Published in final edited form as:
Toxicol Appl Pharmacol. 2010 January 15; 242(2): 182–190. doi:10.1016/j.taap.2009.10.006.

MECHANISM OF PROTECTION BY METALLOTHIONEIN AGAINST ACETAMINOPHEN HEPATOTOXICITY

Chieko Saito[a], **Hui-Min Yan**[a], **Antonio Artigues**[b], **Maria T. Villar**[b], **Anwar Farhood**[c], and **Hartmut Jaeschke**[a]

Chieko Saito: csaito@kumc.edu; Hui-Min Yan: hyan@kumc.edu; Antonio Artigues: aartigues@kumc.edu; Maria T. Villar: mvillar@kumc.edu; Anwar Farhood: afarhood@seton.org; Hartmut Jaeschke: hjaeschke@kumc.edu

[a]Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas 66160

[b]Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160

[c]Department of Pathology, Brackenridge Hospital, Austin, Texas 78701

Abstract

Acetaminophen (APAP) overdose is the most frequent cause of drug-induced liver failure in the US. Metallothionein (MT) expression attenuates APAP-induced liver injury. However, the mechanism of this protection remains incompletely understood. To address this issue, C57BL/6 mice were treated with 100 μmol/kg $ZnCl_2$ for 3 days to induce MT. Twenty-four hours after the last dose of zinc, the animals received 300 mg/kg APAP. Liver injury (plasma ALT activities, area of necrosis), DNA fragmentation, peroxynitrite formation (nitrotyrosine staining), MT expression, hepatic glutathione (GSH) and glutathione disulfide (GSSG) levels were determined after 6 h. APAP alone caused severe liver injury with oxidant stress (increased GSSG levels), peroxynitrite formation and DNA fragmentation, all of which were attenuated by zinc-induced MT expression. In contrast, MT knockout mice were not protected by zinc. Hydrogen peroxide-induced cell injury in primary hepatocytes was dependent only on the intracellular GSH levels but not on MT expression. Thus, the protective effect of MT *in vivo* was not due to the direct scavenging of reactive oxygen species. Zinc treatment had no effect on the early GSH depletion kinetics after APAP administration, which is an indicator of the metabolic activation of APAP to its reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI). However, MT was able to effectively trap NAPQI by covalent binding. We conclude that MT scavenges some of the excess NAPQI after GSH depletion and prevents covalent binding to cellular proteins, which is the trigger for the propagation of the cell injury mechanisms through mitochondrial dysfunction and nuclear DNA damage.

Keywords

Acetaminophen; hepatotoxicity; metallothionein; oxidant stress; liver failure; covalent binding

For Correspondence: Hartmut Jaeschke, Ph.D., Department of Pharmacology, Toxicology & Therapeutics, University of Kansas Medical Center, 3901 Rainbow Blvd, MS 1018, Kansas City, KS 66160, Tel. (913) 588 7969, Fax. (913) 588 7501, hjaeschke@kumc.edu.

CONFLICT OF INTEREST

None of the authors has any conflict of interest to declare.

INTRODUCTION

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug. Although safe at therapeutic levels, an overdose can cause severe liver injury in animals and in humans (Larson et al., 2005). The toxicity depends on the metabolic activation of APAP via cytochrome P-450, which results in the formation of an electrophilic reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Nelson, 1990). NAPQI is rapidly conjugated with glutathione (GSH) and the GSH-APAP adduct is excreted mainly into bile (Nelson, 1990). However, after the cellular GSH content is exhausted, NAPQI covalently binds to cellular proteins (Cohen and Khairallah, 1997) including mitochondrial proteins (Qiu et al., 2001; Tirmenstein and Nelson, 1989). This covalent binding is thought to be responsible for the mitochondrial dysfunction observed after APAP overdose including inhibition of mitochondrial respiration, ATP depletion, release of intermembrane proteins, and mitochondrial oxidant stress and peroxynitrite formation (Jaeschke and Bajt, 2006). The characteristic nuclear DNA damage after APAP overdose (Ray et al., 1990) is initially linked to mitochondrial Bax translocation (Bajt et al., 2008a) and later to oxidant stress/peroxynitrite formation and mitochondrial dysfunction (Cover et al., 2005). The opening of mitochondrial membrane permeability transition (MPT) pores with collapse of the membrane potential and declining ATP levels (Kon et al., 2004) together with the extensive nuclear DNA damage (Shen et al., 1991) are the main reasons for the massive oncotic necrotic cell death after APAP overdose (Gujral et al., 2002).

In addition to the intracellular signaling pathways of APAP-induced liver injury, a potential contribution of inflammatory cells is extensively but controversially discussed (Jaeschke, 2005, 2008; Liu and Kaplowitz, 2006). For example, investigations into the role of neutrophils yielded opposite results using the same neutropenia-inducing antibody (Liu et al., 2006; Cover et al., 2006). We suggested that the different results were caused by the prolonged pretreatment regimen in one of the studies that lead to a preconditioning effect with induction of several acute phase genes, in particular metallothionein (MT) (Jaeschke and Liu, 2007). The upregulation of any of these genes rather than neutropenia could have been the reason for the protection of the anti-neutrophil antibody. However, in order to address this complex problem, it is necessary to understand the mechanism by which some of these individual genes including MT could protect against APAP hepatotoxicity.

MT, a ubiquitous heavy metal binding and cysteine-rich protein (Klaassen et al., 1999), has been suggested to react with free radicals and organic electrophiles (Cagen and Klaassen, 1980). Studies of APAP hepatotoxicity demonstrated enhanced liver injury and mortality in metallothionein-deficient mice (Rofe et al., 1998; Liu et al., 1999). In addition, induction of MT attenuated APAP-induced liver injury (Chengelis et al., 1986; Szymanska et al., 1991). The modulation of toxicity was independent of the P450 levels and the metabolic activation of APAP (Rofe et al., 1998; Liu et al., 1999). In contrast, the protection appeared to be correlated with an antioxidant function of MT (Liu et al., 1999). However, no direct evidence for this mechanism exists *in vivo*. In particular, it remains unclear if MT can actually scavenge reactive oxygen species (ROS) and peroxynitrite, which are critical mediators of the MPT and cell death (Jaeschke et al., 2003). Thus, the main objective of this investigation was to investigate the mechanism by which induction of MT gene expression may protect against APAP hepatotoxicity *in vivo*.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (8–10 weeks old), male age-matched wild type 129S1/SvImJ and male MT-1/MT-2 deficient mice (129S7/SvEvBrd-*Mt1*^{tm1Bri} *Mt2*^{tm1Bri}/J) were purchased from

Jackson Laboratories (Bar Harbor, ME). Animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals". The experimental protocol was approved by the institutional animal care and use committee of Kansas University Medical Center. Some animals received a non-toxic dose of 100 μmol/kg zinc chloride (Fluka Chemical Corp., Milwaukee, WI) dissolved in saline (9.9 μmol/ml) subcutaneously once a day for 3 days (Liu et al., 2009). All animals were fasted overnight and on the 4th day, they received 300 mg/kg APAP (Sigma Chemical Co., St. Louis, MO) dissolved in warm saline (15 mg/ml) by intraperitoneal injection.

Experimental protocols

At 6 h or 24 h after APAP administration, animals were killed by cervical dislocation under isoflurane anesthesia. Blood was drawn from the vena cava into heparinized syringes and centrifuged. The plasma was used for determination of alanine aminotransferase (ALT) activities with the kinetic test kit 68-B (Biotron Diagnostics, Inc., Hernet, CA). Immediately after collecting the blood, the livers were excised and rinsed in saline. A small section from each liver was placed in 10% phosphate buffered formalin to be used for histology. The remaining liver was frozen in liquid nitrogen and stored at −80°C. Total soluble GSH and GSSG were measured in the liver homogenate with a modified method of Tietze as described in detail (Jaeschke and Mitchell, 1990).

Histology and immunohistochemistry

Formalin-fixed tissue samples were embedded in paraffin and 5 μm sections were cut. Replicate sections were stained with hematoxylin and eosin (H&E) for blinded evaluation of the areas of necrosis by the pathologist. The percent of necrosis was estimated by evaluating the number of microscopic fields with necrosis compared to the entire cross section. Additional sections were stained for nitrotyrosine (NT) protein adducts with the DAKO LSAB peroxidase Kit (K684) (DAKO Corp., Carpinteria, CA) according to the manufacturer's instruction using an anti-nitrotyrosine antibody (Molecular Probes, Eugene, OR). For the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, liver sections were stained with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN) as described in the manufacturer's instructions (Gujral et al., 2002).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Expression of selected genes was quantified using qRT-PCR analysis as previously described (Bajt et al., 2008b). Briefly, total RNA was reversed transcribed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo-dT primers (ABI Primer Express software, Foster City, CA). The SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values. Ct values for the various genes were first normalized with that of β-actin in the same sample, and then relative differences between groups were expressed as relative increases setting control as 1.

Western blotting

Metallothionein protein levels were analyzed by western blotting as described in detail (Bajt et al. 2000). A monoclonal mouse anti-metallothionein antibody (DAKO Corp., Carpinteria, CA) was used as the primary antibody. Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech. Inc., Piscataway, NJ) according to the manufacturer's instructions.

