

Original Contribution

Carbon monoxide blocks oxidative stress-induced hepatocyte apoptosis via inhibition of the p54 JNK isoform

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Abstract

Most chronic liver diseases are accompanied by oxidative stress, which may induce apoptosis in hepatocytes and liver injury. Oxidative stress induces heme oxygenase-1 (HO-1) expression. This stress-responsive cytoprotective protein is responsible for heme degradation into carbon monoxide (CO), free iron, and biliverdin. CO is an important intracellular messenger; however, the exact mechanisms responsible for its cytoprotective effect are not yet elucidated. Thus, we investigated whether HO-1 and CO protect primary hepatocytes against oxidative-stress-induced apoptosis. *In vivo*, bile duct ligation was used as model of chronic liver disease. *In vitro*, primary hepatocytes were exposed to the superoxide anion donor menadione in a normal and in a CO-containing atmosphere. Apoptosis was determined by measuring caspase-9, -6, -3 activity and poly(ADP-ribose) polymerase cleavage, and necrosis was determined by Sytox green staining. The results showed that (1) HO-1 is induced in chronic cholestatic liver disease, (2) superoxide anions time- and dose-dependently induce HO-1 activity, (3) HO-1 overexpression inhibits superoxide-anions-induced apoptosis, and (4) CO blocks superoxide-anions-induced JNK phosphorylation and caspase-9, -6, -3 activation and abolishes apoptosis but does not increase necrosis. We conclude that HO-1 and CO protect primary hepatocytes against superoxide-anions-induced apoptosis partially via inhibition of JNK activity. CO could represent an important candidate for the treatment of liver diseases.

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Keywords: NASH; Oxidative stress; Caspase; Apoptosis; Necrosis; Heme oxygenase-1; Carbon monoxide; MAPK; JNK

Introduction

Chronic liver diseases, such as non alcoholic steatohepatitis, chronic cholestasis, and alcoholic and chronic viral hepatitis are

almost invariably accompanied by exposure to reactive oxygen species (ROS) [1–3]. ROS can contribute to hepatocyte cell death and liver injury by either apoptosis or necrosis.

Caspases [4,5] represent the central executioners of apoptosis [6,7]. Apoptosis is an active process characterized by cell shrinkage, chromatin condensation and formation of apoptotic bodies. In contrast, necrosis is passive and associated with ATP depletion, rupture of the plasma membrane and spilling of the cellular content eliciting inflammation [6].

Heme oxygenase-1 (HO-1) catalyzes the oxidation of heme to form equimolar amounts of ferrous iron, biliverdin and carbon monoxide (CO). Biliverdin is rapidly converted into bilirubin by NAD(P)H: biliverdin, reductase. HO-1 is induced by a variety of stimuli mostly associated with oxidative stress

Abbreviations: HO-1, heme Oxygenase-1; Ad5LacZ, adenoviral β -galactosidase; Ad5I κ BAA, adenoviral dominant-negative I κ B- α ; Ad5HO-1, adenovirus overexpressing HO-1; MAPK, Mitogen-activated protein kinase; BSO, L-buthionine-(S,R)-sulfoximine; DEM, diethylmaleate; CO, carbon monoxide; ROS, reactive oxygen species; BDL, bile duct ligation; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; sGC, soluble guanylate cyclase; PARP, poly (ADP-ribose, polymerase); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase.

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[8]. Many studies have suggested that HO-1 acts as an inducible defence against oxidative stress, e.g., in models of inflammation, ischemia–reperfusion, hypoxia and hyperoxia-mediated injury [9]. In the liver, HO-1 induction protected against ischemia–reperfusion injury [10,11] and endotoxemia [12,13]. In addition, overexpression of HO-1 by gene transfer protects against hyperoxia-induced lung injury [14], immune-mediated apoptotic liver damage in mice [15] and CYP2E1-dependent toxicity in HepG2 cells [16]. However, the mechanisms by which HO-1 mediates cytoprotection are not yet elucidated. Protective effects of both biliverdin and CO have been reported and several papers suggest that biliverdin protects against oxidative stress by acting as an antioxidant in different models of liver injury [17,18]. Less is known about the biological actions of CO. Although CO is known to be toxic and lethal at high doses, interest has recently emerged for the role of CO as a signalling and regulatory molecule in cellular processes [19,20]. A cytoprotective effect of CO has been demonstrated in several conditions associated with apoptosis. In the present study, our aim was to investigate the regulation of HO-1 *in vivo* (model of acute inflammation and chronic cholestasis) and *in vitro* in primary cultures of rat hepatocytes. In addition, we investigated whether CO contributes to the protective effect of HO-1 against oxidative-stress-induced apoptosis in primary hepatocytes and the mechanisms involved in the process.

Materials and methods

Animals

Specified pathogen-free male Wistar rats (220–250 g) were purchased from Harlan (Zeist, The Netherlands). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Experiments were performed following the guidelines of the local Committee for Care and Use of laboratory animals.

Animal models

Bile duct ligation

Male Wistar rats were anesthetized and subjected to bile duct ligation (BDL) as a model of chronic cholestasis [21]. At the indicated time points after BDL, rats ($n=4$ per group) were sacrificed and livers were perfused with saline and removed. Control rats ($n=4$) received a sham operation (Sham) or not (control).

Model of acute inflammation

Rats were injected intraperitoneally (IP) with 5 mg/kg body weight lipopolysaccharide (LPS serotype 0127:B8; Sigma, St. Louis, MO, USA), or the same volume of phosphate-buffered saline (PBS; control group; $n=4$ for each experimental group), with or without diethylmaleate (DEM; 4 mmol/kg body weight; (Sigma), 30 min prior to LPS or PBS injection. LPS and DEM were dissolved in sterile PBS. Rats were sacrificed 6 h after LPS injection. Livers were perfused with saline and snap-frozen in liquid nitrogen until further use.

Rat hepatocyte isolation

Hepatocytes were isolated as described previously [22] and cultured in William's E medium (Invitrogen; Breda, The Netherlands) supplemented with 50 µg/ml gentamycin (Invitrogen) without the addition of hormones or growth factors. During the attachment period (4 h), 50 nmol/L dexamethasone (Sigma) and 5% fetal calf serum (Invitrogen) were added to the medium. Cells were cultured in a humidified incubator at 37 °C and 5% CO₂. Hepatocyte viability was always more than 90% and purity more than 95% as determined by trypan blue staining.

