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INDUCTION OF HAEM OXYGENASE CONTRIBUTES TO THE SYNTHESIS OF PRO-INFLAMMATORY CYTOKINES IN RE-OXYGENATED RAT MACROPHAGES: ROLE OF cGMP

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Macrophage activation and the resulting inflammatory response may be a major component of tissue injury upon hypoxia and re-oxygenation. Activation of the haem oxygenase (HO)/carbon monoxide (CO) pathway may be an important regulator of the inflammatory response, through production of cyclic 3′, 5′-monophosphate (cGMP). We have assessed whether HO contributes to the increased production of the pro-inflammatory cytokines TNF-α and IL-6 in re-oxygenated rat peritoneal macrophages.

Hypoxia/re-oxygenation markedly increased levels of HO-1 mRNA and cGMP. The increase in cGMP was reduced by the HO-1 inhibitor tin-protoporphyrin (SnPP-9) given during re-oxygenation. Hypoxia and re-oxygenation also increased IL-6 and TNF-α mRNA expression, as well as IL-6 and TNF-α concentrations in the cell supernatant. These increases were nullified by SnPP-9 and by Methylene Blue, an inhibitor of guanylate cyclase, but were not affected by L-NNA, an inhibitor of NO synthesis. The inhibitory effect of SnPP on the synthesis of cytokines was reversed by co-administration of the stable analogue of cGMP, 8-Br-cGMP.

Our results indicate that activation of haem oxygenase and of the CO/cGMP pathway is a major stimulus for the synthesis and release of pro-inflammatory cytokines in re-oxygenated macrophages. This pathway may play a central role in pathological situations in which local tissue hypoxia/re-oxygenation triggers a systemic inflammatory response, for example in patients with shock.

Tissue injury induced by hypoxia/re-oxygenation shares many features with an inflammatory response. Indeed, post-hypoxic re-oxygenation is associated with an increased expression of inflammatory mediators, and especially of cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6) and interleukin 8 (IL-8).[1–4] Tissue hypoxia/re-oxygenation may also be a major trigger for the synthesis of cytokines and the systemic inflammatory response associated with pathophysiological conditions such as haemorrhage.[5–8]

Although many cell types may contribute to the inflammatory response in hypoxia/re-oxygenation, activated macrophages may play a central role in this response, especially in terms of the production of inflammatory cytokines. Indeed, re-oxygenation markedly increases the synthesis of IL-1 in macrophages.[3] However, the mechanisms by which hypoxia/re-oxygenation leads to the production of pro-inflammatory cytokines in macrophages is not entirely clear.

Haem oxygenase (HO) is the initial and rate-limiting enzyme in haem metabolism.[9] This enzyme catalyzes the degradation of haem into biliverdin, iron and carbon monoxide (CO).[10] At least two isoforms of HO exist: HO-2 is constitutively expressed,[11] while HO-1 is inducible by a large number of agents and by a variety of stress conditions, such as oxidative stress

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and tissue ischaemia/reperfusion.[12–15] Induction of HO-1 enables removal of the potentially toxic molecule haem, as well as generation of biliverdin and bilirubin, which possess antioxidant properties.[16] Moreover, HO generates CO, which shares many properties with nitric oxide (NO). CO stimulates guanylate cyclase and increases cyclic guanosine monophosphate (cGMP) in normoxic[17] or hypoxic conditions.[18] Thus, it is becoming clear that the CO–cGMP pathway may be of major importance in the regulation of cell communication, for example in the vascular wall,[19] but also as a biological signal in the immune system.[20]

cGMP has been shown to act as an important intracellular messenger for macrophage activation, especially in terms of the production of tumor necrosis factor-alpha (TNF-α).[21] Thus, it is possible that increased cGMP levels from activation of haem oxygenase may be also a trigger for the production of pro-inflammatory cytokines in re-oxygenated macrophages.

We designed the present study to assess whether HO-1 induction by hypoxia/re-oxygenation may play a role in the regulation of TNF-α and IL-6 production by rat macrophages.

RESULTS

HO-1 mRNA production by macrophages subjected to hypoxialre-oxygenation conditions

Macrophages kept in normoxic medium showed a low expression of HO-1 (lanes, 2, 4, 6 and 8 in Fig. 1). However, after 6 h of exposure to hypoxia followed by exposition to re-oxygenation for varying periods of time, there was a marked increase in HO-1 expression. Levels of HO-1 mRNA started to increase within 1 h, and reached a maximum at 6–9 h of re-oxygenation (lanes 1, 3, 5 and 7 in Fig. 1). Levels of HO-1 mRNA after 6 h of exposure to hypoxia without re-oxygenation showed no significant difference with macrophages in normoxic medium (data not shown).

