Acetaminophen Toxicity
OPPOSITE EFFECTS OF TWO FORMS OF GLUTATHIONE PEROXIDASE*

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Oleg Mirochnitchenko‡‡, Miriam Weisbrod-Lefkowitz‡‡, Kenneth Reuhl¶, Laishun Chen†,
Chung Yang‡, and Masayori Inouye‡**

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‡ These authors contributed equally to this work.

Acetaminophen is one of the most extensively used analgesics/antipyretics worldwide, and overdose or idiopathic reaction causes major morbidity and mortality in its victims. Research into the mechanisms of toxicity and possible therapeutic intervention is therefore essential. In this study, the response of transgenic mice overexpressing human antioxidant enzymes to acute acetaminophen overdose was investigated. Animals overexpressing superoxide dismutase or plasma glutathione peroxidase demonstrated dramatic resistance to acetaminophen toxicity. Intravenous injection of glutathione peroxidase provided normal mice with nearly complete protection against a lethal dose of acetaminophen. Surprisingly, animals overexpressing intracellular glutathione peroxidase in the liver were significantly more sensitive to acetaminophen toxicity compared with nontransgenic littermates. This sensitivity appears to be due to the inability of these animals to efficiently recover glutathione depleted as a result of acetaminophen metabolism. Finally, the results suggest that glutathione peroxidase overexpression modulates the synthesis of several acetaminophen metabolites. Our results demonstrate the ability of glutathione peroxidase levels to influence the outcome of acetaminophen toxicity.

Acetaminophen is one of the most commonly used analgesics/antipyretics worldwide. Although generally considered a safe drug, it continues to be a cause of death either through overdose, idiosyncratic reaction, or synergism with alcoholic liver disease. Death from acetaminophen overdose is thought to be secondary to liver failure, which is caused by massive hepatic necrosis, the hallmark pathological feature of acetaminophen toxicity. In addition to liver, however, many organ systems may fail under acute overdose such as renal, cardiac, and central nervous systems (1). It is thought that the liver is the target organ for acetaminophen toxicity because this is primarily where the drug is detoxified. Under normal conditions, acetaminophen is mainly metabolized by undergoing sulfation and glucuronidation (2). It has been proposed that a small amount of drug goes through the cytochrome P450 mixed function oxidase system and is metabolized into the reactive intermediates N-acetyl-P-benzoquinoneimine (NAPQI), which is in turn detoxified by reaction with glutathione (3, 4). When large quantities of acetaminophen are consumed, the three detoxification pathways become saturated.

The precise mechanism by which acetaminophen causes cell death remains unknown, although there are two prevailing theories that are controversial today. The first theory, the oxidative stress theory, maintains that acetaminophen metabolites cause oxidative stress in the cell ultimately leading to its demise. The second theory, the covalent binding theory, states that the binding of the highly reactive acetaminophen metabolites to cell macromolecules causes cell death. There is much evidence to substantiate both theories, and the question may be to what extent each plays a role in acetaminophen toxicity (5).

The depletion of cellular glutathione, a natural antioxidant, leaves the cell particularly vulnerable to oxidative insults following acetaminophen overdose. The oxidative stress theory has gained increased recognition as a result of a number of studies, which indirectly and directly demonstrate the presence of reactive oxygen species in cells following acetaminophen administration. Several antioxidants have been shown to protect against acetaminophen toxicity such as β-carotene (6) and α-tocopherol (7). In addition to the studies involving these chemicals, the exogenous administration of antioxidant enzymes such as catalase and superoxide dismutase (8) has been shown to protect dramatically against acetaminophen toxicity. Although it seems clear that oxidative stress plays some role in acetaminophen toxicity, the exact source(s) of the oxidative stress is not known, and the mechanism of the resultant cytotoxicity is also the subject of speculation.

