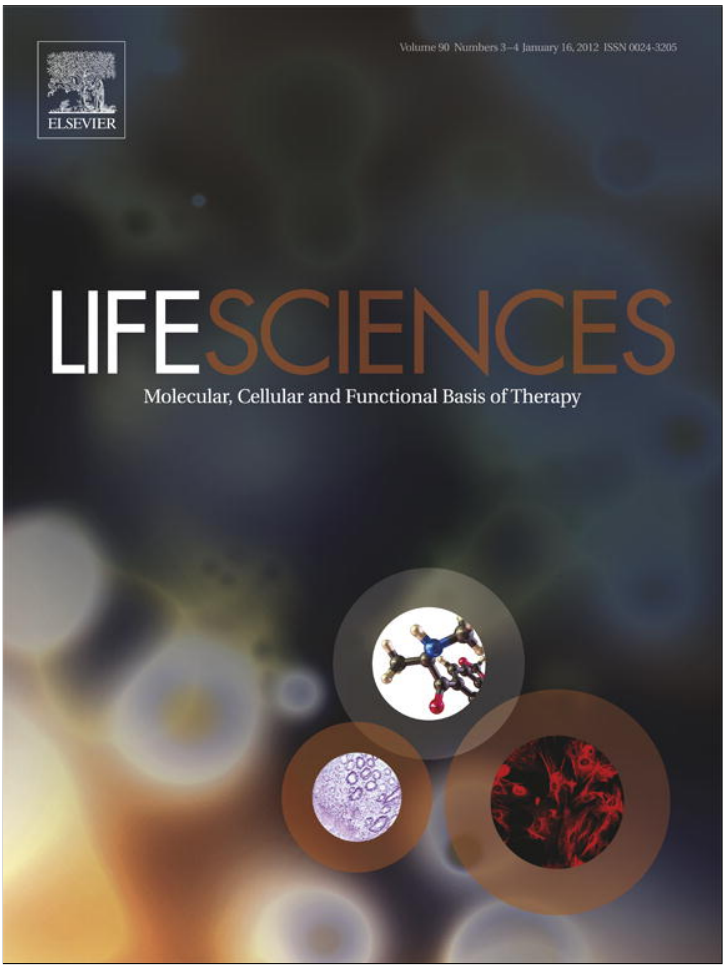

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Effects of Kupffer cell blockade on the hepatic expression of metallothionein and heme oxygenase genes in endotoxemic rats with obstructive jaundice

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ABSTRACT

Aims: Heme oxygenase (HO) and metallothionein (MT) genes are rapidly upregulated in the liver by pro-inflammatory cytokines and/or endotoxin as protection against cellular stress and inflammation. Gadolinium chloride ($GdCl_3$)-induced Kupffer cell blockade has beneficial consequences in endotoxemia following bile duct ligation. Herein we further characterized the effects of Kupffer cell inhibition on the activation of the antioxidant defense system (HO and MT gene expressions, and antioxidant enzyme activities) in response to endotoxemia and obstructive jaundice.
Main methods: The isoform-specific expression of MT and HO genes was assessed (RT-PCR) in rat livers following 3-day bile duct ligation, 2-h lipopolysaccharide treatment (1 mg/kg) or their combination, with or without $GdCl_3$ pretreatment (10 mg/kg, 24 h before endotoxin). Lipid peroxidation, DNA damage and hepatic antioxidant enzyme activities were also assessed.
Key findings: All these challenges induced similar extents of DNA damage, whereas the lipid peroxidation increased only when endotoxemia was combined with biliary obstruction. The MT and HO mRNA levels displayed isoform-specific changes: those of MT-1 and HO-2 did not change appreciably, whereas those of MT-2 and HO-1 increased significantly in 2-h endotoxemia, with or without obstructive jaundice. Among the enzymes reflecting the endogenous protective mechanisms, the catalase and copper/zinc-superoxide dismutase levels decreased, while that of Mn-SOD slightly increased. Interestingly, $GdCl_3$ alone induced lipid peroxidation, DNA damage and MT-2 expression. In response to $GdCl_3$, HO-1 induction was significantly lower in each model.
Significance: Despite its moderate hepatocellular toxicity, the ameliorated stress-induced hepatic reactions provided by $GdCl_3$ may contribute to its protective effects.

Introduction

In surgical practice, biliary obstruction is often accompanied by septic complications. With an earlier experimental biliary obstruction model, an enhanced susceptibility of jaundiced animals to endotoxemia was demonstrated, which was critically linked to activation of the Kupffer cells (KCs) (Lázár et al., 2002; Abrahám et al., 2008). Other manifestations included an increased release of pro-inflammatory cytokines, and characteristic inflammatory and microvascular responses; some of these inflammatory effects could be overcome by the prevention of KC activation.