Mouse hepatocyte isolation

Primary hepatocytes were isolated from overnight fasted mice with a standard collagenase procedure as previously described in detail (Bajt et al., 2004). Untreated and $ZnCl_2$-treated animals were used. Some of the animals were treated with 100 mg/kg phorone i.p. (Sigma) to deplete hepatic GSH levels 90 min before cell isolation. The GSH-depleted cells were then incubated in the presence of the GSH synthesis inhibitor buthionine sulfoximine (1 mM) (Sigma). From the isolation of one mouse liver, a typical yield was about 50–60 $\times 10^6$ hepatocytes. Cell viability, as determined by trypan blue exclusion, was generally >90%, and cell purity was >95% hepatocytes. Cells were plated in six-well plates (6×10^5 cells/well) (Biocoat collagen I cellware plates; Becton Dickinson) in Williams' Medium E (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin/streptomycin, and 1×10^{-7} M insulin and cultured at 37 °C in room air with 5% CO_2. After an initial 4 h attachment period, cultures were washed with phosphate-buffered saline (PBS) and then plain culture medium (controls) or media containing various concentrations of hydrogen peroxide were added. Cell injury was assessed by lactate dehydrogenase (LDH) release into the medium. LDH activities were measured as described (Bajt et al., 2004).

Analysis of MT-NAPQI interactions by mass spectrometry

MT from rabbit liver was purchased from Sigma (M5269) as a lyophilized powder. The protein was resuspended in deionized water to a protein concentration of 1 mg/ml. NAPQI was purchased from Sigma (A7300) and diluted in water to a final concentration of 12 μg/ml. For mass spectrometric measurements, protein samples were desalted on a C18 reverse phase column (Zorbax C18SB Wide pore guard Column, MicroTech Scientific, 1 cm × 0.32 mm), which was connected online to a valve to direct the flow either to waste or to the mass spectrometer (MS). After washing the column with 0.1% (v/v) TFA at a flow of 100 μl/min, the flow was directed to the mass spectrometer and the protein eluted using a 0–60% (v/v) acetonitrile gradient in 0.1% TFA at a flow rate of 20 μl/min. Electrospray ionization (ESI) MS data were acquired in the m/z range 800–2000 on a ThermoFinnigan LTQ FT. The mass spectrometer was under manual control to facilitate switching between two modes of data acquisition, on the Ion Trap (IT) and on the Ion Cyclotron Resonance Fourier Transform cell (ICR FT). Final optimized settings for detection of MT were: ion spray voltage 2.1 kV, capillary temperature 250°C, capillary voltage 34V. The two modes of operation have different sensitivity and mass resolution. The charge state of all protein species detected was calculated from the isotopic distribution of the high resolution spectrum obtained in the ICR FT. However, due to the higher sensitivity of the IT, data reported here are for the acquisition on the IT. Masses of the different protein variants were calculated from the multiple charged protein ions using the deconvolution software included in BioworksBrowser V. 3.1 (ThermoFinningan), the resulting masses were measured with an experimental precision of ± 1.5 daltons.

Statistics

Data are expressed as means ± S.E. Comparison between two groups were performed with Student's *t*-test or one-way ANOVA followed by Bonferroni *t*-test for multiple groups. If the data were not normally distributed, the Mann-Whitney test was applied for comparison of two groups and the Kruskal-Wallis Test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test for multiple groups. $P<0.05$ was considered significant.

RESULTS

Hepatic MT induction by $ZnCl_2$

Treatment with a non-toxic dose of $ZnCl_2$ for 3 days resulted in a 100-fold increase of MT-1 mRNA expression and a 190-fold increase in MT-2 (Figure 1A). No other gene measured

showed a significant change including CuZnSOD, MnSOD, GPx1, catalase, HO-1, HSP70, TNF-α, IL-1β, IL-10, KC, MIP-2, iNOS or ICAM-1 (data not shown). Similar gene expression profiles were recently reported for the same dose of $ZnCl_2$ given over 4 days (Liu et al., 2009). The increase in MT mRNA translated into extensive MT protein expression as indicated by western blot analysis (Figure 1B) and immunohistochemical staining of liver sections (Figure 1C).

Hepatic MT induction protects against APAP toxicity

A dose of 300 mg/kg of APAP induced severe liver injury at 6 h as indicated by the increase in plasma ALT activities and the extensive necrosis in the liver (Figure 2, 3). Pretreatment with $ZnCl_2$ attenuated the increase in plasma ALT levels by 68% and reduced the area of necrosis by 62% compared to APAP alone (Figure 2, 3). A similar protective effect was observed at 24 h (data not shown). To investigate the mechanism of protection, liver sections were stained for nitrotyrosine protein adducts, an indicator for peroxynitrite formation, and the TUNEL assay, an indicator for nuclear DNA fragmentation. Whereas there was no nitrotyrosine or TUNEL staining in untreated controls (data not shown), extensive centrilobular areas stained positive for both nitrotyrosine and DNA fragmentation 6 h after APAP treatment (Figure 3). The staining correlated with the area of necrosis as assessed in H&E stained sections (Figure 3). Pretreatment with $ZnCl_2$ substantially reduced both nitrotyrosine staining and the number of TUNEL-positive cells (Figure 3). Since most peroxynitrite is being formed in mitochondria (Cover et al., 2005), these data suggest that MT induction either prevented the mitochondrial oxidant stress or scavenged peroxynitrite.

MT induction prevents APAP-induced oxidant stress

In a previous study, we demonstrated that ROS (mainly hydrogen peroxide) but not peroxynitrite was responsible for GSSG formation in the liver after APAP overdose (Knight et al., 2002). To assess if MT can scavenge hydrogen peroxide *in vivo*, hepatic GSH and GSSG levels were measured in the liver (Figure 4). At 6 h after APAP treatment, total glutathione (GSH+GSSG) levels only partially recovered (Figure 4A), the GSSG content was significantly increased (Figure 4B) and the GSSG-to-GSH ratio was increased from 0.5% to 2.5% (Figure 4C). The data indicate a significant oxidant stress in these livers consistent with previous reports that identified this GSSG increase as being predominantly in mitochondria (Jaeschke, 1990; Knight et al., 2001). Induction of MT with $ZnCl_2$ improved the recovery of hepatic GSH levels, attenuated the increase in GSSG content and significantly reduced the GSSG-to-GSH ratio (Figure 4A–C). These results suggest that MT induction attenuated the formation of reactive oxygen species *in vivo* rather than scavenged them. To directly assess if MT is able to scavenge reactive oxygen in intact cells, isolated mouse hepatocytes were exposed to various concentrations of hydrogen peroxide (H_2O_2), the ROS most likely responsible for the elevated GSSG levels. H_2O_2 dose-dependently caused cell injury as indicated by LDH release (Figure 5A,B). However, pretreatment with $ZnCl_2$ did not affect the injury in normal cells. In contrast, prior depletion of GSH substantially aggravated H_2O_2 – induced cell death (Figure 5A,B). In agreement with these results, $ZnCl_2$ – pretreatment did not affect cell injury in GSH-depleted hepatocytes (data not shown). Together these data suggest that intracellular GSH was able to detoxify H_2O_2 diffusing into the cell. However, intracellular MT expression did not impact ROS-mediated toxicity indicating that MT, in contrast to GSH, did not detoxify H_2O_2. Overall, the combined *in vitro* and *in vivo* data suggest that MT prevented APAP-induced ROS formation rather than scavenged ROS.

MT induction did not affect NAPQI formation

The fact that MT induction prevented the oxidant stress indicates that MT must have acted upstream of mitochondria. To evaluate if $ZnCl_2$ treatment affected NAPQI formation, the initial

depletion kinetics of GSH was measured. During the first 20 min after APAP injection, the rapid decline of hepatic GSH levels is caused almost exclusively by NAPQI formation (Jaeschke, 1990). Controls and $ZnCl_2$ – treated animals showed a similar rate of GSH loss during the first 20 min (Figure 6). However, livers from $ZnCl_2$ – treated mice had significantly higher basal GSH levels than livers from control animals (Figure 6). The difference was maintained during the first 20 min and disappeared when GSH depletion in controls slowed down more rapidly compared to the $ZnCl_2$ - treated animals (Figure 6). Together, these data suggest that NAPQI formation was not impaired by MT induction but the livers from $ZnCl_2$ - treated animals had a higher capacity to detoxify NAPQI. The slightly higher GSH level may have been a contributing factor but was unlikely the only reason for the effective protection against APAP-induced liver injury.

