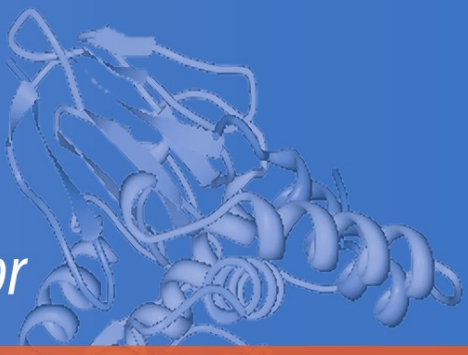
Subcellular Biochemistry 67



Aparajita Dey *Editor*

Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism

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Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism

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ISSN 0306-0225
ISBN 978-94-007-5880-3 ISBN 978-94-007-5881-0 (eBook)
DOI 10.1007/978-94-007-5881-0
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2013931178

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

To the memory of my parents
and
To Prof. Arthur I. Cederbaum,
A mentor par excellence

Preface

This book deals with various clinical aspects of cytochrome P4502E1 (CYP2E1) which is a potent source for oxidative stress. Cytochrome P-450 (CYP) enzymes are proteins that essentially contain a heme moiety and are involved in diverse oxidative metabolism of a wide spectrum of endogenous compounds as well as xenobiotics. Further, they are induced by several stimuli which include pathophysiological conditions, thus emphasizing their critical role in human physiology and diseases.

Ethanol-inducible CYP2E1 which forms the key enzyme in the microsomal ethanol-oxidizing system, besides metabolizing ethanol to acetaldehyde, also catalyzes oxidative metabolism of substrates primarily through acting as a monooxygenase and generating reactive oxygen species in the process. Oxidative stress is critical for pathogenesis of diseases and CYP2E1 is a major contributor for oxidative stress. Several clinical disorders are associated with changes in regulation of CYP2E1 and the consequent abnormalities which include alcoholic liver disease, alcoholic pancreatitis, carcinogenesis, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, obesity, hepatitis C virus infection, reproductive organ toxicity, hepatocellular and cholestatic injury.

The list for the involvement of clinical and metabolic disorders associated with changes in regulation of CYP2E1 is extensive which includes bone loss, cross-tolerance in smokers and people treated with nicotine (e.g., smokers, patients with Alzheimer's disease, ulcerative colitis, neuropsychiatric motor disorders), disorders of central nervous system due to exposure to certain environmental chemicals and changes in the metabolism of protoxicants in the circulatory system.

Changes in regulation of CYP2E1 may occur due to endotoxemia, inflammatory stimuli, complex endocrine regulation by pituitary and testicular hormones, expression of methionine adenosyltransferase genes, nicotine or environmental tobacco smoke exposure, polymorphic gene expression, transcription factor hepatocyte nuclear factor 1 alpha, calmodulin dependent protein kinase, protein kinase C and cAMP dependent protein kinase, drugs such as isoniazid and clofibrate, starvation, insulin, diabetes or alcohol consumption.

The several mechanisms through which CYP2E1 exerts its damaging effects include increased oxidative stress, acetaldehyde formation and accumulation, increased hepatotoxicity of carcinogens like nitrosodimethylamine, urethane and acrylamide, oxidative DNA damage, augmentation of iron induced hepatotoxicity, priming of Kupffer cells to lipopolysaccharide-induced toxicity, affecting therapeutic index of drugs, i.e. potentiating acetaminophen mediated toxicity, increasing polyunsaturated fat mediated injury, depletion of the levels of the major cellular antioxidant glutathione and increase in collagen expression. Other mechanisms for clinical abnormalities associated with CYP2E1 include mitochondrial dysfunction, apoptotic cell death, potentiation of lipopolysaccharide or cisplatin mediated injury, inhibition of microsomal Ca2+−ATPase, formation of carcinogenic etheno-DNA adducts, modulation of the immune response, increases in proinflammatory cytokines, polymorphic gene expression and increased hydroxyethyl radical formation.

Further, CYP2E1 causes metabolic abnormalities through formation of autoantibodies against CYP2E1, necroinflammation, increased degradation of retinoic acid and vitamin A, JNK activation, decrease in proteasome activity with subsequent accumulation of oxidized proteins, formation of cytokeratin aggresomes and Mallory body-like inclusions. Several other mechanisms through which CYP2E1 exerts toxicity include increased formation of Kupffer cell-generated metabolites, which may contribute to Kupffer cell toxicity; elevated c-fos mRNA; oxidative modifications of heat shock protein 60; protein disulfide isomerase; mitochondrial aldehyde dehydrogenases, prohibitin, and other proteins; formation of 3-nitrotyrosine adducts and high molecular weight microsomal ubiquitin conjugates; increased levels of endoplasmic reticulum stress marker tribbles-related protein 3 and chemokine CXCL-2; impairment of insulin signaling; formation of protein adducts of aldehydes such as acetaldehyde, malondialdehyde and 4-hydroxy-nonenal; suppression of activities of antigen-trimming enzymes, thereby decreasing the cleavage of C-extended and N-extended peptides which may potentially result in decreased MHC class I-restricted antigen presentation on virally infected liver cells; impairment of interferon gamma signaling; irreversible inhibition of fatty acid oxidation, potentially through suppression of PPARalpha-regulated pathways; and potentiation of thioacetamide mediated hepatotoxicity.

The first chapter gives an overview of the research on different aspects of CYP2E1 and the aim of the chapter is to acquaint the readers with a general picture regarding CYP2E1 before they delve deeper into further chapters which are specialized research findings discussed in detail by the different experts with respect to the studies being performed in their own respective laboratories. The subsequent chapters deal with some of the research activities dealing with CYP2E1 in major laboratories around the world.

Dr. Arthur I. Cederbaum discusses about the role of the transcription factor Nrf2, the key regulator of cytoprotective enzymes as a protective mechanism against CYP2E1 mediated oxidative stress in a human hepatoma cell line transfected with CYP2E1. Dr. Helmut K. Seitz discusses about the important role of ethanol inducible CYP2E1 in promoting alcohol mediated carcinogenesis. Dr. Samuel W. French deals with CYP2E1 mediated drug metabolism and the consequent drug mediated

hepatitis due to co-administration of ethanol and drugs. Also, epigenetic effects due to induction of CYP2E1 are discussed.

Dr. Ann K. Daly discusses about the role of CYP2E1 as a genetic risk factor for non-alcoholic fatty liver disease- evidences in favour and against it, as documented in studies involving genetic analyses. Drs. Terence M. Donohue and Natalia A. Osna discuss about the role of CYP2E1 in regulating cytokine signaling, antigen presentation, and macromolecular degradation leading to liver injury. Dr. M. Raj Lakshman discusses about the role of CYP2E1 and ethanol mediated oxidative stress in downregulating the hepatic expression of paraoxonase 1, a multifunctional antioxidant enzyme that prevents LDL oxidation and detoxifies the homocysteine metabolite, homocysteinethiolactone. Dr. Vasilis K. Vasiliou discusses about the role of CYP2E1 in ethanol metabolism in the central nervous system, including its regulation and expression and its influence on sensitivity to ethanol in the brain.

Thus, CYP2E1 is implicated in several clinical disorders through diverse mechanisms of injury. It is interesting to explore some of these pathways which shed light on the several other aspects linked with this enzyme. The different biochemical, toxicological and clinical aspects of CYP2E1 and the underlying mechanisms through which CYP2E1 plays a critical and indispensible role in modulating the therapeutic effects of drugs, and in development and pathogenesis of clinical disorders, form the core of the book.

Acknowledgements

First and foremost, I would like to thank Dr. Meran Owen, Senior Editor, Springer London Cell Biology, Molecular Biology for giving me this opportunity to put together a book on 'Cytochrome P4502E1: Its Role in Disease and Drug Metabolism'. This provided me a wonderful chance to review and acquaint myself with the valuable contributions of different experts in the field of CYP2E1 who have carried on studies regarding the different aspects of the enzyme.

I would also like to thank the contributing authors for most graciously accepting my invitation, taking the time out of their busy schedules and putting their painstaking efforts to write the different chapters. In this regard, I would especially thank my postdoctoral mentor Prof. Arthur I. Cederbaum who in a way introduced me to the field of CYP2E1 in a deeper sense and helped me to develop my understanding of the subject. An excellent mentor and a far better human being; he has always been a source of inspiration for me.

My heartfelt thanks to Dr. Helmut K. Seitz, Dr. Terrence M. Donohue, Dr. Ann K. Daly, Dr. M. Raj Lakshman, Dr. Samuel W. French, Dr. Vasilis K. Vasiliou and Dr. Natalia A. Osna for their very kind contributions to the various chapters and sharing their expertise in the field of CYP2E1 with the readers, in turn providing valuable insights into the mechanisms for CYP2E1 mediated toxicity.

I would also like to thank my brothers Abhishek and Abhijit and their families for their unfailing help and support through all these years which formed the foundation for all my professional endeavors. My special thanks to my nephews Aditya, Aniruddha and my niece Aishwarya for their smiles and laughter which always made my days bright and sunny.

My students Kavitha, Mathan, Karthikeyan and Lakshmanan were generous enough to proof read the manuscript, patiently bearing with my busy activities and ensuring the smooth running of the laboratory.

Several others who have been instrumental in my personal and professional success also deserve a note of thanks, many of whom have not been mentioned here.

Chennai, India
21 September 2012

Aparajita Dey

Contents

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Chapter 1
Cytochrome P450 2E1: Its Clinical Aspects and a Brief Perspective on the Current Research Scenario

Aparajita Dey

Abstract Research on Cytochrome P450 2E1 (CYP2E1), a key enzyme in alcohol metabolism has been very well documented in literature. Besides the involvement of CYP2E1 in alcohol metabolism as illustrated through the studies discussed in the chapter, recent studies have thrown light on several other aspects of CYP2E1 i.e. its extrahepatic expression, its involvement in several diseases and pathophysiological conditions; and CYP2E1 mediated carcinogenesis and modulation of drug efficacy. Studies involving these interesting facets of CYP2E1 have been discussed in the chapter focusing on the recent observations or ongoing studies illustrating the crucial role of CYP2E1 in disease development and drug metabolism.

Keywords Cytochrome P450 2E1 • Drugs • Reactive oxygen species • Diseases • Injury

1.1 Introduction

Cytochrome P450 2E1 (CYP2E1) is implicated in several diseases and is a key player in alcohol metabolism and oxidative stress (Gonzalez 2005; Brzezinski et al. 1999; Wu and Cederbaum 2005). CYP2E1 which is induced due to alcohol consumption plays a major role in human health due to its ability to bioactivate numerous hepatotoxins and metabolize alcohol (Koop 1992; Cederbaum 2010). The abundance of expression of CYP2E1 in liver and extrahepatic tissues holds importance

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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*,
Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_1,

keeping in view its role in generating oxidative stress (Joshi and Tyndale 2006a). Further, due to its ability to modulate the effects of drugs, CYP2E1 plays a crucial role in drug metabolism (Lieber 2004; Joshi and Tyndale 2006a).

1.1.1 Purification and Characterization of CYP2E1

The initial purification of CYP2E1 from rabbits led to its discovery in several other animal species and humans (Koop et al. 1982). CYP2E1 is primarily an endoplasmic reticulum resident protein (Lieber 2004; Konishi and Ishii 2007), but recent studies have also shown that CYP2E1 is also present in mitochondria (Bhagwat et al. 1995; Neve and Ingelman-Sundberg 1999; Robin et al. 2001). The localization of hepatic CYP2E1 is predominantly restricted to petrivenous region in liver (Bühler et al. 1991; Lieber 1993). Regulation of CYP2E1 occurs through transcriptional activation, mRNA stabilization, increased mRNA translatability and decreased protein degradation and the principal mechanism which controls the induction process depends on several factors such as the chemical nature of the inducer, the age, and the nutritional and hormonal status of the animal (Koop and Tierney 1990).

1.1.2 The Role of CYP2E1 as a Potent Source for Oxidative Stress

CYP2E1 catalyzes several oxidative biochemical reactions for which it requires NADPH and the incomplete reduction of oxygen in CYP2E1 catalyzed reactions leads to generation of free radical species (Wu and Cederbaum 2003). As a poor coupling link exists between NADPH-cytochrome P450 reductase and CYP2E1, CYP2E1 has been shown to exhibit increased NADPH oxidase activity which leads to generation of reactive oxygen species (ROS) (Ekström and Ingelman-Sundberg 1989; Gorsky et al. 1984; Cederbaum 2010). The ability of CYP2E1 to generate ROS such as the superoxide anion radical and hydrogen peroxide is enhanced in the presence of iron catalysts, and powerful oxidants such as the hydroxyl radical are generated (Lu and Cederbaum 2008). CYP2E1 dependent toxicity is closely linked to oxidative stress injury along with peroxynitrite, tumor necrosis factor alpha (TNF alpha), protein adducts and several other mechanisms which provide the complete picture (Reed 2004).

1.1.3 CYP2E1 and Ethanol-Mediated Oxidative Stress

Ethanol-mediated oxidative stress plays a crucial role in the development of liver injury due to alcohol consumption (Cederbaum 1991). Among several pathways which have been suggested to contribute to the ability of ethanol to induce a state of

oxidative stress, one central pathway seems to be the induction of CYP2E1 by ethanol (Lu and Cederbaum 2008).

Generation of ROS by CYP2E1 is a sequential process, as elegantly described by Koop, 2006 and illustrated in the following section (Koop 2006). The use of oxygen by CYP2E1 to metabolize alcohol, leads to generation of ROS by the following chain of events:

- Ethanol binds to the enzyme.
- As the first electron is passed to the heme of CYP2E1 and oxygen is bound, the electron can move and exist on the oxygen, essentially generating superoxide bound to the heme of CYP2E1. Occasionally, the superoxide will break down, releasing free superoxide and generating the starting enzyme.
- If the second electron is added to the enzyme, then a second form of reduced oxygen is produced that is identical to a heme-bound form of the two electron–reduced oxygen (i.e., peroxide).
- When this product breaks down, it picks up two hydrogens to generate hydrogen peroxide.
- The production of these ROS by CYP2E1 is referred to as an "uncoupled reaction" because the oxygen does not end up in the substrate.
- If the ROS remains bound, then the enzyme will transfer one oxygen atom to the substrate and the other atom becomes water, producing an unstable intermediate (i.e., a gem-diol) product that decomposes to acetaldehyde.

1.1.4 Importance of CYP2E1 in Health and Disease

Several pathophysiological conditions such as alcoholism, diabetes etc. and drug administration lead to the induction of CYP2E1 as illustrated by the studies in the following sections. Numerous targets exist for CYP2E1 mediated injury-DNA, protein, mitochondria, etc., thus disrupting the essential structural and functional integrity of the cell and the organism as a whole. CYP2E1 also modulates the actions of several drugs, thus altering their therapeutic efficacy, and it also activates xenobiotics to their carcinogenic forms, besides its multifarious toxic functions. Some of the drugs exerting their hepatotoxic effects through the involvement of CYP2E1 and discussed in the chapter have been summarized in Table 1.1. All the above stated factors underlie the role of CYP2E1 as an emerging player in health and disease.

Interindividual variability in the expression and functional activity of CYP2E1 has been observed (Neafsey et al. 2009) and genetic polymorphisms in CYP2E1 have been linked to altered susceptibility to several diseases (Trafalis et al. 2010). Also, chronic exposure to CYP2E1 inducers, such as ethanol, isoniazid, various solvents and chemicals, also increase the probability of developing malignancy, especially for carriers of certain CYP2E1 alleles (Trafalis et al. 2010).

