

Chapter 2

Nrf2 and Antioxidant Defense Against CYP2E1 Toxicity

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Abstract The transcription factor Nrf2 regulates the expression of important cytoprotective enzymes. Induction of cytochrome P450 2E1(CYP2E1) is one of the central pathways by which ethanol generates oxidative stress. CYP2E1 can be induced by ethanol and several low molecular weight chemicals such as pyrazole. The chapter discusses biochemical and toxicological effects of CYP2E1 and the effects of Nrf2 in modulating these actions of CYP2E1. Besides ethanol, CYP2E1 metabolizes and activates many other important toxicological compounds. One approach to try to understand basic effects and actions of CYP2E1 was to establish HepG2 cell lines that constitutively express human CYP2E1. Ethanol, polyunsaturated fatty acids and iron were toxic to the HepG2 cells which express CYP2E1 (E47 cells) but not control C34HepG2 cells which do not express CYP2E1. Toxicity was associated with enhanced oxidant stress and could be prevented by antioxidants and potentiated if glutathione (GSH) was removed. The E47 cells had higher GSH levels and a twofold increase in catalase, cytosolic and microsomal glutathione transferase, and heme oxygenase-1 (HO-1) than control HepG2 cells due to activation of their respective genes. These activations were prevented by antioxidants, suggesting that reactive oxygen species (ROS) generated by CYP2E1 were responsible for the up-regulation of these antioxidant genes. This upregulation of antioxidant genes may reflect an adaptive mechanism to remove CYP2E1-derived oxidants. Increases in Nrf2 protein and mRNA were observed in livers of chronic alcohol-fed mice or rats and of pyrazole-treated rats or mice, conditions known to elevate CYP2E1. E47 cells showed increased Nrf2 mRNA and protein expression compared with control HepG2 C34 cells. Upregulation of antioxidant genes in E47 cells is dependent on Nrf2 and is prevented by siRNA-Nrf2. Blocking Nrf2 by siRNA-Nrf2 decreases

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GSH and increases ROS and lipid peroxidation, resulting in decreased mitochondrial membrane potential and loss of cell viability of E47 cells but not C34 cells. Nrf2 is activated and levels of Nrf2 protein and mRNA are increased when CYP2E1 is elevated. These results suggest that Nrf2 plays a key role in the adaptive response against increased oxidative stress caused by CYP2E1 in the HepG2 cells.

Keywords Antioxidants • CYP2E1 • Ethanol hepatotoxicity • HepG2 cells • Nrf2 • Oxidative stress

2.1 Introduction

Alcohol acts through numerous pathways to affect the liver and other organs and to lead to the development of alcoholic liver disease (ALD) (Gonzalez 2005; Novak and Woodcroft 2000; Jimenez-Lopez and Cederbaum 2005; Dey and Cederbaum 2006; Nordmann et al. 1992; Ishii et al. 1997; Cederbaum 2001). Many mechanisms act in concert, reflecting the spectrum of the organism's response to a myriad of direct and indirect actions of alcohol. A summary of some of the most common causes for alcohol toxicity is shown in Table 2.1. One factor playing a central role in many pathways of alcohol-induced damage is the excessive generation of free radicals (Cederbaum 2001; Arteel 2003). Reactive oxygen species (ROS) can damage or cause complete degradation of essential complex molecules in cells, including lipids, proteins, and DNA. Both acute and chronic alcohol exposure can increase production of ROS and enhance peroxidation of lipids, protein, and DNA, as has been demonstrated in a variety of systems, cells, and species, including humans (Toykuni 1999; de Groot 1994). Under certain conditions, such as acute or chronic alcohol exposure, ROS production is enhanced and/or the level or activity of antioxidants is reduced. The resulting state which is characterized by a disturbance in the balance between ROS production on one hand and ROS removal and repair of damaged complex molecules on the other—is called oxidative stress. Many processes and factors are involved in causing alcohol-induced oxidative stress. Table 2.2 presents some of the leading mechanisms which have been proposed to promote alcohol-induced oxidative stress and are briefly discussed below and in more detail in Jimenez-Lopez and Cederbaum (2005); Dey and Cederbaum (2006); Nordmann et al. (1992); Ishii et al. (1997); Arteel (2003).

Changes in the NAD⁺/NADH redox ratio in the cell as a result of alcohol metabolism play an important role in ethanol-induced oxidative stress (Kurose et al. 1997). The production of acetaldehyde as a consequence of ethanol oxidation contributes to oxidative stress because of its reactivity and interactions with proteins and lipids can lead to radical formation and cellular damage. Acetaldehyde produces protein adducts which can be immunogenic and toxic (Niemela 2001). Alcohol has long been known to damage the mitochondrial electron transport chain (Hoek et al. 2002; Bailey 2003). This not only lowers ATP levels but also increases ROS production as a consequence of a reduced respiratory chain (Kurose et al. 1997; Bailey and

Table 2.1 Causes and mechanisms for alcohol mediated oxidative stress and toxicity

<i>A. Suggested causes for alcohol toxicity</i>
Redox state changes (↓NAD ⁺ /NADH)
Acetaldehyde formation
Membrane effects
Immune response
Hypoxia
Kupffer cell activation
Cytokine formation
Mitochondrial damage
Oxidative stress
<i>B. Suggested mechanisms for alcohol-induced oxidative stress</i>
Decrease in antioxidant defense
Mobilization of iron
Metabolic effects-↓NAD ⁺ /NADH; acetaldehyde
Release of chemoattractants
Activation of Kupffer cells-release of cytokines
Mitochondrial injury-↓ATP, ↓Δψ, ↑permeability transition
Hypoxia
Formation of 1-hydroxyethyl radical
Induction of CYP2E1

Table 2.2 Characterization of cytochrome P450 2E1

Cytochrome P4502E1 (CYP2E1)
<i>A. A minor pathway of ethanol metabolism</i>
Produces acetaldehyde, 1-hydroxyethyl radical
Modulates drug metabolism, alcohol drug interactions
Activates hepatotoxins e.g. acetaminophen, benzene, CCl ₄
Activates procarcinogens e.g. nitrosamines, azo-compounds
Loosely coupled CYP-produces superoxide, H ₂ O ₂
<i>B. Induced by</i>
Ethanol
Alcohols, solvents e.g. DMSO, pyridine
Halogenated compounds, imidazoles, azoles
Pathophysiological conditions e.g. obesity, diabetes, fasting
Non-alcoholic fatty liver and nonalcoholic steatohepatitis

Cunningham 1998). Alcohol-induced hypoxia, especially in the pericentral zone of the liver acinus where extra oxygen is necessary to metabolize ethanol also causes reduction of the electron transfer chain which can then generate ROS when oxygen is available after the ethanol has been metabolized. Alcohol causes leakiness in the gut epithelium which allows bacteria to enter the blood. Bacterial secreted lipopolysaccharide (LPS) activates immune cells, especially hepatic Kupffer cells and the elevated production of cytokines, especially tumor necrosis factor alpha (TNFα) elevates oxidative stress (Thurman 1998; Honchel et al. 1992; Takei et al. 2005).

CYP2E1 is a loosely coupled cytochrome P450 which is very reactive in producing superoxide during its catalytic cycle (Gonzalez 2005; Novak and Woodcroft 2000). CYP2E1 is elevated by alcohol which may be a major mechanism for alcohol-induced oxidant stress as discussed below. Alcohol increases levels of cellular iron (Valerio et al. 1996; Stal et al. 1996). Iron promotes the production of powerful oxidants such as the hydroxyl radical by catalyzing the Fenton or the Haber-Weiss reactions. Alcohol can generate an alcohol free radical, the one hydroxyethyl radical as a result of its one electron oxidation by ROS and by CYP2E1 (Albano et al. 1988). Many of these processes operate concurrently, and it is likely that several, indeed many systems contribute to the ability of alcohol to induce a state of oxidative stress.