We are using transgenic animals overexpressing the human antioxidant enzymes glutathione peroxidase, intracellular (GPI) and extracellular (GPP) forms, as well as Cu,Zn-superoxide dismutase (SOD) to investigate their ability to influence acetaminophen toxicity. We report here that GPP and SOD transgenic mice are equally protected against the lethality of acetaminophen overdose. The oxidative stress theory has gained increased recognition as a result of a number of studies, which indirectly and directly demonstrate the presence of reactive oxygen species in cells following acetaminophen administration. Several antioxidants have been shown to protect against acetaminophen toxicity such as β-carotene (6) and α-tocopherol (7). In addition to the studies involving these chemicals, the exogenous administration of antioxidant enzymes such as catalase and superoxide dismutase (8) has been shown to protect dramatically against acetaminophen toxicity. Although it seems clear that oxidative stress plays some role in acetaminophen toxicity, the exact source(s) of the oxidative stress is not known, and the mechanism of the resultant cytotoxicity is also the subject of speculation.

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GSH during acetaminophen intoxication. The results also indicate that GP overexpression influences the synthesis of several oxidized acetaminophen metabolites.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice**—Transgenic mice with human GP or Cu,Zn-SOD genes were produced as described previously (9, 10). Normal and transgenic animals for the experiments were obtained by breeding heterozygous transgenic founders with C57BL/6 × CBA/J F1 mice. SOD and GPI transgenic mice show increased activities of Cu,Zn-SOD and GPI, respectively, in most of the tissues, as reported earlier. For example Cu,Zn-SOD activity in liver of SOD mice is 2.2-fold higher, whereas activity of GPI in liver of GPI mice is 1.3-fold higher than that of nontransgenic mice (11). GPP mice have 50% increased activity of GP in blood plasma, and the same activity in liver as nontransgenic mice. Animals were maintained with 12 h light/dark cycling at 25 °C and fed ad libitum. Nontransgenic littermates were used as controls. Experimental animals were matched for age and gender. Experimental protocol has been approved by the University of Medicine and Dentistry Animal Care and Use Committee.

**Plasma Enzymes and Histopathology**—Lactate dehydrogenase and alanine aminotransferase were measured in plasma samples using an assay kit (Sigma) according to the kit’s protocol. Thin sections were cut by microtome and stained with hematoxylin and eosin and examined by light microscope. Damage was assessed by expert pathologist on a scale from 0 to 4 based on qualitative and quantitative criteria.

**Drug Treatments**—For survival studies, 425 mg/kg acetaminophen (Sigma) were injected intraperitoneally from a 15 mg/ml solution in sterile PBS. Animals were followed for 72 h for survival. To evaluate the effect of intravenously administered GP on animal sensitivity to acetaminophen, bovine GPI (Sigma) was dissolved in sterile PBS to a concentration of 500 units/ml and 1.4 milliunits/kg injected into the tail veins of 11 nontransgenic mice. Approximately equal volumes of sterile PBS were injected into tail veins of 10 additional nontransgenic mice. Intravenous injections were followed immediately by intraperitoneal injections of acetaminophen, 350 mg/kg. The dosage was based on a dose response study in males used in this analysis, that are known to be more sensitive to acetaminophen toxicity.

**Glutathione and Lipid Peroxidation Measurements**—Total (GSH + GSSG) and oxidized (GSSG) glutathione were measured using the recycling method (12). Tissue was homogenized in 10% perchloric acid and frozen at −70 °C until time of assay. Frozen homogenate was then centrifuged for 20 min at 6,000 × g. Acid supernatant was used for GSH assay, acid supernatant reacted with 100 mM N-ethylmaleimide (Sigma) and run through Sep-Pak P18 cartridge (Waters) was used for GSSG determination. Reaction mixtures contained 0.15 mM NADPH and assay buffer (0.6 mM 5,5′-dithio-bis(2-nitro-benzoic acid) in phosphate buffer (0.1 M, pH 7.4) containing 1 mM EDTA, and glutathione reductase (80 milliunits/ml)). Plasma was mixed with equal volumes of 5-sulfosalicylic acid for plasma glutathione measurements (10%, w/v) and displaying less spontaneous locomotor activity.

**Quantitative determinations of 500 units/ml and 1.4 milliunits/kg injected into the tail veins of 11 nontransgenic mice. Approximately equal volumes of sterile PBS were injected into tail veins of 10 additional nontransgenic mice. Intravenous injections were followed immediately by intraperitoneal injections of acetaminophen, 350 mg/kg. The dosage was based on a dose response study in males used in this analysis, that are known to be more sensitive to acetaminophen toxicity.