MT can scavenge and covalently bind NAPQI

Since MT induction did not reduce NAPQI formation but prevented the mitochondrial oxidant stress, which is assumed to be caused by NAPQI binding to mitochondrial proteins, the only remaining intervention point could be the direct scavenging of NAPQI by MT. To test this hypothesis, a solution of purified MT (1 mg/ml) was mixed with NAPQI solution (12 μg/ml) at 4°C and transferred immediately or after 2 h of incubation at 4°C to the loop of an HPLC connected online to a mass spectrometer and analyzed as indicated in Material and Methods (Figure 7). As a control sample, an aliquot of MT kept under the same conditions, but without the addition of NAPQI, was analyzed under the identical conditions. This control sample showed multiple ions in the m/z range 1537–1566, corresponding to the +4 charge state of the protein (Figure 7A). This is consistent with the presence of several isoforms of MT in the commercially available form of the protein. Mixing of MT with NAPQI resulted in the immediate disappearance of the ions at m/z range 1537–1566 with a time-dependent formation of a series of ions at a higher m/z range (1592–1644) (Figure 7B). Four of these ions, at m/z at 1637.6, 1643.7, 1665.4 and 1671.7, are clearly newly formed. Other ions, at m/z at 1592.7, 1611.6 and 1618.3, appear on top of small ions present in the commercial preparation of MT. However, their relative change in intensity indicates that these ions are also the result of the reaction of NAPQI with MT. The dead time between the addition of NAQPI and the injection on the loop of the HPLC to start the chromatographic separation was 20 sec. This reaction is complete following 2 h incubation at 4°C (Figure 7C). Because of the complexity of the resulting mass spectra, the exact pair of shifts in mass can not be determined with absolute certainty. However, given the accuracy of the mass measurement and the fact that the reaction of NAPQI with proteins results in an increase in mass of 149.1 Dalton (Hoos et al., 2007), it is possible to assume that most of the isoforms of MT reacted with one, two or three molecules of NAPQI, resulting in a shift of 149 Dalton (from m/z 1554.6 to m/z 1592.7), 298 Dalton (from m/z 1537.1, 1545.1, 1561.2 and 1565.6 to 1611.6, 1618.3, 1637.6 and 1643.7, respectively) or 447 Dalton (from m/z 1554.7 and 1561.2 to 1565.4 and 1671.7, respectively).

MT induction is responsible for $ZnCl_2$-induced protection against APAP hepatotoxicity

To verify that MT gene expression was indeed the mechanism by which Zn reduced APAP-induced liver injury, 129S1/SvImJ wild type and MT-deficient mice were treated with APAP for 6 h. Based on plasma ALT activities and the area of necrosis, APAP caused severe liver injury, which was significantly attenuated in Zn-treated wild type animals but not in MT-deficient mice (Figure 8). These data support the conclusion that MT gene expression is the main mechanism of protection of the Zn treatment regimen employed in this study.

DISCUSSION

The objective of this investigation was to evaluate the mechanism(s) of protection of MT induction by $ZnCl_2$ against APAP hepatotoxicity. Our data support the conclusion that

$ZnCl_2$ selectively induced MT gene expression, which did not scavenge reactive oxygen but appeared to prevent the oxidant stress by scavenging the reactive metabolite NAPQI.

It is well established that heavy metals including Zn can induce MT gene expression in the liver (Klaassen et al., 1999; Liu et al., 2009). Our data confirmed this observation and showed that both MT-1 and MT-2 mRNA levels were induced in the liver and that this translated into a substantial increase in MT protein expression in hepatocytes. Despite the effective induction of MT by Zn, there is some controversy whether this is the main mechanism by which Zn can protect against drug-induced liver injury. For example, Zn supplementation in the diet attenuated alcoholic liver injury in MT-deficient mice during the 12 week study period suggesting that the protection was independent of MT (Zhou et al., 2005). However, the average oral Zn intake was calculated to be 33 mg/kg/day (505 μmol/kg/day), which is about 5 times higher than the subcutaneous dose used in the present investigation for 3 days. A similar s.c. treatment of Zn did not protect MT-deficient mice against APAP-induced liver injury (Liu et al., 1999). We could confirm these findings (Figure 8) suggesting that MT induction was the principle mechanism of protection. In addition, we showed that 100 μmol/kg/day $ZnCl_2$ selectively induced MT but did not affect a number of other genes, whose upregulation has been shown to be beneficial against APAP-induced liver injury. This includes HO-1 (Chiu et al., 2002), HSP70 (Tolson et al., 2006) and MnSOD (Fujimoto et al., 2009). A recent genomic study using the same dose of Zn reported similar findings (Liu et al., 2009). These data together suggest that the protective effect of $ZnCl_2$ treatment was mainly caused by MT induction in hepatocytes. However, the slightly higher hepatic GSH levels in $ZnCl_2$-treated animals may have also contributed to the protection. This effect of Zn could have been caused by the moderate upregulation of nuclear factor-erythroid 2-related factor 2 (Nrf2) (Liu et al., 2009), which positively regulates GSH synthesizing enzymes (Lu, 2009).

It has been extensively postulated that MT can scavenge reactive oxygen species (Kumari et al., 1998; Sato and Bremner, 1993; Thornalley and Vasak, 1985). However, most studies were done with isolated proteins in the test tube. In an *in vivo* study where it was concluded that MT protected against APAP toxicity by acting as ROS scavenger it remained unclear if MT prevented formation of ROS or scavenged them (Liu et al., 1999). Our *in vivo* data demonstrated that MT induction reduced nitrotyrosine staining and prevented DNA fragmentation, which is dependent on mitochondrial peroxynitrite formation and dysfunction (Bajt et al., 2008a; Cover et al., 2005). These results can be interpreted as if MT acted as an effective scavenger of peroxynitrite in the mitochondria or that MT acted upstream and prevented the formation of ROS. The fact that MT induction reduced hepatic GSSG levels and the GSSG-to-GSH ratio after APAP suggested that the formation of ROS, especially hydrogen peroxide, was prevented. This conclusion is supported by previous findings showing that GSSG levels remained elevated when only peroxynitrite is scavenged but ROS formation is unaffected (Knight et al., 2002). In addition, the experiments with isolated hepatocytes exposed to various concentrations of hydrogen peroxide did not indicate that MT was able to effectively scavenge ROS in the presence or absence of GSH. In contrast, GSH was the critical antioxidant controlling ROS-induced cell death in hepatocytes. This shows that hydrogen peroxide entered the cells and a competent scavenger (GSH) was able to intercept this oxidant in the cytosol. In general, peroxides diffuse into the cell, trigger mitochondrial dysfunction and ultimately cause MPT pore formation and cell necrosis (Nieminen et al., 1995). Although most MT is expressed in the cytosol after induction, MT did not affect cell injury. This indicates that MT is not a relevant scavenger of hydrogen peroxide, which suggests that the reduced hepatic GSSG levels and the reduced GSSG-to-GSH ratio in APAP-treated animals are unlikely to be caused by the scavenging of ROS especially not in the presence of GSH. Thus, our findings indicate that MT acted upstream of the initiation of the mitochondrial oxidant stress.

The main event upstream of mitochondrial protein binding is the formation of the reactive metabolite NAPQI. Inhibition of the metabolic activation is most effective in preventing APAP toxicity. Since NAPQI is detoxified by GSH, the initial decline of the hepatic GSH levels reflects the formation of NAPQI (Jaeschke, 1990). Based on the GSH depletion kinetics, there was no relevant difference between controls and $ZnCl_2$-treated animals suggesting that there was no difference in NAPQI formation. However, it was observed that the basal GSH levels after $ZnCl_2$ were higher than in controls. Although the difference was only 11%, the data indicate a slightly higher capacity to detoxify NAPQI in the $ZnCl_2$-treated livers. This effect may have contributed to the protective effect of $ZnCl_2$ treatment against APAP toxicity. Nevertheless, it is unlikely that this limited amount of additional GSH could have been entirely responsible for the protection against APAP toxicity.

The only remaining explanation for the protective effect of MT after NAPQI formation but before mitochondrial protein binding would be a direct scavenging of NAPQI by MT. Due to its high content of cysteine residues, MT can react with organic electrophiles (Cagen and Klaassen, 1980) including NAPQI (Roberts et al., 1987). Our mass spectrometric data confirm that possibility and suggest a time-dependent covalent binding of 1–3 NAPQI molecules to each MT protein. Since the depletion kinetics of GSH is not affected, these data suggest that MT reacts with NAPQI only when GSH levels are very low. A higher affinity of NAPQI to GSH as compared to the sulfhydryl groups of MT has also been observed with other electrophiles (Cagen and Klaassen, 1980). These findings indicate that MT induction is not a primary detoxification mechanism for organic electrophiles but MT can become important once GSH is depleted as it is after APAP overdose.

Despite the strong support by our experimental data for the proposed mechanisms of MT-mediated protection against APAP hepatotoxicity, there are several issues that need to be discussed. First, why was no protein binding evaluated? We suggest that MT functions as decoy to reduce binding to more vitally important proteins. However, an antibody against APAP-protein adducts would not distinguish between MT-APAP adducts and other protein-APAP adducts and therefore a shift in protein binding would not be detectable. Second, given the high cellular concentrations of GSH and the higher affinity to NAPQI than MT, will there be enough MT to successfully compete with GSH? The average hepatic GSH levels decline by more than 90% within less than 1 h after APAP treatment (Knight et al., 2001). Since the concentration of GSH may even be lower in the centrilobular area and MT contains more than 20 cysteine residues per molecule, the total available sulfhydryl groups from MT can indeed exceed those provided by GSH and thus it is feasible that MT can compete for NAPQI under these conditions. Third, Liu et al. (1999) found no consistent difference in APAP protein adducts between wild-type control and MT-deficient mice, which does not support MT as an important NAPQI scavenger. However, in these studies the effect of endogenous MT induction, which is much lower than Zn-induced MT expression, was studied (Liu et al., 1999). Importantly, most protein adducts are formed within less than 2 hours after APAP administration, i.e. well before endogenous MT is up-regulated in response to APAP-induced stress. Thus, in contrast to pretreatment with Zn where MT is maximally induced before APAP is injected, one would not expect that the low baseline levels of MT and the late up-regulation of endogenous MT can effectively interfere with the early protein binding, which is the initiating event for mitochondrial dysfunction. Since not the overall protein binding but the binding of critical targets in the mitochondria determines the initiation of toxicity (Qiu et al., 1998, 2001; Tirmenstein and Nelson, 1989), mitochondrial not cytosolic protein adducts, as assessed by Liu et al. (1999), are expected to change. The question remains, why does endogenous MT affect APAP toxicity as indicated by the higher susceptibility of MT1/2-deficient mice (Liu et al., 1999). Since this difference in toxicity is only seen in fed but not in starved mice and correlates with lower glycogen and glucose reserves in MT1/2-deficient mice, this may reflect more compromised mitochondrial function in these animals (Rofe et al., 1998). Indeed, support

of the mitochondrial energy supply by high doses of N-acetylcysteine improves ATP levels and liver injury beyond the effects of GSH re-synthesis (Saito et al., 2009). Thus, the complete absence of MT in the knockout mice renders liver cells more susceptible to APAP-induced injury due to a compromised mitochondrial energy metabolism. These effects are fundamentally different compared to MT induction with Zn prior to APAP exposure.