2.2 Systems Producing ROS

The major source of ROS production in the cell is the mitochondrial respiratory chain which utilizes approximately 80–90% of the oxygen consumed. Thus, even though only a small percentage of that oxygen is converted to ROS, the mitochondrial respiratory chain in all cells generates most of the ROS produced in the body (Chance et al. 1979). Another major source of ROS, especially in the liver, is the cytochrome P450 mixed-function oxidases. P450s are responsible for removing or detoxifying a variety of compounds present in our environment and ingested (e.g., foods or drugs), including alcohol. Oxygen activation by P450, necessary for the catalytic function of the enzymes, can also result in the production of ROS. Small amounts of the superoxide anion radical ($O_2^{\cdot-}$) can be produced from decay of the oxygenated P450 complex; while hydrogen peroxide (H_2O_2) can form from either dismutation of $O_2^{\cdot-}$ or from decay of the peroxy P450 complex (White 1991). The extent of ROS generation may vary considerably depending on the compound to be degraded and on the cytochrome P450 molecule involved. CYP2E1 is of particular interest when investigating alcohol-induced oxidative stress because its activity increases after heavy alcohol exposure and because CYP2E1 itself also metabolizes alcohol (Lieber 1997).

2.2.1 Protection Against ROS Toxicity

A variety of enzymatic and nonenzymatic mechanisms have evolved to protect cells against ROS (Halliwell 1999). Antioxidant enzymes involved in the elimination of ROS include superoxide dismutases (SODs), catalase, and glutathione peroxidase. SODs catalyze removal of superoxide radicals. A copper–zinc SOD is present in the cytosol and in the space between the two membranes surrounding the mitochondria, while a manganese-containing SOD is present in the matrix. Catalase and the glutathione peroxidase system both help to remove H_2O_2 . Catalase is found primarily in peroxisomes, it catalyzes a reaction between two H_2O_2 molecules, resulting in the

formation of water and O_2 . Catalase can use H_2O_2 to metabolize alcohols such as ethanol and methanol to acetaldehyde and formaldehyde, respectively. The glutathione peroxidase system consists of several components, including the enzymes glutathione peroxidase and glutathione reductase and the cofactors GSH and NADPH. Together, these molecules effectively remove H_2O_2 . GSH is synthesized from glutamate plus cysteine plus glycine in two steps as catalyzed by gamma glutamyl cysteine synthetase (GCS) and glutathione synthetase. GCS is the rate limiting enzyme in this two step pathway. Because of all its functions, GSH is probably the most important nonenzymatic antioxidant present in cells. Enzymes that help generate GSH are critical to the body's ability to protect itself against oxidative stress. Numerous other nonenzymatic antioxidants are present in cells, most prominently vitamin E (α -tocopherol) and vitamin C (ascorbate). Vitamin E is a major antioxidant found in the lipid phase of membranes and, acts as a powerful terminator of lipid peroxidation.

The effects of ethanol on total hepatic GSH levels are variable, with reports of decreases, no effects, or even an increase (Fernandez-Checa et al. 1997; Polavarapu et al. 1998; Oh et al. 1998). Lowering of mitochondrial GSH by chronic ethanol treatment appears to be a key lesion contributing to ALD (Fernandez-Checa et al. 1997; Colell et al. 1998). Because liver mitochondria lack catalase, mitochondrial GSH in association with glutathione peroxidase is the major mechanism by which H_2O_2 is detoxified by mitochondria. Chronic ethanol intake either in the Lieber–DeCarli model or the intragastric infusion model selectively lowers levels of mitochondrial GSH in hepatocytes (Colell et al. 1998; Garcia-Ruiz et al. 1995). Depletion of mitochondrial GSH by chronic ethanol feeding occurs preferentially in pericentral hepatocytes, where most of the liver injury originates (Colell et al. 1998). Lowering of mitochondrial GSH by ethanol has been suggested to sensitize hepatocytes to $TNF\alpha$ -induced cell death, and replenishment of mitochondrial GSH with S-adenosylmethionine protects hepatocytes from alcohol-treated rats to TNF toxicity (Colell et al. 1998). Bailey et al. (2001) however, found that mitochondrial GSH levels were increased after chronic ethanol feeding in the Lieber–DeCarli model by approximately 25%. This finding was suggested to reflect an adaptive response to counteract ethanol-related increases in mitochondrial production of ROS. Thus, the effects of ethanol on mitochondrial GSH, as with total GSH, remain controversial.

2.2.2 Alcohol, Oxidative Stress, and Cell Injury

What is the evidence that alcohol-induced oxidative stress plays a role in cell injury, particularly damage to the liver cells? Many studies have demonstrated that alcohol increases lipid peroxidation as well as the modification of proteins; however, it is not always clear if these changes are the causes rather than consequences of alcohol-induced tissue injury. Nevertheless, numerous investigations have found that administering antioxidants, agents that reduce the levels of free iron, or agents that replenish GSH levels can prevent or ameliorate the toxic actions of alcohol

(Kono et al. 2001; Iimuro et al. 2000; Sadrzadeh et al. 1994; Wheeler et al. 2001). For example, in the intragastric infusion model, the antioxidant vitamin E; the chemical ebselen, which mimics the actions of glutathione peroxidase; the copper–zinc or manganese SODs; or a GSH precursor—all prevented ALD (Kono et al. 2001; Iimuro et al. 2000; Sadrzadeh et al. 1994; Wheeler et al. 2001).

ALD was associated with enhanced lipid peroxidation, protein modification, formation of the 1-hydroxyethyl radical and lipid radicals, and decreases in the hepatic antioxidant defense, particularly GSH levels (Polavarapu et al. 1998; Kono et al. 2001; Iimuro et al. 2000). Moreover, changes in the animals' diets that helped promote or reduce oxidative stress led to corresponding changes in the extent of liver injury. For example, when polyunsaturated fats were replaced with saturated fats or medium-chain triglycerides, lipid peroxidation as well as ALD were reduced or prevented completely, indicating that both alcohol and polyunsaturated fats must be present for ALD to occur (Nanji et al. 2001). The extent of the ALD was further exacerbated when iron which promotes oxidative stress—was added to these diets (Castillo et al. 1992).

Studies with liver cells grown in culture also showed that alcohol can produce oxidative stress and hepatocyte toxicity (Ishii et al. 1997; Bailey and Cunningham 1998). Studies with hepatocytes isolated from control rats or from rats fed alcohol indicated that alcohol metabolism *via* the enzyme alcohol dehydrogenase results in increased ROS production, hepatocyte injury, and apoptosis. These reactions could be blocked by antioxidants (Ishii et al. 1997). Studies using an established hepatocyte cell line that expresses CYP2E1 demonstrated that adding alcohol, polyunsaturated fatty acids, or iron, as well as reducing GSH, resulted in cell toxicity, increased oxidative stress, and mitochondrial damage (Jimenez-Lopez and Cederbaum 2005; Caro and Cederbaum 2004). These reactions could be prevented by administering antioxidants. HepG2 cells expressing both CYP2E1 and ADH, the VL-17A cells have been very valuable in studies on ethanol-induced oxidative stress, cell injury and alteration of proteasome activity (Clemens 2006). Taken together, these findings indicate that alcohol-induced oxidative stress is a pivotal factor in the development of ALD.

2.3 CYP2E1

CYP2E1 metabolizes a variety of small, hydrophobic substrates and drugs (Gonzalez 2005; Lieber 1997; Yang et al. 1990; Bolt et al. 2003; Tanaka et al. 2000). Possible physiological substrates are acetone and fatty acids such as linoleic and arachidonic acid (Koop 1992; Koop and Casazza 1985). From a toxicological point of view, interest in CYP2E1 revolves around the ability of this enzyme to metabolize and activate many toxicologically important compounds such as ethanol, carbon tetrachloride, acetaminophen, benzene, halothane and many other halogenated substrates. Procarcinogens including nitrosamines and azo compounds are effective substrates for CYP2E1 (Yang et al. 1990; Guengerich et al. 1991). Toxicity by the above

compounds is enhanced after induction of CYP2E1 e.g. by ethanol treatment, and toxicity is reduced by inhibitors of CYP2E1 or in CYP2E1 knockout mice (Lee et al. 1996).