The final consequence of the above microcirculatory inflammatory changes is an oxidative stress reaction hallmarked by lipid peroxidation (LPO) and DNA damage. Tissue damage resulting from either endotoxemia or obstructive jaundice (or their combination), however, also leads to the activation of endogenous protective mechanisms. Among the upregulated proteins, the expression of microsomal heme oxygenase (HO) is triggered by both endotoxemia and biliary obstruction (Froh et al., 2007; Bauer et al., 1998). HO plays a role in the degradation of heme, and also produces carbon monoxide, a vasoactive dilator agent with important free radical scavenger properties (Camhi et al., 1995; Gemsa et al., 1974; Stocker, 1990). Two major isoforms of HO have been characterized: the heme oxygenase 1 isoform (HO-1), which is inducible in response to stressors such as heavy metals, oxidative stress and cytokines, and the constitutive heme oxygenase 2 isoform (HO-2) (for a review, see Wagener et al., 2003). The fact that stress reactions, including endotoxemia, are associated with HO upregulation in the

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KCs suggests that these self-defensive anti-inflammatory reactions are also initiated in this important pro-inflammatory cellular component of the liver (Gemsa et al., 1974; Stocker, 1990; Rizzardini et al., 1994).

Metallothioneins (MTs) are low molecular weight, non-enzymatic proteins that play a homeostatic role in the control and detoxification of heavy metal ions. MTs are constitutively expressed in the liver and are overexpressed in endotoxemia (Kille et al., 1994; Abe et al., 1987; Hur et al., 1999). As one-third of its aminoacid residues are cysteines, MT provides a neutrophilic sink binding electrophiles (Kille et al., 1994; Kojima et al., 1976). MTs may ameliorate the effects of oxidative stress by scavenging free radicals (Sato and Bremner, 1993; Nishimura et al., 2001) and even by preventing their formation (Takano et al., 2004).

In the present study, our primary aim was to characterize the oxidative hepatic injury (DNA damage and LPO) and to examine the antioxidant defense components (catalase (CAT) and manganese (Mn-SOD) and copper/zinc-superoxide dismutase (Cu/Zn-SOD)) during endotoxemia, with or without bile duct ligation (BDL). In order to obtain a broader overview of the changes in endogenous protective mechanisms, we also measured the induction of both isoforms of heme oxygenase (HO-1 and HO-2) and the MTs (MT-1 and MT-2) in the rat liver. Since it has been suggested that depletion of the KCs with gadolinium chloride ($GdCl_3$) reduces the harmful microcirculatory inflammatory reactions and systemic consequences of endotoxemia accompanying obstructive jaundice (Lázár et al., 2002; Abrahám et al., 2008), we hypothesized that KC blockade exerts its protective effect in part by enhancing the above endogenous antioxidant defensive mechanisms.

Materials and methods

The experiments were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals. The study was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged.

Animals

Male outbred Wistar rats from the University of Szeged (weighing 250–300 g) were maintained on standard laboratory diet and tap water ad libitum and housed in an environmentally controlled room under a 12-h light–dark cycle.

Experimental protocol

The animals were allocated randomly to one or other of eight groups. In the first group, endotoxemia was elicited by injecting a low dose (1 mg/kg bw) of lipopolysaccharide (LPS) (*E. coli* 026:B6 LPS B. Difco; Laboratories, Detroit, MI, USA) i.v. through the tail vein, 2 h before sampling (LPS group, $n=5$) (also see at Abrahám et al., 2008). In another group, extrahepatic biliary obstruction was induced by BDL 3 days prior to liver tissue biopsy (BDL group, $n=5$). Briefly, a short midline abdominal incision was made and the common bile duct was ligated with a 4–0 silk suture under light oxygen-ether anesthesia. The abdomen was then closed in two layers and the animals were allowed to recover. In a third group, these challenges were combined (BDL+LPS group). The data were compared with those on sham-operated animals (Sham group, $n=5$). The animals in all groups received 1 ml/kg saline i.v. through the tail vein 24 h before the biopsy.

After characterization of the consequences of the above major challenges, additional experimental groups were used to examine the effects of KC blockade. In this series, the rats were pretreated with $GdCl_3$ (10 mg/kg bw i.v. through the tail vein; Prolabo, Paris, France) (Lázár, 1973; Husztik et al., 1980) 24 h before the liver biopsies; the animals were then challenged with the sham operation (Sham + $GdCl_3$ group, $n=5$), with BDL (BDL + $GdCl_3$ group, $n=5$), with LPS (LPS + $GdCl_3$ group, $n=5$) or with their combination (BDL + LPS + $GdCl_3$ group, $n=5$).

RNA extraction, reverse transcription and PCR amplification

For molecular biological examinations, animals were anesthetized with sodium pentobarbital (45 mg/kg i.p.), and the tissues were removed, frozen immediately in liquid nitrogen and stored at −80 °C. Approximately 100 mg of frozen tissue was homogenized in RNAzol B reagent (Tel-Test, Inc., Friendswood, TX, USA) and total RNA was prepared according to the procedure suggested by the manufacturer. Total RNA was routinely treated with 100 U RNAse-free DNAse I to avoid any DNA contamination. To quantify MT and HO-specific mRNAs, an RT-PCR-based strategy was employed. First-strand cDNA was synthetized by using 5 μg total RNA as template. The RNA was denatured at 90 °C, and mixed with 200 pmol of each dNTP (Sigma, St. Louis, MO, USA), 200 U of M-MuLV reverse transcriptase (Sigma, St. Louis, MO, USA) and 500 pmol of random hexamer primer. The reaction mixture was incubated for 10 min at 37 °C, followed by 1 h at 42 °C. The reaction was stopped by heating at 65 °C for 5 min. 2 μl of reverse transcription product was added to 48 μl of PCR reaction mixture containing 250 μmol of each dNTP, 1x Sigma PCR buffer/$MgCl_2$, 5 U of Taq polymerase (Sigma St. Louis, MO, USA) and 50 pmol of primers specific to the MT-1, MT-2 and HO-1 and HO-2 isoforms and the β-actin gene. Amplification was performed in a PTC 200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). The number of amplification cycles during which PCR product formation was limited by the template concentration was determined in pilot experiments: for β-actin 25, and for MTs and HOs 30 cycles were used. Preliminary experiments were performed for the detection of PCR products before saturation (in the linear amplification phase, data not shown). Based on these pilot studies, the expression of β-actin was assessed by applying 25 cycles while for MT and HO detection 30 cycles were used. The amplified products were electrophoresed on 2% agarose (Sigma, St. Louis, MO, USA) gel.