Intracellular cGMP levels after hypoxia and re-oxygenation

Compared with cells maintained in normoxia, hypoxia followed by re-oxygenation was associated with a significant increase in cGMP, with a maximum effect at 6 h. The time course of the increase in cGMP was similar to that observed with HO-1. The increase in cGMP after hypoxia and re-oxygenation was nullified in the presence of the HO-1 inhibitor (SnPP-9), and this was apparent at all the time points studied (Fig. 2).

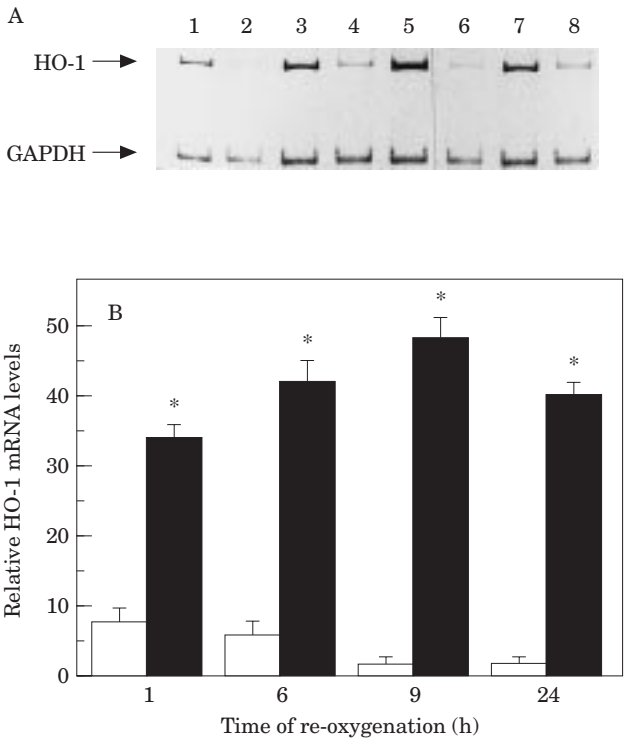

Figure 1. Effect of hypoxia/re-oxygenation on the HO-1 mRNA expression.

(A) Total RNA was extracted from peritoneal macrophages subjected to 6 h hypoxia, followed by 1 (lane 1), 6 (lane 3), 9 (lane 5) or 24 h (lane 7) of reoxygenation, and RT-PCR analysis was performed. Lanes 2, 4, 6 and 8 represent the time-matched normoxic controls.

Relative mRNA levels normalized to GAPDH mRNA derived from densitometric analysis of amounts of HO-1 transcripts expressed under reoxygenation, compared with mRNA from time-matched normoxic controls. Data are mean ± SE of three independent experiments. *$P<0.05$ vs normoxia.

Effects of hypoxialre-oxygenation on IL-6 and TNF-α mRNA production by macrophages

IL-6 mRNA increased four-fold ($P<0.05$) after 1 h of re-oxygenation and remained high until 24 h (lanes 2–5, in Fig. 3). This increase in IL-6 expression was almost eliminated by the HO-1 inhibitor SnPP-9 (1 mM) or by Methylene Blue (MB; 10 μM) (lanes 7–10 in Fig. 3).

The TNF-α mRNA level reached a maximum at 6 h following re-oxygenation and then decreased until 24 h had elapsed (Fig. 4) at all time points studied. In the case of IL-6, treatment with SnPP-9 or MB during re-oxygenation almost eliminated the increase in TNF-α mRNA expression, except for the 1 h test in the case of Methylene Blue (Fig. 4).

In order to evaluate whether cGMP was involved in the regulation of expression of TNF-α and IL-6 mRNA, the cGMP analogue (8-Br-cGMP) was added to the macrophages together with HO-1 inhibitor (SnPP-9). In cells cultured in normoxic conditions,

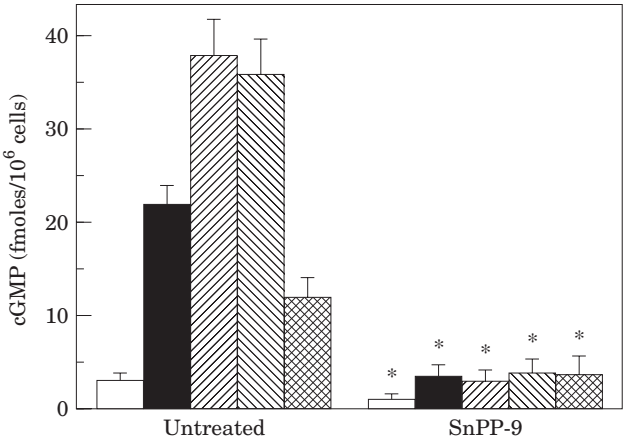

Figure 2. Hypoxia followed by re-oxygenation increases cGMP in peritoneal macrophages.