Table 1.1 Drugs exerting hepatotoxic effects modulated through CYP2E1

Drug	Clinical usage	References
Geldanamycin	Anti-tumour	Dey and Cederbaum (2006)
Acetaminophen	Analgesic	Dai and Cederbaum (1995), Jones et al. (2002), Bae et al. (2001), Knockaert et al. (2011), Cheung et al. (2005), Chen et al. (2008, 2009), Bai and Cederbaum (2004), Lee et al. (2006a), and Abdelmegeed et al. (2010)
Cisplatin	Anti-cancer	Lu and Cederbaum (2006)
Sodium salicylate	Anti-inflammatory	Wu and Cederbaum (2001)
Chlorzoxazone	Muscle relaxant	Dupont et al. (1998), Cheung et al. (2005), Cummings et al. (1999, 2001), Carpenter et al. (1997), Raucy et al. (1997), Upadhya et al. (2000), Orellana et al. (2006), Tindberg and Ingelman-Sundberg (1996), Gebhardt et al. (1997), Lee et al. (2006c), Khemawoot et al. (2007a, b), Howard et al. (2001), Chalasani et al. (2003), Huan and Koop (1999), Varela et al. (2008), Zhukov and Ingelman-Sundberg (1999), Nedelcheva et al. (1999), Lerche et al. (1996), Lejus et al. (2002), Khalighi et al. (1999), Ramaiah et al. (2001), and Fairbrother et al. (1998)
Ciprofibrate	Peroxisome proliferator and anti-hyperlipidemic	Zangar et al. (1995)
Clofibrate	Lipid lowering agent	Cummings et al. (2001), Raucy et al. (2004), and Carpenter et al. (1996)
Isoniazid	Anti-tuberculosis	Park et al. (1993)
Sevoflurane	Anaesthetic	Hase et al. (2000)
Phenobarbital	Anti-convulsant	Lee et al. (2006c)
Pyridine	Precursor to pharmaceuticals	Cummings et al. (2001) and Lash et al. (2007)
Hydrazine	Precursor to pharmaceuticals	Runge-Morris et al. (1996)
Phenelzine	Anti-depressant and anxiolytic	Runge-Morris et al. (1996)

1.2 Research Studies Elucidating the Role of CYP2E1 in Disease and Drug Metabolism

The following sections deal with the studies related to the physiological, pharmacological and toxicological aspects of CYP2E1 which have been performed in laboratories of eminent scientists globally, thus stressing upon the importance of CYP2E1 in health and disease. Although the list of the studies discussed here is not complete and the studies which have not been represented do not account for lesser importance of the

findings but the emphasis is more on ongoing studies with CYP2E1 or studies dealing with the indispensable role of CYP2E1 in drug metabolism and disease development.

I

C. Lieber (1931–2009)

CYP2E1 as an Integral Component of the Microsomal Ethanol Oxidizing System and Its Physiologic and Pathologic Roles

Discovery of the Microsomal Ethanol Oxidizing System

The pioneering work of Dr. Charles Lieber and his group led to the discovery of CYP2E1 as an integral component of the microsomal ethanol oxidizing system (MEOS). The discovery of the proliferation of the smooth endoplasmic reticulum after chronic alcohol consumption, suggested the existence of an additional pathway for ethanol metabolism apart from alcohol dehydrogenase (ADH) which was described by Lieber and DeCarli, as the microsomal ethanol oxidizing system, involving cytochrome P450 (CYP) (Lieber and DeCarli 1968). The MEOS was distinct from the alcohol metabolizing enzymes- ADH and catalase and was a CYP dependent reaction (Teschke et al. 1972, 1974). Further, after chronic ethanol consumption, the activity of the MEOS increased with an associated rise in cytochrome P450 in rodents and humans (Ohnishi and Lieber 1977; Wrighton et al. 1986; Song et al. 1986) and the ethanol inducible cytochrome P450 was designated as CYP2E1 (Nelson et al. 1993).

Contributions of Other CYPs to MEOS

Using HepG2 cells heterologously expressing human CYP2E1, CYP1A2, and CYP3A4 and livers isolated from alcoholic patients and assessing their ethanol oxidation using selective inhibitors-4-methylpyrazole (CYP2E1), furafylline (CYP1A2), and troleandomycin (CYP3A4), it has been observed that the specific activities for ethanol oxidation in human liver microsomes follows the pattern: CYP2E1 > CYP1A2 > CYP3A4 (Salmela et al. 1998). Thus, in human liver microsomes, CYP2E1 plays the major role in the pathogenesis of alcoholic liver disease (Salmela et al. 1998). However, CYP1A2 and CYP3A4 contribute significantly to microsomal ethanol oxidation and may, therefore, also be involved in the pathogenesis of alcoholic liver disease (Salmela et al. 1998). The diseases or pathophysiological conditions associated with induction of CYP2E1 have been summarized in Table 1.2.

Development of Assay for the Measurement of Catalytic Activity of CYP2E1

A highly sensitive, simple assay for the determination of 4-nitrocatechol formed during the CYP2E1-dependent hydroxylation of p-nitrophenol utilizing high-performance liquid chromatography with electrochemical detection has been developed (Mishin et al. 1996).

Ethanol Inducible Hepatic CYP2E1: Evidences from Rodent and Human Models

In human subjects comprised of recently drinking alcoholics (<36 h), acinar regions of liver show elevated CYP2E1 transcripts with mRNA increase occurring mainly in perivenular cells (zone 3) and marked elevations in CYP2E1 protein

Table 1.2 Diseases or pathophysiological conditions associated with induction of CYP2E1 in animal and human models

Disease	References
Alcohol induced liver injury/alcohol induced liver cell toxicity	Salmela et al. (1998), Takahashi et al. (1993), Tsutsumi et al. (1993), Koivisto et al. (1996), Ma et al. (1991), Aleynik et al. (1999), Mi et al. (2000), Aleynik and Lieber (2001), Xu et al. (2005), Lieber et al. (2007b), Dai et al. (1993), Carroccio et al. (1994), Wu and Cederbaum (1993b, 1996, 2000), Kukielka and Cederbaum (1994), Lu et al. (2008, 2010), Bai and Cederbaum (2006), Nieto et al. (2000), Osna et al. (2003, 2005, 2007, 2008, 2009, 2010), Castillo et al. (1992), Gebhardt et al. (1997), Oneta et al. (2002), Wang et al. (2009), Curry-McCoy et al. (2010), Wan et al. (1995), Morimoto et al. (1993, 1994, 1995a, b), Gouillon et al. (1999, 2000), Bardag-Gorce et al. (2000, 2002, 2006), Donohue et al. (2006), Garige et al. (2005), Albano et al. (1996), Dupont et al. (1998), Clot et al. (1997), Vidali et al. (2007), Liu et al. (2001), Veeramachaneni et al. (2008), Nanji et al. (1993, 1994), Roberts et al. (1994, 1995), Jeong et al. (2000), Kim et al. (2006), Baumgardner et al. (2007), Otani et al. (2005), Raucy et al. (2004), Kunitoh et al. (1997), Niemelä et al. (1998, 1999), Esfandiari et al. (2005), Korourian et al. (1999), Rowlands et al. (2003), Ronis et al. (2010), Demeilliers et al. (2002), Robin et al. (2005), Knockaert et al. (2011), Howard et al. (2001, 2003a), Ferguson et al. (2011), Yue et al. (2009), Bailey et al. (2009), Carpenter et al. (1996, 1997), Sampey et al. (2003), Roede et al. (2008), Bradford et al. (2005), Bühler et al. (1991, 1992), Takahashi et al. (1992), Hu et al. (1994), Butura et al. (2009), Simi and Ingelman-Sundberg (1999), Clot et al. (1996), Lytton et al. (1999), Fang et al. (1998), French et al. (1993), and Chandrasekaran et al. (2011, 2012b)
Alcohol mediated neurotoxicity	Vasiliou et al. (2006), Howard et al. (2003a, b), Joshi and Tyndale (2006a, b), Zimatkin et al. (2006), Brzezinski et al. (1999), Yadav et al. (2006), Kapoor et al. (2006), Anandatheerthavarada et al. (1993), Bhagwat et al. (1995), Upadhya et al. (2000), and Tindberg and Ingelman-Sundberg (1996)
Alcohol mediated renal injury	Shankar et al. (2008)
NASH	Lieber et al. (2004a, b), Baumgardner et al. (2008), Leclercq et al. (2000b), Chalasani et al. (2003), Wang et al. (2008), Abdelmegeed et al. (2011), Varela et al. (2008), and Mantena et al. (2009)

(continued)

Table 1.2 (continued)

Disease	References
Diabetes	Wu and Cederbaum (1993a), Arinç et al. (2005), Wang et al. (2000), Sindhu et al. (2006), Zaluzny et al. (1990), Song et al. (1990), Raza et al. (2004), Woodcroft and Novak (1997), Arinç et al. (2005), Leclercq et al. (2000a), and Martínez-Chantar et al. (2002)
Chronic hepatitis C	Vidali et al. (2007), Rigamonti et al. (2009), Osna et al. (2008), and Otani et al. (2005)
NAFLD	Videla et al. (2004), Orellana et al. (2006), and Kathirvel et al. (2009)
Carcinogenesis	Wang et al. (2009), Millonig et al. (2011), Ghanayem et al. (2005a, b, c), Ghanayem (2007), Wang et al. (2002a, b), Hoffler et al. (2003, 2005), Garner et al. (2007), Garro et al. (1981), Lerche et al. (1996), Howard et al. (2001), Ma et al. (1991), Huan and Koop (1999), Roberts et al. (1995), Arinç et al. (2007), Zaluzny et al. (1990), Dey et al. (2002, 2005), Kapoor et al. (2006), Khan et al. (2011), Anandatheerthavarada et al. (1993), and Bhagwat et al. 1995
Methionine deficiency induced steatohepatitis	Martínez-Chantar et al. (2002) and Schattenberg et al. (2005)
Cigarette smoking	Micu et al. (2003), Lee et al. (2006b), Yue et al. (2009), Ferguson et al. (2011), and Joshi and Tyndale (2006a, b)
Maturity onset diabetes of the young (type 3 diabetes)	Cheung et al. (2003)
Hyperglycemia	Chandrasekaran et al. (2012a)
Hyperglycemia and alcoholism	Chandrasekaran et al. (2012b)
Parkinson's disease	Singh et al. (2008)
Alcoholic liver cirrhosis	Khan et al. (2009, 2010, 2011)
Head and neck squamous cell carcinoma	Ruwali et al. (2009)

content in both perivenular and midzonal (zone 2) hepatocytes (Takahashi et al. 1993). The tissue and organ specific expression of CYP2E1 has been summarized in Table 1.3.

Further, as observed in rats fed liquid diets containing 36% of total calories as ethanol, the *in vivo* induction of hepatic CYP2E1 protein by ethanol involves increased enzyme synthesis rather than decreased enzyme degradation (half life of 27–28 h) (Tsutsumi et al. 1993). This enhancement of *de novo* CYP2E1 synthesis most likely entails the ethanol-mediated increase of steady-state levels of CYP2E1 mRNA and/or the stimulation of its translational efficiency (Tsutsumi et al. 1993).

Table 1.3 Tissue and organ specific expression of CYP2E1 including transfected cell lines

Tissue/organ	References
Liver	Lieber and DeCarli (1968), Teschke et al. (1972, 1974), Ohnishi and Lieber (1977), Wrighton et al. (1986), Song et al. (1986), Salmela et al. (1998), Takahashi et al. (1993), Tsutsumi et al. (1993), Kessova et al. (1998, 2001), Ma et al. (1991), Aleynik et al. (1999), Aleynik and Lieber (2001), Lieber et al. (2004a, b, 2007a, b), Kukielka and Cederbaum (1994), Kessova and Cederbaum (2007), Wu and Cederbaum (1993a), Dey and Cederbaum (2007), Lu et al. (2005, 2008, 2010), Lu and Cederbaum (2006), Garro et al. (1981), Gebhardt et al. (1997), Wang et al. (2009), Morimoto et al. (1993, 1994), Morimoto et al. (1995a, b), Wan et al. (1995), Bardag-Gorce et al. (2000, 2002, 2005), Gouillon et al. (1999, 2000), Garige et al. (2005), Curry-McCoy et al. (2010), Vasiliou et al. (2006), Castillo et al. (1992), Albano et al. (1996), Dupont et al. (1998), Liu et al. (2001), Veeramachaneni et al. (2008), Wang et al. (2008), Nanji et al. (1993, 1994), Chang et al. (2006), Tierney et al. (1992), Runge-Morris et al. (1996), Videla et al. (2004), Fernández et al. (2003), Orellana et al. (2006), Varela et al. (2008), Asai et al. (1996), Kunitoh et al. (1997), Jeong et al. (2000), Roberts et al. (1994, 1995), Abdelmegeed et al. (2010, 2011), Khemawoot et al. (2007a), Park et al. (1993), Baumgardner et al. (2007, 2008), Lejus et al. (2002), Arinç et al. (2005, 2007), Wang et al. (2002a), Morgan et al. (2002), Kathirvel et al. (2009, 2010), Niemelä et al. (1999), Esfandiari et al. (2005), Sindhu et al. (2006), Cheng et al. (2003), Leclercq et al. (2000a, b), Korourian et al. (1999), Morgan (1993), Sewer et al. (1998), Sewer and Morgan (1998), Chen et al. (1999), Ronis et al. (2010), Robin et al. (2005), Martínez-Chantar et al. (2002), Raza et al. (2004), Howard et al. (2001), Micu et al. (2003), Lee et al. (2006b), Ferguson et al. (2011), Starkel et al. (2000), Vasiliou et al. (2006), Zaluzny et al. (1990), Bradford et al. (2005), Mantena et al. (2009), Bailey et al. (2009), Cheung et al. (2003, 2005), Chalasani et al. (2003), Andersen et al. (1998), Sampey et al. (2003), Roede et al. (2008), Carpenter et al. (1996), Wang et al. (2000), Ramaiah et al. (2001), Chilakapati et al. (2007), Lee et al. (2006c), Bühler et al. (1991, 1992), Takahashi et al. (1992), Hu et al. (1994), Johansson et al. (1990), Ronis et al. (1991), Neve and Ingelman-Sundberg (2001), Terelius et al. (1991), Albano et al. (1995), Gut et al. (1996), Nedelcheva et al. (1999), Clot et al. (1996), Lytton et al. (1999), Fang et al. (1998), Eliasson et al. (1992), Zhukov et al. (1993), French et al. (1993), Qu et al. (2009), Niemelä et al. (1998), and Zaluzny et al. (1990)
Prenatal liver	Khalighi et al. (1999) and Brzezinski et al. (1999)
Fetal liver	Carpenter et al. (1997) and Wu and Cederbaum (1993b)
Liver mitochondria	Lieber et al. (2007b), Demeilliers et al. (2002), Robin et al. (2001), and Mantena et al. (2009)

(continued)

Table 1.3 (continued)

