotes angiogenesis. *J. Cell Biochem.,* **68,** 121 – 127.

DURANTE, W., KROLL, M.H., CHRISTODOULIDES, N., PEYTON, K.J. & SCHAFER, A.I. (1997). Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ. Res.,* **80,** 557 – 564.

FORESTI, R., CLARK, J.E., GREEN, C.J. & MOTTERLINI, R. (1997). Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. *J. Biol. Chem.,* **272,** 18411 – 18417.

FREI, B., STOCKER, R. & AMES, B.N. (1988). Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Natl. Acad. Sci. U.S.A.,* **85,** 9748 – 9752.

FU, J.-Y., MASFERRER, J.L., SEIBERT, K., RAZ, A. & NEEDLEMAN, P. (1990). The induction and suppression of prostaglandin H_2 synthase (cyclooxygenase) in human monocytes. *J. Biol. Chem.,* **265,** 16737 – 16740.

GREEN, L.C., WAGNER, D., GLOGOWSKI, P.L., SKIPPER, J.S., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite and (^{15}N) in biological fluids. *Anal. Biochem.,* **126,** 131 – 138.

HABIB, A., BERNARD, C., LEBRET, M., CRÉMINON, C., ESPOSITO, B., TEDGUI, A. & MACLOUF, J. (1997). Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. *J. Immunol.,* **158,** 3845 – 3851.

HAIDER, A., FERRERI, N.R. & ABRAHAM, N.G. (1999). The regulatory role of heme oxygenase on the cyclooxygenase pathway. *FASEB J.,* **13** (Part II), A781.

HARTSFIELD, C.L., ALAM, J., COOK, J.L. & CHOI, A.M.K. (1997). Regulation of heme oxygenase-1 gene expression in vascular smooth muscle cells by nitric oxide. *Am. J. Physiol.,* **273,** L980 – L988.

HERSCHMAN, H., GILBERT, R., REDDY, S. & XIE, W. (1997). Coordinate regulation of the inducible forms of prostaglandin synthase and nitric oxide synthase in fibroblasts and macrophages. In *Eicosanoids and other bioactive lipids in cancer, inflammation, and radiation injury*. ed. Honn, K.V., pp. 177 – 182. New York: Plenum Press.

HLA, T. & NEILSON, K. (1992). Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 7384 – 7388.

KELNER, M.J. & UGLIK, S.F. (1994). Mechanism of prostaglandin E_2 release and increase in PGH_2/PGE_2 isomerase activity by PDGF: involvement of nitric oxide. *Arch. Biochem. Biophys.,* **312,** 240 – 243.

KIM, Y.M., BERGONIA, H.A., MÜLLER, C., PITT, B.R., WATKINS, W.D. & LANCASTER, J.R.J. (1995). Loss and degradation of enzyme-bound heme induced by cellular nitric oxide synthesis. *J. Biol. Chem.,* **270,** 5710 – 5713.

KNOWLES, R.G. & MONCADA, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.,* **298,** 249–258.

KRÖNCKE, K.-D., FEHSEL, K. & KOLB-BACHOFEN, V. (1997). Nitric oxide: cytotoxicity versus cytoprotection–How, why, when and where? *Nitric Oxide: Biol. Chem.,* **1,** 107–120.

KUJUBU, D.A., REDDY, S.T., FLETCHER, B.S. & HERSCHMAN, H.R. (1993). Expression of the protein product of the prostaglandin synthase-2/TIS10 gene in mitogen stimulated Swiss 3T3 cells. *J. Biol. Chem.,* **268,** 5425–5430.

KURATA, S.-I., MATSUMOTO, M., TSUJI, Y. & NAKAJIMA, H. (1996). Lipopolysaccharide activates transcription of the heme oxygenase gene in mouse M1 cells through oxidative activation of nuclear factor kB. *Eur. J. Biochem.,* **239,** 566–571.

LANIADO-SCHWARTZMAN, M., ABRAHAM, N.G., CONNERS, M., DUNN, M.W., LEVERE, R.D. & KAPPAS, A. (1997). Heme oxygenase induction with attenuation of experimentally induced corneal inflammation. *Biochem. Pharmacol.,* **53,** 1069–1075.

MCCOUBREY, W.K., HUAG, T.J. & MAINES, M.D. (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur. J. Biochem.,* **247,** 725–732.

MEADE, E.A., MCINTYRE, T.M., ZIMMERMAN, G.A. & PRESCOTT, S.M. (1999). Peroxisome proliferators enhance cyclooxygenase-2 expression in epithelial cells. *J. Biol. Chem.,* **274,** 8328–8334.

MONCADA, S. & HIGGS, A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.,* **329,** 2002–2012.

MOSLEY, K., WEMBRIDGE, D.E., CATTELL, V. & COOK, H.T. (1998). Heme oxygenase is induced in nephrotoxic nephritis and hemin, a stimulator of heme oxygenase synthesis, ameliorates disease. *Kidney Int.,* **53,** 672–678.