Peritoneal macrophages were maintained in normoxia (□) or subjected to 6 h hypoxia (H), followed by 1 (■), 6 (▨), 9 (▧) or 24 (▩) h re-oxygenation (R), in the absence (untreated) or in the presence of tin-protoporphyrin (SnPP-9) 1mM. Data are mean ± SE of three experiments performed in duplicate. *P<0.05 vs hypoxia/re-oxygenation without SnPP.

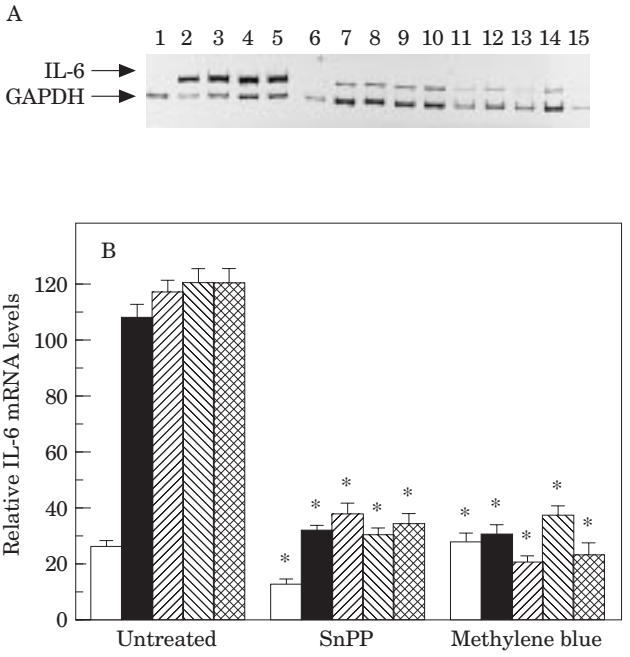

Figure 3. Effect of the HO-1 inhibitor: SnPP-9 or the guanylate cyclase inhibitor: Methylene Blue on IL-6mRNA expression in peritoneal macrophages.

(A) Lane 1: macrophages maintained in normoxic conditions; lane 2–5: macrophages subjected to 6 h of hypoxia followed by 1, 6, 9 or 24 h re-oxygenation, respectively). Lanes 6–10: macrophages treated with SnPP-9 (1mM) (Lane 6: normoxic conditions; lanes 7–10: hypoxia followed by 1, 6, 9 or 24 h re-oxygenation, respectively). Lanes 11–15: macrophages treated with Methylene Blue (10 μM (lane 11: normoxic conditions; lanes 12–15 hypoxia followed by 1, 6, 9 or 24 h re-oxygenation, respectively). RT-PCR analysis was performed as indicated in Material and Methods.

(B) Relative mRNA levels normalized to GAPDH mRNA derived from densitometric analysis of amounts of IL-6 transcripts in peritoneal macrophages maintained in normoxia (□) or subjected to 6 h hypoxia (H), followed by 1 (■), 6 (▨), 9 (▧) or 24 (▩) h re-oxygenation (R), in the absence (untreated) or in the presence of SnPP or Methylene Blue. Data are mean ± SE of three independent experiments. *P<0.05 vs untreated.

8-Br-cGMP increased IL-6 and TNF-α mRNA levels to an extent similar to that observed following hypoxia/re-oxygenation (lane 1 and 6, in Figs 5A, 5B). Moreover, addition of 8-Br-cGMP in cells treated with SnPP-9 (lanes 7–10 in Figs 5A, 5B) increased IL-6 and TNF-α mRNA levels to the same extent as that in cells subjected to hypoxia/re-oxygenation (lanes 2–5 in Fig. 5A, 5B).

TNF-α and IL-6 mRNA are not induced by NO in macrophages subjected to hypoxia/re-oxygenation

In order to assess whether NO was able to regulate the mRNA synthesis of TNF-α and IL-6 following hypoxia/re-oxygenation, macrophages were exposed to hypoxia for 6 h and submitted to re-oxygenation for varying periods of time in the absence or presence of 2.5 mM L-NNA, an inhibitor of NO synthesis. As shown in Figure 6, mRNA for IL-6 and TNF-α remained elevated even in the presence of L-NNA which suggested that NO was not involved as a transmitter of the signal of TNF-α and IL-6 synthesis.

Effects of cGMP analogue and inhibitors of HO-1 and NOS on TNF-α and IL-6 production in the supernatant of macrophages subjected to hypoxia/re-oxygenation

The TNF-α and IL-6 concentrations were measured in the supernatant of the peritoneal macrophages subjected to hypoxia/re-oxygenation. The level of TNF-α was maximal at 9 h and had decreased at 24 h, whereas the IL-6 concentration was highest 24 h after re-oxygenation (Figs 7 and 8). As with mRNA expression, pre-treatment of cells prior to re-oxygenation with SnPP-9 or MB nullified the increase in TNF-α and IL-6 concentrations (Figs 7 and 8). In cells under normoxic conditions, 8-Br-cGMP increased IL-6 and TNF-α concentrations to an extent similar to that of hypoxia/re-oxygenation. Moreover, 8-Br-cGMP fully reversed the inhibitory effect of SnPP in cells subjected to hypoxia/re-oxygenation (Figs 7 and 8).