Tissue/organ	References
Hepatocytes	Mi et al. (2000), Wu and Cederbaum (2000, 2001), Caro and Cederbaum (2001), Wang et al. (2009), Clot et al. (1997), Zangar et al. (1995), Osna et al. (2009), Kraner et al. (1993), Raucy et al. (2004), Woodcroft and Novak (1997, 1999), Woodcroft et al. (2002), Abdelmegeed et al. (2005), Abdel-Razzak et al. (1993), Morgan et al. (1994), Robin et al. (2005), Lash et al. (2007), Sidhu et al. (2001), Ronis et al. (1991), Butura et al. (2009), Johansson et al. (1991), and Eliasson et al. (1992)
Rat hepatocyte cell line RALA255-10G	Liu et al. (2002), Jones et al. (2002), Singh et al. (2009), and Schattenberg et al. (2004, 2005)
Co-culture of rat liver epithelial cells (RLECs) and hepatocytes	Lerche et al. (1996)
HepG2 cells	Salmela et al. (1998), Xu et al. (2003a, b, 2005), Dai et al. (1993), Carroccio et al. (1994), Wu and Cederbaum (1996, 2001), Chen and Cederbaum (1998), Dey and Cederbaum (2006), Dey et al. (2006), Caro et al. (2009), Chen et al. (1997), Caro and Cederbaum (2001, 2002a, b, 2003), Lu and Cederbaum (2006), Dai and Cederbaum (1995), Bai and Cederbaum (2004), Dey et al. (2006), Bardag-Gorce et al. (2006), Garige et al. (2005), Donohue et al. (2006), Osna et al. (2003, 2005, 2007, 2008, 2009), Lagadic-Gossmann et al. (2000), Kim et al. (2006), Qu et al. (2009), and Chandrasekaran et al. (2011, 2012a, b)
HepG2 mitochondria	Bai and Cederbaum (2006) and Kim et al. (2006)
Kupffer cells	Koivisto et al. (1996) and Koop et al. (1991)
Hepatic stellate cells	Nieto et al. (1999, 2000)
Co-culture of HepG2 cells and hepatic stellate cells	Nieto et al. (2002a, b)
HeLa cells	Huan and Koop (1999) and Huan et al. (2004)
Huh 7 cells	Otani et al. (2005) and Osna et al. (2010)
FGC-4 hepatoma cells	Rowlands et al. (2003)
Fao rat hepatoma cells	Simi and Ingelman-Sundberg (1999) and Zhukov and Ingelman-Sundberg (1997)
RAW 264.7 macrophages	Cao et al. (2005)
Kidney	Wu and Cederbaum (1994), Runge-Morris et al. (1996), Chen et al. (1999), Cummings et al. (1999, 2001), Roberts et al. (1994), Arinç et al. (2007), and Zaluzny et al. (1990)
Monkey kidney cell line COS-7	Knockaert et al. (2011)
Renal proximal tubule cells (RPTCs)	Shankar et al. (2008)
Blood	Oneta et al. (2002), Sutti et al. (2010a, b), Daly et al. (2006), Fairbrother et al. (1998), Vidali et al. (2007), Rigamonti et al. (2009), and Khemawoot et al. (2007b)

(continued)

Table 1.3 (continued)

Tissue/organ	References
Lymphocytes	Soh et al. (1996), Song et al. (1990), Chalasani et al. (2003), Raucy et al. (1997), and Dey et al. (2002, 2005)
B-lymphoblastoid cells	Asai et al. (1996)
Mononuclear cells	Hase et al. (2000) and Yano et al. (2001)
Pancreas	Kessova et al. (1998)
Lung	Arinç et al. (2007) and Wang et al. (2002a)
Nasal tissue including olfactory epithelial cells	Wang et al. (2002a)
Brain	Vasiliou et al. (2006), Vaglini et al. (2004), Farin and Omiecinski (1993), Yadav et al. (2006), Johri et al. (2007), Roberts et al. (1994), Pardini et al. (2008), Viaggi et al. (2009), Correa et al. (2009), and Zimatkin et al. (2006)
Neuronal and glial cells	Kapoor et al. (2006)
C6 glioma cells	Bae et al. (2001) and Lee et al. (2006a)
Brain (Cortical glial cells)	Tindberg et al. (1996)
Brain (frontal, cortical and pyramidal neurons, cerebellar Purkinje cells)	Lee et al. (2006c)
Brain (olfactory bulbs, frontal cortex, hippocampus, cerebellum, olfactory tubercle, brain stem)	Howard et al. (2003a)
Brain (frontal cortex and cerebellum)	Joshi and Tyndale (2006a)
Brain (frontal cortex, hippocampus, cerebellum)	Joshi and Tyndale (2006b)
Brain (cortex, hippocampus, hypothalamic nuclei, basal ganglia, reticular nucleus and brain stem)	Anandatheerthavarada et al. (1993)
Brain (cortex, hippocampus)	Upadhya et al. (2000)
Brain mitochondria	Bhagwat et al. (1995)
Brain (hippocampus)	Tindberg and Ingelman-Sundberg (1996)
Maternal brain	Carpenter et al. (1997)
Prenatal brain	Boutelet-Bochan et al. (1997) and Brzezinski et al. (1999)
Reticulocytes	Kocarek et al. (2000)
Human umbilical vein endothelial cells (HUVEC)	Farin et al. (1994)
Placenta	Carpenter et al. (1997)
Primary and human papillomavirus immortalized oral and cervical epithelial cells	Farin et al. (1995)

(continued)

Table 1.3 (continued)

Tissue/organ	References
Reticulocytes	Kocarek et al. (2000)
Squamous epithelial cells of the cheek mucosa, tongue, esophagus, forestomach	Shimizu et al. (1990)
Esophageal mucosa	Millonig et al. (2011)
Alimentary tract (duodenal and jejunal villous cells, surface epithelium of proximal colon)	Shimizu et al. (1990)
Upper gastrointestinal tract	Roberts et al. (1994)

Presence of CYP2E1 in Kupffer Cells

The content of CYP2E1 in Kupffer cells is several times lower than in hepatocytes and is located in the endoplasmic reticulum of Kupffer cells *in vivo* suggesting that it is possibly the major pathway for ethanol metabolism in Kupffer cells (Koivisto et al. 1996). The induction of CYP2E1 by ethanol in Kupffer cells isolated from rats fed ethanol-containing Lieber-DeCarli diets for 3 weeks suggests its role in causing significant changes in intracellular acetaldehyde concentrations which, together with increased lipid peroxidation, may contribute to the development of alcoholic liver injury (Koivisto et al. 1996). The mechanisms associated with the toxic actions of CYP2E1 have been summarized in Table 1.4.

Oxidant generation after CYP2E1 overexpression in RAW 264.7 macrophages transfected with CYP2E1 and possessing stable increased CYP2E1 expression (E2) appears to be central to macrophage priming and their sensitization to lipopolysaccharide (LPS) stimuli (Cao et al. 2005). Accordingly, CYP2E1 priming could explain the sensitization of Kupffer cells to LPS activation by ethanol, a crucial early step in alcoholic liver disease (Cao et al. 2005).

Presence of CYP2E1 in Extrahepatic Tissues

Immunoreactive CYP2E1 is detectable only in duodenal and jejunal villous cells in rats fed a control diet consisting of carbohydrate for 3 weeks (Shimizu et al. 1990). The content of CYP2E1 increases in duodenal and jejunal villi, and the enzyme is also detectable in squamous epithelial cells of the cheek mucosa, tongue, esophagus, and forestomach, and in surface epithelium of the proximal colon in rats pair-fed liquid diets containing 36% of total calories as ethanol (Shimizu et al. 1990). Thus, the presence of CYP2E1 in the alimentary tract, when considered together with the xenobiotic activation properties of CYP2E1, may partly explain why alcohol abuse is a risk factor for cellular damage or cancer or both in those alimentary tract tissues in which CYP2E1 is inducible by chronic ethanol intake (Shimizu et al. 1990).

In pancreatic and hepatic microsomes isolated from rats administered ethanol, ethanol induces CYP2E1 protein and p-nitrophenol hydroxylase activity, which implicates its role in the pathogenesis of pancreatitis and/or pancreatic cancer (Kessova et al. 1998).

Table 1.4 Mechanisms associated with CYP2E1 mediated injury

Integral component of the microsomal ethanol oxidizing system (Lieber and DeCarli 1968)
Acetaldehyde generation (Correa et al. 2009; Vasiliou et al. 2006; Zimatkin et al. 2006; Niemelä et al. 1998; Koivisto et al. 1996; Garige et al. 2005; Donohue et al. 2006; Kunitoh et al. 1997; Niemelä et al. 1999; Jeong et al. 2000; Carpenter et al. 1996)
ROS generation (Lu et al. 2008, 2010; Xu et al. 2005; Lieber et al. 2004b, 2007a; Chen and Cederbaum 1998; Caro and Cederbaum 2002a; Bardag-Gorce et al. 2006; Roede et al. 2008; Kathirvel et al. 2009, 2010; Garige et al. 2005; Osna et al. 2003; Zhukov and Ingelman-Sundberg 1999; Jones et al. 2002; Chen et al. 1997; Xu et al. 2003b; Lu and Cederbaum 2006; Nieto et al. 1999, 2002a, b; Chen et al. 2008, 2009; Liu et al. 2002; Schattenberg et al. 2004; Chandrasekaran et al. 2011, 2012a, b; Dai et al. 1993; Nieto et al. 2002a; Bailey et al. 2009)
Lipid peroxidation (Morimoto et al. 1995a, b; Leclercq et al. 2000b; Martínez-Chantar et al. 2002; Khalighi et al. 1999; Niemelä et al. 1998; Liu et al. 2002; Koivisto et al. 1996; Wang et al. 2008; Xu et al. 2003a; Lieber et al. 2007b; Dai et al. 1993; Chen and Cederbaum 1998; Chen et al. 1997; Caro and Cederbaum 2001, 2002a, b, 2003; Wu and Cederbaum 2001; Nieto et al. 2002a, b; Wang et al. 2009; Garige et al. 2005; Castillo et al. 1992; Albano et al. 1996; Wang et al. 2008; Nanji et al. 1994; Hu et al. 1994; Ronis et al. 1991; French et al. 1993; Sampey et al. 2003; Singh et al. 2009; Dey et al. 2002; Chandrasekaran et al. 2011, 2012a)
Mitochondrial oxidative stress and damage (Bansal et al. 2010; Xu et al. 2005; Caro and Cederbaum 2002b; Bai and Cederbaum 2004, 2006; Kim et al. 2006; Otani et al. 2005; Demeilliers et al. 2002; Knockaert et al. 2011; Raza et al. 2004; Robin et al. 2005)
Protein adduct formation (Bai and Cederbaum 2004; Sampey et al. 2003; Roede et al. 2008; Niemelä et al. 1998, 1999; Jeong et al. 2000; French et al. 1993)
Involved in priming of macrophages and their sensitization to lipopolysaccharide stimuli (Cao et al. 2005)
Oxidative damage and inactivation of microsomal Ca2+-ATPase resulting in elevated calcium level (Caro et al. 2009)
Increased influx of intracellular Ca2+ and activation of Ca2+ dependent proteases (Caro and Cederbaum 2002a)
Increase in collagen expression (Nieto et al. 1999, 2000, 2002a, b; Lu et al. 2010)
Upregulation of COX-2 and prostaglandin E2 (Nieto et al. 2000)
Fibrogenesis (Castillo et al. 1992; Nieto et al. 2002a; Rigamonti et al. 2009; Niemelä et al. 1999; French et al. 1993)
DNA adduct formation (Wang et al. 2009; Millonig et al. 2011; Ghanayem et al. 2005c; Gut et al. 1996)
DNA damage (Demeilliers et al. 2002; Bailey et al. 2009; Bradford et al. 2005; Kukielka and Cederbaum 1994; Ghanayem et al. 2005b; Bansal et al. 2010; Bae et al. 2001; Tumer et al. 2010)
Depletion of glutathione (Xu et al. 2005; Robin et al. 2005; Bansal et al. 2010; Chen et al. 2008; Otani et al. 2005; Martínez-Chantar et al. 2002; Roede et al. 2008; Curry-McCoy et al. 2010; Lieber et al. 2007b)
Downregulation of regulator for fatty acid oxidation-PPAR alpha (Lu et al. 2008; Abdelmegeed et al. 2011)
Significantly greater 18:1/18:0 fatty acids (Morimoto et al. 1995a)
Nitrosative stress (Dey and Cederbaum 2007; Lu et al. 2005; Kathirvel et al. 2010; Mantena et al. 2009; Bailey et al. 2009; Osna et al. 2003)
Decreased levels of ubiquitin pathway proteins or genes (Gouillon et al. 1999; Bardag-Gorce et al. 2006; Tierney et al. 1992)

(continued)

Table 1.4 (continued)

Inhibition of proteasome activity (Osna et al. 2007, 2008, 2009, 2010; Gouillon et al. 2000; Bardag-Gorce et al. 2000, 2006; Donohue et al. 2006)
Accumulation of oxidized proteins (Bardag-Gorce et al. 2000)
Protein carbonyl formation (Bardag-Gorce et al. 2006; Roede et al. 2008)
4-hydroxy-2-nonenal adduct formation (Bardag-Gorce et al. 2006; Niemelä et al. 1998, 1999; Wang et al. 2009; French et al. 1993; Sampey et al. 2003; Chandrasekaran et al. 2011, 2012a; Clot et al. 1996)
Induction of cytokeratin 8 and cytokeratin 18 (Bardag-Gorce et al. 2006; Butura et al. 2009)
Formation of cytokeratin aggresomes (Bardag-Gorce et al. 2005, 2006)
In vitro Mallory body like inclusion formation (Bardag-Gorce et al. 2006)
Marked decreases in Gal beta 1,4 GlcNAc alpha 2,6-sialyl transferase (2,6-ST) levels (Garige et al. 2005)
Suppression of IFN gamma signal transduction (Osna et al. 2005)
Reduction of STAT1 phosphorylation (Osna et al. 2003, 2005)
Triglyceride accumulation (Martínez-Chantar et al. 2002; Ronis et al. 2010; Chalasani et al. 2003; Roede et al. 2008; Lu et al. 2008; Morimoto et al. 1995a)
Induction of TNF alpha (Lieber et al. 2004a, b; Ronis et al. 2010; Abdelmegeed et al. 2011; Fang et al. 1998)
Increased CCl4 mediated toxicity (Martínez-Chantar et al. 2002; Tierney et al. 1992)
Inhibition of fatty acid oxidation (Chen et al. 2009; Lu et al. 2008)
Inhibition of PPAR alpha activity (Chen et al. 2009; Abdelmegeed et al. 2011)
Down regulation of insulin signalling leading to insulin resistance (Chalasani et al. 2003; Schattenberg et al. 2005; Lieber et al. 2004b; Kathirvel et al. 2009)
Impaired protein methylation (Osna et al. 2010)
Behavioural changes (Vasiliou et al. 2006; Correa et al. 2009)
Hydroxyethyl radical generation (Albano et al. 1996; Dupont et al. 1998; Clot et al. 1996, 1997)
Increased IgG complex formation with hydroxyethyl radical formation (Dupont et al. 1998)
Development of auto-antibodies against CYP2E1 (Albano et al. 1996; Vidali et al. 2007; Rigamonti et al. 2009; Sutti et al. 2010a, b; Lytton et al. 1999)
Necroinflammation (Rigamonti et al. 2009; Sutti et al. 2010b)
Enhanced retinoic acid catabolism (Liu et al. 2001)
JNK activation (Liu et al. 2002; Wang et al. 2008; Bae et al. 2001; Singh et al. 2009)
Decrease in arachidonic acid content (Nanji et al. 1993)
Decrease in phospholipase A and C activities (Nanji et al. 1993)
Decreased Akt phosphorylation (Schattenberg et al. 2005)
Reduction in glycogen storage (Kathirvel et al. 2009)
Increased glucose synthesis (Kathirvel et al. 2009)
Nitrosylation of catalase and superoxide dismutase (Kathirvel et al. 2010)
Inflammation (Morimoto et al. 1994; Abdelmegeed et al. 2011; Niemelä et al. 1999; Tindberg et al. 1996; Sampey et al. 2003)
Fat accumulation (Lu et al. 2008; Kathirvel et al. 2009)
Steatosis (Esfandiari et al. 2005; Bailey et al. 2009; Sampey et al. 2003; Lieber et al. 2004a; Lu et al. 2010; Curry-McCoy et al. 2010; Wang et al. 2008; Abdelmegeed et al. 2011; Baumgardner et al. 2008; Videla et al. 2004; Orellana et al. 2006)
Endoplasmic reticulum stress (Esfandiari et al. 2005; Dey et al. 2006; Ronis et al. 2010)
Oxidative or nitrosative modification of mitochondrial proteins (Kim et al. 2006; Mantena et al. 2009; Sampey et al. 2003)

(continued)

Table 1.4 (continued)

Ubiquitin mediated protein degradation (Abdelmegeed et al. 2010)
Decreased circulating 1, 25-dihydroxycholecalciferol (1,25 (OH) 2 D3) (Shankar et al. 2008)
Diminished antioxidant capacity (Videla et al. 2004; Abdelmegeed et al. 2010; Fernández et al. 2003; Otani et al. 2005; Roede et al. 2008)
Cytokine activation (Fang et al. 1998; Starkel et al. 2000)
Inhibition of t-retinoic acid synthesis (Khalighi et al. 1999)

Aggravation of Ethanol Induced Hepatotoxicity due to Other Hepatotoxins

Co-administration of ethanol and the hepatocarcinogen N-nitrosodimethyl amine (NDMA) to rats results in much greater hepatotoxicity than either agent alone (Ma et al. 1991). The addition of ethanol inhibits CYP-dependent demethylation and denitrosation of NDMA in liver microsomes, whereas both activities are enhanced markedly by chronic ethanol administration (Ma et al. 1991). Further, the study suggests the involvement of alcohol-inducible CYP2E1 in both NDMA bioactivation (demethylation and denitrosation) reactions (Ma et al. 1991). Thus, bioactivation plays a crucial role in the hepatotoxicity of NDMA and its aggravation by chronic alcohol consumption (Ma et al. 1991).

