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Role of NAD(P)H:Quinone Oxidoreductase 1 in Clofibrate-Mediated Hepatoprotection from Acetaminophen¹

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Abstract

Mice pretreated with the peroxisome proliferator clofibrate (CFB) are resistant to acetaminophen (APAP) hepatotoxicity. Whereas the mechanism of protection is not entirely known, CFB decreases protein adducts formed by the reactive metabolite of APAP, N-acetyl-*p*-benzoquinone imine (NAPQI). NAD(P)H:quinone oxidoreductase 1 (NQO1) is an enzyme with antioxidant properties that is responsible for the reduction of cellular quinones. We hypothesized that CFB increases NQO1 activity, which in turn enhances the conversion of NAPQI back to the parent APAP. This could explain the decreases in APAP covalent binding and glutathione depletion produced by CFB without affecting APAP bioactivation to NAPQI. Administration of CFB (500 mg/kg, i.p.) to male CD-1 mice for 5 or 10 days increased NQO1 protein and activity levels. To evaluate the capacity of NQO1 to reduce NAPQI back to APAP, we utilized a microsomal activating system. Cytochrome P450 enzymes present in microsomes bioactivate APAP to NAPQI, which binds the electrophile trapping agent, N-acetyl cysteine (NAC). We analyzed the formation of APAP-NAC metabolite in the presence of human recombinant NQO1. Results indicate that NQO1 is capable of reducing NAPQI. The capacity of NQO1 to ameliorate APAP toxicity was then evaluated in primary hepatocytes. Primary hepatocytes isolated from mice dosed with CFB are resistant to APAP toxicity. These hepatocytes were also exposed to ES936, a high affinity, irreversible inhibitor of NQO1 in the presence of APAP. Concentrations of ES936 that resulted in over 94% inhibition of NQO1 activity did not increase the susceptibility of hepatocytes from CFB treated mice to APAP. Whereas NQO1 is mechanistically capable of reducing NAPQI, CFB-mediated hepatoprotection does not appear to be dependent upon enhanced expression of NQO1.

Keywords

acetaminophen; clofibrate; peroxisome proliferators; hepatoprotection; NQO1; NAPQI

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Conflict of Interest Statement

There are no conflicts of interest associated with the preparation of this manuscript.

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Introduction

Peroxisome proliferators are diverse compounds that afford protection against chemical-induced hepatotoxicity. Clofibrate (CFB) has been used extensively to investigate the mechanistic basis of this protection. Pretreatment of mice for 10 days with CFB completely prevents acetaminophen (APAP)-induced hepatotoxicity (Nicholls-Grzemski *et al.*, 1992; Manautou *et al.*, 1994). APAP toxicity is highly dependent upon bioactivation by cytochrome P450 enzymes to the reactive intermediate N-acetyl-*p*-benzoquinoneimine (NAPQI), depletion of hepatocellular glutathione (GSH), adduct formation to target hepatic proteins and generation of oxidative stress. The protection afforded by CFB does not involve alterations in metabolism of APAP or basal GSH content, both of which are major determinants of APAP toxicity (Manautou *et al.*, 1994; Nicholls-Grzemski *et al.*, 2000b). However, CFB does diminish the selective binding of NAPQI to target hepatic proteins as well as GSH depletion and enhances cellular antioxidant capacity (Manautou *et al.*, 1994; Nicholls-Grzemski *et al.*, 2000a). The precise mechanism of this hepatoprotective effect is not known.

NAD(P)H:quinone oxidoreductase 1 (NQO1) is a flavoprotein ubiquitously expressed in all tissues (Lind *et al.*, 1990). NQO1 exists as a homodimer requiring either NADH or NADPH as reducing equivalents. NQO1 acts as a cytoprotectant by catalyzing the reduction of quinones and semiquinones to relatively stable hydroquinones (Ernster, 1967). Reduction of semiquinones impedes redox cycling and formation of reactive oxygen species. In addition to quinone reduction, NQO1 directly scavenges superoxide and enhances the effectiveness of existing cellular antioxidants, such as α -tocopherol quinone and coenzyme Q₁₀ (Landi *et al.*, 1997; Siegel *et al.*, 1997; Siegel *et al.*, 2004). The antioxidant capacity of NQO1 is also emerging as an important compensatory response to liver injury. Recent studies from our laboratory documented the induction of hepatic NQO1 protein and activity in human cases of APAP overdose, which might be an adaptive response that limits progression of hepatotoxicity (Aleksunes *et al.*, 2006).