Experimental design

Experiments were started 24 h after the isolation of hepatocytes. Cells were exposed to the intracellular superoxide anion donor menadione (2-methyl-1,4-naphthoquinone; 50 µmol/L; Sigma) [23], hydrogen peroxide (H₂O₂; 1 mmol/L; Merck, Haarlem, The Netherlands), or a cytokine mixture [24] composed of 20 ng/ml murine TNF-α (R&D Systems, UK), 10 ng/ml human interleukin-1β (R&D Systems), and 10 ng/ml rat interferon-γ (R & D Systems) for the indicated time. Activation of ERK1/2 MAPK was inhibited using the MEK inhibitor U0126 at 10 µmol/L (Promega, Madison, WI, USA). 1*H*-(1,2,4)oxadiazolo-(4,3-*a*)quinoxalin-1-one (ODQ; 50 µmol/L; Sigma) was used to inhibit soluble guanylate cyclase (sGC). Depletion of intracellular reduced glutathione was achieved using D,L-buthionine-(*S,R*)-sulfoximine (BSO; 200 µmol/L; Sigma) and diethylmaleate (1 mmol/l; Sigma). In some experiments, caspase activity was inhibited using a cocktail of caspase inhibitors composed of 5 µmol/L Z-DEVD FMK (caspase-3 inhibitor; R & D Systems), 5 µmol/L Z-IETD-FMK (caspase-8 inhibitor; R & D Systems; R & D Systems), 5 µmol/L Z-VEID-FMK (caspase-6 inhibitor; R & D Systems), and 5 µmol/L Z-LEHD-FMK (caspase-9 inhibitor; R & D Systems).

Hepatocytes were exposed to the adenoviruses Ad5HO-1, Ad5IκBAA; or Ad5LacZ (multiplicity of infection of 10) 15 h before exposure to the superoxide anion donor menadione or cytokine mixture.

Parallel experiments were performed in a normal and in a CO-containing culture atmosphere. Hepatocytes were placed in the CO-containing atmosphere after attachment, approximately 15 h before the start of the experiments. Each experimental condition was performed in triplicate wells. Each experiment was performed at least three times using hepatocytes from different isolations.

Carbon monoxide exposure

CO at a concentration of 1% (10,000 parts per million; ppm) in compressed air was mixed with compressed air containing 5% CO₂ before being delivered into the culture incubator, yielding a final concentration of 400 ppm CO. The incubator was humidified and maintained at 37 °C. A CO analyzer was used to determine CO levels in the chamber. After the chamber had stabilized, no oscillations were measured in the CO concentration.

Table 1
Oligonucleotide primers and probes used for quantitative real time PCR

Primers (rat)	Real-time PCR: primers and probe 5'→3'
18S	CGG CTA CCA CAT CCA AGG A (sense) CCA ATT ACA GGG CCT CGA AA (antisense) CGC GCA AAT TAC CCA CTC CCG A (probe)
HO-1	CAC AGG GTG ACA GAA GAG GTC AA (sense) CTG GTC TTT GTG TTC CTC TGT CAG (antisense) CAG CTC CTC AAA CAG CTC AAT GTT GAG C (probe)

Caspase enzyme activity assay

Caspase-3 enzyme activity was assayed as described previously [25].

Sytox green nuclear staining

Rupture of the plasma membrane distinguishes necrotic from apoptotic cell death [26]. To determine necrosis, hepatocytes were incubated 15 min with Sytox green (Molecular Probes, Eugene, OR, USA) nucleic acid stain, which penetrates only cells with compromised plasma membranes but does not cross the membranes of viable cells or apoptotic bodies. Fluorescent nuclei were visualized using an Olympus CKX41 microscope at 450–490 nm.

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

RNA was isolated using Tri-reagent (Sigma) 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; Sigma).

Quantitative real-time PCR

Reverse transcription was performed on 2.5 µg of total RNA using random primers in a final volume of 50 µl. Real-time detection was performed on the ABI PRISM 7700 (PE Applied Biosystems). Samples were heated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Each sample was analyzed in duplicate. 18 S mRNA levels were used as an endogenous control. Real-time PCR primers and probes are listed in Table 1.

Western blot analysis

Western blot analysis of cell lysates was performed by SDS-PAGE followed by transfer to Hybond ECL nitrocellulose membrane (Amersham). An antibody against GAPDH (Calbiochem) and Ponceau S staining were used to ensure equal protein loading and electrophoretic transfer.

Caspase cleavage was detected using polyclonal rabbit antibodies recognizing only cleaved caspase-9, -6, and -3. A monoclonal antibody was used to detect HO-1 (Stressgen). Poly (ADP-ribose) polymerase (PARP) cleavage was detected using a rabbit anti-PARP polyclonal antibody. PARP (116 kDa) is a substrate of caspase-3 yielding a product of 89 kDa and is considered a late marker for apoptosis. After Western blot analysis for p-ERK1/2 and p-JNK using monoclonal antibodies (Santa Cruz Biotechnology), blots were stripped using 0.1% SDS in PBS/Tween 20 at 65 °C for 30 min and incubated with antibodies against total-ERK1/2 or total-JNK. All antibodies were obtained from Cell Signaling Technology (Beverly, MA), unless indicated otherwise and used at 1:1000 dilution. Quantity One system (Bio-Rad) was used to quantify Western blots.