Further, the pro-vitamin A carotenoid beta-carotene potentiates the induction of CYP2E1 protein and catalytic activity by ethanol in rat liver and also increases CYP4A1, which may, at least in part, explain the associated hepatotoxicity (Kessova et al. 2001). The agents acting as co-inducers of CYP2E1 have been summarized in Table 1.5.

Ethanol Induced Hepatotoxicity and Protective Agents

Ethanol mediated increases in CYP2E1 content and MEOS is significantly reduced with the addition of carbonyl iron in livers of rats fed ethanol (Aleynik et al. 1999). This iron-induced decrease is corrected by Polyenylphosphatidylcholine (PPC), a 94–96% pure mixture of linoleate-rich polyunsaturated phosphatidylcholines that protects against alcohol-induced liver injury (Aleynik et al. 1999). Further, in the absence of iron, the ethanol-mediated induction of CYP2E1 and its corresponding enzyme activities and oxidative stress are significantly less with PPC (Aleynik et al. 1999). In addition, PPC attenuates alcohol-induced apoptosis of rat hepatocytes; this effect may provide a mechanism for PPC's protection against liver injury, possibly in association with its antioxidative action via the down-regulation of ethanol-mediated CYP2E1 induction (Mi et al. 2000).

Dilinoleoylphosphatidylcholine (DLPC) is the major component of PPC, and DLPC significantly decreases CYP2E1 content and its corresponding activities in rats fed ethanol diet and thus could serve a therapeutic target for the prevention of alcoholic liver disease (Aleynik and Lieber 2001). Further, DLPC decreases the cytotoxicity (apoptosis) induced by alcohol in HepG2 cells expressing CYP2E1, a protective action due, at least in part, to an attenuation of the alcohol-induced oxidative stress (diminished hydrogen peroxide production), the alteration in the

Table 1.5 Agents acting as co-inducers of CYP2E1

Agent	References
Ethanol and N-nitrosodimethylamine	Ma et al. (1991)
Ethanol and beta-carotene	Kessova et al. (2001)
Ethanol and extremely low carbohydrate diet	Rowlands et al. (2003)
Ethanol and polyunsaturated fatty acids	Morimoto et al. (1993, 1994) and Nanji et al. (1993)
Ethanol plus (Fe-nitrilotriacetic acid (Fe-NTA)) plus arachidonic acid	Bardag-Gorce et al. (2006)
Ethanol and lycopene	Veeramachaneni et al. (2008)
Ethanol and undernutrition	Baumgardner et al. (2007)
Ethanol and hepatitis C virus core protein	Otani et al. (2005)
Ethanol and castration	Niemelä et al. (1999)
Ethanol and folate deficiency	Esfandiari et al. (2005)
Ethanol and carbohydrate deficiency	Korourian et al. (1999)
Ethanol and nicotine	Ferguson et al. (2011), Yue et al. (2009), and Howard et al. (2001, 2003a)
Ethanol and environmental tobacco smoke and hypercholesterol (Apoprotein E deficiency)	Bailey et al. (2009)
Ethanol and high glucose	Chandrasekaran et al. (2012b)
Streptozocin and 4-methyl pyrazole	Wu and Cederbaum (1993a)
Streptozocin and thioacetamide	Wang et al. (2000)
Pyrazole and lipopolysaccharide	Lu et al. (2005)
Pyrazole and obesity	Dey and Cederbaum (2007)
Arachidonic acid and iron (Fe-NTA)	Caro and Cederbaum (2001)
Acetone and obesity	Dey and Cederbaum (2007) and Leclercq et al. (2000a)
Fasting and obesity	Leclercq et al. (2000a)
4-methyl pyrazole and obesity	Leclercq et al. (2000a)
Pyridine plus Thioacetamide and diet restriction	Ramaiah et al. (2001)

mitochondrial membrane potential and partial restoration of mitochondrial glutathione (GSH) (Xu et al. 2005). Moreover, in a high-fat diet (HF) rat model, the combination of S-adenosylmethionine plus DLPC decreases liver triacylglycerols and CYP2E1 mRNA and CYP2E1 protein, accompanied by a reduction of hepatic 4-HNE, reflecting control of oxidative stress (Lieber et al. 2007a). The agents conferring protection against CYP2E1 mediated toxicity have been summarized in Table 1.6.

Lycopene, a carotenoid with high anti-oxidant capacity, protects HepG2 cells expressing CYP2E1 (HepG2 cells transfected with pCI-neo/2E1 (2E1)) against arachidonic acid (AA) toxicity. This is due, at least in part, to inhibition of hydrogen peroxide production and of the resulting lipid peroxidation, confirming the potent anti-oxidant properties of lycopene and its suitability for clinical studies (Xu et al. 2003a). Further, lycopene opposes the ethanol-induced oxidative stress and apoptosis in 2E1 cells (Xu et al. 2003b).

Table 1.6 Agents conferring protection against CYP2E1 mediated injury

Agent	References
Polyenylphosphatidylcholine	Aleynik et al. (1999)
Dilinoleoylphosphatidylcholine	Aleynik and Lieber (2001)
S-adenosyl methionine	Lieber et al. (2007a), Martínez-Chantar et al. (2002), Osna et al. (2010), and Esfandiari et al. (2005)
Lycopene	Xu et al. (2003a, b)
Medium chain triglycerides	Lieber et al. (2007b)
Acarbose	Lieber et al. (2004a)
Chlormethiazole	Gebhardt et al. (1997), Gouillon et al. (2000), Hu et al. (1994), Simi and Ingelman-Sundberg (1999), Tindberg and Ingelman-Sundberg (1996), Tindberg et al. (1996), Lytton et al. (1999), Fang et al. (1998), and Wang et al. (2009)
cAMP	Gouillon et al. (1999)
Propofol	Lejus et al. (2002)
Insulin	Sidhu et al. (2001, 2006) and Woodcroft and Novak (1997, 1999)
Endotoxin	Morgan (1993), Sewer et al. (1998), Sewer and Morgan (1998), and Cheng et al. (2003)
Interleukins 1&6	Morgan et al. (1994)
Diallyl sulfide	Morimoto et al. (1995a, b), Albano et al. (1996), Ronis et al. (2010), Martínez-Chantar et al. (2002), Zimatkin et al. (2006), Ramaiah et al. (2001), Bardag-Gorce et al. (2006), Osna et al. (2005, 2007, 2008), and Albano et al. (1996)
Phenethyl isothiocyanate	Morimoto et al. (1995a, b), Albano et al. (1996), and Zimatkin et al. (2006)
YH439	Jeong et al. (2000) and Bae et al. (2001)
Isoniazid	French et al. (1993)
SKF-525A	Cummings et al. (2001)
4-methyl pyrazole	Ronis et al. (2010), Donohue et al. (2006), Osna et al. (2007), and Huan and Koop (1999)

Further, the alpha-glucosidase inhibitor acarbose which is beneficial in the prevention of type 2 diabetes has been found to decrease steatosis and inflammation, accompanied by decreases in protein and mRNA expression of the hepatic inflammatory cytokine TNF-alpha, CYP2E1, and collagen in a rat model of non-alcoholic steatohepatitis (NASH) (Lieber et al. 2004a).

In rats fed either 32% of calories as dietary long-chain triglycerides (LCT) (alcohol), or 16% as LCT + 16% as medium-chain triglycerides (MCT) (alcohol-MCT 16%), or 32% as MCT only (alcohol-MCT 32%), both alcohol and alcohol-MCT 16% groups have a significant increase in mitochondrial and microsomal CYP2E1 (Lieber et al. 2007b). When MCT replaces all the fat, like in the alcohol-MCT 32% group, CYP2E1 is significantly reduced both in mitochondria and microsomes (Lieber et al. 2007b). Thus, mitochondria participate in the induction of CYP2E1 by alcohol and contribute to lipid peroxidation and GSH depletion and a diet rich in MCT is beneficial in ameliorating injury.

CYP2E1 and Rodent NASH Model

Rats fed high fat diet reproduce the key features of human NASH which is frequently associated with obesity and diabetes and exhibit insulin resistance and increased hepatic TNF alpha, collagen type 1, alpha1(I) procollagen and CYP2E1 mRNA. In addition, these rats show CYP2E1 induction and oxidative stress with increased 4-hydroxynonenal formation (Lieber et al. 2004b). Thus, NASH in a rodent model is associated with upregulation of CYP2E1.

Arthur I. Cederbaum

Characterization of Biochemical & Toxicolological Actions of CYP2E1

Studies in Dr. Cederbaum's laboratory are mainly directed towards characterization of biochemical and toxicological properties of CYP2E1.

Establishment of CYP2E1 Over-Expressing HepG2 Cell Lines

A human-hepatoma-derived cell line clone MV2E1-9, stably and constitutively expressing the coding sequence of the human CYP2E1 in HepG2, was established by recombinant retroviral expression (Dai et al. 1993). MV2E1-9 metabolized p-nitrophenol, dimethylnitrosamine, aniline, and ethanol and exhibited several fold higher rates of superoxide and H2O2 production and lipid peroxidation when compared to control clones (Dai et al. 1993). Ethanol increases the content of CYP2E1 and catalytic oxidation of CYP2E1 substrates in MV2E1-9 cells, possibly through protein stabilization (Carroccio et al. 1994). The list of potent carcinogens such as dimethylnitrosamine which are metabolized by CYP2E1 have been summarized in Table 1.7.

Ethanol and other substrates such as dimethyl sulfoxide, carbon tetrachloride, isoniazid, and N,N-dimethylnitrosamine exhibit cytotoxic effects in another model for transduced HepG2 cells- HepG2 E9 cells, which express CYP2E1 (Wu and Cederbaum 1996). Further, other transduced HepG2 subclonal cells that overexpress CYP2E1- Hep G2-CI2E1-43 and -47 (E47) cells exhibited slower growth rate than parental HepG2 cells or control subclones that do not express CYP2E1, but remained fully viable (Chen and Cederbaum 1998). Low lipid peroxidation levels are observed in E47 cells, reflective of the ability of CYP2E1 to generate ROS even in the absence of added metabolic substrate (Chen and Cederbaum 1998).

CYP2E1 Mediated Hepatotoxicity: Underlying Mechanisms

CYP2E1 exerts its hepatoxic actions through several mechanisms and some of these mechanisms which have been studied in Dr. Cederbaum's laboratory have been discussed in this section. DNA strand cleavage occurs due to the production of hydroxyl radicals by rat liver microsomes and is further increased after chronic ethanol treatment (Kukielka and Cederbaum 1994). Further, this increased microsomal DNA cleavage in the presence of NADPH and NADH is partially due to induction of CYP2E1, as observed due to inhibition of the process in the presence of anti-(CYP2E1) IgG and inhibitors of CYP2E1, such as diethyl dithiocarbamate and tryptamine (Kukielka and Cederbaum 1994). Further, incubation of hepatocytes isolated from rats treated with pyrazole, with ethanol or arachidonic acid results in the release of cytochrome c and activation of caspase 3,

Table 1.7 CYP2E1 and carcinogenesis

Potent carcinogens	References
Thioacetamide	Wang et al. (2000), Ramaiah et al. (2001), and Chilakapati et al. (2007)
Ethanol	Wang et al. (2009) and Millonig et al. (2011)
Acrylamide	Ghanayem et al. (2005a, b, c) and Ghanayem (2007)
Methacrylonitrile	Wang et al. (2002a)
Acrylonitrile	Wang et al. (2002b)
Urethane	Hoffler et al. (2003, 2005)
1-bromopropane	Garner et al. (2007)
Dimethylnitrosamine	Garro et al. (1981), Arinç et al. (2007), Ma et al. (1991), Dey et al. (2002, 2005), Kapoor et al. (2006), Khan et al. (2011), Anandatheerthavarada et al. (1993), Bhagwat et al. (1995), Huan and Koop (1999), Roberts et al. (1995), and Zaluzny et al. (1990)
N-methyl formamide	Lerche et al. (1996)
Diethylnitrosamine	Lerche et al. (1996)
Nicotine	Howard et al. (2001)

which contributes towards the apoptotic effects of CYP2E1 in the liver cells (Wu and Cederbaum 2000). Heat shock proteins (Hsps) are crucial for the stability and function of numerous proteins and geldanamycin, an inhibitor of Hsp90, causes pronounced oxidative stress and apoptosis in E47 cells suggesting that the inhibition of the molecular chaperone Hsp90 promotes CYP2E1 mediated oxidative stress in liver cells (Dey and Cederbaum 2006). CYP2E1 oxidatively damages and inactivates the microsomal Ca2+-ATPase in CYP2E1 over-expressing E47 cells accounting for the elevated calcium level during CYP2E1 toxicity, suggesting that this may contribute to elevated cytosolic calcium and CYP2E1-potentiated injury (Caro et al. 2009). Studies showing CYP2E1 mediated apoptotis or necrosis have been summarized in Table 1.8.

Antioxidant Depletion Promotes CYP2E1 Mediated Liver Injury: Crucial Role for Oxidative Stress as a Major Mechanism for the Deleterious Effects of CYP2E1

Inhibition of GSH synthesis by treatment with buthionine sulfoximine, results in rapid decline of GSH levels in E47 cells and elevated lipid peroxidation which are not observed in control cells, which is most likely a reflection of CYP2E1-catalyzed formation of ROS (Chen and Cederbaum 1998). Thus, under conditions of CYP2E1 overexpression, two modes of CYP2E1-dependent toxicity can be observed in HepG2 cells: a slower growth rate when cellular GSH levels are maintained and a loss of cellular viability when cellular GSH levels are depleted (Chen and Cederbaum 1998). Further, chronic alcohol consumption induces liver injury in Cu, Zn-superoxide dismutase-deficient mice (Sod1−/−), with extensive centrilobular necrosis, inflammation and mitochondrial dysfunction (Kessova and Cederbaum 2007).

Table 1.8 Modes of cell death associated with CYP2E1 mediated injury

Mode of cell death	References
Apoptosis	Aleynik and Lieber (2001), Wang et al. (2008), Donohue et al. (2006), Ronis et al. (2010), Esfandiari et al. (2005), Bae et al. (2001), Abdelmegeed et al. (2011), Jones et al. (2002), Xu et al. (2003b), Dey and Cederbaum (2006), Lu et al. (2005), Chandrasekaran et al. (2011, 2012a, b), Chen et al. (1997), Chen and Cederbaum (1998), and Mi et al. (2000)
Necrosis	Kessova and Cederbaum (2007), Lu et al. (2005), Niemelä et al. (1999), Korourian et al. (1999), Ronis et al. (2010), French et al. (1993), Sampey et al. (2003), and Jones et al. (2002)

Regulation of Hepatic CYP2E1 Mediated by Pathophysiological Conditions Such as Obesity, Diabetes and Chemical Inducers

Treatment of rats with the chemical inducer-4-methylpyrazole and streptozotocin which is commonly used to induce diabetes, increases CYP2E1 protein and catalytic activity and the values are additive for each inducer alone suggesting that diabetes may increase the susceptibility to toxins which are activated by CYP2E1, more so if pre-exposure to chemical inducers similar to 4-methylpyrazole, e.g., ethanol, isoniazid occurs (Wu and Cederbaum 1993a).