**Statistics**—All results are expressed as mean ± S.E. Statistical differences between means were evaluated using the Student’s t test.

**RESULTS**

**Effect of GP Overexpression on Survival and Hepatotoxicity after Acetaminophen Administration**—Transgenic mice overexpressing SOD, GPI, GPP, and nontransgenic controls received 425 mg/kg acetaminophen intraperitoneally in a single dose. SOD mice were dramatically protected from toxicity, with 25% mortality as compared with 75% mortality in nontransgenic controls (Fig. 1A). Similar protection has been reported for exogenous administration of liposome-encapsulated SOD to rats immediately before acute acetaminophen overdose (18). Animals overexpressing extracellular GP in the blood also demonstrated dramatic protection against acetaminophen, again suffering only 25% mortality. Surprisingly, animals overexpressing GP in the liver tissue itself displayed a completely opposite phenotype-enhancement of acetaminophen toxicity. All GPI animals receiving acetaminophen died within 5.5 h of treatment. Control mortality was only 75% and occurred over a much extended period of time, with the last death occurring at 36 h post-injection. The behavior of the animals corresponded to survival pattern, with more sensitive animals feeding poorly and most of the animals suffering less spontaneous locomotor activity.

To confirm that the response of the GPI transgenic mice was caused by increased GP activity in the blood, 1.4 milliunits/kg GP was injected into tail veins of 11 nontransgenic mice and equal volumes of PBS into tail veins of 10 additional animals, immediately before an intraperitoneal injection of a lethal dose of acetaminophen. The dose of GP was selected because it
achieves roughly the same activity of GP in the blood as is normally found in GPP transgenic mice. Almost 90% of GP-injected animals survived beyond 72 h, whereas none of the PBS-injected animals survived beyond 6 h (Fig. 1B). Increased GP activity in the blood appears to be responsible for the reduced mortality seen in both GP-injected as well as GPP transgenic mice.

By histopathologic criteria there were no differences between groups at 4 h, with all animals sustaining equally severe damage. At 8 h, histopathology generally agreed with survival data, with SOD and GPP animals displaying less damage than controls and GPI displaying more severe damage (Fig. 2). Liver necrosis was also evaluated by measurement of blood alanine aminotransferase activity. Levels of alanine aminotransferase at 8 h were as follows: 5,477 ± 2,035 units/liter, 802 ± 465 units/liter and 3,119 ± 1,594 units/liter for GPI, GPP, and nontransgenic mice, respectively. Data obtained indicate that GPI mice had the highest increase in alanine aminotransferase, whereas GPP mice had the smallest elevation of alanine aminotransferase compared with nontransgenic mice.

Studies of Glutathione Metabolism and Lipid Peroxidation—Hepatic glutathione has a well established pattern of depletion and recovery under conditions of acetaminophen overdose. All animal groups suffered approximately 95% depletion of total GSH at 1 h, as seen in Fig. 3A. At 8 h, when total GSH content returned to baseline in all other groups, GPI mice had significantly lower total GSH than SOD, controls, and GPP mice (37, 32, and 26% lower, respectively). This difference might indicate the inability of GPI mice to efficiently replenish liver GSH, leading to an increase of the sensitivity of these animals to acetaminophen toxicity. However, we could not exclude another possibility that the effect observed in GPI mice might be the result of earlier necrosis developed in these animals, because liver damage in GPI mice was higher than in all other groups at this time point. Interestingly, a comparison of liver GSH content among the different groups reveals a significantly lower proportion of GSH (higher proportion of GSSG) in SOD mice relative to all other groups at 1 h (Fig. 3B). Because GPP animals overexpress the plasma form of GP, plasma levels of total glutathione were measured to determine the degree of oxidative stress in the blood. Similar to what was observed in the liver, total glutathione in the plasma of SOD, GPI, and control animals was almost completely depleted by 1 h (Fig. 3C). In contrast to the liver, however, plasma glutathione levels at 4 h remained low in all groups, except for GPP, in which clear tendencies for recovery were observed. By 8 h, levels of plasma glutathione in SOD and GPP animals returned to baseline, whereas control animals were still at 73% and GPI at 60% of baseline levels. Significantly, although depleted from baseline levels, plasma total GSH was substantially higher in GPP
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Acetaminophen Metabolism in GP Transgenic Mice—Altered metabolism of acetaminophen in the presence of an increased level of GP may explain the seemingly paradoxical response of GP transgenic mice to an overdose of acetaminophen. The significant increase in lipid peroxidation in the livers of SOD mice following an overdose of acetaminophen are most likely the result of increased production of \( \text{H}_2\text{O}_2 \). This observation correlates with the decreased GSH content in these animals compared with nontransgenic and GP mice at earlier time points. Importantly, we did not observe the expected decrease in hepatic lipid peroxidation in GP transgenic mice compared with nontransgenic animals following acetaminophen administration. These data indicate absence of correlation between survival of different groups of animals and the level of liver lipid peroxidation as well as inability of both types of GPs to decrease the level of liver peroxidation measured as TBARS. The data are consistent with a number of studies that have found a dissociation between lipid peroxidation and acetaminophen toxicity (19).