Primers and measurement of MT and HO mRNA levels

For the amplification of rat MT and HO mRNAs, isoform-specific primers were designed on the basis of the databank entries M11794 and AY341880 for the MT-1/2 isoforms, and NM_012580 and NM_024387 for the HO-1/2 isoforms. For normalization of the amounts of MT and HO mRNAs, the β-actin mRNA level was used as internal standard. The sequences of the primers β-actin-3 and 4 were derived from GeneBank entry M24113. Images of ethidium bromide-stained agarose gels were digitalized with a GDS 7500 Gel Documentation System and analyzed with GelBase/GelBlot™ Pro Gel Analysis Software (UVP Inc., San Gabriel, CA, USA). The relative levels of MT and HO mRNAs are expressed as the ratio MT/β-actin or HO/β-actin. For each experimental treatment, 5 animals were used to prepare RNA. RT-PCR reactions for each animal were performed in triplicate to increase the reliability of the measurements.

MT-1 F: 5′ ATGGACCCCAACTGCTCCTG 3′,
MT-1 R: 5′ TGGAGGTGTACGGCAAGACT 3′,
MT-2 F: 5′ AACTGCTCCTGTGCCACAGG 3′,
MT-2 R: 5′ GAAAAAAGTGTGGAGAACCG 3′,
HO-1 F: 5′ TGG GCT CCC TAT ACC AGA TC 3′,
HO-1 R: 5′ ATG CCCTTC TTC CAG TGG GG 3′,
HO-2 F: 5′ TTT TAA GCT TGC CAC CAC TG 3′,
HO-2 R: 5′ CCT GGT TCT CCC AGT CTT CA 3′,
β-actin-3: 5′ GCAAGAGAGGTATCCTGACC 3′,
β-actin-4: 5′ CCCTCGTAGATGGGCACAGT 3′.

Catalase activity

CAT activity was determined spectrophotometrically at 240 nm by the method of Beers and Sizer (1952) and expressed in Bergmeyer units (BU) (1 BU = decomposition of 1 g H_2O_2/min at 25 °C).

Superoxide dismutase

SOD activity was determined on the basis of the inhibition of epinephrine-adrenochrome autoxidation (Misra and Fridovich, 1972; Beauchamp and Fridovich, 1971). Mn-SOD activity was measured by the autoxidation method in the presence of 5×10^{-3} M KCN. Cu/Zn-SOD activity was calculated by deduction of the Mn-SOD activity from the total SOD activity.

Lipid peroxidation

LPO was estimated from the formation of thiobarbituric acid-reactive substances and were determined by using a modification of a method described by Serbinova et al. (1992) and expressed as nanomoles per gram of wet tissue.

DNA single-strand breaks

The alkaline fluorescence analysis of DNA unwinding was used to determine single-strand DNA breaks. DNA samples were prepared from the livers of control and treated animals by using the salting-out method of Miller et al. (1988). To quantify undamaged double-stranded DNA, samples were divided into three sets (F, F_{min} and F_{max}) and the DNA fluorescence was determined under different conditions. To measure F values, the DNA was kept at pH 12.4 to permit its partial unwindin. To determine F_{min}, the DNA samples were kept at pH 12.4, but before the incubation period, they were sonicated for 1 min. For F_{max} determination, the DNA samples were kept at pH 10, which is below the pH needed to induce the unwinding of the DNA. Samples were incubated on ice for 30 min, followed by a 15-min incubation at 15 °C. Fluorescence was measured after the addition of ethidium bromide at 0.67 μg/ml, with an excitation wavelength of 520 nm and an emission wavelength of 590 nm. The results are expressed as D (percentage of double-stranded DNA) $=(F-F_{min})/(F-F_{max})\times100$.

Statistical analysis

Data in all figures are expressed as means ± standard error of the mean (SEM). Data analysis was performed with a statistical software package: SigmaStat version 3.1 (Jandel Corporation, San Rafael, CA, USA). Changes in variables within and between groups were analyzed by two-way ANOVA followed by the Holm-Sidak test. P values <0.05 were considered statistically significant.

Results

HO-1 appeared to be highly inducible by all of the stimuli employed (Fig. 1A and B). Specifically, BDL caused an approximately 4-fold elevation, whereas LPS alone led to a nearly 10-fold elevation in this parameter. When LPS was combined with BDL, a similar extent of HO-1 induction was observed to that seen with LPS alone. In the sham-operated animals treated with $GdCl_3$, no changes in this parameter were detected, but the LPS or BDL + LPS-induced increases in HO-1 were significantly reduced following this intervention. HO-2 induction, however, led to a significant elevation (approximately 2-fold) only in the LPS-treated groups, where the alleviating effect of $GdCl_3$ was also evident (Fig. 1A and C).