Addition of L-NNA to the cultures did not affect the production of either TNF-α or IL-6; this finding was comparable to that obtained in media of cells subjected to hypoxia/re-oxygenation only (Figs 7 and 8).

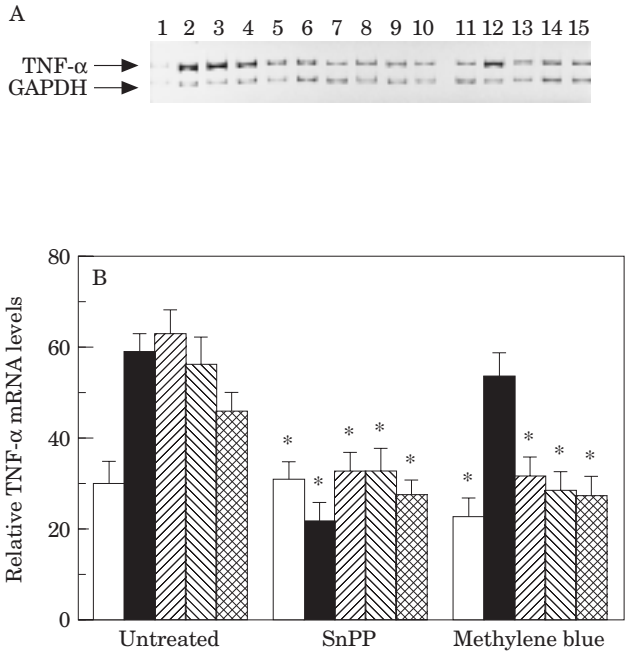

Figure 4. Effect of the HO-1 inhibitor: SnPP-9 or the guanylate cyclase inhibitor: Methylene Blue on TNF-α mRNA expression in peritoneal macrophages.

A. Lanes 1–5: lane 1, macrophages maintained in normoxic conditions; lanes 2–5, macrophages subjected to 6 h of hypoxia followed by 1, 6, 9 or 24 h of re-oxygenation, respectively. Lanes 6–10, macrophages treated with SnPP-9 (1 mM): (lane 6, normoxic conditions; lanes 7–10, hypoxia followed by 1, 6, 9 or 24 h of re-oxygenation, respectively. Lanes 11–15, macrophages treated with Methylene Blue (10 μM): lane 11, normoxic conditions; lanes 12–15, hypoxia followed by 1, 6, 9 or 24 h of re-oxygenation, respectively. RT-PCR analysis was performed as indicated in Material and Methods. B. Relative mRNA levels normalized to GAPDH mRNA derived from densitometric analysis of amounts of TNF-α transcripts in peritoneal macrophages maintained in normoxia (□) or subjected to 6 h of hypoxia (H), followed by 1 (■), 6 (▨), 9 (▧) or 24 (▩) h of re-oxygenation (R), in the absence (untreated) or in the presence of SnPP or Methylene Blue. Data are mean ± SE of three independent experiments. *$P<0.05$ vs untreated.

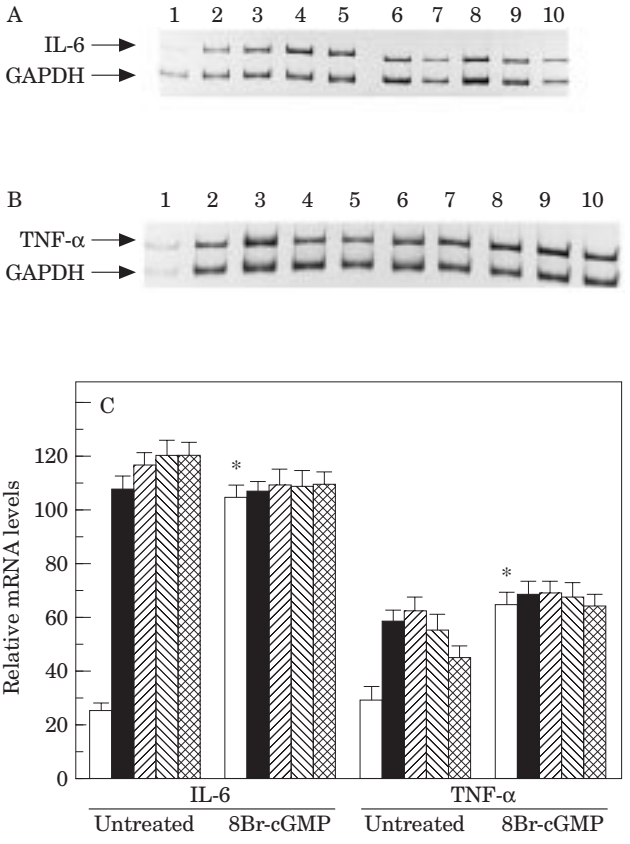

Figure 5. Effect of 8Br-cGMP on IL-6 and TNF-α mRNA expression in peritoneal macrophages.