Pyrazole and 4-methylpyrazole, inducers for hepatic CYP2E1 induce renal CYP2E1, through a post-transcriptional mechanism-possibly involving increased protein stabilization (Wu and Cederbaum 1994). Further, acetone- or pyrazole-mediated induction of CYP2E1 potentiates liver injury in obesity (Dey and Cederbaum 2007). Acetone- or pyrazole-treated obese mice liver exhibit elevated CYP2E1 levels, increased oxidative stress parameters, and greater liver injury (Dey and Cederbaum 2007). Thus, obesity contributes to oxidative stress and liver injury which is potentiated due to the induction of CYP2E1 (Dey and Cederbaum 2007).

Ethanol Mediated Induction of Neonatal CYP2E1

Fetal rat liver is characterized by absence of CYP2E1 because activation of the gene occurs shortly after birth and ethanol induces CYP2E1 in adult rats (Wu and Cederbaum 1993b). Further, consumption of an ethanol-containing liquid diet in pregnant rats starting on the 9th day of gestation induces CYP2E1 content and catalytic activities with no elevations in CYP2E1 mRNA in hepatic microsomes from neonates of mothers compared with controls (Wu and Cederbaum 1993b).

Ethanol Mediated Inducibility of CYP2E1 and Its Hepatotoxic Actions: Effects in CYP2E1 Knock 'out' and Knock 'in' Models

CYP2E1 has been shown to play a role in experimental alcoholic fatty liver in an oral ethanol-feeding model (Lu et al. 2008). In wild type mice administered ethanol, macrovesicular fat accumulation and accumulation of triglyceride, induced CYP2E1 in liver, higher oxidative stress, downregulation of a target gene of the fatty acid oxidation regulator-Peroxisome proliferator-activated receptor alpha (PPARalpha), acyl CoA oxidase are observed but not in CYP2E1-knockout mice (Lu et al. 2008). Further,

it suggests that CYP2E1-derived oxidative stress may inhibit oxidation of fatty acids by preventing up-regulation of PPAR alpha by ethanol, resulting in fatty liver.

In a study using CYP2E1 knock out (KO) mice, and humanized CYP2E1 knock-in (KI) mice (in which the human 2E1 has been added back to mice deficient in the mouse CYP2E1) fed a high-fat Lieber-DeCarli ethanol liquid diet, ethanol-induced steatosis and oxidant stress are blunted in the KO mice (no liver injury) but restored in the KI mice (Lu et al. 2010). Significant liver injury is produced in the ethanol-fed KI mice, accompanied by elevated levels of the human CYP2E1 compared to levels of the mouse CYP2E1 in wild type mice and increased levels of collagen type 1 and smooth muscle actin (Lu et al. 2010). Thus, CYP2E1 plays a major role in ethanol-induced fatty liver and oxidant stress and it is the human CYP2E1 that restores the injurious effects of ethanol, suggesting that results for fatty liver and oxidant stress from rodent models of ethanol intake and mouse CYP2E1 can be extrapolated to human models of ethanol intake and to human CYP2E1 (Lu et al. 2010).

CYP2E1 Potentiates Injury Mediated by Several Hepatotoxins

Arachidonic acid (AA), a representative polyunsaturated fatty acid, causes a concentration- and time-dependent toxicity (apoptosis) to HepG2-MV2E1-9 cells which could be mediated by lipid peroxidation occurring in the cells over-expressing CYP2E1 due to elevated production of ROS (Chen et al. 1997). Further, pyrazole administration to rats increases lipopolysaccharide (LPS)-induced necrosis in liver which appears to involve an increase in oxidative and nitrosative stress generated by the combination of LPS plus elevated CYP2E1 levels (Lu et al. 2005).

The combination of Fe-nitrilotriacetic acid (Fe-NTA) and AA produce synergistic injury, increased lipid peroxidation, and damaged mitochondria in E47 cells (Caro and Cederbaum 2001). Similarly, hepatocytes isolated from pyrazole-treated rats exhibit more Fe + AA toxicity suggesting that low concentrations of Fe and AA can act as priming or sensitizing factors for CYP2E1-induced injury in HepG2 cells, and such interactions may play a role in alcohol-induced liver injury (Caro and Cederbaum 2001).

CYP2E1- and oxidative stress-dependent toxicity with iron and arachidonic acid combination in E47 cells has been shown to be mediated by the activation of lipid peroxidation, followed by an increased influx of extracellular Ca2+ and activation of Ca2+-dependent proteases (Caro and Cederbaum 2002a). Further, CYP2E1-dependent mitochondrial damage, in the presence of extracellular calcium as observed in E47 cells preloaded with AA and then incubated with Fe-NTA reveals CYP2E1-dependent, necrotic, and lipid peroxidation-dependent toxicity which is preceded by oxidative damage to mitochondria and the permeability transition (Caro and Cederbaum 2002b). Further, Ca(2+) mobilization and the activation of calpain (calcium dependent cysteine protease) contributes to the more rapid onset of mitochondrial damage, while oxidative damage and lipid peroxidation are involved in the Ca(2+)-independent later onset of mitochondrial damage (Caro and Cederbaum 2002b).

The release of stored calcium by AA+Fe-NTA, induced by lipid peroxidation, initially activates calpain and phospholipase A2 (PLA2) activity, PLA2 activation is

crucial for a subsequent increased influx of extracellular Ca2+, and the combination of increased PLA2 and calpain activity, increased calcium and oxidative stress cause mitochondrial damage, that ultimately produces the rapid toxicity of AA+Fe in E47 cells (Caro and Cederbaum 2003).

Role of CYP2E1 in Modulating the Hepatotoxicity of Drugs

The analgesic and anti-pyretic drug acetaminophen at high concentrations (above 5 mM) and in the presence of depleted intracellular GSH, causes severe cytotoxicity in MVh2E1-9 cells due to the ability of human CYP2E1 to activate acetaminophen to reactive metabolites which form covalent protein adduct, suggesting the ability of CYP2E1 to modulate the therapeutic index of drugs and cause drug toxicity (Dai and Cederbaum 1995). Further, adenovirus-mediated overexpression of human CYP2E1 in HepG2 cells activates acetaminophen to reactive metabolites which damage mitochondria, form protein adducts, and result in toxicity to HepG2 cells (Bai and Cederbaum 2004).

Sodium salicylate, an anti-inflammatory drug serves as a substrate of CYP2E1, increases CYP2E1 protein and potentiates AA induced lipid peroxidation and toxicity in CYP2E1 over-expressing E47 cells and hepatocytes isolated from pyrazole treated rats, thus suggesting the possible limitations in the use of salicylate and salicylate precursors such as acetylsalicylic acid with certain other drugs (Wu and Cederbaum 2001). In E47 cells and mice subjected to acetone administration, elevated CYP2E1 enhances the anti-cancer drug cisplatin-induced hepatotoxicity, possibly through increased ROS generation and subsequent oxidative stress (Lu and Cederbaum 2006).

CYP2E1 Mediated Oxidative Stress and Endoplasmic Reticulum Dysfunction

CYP2E1 mediated oxidative stress has been shown to downregulate the expression of endoplasmic reticulum resident proteins GRP78 and GRP94 proteins in HepG2 cells and is an important mechanism in causing ER dysfunction in these cells as E47 cells exhibit decreased mRNA and protein expression of GRP78 and GRP94 along with the accumulation of ubiquinated and aggregated proteins (Dey et al. 2006).

Establishment of HepG2 Cells Over-Expressing Mitochondrial CYP2E1: An Insight into the Mechanisms of Liver Injury due to Mitochondrial CYP2E1

HepG2 lines overexpressing CYP2E1 in mitochondria (mE10 and mE27 cells) were established by transfecting a plasmid containing human CYP2E1 cDNA lacking the hydrophobic endoplasmic reticulum targeting signal sequence into HepG2 cells followed by G418 selection (Bai and Cederbaum 2006). A 40-kDa catalytically active NH2-terminally truncated form of CYP2E1 (mtCYP2E1) detected in the mitochondrial compartment is induced due to chronic alcohol consumption and induces oxidative stress in the mitochondria, damaged mitochondria membrane potential, and causes a loss of cell viability (Bai and Cederbaum 2006).

CYP2E1 and Liver Fibrosis: Use of Hepatic Stellate Cells as an Important Tool to Understand the Mechanisms Involved

In a rat hepatic stellate cell line (HSC-T6) expressing CYP2E1, elevated production of ROS occurs which is associated with both elevation and stabilization of alpha 2 collagen type I (COL1A2) messenger RNA levels thus elucidating signaling pathways responsible for oxidant stress-mediated collagen gene induction (Nieto et al. 1999).

In a hepatic stellate cell line over-expressing the ethanol-inducible CYP2E1 (E5 cells), addition of AA elevates cyclooxygenase-2 (COX-2) levels and production of prostaglandin E(2); and ethanol and AA up-regulate alpha 2 collagen type I (COL1A2) gene expression (Nieto et al. 2000). Further, inhibition of COX-2 blocks the effect of AA, but not of ethanol, on COL1A2 expression suggesting that CYP2E1 activates COX-2 expression, and the oxidation of AA by COX-2 is responsible for the increase in COL1A2 (Nieto et al. 2000).

The fibrogenic effects of CYP2E1-dependent generation of ROS are observed in a co-culture of E47 cells with hepatic stellate cells (Nieto et al. 2002a). An increase in H(2)O(2), lipid peroxidation, and collagen type I protein in stellate cells co-cultured with E47 cells occurs (Nieto et al. 2002a). Similar results are observed in co-cultures of hepatocytes isolated from pyrazole-treated rats and primary stellate cells (Nieto et al. 2002a).

The activation of hepatic stellate cells in the presence of CYP2E1-derived ROS as apparent through co-incubation of primary stellate cells with E47 cells is characterized by morphologic changes and loss of lipid droplets (Nieto et al. 2002b). A more pronounced increase in alpha-smooth muscle actin (alpha-sma), intracellular and secreted collagen type I protein, and intra- and extracellular H(2)O(2) and lipid peroxidation products in the cells under co-culture occurs (Nieto et al. 2002b). Thus, hepatocytes containing CYP2E1 release diffusible mediators including ROS, which can activate HSC and besides perturbing the homeostasis of hepatocytes, CYP2E1-derived diffusible oxidants may also interact with stellate cells and contribute to hepatic fibrosis (Nieto et al. 2002b).

The above studies are amongst several other ongoing studies involving in vitro and in vivo models elucidating the role of CYP2E1 as a key player in alcohol induced liver injury from Dr. Cederbaum's laboratory.

Helmut K. Seitz

CYP2E1 and Ethanol Mediated Carcinogenesis

Chronic ethanol ingestion in rats results in an increase in hepatic microsomal dimethylnitrosamine (DMN) demethylase activity and in an increase in hepatic microsomal activation of DMN to a mutagen (Garro et al. 1981). These effects of ethanol on DMN metabolism are detectable *in vitro* at DMN concentrations as low as 0.3–1 mM and as high as 100 mM (Garro et al. 1981). This ability of ethanol to increase the rate of DMN metabolism over such a broad range of DMN concentrations is in marked contrast to the effects of other microsomal enzyme inducers, such as phenobarbital and 3-methylcholanthrene, which increase the rate of DMN metabolism only at relatively high DMN concentrations and repress its metabolism at low DMN concentrations (Garro et al. 1981). The cytochrome P-450 content of hepatic microsomes from rats chronically fed the ethanol-containing diet generally is increased by 25–50% relative to microsomes from control rats and DMN demethylase activity is likely to be related to cytochrome P-450 levels rather than to microsomal protein levels (Garro et al. 1981). Thus, nitrosamine metabolism is catalyzed by CYP2E1 and that chronic alcohol consumption increases the activation of nitrosamines which is important in carcinogenesis (Garro et al. 1981).

Significantly increased levels of carcinogenic etheno-DNA adducts are formed by the reaction of the major lipid peroxidation product, 4-hydroxynonenal (4-HNE) with nucleobases in hepatocytes of alcoholic liver disease (ALD) patients and in the liver of alcohol-fed lean (Fa/?) and obese (fa/fa) Zucker rats (Wang et al. 2009). The protein-bound 4-HNE strongly correlates with CYP2E1 expression in patients with ALD (Wang et al. 2009). The increased level of etheno-DNA adducts detected in ALD patients and the rodent models correlates significantly with CYP2E1 expression (Wang et al. 2009). Further, the role of CYP2E1 in the formation of etheno-DNA adducts is explicitly proved as ethanol increases etheno-DNA adducts in the nuclei of HepG2 cells stably transfected with human CYP2E1 (E47 cells) in a concentration-dependent and time-dependent manner which is significantly blocked with chlormethiazole (Wang et al. 2009). Thus, ethanol-mediated induction of hepatic CYP2E1 leading to the formation of highly miscoding lipid peroxidation-derived DNA lesions may play a central role in hepatocarcinogenesis in patients with ALD (Wang et al. 2009).

In non-tumorous esophageal biopsies of patients with upper aerodigestive tract cancer, chronic alcohol ingestion results in a significant induction of CYP2E1 which correlates with the amount of alcohol consumed (Millonig et al. 2011). Furthermore, a significant correlation between CYP2E1 and the generation of the carcinogenic exocyclic etheno-DNA adducts 1,N(6)-ethenodeoxyadenosine and 3,N(4)-ethenodeoxycytidine is observed (Millonig et al. 2011). Non-smokers and non-drinkers have the lowest rate of cell proliferation, CYP2E1 expression and DNA lesions (Millonig et al. 2011). Thus, ethanol mediated induction of CYP2E1 in the esophageal mucosa in a dose dependent manner occurs in human beings and explains, at least in part, the generation of carcinogenic DNA lesions in this target organ (Millonig et al. 2011).

Drug Mediated Inhibition of Ethanol Inducible CYP2E1

Chlormethiazole, a sedative and anticonvulsive drug used in the treatment of alcohol withdrawal and a potent inhibitor of alcohol-inducible rat hepatic CYP2E1, has been shown to be an effective inhibitor of the metabolism of CYP2E1 probe chlorzoxazone and thus of CYP2E1 activity in alcoholic and control patients; and human liver microsomes (Gebhardt et al. 1997). Therefore, detoxification treatment of alcohol with chlormethiazole may prove to be beneficial in alleviating the detrimental effects of CYP2E1 induction after chronic ethanol consumption (Gebhardt et al. 1997).

Ethanol Mediated Regulation of Human CYP2E1: Dynamics Involved

A significant CYP2E1 induction occurs 1 week following the ingestion of alcohol in human beings and is increased further after 4 weeks (Oneta et al. 2002). The disappearance of CYP2E1 is found to be significant 3 days following ethanol withdrawal in alcoholics and further decreases up to day 8 (Oneta et al. 2002). Thereafter, no significant changes occur and CYP2E1 activities are comparable with those in patients with non-alcoholic liver disease (Oneta et al. 2002). Therefore, a significant and quick induction of CYP2E1 activity occurs at moderate alcohol consumption, which may be of importance in the pathogenesis of alcoholic liver disease, of ethanol, drug and vitamin A interactions and in alcohol associated carcinogenesis (Oneta et al. 2002).