In summary, we demonstrated that $ZnCl_2$ treatment effectively attenuated the APAP-induced mitochondrial oxidant stress and peroxynitrite formation, nuclear DNA damage and necrotic cell death (Figure 9). Since MT1/2-deficient mice were not affected by Zn treatment, the protective effect was mainly caused by the induction of MT protein expression in hepatocytes. The slightly higher GSH levels observed in $ZnCl_2$-treated animals may have also contributed to the protection. The induction of MT did not affect the metabolic activation of APAP but attenuated the oxidant stress. However, lower hepatic GSSG levels after APAP and no evidence that MT was able to scavenge ROS *in vitro* suggested a target upstream of mitochondria. MT was able to effectively react with NAPQI once GSH was depleted. Thus, our data are consistent with the conclusion that MT acts as a second line of defense behind GSH to scavenge NAPQI and prevent the binding to cellular proteins including mitochondrial proteins. This will attenuate the mitochondrial oxidant stress and peroxynitrite formation, which are critical mediators of APAP-induced liver injury. Since MT can be induced by various cellular stresses it could be target for drug toxicity prophylaxis. As such, MT expression could also be an unrecognized mechanism of protection of certain interventions such as a neutropenia antibody (Jaeschke and Liu, 2007).

Acknowledgments

This investigation was supported in part by National Institutes of Health Grants R01 DK070195 and R01 AA12916 to H.J., and by grants P20 RR016475 and P20 RR021940 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health.

REFERENCES

Bajt ML, Farhood A, Lemasters JJ, Jaeschke H. Mitochondrial bax translocation accelerates DNA fragmentation and cell necrosis in a murine model of acetaminophen hepatotoxicity. J. Pharmacol. Exp. Ther 2008a;324:8–14. [PubMed: 17906064]

Bajt ML, Knight TR, Lemasters JJ, Jaeschke H. Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by N-acetyl cysteine. Toxicol. Sci 2004;80:343–349. [PubMed: 15115886]

Bajt ML, Lawson JA, Vonderfecht SL, Gujral JS, Jaeschke H. Protection against Fas receptor-mediated apoptosis in hepatocytes and nonparenchymal cells by a caspase-8 inhibitor in vivo: evidence for a postmitochondrial processing of caspase-8. Toxicol. Sci 2000;58:109–117. [PubMed: 11053547]

Bajt ML, Yan HM, Farhood A, Jaeschke H. Plasminogen activator inhibitor-1 limits liver injury and facilitates regeneration after acetaminophen overdose. Toxicol. Sci 2008b;104:419–427. [PubMed: 18469330]

Cagen SZ, Klaassen CD. Binding of glutathione-depleting agents to metallothionein. Toxicol. Appl. Pharmacol 1980;54:229–237. [PubMed: 7423489]

Chengelis CP, Dodd DC, Means JR, Kotsonis FN. Protection by zinc against acetaminophen induced hepatotoxicity in mice. Fundam. Appl. Toxicol 1986;6:278–284. [PubMed: 3084325]

Chiu H, Brittingham JA, Laskin DL. Differential induction of heme oxygenase-1 in macrophages and hepatocytes during acetaminophen-induced hepatotoxicity in the rat: effects of hemin and biliverdin. Toxicol. Appl. Pharmacol 2002;181:106–115. [PubMed: 12051994]

Cohen SD, Khairallah EA. Selective protein arylation and acetaminophen-induced hepatotoxicity. Drug Metab. Rev 1997;29:59–77. [PubMed: 9187511]

Cover C, Mansouri A, Knight TR, Bajt ML, Lemasters JJ, Pessayre D, Jaeschke H. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J. Pharmacol. Exp. Ther 2005;315:879–887. [PubMed: 16081675]

Cover C, Liu J, Farhood A, Malle E, Waalkes MP, Bajt ML, Jaeschke H. Pathophysiological role of the acute inflammatory response during acetaminophen hepatotoxicity. Toxicol. Appl. Pharmacol 2006;216:98–107. [PubMed: 16781746]

Fujimoto K, Kumagai K, Ito K, Arakawa S, Ando Y, Oda S, Yamoto T, Manabe S. Sensitivity of liver injury in heterozygous Sod2 knockout mice treated with troglitazone or acetaminophen. Toxicol. Pathol 2009;37:193–200. [PubMed: 19332662]

Gujral JS, Knight TR, Farhood A, Bajt ML, Jaeschke H. Mode of cell death after acetaminophen overdose in mice: apoptosis or oncotic necrosis? Toxicol. Sci 2002;67:322–328. [PubMed: 12011492]

Hoos JS, Damsten MC, de Vlieger JS, Commandeur JN, Vermeulen NP, Niessen WM, Lingeman H, Irth H. Automated detection of covalent adducts to human serum albumin by immunoaffinity chromatography, on-line solution phase digestion and liquid chromatography-mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci 2007;859:147–156.

Jaeschke H. Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol. J. Pharmacol. Exp. Ther 1990;255:935–941. [PubMed: 2262912]

Jaeschke H. Role of inflammation in the mechanism of acetaminophen-induced hepatotoxicity. Expert Opin. Drug Metab. Toxicol 2005;1:389–397. [PubMed: 16863451]

Jaeschke H. Innate immunity and acetaminophen-induced liver injury: why so many controversies? Hepatology 2008;48:699–701. [PubMed: 18752320]

Jaeschke H, Bajt ML. Intracellular signaling mechanisms of acetaminophen-induced liver cell death. Toxicol. Sci 2006;89:31–41. [PubMed: 16177235]

Jaeschke H, Knight TR, Bajt ML. The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. Toxicol. Lett 2003;144:279–288. [PubMed: 12927346]

Jaeschke H, Liu J. Neutrophil depletion protects against murine acetaminophen hepatotoxicity: another perspective (letter). Hepatology 2007;45:1588–1589. [PubMed: 17539019]

Jaeschke H, Mitchell JR. Use of isolated perfused organs in hypoxia and ischemia/reperfusion oxidant stress. Methods Enzymol 1990;186:752–759. [PubMed: 2233332]

Klaassen CD, Liu J, Choudhuri S. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annu. Rev. Pharmacol. Toxicol 1999;39:267–294. [PubMed: 10331085]

Knight TR, Kurtz A, Bajt ML, Hinson JA, Jaeschke H. Vascular and hepatocellular peroxynitrite formation during acetaminophen toxicity: role of mitochondrial oxidant stress. Toxicol. Sci 2001;62:212–220. [PubMed: 11452133]

Knight TR, Ho YS, Farhood A, Jaeschke H. Peroxynitrite is a critical mediator of acetaminophen hepatotoxicity in murine livers: protection by glutathione. J. Pharmacol. Exp. Ther 2002;303:468–475. [PubMed: 12388625]

Kon K, Kim JS, Jaeschke H, Lemasters JJ. Mitochondrial permeability transition in acetaminophen-induced necrotic and apoptotic cell death to cultured mouse hepatocytes. Hepatology 2004;40:1170–1179. [PubMed: 15486922]

Kumari MV, Hiramatsu M, Ebadi M. Free radical scavenging actions of metallothionein isoforms I and II. Free Radic. Res 1998;29:93–101. [PubMed: 9790511]

Larson AM, Polson J, Fontana RJ, Davern TJ, Lalani E, Hynan LS, Reisch JS, Schiødt FV, Ostapowicz G, Shakil AO, Lee WM. Acute Liver Failure Study Group. Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology 2005;42:1364–1372. [PubMed: 16317692]

Liu J, Zhou ZX, Zhang W, Bell MW, Waalkes MP. Changes in hepatic gene expression in response to hepatoprotective levels of zinc. Liver Int 2009;29:1222–1229. [PubMed: 19490425]

Liu J, Liu Y, Hartley D, Klaassen CD, Shehin-Johnson SE, Lucas A, Cohen SD. Metallothionein-I/II knockout mice are sensitive to acetaminophen-induced hepatotoxicity. J. Pharmacol. Exp. Ther 1999;289:580–586. [PubMed: 10087053]

Liu ZX, Han D, Gunawan B, Kaplowitz N. Neutrophil depletion protects against murine acetaminophen hepatotoxicity. Hepatology 2006;43:1220–1230. [PubMed: 16729305]

Liu ZX, Kaplowitz N. Role of innate immunity in acetaminophen-induced hepatotoxicity. Expert Opin. Drug Metab. Toxicol 2006;2:493–503. [PubMed: 16859400]