A. IL-6 mRNA levels analysed by RT-PCR. Lanes 1–5, untreated: Lane 1, normoxic conditions; lanes 2–5, hypoxia followed by 1, 6, 9 or 24 h of re-oxygenation, respectively. Lanes 6–10, macrophages treated with SnPP-9 (1 mM) with addition of 8Br-cGMP (1 mM): lane 6, normoxia; lanes 7–10 hypoxia followed by 1, 6, 9 or 24 h of re-oxygenation, respectively. B. TNF-α mRNA levels analysed by RT-PCR. Lanes 1–5 untreated: lane 1, normoxic conditions; lanes 2–5, hypoxia followed by 1, 6, 9 or 24 h of re-oxygenation, respectively. Lanes 6–10, macrophages treated with SnPP-9 (1 mM) with addition of 8Br-cGMP (1 mM): lane 6, normoxic conditions; lanes 7–10, hypoxia followed by 1, 6, 9 or 24 h of re-oxygenation, respectively. C. Relative mRNA levels normalized to GAPDH mRNA derived from densitometric analysis of amounts of IL-6 and TNF-α transcripts in peritoneal macrophages maintained in normoxia (□) or subjected to 6 h of hypoxia (H), followed by 1 (■), 6 (▨), 9 (▧) or 24 (▩) h of re-oxygenation (R), in the absence (untreated) or in the presence of SnPP-9 with 8Br-cGMP. Data are mean ± SE of three independent experiments. *$P<0.05$ vs untreated.

DISCUSSION

The present in vitro study has shown that the induction of HO-1 and the resulting activation of the CO/cGMP pathway plays a central role in the synthesis and release of pro-inflammatory cytokines in peritoneal macrophages exposed to hypoxia and then to re-oxygenation. This constitutes a new mechanism by which macrophages respond to hypoxia through an increased inflammatory response.

It has been clearly demonstrated that re-oxygenation could stimulate macrophages to release several pro-inflammatory cytokines.[3] Although there is evidence that this increase in cytokines synthesis is mediated by oxygen-derived free radicals,[3,23] the mechanisms of activation or regulation of cytokines synthesis in this situation are still incompletely understood. Since recent studies showed that HO-1 activity was induced in various tissues following hypoxia/re-oxygenation,[11–14] we investigated the functional role of HO-1 in controlling the synthesis of TNF-α and IL-6 by peritoneal macrophages following hypoxia/re-oxygenation in vitro.

In our study, we observed high levels of mRNA expression for HO-1 in peritoneal macrophages after hypoxia and re-oxygenation, these levels may be secondary to an increased oxidative stress upon re-oxygenation. Yamaguchi *et al.* have shown that the

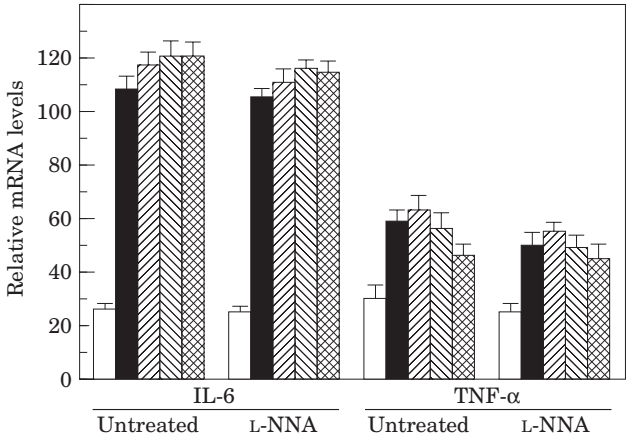

Figure 6. Effect of N^G-nitro L-arginine (L-NNA) as an inhibitor of nitric oxide synthesis on IL-6 and TNF-α mRNA expression in peritoneal macrophages.

Relative mRNA levels were normalized to GAPDH mRNA derived from densitometric analysis of amounts of IL-6 and TNF-α transcripts in peritoneal macrophages maintained in normoxia (□) or subjected to 6 h of hypoxia (H), followed by 1 (■), 6 (▨), 9 (▧) or 24 (▩) h of re-oxygenation (R), in the absence (untreated) or in the presence of L-NNA (2.5 mM). Data are mean ± SE of three independent experiments. *$P<0.05$ vs untreated.