Ann K. Daly

Genetic Polymorphisms in CYP2E1

To investigate whether interindividual variation in CYP2E1 levels can be explained by genetic polymorphism, DNA samples from 40 healthy individuals were analyzed for polymorphisms in the CYP2E1 coding sequence and promoter region (Fairbrother et al. 1998). Polymorphisms were detected at positions −316 (A to G), −297 (T to A), −35 (G to T), 1107 (G to C; intron 1), 4804 (G to A Val179Ile; exon 4) and 10157 (C to T; exon 8) (Fairbrother et al. 1998). All individuals positive for either A(−316)G, G(−35)T, G(4804)A or the previously described RsaI polymorphism at −1,019 were also positive for T(−297)A, which had the highest allele frequency of the observed polymorphisms (0.20). A(−316)G, G(−35)T and G(4804)A were detected at allele frequencies of 0.022, 0.052 and 0.013, respectively (Fairbrother et al. 1998). The functional significance of the upstream polymorphisms was examined by preparing constructs of positions −549 to +3 of CYP2E1 containing the observed combinations of the polymorphisms fused to luciferase reporter genes and transfecting HepG2 cells (Fairbrother et al. 1998). For the G(−35)T/T(−297)A construct, a 1.8-fold increase in luciferase activity compared with the wild-type sequence ($P = 0.06$) and 2.5-fold compared with T(−297)A only ($P = 0.025$) was observed (Fairbrother et al. 1998). No significant difference in activity was observed between the other constructs. The significance of the predicted Val179Ile base change from G(4804)A was determined by expression of the wild-type and mutated full length cDNAs in lymphoblastoid cells (Fairbrother et al. 1998). No significant difference in kinetic constants for chlorzoxazone hydroxylation between mutant and wild-type was observed (Fairbrother et al. 1998). The study demonstrates six novel CYP2E1 polymorphisms, including three upstream of the promoter, but with the possible exception of G(−35)T, none appeared to be of functional significance (Fairbrother et al. 1998).

Methods for Detecting CYP2E1 Polymorphisms

Protocols for the extraction of DNA from human blood and for genotyping for a number of common cytochrome P450 polymorphisms using either polymerase chain reaction (PCR)-restriction fragment length polymorphism or PCR-single-strand conformational polymorphism (SSCP) analysis have been established (Daly et al. 2006). General guidelines for performing amplification using PCR have been established with electrophoresis protocols for analysis of restriction digests of PCR products with agarose and polyacrylamide gels including the use of polyacrylamide-based gels for SSCP analysis (Daly et al. 2006). Protocols for the following specific isoforms and alleles are also discussed: CYP1A1 (*2B and *4 alleles), CYP2C8 (*3 and *4 alleles), CYP2C9 (*2, *3, and *11 alleles), CYP2C19 (*2 and *3 alleles), CYP2D6 (*3, *4, *5, and *6 alleles), CYP2E1 (*5A, *5B, and *6 alleles), and CYP3A5 (*3 allele) (Daly et al. 2006).

Samuel W. French

Polyunsaturated Fats Aggravate Alcohol Mediated Liver Injury: CYP2E1, a Key Player

Increasing the contents of linoleic acid or polyunsaturated fatty acids (PUFAs) in the diet with intragastric tube feeding model worsens the pathology where CYP2E1

is increased posttranslationally by high blood alcohol level (Morimoto et al. 1993). Thus, CYP2E1 induction plays a central role in the pathogenesis of ALD which is worsened by increase in dietary PUFA content (Morimoto et al. 1993). Further, fish oil diet, like corn oil, potentiates ethanol-induced liver injury which includes severe inflammation and focal fibrosis, fatty liver, and increased microsomal NADPH peroxidation and these pathological changes are related to CYP2E1 induction and the presence of polyunsaturated fatty acids in the diet (i.e., either n-6 or n-3) (Morimoto et al. 1994).

Fatty Acid Metabolism and Hepatic CYP2E1

Peroxisome proliferator-activated receptor (PPAR) and retinoid x receptor (RXR) play important roles in fatty acid metabolism (Wan et al. 1995). In contrast to the unchanged levels of RAR and RXR isoforms and catalase, the levels of PPAR and CYP2E1 mRNAs are down- and up-regulated by chronic dosage of ethanol in rat liver, respectively (Wan et al. 1995). The levels of CYP2E1 mRNAs correlate positively with blood alcohol levels (BAL) (Wan et al. 1995). The level of PPAR mRNA and the content of PUFA decreases in ethanol-fed rat livers and the authors conclude that decreased PPAR gene expression in ethanol-fed rats might result from a decrease in the content of polyunsaturated fatty acid in the liver (Wan et al. 1995).

The CYP2E1 inhibitors, diallyl sulfide (DAS) and phenethyl isothiocyanate (PIC) ameliorate both the ethanol-induced changes in fatty acids and the shift in succinic dehydrogenase in livers in rats fed ethanol intragastrically (Morimoto et al. 1995a). Rats fed ethanol without the CYP2E1 inhibitors have significantly greater hepatic total fatty acids and triglyceride fractions, significantlylower ratio of fatty acids with 20:4/18:2 composition and greater 18:1/18:0 fatty acids and many of these effects are inhibited with CYP2E1 inhibitors (Morimoto et al. 1995a). Thus, the changes in the fatty acid composition due to ethanol ingestion are due to CYP2E1-dependent lipid peroxidation and fatty acid metabolism.

Alcohol Mediated CYP2E1 Induction and Lipoperoxidative Liver Injury

In rats fed ethanol intragastrically, ingestion of the CYP2E1 inhibitor PIC decreases ethanol mediated increases in microsomal CYP2E1 protein levels and catalytic activity, and microsomal reduced form of nicotinamide-adenine dinucleotide (NADPH)-dependent lipid peroxidation (Morimoto et al. 1995b). Both DAS and PIC decrease CYP2E1 mRNA (Morimoto et al. 1995b). The lobular distribution of CYP2E1 in liver changes from the centrilobular to a diffuse pattern, with an increase in the periportal region when the CYP2E1 inhibitors are co-administered with ethanol, and this change correlates with the change in the distribution of fat in the lobule (Morimoto et al. 1995b). Thus, a link exists between CYP2E1 induction by ethanol, consequent lipid peroxidation and the early phase of ethanol-induced liver injury in this rat model.

CYP2E1 and Mallory Body Formation

The formation of Mallory body (MB), aggregates of proteins, principally cytokeratin is a complex phenomenon seen in chronic liver disease and CYP2E1 may

play a role in preventing MB formation since it is involved in the elimination of toxic drugs and chemicals (Bardag-Gorce et al. 2005). When mice are fed with diethyl-1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate (DDC), a suicide inhibitor of CYP2E1 for 10 weeks, Mallory bodies (MBs) develop in the liver at the end of this period (Bardag-Gorce et al. 2005). When DDC feeding is combined with CMZ (an efficient *in vivo* CYP2E1 inhibitor), more MBs form associated with decreased levels of hepatic CYP2E1 protein compared to DDC feeding alone (Bardag-Gorce et al. 2005). When CYP2E1 knockout mice and CYP2E1 over-expressed mice are fed with DDC or DDC and CMZ for 10 weeks, MB formation increases markedly in the liver of CYP2E1 knockout mice when fed with DDC only (Bardag-Gorce et al. 2005). CYP2E1 over-expressed mice show an increase in MB formation when the mice are fed with the combination of DDC and CMZ where the amount of CYP2E1 is reduced to levels seen in wild type mice (Bardag-Gorce et al. 2005). Thus, CYP2E1 inhibits MB formation by increasing the rate of elimination of DDC and/or its toxic intermediates (Bardag-Gorce et al. 2005).

cAMP: Its Protective Effects on Ethanol Mediated Liver Injury Through Decrease in CYP2E1 Synthesis, with Ubiquitin and Proteosomal Enzymes as Co-Players

The factors which govern CYP2E1 degradation and turnover include cAMP, ubiquitin, proteasomal enzymes and CYP2E1 mRNA (Gouillon et al. 1999). The cAMP treatment of rats fed ethanol ameliorates the increased liver fat storage and changes in the hepatic fatty acid composition (Gouillon et al. 1999). The amounts of ubiquitin and ubiquitin conjugates and level of ubiquitin mRNA are markedly reduced by ethanol treatment (Gouillon et al. 1999). cAMP ameliorates the inhibition of the proteasomal enzyme proteolysis caused by ethanol feeding (Gouillon et al. 1999). The ethanol-induced increase in the CYP2E1 protein is partially inhibited by cAMP treatment (Gouillon et al. 1999). cAMP treatment decreases CYP2E1 mRNA levels in both ethanol-fed and pair fed control rats (Gouillon et al. 1999). Thus, cAMP treatment partially protects the liver from ethanol-induced fatty liver by reducing CYP2E1 induction through cAMP's effects on CYP2E1 synthesis (Gouillon et al. 1999).

Chlormethiazole: An Effective *In Vivo* Inhibitor of Alcohol Inducible Hepatic CYP2E1 Activity

Administration of the sedative and hypnotic drug chlormethiazole to rats fed ethanol intragastrically for 2 months have significantly less liver injury (Gouillon et al. 2000). Chlormethiazole inhibits the increase in the ethanol-induced CYP2E1 activity *in vivo*, but does not affect the level of increased CYP2E1 apoprotein (Gouillon et al. 2000). Likewise, the reduction in proteasome proteolytic enzyme activity produced by ethanol feeding is blunted in chlormethiazole-fed rats (Gouillon et al. 2000). Thus, chlormethiazole treatment partially protects the liver from injury by inhibiting CYP2E1 activity *in vivo* (Gouillon et al. 2000).

Oxidative Protein Damage and Alcohol Induced Liver Injury: A Consequence of Inhibition of Proteasome Activity by CYP2E1

The proteasomal chymotrypsin-like activity is not reduced in ethanol-fed CYP2E1 knockout mice liver (Bardag-Gorce et al. 2000). The 26S proteasomal activity is decreased more by ethanol feeding than the 20S proteasomal fraction in ethanol-fed wild-type mice liver (Bardag-Gorce et al. 2000). Thus, CYP2E1 induction is responsible for the decrease in proteasome activity seen in the wild-type mice which leads to the accumulation of oxidized proteins in the ethanol treated wild type but not in the knockout mice which are increased as the result of free radicals generated by CYP2E1 metabolism of ethanol (Bardag-Gorce et al. 2000).

CYP2E1 and Proteasome: A Reciprocal Relationship

CYP2E1 degradation *in vivo* using a potent proteasome inhibitor PS-341 has been characterized (Bardag-Gorce et al. 2002). Ethanol is withdrawn at the same time that PS-341 is injected, 24 h before the rats are sacrificed (Bardag-Gorce et al. 2002). Ethanol treatment induces a threefold increase in CYP2E1 and inhibition of liver proteasomal chymotrypsin-like activity (ChT-L) (Bardag-Gorce et al. 2002). When ethanol is withdrawn, CYP2E1 decreases to control levels and the proteasomal ChT-L activity returns to control levels (Bardag-Gorce et al. 2002). In ethanol-withdrawn rats injected with PS-341, CYP2E1 remains at the induced level and the ChT-L activity is significantly inhibited before withdrawal (Bardag-Gorce et al. 2002). Thus, the proteasome is responsible for ethanol-induced CYP2E1 degradation *in vivo* (Bardag-Gorce et al. 2002).

Inhibition of Proteasome Activity by Alcohol Inducible CYP2E1: Involvement of HNE Adducts

In the arachidonic acid plus Fe-NTA plus ethanol treated E47 cells over expressing CYP2E1, CYP2E1 is induced accompanied by a higher level of ROS and carbonyl protein formation, and decreased proteasome activity (Bardag-Gorce et al. 2006). The decrease in proteasome activity in E47 cells is prevented by CYP2E1 inhibition with DAS (Bardag-Gorce et al. 2006). Down regulation of the proteasome subunit, as well as ubiquitin pathway proteins occurs in the ethanol-treated E47 cells (Bardag-Gorce et al. 2006). Increased 4-HNE adducts are observed in the E47 cells treated with ethanol (Bardag-Gorce et al. 2006). Furthermore, the immunoprecipitated 4-HNE modified proteins from these cells stain positive with antibodies to the proteasome subunit alpha 6 (Bardag-Gorce et al. 2006). Thus, ethanol mediated induction of CYP2E1 generates oxidative stress that is responsible for the decrease in proteasome activity (Bardag-Gorce et al. 2006).

Cytokeratin 8 and 18 are induced by ethanol treatment of E47 cells and polyubiquitinated forms of these proteins are found in the polyubiquitin smear (Bardag-Gorce et al. 2006). Cytokeratin aggresomes and Mallory body-like inclusions form in the ethanol-treated E47 cells, indicating that the ubiquitinated cytokeratins accumulate as a result of the inhibition of the proteasome by ethanol treatment when oxidation due to ethanol induced oxidative stress occurs (Bardag-Gorce et al. 2006).

Thus, ethanol causes Mallory body-like cytokeratin inclusions in transformed human liver cells over-expressing CYP2E1 *in vitro* (Bardag-Gorce et al. 2006).

II

M. Raj Lakshman

Ethanol Mediated Decrease in Gal Beta l, 4GlcNAc Alpha 2,6-Sialyltransferase Activity; Role of CYP2E1 Mediated Acetaldehyde Generation and Lipid Peroxidation

Gal beta l, 4GlcNAc alpha 2,6-sialyltransferase (2,6-ST) mediates the addition of alpha 2,6-linked sialic acid to glycoproteins in the Golgi compartment (Garige et al. 2005). Down-regulation of its gene and consequent impaired activity of 2,6-ST seems to be the major cause for the appearance of asialoconjugates in the blood of long-term alcoholics (Garige et al. 2005). Long-term ethanol feeding in rats causes marked decreases of 2,6-ST activity and mRNA level in liver that are due to the decreased stability of its mRNA (Garige et al. 2005). Similar actions of ethanol on 2,6-ST mRNA levels are observed in human liver HepG2 cells stably transfected with ethanol-inducible human CYP2E1 (CYP2E1 cells), or with high alcohol dehydrogenase (HAD cells) but not in wild-type HepG2 cells lacking CYP2E1 expression (Garige et al. 2005). However, incubation of wild-type cells with acetaldehyde causes a dramatic decrease in the 2,6-ST mRNA levels (Garige et al. 2005). Furthermore, exposure of CYP2E1 cells to 4-HNE strongly decreases 2,6-ST mRNA level (Garige et al. 2005). Thus, 2,6-ST gene is highly sensitive to ethanol action in human liver cells either *via* its oxidation product, acetaldehyde, or *via* ROS leading to the generation of a more reactive aldehyde such as 4-HNE (Garige et al. 2005). It is interesting to note that ethanol-inducible CYP2E1 is involved in all the above stated processes, thus suggesting the CYP2E1 mediated regulation of sialyl transferases and hence dysregulated sialyl transferase machinery in alcoholic condition.

Terrence M. Donohue

In Vitro CYP2E1 Mediated Metabolism of Ethanol Leads to Inhibition of Proteasome in Liver Cells

HepG2 cells are transfected with recombinant plasmids, one carrying the murine ADH gene and the other containing the gene encoding human CYP2E1 (Donohue et al. 2006). One of recombinant clones called VL-17A exhibits ADH and CYP2E1 specific activities comparable to those in isolated rat hepatocytes (Donohue et al. 2006). VL-17A cells oxidize ethanol and generate acetaldehyde, the levels of which depend upon the initial ethanol concentration (Donohue et al. 2006). Compared with unexposed VL-17A cells, ethanol exposure increases the cellular redox and causes cell toxicity (Donohue et al. 2006). Exposure of VL-17A cells to 100 mM ethanol significantly elevates caspase 3 activity, an indicator of apoptosis, but not in parental HepG2 cells (Donohue et al. 2006). Because ethanol consumption causes a decline in hepatic protein catabolism, the influence of ethanol exposure on proteasome activity in HepG2, VL-17A (CYP2E1(+)) and (ADH(+)), E-47 (CYP2E1(+)) and VA-13 (ADH(+)) cells has been investigated (Donohue et al. 2006). Exposure to 100 mM ethanol causes a 25% decline in the chymotrypsin-like activity of the

proteasome in VL-17A cells (Donohue et al. 2006). This inhibitory effect on the proteasome is blocked when ethanol metabolism is blocked by 4-MP (Donohue et al. 2006). Thus, recombinant VL-17A cells, which express both ADH and CYP2E1, exhibit hepatocyte-like characteristics in response to ethanol (Donohue et al. 2006). Furthermore, the metabolism of ethanol by these cells *via* ADH and CYP2E1 is sufficient to bring about an inhibition of proteasome activity that may lead to apoptotic cell death (Donohue et al. 2006).