In the current study, we investigated the role of NQO1 in the protection against APAP hepatotoxicity afforded by CFB. Treatment with peroxisome proliferators including CFB increases NQO1 activity in mouse liver (Lundgren *et al.*, 1987; Sohlenius *et al.*, 1993). First, we established that 5- and 10-day CFB treatment increases NQO1 protein and enzyme activity in our mouse model. Second, the capacity of NQO1 to reduce NAPQI availability was determined using an *in vitro* microsomal APAP activation assay. Third, cultured primary hepatocytes from CFB treated mice were used to examine the role of NQO1 induction in the hepatoprotective effect of CFB. The susceptibility of hepatocytes from CFB treated mice to APAP was assessed in the presence of ES936, an irreversible inhibitor of NQO1 (Winski *et al.*, 2001). The results of this study suggest that NQO1 has the capacity to enzymatically reduce NAPQI, but that NQO1 induction is not a central component of CFB-mediated hepatoprotection.

Methods

Chemicals

ES936 was graciously provided by David Ross (University of Colorado Health Sciences Center, Denver, CO) (Winski *et al.*, 2001). Type II collagenase derived from *Clostridium histolyticum* was purchased from Worthington Biochemical (Fairlawn, NJ). All other chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO) and were reagent grade or better.

Animals

Outbred male CD-1 mice aged 10–12 weeks were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in community cages with free access to water and feed (rodent diet No. 5001, PMI Feeds, St. Louis, MO). The vivarium was maintained on a 12-hour dark/light cycle with controlled temperature and humidity. Groups of mice ($n = 3-4$) received daily dosing of CFB (500mg/kg) or corn oil vehicle (5ml/kg), i.p. for 5 or 10 days. Livers were removed, snap frozen in liquid nitrogen, and stored at -80°C until assayed. Primary hepatocytes were also isolated (as described below) from mice dosed with CFB (500mg/kg) or corn oil vehicle (5ml/kg) for 5 or 10 days. The University of Connecticut Institutional Animal Care and Use Committee approved all experimental animal protocols.

Western Blot Analysis of Hepatic NQO1

Livers were homogenized in sucrose-Tris buffer (10 mM Tris base and 150 mM sucrose, pH 7.5) and centrifuged at 10,000g, 4°C for 20 min to remove debris. The resulting supernatants were centrifuged at 120,000g, 4°C for 1 h. Cytosolic fractions were collected and stored at -80°C until assayed. Protein concentrations were determined using the Bio-Rad DC Protein Assay kit (Hercules, CA). Cytosolic proteins (40 μg) were loaded on 12% SDS-polyacrylamide electrophoresis slab gels using a 4% stacking gel followed by electrotransfer to PVDF-Plus membrane (Micron Separations, Westborough, MA). Blots were blocked in 5% nonfat dry milk/phosphate-buffered saline-Tween 20 for 1 h. Primary antibodies obtained from Abcam Inc. (Cambridge, MA) were diluted 1:4000 (NQO1, ab2346) or 1:5000 (β -actin, ab8227) in blocking solution and incubated for 1 h. Immunostained blots were then incubated with peroxidase-conjugated secondary antibodies diluted 1:2000 in blocking buffer for an additional h. Immunoreactive bands were detected using a ECL Chemiluminescent kit (Amersham Life Science, Arlington Heights, IL). Proteins were visualized by exposure to Fuji Medical X-ray film (Fisher Scientific, Pittsburgh). The immunoreactive intensity of proteins was quantified using a PDI Image Analyzer (Protein and DNA ImageWare System; PDI, Inc., Huntington Station, NY). Equivalent protein loading was confirmed with immunoblots for β -actin.