Molecular oxygen itself is likely to be a most important substrate for CYP2E1. CYP2E1, relative to several other P450 enzymes, displays high NADPH oxidase activity as it appears to be poorly coupled with NADPH-cytochrome P450 reductase (Gorsky et al. 1984; Ekstrom and Ingelman-Sundberg 1989). CYP2E1 was the most efficient P450 enzyme in the initiation of NADPH-dependent lipid peroxidation in reconstituted membranes among five different P450 forms investigated. Furthermore, anti-CYP2E1 IgG inhibited microsomal NADPH oxidase activity and microsomal lipid peroxidation dependent on P450 (Ekstrom and Ingelman-Sundberg 1989; Lu and Cederbaum 2008). Microsomes isolated from rats fed ethanol chronically were about twofold to threefold more reactive in generating superoxide radical and H_2O_2 and hydroxyl radical and undergoing lipid peroxidation compared to microsomes from pair-fed controls (Rashba-Step et al. 1993). CYP2E1 levels were elevated about threefold to fivefold in the liver microsomes after feeding rats the Lieber-DeCarli diet for 4 weeks. The enhanced effectiveness of microsomes isolated from the ethanol-fed rats in generating ROS was prevented by addition of chemical inhibitors of CYP2E1 and by polyclonal antibody raised against CYP2E1, confirming that the increased activity in these microsomes was due to CYP2E1. Table 2.2 summarizes some properties of CYP2E1.

Many of the substrates for CYP2E1 can induce their own metabolism. This was initially observed with ethanol, which is a substrate for CYP2E1 and elevates CYP2E1 levels (Lieber 1997; 1999). A variety of heterocyclic compounds such as imidazole, pyrazole, 4-methylpyrazole, thiazole, isoniazid have been shown to elevate CYP2E1 levels as do solvents such as dimethylsulfoxide, various alcohols, benzene and acetone (Song et al. 1986). CYP2E1 can also be induced under a variety of metabolic or nutritional conditions. For example, CYP2E1 levels were elevated in chronically obese, overfed rats and in rats fed a high-fat diet (Lieber 1997, 1999). Diabetes has been reported to increase the expression of CYP2E1 mRNA and protein levels several fold largely by stabilizing CYP2E1 mRNA (Song et al. 1987; Woodcroft et al. 2002; Bellward et al. 1988). This may be related to actions of insulin (Woodcroft et al. 2002). The carbohydrate content of the diet influences CYP2E1 levels as a low carbohydrate diet increased the induction of CYP2E1 by ethanol and high fat/low carbohydrate diets resulted in the highest levels of CYP2E1 induced by ethanol (Yoo et al. 1991).

Nonalcoholic steatohepatitis (NASH) is a condition characterized by hepatomegaly, elevated serum aminotransferase levels, and a histologic picture similar to alcoholic hepatitis. Oxidative stress and lipid peroxidation are among the critical factors involved in the genesis and probably the progression of NASH (Day 2006). In a mouse model of NASH, hepatic CYP2E1 was upregulated, and this was associated with a dramatic increase in total lipid peroxide levels that were substantially inhibited by anti-CYP2E1 (Weltman et al. 1996). The induction of CYP2E1 by low molecular weight chemicals mentioned above, including pyrazole and ethanol, is largely due to a posttranscriptional mechanism in which the inducer stabilizes CYP2E1 against proteasome-mediated

degradation (Song et al. 1986; Koop and Tierney 1990; Roberts 1997). The half life of CYP2E1 has been shown to increase from less than 5–7 h in the absence of inducer to more than 24 h in the presence of inducers such as acetone *in vivo* (Song et al. 1989) or pyrazole *in vitro* (Yang and Cederbaum 1997).

2.3.1 CYP2E1 and Alcohol-Induced Liver Injury

Since CYP2E1 can generate ROS during its catalytic cycle and its levels are elevated by chronic treatment with ethanol, CYP2E1 has been suggested as a major contributor to ethanol-induced oxidant stress, and to ethanol-induced liver injury even though it is only a minor pathway of alcohol oxidation as alcohol dehydrogenase is the major pathway. In the intragastric model of ethanol feeding, prominent induction of CYP2E1 occurs as does significant liver injury (Morimoto et al. 1994; Nanji et al. 1994). In this model, large increases in microsomal lipid peroxidation have been observed and the ethanol-induced liver pathology has been shown to correlate with CYP2E1 levels and elevated lipid peroxidation (Morimoto et al. 1994; Nanji et al. 1994). Experimentally, a decrease in CYP2E1 induction was found to be associated with a reduction in alcohol-induced liver injury (Morimoto et al. 1994). CYP2E1 inhibitors such as diallylsulfide, phenethylisothiocyanate, and chlormethiazole (Gouillon et al. 2000), blocked the lipid peroxidation and ameliorated the pathologic changes in ethanol-fed rats. A CYP2E1 transgenic mouse model was developed that overexpressed CYP2E1 (Morgan et al. 2002). When treated with ethanol, the CYP2E1 over-expressing mice displayed higher transaminase levels and histological features of liver injury compared with the control mice. Infection of HepG2 cells with an adenoviral vector which expresses human CYP2E1 potentiated acetaminophen toxicity as compared to HepG2 cells infected with a LacZ expressing adenovirus. Administration of CYP2E1 adenovirus *in vivo* to mice produced significant liver injury compared to the LacZ-infected mice as reflected by histopathology, markers of oxidative stress and elevated transaminase levels (Bai and Cederbaum 2006). Bradford et al. (2005) using CYP2E1 and NADPH oxidase knockout mice concluded that CYP2E1 was required for ethanol induction of oxidative stress to DNA, whereas NADPH oxidase was required for ethanol-induced liver injury. As mentioned earlier, it is likely that several mechanisms contribute to alcohol-induced liver injury, and that ethanol-induced oxidant stress is likely to arise from several sources, including CYP2E1, mitochondria and activated Kupffer cells.

2.3.2 Biochemical and Toxicological Properties of CYP2E1 in HepG2 Cells

One approach our laboratory utilized to try to understand basic effects and actions of CYP2E1 was to establish cell lines that constitutively express human CYP2E1.

HepG2 cell lines, which overexpress CYP2E1, were established either by retroviral infection methods (MV2E1-9 cells, or E9 cells) or by plasmid transfection methods (E47 cells) (Dai et al. 1993; Chen and Cederbaum 1998). The toxicity of acetaminophen, ethanol, polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA) and iron was characterized in the E9 and E47 cell lines (Dai and Cederbaum 1995; Chen et al. 1997; Sakurai and Cederbaum 1998; Wu and Cederbaum 1996, 1999). Concentrations of ethanol or AA which were toxic to the CYP2E1-expressing cells had no effect on control MV2E1 or E9 HepG2 cells not expressing CYP2E1. Toxicity to CYP2E1-expressing cells was found when GSH was depleted by treatment with 1-buthionine sulfoximine (BSO) (Wu and Cederbaum 2001). Inhibitors of CYP2E1 prevented the toxicity by the above treatments. Antioxidants such as vitamin E, trolox, N-acetylcysteine, and thiourea prevented toxicity found when the CYP2E1-expressing E9 HepG2 cells were treated with either ethanol or AA. The above treatments of CYP2E1-expressing cells with ethanol, AA, iron or BSO resulted in an increase in oxidative stress to the cells as reflected by increased lipid peroxidation and enhanced dichlorofluorescein fluorescence.