Lu SC. Regulation of glutathione synthesis. Mol. Aspects Med 2009;30:42–59. [PubMed: 18601945]

Nelson SD. Molecular mechanisms of the hepatotoxicity caused by acetaminophen. Semin. Liver Dis 1990;10:267–278. [PubMed: 2281334]

Nieminen AL, Saylor AK, Tesfai SA, Herman B, Lemasters JJ. Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to t-butylhydroperoxide. Biochem. J 1995;307:99–106. [PubMed: 7718000]

Qiu Y, Benet LZ, Burlingame AL. Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. J. Biol. Chem 1998;273:17940–17953. [PubMed: 9651401]

Qiu Y, Benet LZ, Burlingame AL. Identification of hepatic protein targets of the reactive metabolites of the non-hepatotoxic regioisomer of acetaminophen, 3'-hydroxyacetanilide, in the mouse *in vivo* using two-dimensional gel electrophoresis and mass spectrometry. Adv. Exp. Med. Biol 2001;500:663–673. [PubMed: 11765013]

Ray SD, Sorge CL, Raucy JL, Corcoran GB. Early loss of large genomic DNA *in vivo* with accumulation of Ca^{2+} in the nucleus during acetaminophen-induced liver injury. Toxicol. Appl. Pharmacol 1990;106:346–351. [PubMed: 2256122]

Roberts DW, Pumford NR, Potter DW, Benson RW, Hinson JA. A sensitive immunochemical assay for acetaminophen-protein adducts. J. Pharmacol. Exp. Ther 1987;241:527–533. [PubMed: 3572810]

Rofe AM, Barry EF, Shelton TL, Philcox JC, Coyle P. Paracetamol hepatotoxicity in metallothionein-null mice. Toxicology 1998;125:131–140. [PubMed: 9570328]

Saito C, Zwingmann C, Jaeschke H. Novel mechanisms of protection against acetaminophen hepatotoxicity in mice by glutathione and *N*-acetylcysteine. Hepatology. 2009in press

Sato M, Bremner I. Oxygen free radicals and metallothionein. Free Radic. Biol. Med 1993;14:325–337. [PubMed: 8458590]

Shen W, Kamendulis LM, Ray SD, Corcoran GB. Acetaminophen-induced cytotoxicity in cultured mouse hepatocytes: correlation of nuclear Ca^{2+} accumulation and early DNA fragmentation with cell death. Toxicol. Appl. Pharmacol 1991;111:242–254. [PubMed: 1957310]

Szymanska JA, Swietlicka EA, Piotrowski JK. Protective effect of zinc in the hepatotoxicity of bromobenzene and acetaminophen. Toxicology 1991;66:81–91. [PubMed: 1996469]

Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim. Biophys. Acta 1985;827:36–44. [PubMed: 2981555]

Tirmenstein MA, Nelson SD. Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver. J. Biol. Chem 1989;264:9814–9819. [PubMed: 2524496]

Tolson JK, Dix DJ, Voellmy RW, Roberts SM. Increased hepatotoxicity of acetaminophen in Hsp70i knockout mice. Toxicol. Appl. Pharmacol 2006;210:157–162. [PubMed: 16280147]

Zhou Z, Wang L, Song Z, Saari JT, McClain CJ, Kang YJ. Zinc supplementation prevents alcoholic liver injury in mice through attenuation of oxidative stress. Am. J. Pathol 2005;166:1681–1690. [PubMed: 15920153]

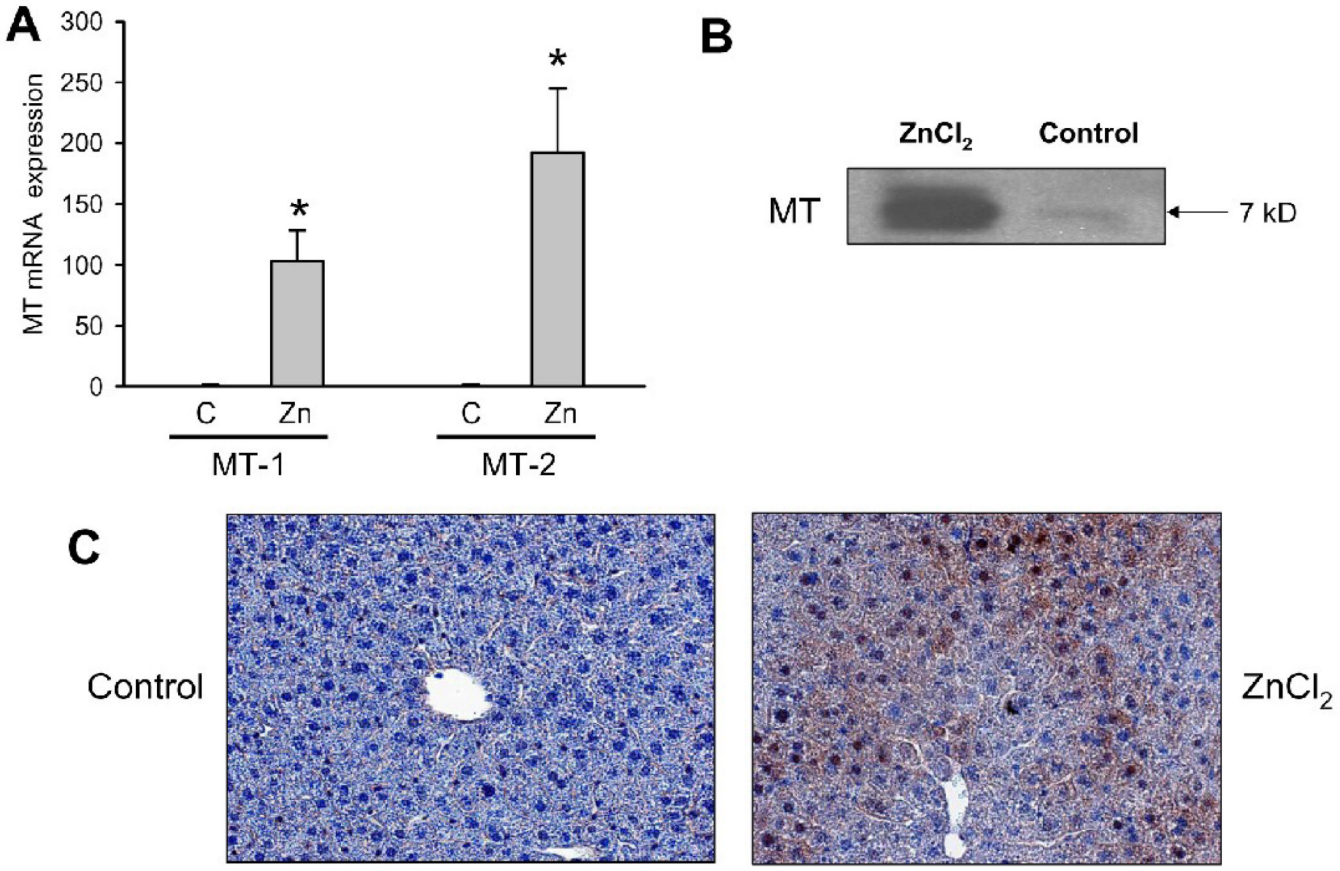

Figure 1. Zinc pre-treatment induced metallothionein

A. Quantitative real-time PCR was used to analyze for MT-1 and MT-2 mRNA expression in livers of animals treated with saline (6 ml/kg) (controls, C) or 100 μmol/kg $ZnCl_2$ for 3 days. MT mRNA is expressed as the MT-to-actin ratio. Data represent mean ± SE of n = 4 animals *P<0.05 (compared to C)