NQO1 Activity Assay

NQO1 activity was evaluated in liver cytosol by measuring the colorimetric reduction of 2,6-dichlorophenolindophenol (DCPIP) over one min as described by Prochaska and Santamaria (1998). Samples from *in vivo* experiments were prepared by homogenizing frozen livers in sample buffer (25mM Tris-HCl, 250mM sucrose, and 1 μM FAD, pH 7.4). Homogenates were centrifuged as described above and the resulting cytosolic fractions were immediately assayed for NQO1 activity. Human recombinant NQO1 (Sigma-Aldrich Corporation, St. Louis, MO) was analyzed for activity following solubilization of the lyophilized protein in sample buffer. Samples from cultured hepatocytes were prepared by extracting the cytosol using digitonin buffer (0.08% digitonin, 17mM MOPS, 250mM sucrose, 2.5mM EDTA, and 1 μM FAD pH7.4) (Mackall *et al.*, 1979). Briefly, media was removed from hepatocytes and replaced with digitonin buffer. Following 10 min incubation at room temperature, the cells were scraped and placed on ice for 15 min. This solution was centrifuged at 14,000xg for 15 min at 25°C and the cytosolic fraction was immediately assayed for NQO1 activity.

In vitro Activation of APAP

The *in vitro* microsomal activation procedure for generating NAPQI was performed according to Manautou *et al.* 1994 with modifications. Liver microsomes from naive male CD-1 mice were prepared and incubated for 30 min with 0.83mM NADP, 15mM MgCl_2 , 20mM glucose-6-phosphate, 4IU glucose-6-phosphate dehydrogenase and 20mM APAP to generate NAPQI. Microsomal incubations were supplemented with 1.0mM N-acetyl cysteine (NAC) as the

trapping agent for NAPQI. The APAP-mercapturate conjugate (APAP-NAC) was then extracted with methanol for analysis by HPLC. All reactions were run in duplicate. Increasing concentrations (0.1 – 10.0 units) of human recombinant NQO1 were added to the NAPQI generating system to examine the capacity of NQO1 to reduce NAPQI back to APAP. Human recombinant NQO1 (hNQO1) was utilized because it is commercially available and its substrate specificity and enzymatic rates are similar to the mouse protein (Faig *et al.*, 2000). Concentrations of hNQO1 protein added to the microsomal incubations were selected to reflect the range of enzyme activities measured in vehicle and CFB treated mouse livers. Although the APAP microsomal activating system contains an NADPH regenerating system, all reactions were supplemented with 200 μ M NADPH to ensure that the enzymatic capacity of NQO1 was not limited by insufficient amounts of reducing equivalents. Cofactor concentrations and the reconstitution buffer were adopted from the NQO1 activity assay protocol. Dicumarol (0.2 – 20 μ M) was added to additional incubations to inhibit NQO1 activity for the purpose of assessing non-enzymatic reduction of NAPQI. The use of dicumarol as an NQO1 inhibitor in these bioactivating reactions allowed us to reserve our limited supply of ES936 for the toxicity studies in cultured hepatocytes. ES936 is another NQO1 inhibitor more suitable for *in vivo* and cell-based studies. A catalytically mutant form of human recombinant NQO1 protein (NQO1*2) was used as a negative control (Gaedigk *et al.*, 1992). The NQO1*2 mutant protein was provided by David Ross (University of Colorado Health Sciences Center, Denver, CO).

Isolation and Culturing of Primary Mouse Hepatocytes

Mouse hepatocytes from vehicle and CFB treated mice were isolated using a collagenase perfusion method described by Davila and Morris (1999). Following percoll enrichment, hepatocyte viability was typically greater than 95% as assessed by trypan-blue dye exclusion. Cells in standard media were plated in 12-well rat collagen coated culture plates (Fisher Scientific, Pittsburgh, PA) at 3.0×10^5 cells per well. The entire hepatocyte isolation and plating procedure was completed within 2h. Hepatocytes were allowed to incubate for 2h in a humidified environment (95%) and 5% CO₂ to facilitate cell attachment to the collagen matrix.

Cell Culture Treatments

Following the initial 2h incubation for hepatocyte attachment to the collagen matrix, cells from vehicle- and CFB-treated mice were exposed to serum-free media with or without 200nM ES936 for 1h to inhibit NQO1 activity (Dehn *et al.*, 2003). After pre-incubation, hepatocytes were then exposed to serum free media with or without 200nM ES936 and 0.5mM APAP for 4 additional h. Both ES936 and APAP were freely miscible in media and the inclusion of ES936 did not alter the pH.