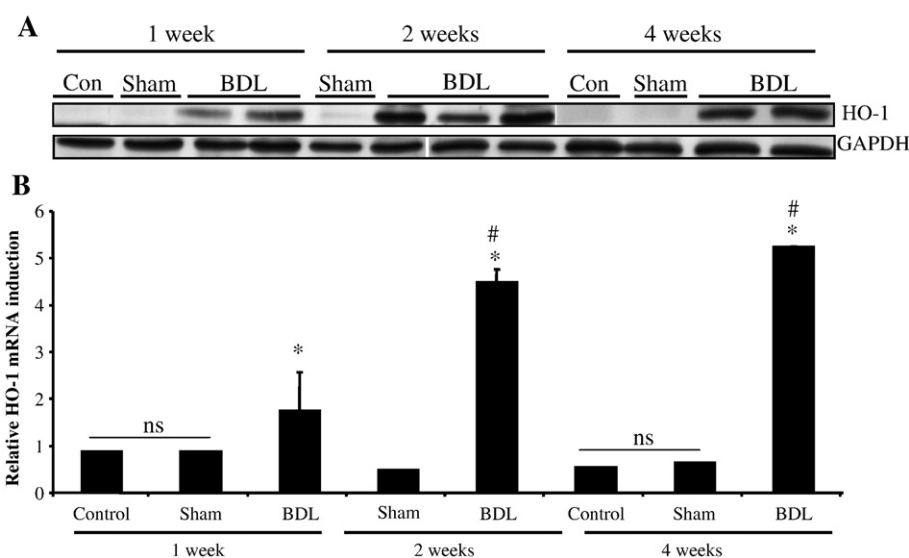


Fig. 1. HO-1 expression is induced in chronic cholestasis. Rats were subjected to bile duct ligation (BDL) as a model of cholestasis. At indicated time points after BDL, rats ($n=4$ per group) were sacrificed and livers were perfused with saline and removed. Control rats received a sham operation (Sham) or not (Con). HO-1 expression is induced in BDL at protein level (as determined by Western blot (A)) and at mRNA level (as determined by real-time PCR (B)). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control in Western blots and 18 S mRNA levels were used as an endogenous control in quantitative real time-PCR. (*): $p<0.05$ compared to control in each group; (#): $p<0.05$ compared to BDL 1 week group; (ns): not significant.

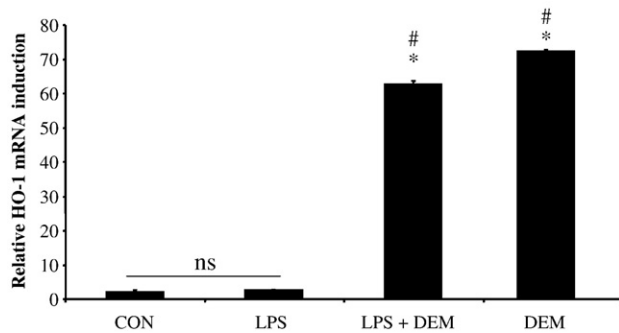


Fig. 2. Acute inflammation does not induce HO-1 expression in the liver. Rats were injected intraperitoneally (IP) with 5 mg/kg body weight lipopolysaccharide (LPS) or the same volume of phosphate-buffered saline (PBS; control group; Con) with or without diethylmaleate (DEM; 4 mmol/kg body weight) 30 min prior to LPS or PBS injection. After 6 h of LPS injection, rats were anesthetized with pentobarbital (60 mg/kg, IP). However, DEM-induced oxidative stress, superposed on acute inflammation, strongly increases HO-1 mRNA level in acute inflammation *in vivo*, as determined by real-time PCR. In the figure, a representative set of a group ($n=4$) is shown. (*): $p<0.05$ compared to control; (#): $p<0.05$ compared to LPS group; (ns): not significant.

Adenoviral constructs

Recombinant, replication-deficient adenovirus Ad5I κ BAA was used to inhibit NF- κ B activation as previously described [27]. Functionality of the Ad5I κ BAA virus was demonstrated by its ability to sensitize hepatocytes to cytokine-induced hepatocyte apoptosis. Ad5LacZ containing the Escherichia coli β -galactosidase gene was used as a control virus. The Ad5HO-1 adenovirus was a kind gift of Professor Augustine Choi, University of Pittsburg, Pittsburg, Pennsylvania, USA and has been previously described [28,29].

Statistical analysis

All data are expressed as the mean of at least three independent experiments \pm S.D. Statistical significance was deter-

mined by the Mann–Whitney U test; $p<0.05$ was considered statistically significant.

Results

Regulation of HO-1 *in vivo*

To investigate the regulation of HO-1 *in vivo*, models of chronic cholestatic liver disease (BDL) and acute inflammation were used. Chronic cholestasis increased HO-1 mRNA level about six-fold compared to controls in a time-dependent manner (Fig. 1).

In contrast, acute inflammation does not increase HO-1 expression, although iNOS expression was highly elevated in the same livers at protein and at mRNA levels as previously demonstrated [30]. DEM-induced oxidative stress, superposed on acute inflammation, strongly increases HO-1 mRNA level in acute inflammation *in vivo*. Induction of oxidative stress with DEM alone also increases HO-1 mRNA in the liver (Fig. 2). The induction of HO-1 by DEM alone or in combination with LPS correlates with results obtained in rat intestine in the same model [31].

Regulation of HO-1 *in vitro*

To confirm our *in vivo* data we investigated the regulation of HO-1 expression *in vitro* in primary rat hepatocytes.

The superoxide anion donor menadione induces HO-1 expression in a time- (Fig. 3A) and concentration-dependent manner (Fig. 3B). Exposure to H₂O₂ does not induce HO-1 expression in hepatocytes (Fig. 4A). In contrast, cytokine mixture only slightly induces HO-1 expression *in vitro* (Fig. 5), although iNOS expression was highly induced under the same circumstances as shown previously [30]. Depletion of glutathione, using BSO, an inhibitor of glutamate cysteine ligase, the rate-limiting step in GSH biosynthesis, or DEM further enhances the induction of HO-1 mRNA and HO-1 protein level *in vivo* (Fig. 2) and *in vitro* (Figs. 4 and 5) in all tested conditions.

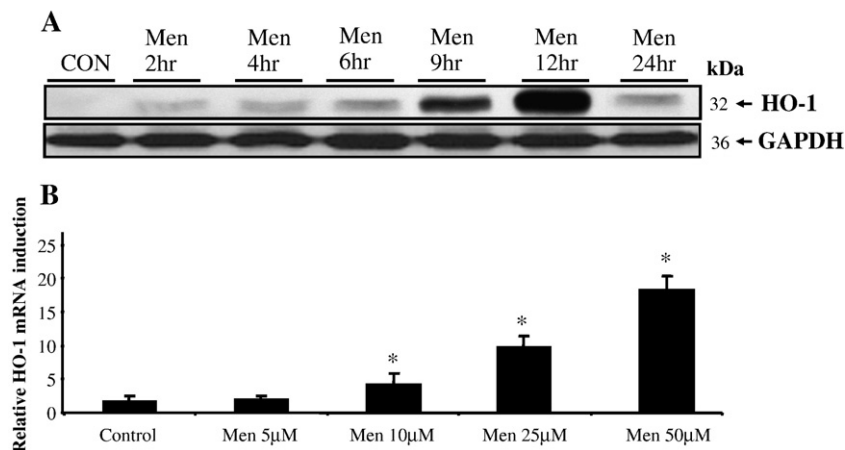


Fig. 3. The intracellular superoxide anion donor menadione induces HO-1 expression in a time- and concentration-dependent manner *in vitro*. Cells were exposed to menadione (Men; 50 μ mol/L) and harvested at different time points (A) or exposed to different concentrations of menadione for 9 hrs (B). HO-1 expression was determined at (A) protein level by Western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control and at (B) mRNA level by real-time PCR (9 h). 18S mRNA levels were used as an endogenous control. (*): $p<0.05$ compared to control.