Work from several laboratories has indicated that mitochondrial damage may represent a common early event in cell injury caused by toxic agents. Mitochondrial damage is initially manifested by a decrease in mitochondrial membrane potential ($\Delta\psi_m$) followed by ATP depletion (Orrenius et al. 1996). Mitochondrial damage appears to be an important event in CYP2E1-mediated toxicity. Mitochondrial membrane potential was assessed by flow cytometry after double staining with rhodamine 123 and propidium iodide (PI). Exposure of E47 cells to BSO (Mari et al. 2002), AA (Perez and Cederbaum 2001), and Fe+AA (Caro and Cederbaum 2001) increased the percentage of cells that showed low rhodamine 123 fluorescence but were not stained with PI. This population refers to cells that are still viable but with damaged mitochondria, showing that these CYP2E1-dependent models of toxicity affect mitochondria before the onset of cell death (i.e., early event). This early mitochondrial damage was prevented by antioxidants, linking oxidative stress to mitochondrial damage. If the decrease of mitochondrial membrane potential depends on the opening of the permeability transition pore, then a specific inhibitor of the pore opening should decrease the loss of mitochondrial membrane potential induced by the toxic agents. Cyclosporin A inhibited the loss of $\Delta\psi_m$ and the toxicity in CYP2E1-expressing cells exposed to AA, AA+Fe, and BSO (Caro and Cederbaum 2002; Wu and Cederbaum 2002) suggesting a role for the permeability transition on mitochondrial depolarization and subsequent toxicity.

2.3.3 Upregulation of Antioxidant Defense in CYP2E1 Expressing Liver Cells

While much of the focus on CYP2E1 has been from a toxicological point of view, the possibility the hepatocyte attempts to respond to increased levels of CYP2E1 by upregulation of protective factors has not been examined. Whether CYP2E1

over-expression could mediate an effect on GSH homeostasis and modulate the levels of other antioxidant enzymes important for the removal of ROS was evaluated. E47 cells had a significant 30% increase in total GSH as compared to C34 cells (Mari and Cederbaum 2000). There was no difference in the activity of glutathione reductase or superoxide dismutase between C34 and E47 cells. There was a 30% decrease in glutathione peroxidase activity in CYP2E1-expressing cells. However, there was a twofold increase in the activities of total GST and catalase in E47 cells compared to C34 cells. Northern blot analysis and nuclear run-on experiments indicated that increases in catalase and GST were due to activation of their respective genes (Mari and Cederbaum 2001). These activations in the E47 cells were prevented by antioxidants, suggesting that ROS generated by CYP2E1 were responsible for the upregulation of these antioxidant genes. GCS is composed of a heavy catalytic subunit (GCS_c) and a lighter regulatory subunit (GCS_r) (Lu 1999). The CYP2E1-expressing E47 cells showed a twofold increase in mRNA levels of both transcripts and nuclear run-on experiments revealed increased capacity of the E47 cells to transcribe the GCS_c gene. The upregulation of these antioxidant genes may reflect an adaptive mechanism to remove CYP2E1-derived oxidants. Many of the effects found in the transformed HepG2 cells with respect to biochemical and toxicological actions of CYP2E1 were also found in intact rat hepatocytes, including a CYP2E1-dependent elevation of hepatic GSH levels and increases in GCS_c and GCS_r mRNA levels (Nieto et al. 2003).

Hemeoxygenase (HO-1) is the rate-limiting enzyme in the conversion of heme into biliverdin, carbon monoxide (CO), and free iron (Fe^{2+}) (Choi and Alam 1996). Up-regulation of HO-1 may be among the most critical cytoprotective mechanisms that are activated during cellular stress (Maines 1997). Studies to evaluate whether CYP2E1 derived oxidant stress up-regulates HO-1, in analogy to experiments described above for GCS and what are the functional consequences of this upregulation were carried out (Gong et al. 2003, 2004). HO-1 induction was observed in the livers of chronic alcohol-fed mice or pyrazole-treated rats, conditions known to elevate CYP2E1 levels. Increased levels of HO-1 mRNA and protein were observed in HepG2 cells over expressing CYP2E1 (E47 cells) compared with control C34 HepG2 cells (Fig. 2.1b, c). Expression of CYP2E1 in HepG2 cells transcriptionally activated the HO-1 gene, increasing HO-1 mRNA and protein expression and activity of a HO-1 reporter construct. CYP2E1 inhibitors such as diallyldisulfide, 4-methylpyrazole and dimethylsulfoxide blocked the increased production of ROS (Fig. 2.1a) as well as HO-1 induction (Fig. 2.1b, c). The phosphorylated form of ERK MAPK but not that of p38 or JNK MAPK was increased in E47 cells compared with the control C34 HepG2 cells. PD98059, a specific inhibitor of ERK MAPK, blocked the activity of a HO-1 reporter in E47 cells but not in C34 cells. These results suggest that increased CYP2E1 activity leads to induction of the HO-1 gene, and the ERK MAPK pathway is important in mediating this process. This induction may serve as an adaptive mechanism to protect the E47 cells against the CYP2E1-dependent oxidative stress.

The possible functional significance of this increase in HO-1 in protecting against CYP2E1-dependent toxicity was evaluated (Gong et al. 2004). Treatment with AA

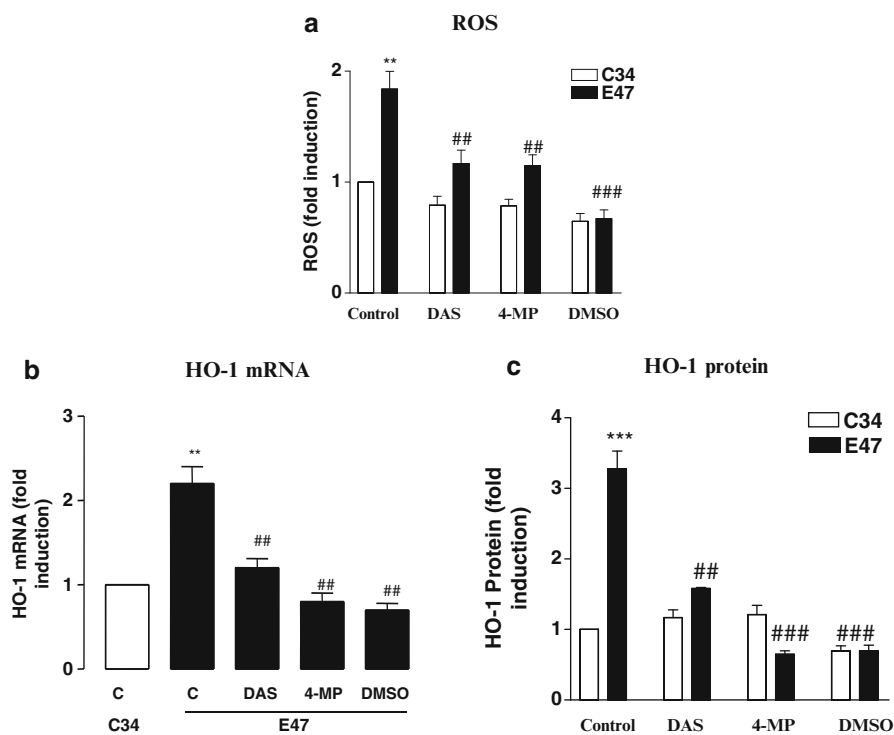


Fig. 2.1 Effect of inhibitors of CYP2E1 on (a) ROS production in E47 and C34 cells and (b, c) the induction of HO-1 mRNA and protein. E47 and C34 cells were incubated with 0.2 mM diallyldisulfide (DAS), 1 mM 4-methylpyrazole (4-MP) and 200 mM dimethylsulfoxide (DMSO) and ROS production (DCF fluorescence), HO-1 mRNA (Northern blot) and HO-1 protein (Western blot) assayed

and BSO caused loss of cell viability (40 and 50%, respectively) in E47 cells. Chromium mesoporphyrin (CrMP), an inhibitor of HO activity, significantly potentiated this cytotoxicity. ROS production, lipid peroxidation, and the decline in mitochondrial membrane potential produced by AA and BSO were also enhanced in the presence of CrMP in E47 cells. Infection with an adenovirus expressing rat HO-1 protected E47 cells from AA toxicity, increasing cell viability and reducing LDH release. HO-1 catalyzes formation of CO, bilirubin, and iron from the oxidation of heme. Bilirubin was not protective whereas iron catalyzed the AA toxicity. The carbon monoxide (CO) scavenger hemoglobin enhanced AA toxicity in E47 cells analogous to CrMP, whereas exposure to exogenous CO partially reduced AA toxicity and the enhanced AA toxicity by CrMP. Addition of exogenous CO to the cells inhibited CYP2E1 catalytic activity, as did overexpression of a rat HO-1 adenovirus. These results suggest that induction of HO-1 protects against CYP2E1-dependent toxicity and this protection may be mediated in part via production of CO and CO inhibition of CYP2E1 activity and oxidative stress.