Cytotoxicity Measurement

Cellular toxicity was determined by lactate dehydrogenase (LDH) activity in media using an *In Vitro* Toxicology Assay Kit (Sigma-Aldrich Corporation, St. Louis, MO). Following the 4h ES936 and APAP exposure, media was collected, replaced by lysis buffer, and cells were incubated for 45 min. The plates were centrifuged at 250 x g for 4 min and the cell lysates were collected. All samples (media and cell lysates) were frozen at -20°C until assayed as directed by the manufacturer. Results are expressed as percent of total LDH activity released into the media.

Statistical Analysis

Results are expressed as means $\pm$ standard error of the mean (SE) for 3 to 6 mice per treatment group. Cell culture experiments were conducted in triplicate. Statistical analysis of the data

was performed using a student's T test or ANOVA followed by post-hoc analysis. Differences were considered significant at $p < 0.05$.

Results

Hepatic NQO1 Protein Expression and Enzyme Activity Following CFB Treatment

Hepatic NQO1 protein expression and activity were assessed in male CD-1 mice following daily administration of CFB for 5 or 10 days. Protein expression was induced with the 5- and 10-day CFB regimens by 30% and 77%, respectively (Figure 1A). Equal protein loading was confirmed by β -actin staining. Correspondingly, NQO1 activity increased by approximately 450% after 5 days, and 330% after 10 days of CFB treatment (Figure 1B).

Effect of NQO1 on NAPQI Availability in a Microsomal Activating System for APAP

To investigate the potential for NAPQI reduction by NQO1, we utilized an *in vitro* microsomal activating system for APAP. Incubations of mouse microsomes, cofactors, APAP, and an electrophile trapping agent were allowed to react for 30 min. Cytochrome P450s present in microsomes bioactivate APAP to form NAPQI, which then spontaneously binds to the trapping agent NAC. The resulting stable adduct, APAP-NAC, is quantified by HPLC. It was hypothesized that less APAP-NAC would be detected in the presence of NQO1 added exogenously to the microsomal incubations (Figure 2).

To evaluate the capacity of NQO1 to reduce NAPQI availability, APAP-NAC content was measured in microsomal incubations supplemented with human recombinant NQO1 (hNQO1). Concentrations of hNQO1 added to microsomal incubations were selected for their *in vivo* relevance (Figure 3). The NQO1 activity of 0.1U and 1.0U are roughly comparable to *in vivo* levels of NQO1 in vehicle and CFB treated mice, respectively. Increasing concentrations of hNQO1 added to the microsomal incubations decreased levels of APAP-NAC, indicating reduced availability of NAPQI (Figure 4A). Incubations with 10.0U hNQO1 decreased APAP-NAC levels below detectable limits.

To address potential non-enzymatic interactions between NQO1 and NAPQI, parallel incubations were conducted with dicumarol, an inhibitor of NQO1 (Ernster *et al.*, 1962). Dicumarol has been used extensively to inhibit NQO1 in cell free systems (Prochaska and Santamaria, 1988). Dicumarol produced a concentration-dependent effect on these microsomal reactions (Figure 4B). Incubations with 20 μ M dicumarol completely prevented the reduction of APAP-NAC levels afforded by 10U hNQO1. These results suggest that reduced APAP-NAC levels in the presence of hNQO1 are due to enzyme catalysis. An alternative explanation for reduced APAP-NAC levels in the presence of hNQO1 could be nonspecific binding of NAPQI to hNQO1 protein. In order to address this, we used a catalytically suppressed, mutant form of the protein (hNQO1*2) (Figure 4C) (Gaedigk *et al.*, 1998). This atypical form of NQO1*2 results from a single nucleotide change in the NQO1 sequence, coding for a proline to serine substitution (Traver *et al.*, 1992). NQO1*2 protein equivalent to 0.1, 1.0, and 10 units of hNQO1 were added to individual microsomal reactions. Addition of NQO1*2 to the incubations did not result in decreased APAP-NAC levels, suggesting that NQO1 is not acting as a preferential target for NAPQI covalent binding. Instead, NQO1 appears to be enzymatically reducing NAPQI back to APAP.