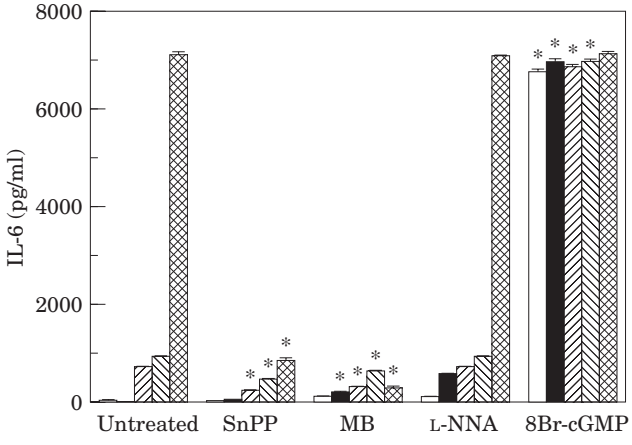

Figure 7. Effect of 8Br-cGMP and of inhibitors of HO-1, NOS on IL-6 production in media of macrophages subjected to hypoxia/re-oxygenation.

Concentrations of IL-6 were analysed in the supernatant of peritoneal macrophages maintained in normoxia (□) or subjected to 6 h of hypoxia (H), followed by 1 (■), 6 (▨), 9 (▧) or 24 (▩) h of re-oxygenation (R), in the absence (untreated) or in the presence of SnPP-9, Methylene Blue, L-NNA or SnPP-9 with 8Br-cGMP. Data are mean ± SE of three independent experiments. *$P<0.05$ vs untreated.

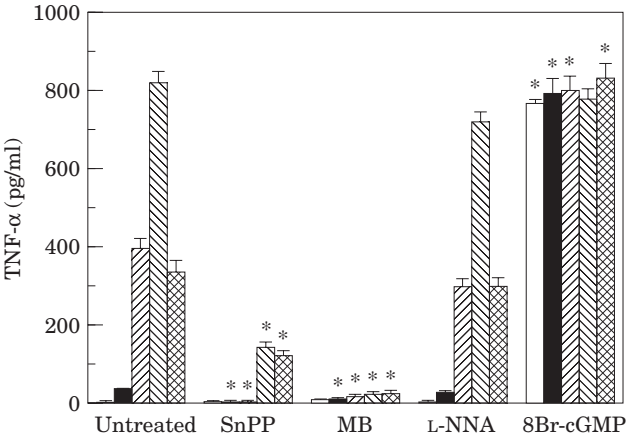

Figure 8. Effect of 8Br-cGMP and of inhibitors of HO-1, NOS on TNF-α production in media of macrophages subjected to hypoxia/re-oxygenation.

Concentrations of TNF-α were analysed in the supernatant of peritoneal macrophages maintained in normoxia (□) or subjected to 6 h of hypoxia (H), followed by 1 (■), 6 (▨), 9 (▧) or 24 (▩) h of re-oxygenation (R), in the absence (untreated) or in the presence of SnPP, Methylene Blue, L-NNA or SnPP-9 with 8Br-cGMP. Data are mean ± SE of three independent experiments. *$P<0.05$ vs untreated.

HO-1 activity was also increased after ischaemia/reperfusion treatment following the increase in HO-1 mRNA.[24] Evidence that HO-1 induction plays a key role in the synthesis of TNF-α and IL-6 is supported by the following observations: first, HO-1 induction by hypoxia/re-oxygenation was correlated with the ability of peritoneal macrophages to release TNF-α and IL-6; second, the selective inhibitor of HO-1: SnPP virtually eliminated the TNF-α and IL-6 gene expression, as well as the respective increase of these cytokines in media of cultured macrophages. Moreover, evidence that this HO-1-induced expression of cytokines is mediated by activation of guanylate cyclase and increase in intracellular cGMP is supported by the following observations: first, the inhibitor of guanylate cyclase, Methylene Blue, inhibited the re-oxygenation-induced increase in cytokines synthesis to an extent similar to that of SnPP-9; second, the inhibitory effect of SnPP-9 on the synthesis of cytokines was reversed by co-treatment with 8-Br-GMPc, a stable analogue of cGMP. The fact that cGMP regulates TNF-α and IL-6 synthesis is consistent with previous experiments. cGMP may well be a trigger for the increased production of TNF-α in activated macrophages,[20] and treatment of cells with cGMP analogues induces *c-fos* mRNA expression, which is an important transcriptional factor for IL-6 gene expression.[25] In addition, recent studies have indicated that human pulmonary macrophages cultured in the presence of lipopolysaccharide showed an increase in intracellular cGMP and were able to release TNF-α.[26] Taken together, these studies and the present work demonstrate that cGMP is an important regulator of TNF-α and IL-6 gene expression.