2.4 Nrf2 Signaling

The transcription factor Nrf2 regulates the expression of many cytoprotective enzymes which results in cellular protection against a variety of insults produced by electrophilic and oxidative chemicals (Nguyen et al. 2003, 2004). Nrf2 has been shown to be protective against a variety of drugs which can cause hepatotoxicity, lung injury, neurotoxicity, carcinogenesis and inflammation (e.g. Table I of Ref. (Osburn and Kensler 2008)). Nrf2 is a member of the Cap-N-Collar transcription factor family and recognizes the antioxidant response element (ARE) in the promoter of target genes (Yu and Kensler 2005). Normally, under basal conditions Nrf2 is bound to Keap1 in the cytoplasm (Tong et al. 2006). Binding of Nrf2 to Keap1 promotes ubiquitination of Nrf2 followed by its proteasomal degradation. Exposure to many chemicals, oxidants and cellular stresses leads to increased production of Nrf2 and subsequent entry of newly synthesized Nrf2 into the nucleus. Once inside the nucleus, Nrf2 dimerizes with small Maf proteins which leads to binding to antioxidant response elements (AREs) present in the promoter of Nrf2 – target genes, followed by transcriptional activation of these genes. As described in (Osburn and Kensler 2008; Kensler et al. 2007), classes of Nrf2 – regulated genes include electrophile conjugating enzymes, antioxidative enzymes, GSH homeostasis, production of reducing equivalents and components of the proteasome. The protein products of these genes provide multiple layers of protection during cellular insults, collectively favoring cell survival. Examples of some enzymes which are transcriptionally regulated by Nrf2 include glutathione transferases (GSTs), NADPH–quinine oxidoreductase, UDP-glucuronyltransferases; antioxidant enzymes such as glutathione reductase, SOD1; several subunits of the 20S proteasome; the catalytic and regulatory subunits of the limiting enzyme in glutathione synthesis, GCS. Activation of these enzymes by various chemicals and oxidants has been shown to be Nrf2-dependent in a variety of *in vitro* and *in vivo* systems. The development of the Nrf2 knockout mouse (Enomoto et al. 2001) has been most valuable in demonstrating the key role of Nrf2 in upregulating these enzymes and in protecting against toxicity.

In view of the importance of Nrf2 in upregulating many critical protective enzymes, there has been considerable interest in efforts to activate Nrf2 signaling by administration of low molecular weight molecules. Dithiolethiones such as Oltipraz have been shown to have cancer chemopreventive activity and protect against aflatoxin-mediated hepatotoxicity in rats (Kensler et al. 1987). Isothiocyanates such as sulforaphane were shown to be potent inducers of Nrf2 and possess chemopreventive activity (Hu et al. 2006). An Nrf2-inducing synthetic triterpenoid, 1-{2-cyano-3-12 dioxooleana-1,9(11)-dien-28-oyl} imidazole (CDDO-Im), has recently been identified with approximately 100-fold greater potency than dithiolethiones in chemoprevention of aflatoxin-mediated hepatocarcinogenesis in rats (Yates et al. 2006). It is likely that much effort to develop safe and effective small molecules to activate Nrf2 will be an important area of research.

2.4.1 *Nrf2 and CYP2E1-Induced Toxicity*

Several of the antioxidant genes found to be upregulated in CYP2E1 expressing liver cells e.g. GCS, GST, HO-1 are activated by Nrf2. The possibility that Nrf2 was activated in the CYP2E1 expressing liver cells and played a role in the upregulation of GCS, GST, HO-1 in these cells was evaluated (Gong and Cederbaum 2006a, b). Initial experiments *in vivo* showed that chronic feeding of ethanol to mice or rats increased the Nrf2 protein level and the Nrf2mRNA level about twofold (Fig. 2.2a, b). These increases were associated with a fourfold increase in CYP2E1 protein levels (Fig. 2.2a). Similarly, using a different inducer of CYP2E1, pyrazole, also showed that a 2.5–3 fold increase in CYP2E1 protein in rats and mice, was associated with 2.6–3.5 fold increases in Nrf2 protein levels (Fig. 2.2c). Thus, Nrf2 levels are elevated after *in vivo* feeding of mice or rats with ethanol or injection of pyrazole, treatments which also elevate CYP2E1 levels. Basal levels of Nrf2 protein and mRNA (Gong and Cederbaum 2006a) were higher in the CYP2E1-expressing E47 cells compared to the C34 cells. Besides an increase in total Nrf2 protein in E47 cell extracts, there was a twofold increase in nuclear Nrf2 levels in the E47 cells. Nrf2 DNA binding activity to a consensus ARE probe was also increased using nuclear extracts from the E47 cells. Nuclear run-on assay showed that the transcription of Nrf2 mRNA is 2.3 fold higher in the E47 cells; stability of the Nrf2 mRNA was similar in E47 and C34 cells suggesting that the increase in Nrf2 mRNA levels in the E47 cells is caused by increased Nrf2mRNA transcription. Inhibitors of CYP2E1 such as 4-methylpyrazole or DMSO blocked the elevated ROS production in the E47 cells in association with a decrease in the elevated Nrf2 levels. Moreover, the ROS scavenger N-acetylcysteine also blocked the increase in ROS production in conjunction with lowering the elevated Nrf2 levels. These results suggest that increased production of ROS by CYP2E1 plays a role in the elevation of Nrf2 in the E47 cells.

To study whether upregulation of the antioxidant proteins GCS_c, HO-1, and catalase in E47 cells is mediated by Nrf2, siRNA-Nrf2 was used to block the effects of Nrf2. The non-target siRNA, siRNA-control(Si-c) was used as a control. The transfection efficiency of siRNA-Nrf2 and siRNA-control in C34 and E47 cells was similar ($50.1 \pm 4.6\%$). After transfection of siRNA-Nrf2, Nrf2 mRNA levels were decreased in C34 cells, and more dramatically in E47 cells compared with C34 or E47 cells transfected with siRNA-control. GCS_c and HO-1 mRNA levels also showed some decreases in C34 cells when transfected with siRNA-Nrf2. Importantly, the increased GCS_c and HO-1 mRNA expression in E47 cells was completely blocked after transfection with siRNA-Nrf2. Although catalase mRNA was induced in E47 cells, siRNA-Nrf2 had no effect on catalase mRNA levels in both C34 and E47 cells, indicating that Nrf2 is not a critical transcriptional factor for expression of catalase in the HepG2 cells as it is for the expression of GCS or HO-1. As found with the mRNA levels, Nrf2, GCS_c, and HO-1 protein levels were decreased in a time-dependent manner in C34 cells and more dramatically in E47 cells by