Role of NQO1 in Protecting Primary Hepatocytes from APAP

Primary hepatocytes were isolated from male CD-1 mice dosed *in vivo* with CFB or vehicle for 10 days. Similar to the above *in vivo* findings, treatment with CFB for 10 days induced hepatocellular NQO1 activity by 120% (Figure 5A; CFB-media versus vehicle-media). NQO1

activity in hepatocytes from vehicle- and CFB-treated mice exposed to 200nM of the NQO1 inhibitor ES936 was decreased to 7% or less of control values.

The susceptibility of hepatocytes to APAP was assessed by lactate dehydrogenase (LDH) activity, measured as percent of total released in media. Peroxisome proliferator-mediated cytoprotection was confirmed by exposing hepatocytes from vehicle or CFB treated mice to 500 μ M APAP. Hepatocytes from CFB treated mice were protected from APAP, as evidenced by a reduction in LDH activity from 66% in vehicle (Figure 5B; vehicle-APAP) to 31% in CFB pretreated hepatocytes (Figure 5C; CFB-APAP). Exposure to ES936 by itself did not produce cytotoxicity in hepatocytes from either vehicle or CFB treated mice (<5% LDH activity). Co-exposure to 200nM ES936 and 500 μ M APAP did not alter LDH release by APAP only in hepatocytes from CFB or vehicle treated mice (Figure 5C; CFB-APAP versus CFB-APAP-ES936). Furthermore, cytotoxicity studies were also performed using hepatocytes from 5-day CFB treated mice exposed to ES936 and APAP. Similarly, no differences in LDH activity were seen with the use of ES936 after 5 days of CFB treatment (data not shown). These results indicate that inhibition of NQO1 activity does not alter the susceptibility of hepatocytes to APAP, thus enhanced NQO1 activity is not a central component of peroxisome proliferator-mediated hepatoprotection.

Discussion

Toxicity resulting from an APAP overdose produces centrilobular liver necrosis in both rodents and humans. Although the sequence of biochemical changes associated with APAP toxicity is not entirely known, the degree of APAP covalent binding to target hepatic proteins is directly proportional to the severity of toxicity (reviewed by Hinson *et al.*, 2004). In addition, APAP toxicity increases production of reactive oxygen and nitrogen species, often accompanied by lipid peroxidation. The oxidative stress component of APAP toxicity is indicated by the profound hepatoprotection afforded by co-administration of a number of antioxidants including ascorbic acid, cysteamine, and α -tocopherol (Lake *et al.*, 1981; Fairhurst *et al.*, 1982; Miller and Jollow, 1986; Amimoto *et al.*, 1995). Interestingly, some of these antioxidants are known to modulate APAP toxicity without noticeable changes in protein covalent binding. This indicates that chemical hepatoprotection could involve pre- and post-covalent binding interventions.

The role of NQO1 as a central component of CFB-mediated hepatoprotection is intriguing because it could explain the protective effect from several mechanistically diverse toxicants. In addition to APAP, CFB protects from bromobenzene, carbon tetrachloride, and paraquat liver injury (Nicholls-Grzemeski *et al.*, 1996; Manautou *et al.*, 1998). These toxicants all produce oxidative stress, leading to cellular damage. NQO1 is capable of reducing oxidative stress by scavenging superoxide and enhancing endogenous antioxidants (Landi *et al.*, 1997; Siegel *et al.*, 1997; Siegel *et al.*, 2004). Whereas toxicity from carbon tetrachloride and paraquat is primarily associated with oxidative stress due to formation of radicals, APAP and bromobenzene additionally generate reactive quinone metabolites that bind to proteins (Koen and Hanzlik, 2002; Guo *et al.*, 2004). Reduction of these highly reactive quinones by NQO1 may reduce protein adduct formation, thereby decreasing toxicity. Mechanistically, enhanced expression of NQO1 could explain the substantial hepatoprotection afforded by CFB through enhanced antioxidant capacity and direct reduction of reactive quinones.