In theory, several metabolites of haem oxygenase could contribute to the observed increase in pro-inflammatory cytokine synthesis, including bilirubin, biliverbin and CO. However, bilirubin possesses anti-oxidant properties,[15,24] and thus should tend to decrease cytokine synthesis, since such synthesis is

activated by the oxidative stress.[3] Otterbein *et al*. have demonstrated that HO-1 may help increase antioxidant defences by increasing the production of bilirubin.[27] Since CO is a potent activator of guanylate cyclase,[28,29] the fact that the HO-1-induced synthesis of cytokines involves the guanylate cyclase pathway strongly suggests that CO is indeed the metabolite of HO-1 responsible for this effect.

Although the major source of CO arises from the degradation of haem by haem oxygenase, CO may also be produced from other sources, such as lipid peroxidation.[30] Thus, since lipid peroxidation does occur upon re-oxygenation mainly because of the production of oxygen-derived free radicals, it is possible that CO produced upon re-oxygenation may be derived both from haem oxygenase and from lipid peroxidation. However, the marked inhibitory effect of SnPP-9, as observed in our experiments, suggests that most of the CO-induced stimulation of cytokines synthesis derives from haem oxygenase.

The characteristics and mechanisms of guanylate cyclase activation by CO resemble those by NO. In our experiments, however, we found no effect of an inhibitor of NO synthesis on TNF-α and IL-6 synthesis. Different results were obtained in models of LPS-induced TNF-α production, in which NO tends to augment the production of inflammatory mediators including TNF-α, although the mechanism of this effect does not seem to be dependent on cGMP.[31,32] One possible explanation is that the induction of type II NO synthase is less marked in situations of hypoxia/re-oxygenation than after LPS, and thus that the respective contributions of CO and NO to the overall activation of guanylate cyclase is different in the two situations.

In conclusion, our study demonstrates for the first time that activation of haem oxygenase, and the resulting activation of the CO/cGMP pathway, is an important element of the inflammatory response which accompanies hypoxia and re-oxygenation in macrophages. This haem oxygenase pathway may play a central role in pathological situations in which local tissue hypoxia/re-oxygenation triggers a systemic inflammatory response, for example during shock with gut ischaemia.

MATERIALS AND METHODS

Animals

Adult male Wistar rats, each weighing 300–320 g, were kept at 25°C with 12 h light/dark cycles. Animals were fasted 16–20 h before experimentation and allowed water *ad libitum*.

Preparation of peritoneal macrophages

Peritoneal macrophages were harvested by five consecutive washes of the peritoneal cavity with 10 ml of ice-cold medium containing 5% fetal calf serum, 100 U/ml penicillin and 1% glutamine. Cells were washed once with RPMI medium and centrifuged at $280 \times g$ for 15 min at 4°C. The cell pellet was resuspended in RPMI 1640 medium (5% fetal calf serum, 100 U/ml penicillin G, 50 µg/ml gentamicin, 1% glutamine) and placed into 25-cm^2 culture flasks (20×10^6 cells/flask). After 12 h of incubation (37°C, 5% CO_2), the supernatant was removed to eliminate contaminating non-adherent cells. Macrophages were then immersed in 4 ml of freshly prepared medium. Adherent cells were found to be >99% viable (Trypan Blue exclusion test).

Cellular RNA extraction

Total RNA was extracted from peritoneal macrophages by a one-step method. Briefly, cells were lysed with a denaturing solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and 100 mM 2-mercaptoethanol. Total cellular RNA was obtained by using acid guanidium thiocyanate–phenol–chloroform extraction. Total RNA concentration was measured by spectrophotometry (absorbance at 260 and 280 nm). The RNA preparations were controlled by minigel agarose electrophoresis with visualization of the 18S and 28S ribosomal RNA bands after ethidium bromide staining.

Measurement of cytokine gene expression by quantitative RT-PCR

Two mg of total RNA were reverse transcribed in 30 µl (final volume) of a reaction buffer made with 30 mM Tris HCl pH 8.3, 10 mM DTT, 85 mM KCl, 3 mM $MgCl_2$, 10 mM of each of dNTP, 60 U RNasin (Promega, Charbonnières, France), 400 U reverse transcriptase (from murine Moloney virus) and 8.3 pmol random hexamer primer. The mixture was incubated for 60 min at 37°C and then heated at 95°C for 5 minutes. 10-µl aliquots of the RT product were used for the PCF amplification. They were brought to a volume of 50 µl using a PCR mix of 10 µl of 10 × PCR buffer, 10 µl dNTPs (2 mM each), 10 µl $MgCl_2$ 2.25 mM, 1.25 U DNA Taq polymerase and 10 µl of 3′ and 5′-DNA specific primers (2 mM each). Primers sequences were chosen from separate exons of the rat genes so that the RNA-associated PCR product could readily be distinguished from any PCR products induced by contaminating genomic DNA. Primers were chosen to have a GC content of 40–60%. The primer sequences and the sizes of the expected PCF products were:

Gene	Primer sequences (5′→3′)	Size (bp)
IL-6	Upper AGCCAGAGTCATTCAGAGCA	325
	Lower AGTTCTTAGAGAACAACATA	
TNF-α	Upper GTAGCCCCACGTCGTAGCAAA	346
	Lower CCCTTCTCCAGCTGGAAGAC	
HO-1	Upper GAATTCAGCATGCCCCAGGAT	615
	Lower TCTAGACTAGCTGGATGTTGA	
GAPDH	Upper CAGAGCAAGAGAGGCATCCT	535
	Lower GGCAGCTCATAGCTCCTTCTC	

Each PCR cycle consisted of a heat denaturation step at 94°C for 4 min, a primer annealing step at 56°C for 30 s and a polymerization step at 72°C for 1 min in an Omnigene thermal cycler (Hybaid, Seralabo, France). The number of PCR cycles was 20 for IL-6 and 24 for TNF-α. The GAPDH gene was used as an internal control for standardization of PCR product. PCR products were electrophoresed through 7.5% polyacrylamide gel. Molecular weight markers were from Pharmacia. Gels were deionized water containing 0.5 mg/ml ethidium bromide, illuminated with UV light and photographed using Polaroid films. They were measured by quantitative scanning densitometry of autoradiographs (Biocom densitometer scientific instrument, Les Ulis, France).

Measurement of cytokine production

At the end of experiments, culture supernatants were centrifuged (20 min at $2000 \times g$), filtered under sterile conditions and stored at −80°C before use.

TNF-α was measured by enzyme immunoassay (Biosource International), with a detection threshold of <0.7 pg/ml. IL-6 activity was determined spectrophotometrically by assessing the 72 h proliferation of the IL-6-dependent murine hybridoma cell line B-9, using serial dilutions of supernatants in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were incubated at 35°C for an additional 4 h period. The supernatants were removed and 100 µl isopropanol–HCl was added to cells that were incubated overnight in the dark at room temperature. The absorbance was read at 550 nm. A standard curve with 200 units/ml human recombinant IL-6 was prepared by plotting. The absorbance values versus the logarithms of the concentration of the standards. Unknown values were determined by comparison with the standard curve.

Measurement of cGMP

cGMP was extracted from the cells by rapid aspiration of the medium, followed by washing of the cells with ice-cold phosphate-buffered saline, and by addition of 65% (V/V) ice-cold ethanol. The cells were harvested and centrifuged at $2000 \times g$ for 5 min at 4°C. Supernatants were evaporated at 60°C in a vacuum oven until completely dry and stored at −80°C until assay. The cellular cGMP content was measured by enzyme immunoassay (Biotrak, Amersham International PLC).

Experimental protocol

After 12 h of culture in normoxic medium (RPMI 1640), macrophages were subjected to 6 h of hypoxia followed by for 1 h, 6, 9 and 24 h of re-oxygenation.[3] Hypoxia was induced by placing the cells at 37°C in an anaerobic chamber (Forma Scientific). This leads to a >99% decrease in pO_2 (pO_2: normoxia, 95 Torr; hypoxia, <0.5 Torr). Re-oxygenation was achieved by replacing the hypoxic medium by an oxygenated medium and by returning macrophages in the normal cell incubator. Controls consisted in macrophages incubated in RPMI 1640 in normoxic conditions.

To examine the role of CO or NO in modulating macrophage cGMP levels, the following reagents were used: tin-protoporphyrin (SnPP-9), an inhibitor of HO-1 and CO synthesis (1 mM),[22] N^G-nitro L-Arginine (L-NNA), an inhibitor of NO synthesis (2.5 mM),[22] Methylene Blue (MB), an inhibitor of guanylate cyclase (10 µM)[20] and 8-Br-cGMP, a stable analogue of cGMP (1 mM).[25,26] Reagents were added at the time of re-oxygenation and remained present in the medium throughout the period of re-oxygenation. These agents have no effect on cell viability because adherent cells were found to be 99% viable (Trypan Blue exclusion test).

Reagents

SnPP-9 was purchased from Porphyrin Products (Logan UT), L-NNA and MB were obtained from Sigma Chimie, La Verpillère, France and 8-Br-cGMP from Boehringer Mannheim. RNasin and Taq polymerase were obtained from Promega, Charbonnières, France. Reverse transcriptase was obtained from Gibco BRL, Cergy-Pontoise, France. Random hexamer primers were obtained from Pharmacia, Saint Quentin en Yvelines, France. The PCR primers were obtained from Bioprobe Systems, Montreuil sous Bois, France.

Data analysis

Data are presented as means ± SE. Results were compared using the Student's *t*-test, or ANOVA when appropriate, and a P value less than 0.05 was considered statistically significant.

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