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ScienceDirect
Toxicology 230 (2007) 197–206
TOXICOLOGY
www.elsevier.com/locate/toxicol

Role of NAD(P)H:quinone oxidoreductase 1 in clofibrate-mediated hepatoprotection from acetaminophen☆

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Received 31 July 2006; received in revised form 9 November 2006; accepted 12 November 2006
Available online 19 November 2006

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 amelioriate 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, and 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

1. 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

☆ Presented at the Society of Toxicology Meeting, New Orleans, LA, USA, March 2005.

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doi:10.1016/j.tox.2006.11.052

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 Q_{10} (Landi et al., 1997; Siegel et al., 1997, 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.

2. Methods

2.1. 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.

2.2. 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 (500 mg/kg) or corn oil vehicle (5 ml/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 (500 mg/kg) or corn oil vehicle (5 ml/kg) for 5 or 10 days. The University of Connecticut Institutional Animal Care and Use Committee approved all experimental animal protocols.

2.3. 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 10,000 × *g*, 4 °C for 20 min to remove debris. The resulting supernatants were centrifuged at 120,000 × *g*, 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% non-fat 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, PA). 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.