B. Western blot analysis of MT protein in controls and $ZnCl_2$-treated animals.

C. Immunohistochemical analysis of MT expression in control liver compared to the liver of a $ZnCl_2$-treated animal.

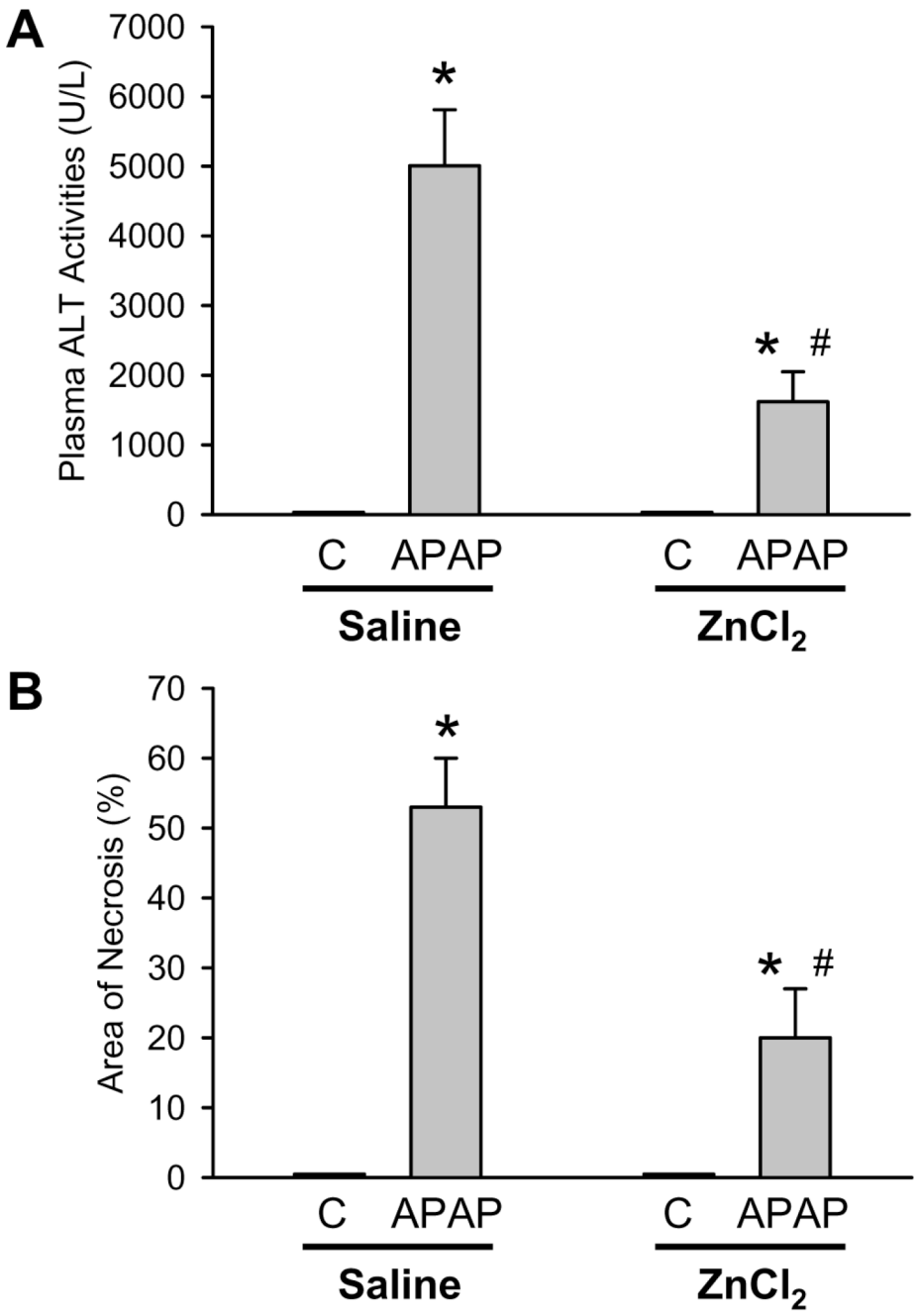

Figure 2. Metallothionein induction protects against acetaminophen hepatotoxicity

Liver injury was evaluated by measuring plasma alanine aminotransferase (ALT) activities (panel A) and quantitating the area of necrosis (panel B) in untreated animals (C) and 6 h after injection of 300 mg/kg acetaminophen (APAP). Animals were either treated with saline (6 ml/kg) or 100 μmol/kg $ZnCl_2$ for 3 days. APAP was injected 24 h after the last dose of Zn/saline. Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to C) $^{\#}$P<0.05 (compared to APAP/saline)

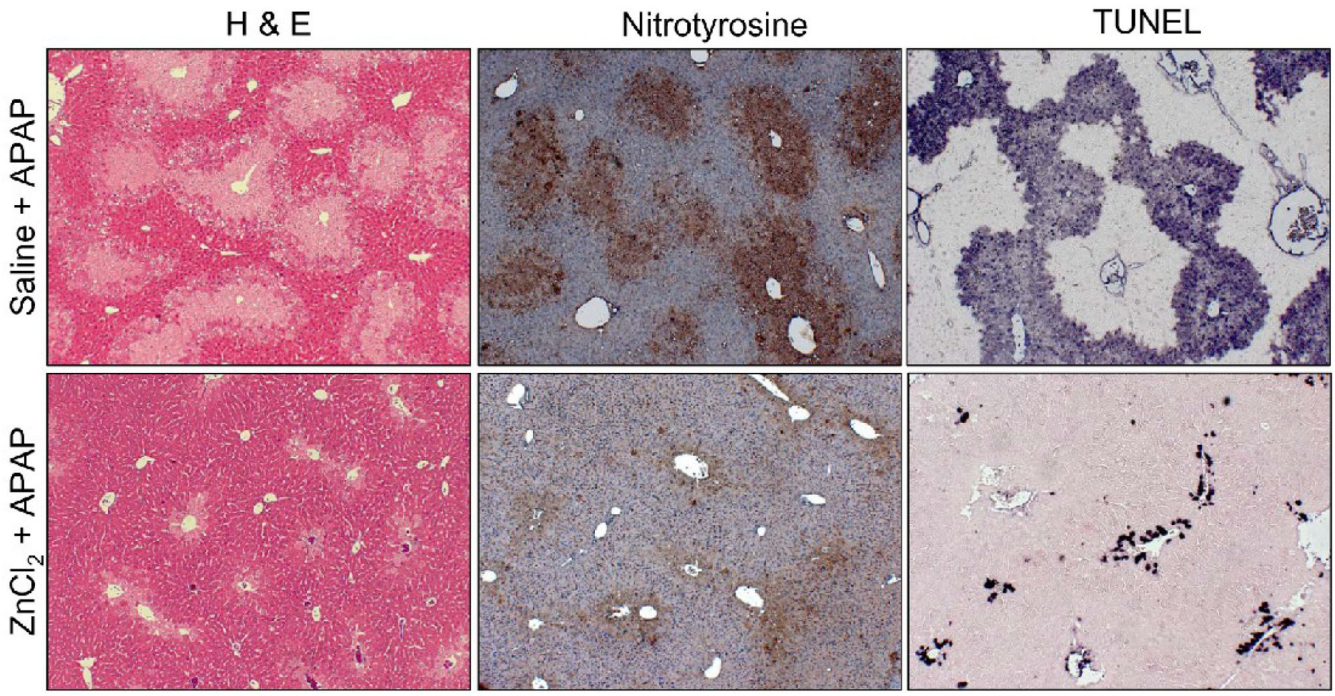

Figure 3. Histological assessment of liver injury, peroxynitrite formation and DNA damage

Representative liver sections of animals treated for 6 h with 300 mg/kg acetaminophen were stained with H&E (assess area of necrosis), a nitrotyrosine antibody (marker of peroxynitrite formation) and the TUNEL assay (marker for DNA strandbreaks). Animals were either treated with saline (6 ml/kg) or 100 μmol/kg $ZnCl_2$ for 3 days. APAP was injected 24 h after the last dose of Zn/saline. (magnification of all graphs: ×50)

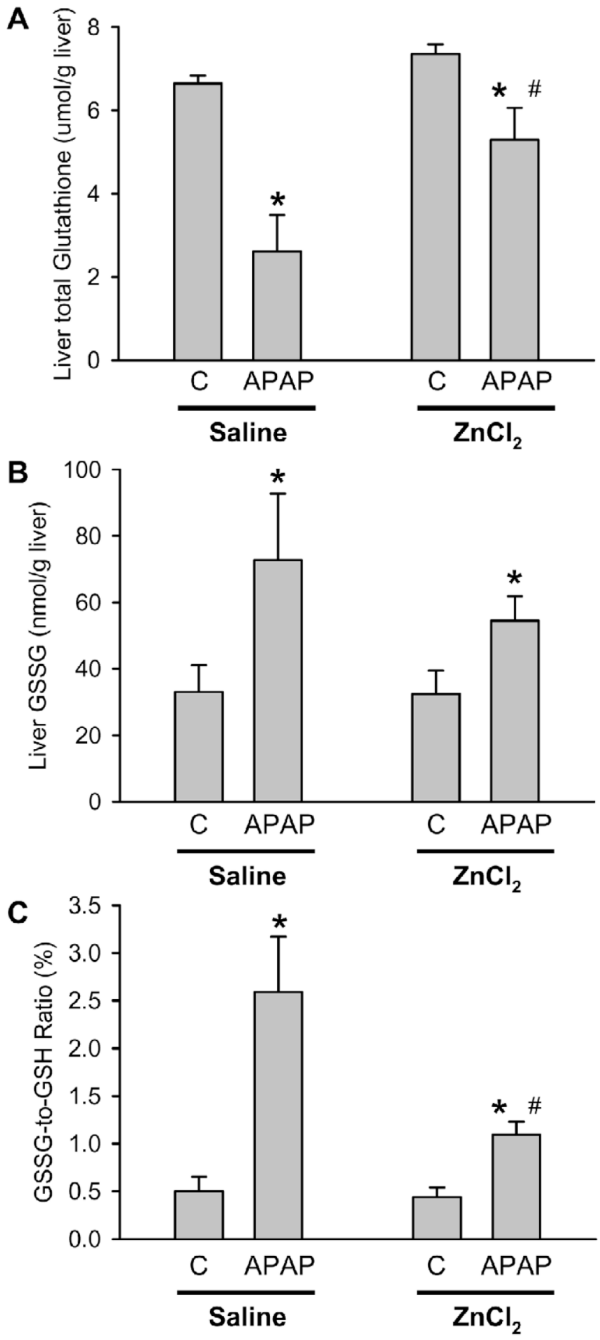

Figure 4. Hepatic glutathione levels and oxidant stress

Total glutathione (GSH+GSSG) (panel A) and glutathione disulfide (GSSG) (panel B) levels were measured and the GSSG-to-GSH ratio (panel C) was calculated before and 6 h after treatment with 300 mg/kg APAP. Animals were either treated with saline (6 ml/kg) or 100 μmol/kg $ZnCl_2$ for 3 days. APAP was injected 24 h after the last dose of Zn/saline. Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to C) $^{\#}$P<0.05 (compared to APAP/saline)