No increases in the expression of the MT-1 gene were found in response to the different stimuli (Fig. 2A and B), but an enhanced MT-2 gene expression (approximately 3.5-fold) could be demonstrated after LPS alone or when LPS was combined with BDL. No alterations in this parameter were observed in the vehicle-treated animals subjected to 3-day biliary obstruction. Significant and similar degrees of

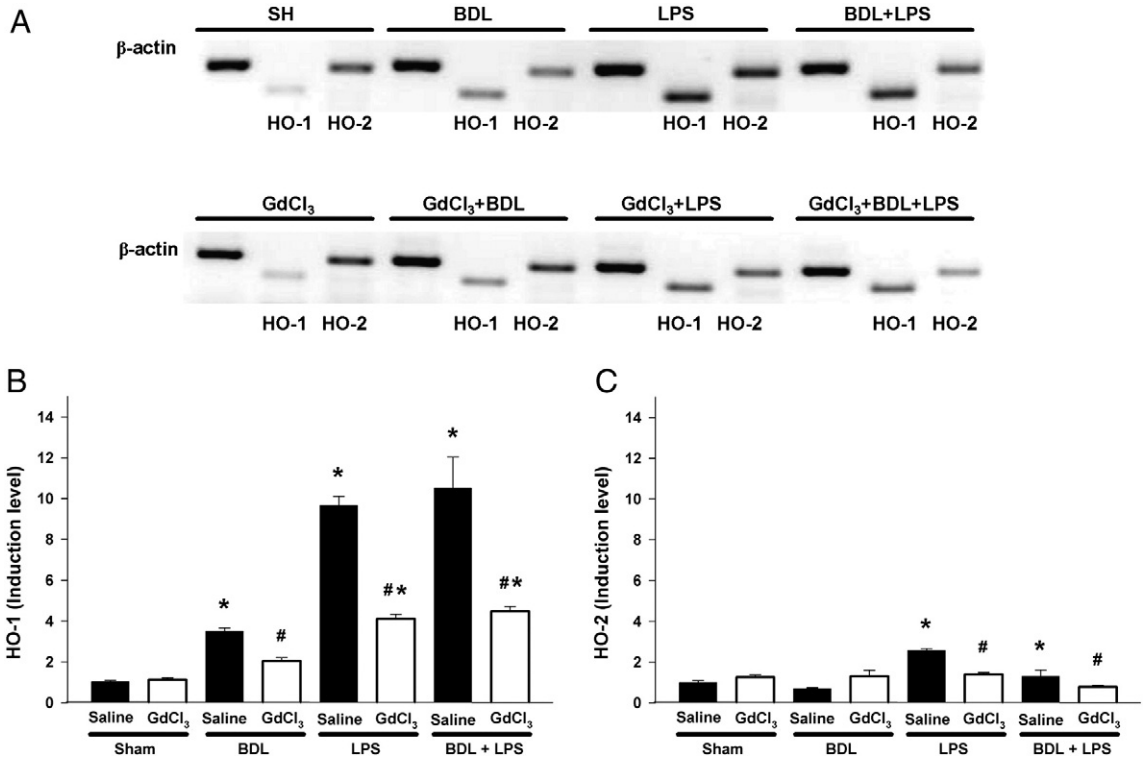

Fig. 1. Representative RT-PCR amplification (A) demonstrating the hepatic expressions of heme oxygenase-1 and 2 (HO-1 and HO-2) in response to sham operation (Sham), endotoxemia induced with lipopolysaccharide (LPS), bile duct ligation (BDL) or the combination of these challenges (BDL + LPS), and the effects of $GdCl_3$ pretreatment. The induction of HO-1 (B) and HO-2 (**C**) in response to the above challenges. Black bars denote the saline-treated animals, while the $GdCl_3$-pretreated animals are represented by white bars. Data are presented as means ± SEM. * $P<0.05$ vs Sham; # $P<0.05$ vs Saline.