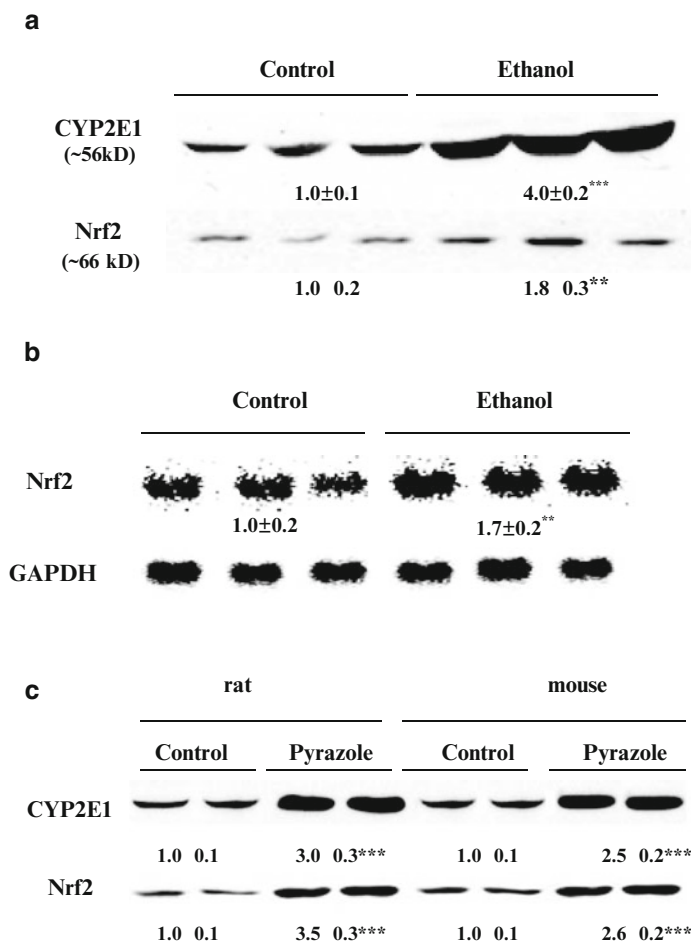


Fig. 2.2 Nrf2 protein (a, c) and mRNA (b) are elevated by chronic ethanol feeding to mice (a, b) or by acute pyrazole injection in rats or mice (c), conditions which elevate CYP2E1 protein levels (a, c). Numbers under the blots refer to (a) CYP2E1 or Nrf2/ β -actin ratio; (b) Nrf2/GAPDH ratio; (c) CYP2E1 or Nrf2/ β -actin ratio

siRNA-Nrf2 (immunoblots shown in Fig. 2.3a and quantified in Fig. 2.3b). The decline in GCS_c and HO-1 proteins parallels the decline in Nrf2 protein in both cell lines, suggesting an association between Nrf2 levels and these two antioxidant proteins. Catalase protein level in C34 and E47 cells transfected with siRNA-Nrf2 was unchanged compared with siRNA-control (Fig. 2.3a, b). CYP2E1 protein levels in the E47 cells were unchanged by transfection of siRNA-control or siRNA-Nrf2 (Fig. 2.3a).

Since SiRNA-Nrf2 blocks the induction of some antioxidant genes (e.g., GCS_c and HO-1) by CYP2E1, the effects of siRNA-Nrf2 on the content of GSH, ROS levels, and lipid peroxidation were determined. E47 cells transfected with siRNA-control

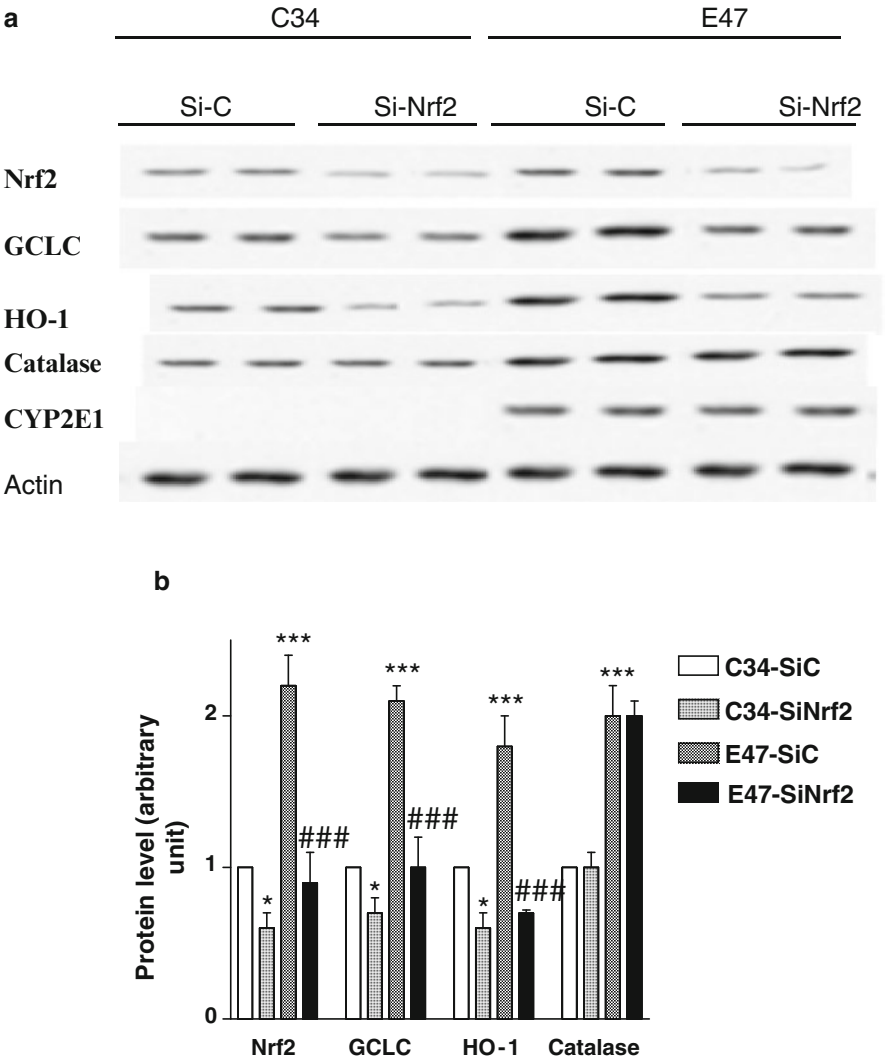


Fig. 2.3 SiRNA against Nrf2 decreases the elevated protein levels of Nrf2, GCS (*GCLC*) and HO-1 but not catalase in E47 cells. The SiRNA against Nrf2 had no effect on CYP2E1 protein levels in the E47 cells. 2.5 million E47 or C34 cells per well were transfected with either Si-C or Si-Nrf2. After 30 h, cells were collected, washed, lysed and immunoblotted for detection of the indicated enzymes

had higher levels of GSH compared with C34 cells transfected with siRNA-control consistent with upregulation of the rate-limiting enzyme in GSH synthesis, GCS_c. siRNA-Nrf2 did not significantly decrease GSH levels in C34 cells but significantly lowered GSH levels in E47 cells compared with C34 or E47 cells transfected with siRNA-control. The ROS level in E47 cells transfected with siRNA-control was

higher than C34 cells transfected with siRNA-control. siRNA-Nrf2 did not significantly increase ROS levels in C34 cells but significantly increased ROS levels in E47 cells. Lipid peroxidation was determined *via* assay for thiobarbituric acid reactive substances (TBARS). The TBARS level in E47 cells transfected with siRNA-control was higher than in C34 cells transfected with siRNA-control. siRNA-Nrf2 did not significantly increase TBARS level in C34 cells, but significantly increased the TBARS level in E47 cells. These results suggest that Nrf2 plays an important role in preventing CYP2E1-induced oxidative stress in the E47 cells.

Damage to the mitochondria and decreases in mitochondrial membrane potential are targets of CYP2E1-oxidative stress (Jimenez-Lopez and Cederbaum 2005; Caro and Cederbaum 2004; Lu and Cederbaum 2008; Wu and Cederbaum 2002). Mitochondrial membrane potential was assayed *via* flow cytometry after double-staining with rhodamine 123 and propidium iodide. Most of the C34 and E47 cells transfected with siRNA-control appear on the low propidium iodide and high rhodamine 123 fluorescence field, indicative of intact, viable cells with high mitochondrial membrane potential. siRNA-Nrf2 did not change the flow cytometry graph pattern of C34 cells, but it decreased the mitochondrial membrane potential of E47 cells as the number of cells in the hypodiploidM1 zone, indicative of apoptotic cells, increased from 5.52 to 17.34% ($P < .01$). The cells with lower mitochondrial membrane potential are still viable (low propidium iodide staining), suggesting that the loss in membrane potential caused by siRNA-Nrf2 occurs before the loss of cell viability.