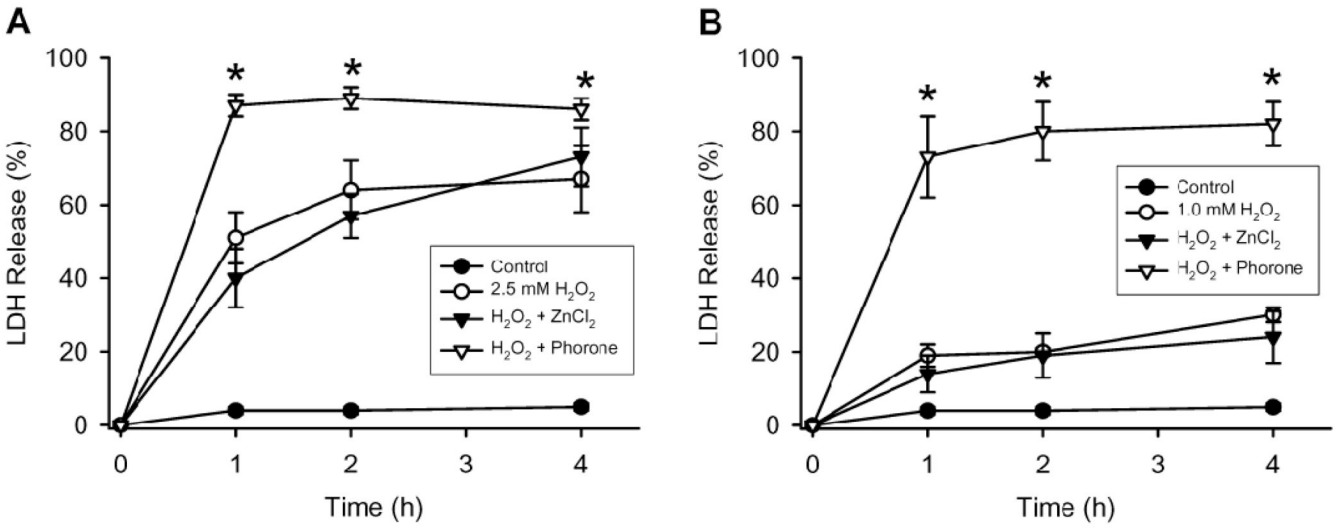

Figure 5. Effect of MT induction and GSH depletion on hydrogen peroxide-induced cell injury

Primary cultured murine hepatocytes were exposed to various concentrations of hydrogen peroxide (H_2O_2) or vehicle (culture medium) for up to 4 h and cell injury was measured as % of released lactate dehydrogenase (LDH). Animals used for cell isolation were either untreated or received 100 μmol/kg $ZnCl_2$ for 3 days or 100 mg/kg phorone 90 min before isolation. All cells isolated from phorone-treated animals were cultured in the presence of 1 mM buthionine sulfoximine to inhibit GSH synthesis. All data represent means ± SE of 4 independent time course experiments. *$P<0.05$ (compared to H_2O_2 alone)

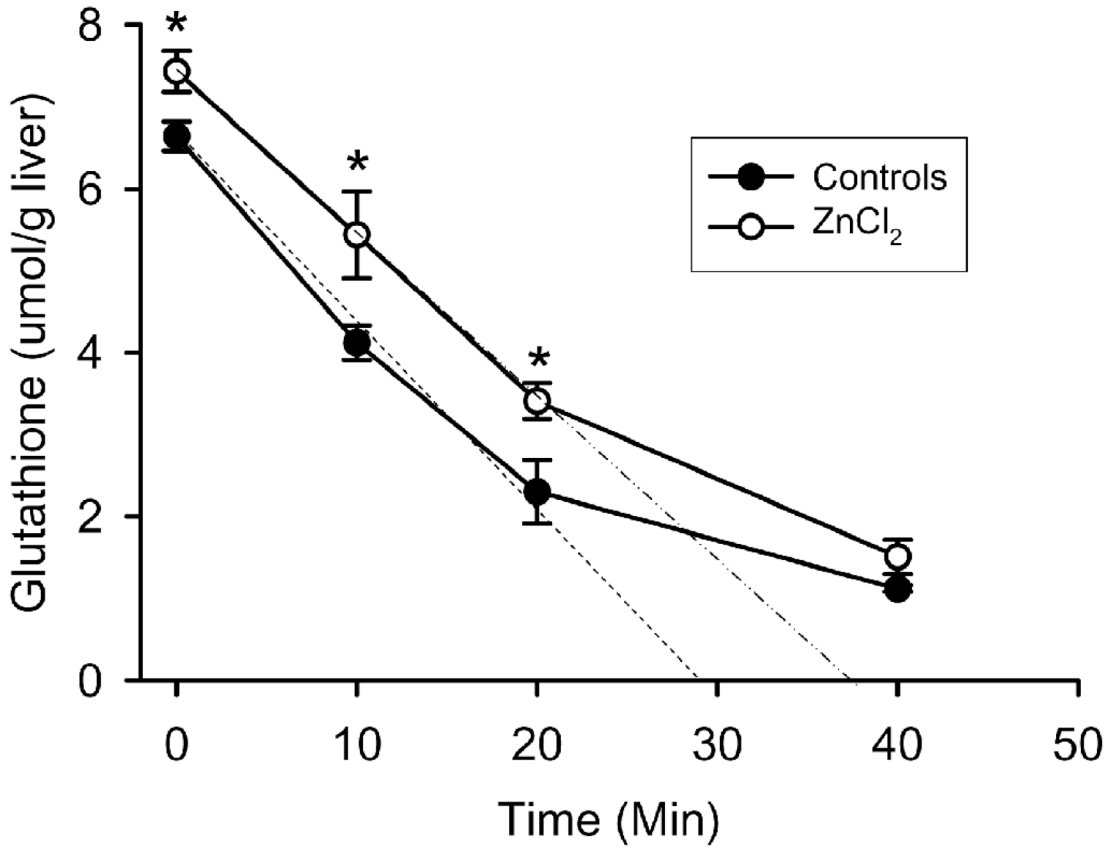

Figure 6. MT induction does not affect the early GSH depletion kinetics

Hepatic glutathione levels (GSH+GSSG) were measured at early time points (0 – 40 min) after administration of 300 mg/kg acetaminophen. Animals were either treated with saline (6 ml/kg) or 100 μmol/kg $ZnCl_2$ for 3 days. APAP was injected 24 h after the last dose of Zn/saline. Data represent means ± SE of n = 5 animals per group. *P<0.05 (compared to controls)

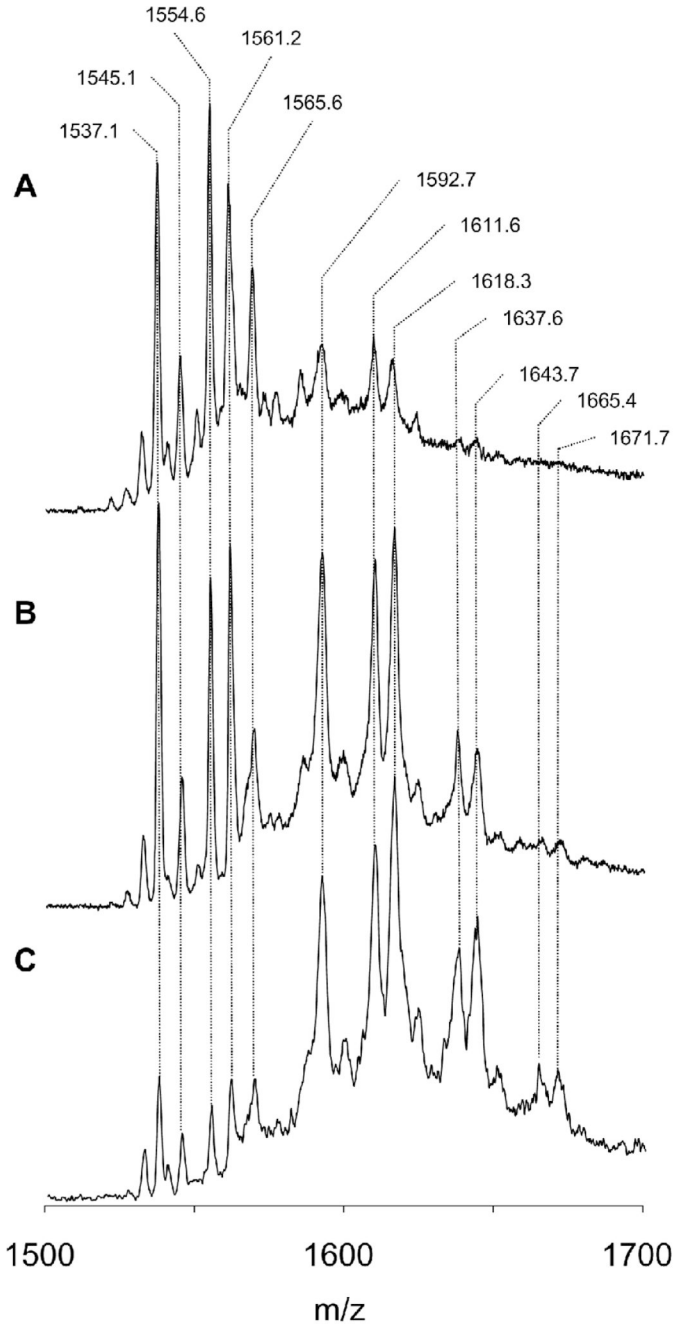

Figure 7. LC ESI MS mass spectrum of liver metallothionein

Rabbit liver MT was analyzed by reverse phase chromatography/mass spectrometry as described in the experimental procedures. The figure shows the +4 charge state protein ions obtained on the IT of the control sample. An aliquot of MT that was not reacted with NAPQI (**A**), or following the addition of NAPQI and incubation at 4°C for 20 sec (**B**) or 2h (**C**). For each panel, data are reported as percentage relative to the most intense ion. Thus, the progress of the reaction is indicated by a change in the relative intensities of the ions present at each experimental time. There is a decrease of the intensity of the ions in the m/z range 1537–1565 with a concomitant increase of the ions in the m/z range 1592–1671.