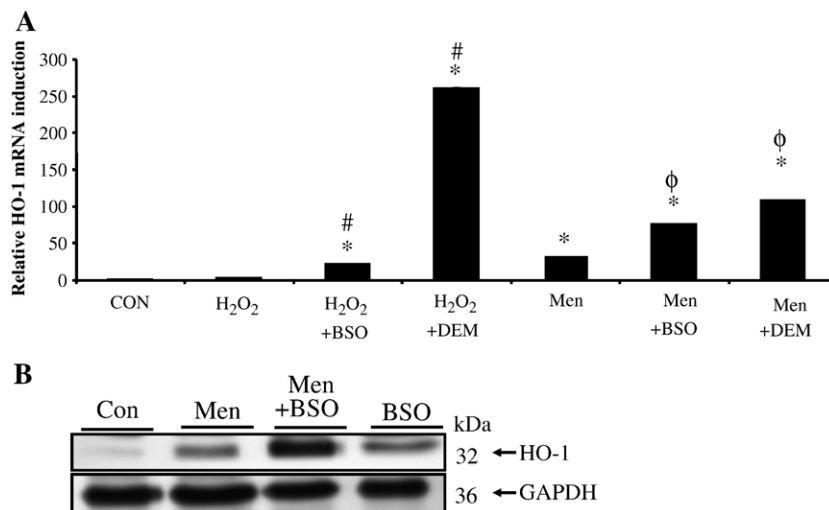


Fig. 4. HO-1 expression is not increase by hydrogen peroxide *in vitro*. D,L-Buthionine-(S,R)-sulfoximine (BSO; 200 $\mu\text{mol/L}$) was added 15 h before and 30 min before the start of the experiment. Diethylmaleate (DEM; 1 mol/L) was added 30 min before the start of the experiment. After that, cells were exposed to hydrogen peroxide (H_2O_2 ; 1 mmol/L; 6 h) or the superoxide anion donor menadione (Men; 50 $\mu\text{mol/L}$; 9 h). (A) HO-1 mRNA level was increased by the superoxide anion donor menadione, BSO, and DEM but not by hydrogen peroxide as determined by quantitative real-time PCR. (B) At protein level, HO-1 expression is induced by menadione and BSO as determined by Western blot. (*): $p < 0.05$ compared to control. (#): $p < 0.05$ compared to H_2O_2 group. (ϕ): $p < 0.05$ compared with menadione (Men)-treated cells.

Heme oxygenase-1 overexpression protects against oxidative stress

To investigate whether HO-1 plays a role in the protection against oxidative stress, HO-1 was overexpressed in primary rat hepatocytes by transfection with Ad5HO-1 15 h before exposure to menadione. Although metalloporphyrins such as hemin or chromium mesoporphyrin are widely used to induce or reduce expression of HO-1, respectively, in our hands they showed serious toxic side effects. This confirms recent reports that metalloporphyrins induce oxidative stress and therefore cannot be used in combination with menadione [32]. In addition, it has recently been reported that metalloporphyrins interfere with caspase activity, independent of its HO-1-inducing effect [33]. To avoid these confounding side effects, we used an adenoviral construct to achieve overexpression of HO-1.

As shown in Fig. 6A, Ad5HO-1 infection dose-dependently increased HO-1 expression at mRNA and protein level in hepatocytes at the time of menadione exposure. Overexpression of HO-1 protected against menadione-induced caspase-3 activity and processing compared with control and Ad5LacZ-infected hepatocytes (Fig. 6B). Hepatocytes infected with only the Ad5LacZ virus or Ad5HO-1 virus and not exposed to menadione did not display elevated caspase-3 activity (Fig. 6B).

Carbon monoxide protects primary rat hepatocytes against oxidative-stress-induced apoptosis

Because HO-1 overexpression protects primary rat hepatocytes against superoxide anions-induced apoptosis, we investigated whether CO contributes to the protective effect of HO-1 expression. CO suppresses menadione-induced PARP cleavage

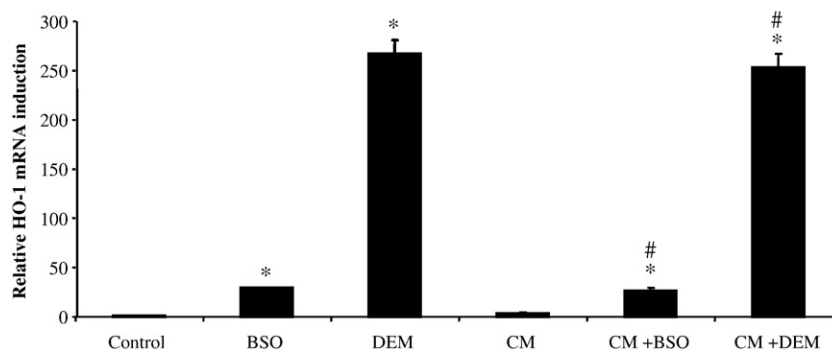


Fig. 5. Cytokines slightly induce HO-1 expression in primary hepatocytes *in vitro*. D,L-Buthionine-(S,R)-sulfoximine (BSO; 200 $\mu\text{mol/L}$) was added 15 h before and 30 min before the start of the experiment. Diethylmaleate (DEM; 1 mol/L) was added 30 min before the start of the experiment. Then cells were exposed to cytokine mixture (CM; 20 ng/ml murine TNF- α , 10 ng/ml human interleukin-1 β , 100 U/ml rat interferon- γ) for 10 h. HO-1 mRNA level was increased by BSO and DEM but only a minor increase was observed after exposure to CM as determined by quantitative real time-PCR. (*): $p < 0.05$ compared to control group; (#): $p < 0.05$ compared to CM-treated group.