Deficiency of SOD Is Associated with Decreased Ethanol Metabolism and Lack of Alcohol Mediated Inducibility of CYP2E1

Ethanol-fed SOD(−/−) mice exhibit lower ADH activity and lack of CYP2E1 inducibility, thereby causing decreased ethanol metabolism compared with wild-type mice (Curry-McCoy et al. 2010). Further, the other atypical responses to ethanol, including the absence of ethanol-induced steatosis and enhanced GSH levels, appear to be linked to enhanced oxidative stress due to lack of antioxidant enzyme capacity.

Natalia A. Osna/Terrence M. Donohue

Regulation of CYP2E1 and Proteasome Activity by IFNgamma

Interferon gamma (IFNgamma) initiates signal transduction, which alters the activities of CYP2E1 and iNOS, thereby producing ROS in VL-17A (ADH(+), CYP2E1(+)) and E-47 (CYP2E1(+)) cells (Osna et al. 2003). One of these oxidants, possibly peroxynitrite, may be directly involved in proteasome activation (Osna et al. 2003). Ethanol metabolism by VL-17A cells suppresses IFNgamma-mediated induction of proteasome activity, in part, by preventing STAT1 phosphorylation (Osna et al. 2003).

CYP2E1 and ADH Mediated Metabolism of Ethanol Leads to Reduction in STAT1 Phosphorylation

IFNgamma signal transduction is suppressed by ethanol in VL-17A cells (Osna et al. 2005). The mechanisms by which STAT1 phosphorylation is blocked by ethanol treatment in VL-17A cells have been evaluated (Osna et al. 2005). Reduction of STAT1 phosphorylation by 100 mM ethanol is prevented in the presence of 4MP, DAS, or uric acid, indicating that the oxidative products from ethanol metabolism are partly responsible for suppression of STAT1 phosphorylation (Osna et al. 2005). Ethanol exposure decreases STAT1 tyrosine phosphorylation, whereas serine phosphorylation on the protein is unchanged (Osna et al. 2005). These effects of ethanol are mimicked by the peroxynitrite (PN) donor, SIN-1, which also blocks tyrosine, but not serine phosphorylation, on STAT1 (Osna et al. 2005). Further, under conditions of ethanol-elicited oxidative stress, peroxynitrite (PN) prevents STAT1 phosphorylation by stabilization of SOCS1, and possibly by nitration of tyrosine residues in STAT1 protein cells in expressing either ADH (VA-13 cells) or CYP2E1 (E-47 cells) (Osna et al. 2005).

CYP2E1 Mediated Metabolism of Ethanol Leads to Oxidative Stress Which Suppresses HCV Mediated Low Oxidative Stress and Inhibits Proteasome Activity

Proteasome activity in inducible Hepatitis C core-positive 191–20 cells is 20% higher than that in core-negative cells and is enhanced threefold in CYP2E1-expressing L14

cells (Osna et al. 2008). Exposure of core-positive cells to glutathione ethyl ester, catalase, or the CYP2E1 inhibitor DAS partially reverses the elevation of proteasome activity in core-positive cells, whereas ethanol exposure suppresses proteasome activity (Osna et al. 2008). Thus, proteasome activity is up-regulated by low levels of HCV core-induced oxidative stress but down-regulated by high levels of ethanol-elicited stress (Osna et al. 2008).

Suppression of Activities of Antigen-Trimming Enzymes Through Ethanol Metabolism: Involvement of CYP2E1

Processing of peptides for antigen presentation is catalyzed by antigen-trimming enzymes, including the proteasome and leucine aminopeptidase (Osna et al. 2007). Oxidative stress suppresses proteasome function (Osna et al. 2007). Ethanol exposure to VL-17A cells increases CYP2E1 and decreases proteasome peptidase activities. The latter effect is prevented by treatment of cells with inhibitors, 4-MP and DAS. Ethanol metabolism suppresses activities of antigen-trimming enzymes, thereby decreasing the cleavage of C-extended and N-extended peptides in ethanol-metabolizing VL-17A cells or WIF-B cells over-expressing CYP2E1 (Osna et al. 2007). This defect may potentially result in decreased MHC class I-restricted antigen presentation on virally infected liver cells (Osna et al. 2007).

The proteasome is a major enzyme that cleaves proteins for antigen presentation (Osna et al. 2009). Cleaved peptides traffic to the cell surface, where they are presented in the context of major histocompatibility complex (MHC) class I (Osna et al. 2009). Recognition of these complexes by cytotoxic T lymphocytes is crucial for elimination of cells bearing "nonself" proteins (Osna et al. 2009). In primary mouse hepatocytes, even in the absence of IFNgamma, a similar decline in proteasome activity and antigen presentation after ethanol exposure has been observed (Osna et al. 2009). Proteasome function is directly suppressed by ethanol metabolism and indirectly by preventing the activating effects of IFNgamma (Osna et al. 2009). Ethanol-elicited reduction in proteasome activity contributes to the suppression of the ovalbumin peptide SIINFEKL-H2Kb presentation on the surface of mouse hepatocyte cell line (CYP2E1/ADH-transfected HepB5 cells) (Osna et al. 2009).

Suppression of Proteasome Activity by Impaired Methylation of Proteasome Subunits in CYP2E1 Expressing Liver Cells

The chymotrypsin-like proteasome activity in Huh7 cells stably transfected with CYP2E1 plasmid, hepatocytes isolated from mice or prepared liver cytosols and nuclear extracts or purified 20S proteasome under conditions that maintain or prevent protein methylation has been determined (Osna et al. 2010). Reduction of proteasome activity of hepatoma cell and hepatocytes by ethanol or tubercidin is prevented by simultaneous treatment with S-adenosylmethionine (SAM) (Osna et al. 2010). Moreover, the tubercidin-induced decline in proteasome activity occurs in both nuclear and cytosolic fractions (Osna et al. 2010). *In vitro* exposure of cell cytosolic fractions or highly purified 20S proteasome to low SAM:S-adenosylhomocysteine (SAH) ratios in the buffer also suppresses proteasome function, indicating that one or more methyltransferase(s) may be associated with

proteasomal subunits (Osna et al. 2010). Impaired methylation of proteasome subunits suppresses proteasome activity in liver cells indicating an additional, yet novel mechanism of proteasome activity regulation by ethanol (Osna et al. 2010).

Vasilis K. Vasiliou
Brain CYP2E1 and Ethanol Sensitivity
The role of CYP2E1 and catalase in ethanol metabolism and sensitivity, using transgenic knockout Cyp2e1(−/−) mice, acatalasemic (Cs/Cs) mice, and double mutant Cyp2e1(−/−)/Cs/Cs mice has been investigated (Vasiliou et al. 2006). Cs/Cs, Cyp2e1(−/−) and Cyp2e1(−/−)/Cs/Cs mice exhibit longer ethanol-induced sleep times, especially at higher ethanol doses (Vasiliou et al. 2006). This infers that there is less acetaldehyde produced in the brains of these animals and is in opposition to the idea that increased acetaldehyde increases the actions of ethanol (Vasiliou et al. 2006). The Cyp2e1(−/−) animals produce lower whole blood levels of acetaldehyde than wild-type controls; however, this difference is seen only at higher doses of ethanol (Vasiliou et al. 2006). The amount of acetaldehyde produced following the incubation of ethanol with liver and brain microsomes is greater in tissues derived from wild type mice with normal CYP2E1 expression than in those from Cyp2e1(−/−) mice (Vasiliou et al. 2006). Although the contribution of CYP2E1 and catalase in ethanol oxidation may be of little significance, these enzymes appear to play a significant role in ethanol sensitivity in the brain (Vasiliou et al. 2006).

H. Tsukamoto
Development of Intragastric Model for Alcohol Consumption and Liver Injury: Role of CYP2E1
Adult male Wistar rats intragastrically infused with a high-fat diet and ethanol or glucose for 16 weeks exhibit hepatic lipid peroxidation and fibrogenesis (Castillo et al. 1992). A 52% higher basal lipid peroxidation is observed in microsomes from alcohol-fed rats compared to the controls. Enhancement of lipid peroxidation induced by CC14 or iron is also accentuated twofold in the microsomes from the former rats (Castillo et al. 1992). All these increases in basal, CC14- induced, and iron-catalyzed lipid peroxidation in microsomes from alcohol-fed rats are completely blocked by addition of anti-CYP2E1 IgG but not by nonimmune control IgG (Castillo et al. 1992). These results are also complemented by a severalfold increase in the level of CYP2EI protein and catalytic activity (Castillo et al. 1992). Thus, CYP2E1 plays an important role in the enhanced microsomal lipid peroxidation in experimental alcoholic liver disease and to the increased vulnerability of the microsomes from alcohol-fed rats to CC14 and iron-induced oxidative stress.

E. Albano
CYP2E1 Mediated Ethanol Metabolism Leads to Generation of Hydroxyethyl Radicals and Formation of Auto-Antibodies
Treatment of rats with DAS and PIC significantly decreases the trapping of hydroxyethyl free radicals in liver microsomes incubated *in vitro* with ethanol (Albano et al. 1996). Furthermore, these inhibitors also greatly reduce the production of hydroxyethyl radical-derived epitopes detectable *in vivo* in the liver of ethanol-fed rats

(Albano et al. 1996). The actions of DAS and PIC on the formation of hydroxyethyl radicals parallel their inhibitory effect on lipid peroxidation (Albano et al. 1996). Thus, these results indicate a link between the induction of CYP2E1 by ethanol, the formation of hydroxyethyl radicals and the stimulation of lipid peroxidation (Albano et al. 1996). The pathological scores in the livers of rats fed with ethanol plus or minus DAS and PIC also correlate with levels of hydroxyethyl radical-derived epitopes (Albano et al. 1996). Rats fed intragastrically with ethanol develop antibodies and the formation of these antibodies is greatly reduced by DAS and PIC (Albano et al. 1996). Thus, CYP2E1 plays an important role in the generation of hydroxyethyl radicals during chronic alcohol feeding and that ethanol-derived free radicals might play a role in the onset of liver injury in this model of alcohol administration (Albano et al. 1996).

In alcoholic patients, oxidation of chlorzoxazone, a CYP2E1 probe increases consistently with CYP2E1 induction by ethanol (Dupont et al. 1998). IgG reacting with hydroxyethyl free radical-protein adducts significantly increases in alcoholics with induced CYP2E1 activity (Dupont et al. 1998). Further, chlorzoxazone oxidation is significantly lowered in alcoholics without clinical and biochemical signs of liver disease as compared to patients with alcoholic liver disease (Dupont et al. 1998). Thus, CYP2E1 activity greatly influences the formation of hydroxyethyl radicals in humans and plays a possible role in the development of alcoholic liver disease (Dupont et al. 1998).

CYP2E1-hydroxyethyl radical adducts are present in the plasma membranes of isolated rat hepatocytes incubated *in vitro* with ethanol or obtained from ethanol-treated animals (Clot et al. 1997). Further, cytotoxicity is observed in ethanol-treated hepatocytes incubated with immunoglobulin G from patients with ALD and normal human blood mononuclear cells (Clot et al. 1997). The observed cytotoxicity is blocked by preabsorbing the sera with human albumin complexed with hydroxyethyl radicals, which also eliminates the antibody reaction with the plasma membranes (Clot et al. 1997). Thus, hydroxyethyl radicals bound to CYP2E1 on hepatocyte plasma membranes can target immune reactions triggered by alcohol abuse (Clot et al. 1997).

Formation of CYP2E1 Autoantibodies in Hepatitis C Patients

Auto-antibodies against CYP2E1 are significantly increased in chronic hepatitis C (CHC) patients with and without alcohol consumption (Vidali et al. 2007). Further, anti-CYP2E1 auto-reactivity is significantly associated with the severity of periportal/periseptal interface hepatitis (Vidali et al. 2007). Further, anti-CYP2E1 IgG associated with CHC recognizes CYP2E1 exposed on the outer side of hepatocyte plasma membranes (Vidali et al. 2007). Thus, hepatitis C virus (HCV) infection favours the breaking of self-tolerance against CYP2E1 that might contribute to hepatocyte injury (Vidali et al. 2007).

The antigen specificity and the possible origin of circulating auto-antibodies targeting conformational antigens on CYP2E1 detectable in CHC patients have been characterized (Sutti et al. 2010a, b). In CHC patients, cross-reactivity between CYP2E1 and specific sequences in the NS5b protein of HCV promotes the

development of auto-antibodies targeting conformational epitopes on the CYP2E1 surface that might contribute to hepatic injury (Sutti et al. 2010a). The development of anti-CYP2E1 auto-antibodies targeting conformational CYP2E1 epitopes is associated with more severe liver damage-necro-inflammatory and fibrosis in CHC (Sutti et al. 2010b).

Post-orthotropic Liver Transplantation Recurrent Hepatitis C and CYP2E1

As autoimmune reactions are increasingly detected after orthotopic liver transplantation (OLT), the prevalence and significance of anti-CYP2E1 autoantibodies in patients with post-OLT recurrent hepatitis C has been studied (Rigamonti et al. 2009). IgG against recombinant human CYP2E1 above the control threshold is detected in sera collected immediately before and after OLT (Rigamonti et al. 2009). Although anti-CYP2E1 reactivity is not modified by OLT, the patients with persistently elevated anti-CYP2E1 IgG show significantly higher prevalence of recurrent hepatitis with severe necroinflammation and fibrosis than those persistently negative or positive only either before or after OLT (Rigamonti et al. 2009). Moreover, the probability of developing severe necroinflammation is significantly higher in persistently anti-CYP2E1-positive subjects (Rigamonti et al. 2009). The persistence of anti-CYP2E1 IgG, together with a history of acute cellular rejection and donor age >50 years, is an independent risk factor for developing recurrent hepatitis C with severe necroinflammation (Rigamonti et al. 2009). Thus, autoimmune reactions involving CYP2E1 might contribute to hepatic damage in a subgroup of transplanted patients with recurrent hepatitis C (Rigamonti et al. 2009).

Xiang-Dong Wang

CYP2E1 and Retinoic Acid Metabolism

Investigations into the role of CYP2E1 in the metabolism of retinoic acid (RA) show that the incubation of the liver microsomal fraction isolated from ethanol-treated rats with RA results in greater disappearance of RA and increased appearance of 18-hydroxy-RA and 4-oxo-RA compared with control rat liver microsomal fractions (Liu et al. 2001). The enhancement of RA catabolism by ethanol is inhibited by CYP2E1 inhibition in a dose-dependent fashion (Liu et al. 2001). Thus, ethanol-induced CYP2E1 plays a major role in the degradation of RA, which may provide a possible biochemical mechanism for chronic and excessive ethanol intake as a risk for both hepatic and extrahepatic cell proliferation and carcinogenesis (Liu et al. 2001).

The Antioxidant Lycopene Mediated CYP2E1 Induction

Lycopene supplementation at the higher dose significantly induces hepatic CYP2E1 protein, TNFalpha mRNA, and the incidence of inflammatory foci in the alcohol-fed rats (Veeramachaneni et al. 2008). Therefore, an interaction between chronic alcohol ingestion and lycopene supplementation occurs and suggests a need for caution among individuals consuming high amounts of both alcohol and lycopene (Veeramachaneni et al. 2008).

