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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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 (GdCl₃)-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 GdCl₃ 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 GdCl₃ pretreatment. HO induction was significantly lower in each model.

Significance: Despite its moderate hepatocellular toxicity, the ameliorated stress-induced hepatic reactions provided by GdCl₃ may contribute to its protective effects.

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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; Ábrahám 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 hallmarkied 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...
KCs suggests that these self-defense 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 (Husztik et al., 1980) 24 h before the liver biopsies; the animals were sacrificed by intraperitoneal injection of 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 synthesized 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/MgCl₂, 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, isomorph-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/GelBiot™ 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.

CAT 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 μmol H₂O₂/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 $5 \times 10^{-3}$ 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 ($F$, $F_{\text{min}}$, and $F_{\text{max}}$) 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 unwinding. To determine $F_{\text{min}}$, the DNA samples were kept at pH 12.4, but before the incubation period, they were sonicated for 1 min. For $F_{\text{max}}$ 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) = $(F - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}}) \times 100$.

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.](image)
increase in MT-2 induction were found in all GdCl₃-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.). GdCl₃ itself resulted in some reduction (by ~10%) in CAT activity. However, when GdCl₃ 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); GdCl₃ 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 GdCl₃ (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 GdCl₃ 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). GdCl₃ 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.

Discussion

Our earlier experiments demonstrated the effects of obstructive jaundice and endotoxemia, and the beneficial systemic and microcirculatory consequences of KC blockade with GdCl₃ (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
The effect of KC blockade on 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 potent 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 GdCl3 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 regulatory elements (Alam and Cook, 2007). Consequently, the effects of GdCl3 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 (Greg et al., 1988).

In response to GdCl3 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 GdCl3 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 GdCl3, 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

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 GdCl3 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 GdCl3 pretreatment (white bars). Data are presented as means±SEM. *P<0.05 vs Sham; #P<0.05 vs Saline.
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 GdCl₃ 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. GdCl₃ alone caused elevations in both parameters (particularly in LPO), which lends support to earlier observations regarding the hepatotoxic effects of GdCl₃ (Rüttinger et al., 1996; Paxon 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 GdCl₃ was revealed in its partial restoration of GdCl₃ was revealed in its partial restoration of protective effect of GdCl₃ and MTs still exists 24 h after the treatment, and (3) if so, how it influences the free radical-scavenging capacity of MT.

Conclusions

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 GdCl₃ 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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