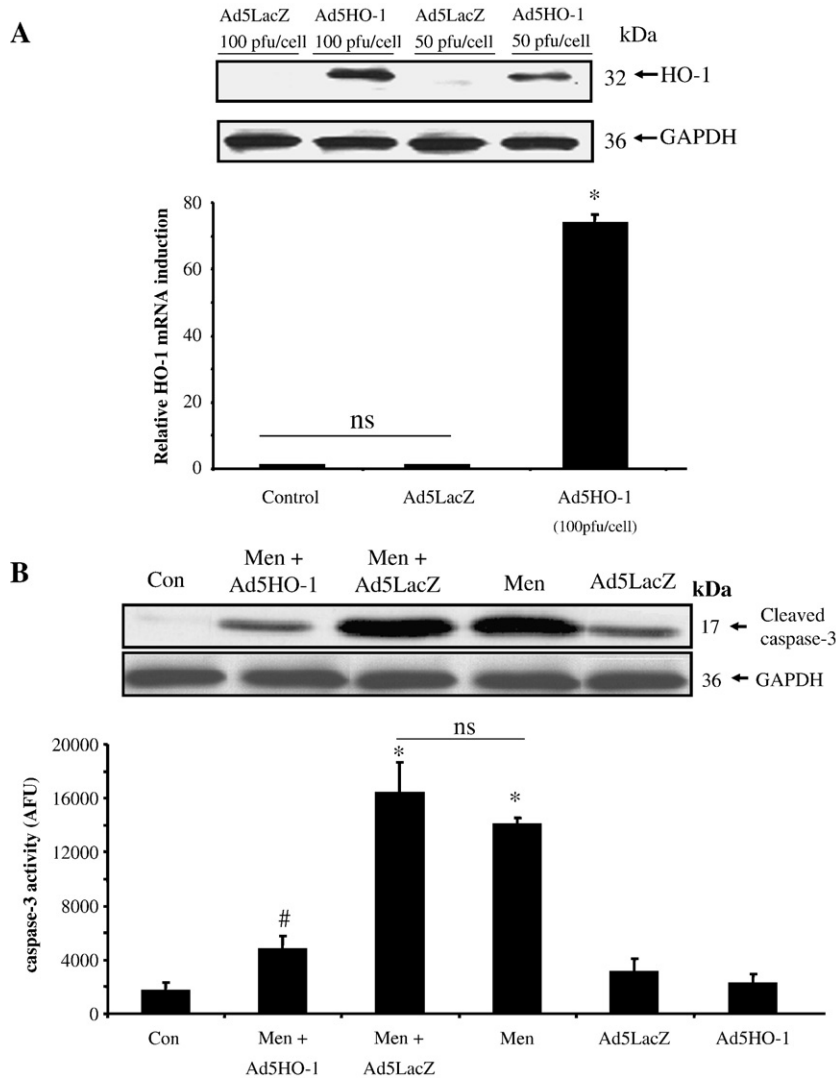


Fig. 6. Overexpression of HO-1, using the adenovirus Ad5HO-1, leads to protection of primary hepatocytes against oxidative stress-induced apoptosis. Cells were exposed to Ad5HO-1 or Ad5LacZ (virus control) 15 h before the addition of menadione (Men; 50 μ mol/L; 9 h). (A) Ad5HO-1 highly induces HO-1 expression dose-dependently at protein level (top panels) and at mRNA level (up to 70-fold as determined by real-time PCR, bottom graph) compared with Ad5LacZ and control. (*): $p < 0.05$; (ns): not significant. (B) Overexpression of HO-1 prevents caspase-3 activity (bottom graph) and processing (Western blot, top panels). (*): $p < 0.05$ compared to control; (#): $p < 0.05$ compared to menadione-treated cells; (ns): not significant.

and apoptosis in primary hepatocytes, indicating that CO has a protective role against superoxide-anions-induced apoptosis (Figs. 7). In addition, CO inhibits menadione-induced caspase -9, -6 and -3 activation (Fig. 8), in accordance with our previous finding that menadione-induced apoptosis is dependent on caspase activation in primary hepatocytes [27]. CO did not induce necrosis in primary rat hepatocytes (data not shown), suggesting that CO is really protective against oxidative stress-induced apoptosis and does not switch the balance between apoptosis and necrosis.

The protective effect of CO is not dependent on soluble guanylate cyclase

To investigate whether the sGC is involved in the protective effect of CO against menadione-induced apoptosis in primary

hepatocytes, cells were exposed to the sGC inhibitor ODQ with or without menadione in the presence of CO. The ability of CO to inhibit menadione-induced apoptosis was not reversed by ODQ (Fig. 9), indicating that the antiapoptotic effect of CO is not dependent on the activation of the sGC or the generation of cGMP. In the absence of CO, ODQ had no effect on primary hepatocytes.

The NF- κ B pathway is not involved in the protective effect of CO against menadione-induced apoptosis

In our study, inhibition of the NF- κ B pathway by recombinant adenovirus expressing dominant negative I κ B did not reverse the protective effect of CO against superoxide anions-induced apoptosis (Fig. 10A). Functionality of the virus was demonstrated by sensitizing hepatocytes to cytokine-induced apoptosis (Fig. 10B).

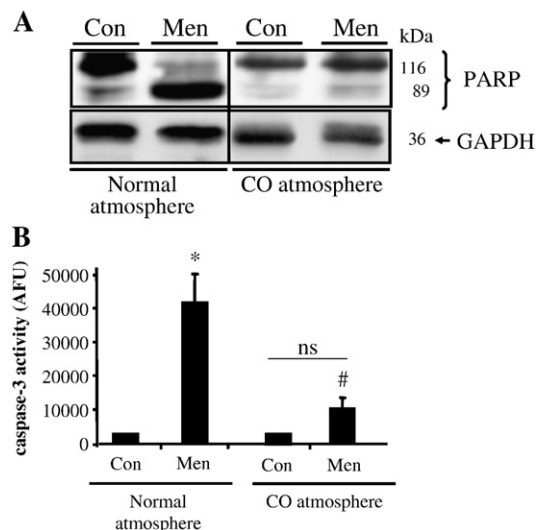


Fig. 7. Carbon monoxide prevents oxidative stress-induced apoptosis in hepatocytes. Cells were incubated with menadione (Men; 50 $\mu\text{mol/L}$; 9 h) or not (Con; untreated cells) in the presence and absence of carbon monoxide (CO; 400 parts per million; 9 h). (A) Western blot: CO inhibits poly(ADP-ribose) polymerase (PARP) cleavage induced by menadione. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control. (B) Caspase-3 activity assay: CO blocks menadione-induced apoptosis (expressed as caspase-3 activity). (*): $p=0.009$ compared to control. (#): $p=0.009$ compared to menadione-treated cells in a normal atmosphere; (ns): not significant.