C34 and E47 cells were transfected with siRNA-control or siRNA-Nrf2. After transfection, ROS level and cell viability was determined at 0, 1, 2, and 3 days. siRNA-Nrf2 only had a small tendency to increase the ROS level and decrease the viability (Fig. 2.4a, b) of C34 cells ($P > .05$), but significantly increased the ROS level and decreased the viability (Fig. 2.4a, b) of E47 cells in a time-dependent manner ($P < .01$) compared with C34 and E47 cells transfected with siRNA-control. There appears to be close time dependence between the increase in ROS production caused by siRNA-Nrf2 and the decrease in E47 cell viability. Thus, Nrf2 plays a key role in protecting E47 cell mitochondria against CYP2E1-generated ROS and in maintaining E47 cell viability.

2.4.2 *Nrf2 Protects HepG2 Cells Against CYP2E1 Plus Arachidonic Acid Toxicity*

Polyunsaturated fatty acids such as AA play an important role in alcoholic liver injury, serving as a source for lipid peroxidation. AA induces toxicity in E47 cells to a much greater extent than in C34 cells (Perez and Cederbaum 2001; Caro and Cederbaum 2002; Wu and Cederbaum 2002). This toxicity is prevented by inhibitors of CYP2E1 and antioxidants. Experiments were carried out to evaluate whether Nrf2 can protect against AA plus CYP2E1 dependent toxicity (Gong and Cederbaum 2006b). As described above, blocking Nrf2 with siRNA-Nrf2 alone did not cause

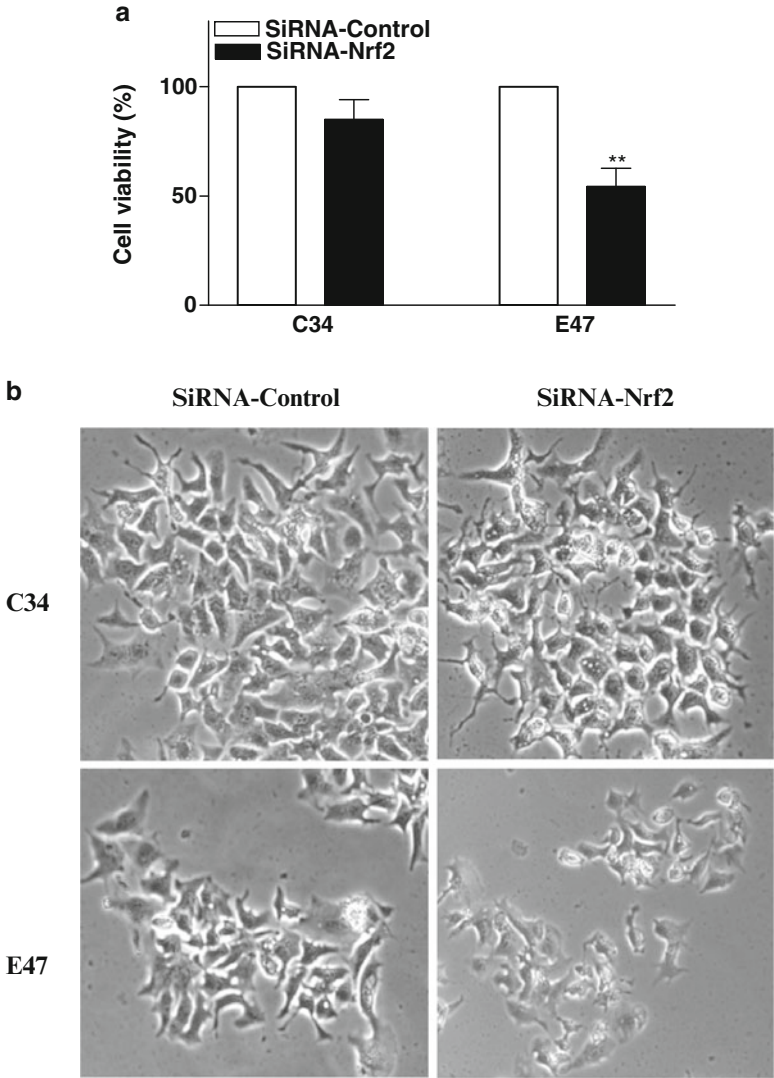


Fig. 2.4 SiRNA against Nrf2 causes toxicity in E47 cells to a greater extent than in C34 cells as shown by a MTT reduction assay (**a**) or morphology (**b**)

toxicity in C34 cells but did cause a mild toxicity in E47 cells (cell viability $80 \pm 7.0\%$, $p < 0.05$). AA treatment did not affect cell viability of C34 cells transfected with siRNA-control but caused moderate toxicity in E47 cells transfected with siRNA-control. Blocking Nrf2 by siRNA-Nrf2 did not significantly affect cell viability of C34 cells treated with AA, however, siRNA-Nrf2 dramatically enhanced the toxicity of AA and BSO in E47 cells, as cell viability decreased from $62.5 \pm 8.5\%$ to $10.7 \pm 1.2\%$. AA treatment decreased the GSH level of E47 cells transfected with

siRNA-control and increased the levels of ROS and TBARS. Blocking Nrf2 with siRNA-Nrf2 significantly enhanced these changes. siRNA-Nrf2 also enhances the decline of mitochondrial potential caused by AA in E47 cells.

To study whether AA treatment can activate Nrf2, Nrf2 protein nuclear translocation and Nrf2-AREbinding activity in E47 cells was studied. The level of Nrf2 protein in the nuclei of E47 cells was significantly increased, whereas Nrf2 protein in the cytoplasm was significantly decreased after AA treatment, suggesting the translocation of Nrf2 from the cytoplasm into the nucleus. Nrf2 binding activity was determined by electrophoretic mobility shift assay with a double strand DNA containing the ARE sequence as probe. ARE binding activity was increased after AA treatment compared with untreated control E47 cells. Nrf2 antibody can supershift this complex, indicating that it contains Nrf2. Together, these results suggest that Nrf2 is activated by AA treatment in E47 cells.

In the untreated E47 cells, siRNA-Nrf2 decreased Nrf2, GCS_c, and HO-1 basal mRNA and protein expression levels, validating the effectiveness of the siRNA in decreasing Nrf2 and its antioxidant-responsive genes. AA treatment increased the Nrf2 mRNA and protein level as well as GCS_c and HO-1 mRNA and protein levels in E47 cells transfected with siRNA-control. siRNA-Nrf2 blocked this response of E47 cells to AA treatment, as Nrf2, GCS_c, and HO-1 mRNA and protein levels in E47 cells transfected with siRNA-Nrf2 were all lower than E47 cells transfected with siRNA-control in response to AA treatment. The increase in Nrf2 protein levels likely reflects an adaption by the E47 cells to the increased oxidative stress produced by AA in the E47 cells. If so, antioxidants should prevent the AA-induced increase in Nrf2 protein levels. Treatment with 50 μ M-tocopherol or 2 mM glutathione ethyl ester or 5 mM *N*-acetylcysteine indeed lowers the AA-induced levels of Nrf2 protein to the basal levels found in the absence of AA. Thus Nrf2 is important in protecting E47 cells against AA toxicity.

2.4.3 *Nrf2 and Ethanol-Induced Toxicity*

Increases in Nrf2 protein and Nrf2 mRNA levels have been observed in liver and hepatocytes of chronic ethanol-fed mice and rats (Gong and Cederbaum 2006a). The mechanism for this increase was not studied but presumably relates to ethanol-induced oxidative stress. Lamlé et al. (2008) established a central role for Nrf2 in protecting mice against ethanol-induced liver injury. Nrf2 knockout mice and wild type control mice were fed a Lieber-DeCarli ethanol liquid diet for 4–7 days. The Nrf2^{-/-} mice displayed increased mortality, marked steatosis and elevated liver pathology and serum alanine aminotransferase levels (Lamlé et al. 2008). Toxicity was associated with accumulation of the initial metabolite produced by the oxidation of ethanol, acetaldehyde, a decline in hepatic GSH and ATP levels and an aggravated inflammatory response mediated by Kupffer cells. Surprisingly hepatic oxidative stress was minimally elevated in the ethanol-fed Nrf2^{-/-} mice and was similar to that of the ethanol-fed wild type mice. Moreover, *N*-acetylcysteine, an antioxidant and

GSH precursor, did not reduce the ethanol-induced fatty liver in the Nrf2^{-/-} mice (Lamlé et al. 2008). No explanation for the lack of an increase in oxidative stress by ethanol in the Nrf2 knockout mice was presented by the authors. Unfortunately, no data as to whether CYP2E1 was induced by ethanol in the wild type and in the Nrf2 knockout mice or whether CYP2E1 plays any role in the ethanol toxicity in the knockout mice was shown. Thus, while further studies are necessary to identify mechanisms by which Nrf2 ablation enhances ethanol toxicity, this interesting study clearly establishes a critical role for Nrf2 in the protection against ethanol-induced liver injury.