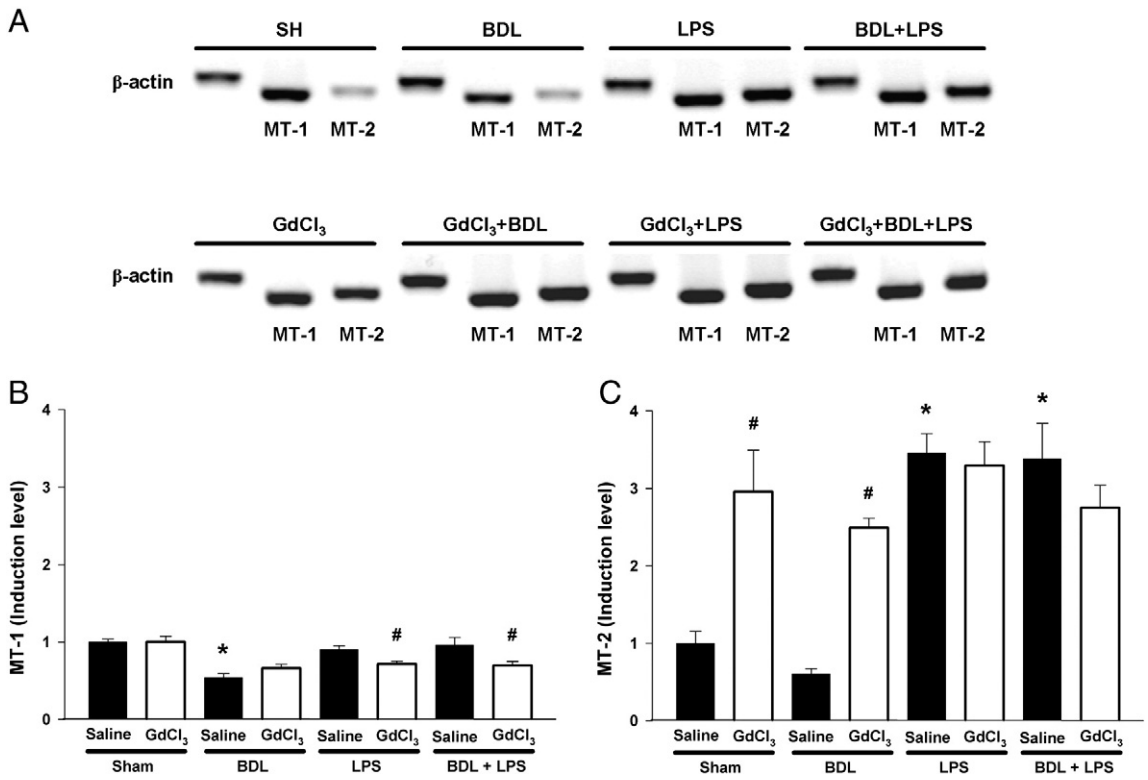

Fig. 2. Representative RT-PCR amplification of metallothionein-1 and 2 (MT-1 and 2), using total RNA as a template prepared from rat liver (A) and induction of MT-1 (B) and MT-2 (C) in response to sham operation (Sham), endotoxemia induced with lipopolysaccharide (LPS), bile duct ligation (BDL) or the combination of these challenges (BDL + LPS) (black bars), and the effects of $GdCl_3$ pretreatment (white bars). Data are presented as means ± SEM. * $P<0.05$ vs Sham; # $P<0.05$ vs Saline.

increase in MT-2 induction were found in all $GdCl_3$-treated groups, irrespective of the different challenges applied (Fig. 2A and C).

As compared with the sham-operated animals, BDL and BDL + LPS caused severe (>50%) reductions in CAT activity, whereas LPS alone gave rise to an approximately 30% decrease in this parameter (Fig. 3.). $GdCl_3$ itself resulted in some reduction (by ~10%) in CAT activity. However, when $GdCl_3$ was applied in the presence of endotoxemia (or endotoxemia combined with BDL), it caused a partial restoration in CAT levels.

An approximately 2-fold increase in Mn-SOD activity was found in all of the challenged groups (Fig. 4A), but the Cu/Zn-SOD activity did not change significantly (Fig. 4B); $GdCl_3$ did not affect these alterations.

A moderately enhanced and comparable degree of DNA damage (approximately 4-fold) was observed in all challenged groups with or without KC blockade elicited by the heavy metal salt $GdCl_3$ (Fig. 5A), and a similar degree of DNA damage was also evidenced in response to this treatment alone. A lower level of DNA breakage was observed only in the animals challenged with BDL in the presence of $GdCl_3$ treatment. The MDA content in the liver was increased significantly (4-fold) only in the most severe condition, when endotoxemia was combined with biliary obstruction (Fig. 5B). $GdCl_3$ enhanced the LPO (2–3-fold) in all groups (including the sham-operated animals) and the degree of LPO again appeared to be independent of the type and severity of the challenge.

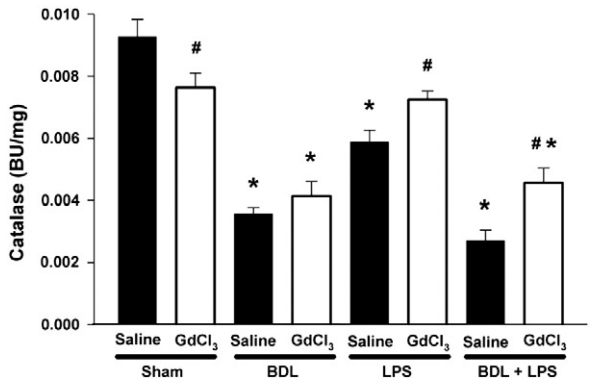

Fig. 3. Changes in activity of catalase (CAT) in response to sham operation (Sham), endotoxemia induced with lipopolysaccharide (LPS), bile duct ligation (BDL) or the combination of these challenges (BDL + LPS) (black bars), and the effects of $GdCl_3$ pretreatment (white bars). Data are presented as means ± SEM. * $P<0.05$ vs Sham; # $P<0.05$ vs Saline.