Carbon monoxide blocks phosphorylation of the proapoptotic MAP kinase JNK

Menadione-induced caspase activation and apoptosis are dependent on JNK activity in primary rat hepatocytes [27]. Because CO prevents caspase -9, -6, and -3 activation, we also investigated the effect of CO on MAPK phosphorylation.

As shown in Fig. 11A, CO induces ERK phosphorylation at 2 h. To investigate the role of ERK in the protective role of CO against menadione-induced apoptosis, hepatocytes were exposed to CO in the presence of the ERK1/2 inhibitor U0126. Inhibition of ERK1/2 did not reverse the protective effect of CO (Fig. 11B). The functionality of U0126 was shown by its ability to reduce ERK1/2 phosphorylation as shown by Western blot (Fig. 11C). On the other hand, CO inhibits predominantly the menadione-induced phosphorylation of the p54 JNK isoform (Fig. 11A). Because inhibition of JNK activity, using the inhibitor SP600125, prevents menadione-induced caspase activation and apoptosis [27], our results indicate that the protective effect of CO against superoxide anions-induced apoptosis is at least partly due to inhibition of JNK activation. To distinguish between the possibility that JNK activation is the cause of apoptosis and the possibility that JNK activation is the result of apoptosis, we determined JNK phosphorylation in response to menadione in the presence of a cocktail of caspase inhibitors (caspase-3,6,8,9 inhibitors). Inhibition of apoptosis did not change menadione-induced JNK phosphorylation (data not shown).

Discussion

In this study we demonstrated that HO-1 expression is induced in different models of oxidative stress in the liver both *in vitro* and

in vivo, including chronic cholestasis. Interestingly, acute inflammation, induced by endotoxin administration, is a very weak inducer of HO-1, although in this model NF- κ B-regulated genes such as iNOS are highly induced as shown previously [30]. A similar reciprocal regulation of AP-1-responsive genes such as HO-1 and NF- κ B-responsive genes like iNOS was observed in intestinal epithelial cells [31] and is in accordance with other reports [39]. We have demonstrated that this switch from an NF- κ B-regulated stress response to an AP-1-regulated stress response in hepatic inflammation is controlled by the extent of oxidative stress. An important regulator of this switch might be the content of reduced glutathione (GSH) in the cell: GSH depletion inhibits NF- κ B-mediated cytokine-inducible iNOS expression and simultaneously increases oxidative stress and the expression of oxidative-stress-inducible genes such as HO-1 [30,31]. These results suggest that TNF/CM exposure (*in vitro*) or LPS exposure (*in vivo*) in the presence of sufficient GSH levels does not induce important oxidative stress and hence no AP-1 activation and HO-1 induction. This switch might be an adaptation to the type of stress. NF- κ B-regulated protective genes include antiapoptotic genes, for example iNOS, which protect against proapoptotic inflammatory cytokines such as TNF- α , whereas AP-1-regulated genes, e.g. HO-1, are involved in the protection against various forms of oxidative stress. *In vitro*, in primary cultures of rat hepatocytes, HO-1 expression is induced by the superoxide anion donor menadione but not by H_2O_2 . Hepatocytes have a high capacity to detoxify H_2O_2 [27], and it is likely that this molecule is hardly sensed by hepatocytes. This also correlates with our previous finding that 1 mmol/L H_2O_2 does not induce cell death. The induction of HO-1, both *in vivo* and *in vitro*, was much more pronounced after depletion of the antioxidant glutathione. This correlates with our *in vivo* model of acute inflammation demonstrating that acute inflammation is not accompanied by extensive oxidative stress and depletion of antioxidants.

Numerous studies have shown the cytoprotective and antiapoptotic properties of HO-1 against oxidative stress [34]. Although the superoxide anion donor menadione induces HO-1 expression in primary hepatocytes, this induction does not prevent apoptosis, probably because HO-1 induction is not high and/or rapid enough. In support of this we observed that overexpression of

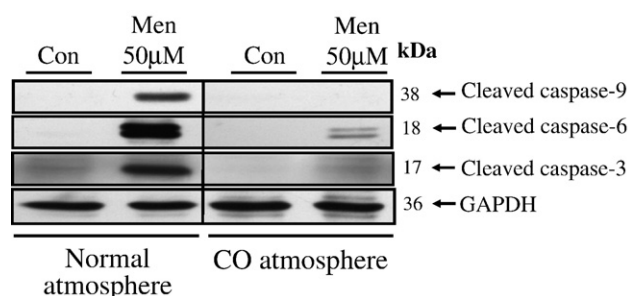


Fig. 8. Carbon monoxide (CO) prevents menadione-induced caspase activation in hepatocytes. Cells were incubated with menadione (Men; 50 $\mu\text{mol/L}$; 9 h) and without (Con; untreated cells) in the presence and absence of carbon monoxide (400 parts per million, 9 h). Western blot: CO inhibits caspase-9, -6, and -3 cleavage induced by menadione. GAPDH was used as a protein loading control.

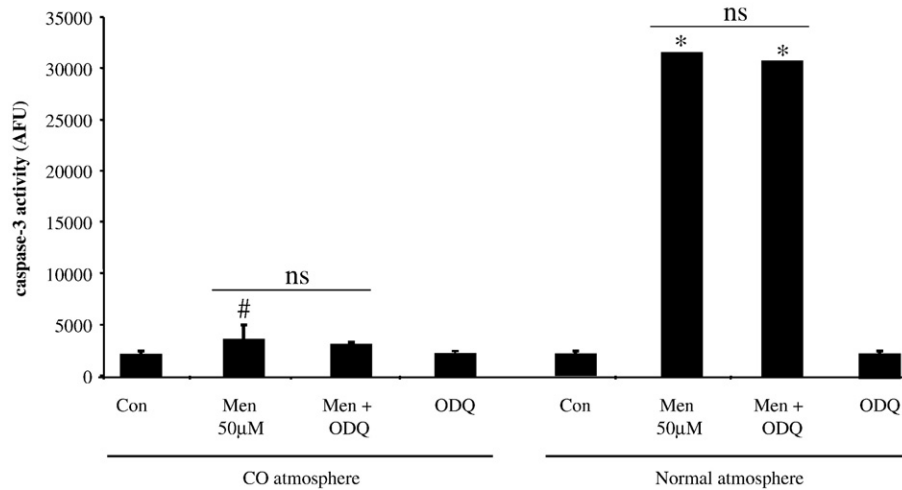


Fig. 9. The protective effect of carbon monoxide (CO) is not dependent on the activation of soluble guanylate cyclase (SGC). Cells were exposed to (1H-[1,2,4]oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ), a sGC inhibitor, with or without menadione (Men; 50 μmol/L; 9 h) in the presence of carbon monoxide (CO, 400 ppm; 9 h). Caspase-3 activity assay: The ability of CO to inhibit menadione-induced apoptosis was not reversed by ODQ. (*): $p < 0.05$ compared to control; ([#]): $p < 0.05$ compared to menadione-treated cells in a normal atmosphere; (ns): not significant.