MOTTERLINI, R., GONZALES, A., FORESTI, R., CLARK, J.E., GREEN, C.J. & WINSLOW, R.M. (1998). Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. *Circ. Res.,* **83,** 568–577.

NAKAGAMI, T., TOYOMURA, K., KINOSHITA, T. & MORISAWA, S. (1993). A beneficial role of bile pigments as an endogenous tissue protector: anti-complement effects of biliverdin and conjugated bilirubin. *Biochim. Biophys. Acta,* **1158,** 189–193.

NUMAZAWA, S., YAMADA, H., FURUSHO, A., NAKAHARA, T., OGURO, T. & YOSHIDA, T. (1997). Cooperative induction of c-fos and heme oxygenase gene products under oxidative stress in human fibroblastic cells. *Exp. Cell Res.,* **237,** 434–444.

OTTERBEIN, L.E., KOLLS, J.K., MANTELL, L.L., COOK, J.L., ALAM, J. & CHOI, A.M. (1999). Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. *J. Clin. Invest.,* **103,** 1047–1054.

POSS, K.D. & TONEGAWA, S. (1997a). Heme oxygenase 1 is required for mammalian iron reutilization. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 10919–10924.

POSS, K.D. & TONEGAWA, S. (1997b). Reduced stress defense in heme oxygenase 1-deficient cells. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 10925–10930.

PRADELLES, P., GRASSI, J. & MACLOUF, J. (1985). Enzyme immunoassay of eicosanoids using acetylcholine esterase as label: an alternative to radioimmunoassay. *Anal. Chem.,* **57,** 1170–1173.

REDDY, S.T. & HERSCHMAN, H.R. (1994). Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. *J. Biol. Chem.,* **269,** 15473–15480.

SEIBERT, K., ZHANG, Y., LEAHY, K., HAUSER, S., MASFERRER, J., PERKINS, W., LEE, L. & ISAKSON, P. (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 12013–12017.

SHIBAHARA, S., MULLER, R.M. & TAGUCHI, H. (1987). Transcriptional control of rat heme oxygenase by heat shock. *J. Biol. Chem.,* **262,** 12889–12892.

SOARES, M.P., LIN,Y., ANRATHER, J., CSIZMADIA, E., TAKIGAMI, K., SATO, K., GREY, S.T., COLVIN, R.B., CHOI, A.M., POSS, K.D. & BACH, F.H. (1998). Expression of heme oxygenase-1 can determine cardiac xenograft survival. *Nature Med.,* **4,** 1073–1077.

STEINHAUSER, M.L., KUNKEL, S.L., HOGABOAM, C.M., EVANOFF, H., STRIETER, R.M. & LUKACS, N.W. (1998). Macrophage/fibroblast coculture induces macrophage inflammatory protein-1alpha production mediated by intercellular adhesion molecule-1 and oxygen radicals. *J. Leukoc. Biol.,* **64,** 636–641.

STOCKER, R. & PETERHANS, E. (1989). Antioxidant properties of conjugated bilirubin and biliverdin: biologically relevant scavenging of hypochlorous acid. *Free Rad. Res. Comms.,* **6,** 57–66.

TERRY, C.M., CLIKEMAN, J.A., HOIDAL, J.R. & CALLAHAN, K.S. (1998). Effect of tumor necrosis factor-α and interleukin-1α on heme oxygenase-1 expression in human endothelial cells. *Am. J. Physiol.,* **274,** H883–H891.

TETSUKA, T., DAPHNA-IKEN, D., SRIVASTAVA, S.K. & MORRISON, A.R. (1995). Regulation of heme oxygenase mRNA in mesangial cells: prostaglandin E_2 negatively modulates interleukin-1 induced heme oxygenase-1 mRNA. *Biochem. Biophys. Res. Commun.,* **212,** 617–623.

WAGENER, F.A.D.T.G., FELDMAN, E., DE WITTE, T. & ABRAHAM, N.G. (1997). Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells. *Proc. Soc. Exp. Biol. Med.,* **216,** 456–463.

WILLIS, D., MOORE, A.R., FREDERICK, R. & WILLOUGHBY, D.A. (1996). Heme oxygenase: a novel target for the modulation of the inflammatory response. *Nature Med.,* **2,** 87–90.

WILLIS, D., TOMLINSON, A., FREDERICK, R., PAUL, C.M. & WILLOUGHBY, D.A. (1995). Modulation of heme oxygenase activity in rat brain and spleen by inhibitors and donors of nitric oxide. *Biochem. Biophys. Res. Commun.,* **214,** 1152–1156.

ZHANG, S.Z., LIPSKI, M.M., TRUMP, B.F. & HSU, I.C. (1990). Neutral red (NR) assay for cell viability and xenobiotic-induced cytotoxicity in primary cultures of human and rat hepatocytes. *Cell Biol. Toxicol.,* **6,** 219–234.

(Received November 22, 1999
Accepted February 9, 2000)