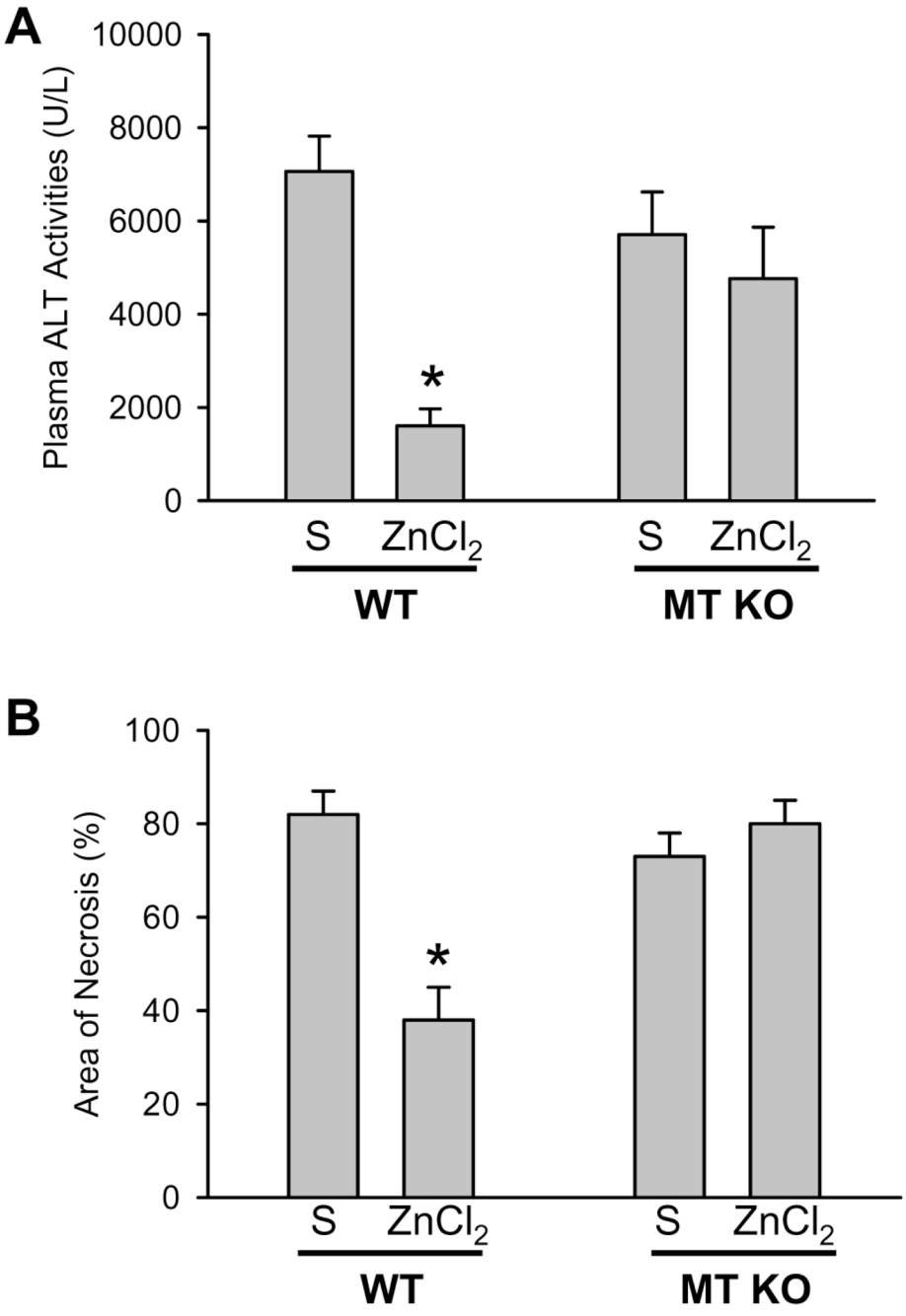

Figure 8. $ZnCl_2$ protects wild type but not MT-deficient against acetaminophen hepatotoxicity

Liver injury was evaluated by measuring plasma alanine aminotransferase (ALT) activities (panel A) and quantitating the area of necrosis (panel B) in wild type and MT-1/MT-2-deficient (MT KO) mice 6 h after injection of 300 mg/kg acetaminophen (APAP). Animals were either treated with saline (6 ml/kg) (S) or 100 μmol/kg $ZnCl_2$ for 3 days. APAP was injected 24 h after the last dose of Zn/saline. Data represent means ± SE of n = 4–6 animals per group. *P<0.05 (compared to APAP/saline)

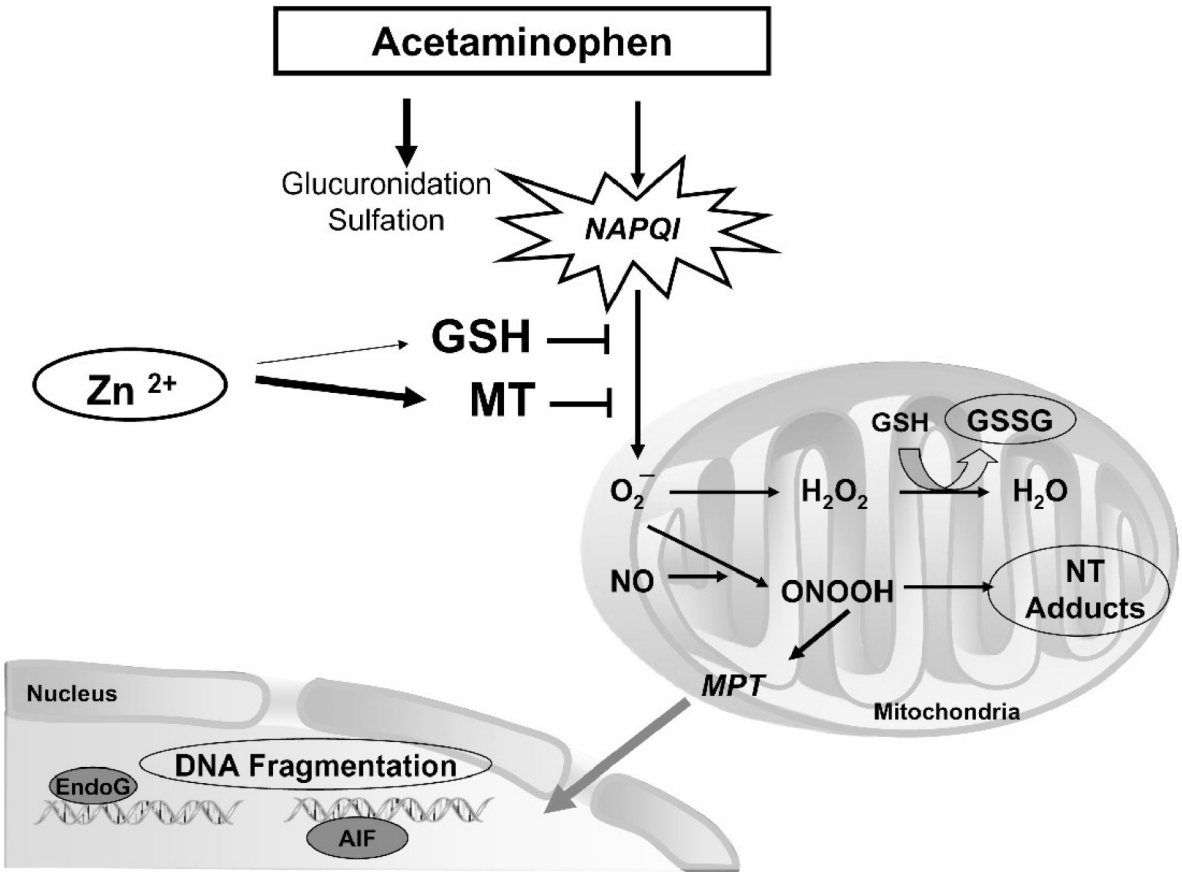

Figure 9. Mechanisms of protection by metallothionein against acetaminophen hepatotoxicity

See text for details. Abbreviations: AIF, apoptosis-inducing factor; EndoG, endonuclease G; GSSG, glutathione disulfide; MPT, mitochondrial membrane permeability transition pore; MT, metallothionein; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NT adducts, nitrotyrosine protein adducts; ONOOH, peroxynitrite;