Discussion

Our earlier experiments demonstrated the effects of obstructive jaundice and endotoxemia, and the beneficial systemic and mircocirculatory consequences of KC blockade with $GdCl_3$ (Lázár et al., 2002; Abrahám et al., 2008). We observed that the effects of BDL are mostly characterized by hepatic perfusion failure and structural damage, whereas in endotoxemia the inflammatory reactions (hallmarked by increased leukocyte accumulation) predominate. Most of the above reactions and also the production of interleukin-6 and tumor necrosis factor alpha (TNF-α) were enhanced when obstructive jaundice was followed by LPS injection (Hoffmann et al., 1994; Busam et al., 1990). In the present study, we set out to characterize the background of these inflammatory reactions at the level of oxidative stress, examining the changes in the activities of antioxidant enzymes and the degree of DNA damage during the above challenges and after KC blockade, and additionally the expression of the HO and MT genes at the transcriptional level and in an isoform-specific manner.

The sensitivity of the liver to inflammatory conditions is illustrated by the fact that the MT and HO gene expressions are more inducible in the liver than in other organs (Hur et al., 1999; De et al., 1990; Guo et al., 2001). As the production of these proteins is mostly regulated at the transcriptional level, the expressions of their genes and not their protein levels were subjected to examination in our present

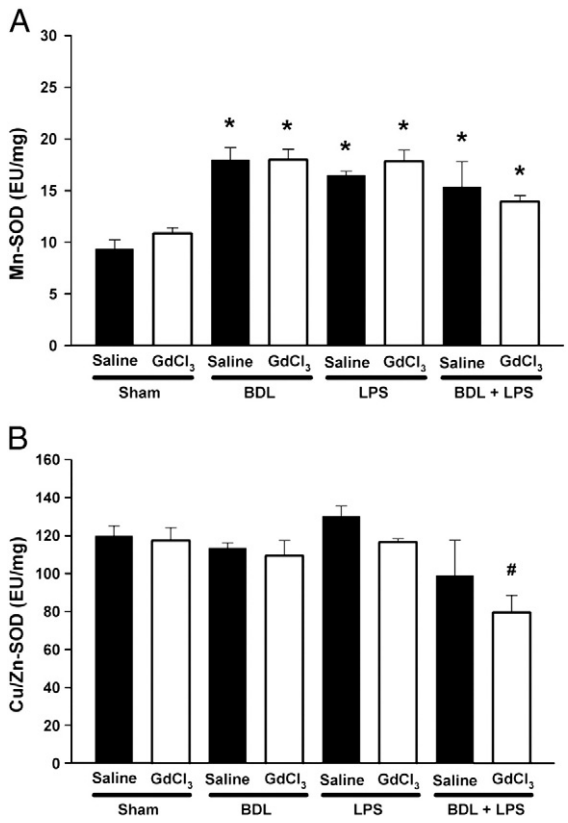

Fig. 4. Changes in activity of manganese-superoxide dismutase (Mn-SOD) (A) and copper/zinc-superoxide dismutase (Cu/Zn-SOD) (B) in response to sham operation (Sham), endotoxemia induced with lipopolysaccharide (LPS), bile duct ligation (BDL) or the combination of these challenges (BDL + LPS) (black bars), and the effects of $GdCl_3$ pretreatment (white bars). Data are presented as means ± SEM. * $P<0.05$ vs Sham; # $P<0.05$ vs Saline.

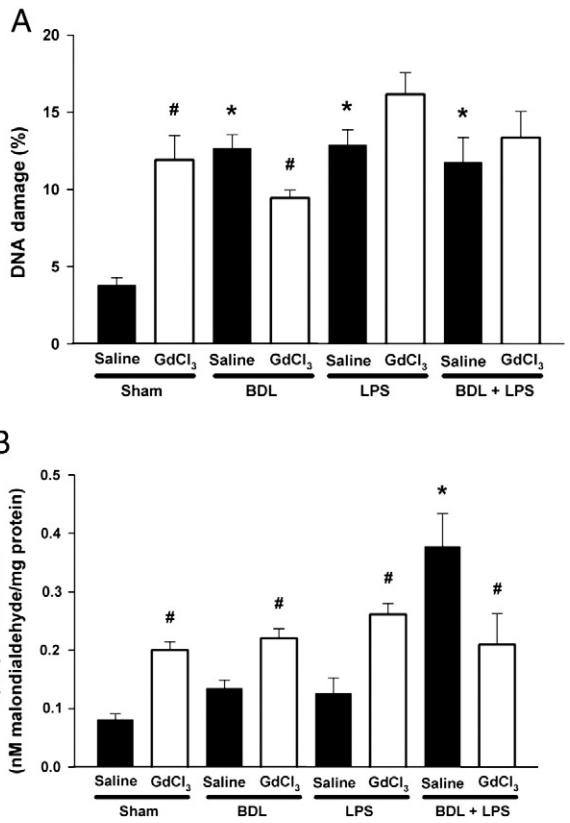

Fig. 5. Extents of deoxyribonucleic acid (DNA) damage (A) and lipid peroxidation (B) in response to sham operation (Sham), endotoxemia induced with lipopolysaccharide (LPS), bile duct ligation (BDL) or the combination of these challenges (BDL + LPS) (black bars), and the effects of $GdCl_3$ pretreatment (white bars). Data are presented as means ± SEM. * $P<0.05$ vs Sham; # $P<0.05$ vs Saline.