Nrf2 was also shown to be protective against ethanol toxicity *in vitro* (Yao et al. 2007). After incubation with 100 mM ethanol, human hepatocytes displayed elevated enzyme release, GSH depletion and elevated lipid peroxidation. The flavonoid quercetin protected against this ethanol toxicity due to induction of HO-1. This induction was mediated *via* quercetin promoted activation of Nrf2 translocation into the nucleus followed by induction of the HO-1 gene (Yao et al. 2007). Interestingly, inhibitors of p38 MAPkinase and especially ERK blocked this Nrf2 translocation and induction of HO-1. Activation of an ERK MAPkinase pathway by oxidants was similarly found to be important in the induction of HO-1 in HepG2 liver cells expressing CYP2E1 (Gong et al. 2003). How ERK is regulating Nrf2 translocation into the nucleus remains unclear, however, this study shows that Nrf2, *via* induction of HO-1, protects against ethanol toxicity in human hepatocytes.

GCS transcription is induced by ethanol and activation of Nrf2 plays a role in this upregulation in HepG2 cells expressing CYP2E1 (Gong and Cederbaum 2006a). A recent study (Kimura et al. 2009) indicated that NF- κ B activation may also be important for ethanol-induced expression of the GCS_c catalytic subunit. Using luciferase reporter assays, an ethanol-responsive element spanning bases -1,432 to -832 was observed in the human GCS_c promoter in hepatocytes and HepG2 cells transfected with CYP2E1. This region lacked an ARE but had a NF- κ B element; treatment with ethanol increased NF- κ B DNA binding activity in CYP2E1-expressing HepG2 cells (Kimura et al. 2009). Thus, the upregulation of the GCS_c promoter by ethanol was dependent on CYP2E1 and was mediated by not only Nrf2 but also NF- κ B. Whether NF- κ B contributes to upregulation of other genes classically known to be Nrf2-sensitive would be interesting to study.

Fetal alcohol syndrome is a major toxic effect of alcohol abuse. A recent study (Dong et al. 2008) determined the role of Nrf2 in protecting against ethanol toxicity to mouse embryos. Maternal ethanol treatment elevated Nrf2 protein and Nrf2-ARE binding in mouse embryos, in association with moderate increases in several Nrf2 downstream target antioxidant genes and proteins such as SOD1, SOD2, SOD3, catalase, glutathione reductase, thioredoxin, and glutathione peroxidase 1 and 3 (Dong et al. 2008). Pre-treatment with the powerful Nrf2 inducer 3H-1,2dithiole-3-thione (D3T) more significantly elevated Nrf2 levels, binding, and expression of the above antioxidant mRNAs and proteins. D3T treatment decreased the ethanol-induced increase in generation of ROS and apoptosis in mouse embryos. These results suggest that Nrf2 may protect against ethanol-induced oxidative stress and toxicity in mouse embryos by upregulating endogenous antioxidants.

2.5 Conclusions

Ethanol-induced oxidative stress plays a major role in mechanisms by which ethanol causes liver injury. Induction of CYP2E1 is one central pathway by which ethanol generates oxidative stress. Biochemical and toxicological properties of CYP2E1 have been studied in HepG2 cells engineered to constitutively express high levels of human CYP2E1. Ethanol and other prooxidants were toxic to the HepG2 cells expressing CYP2E1, E47 cells, but not control cells which did not express CYP2E1 (C34 cells). This toxicity was due to elevated oxidative stress. The E47 cells upregulated several antioxidant genes including GCS_c, GCS_r, HO-1, cytosolic and microsomal GST, and catalase to help protect against the CYP2E1-dependent oxidant stress. With the exception of catalase, this upregulation is dependent on the transcription factor Nrf2. Levels of Nrf2 protein and mRNA are elevated in livers from ethanol-fed mice and rats and after induction of CYP2E1, and in the E47 cells. The upregulation of antioxidant genes in the E47 cells is dependent on Nrf2 since the upregulation is prevented by siRNA-Nrf2. Blocking Nrf2 by siRNA-Nrf2 decreases glutathione and increases ROS and lipid peroxidation, resulting in decreased mitochondrial membrane potential and loss of cell viability of E47 cells but not C34 cells. These results show that Nrf2 is activated and that levels of Nrf2 protein and mRNA are increased when CYP2E1 is elevated, and suggest that Nrf2 plays a key role in the adaptive response against increased oxidative stress caused by CYP2E1 in HepG2 liver cells.

There are considerable efforts to develop effective small molecules which can activate Nrf2 e.g. dithiolethiones, isothiocyanates, CDDO-IM (Osburn and Kensler 2008; Yu and Kensler 2005; Kensler et al. 1987; Hu et al. 2006; Yates et al. 2006). The ability of such agents to protect against CYP2E1 – dependent and alcohol-induced hepatotoxicity has not been studied and this should be a high priority. As discussed above, CYP2E1 activates many important toxicological-relevant drugs such as acetaminophen, benzene, many halogenated components (e.g. CCl₄), anesthetics (e.g. halothane) and procarcinogens, hence, the possible protection against CYP2E1-drug toxicity by chemical activators of Nrf2 would be important to document and characterize. CYP2E1 is elevated under a variety of pathophysiological conditions such as obesity, diabetes, non-alcoholic fatty liver disease and may play a role in the hepatotoxicity observed in these conditions. Oxidant stress is elevated in these conditions. The possible amelioration of liver injury by chemical activators of Nrf2 would appear to be of clinical translational relevance. Clearly, the possible protection afforded by chemical activators of Nrf2 against acute or binge or chronic alcohol toxicity should be assessed. Indeed, a recent study (Dong et al. 2008) showed that Nrf2 was protective against ethanol toxicity to mouse embryos; the Nrf2 inducer 3H-1,2 dithiole-3-thione decreased the ethanol elevation of ROS and apoptosis in mouse embryos. Since fetal alcohol syndrome is a major complication of alcohol consumption during pregnancy, can activation of Nrf2 be protective against e.g. binge drinking during pregnancy? Although this review has focused on Nrf2 protection against CYP2E1 and alcohol-dependent hepatotoxicity, other organs are also

damaged e.g. alcohol-induced pancreatitis, lung injury, brain injury, heart damage, and whether Nrf2 is activated in these organs by alcohol or inducers of CYP2E1 and can be protective has not been evaluated.

A number of single nucleotide polymorphisms (SNP) have been found in the promoter region of the human Nrf2 gene (Marzec et al. 2007). One of these SNPs resulted in a decreased *in vitro* binding of Nrf2 to an ARE promoter and individuals with this SNP were more likely to develop acute lung injury following major trauma (Marzec et al. 2007). The possible pathophysiological significance of individuals with certain Nrf2 SNPs in developing enhanced sensitivity to CYP2E1, to conditions such as obesity, diabetes, nonalcohol fatty liver disease and to alcohol liver disease would be an exciting highly important future area to study.

Further understanding the biochemical actions of Nrf2 and perhaps other members of the Cap 'n' Collar family of transcription factors could lead to new insights regarding the role of oxidative stress in alcohol liver disease and CYP2E1-dependent toxicity. This could lead to more rational therapeutic approaches to preventing or ameliorating these toxicities. For example, compounds such as sulforaphane which is present in cruciferous vegetables, not only activate Nrf2, but also inhibit CYP2E1, hence, have the potential to be highly effective in minimizing alcohol hepatotoxicity based upon these dual actions.

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