Acetaminophen Oxidation in Vitro—Based on the above results, it was hypothesized that intracellular GP could use acetaminophen as electron donor, thus converting acetaminophen to acetaminophen free radical. The latter could be produced from critical cellular molecules. This process in liver overexpressing GP might lead to the increase in toxicity and exacerbation of damage in transgenic mice. To test this hypothesis, we exposed acetaminophen to the purified intracellular glutathione peroxidase in the presence and absence of \( \text{H}_2\text{O}_2 \). Two approaches were used to test the ability of GP to form NAPQI from acetaminophen. In the first series of experiments, a mixture of acetaminophen and GP was treated with \( \text{H}_2\text{O}_2 \), and NAPQI formation was measured by fluorescence spectroscopy. As shown in Fig. 5A, commercially available NAPQI exhibits readily detectable fluorescence emission with a maximum at 440–450 nm using 306 nm as the excitation wavelength (16). Prolonged exposure of acetaminophen to \( \text{H}_2\text{O}_2 \) leads to the appearance of a similar, but weaker fluorescence emission, probably reflecting autooxidation of the drug (Fig. 5B). Addition of 0.1–10 units of GP to the reaction mixture did not increase but decreased the spontaneous oxidation (Fig. 5C). Though we do not know the exact reason for this effect at present, GPI at least was not able to augment NAPQI formation as other peroxidases. In a second indirect approach, GP activity was analyzed by following oxidation of NADPH to NADP in the presence of acetaminophen and \( \text{H}_2\text{O}_2 \) according to the method of Keller and Hinson (17). We did not observe any effect of acetaminophen (0.01 to 1 mM) on the rate of NADPH oxidation.

Lipid peroxidation was assessed by measurement of thiobarbituric acid reactive substances (TBARS). After 4 h, TBARS were elevated in all groups (Fig. 3D), but after 8 h, they returned to baseline, except for SOD mice. The significant increases in lipid peroxidation in the livers of SOD mice following an overdose of acetaminophen are most likely the result of increased production of \( \text{H}_2\text{O}_2 \). This observation correlates with the decreased GSH content in these animals compared with nontransgenic and GP mice at earlier time points. Importantly, we did not observe the expected decrease in hepatic lipid peroxidation in GP transgenic mice compared with nontransgenic animals following acetaminophen administration. These data indicate absence of correlation between survival of different group of animals and the level of liver lipid peroxidation as well as inability of both types of GPs to decrease the level of liver peroxidation measured as TBARS. The data are consistent with a number of studies that have found a dissociation between lipid peroxidation and acetaminophen toxicity (19).