HO-1 prior to menadione protects against oxidative stress-induced apoptosis. Metalloporphyrins such as hemin or chromium mesoporphyrin are widely used as inducers or inhibitors, respectively, of HO-1. In our hands these compounds showed serious side effects, confirming recent reports that describe aspecific effects of these compounds, in particular when studying phenomena related to oxidative stress and apoptosis [32,33].

In this study we provided evidence that CO, one of the products of HO-1, protects against superoxide anions-induced

apoptosis. Previously, we showed that superoxide anions-induced apoptosis is dependent on caspase activation [27]. In accordance with these findings, we demonstrated that CO is a potent inhibitor of caspase activation, PARP cleavage, and apoptosis in primary hepatocytes exposed to the superoxide anion donor menadione. Because CO inhibits caspase-9 activation, we speculate that CO prevents disruption of mitochondria and subsequent apoptosis. Indeed, it has been shown that CO blocks mitochondrial cytochrome *c* release [35,36]. We also showed

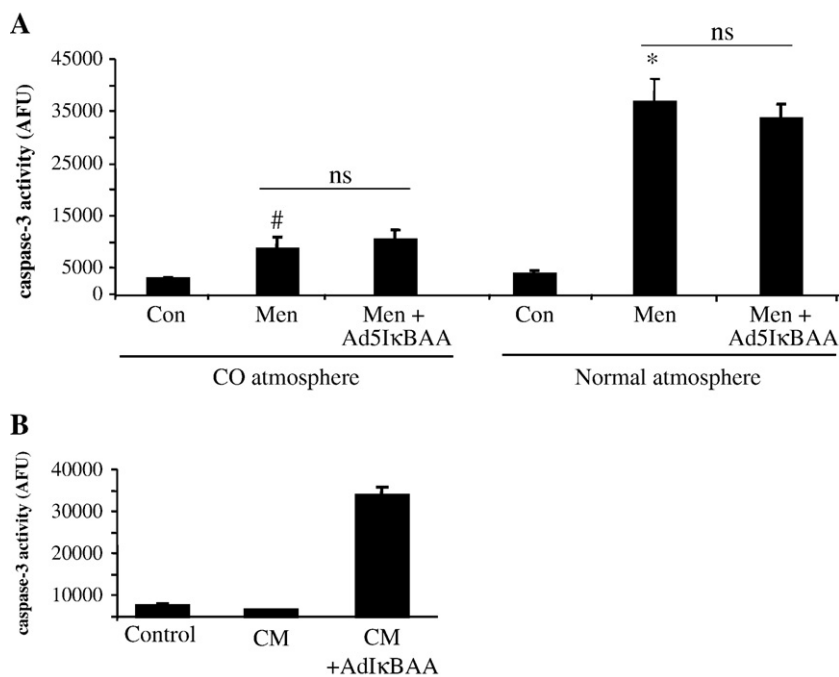


Fig. 10. The NF-κB pathway is not involved in the protective effect of CO against oxidative stress-induced-apoptosis. (A) Caspase-3 activity assay: Inhibition of the NF-κB pathway, using the Ad5IκB 15 h before menadione addition, did not reverse the protective effect of carbon monoxide (CO; 400 ppm; 9 h) against menadione-induced apoptosis. (B) Functionality of the Ad5IκBAA virus was demonstrated by its ability to sensitize hepatocytes to cytokine-induced apoptosis. (*): $p < 0.05$ compared to control; ([#]): $p < 0.05$ compared to menadione-treated cells in a normal atmosphere; (ns): not significant.