models. Such regulation of these genes was apparent in these observations: only the induction of the HO-1 and MT-2 isoforms changed considerably, with the induction level dependent on the stimulus applied. As concerns HO-1, its increased expression is regarded as a sign of the activation of an endogenous protective reaction in response to oxidative stress (Tüzüner et al., 2004). In our study, a moderately increased induction of hepatic HO-1 was observed even as late as 3 days after the biliary obstruction. It is noteworthy, however, that the level of HO-1 induction was higher in the acute phase of endotoxemia than in the later phase of obstructive jaundice. The HO-2 changes demonstrated a similar tendency, with markedly lower induction levels. It is reasonable to assume that endotoxemia alone results in a close to maximal induction of the HO gene, since these changes could not be further enhanced when the BDL and LPS challenges were combined. Furthermore, our measurements were performed at a stage of endotoxemia when the TNF-α levels had attained their maximal values (Abrahám et al., 2008); the elevations in level of this proinflammatory cytokine are effectively prevented by this method of KC blockade (Abrahám et al., 2008; Rizzardini et al., 1998). Since TNF-α is a potential enhancer of HO-1 mRNA expression (Oguro et al., 2002), the lower extent of HO-1 induction may also result from the reducing effect of KC blockade on the TNF-α expression. The HO expression was found to be reduced by $GdCl_3$ in this study, in accord with others (Paxian et al., 2001; Rizzardini et al., 1998), and the magnitudes of the effects elicited by GdCl3 in attenuating the induction of the different HO isoforms were found to be different. These genes differ in their promoter regions or in the positions and amounts of their potential common regulating elements (Alam and Cook, 2007). Consequently, the effects of $GdCl_3$ on the expressions of the distinct HO isoforms can differ in extent.

Another manifestation of the stress-induced hepatic response is induction of the MT gene, which has been described in the early phase of endotoxemia (Giralt et al., 1993) and of biliary obstruction (Brambila et al., 2000). To the best of our knowledge, the isoform-specific changes in hepatic MT expression observed under the above circumstances in the present study are described for the first time here. Similar to the HO-1 changes, the induction of MT-2 (but not MT-1) seen after acute endotoxemia was not enhanced when LPS was combined with BDL. The dependence of the expression of MT-2 on the time course of BDL cannot be ruled out (and could not be assessed in the present study); nonetheless, the induction of this gene was not found to be elevated 3 days after BDL. Further studies are needed to elucidate why additive effects of these challenges are not observed for the above gene (HO and MT) expressions, even though LPS has been shown to worsen the consequences of BDL both experimentally (Harry et al., 1999) and in clinical practice (Greig et al., 1988).

In response to $GdCl_3$ treatment, elevations in MT induction have been observed by others (Andrés et al., 2003; Harstad and Klaassen, 2002; Sauer and Sipes, 1995) and these changes were virtually independent of the noxious stimulus in our study. It is conceivable that the mechanisms of MT induction in endotoxemia and after $GdCl_3$ are different. It is likely that the MT mRNA expression elevation is related to the injury caused by free radicals in the case of endotoxemia, whereas MT induction is a direct consequence of the metal ion overload caused by the heavy metal ion Gd^{3+}. It is also noteworthy that an overload of the heavy metal salt $GdCl_3$, which is most probably a specific trigger of MT induction, caused a similar degree of MT-2 induction to that seen when the challenges were combined. The additive effects of BDL plus LPS are therefore missing either because this timeframe of cholestasis does not influence the MT expression (irrespectively of the presence or absence of endotoxemia) or because MT induction reaches a maximal level which cannot further be enhanced by the combination of BDL with LPS. A milder trigger of MT

induction evoked by a lower dose of LPS may provide a specific answer to this question.

The MT proteins are believed to be protective in nature, representing an intrinsic protective mechanism in endotoxemia (Takano et al., 2004) as well as against heavy metal-induced oxidative damage (Chubatsu and Meneghini, 1993) and against cadmium toxicity (Kawagoe et al., 2005; Liu and Klaassen, 1996; Michalska and Choo, 1993; Masters et al., 1994). The exact consequences of MT-2 induction are also a potential subject of further examinations, targeting the questions of whether (1) only the MT-2 expression remains elevated, (2) binding between $GdCl_3$ and MTs still exists 24 h after the treatment, and (3) if so, how it influences the free radical-scavenging capacity of MT.

In the present experiments, all of the noxious stimuli led to a similar, albeit moderate degree of hepatic DNA single-strand break formation. An increased level of LPO was observed only when BDL and LPS were combined. $GdCl_3$ alone caused elevations in both parameters (particularly in LPO), which lends support to earlier observations regarding the hepatotoxic effects of $GdCl_3$ (Rüttinger et al., 1996; Paxian et al., 2001). Our models demonstrate that the free radical-derived hepatotoxicity overwhelms the endogenous hepatic protective antioxidant mechanisms. This is manifested in the characteristic changes in the CAT and Mn-SOD activities, which may reflect the cumulative effect of free radical toxicity, referring also to the time frame of the challenges. With respect to the CAT activity, BDL and BDL combined with LPS comprise a stronger signal than acute endotoxemia alone. Our results support observations that endotoxemia leads to a decrease in hepatic CAT activity (Llesuy et al., 1994; Spolarics, 1996; Zhong et al., 2002) and we additionally observed a simultaneous increase in Mn-SOD activity. Most BDL studies have yielded similar results concerning CAT activity, but usually the total SOD activities are determined (El-Sayed et al., 2003; Padillo et al., 2004; Singh et al., 1992; Orellana et al., 2000). In our study with BDL, the mitochondrial Mn-SOD activity was found to increase, with a simultaneous decrease in the cytosolic Cu/Zn-SOD level. We also determined the changes in other components of the superoxide targeting antioxidant machinery, the levels of reduced glutathione and glutathione peroxidase, but these varied only insignificantly in response to any of the challenges applied (data not shown). Despite its mentioned toxic effects, a moderate protective effect of $GdCl_3$ was revealed in its partial restoration of the CAT levels in our two models involving endotoxemia.