NQO1 was first identified as a protective enzyme against quinone toxicity when dicumarol inhibited the detoxification of menadione in rat liver (reviewed by Ernster, 1987). Subsequent studies suggest that NQO1 detoxifies a wide range of toxic quinone-related compounds including naphthoquinones, quinone epoxides, and benzoquinones. Several studies have investigated the role of NQO1 in the detoxification of NAPQI. The effect of dicumarol on

NAPQI-mediated toxicity was examined in isolated rat hepatocytes (Rundgren *et al.*, 1988). Addition of dicumarol did not enhance toxicity, leading the authors to conclude that NQO1 was not involved in NAPQI detoxification. It is important to note that this study relied upon direct application of exogenous NAPQI to assess APAP toxicity. Given the highly reactive nature of NAPQI, it is unclear if the cellular accessibility or kinetics of exogenous NAPQI accurately depicts APAP bioactivation within hepatocytes. A subsequent study of NQO1 inhibition *in vivo* demonstrated that dicumarol enhances APAP toxicity (Lee *et al.*, 1999). Pretreatment of mice with dicumarol enhanced the pathology and mortality associated with APAP toxicity. It is difficult to determine the significance of these observations in light of other reported effects of dicumarol besides NQO1 inhibition. Dicumarol is a competitive inhibitor of NQO1 that additionally uncouples mitochondrial oxidation and inhibits APAP glucuronidation, potentially enhancing APAP toxicity through mechanisms independent of NQO1 inhibition (Wilson and Merz, 1967; Bolanowska and Gessner, 1978). The current study addresses these concerns through *in vitro* microsomal activation of APAP, where glucuronidation and uncoupling of oxidative phosphorylation are not confounding effects in assessing the capacity of NQO1 to reduce NAPQI. Furthermore, APAP toxicity studies in cultured hepatocytes were conducted using ES936, a highly selective, irreversible NQO1 inhibitor to reduce possible NQO1-independent interactions attributed to the use of dicumarol.

Measurement of NQO1 activity in mouse liver cytosol following 5- and 10- day CFB treatment confirmed previous observations that peroxisome proliferators enhance NQO1 activity in mouse liver (Lundgren *et al.*, 1987; Sohlenius *et al.*, 1993). To evaluate the capacity of NQO1 to reduce NAPQI availability, APAP-NAC concentrations from microsomal incubations supplemented with human recombinant NQO1 (hNQO1) were quantified. The results of these experiments suggest that hNQO1 is capable of reducing NAPQI back to APAP, which would explain the diminished APAP covalent binding and GSH depletion seen *in vivo* in CFB-pretreated mice in the absence of changes in APAP bioactivation and glucuronidation (Manautou *et al.*, 1994). To address potential non-enzymatic interactions between NQO1 and NAPQI, parallel incubations were conducted with an inhibitor of NQO1, dicumarol (Prochaska and Santamaria, 1988). The inclusion of dicumarol prevented the reduction of APAP-NAC formation by hNQO1 in a concentration dependent manner. Furthermore, the potential for NQO1 becoming a preferential target of NAPQI covalent binding instead of the trapping agent NAC was addressed using a catalytically suppressed, mutant form of the protein (NQO1*2). Inclusion of NQO1*2 to the reaction mixture resulted in APAP-NAC levels comparable to control incubations lacking recombinant NQO1, suggesting that the protein is not acting as a reservoir for APAP binding.

Whereas the above studies provide a proof of concept that NQO1 reduces NAPQI, they do not address whether or not NQO1 protects against APAP *in vivo*. If NQO1 is a central component of CFB-mediated protection from APAP, exposure to an NQO1 inhibitor would restore the susceptibility of the liver to APAP toxicity in CFB treated mice. Dicumarol has long been used to inhibit NQO1 activity. However, given the complexities of using dicumarol *in vivo* and in cultured hepatocytes, we utilized ES936 instead. Limited availability of this highly specific inhibitor of NQO1 ES936 precluded its use in the intact animal. Sufficient quantities were only available to conduct experiments with freshly isolated hepatocytes. Cultured primary hepatocytes from mice treated with CFB are resistant to a number of toxicants including APAP (Nicholls-Grzemeski *et al.*, 2000b). We showed that cultured hepatocytes from CFB treated mice have enhanced NQO1 activity, consistent with the CFB effect *in vivo*. Furthermore, ES936 was able to effectively block NQO1 activity to at least 7% of control in these cultured hepatocytes (from both vehicle and CFB treated mice). However, exposure to ES936 did not enhance the hepatocellular toxicity of APAP in cells from CFB or vehicle treated mice. These studies are significant since they rule out the contribution of an enzyme whose induction might

have explained the decreases in APAP covalent binding and GSH depletion by CFB treatment in the absence of changes in NAPQI formation by cytochrome P450 enzymes, while also explaining the protection against other hepatotoxicants like carbon tetrachloride by scavenging reactive oxygen species and maintaining favorable cellular antioxidant status.