Acetaminophen metabolism was evaluated by measurements of specific metabolites in the serum of animals following administration of acetaminophen. Overall there are significant similarities in the patterns of changes of acetaminophen metabolites in all animals tested (Fig. 4). Nevertheless, the concentration of free acetaminophen was somewhat higher in the serum of GPP mice at 30 min, and the concentration of acetaminophen-glucuronide and mercapturate were lower in GPP mice at 50 min compared with normal and GPI mice (Fig. 4A, B, and F). Furthermore, the concentrations of the mercapturic acid metabolites of acetaminophen were much higher in GPI animals at 25–60 min concomitantly with a lower concentration of acetaminophen-GSH. These data indicate that GP overexpression influences the level of several acetaminophen metabolites. The rate of acetaminophen oxidation by cytochrome P450 in liver microsomal fractions from normal and GPI mice was the same (data not shown).

Acetaminophen Oxidation in Vitro—Based on the above results, it was hypothesized that intracellular GP could use acetaminophen as electron donor, thus converting acetaminophen to acetaminophen free radical. The latter could be reduced back to acetaminophen by a variety of processes, including potentially toxic reactions such as abstracting hydrogen atoms from critical cellular molecules. This process in liver overexpressing GPI might lead to the increase in toxicity and exacerbation of damage in transgenic mice. To test this hypothesis, we exposed acetaminophen to the purified intracellular glutathione peroxidase in the presence and absence of \( \text{H}_2\text{O}_2 \). Two approaches were used to test the ability of GP to form NAPQI from acetaminophen. In the first series of experiments, a mixture of acetaminophen and GP was treated with \( \text{H}_2\text{O}_2 \), and NAPQI formation was measured by fluorescence spectroscopy. As shown in Fig. 5A, commercially available NAPQI exhibits readily detectable fluorescence emission with a maximum at 440–450 nm using 306 nm as the excitation wavelength (16). Prolonged exposure of acetaminophen to \( \text{H}_2\text{O}_2 \) leads to the appearance of a similar, but weaker fluorescence emission, probably reflecting autooxidation of the drug (Fig. 5B). Addition of 0.1–10 units of GP to the reaction mixture did not increase but decreased the spontaneous oxidation (Fig. 5C). Though we do not know the exact reason for this effect at present, GP at least was not able to augment NAPQI formation as other peroxidases. In a second indirect approach, GP activity was analyzed by following oxidation of NADPH to NADP in the presence of acetaminophen and \( \text{H}_2\text{O}_2 \) according to the method of Keller and Hinson (17). We did not observe any effect of acetaminophen (0.01 to 1 mM) on the rate of NADPH oxidation.
DISCUSSION

Acetaminophen toxicity is one of many human disease processes widely believed to involve reactive oxygen species. The success in using various antioxidants to protect against acetaminophen toxicity is important not only for its therapeutic application but because it may shed light on the mechanism of hepatotoxicity. Because of the severity and high costs of poor clinical outcomes of acetaminophen poisoning and because acetaminophen may serve as a model for other diseases involving oxidative stress, research into mechanisms of toxicity and candidates for therapeutic intervention is quite important.

GP is a critical antioxidant enzyme for the detoxification of peroxides. Its particular kinetics and relatively low substrate specificity makes it a very efficient reducer of peroxides (24), far more so than catalase, which is the other enzyme that detoxifies H$_2$O$_2$. The importance of GP has been shown in many studies including several that demonstrate marked protection against oxidative damage in cells by overexpression of GP through transfection (25, 26). GP has been largely overlooked despite its promise as a candidate for therapeutics in diseases involving oxidative stress, research into mechanisms of toxicity and candidates for therapeutic intervention is quite important.

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Overexpression of the plasma form of GP and of intracellular SOD are equally protective against a lethal overdose of acetaminophen. That SOD is protective, both in this and a previous study (10), implicates the superoxide anion in the mechanism of acetaminophen toxicity. Indeed, O$_2^-$ was found to be increased more than 2-fold in mouse liver microsomes following a large dose of acetaminophen in the studies of Lores Arnaiz et al. (27). The enhanced sensitivity of GPI transgenic mice to acetaminophen toxicity was an unexpected finding in our study. Intracellular overexpression of GPI, which is able to detoxify the final products of oxidative stress and is significantly more efficient in protecting cells in vitro against reactive oxygen species than SOD and catalase (28), would be expected to confer protection on the animals. Nevertheless, overexpression of GPI was unable to protect against liver damage, and moreover, significantly increased mortality of animals. The explanation of this effect most likely lies in a unique situation, i.e. depletion of the important intracellular antioxidant GSH by the antioxidant defense enzyme GPI, an action that compounds GSH depletion by acetaminophen metabolites and reactive oxygen species and could account for the slow recovery of GSH in GPI mice. We did not observe any difference in GSH depletion even at a short period of time, such as 5, 10, and 30 min after acetaminophen administration (data not shown). There were significant differences in GSH recovery in the liver and blood in GPI mice compared with all other groups of mice at a later time point. Importantly, this time period (4–8 h) correlates with the highest lipid peroxidation, which requires active antioxidant enzymatic activity for detoxification. Insufficient amounts of intracellular GSH might explain why the level of lipid peroxidation in the liver was not affected by GPI overexpression as well.