The characteristics of the induction of the HO and MT genes are rather diverse. One of the main differences is that the HO genes are distinct in their genetic origin, whereas the MT genes are replicates of the same gene. The HO proteins are therefore different in structure and molecular weight, and hence the amounts produced can be adequately determined and compared. In contrast, the MT proteins are much more identical in structure (higher than 80% identity), and hence their induction level can be determined more easily than expression differences at the protein level. Nonetheless, there are common inductors of MT and HO genes. As such, heavy metals and molecules playing roles in oxidative stress, e.g. hydrogen peroxide and nitric oxide, bring about the induction of both genes, because they have similar metal- and antioxidant-responsive elements in their promoter regions (Alam and Cook, 2007; Dalton et al., 1994). As a result, some of the challenges applied in this study (e.g. BDL + LPS) induced certain isoforms of these genes (HO-1 and MT-2) simultaneously. Other stressors such as BDL, however, caused induction of only the HO-1 gene without affecting the induction of the MT gene. It has been shown in earlier studies that HO has both constitutive and inducible isoforms and the inducible HO-1 isoform therefore expectedly reached higher expression levels in this study (for a review, see Wagener et al., 2003). Both isoforms of MT, however, are expressed constitutively in the liver and may therefore differ in tissue specificity and stress-dependent inducibility (for a review, see Thirumoorthy et al., 2007).

The specific roles of the distinct isoforms of HO and MT are not fully understood, but can most probably be attributed to the differences in tissue localization or the specific inducibility of the actual encoding gene. Likewise, since HO-1 is produced mostly in the Kupffer cells (Goda et al., 1998), the alleviating effect of this enzyme may lie in the antioxidant and immune response modulator properties of its products. This may represent an endogenous protective mechanism against environmental stress and a good indicator of this response to the stress reaction induced by BDL or endotoxemia. HO-2 is produced in the hepatic parenchymal cells (Goda et al., 1998), and hence it may have a role in regulating sinusoidal endothelium-related functions (such as synosoidal perfusion). The expression of MT, however, did not show any increase after BDL. In another study, where the time sequence of MT expression could be assessed, the highest levels of MT expression occurred in earlier phases of biliary obstruction, which was attributed not to the hepatocellular damage caused by BDL, but to the increased Zn levels caused by cholestasis (see Brambila et al., 2000). Owing to methodological difficulties, the distinct roles of the different isoforms of MT have not been clarified yet and cannot be judged on the basis of our present results either. The available literature data suggest that the possible roles of MT may include the detoxification of metals in response to cholestasis and the elimination of free radical-mediated injury in endotoxemia.

The findings in previous reports and the present data reveal that the role of $GdCl_3$ is rather controversial. Besides reducing the KC phagocytic activity and enhancing KC apoptosis, $GdCl_3$ also affects the hepatocyte function, with concomitant reductions of the hepatic NO, PGE2 and cAMP levels (Ding et al., 2003). The toxicity of Gd^{3+} depends on its dose and on its chemical form (hydroxylated/protein complex form) (Ding et al., 2003) In other studies, $GdCl_3$ alone brought about enhanced TNF-alpha and interleukin-6 release and caused a microcirculatory perfusion failure (Rüttinger et al., 1996). The present study reveals some toxic aspects of $GdCl_3$, such as enhanced LPO and DNA damage, independently of the various challenges, but no microcirculatory deterioration. On the other hand, most reports attribute protective features to $GdCl_3$ such as an alleviation of cadmium chloride (Harstad and Klaassen, 2002) or retinol (Sauer and Sipes, 1995) toxicity. We earlier reported that $GdCl_3$ ameliorates the microcirculatory and structural consequences of endotoxemia and obstructive jaundice, also reducing inflammatory cytokine release (TNF and IL-6) (Lázár et al., 2002; Abrahám et al., 2008). The present study has demonstrated further alleviating effects of $GdCl_3$, e.g. the stress-induced induction of HO-1 is reduced and the CAT levels are partially restored. We believe that the final outcome of the challenges applied corresponds strongly with the functional and structural impairment of the liver.

Conclusion

Accordingly, ameliorated hepatic injury (structural, functional and microcirculatory), together with the reduced stress-induced induction of the HO gene, reflect the obvious predominance of the positive effects of $GdCl_3$ in the present models. Taken together, the present findings reveal that the inhibition of KC functions exerts a beneficial influence on the complications of obstructive jaundice. Reduced expressions of HO-1 and CAT may comprise further evidence of these protective effects. Compounds targeting KC inactivation with no toxic side-effects may beneficially affect the clinical outcome of inflammatory complications of obstructive jaundice.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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