In conclusion, this study demonstrates that NQO1 is mechanistically capable of reducing NAPQI availability. However, enhanced NQO1 activity in mouse liver is not sufficient to explain CFB-mediated hepatoprotection from APAP.

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Figure 1.

Measurement of NQO1 protein expression and activity in mouse liver cytosol following 5 or 10 day treatment with CFB. Asterisks (*) represent a statistical difference from vehicle where $p < 0.05$ ($n = 3-4$). A. NQO1 protein expression following 5 or 10 day treatment with CFB. The data are presented as normalized optical density ($OD \times mm^2$ percent of control) $\pm$ SE. β -actin protein expression was measured as a loading control. B. NQO1 activity following 5 or 10 day treatment with CFB. The data are presented as mean specific activity (nmoles DCPIP reduced/min/mg protein) $\pm$ SE.

Figure 2.

Proposed mechanism of NAPQI detoxification by NQO1 through quinone reduction. APAP is bioactivated by cytochrome P450 to the reactive intermediate NAPQI, which readily binds to the electrophile trapping agent NAC forming the stable metabolite, APAP-NAC. Enhanced levels of hepatic NQO1 may detoxify NAPQI by reducing it back to the parent APAP compound.

Figure 3.

Measurement of human recombinant NQO1 (hNQO1) activity was carried out to determine protein amounts relevant to hepatic NQO1 activity *in vivo*. Results indicate that 0.1, 1.0, and 10.0 units of hNQO1 roughly correspond to vehicle, CFB treated, and supraphysiological levels of hepatic NQO1 activity, respectively. The data are presented as mean specific activity (nmoles DCPIP reduced/min/mg protein) $\pm$ SE. Asterisks (*) represent a statistical difference from reactions containing 0.1U NQO1 where $p < 0.05$ ($n = 3$).

Figure 4.

Indirect measurement of NAPQI availability in the presence of increasing concentrations of human recombinant NQO1 (hNQO1). APAP is metabolically activated by microsomes (micros) to the reactive intermediate NAPQI. NAC included in the microsomal reaction mixture binds NAPQI to form the stable conjugate APAP-NAC. Asterisks (*) represent a statistical difference from microsomal control incubations (white bar) (n=3)

A. Analysis of APAP-NAC formation rates in microsomal incubations supplemented with increasing amounts of hNQO1.

B. Analysis of APAP-NAC formation rates in microsomal incubations supplemented with 10.0 units of hNQO1 and increasing amounts of dicumarol (Dic).

C. Analysis of APAP-NAC formation rates in microsomal incubations supplemented with increasing amounts of a catalytically mutant form of NQO1 (NQO1*2).

Figure 5.

Effect of NQO1 inhibition on CFB-mediated cytoprotection. Asterisks (*) represent a statistical difference from vehicle only treatment and daggers (†) represent a difference from CFB only treatment where $p < 0.05$ ($n = 3$ wells per treatment)

A. Measurement of NQO1 activity in cytosol from hepatocytes incubated in the presence of the NQO1 inhibitor ES936. Hepatocytes were isolated from mice dosed with vehicle or CFB and then incubated in the presence or absence of ES936. The data are presented as mean specific activity (nmoles DCPIP reduced/min/mg protein) $\pm$ SE.

B. Effect of NQO1 inhibition on APAP toxicity in hepatocytes isolated from vehicle treated mice. The data are presented as percent of total LDH activity released into the media $\pm$ SE.

C. Effect of NQO1 inhibition on APAP toxicity in hepatocytes isolated from CFB treated mice. The data are presented as percent of total LDH activity released into the media $\pm$ SE.