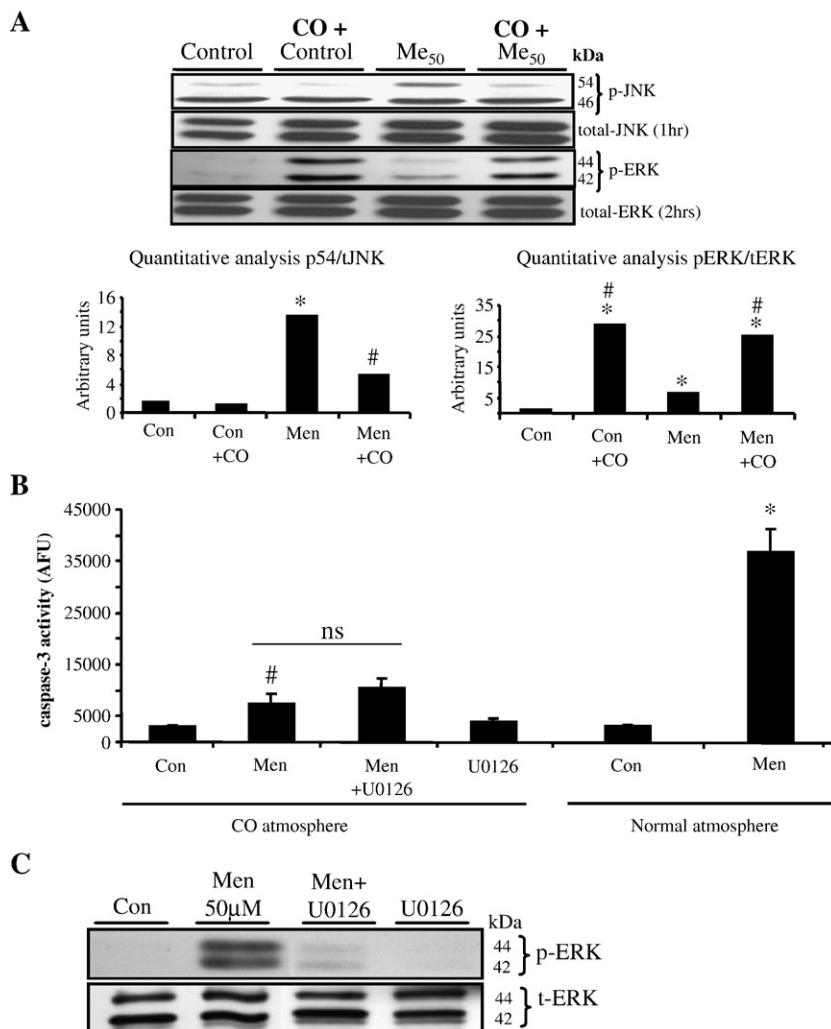


Fig. 11. Carbon monoxide (CO) induces ERK1/2 and inhibits JNK phosphorylation. Cells were exposed to menadione (Men; 50 μ mol/L; 1–2 h (A) or 9 h (B)) in the presence and absence of carbon monoxide (CO, 400 ppm, 1–2 h (A) or 9 h (B)). (A) Western blots were performed against phosphorylated-JNK, total-JNK, phosphorylated-ERK1/2, and total-ERK1/2. Total-JNK and total-ERK were used as loading controls. CO induces p-ERK1/2 at 2 h. In contrast, CO prevents menadione-induced p54 phosphorylation (1 h). Western blots were quantified using the Quantity One system. Statistical analysis was performed using the quantified values of Western blots from at least three different isolations. Relative protein amount of p54/pERK normalized using tJNK/tERK, respectively, (bottom graphs). (B) Caspase-3 activity assay: Cells were exposed to the ERK1/2 inhibitor U0126 30 min before and during the incubation with menadione (Men; 50 μ mol/L, 9 h) in the presence of CO (400 ppm). Inhibition of ERK1/2 activation does not prevent the protective effect of CO. (C) Western blot: Inhibition of ERK1/2 activation by the specific ERK1/2 inhibitor U0126. Statistical analysis: (*): $p < 0.05$ compared to control; (#): $p < 0.05$ compared to menadione-treated cells in a normal atmosphere; ns, not significant.

that CO does not shift the balance from apoptotic cell death to necrotic cell death. This is not a trivial finding as we have shown previously [27]. Therefore, CO really protects hepatocytes against superoxide anions-induced cell death. Our findings are in accordance with studies using different inducers of hepatocyte cell death, including glucose-deprivation in BNL CL.2 hepatoma cells or by Fas [37] and TNF- α in hepatocytes [38]. In addition, a protective effect of CO has been demonstrated in a wide range of different cell types [35,39,40]. In contrast, a proapoptotic effect of CO has been observed for murine thymocytes and bovine pulmonary arterial endothelial cells [41,42]. These data indicate that CO may regulate apoptosis and cytotoxicity in a cell-specific manner.

It has been suggested that the cytoprotective effect of CO is due to activation of soluble guanylate cyclase; however, we saw

no effect of sGC inhibition on the protective effect of CO. Previous studies are contradictory with regard to the role of sGC in the antiapoptotic effect of CO, both confirming [40] and contradicting [35] our findings. Again, different signaling pathways may be involved in the protective effect of CO in different cell types. NF- κ B inhibition did not influence the protective effect of CO against superoxide anions-induced apoptosis in hepatocytes, although it was reported that the protective effect of CO was dependent on NF- κ B activation in hepatocytes exposed to TNF- α [38].

In this study we demonstrated that CO inhibits JNK activation, in particular the p54 isoform of JNK. A similar inhibitory effect of CO on JNK phosphorylation has been described in a model of sepsis *in vivo* [43]. In a previous study we demonstrated that superoxide anions-induced apoptosis is dependent on JNK

activity because inhibition of JNK activity prevents menadione-induced caspase activation and apoptosis in primary hepatocytes [27]. However, we cannot conclude beyond any doubt that the inhibitory effect of CO on JNK activity is responsible for the protective effect of CO because CO appears to inhibit mainly the phosphorylation of the p54 isoform of JNK, whereas the pharmacological inhibitor SP600125 inhibits both the p54 and the p46 isoforms of JNK.

Menadione-induced JNK activation was not changed in the presence of a cocktail of caspase inhibitors. Together with our previous findings [27] that inhibition of JNK activity using SP600125 abolishes menadione-induced caspase activation and apoptosis and that JNK activation precedes caspase activation and apoptosis by at least 6–8 h, these results indicate that reduced JNK activation is the *cause* of reduced apoptosis and not the *result* of reduced apoptosis.

The mechanisms by which JNK exerts its proapoptotic properties are probably diverse and not yet completely elucidated. Many studies suggest that JNK triggers the mitochondrial death pathway, including phosphorylation and activation of proapoptotic bcl-2 family members [44–50]. Recently, a role for JNK in the degradation of the antiapoptotic protein c-FLIP has been reported [51].

Another finding of the present study is that CO induces ERK1/2 MAPK phosphorylation. ERK1/2 activity is antiapoptotic in hepatocytes and its activation attenuates cell death. However, although significant, the magnitude of the protective effect of ERK1/2 activity against superoxide anions-induced apoptosis is moderate [27]. Apparently, the antiapoptotic action of CO is due mainly to its inhibitory effect on JNK activation and not to its stimulatory action on ERK1/2 activation. Regulation of MAP kinase signaling pathways by CO in a variety of cell types has been reported in other studies [36,52]. In addition, it has been reported that CO mediates protection against CYP2E1-dependent cytotoxicity in part via inhibition of CYP2E1 activity [16].

In this study we focused on the mechanism of the protective action of CO. Biliverdin, another product of HO-1, is also protective against oxidative-stress-induced injury in part due to its antioxidant properties [17]. Indeed, a cooperative protective effect of biliverdin and carbon monoxide has been shown in immune-mediated liver injury in mice [18].

In conclusion, HO-1 is induced by oxidative stress in primary hepatocytes *in vivo* and *in vitro*. HO-1 protects primary hepatocytes against superoxide-anions-induced apoptosis, and this protection may be mediated in part via CO production. CO inhibits the activity of the proapoptotic JNK MAPK and induces ERK1/2 MAPK antiapoptotic activity. Modulation of HO-1 expression, or the levels of its product CO, may become an important therapeutic target in liver disorders caused by exposure to excessive oxidative stress.

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