The protection of GPP animals is surprising in view of the traditional belief that the major target organ of acetaminophen
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A

Relative intensity

\[ \lambda, \text{ nm} \]

B

Relative intensity

\[ \lambda, \text{ nm} \]

C

Relative intensity

\[ \lambda, \text{ nm} \]

FIG. 5. Fluorescence spectrum of NAPQI (A) and reaction mixtures containing 50 mM sodium phosphate (pH 7.7), 0.5 mM acetaminophen, 36 mM cetyltrimethylammonium bromide, 0.2 mM H$_2$O$_2$ in the absence (B) and presence (C) of 2 units of GPI.

toxicity is liver and that metabolism and thus toxic by-products would be localized there. Several possibilities may contribute to acetaminophen resistance in GPP animals. First, toxic metabolites of acetaminophen and/or peroxides may be released from the liver into the blood and these are efficiently detoxified by the increased plasma levels of GP. Circulation of toxic metabolites could explain why, in some cases of acetaminophen toxicity, organs besides the liver and kidney are affected (sometimes even in the absence of severe hepatic necrosis) (31). Another possibility is that secondary factors following acetaminophen toxicity are influenced by overexpression of plasma GP. For example, overexpression of extracellular GP can protect against damage by increased scavenging of the released reactive oxygen species as well as by inhibition of activation of inflammatory leukocytes, which play an important role in acetaminophen-induced hepatotoxicity (30). An additional important factor could be related to the difference in electron donors utilized by GPP and GPI. It was recently shown that GPP, in contrast to GPI, uses thioredoxin and glutaredoxin significantly more efficiently than GSH (31). Thus, GPP mice were able to detoxify blood reactive oxygen species under conditions of severe oxidative stress caused by scavenging of GSH. On the other hand, increased levels of lipid peroxides will not lead to increased GSH oxidation by elevated levels of GPP. Indeed, GPP animals showed the least depletion of glutathione in the plasma (Fig. 3C) and thus were experiencing less oxidative stress in blood than any of the other three groups. Details of the critical role of GSH presence in extracellular pools, including blood, in detoxification and protection against chemical and oxidant-induced injuries are described in a recent review by Smith et al. (32).

In summary, SOD and GPP transgenic mice demonstrated marked resistance to acetaminophen overdose. In contrast, GPP animals showed significantly increased sensitivity to acetaminophen as compared with controls. These animals had a delay in restoration of the level of glutathione, whereas GPP mice were characterized by least depletion and most efficient restoration. Our study indicates that the phenotype may be independent of lipid peroxidation with regard to acetaminophen toxicity, consistent with a number of studies that have found no correlation between toxicity and lipid peroxidation (24). Increased toxicity most likely does not involve elevated peroxidative activity of GPP using acetaminophen as cofactor, because this enzyme was not able to form NAPQI from acetaminophen in vitro. At this point we do not know the mechanism by which GPs were able to affect the profile of oxidized acetaminophen metabolites, especially mercapturate. The blood levels of these metabolites are the result of several processes that might be affected by the level of GP expression. Our data also suggest that in addition to hepatocellular damage, which has been the accepted hallmark feature of acetaminophen toxicity, events in the blood are also crucial for organismal well being and survival in the condition of an acetaminophen overdose. This finding may have important implications for therapeutic intervention in patients suffering from acetaminophen toxicity.

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