High Fat Diet Induced NASH and CYP2E1 Induction

The key histological features of NASH, including steatosis, inflammatory cell infiltration, and ballooning degeneration of hepatocytes, are induced in rats fed high-fat diet (HFD), with increased hepatic TNFalpha mRNA expression (Wang et al. 2008). HFD-fed rats have elevated lipid peroxidation products and CYP2E1 protein in the liver (Wang et al. 2008). HFD feeding increases both hepatic phosphorylated JNK and apoptotic parameters (Wang et al. 2008). Thus, the increased oxidative stress and its associated JNK activation as well as an imbalance of pro- and anti-apoptotic proteins in the Bcl-2 family all contribute to high hepatocyte apoptosis that may play an important role in the pathogenesis of NASH in this model, which is associated with induction of CYP2E1 (Wang et al. 2008).

A.A. Nanji

A Close Relationship Between CYP2E1 and Conjugated Dienes: *In Vivo* and *In Vitro* Studies

A significant correlation is observed between the level of CYP2E1 and the decrease in arachidonic acid (AA) in rats fed ethanol and corn oil intragastrically (Nanji et al. 1993). The decrease in AA also correlates with the increase in conjugated dienes (Nanji et al. 1993). Phospholipase C (PLC) and phospholipase A (PLA) activities are both significantly increased with correlated decline in AA in the corn oil plus dextrsose and ethanol plus corn oil fed rats (Nanji et al. 1993). The correlations observed between the decrease in microsomal AA and CYP2E1 induction and conjugated diene formation suggest that these processes may be interlinked especially in regard to generation of lipid peroxides that may play a role in alcoholic liver injury (Nanji et al. 1993).

The role of changes in CYP2E1 and lipid peroxidation in relation to development of severe liver injury in fish oil-ethanol-fed rats has been studied (Nanji et al. 1994). Greater pathological changes and significantly higher CYP2E1 protein levels are evident in the rats fed fish oil and ethanol than in the corn oil and ethanol group (Nanji et al. 1994). Higher levels of eicosapentaenoic and docosahexaenoic acids and lower levels of AA are detected in liver microsomes from rats fed fish oil (fish oil and ethanol/fish oil and dextrose) compared with corn oil and ethanol group (Nanji et al. 1994). A significant correlation is obtained between CYP2E1 protein and conjugated diene levels (Nanji et al. 1994). The markedly increased CYP2E1 induction and lipid peroxidation in the fish oil and ethanol group provides one possible explanation for the greater severity of liver injury in this group (Nanji et al. 1994).

D.J. Waxman

Metabolism of Organic Solvents and Development of Assay for the Catalytic Activity of CYP2E1

The ability of CYP2E1 to catalyze the oxidative metabolism of many solvents and other small organic molecules has been utilized in developing a spectrophotometric method for determination of CYP2E1 activity by monitoring the formation of p-nitrocatechol from p-nitrophenol by cDNA-expressed CYP2E1 or isolated liver microsomes (Chang et al. 2006). The mehod holds importance in assessing the content of catalytically active CYP2E1 through an enzymatic assay.

D.R. Koop

CYP2E1 and Kupffer Cells

CYP2E1 is immunochemically detectable at low levels in Kupffer cell homogenates from untreated rats and is greatly induced by acetone-treatment (Koop et al. 1991). The presence of CYP2E1 in Kupffer cells from untreated rats is confirmed by inhibition of benzene hydroxylation with anti-CYP2E1 immunoglobulin G (Koop et al. 1991). Benzene hydroxylase activity is induced to a similar extent and similar specific activity is observed in Kupffer cells and hepatocytes isolated from acetone-treated rats (Koop et al. 1991). Further, the induction in benzene hydroxylation in Kupffer cells is inhibited by anti-P4502E1 antibody (Koop et al. 1991). The presence and inducibility of CYP2E1 in Kupffer cells suggests that, under conditions where CYP2E1 is induced, Kupffer cell-generated metabolites may contribute to Kupffer cell toxicity, as well as general hepatic injury (Koop et al. 1991).

Differential Degradation of Hepatic CYP2E1 Protein and the Central Role of Ubiquitin

Different modes of inactivation of hepatic CYP2E1 by different agents has been investigated (Tierney et al. 1992). Carbon tetrachloride (CCl4) is metabolized by CYP2E1 to trichloromethyl free radicals which exert hepatotoxic effects. Treatment of mice with CCl4 at the point of maximal induction of CYP2E1 after a single oral dose of acetone results in the complete loss of CYP2E1-dependent p-nitrophenol hydroxylation and a 75% loss of immunochemically detectable protein within 1 h of administration (Tierney et al. 1992).

Further, treatment with the CYP inhibitor 1-aminobenzotriazole at the point of maximal induction causes a complete loss of CYP2E1-dependent p-nitrophenol hydroxylation but only a 12% loss of immunochemically detectable protein 1 h after administration (Tierney et al. 1992).

Treatment of mice with CYP2E1 inhibitor 3-amino-1,2,4-triazole causes a rapid loss of both catalytic activity and microsomal p-nitrophenol hydroxylase activity (Tierney et al. 1992). However, unlike CCl4 treatment, the activity and enzyme level rebounds 5 and 9 h after treatment (Tierney et al. 1992).

The CYP2E1 ligand, 4-MP, administered at the point of maximal induction maintains the acetone-induced catalytic and immunochemical level of CYP2E1 (Tierney et al. 1992). Thus, differentially modified forms of CYP2E1 show a characteristic susceptibility to degradation (Tierney et al. 1992). While there are many potential pathways for protein degradation, the loss of CYP2E1 is associated with increased formation of high molecular weight microsomal ubiquitin conjugates (Tierney et al. 1992). The formation of ubiquitin-conjugated microsomal protein which correlates with CYP2E1 loss suggests that ubiquitination may represent a proteolytic signal for the rapid and selective proteolysis of certain labilized conformations of CYP2E1 from the endoplasmic reticulum (Tierney et al. 1992).

Regulation of CYP2E1 by the Broad-Spectrum Antibiotic Tetracycline: Induction, Stability and Degradation

A tetracycline (Tc)-controlled gene expression system comprised of subcloned rabbit CYP2E1 cDNA that quantitatively controls gene expression in eukaryotic

cells has been used to express CYP2E1 in HeLa cells in culture (Huan and Koop 1999). A time-dependent induction and more than 100-fold induction of CYP2E1 is observed after removal of Tc (Huan and Koop 1999). At maximal levels of expression, the enzyme catalyzes the formation of much higher amounts of 6-hydroxychlorzoxazone (Huan and Koop 1999). In addition, the level of the enzyme could be modulated by the concentration of Tc in the media (Huan and Koop 1999). In the absence of Tc, exposure of cells to NDMA causes a significant dose-dependent decrease in cell viability (Huan and Koop 1999). A rapid turnover of CYP2E1 with a half-life of 3.9 h is observed 72 h after removal of Tc (Huan and Koop 1999). Addition of the ligand, 4-MP, and the suicide substrate, 1-aminobenzotrizole, decreases the degradation of CYP2E1 (Huan and Koop 1999). This cell line offers a useful system to examine the role of CYP2E1 in the cytotoxicity of xenobiotics and to investigate post-translational regulation of the enzyme (Huan and Koop 1999).

The degradation of ethanol-inducible CYP2E1 expressed in tetracycline (Tc)-inducible HeLa cell linehas been characterized (Huan et al. 2004). A half-life of 3.8 h is observed for CYP2E1 (Huan et al. 2004). Lactacystin and other selective proteasome inhibitors including N-benzyloxycarbonyl-leucyl-leucyl-leucinal (MG132) and N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-norvalinal (MG115) significantly inhibit CYP2E1 degradation (Huan et al. 2004). Further, the turnover of CYP2E1 is slightly inhibited by calpain inhibitors (Huan et al. 2004).

R.F. Novak

Peroxisome Proliferation and Induction of CYP2E1: The Dynamics Involved

Treatment of primary cultured rat hepatocytes with the peroxisome proliferator and anti-hyperlipidemic drug ciprofibrate (CIPRO) for the time period of 24 h causes a significant increase in CYP2E1 mRNA at a wide range of dosage (30–300 μM) of CIPRO (Zangar et al. 1995). CYP2E1 mRNA levels are maximally elevated between two and threefold at both 24 and 48 h and return to basal levels by 72 h with 30 μM CIPRO (Zangar et al. 1995).

Insulin Mediated Regulation of CYP2E1

Insulin itself, in the absence of other diabetes-induced metabolic or hormonal alterations, affects CYP2E1 expression in primary cultured rat hepatocytes (Woodcroft and Novak 1997). Decreasing the concentration of insulin in the culture medium provides a method by which CYP2E1 levels can be increased in primary cultured hepatocytes to facilitate mechanistic studies on the regulation of CYP2E1 expression (Woodcroft and Novak 1997).

The signaling pathways involved in insulin and glucagon regulation of CYP2E1 expression are examined in primary cultured rat hepatocytes (Woodcroft and Novak 1999). Glucagon (100 nM) opposes the effects of insulin (1 nM) on CYP2E1 mRNA expression and conversely, insulin blocks the effects of glucagon (Woodcroft and Novak 1999). Thus, the regulation of CYP2E1 expression occurs via mutually antagonistic signaling pathways involving insulin and glucagon (Woodcroft and Novak 1999).

The ketone body and insulin mediated regulation of CYP2E1 expression has been studied in primary cultured rat hepatocytes (Woodcroft et al. 2002). The ketone bodies-hydroxybutyrate and acetoacetate (AC), alone or in combination, either fail to affect or decrease CYP2E1 mRNA levels by up to 90% relative to untreated hepatocytes (Woodcroft et al. 2002). Insulin produces a concentration-dependent decrease in CYP2E1 mRNA levels, and transcriptional and posttranscriptional mechanisms are involved in the insulin-mediated regulation of CYP2E1 and implicate phosphatidylinositol 3-kinase, p70 S6 kinase, and Src kinase in mediating these effects (Woodcroft et al. 2002).

Regulation of CYP2E1 by Ketone Bodies

The ketone body acetoacetate decreases CYP2E1 mRNA expression through inhibition of gene transcription while simultaneously elevating CYP2E1 protein levels through increased translation and decreased protein degradation in primary cultured rat hepatocytes (Abdelmegeed et al. 2005).

Structural Elucidation of CYP2E1 mRNA for Understanding Its Translational Efficiency

Regulation of hepatic CYP2E1 by xenobiotic or physiological stimuli is largely mediated through post-transcriptional mechanisms that may include altered CYP2E1 mRNA translation and/or protein degradation (Kocarek et al. 2000). Approximately 30–40% of CYP2E1 mRNA is not associated with polysomes and therefore not actively engaged in protein synthesis (Kocarek et al. 2000). To examine the CYP2E1 mRNA molecule for sequences that might affect its translational efficiency, a series of CYP2E1 recombinant RNAs (rcRNAs) with modified 5′ or 3′ untranslated regions (UTRs) is translated *in vitro* using the rabbit reticulocyte lysate system (Kocarek et al. 2000). It is concluded that the secondary structure in the 5′ UTR of CYP2E1 mRNA is at least partially responsible for the inefficient translation of this mRNA (Kocarek et al. 2000). The poly(A) tail and sequences contained within the 3′ UTR appear to be important for protecting CYP2E1 mRNA from RNase activity associated with the translation machinery (Kocarek et al. 2000).

Renal and Hepatic Injury Mediated by Hydrazine Derived Antidepressants: Role of CYP2E1

The hepatic and renal toxicity associated with hydrazine treatment has been linked to free radical damage resulting from oxidative metabolism by CYP2E1 (Runge-Morris et al. 1996). Treatment with hydrazine or the therapeutic hydrazine phenelzine significantly increases the expression of rat renal CYP2E1 protein through a posttranscriptional mechanism (Runge-Morris et al. 1996).

K. Miyamoto

Obesity and CYP2E1

The induction of CYP2E1 in obese Zucker rats and its effect on the disposition kinetics of chlorzoxazone (CZX) has been investigated (Khemawoot et al. 2007a). In Zucker rats fed a normal diet (ND), normal Zucker rats fed a high-fat diet (HF), and genetically obese Zucker rats fed a normal diet (OB) and administered CZX, the values of the area under the plasma concentration-time curve from 0 to infinity

(AUC(infinity)) of CZX are in the order of ND > HF > OB rats (Khemawoot et al. 2007a). The AUC (infinity) values of total 6-hydroxychlorzoxazone (6OHCZX-T), which is considered to be a CYP2E1 metabolic marker, are in the opposite order (Khemawoot et al. 2007a). The values of the AUC (infinity) ratio (6OHCZX-T/CZX) in ND, HF and OB rats are approximately 0.2, 0.3 and 0.4, respectively (Khemawoot et al. 2007a). Induction of CYP2E1 protein is greater in both liver and fat of OB rats than in those of HF rats (Khemawoot et al. 2007a). Microsomal activity of CYP2E1 in liver and fat is also in the order of OB > HF > NM rats (Khemawoot et al. 2007a). Thus,CYP2E1 may be induced in liver and fat of obese patients, thereby potentially altering the disposition kinetics of not only CZX, but also other lipophilic drugs metabolized by CYP2E1 (Khemawoot et al. 2007a).

Circadian Rhythm of CYP2E1 and Its Effects on Drug Metabolism

Microsomal CYP2E1 shows a substantial circadian variation in rats, and this is associated with a decrease of chlorzoxazone half life, and an increase of 6-hydroxychlorzoxazone production (Khemawoot et al. 2007b). Therefore, the temporal variations of therapeutic response and toxicological effects may have to be taken into consideration for other xenobiotics that are predominantly metabolized by CYP2E1, particularly those with a short half-life (Khemawoot et al. 2007b).

B.J. Song

Translational Induction of CYP2E1 by the Anti-tuberculosis Drug Isoniazid

The induction of CYP2E1 by isoniazid is not accompanied by an increased level of CYP2E1 mRNA, and is completely blocked by pretreatment with cycloheximide or sodium fluoride, inhibitors of mRNA translation (Park et al. 1993). CYP2E1 induction by isoniazid is due to activation of CYP2E1 mRNA translation and the hydrazide group on the pyridine ring of isoniazid is important both in the selective induction of CYP2E1 and for magnitude of effect (Park et al. 1993).

Ethanol Mediated Protein Stabilization of CYP2E1

Chronic ethanol administration to rats induces hepatic and extra-hepatic CYP2E1 protein, which reverts to normal levels 12 h after ethanol withdrawal, unaccompanied by changes in CYP2E1 mRNA, suggesting ethanol mediated post-translational regulation of CYP2E1 (Roberts et al. 1994). CYP2E1 possesses a half-life of 6 h or less in the liver and is rapidly degraded following the removal of ethanol (Roberts et al. 1994). Similar patterns of CYP2E1 turnover are also observed in other tissues such as kidney, brain and upper gastro-intestinal tract, suggestive of a similar mode of regulation (Roberts et al. 1994). Thus, ethanol mediated induction of CYP2E1 is through a post translational mechanism involving protein stabilization.

Acetaldehyde-Protein Adduct Formation by Ethanol Inducible Hepatic CYP2E1: A Mechanism for Alcohol Induced Hepatotoxicity

Immunoblot analysis from livers isolated from rats fed an isocaloric control or alcohol liquid diet with and without cotreatment of YH439, an inhibitor of CYP2E1 gene transcription reveals that the acetaldehyde-protein adduct, absent in the pair-fed control, is evident in alcohol-fed rats but is markedly reduced by YH439 treatment (Jeong et al. 2000). The 37-kDa adduct is predominantly localized in the pericentral

region of the liver where CYP2E1 protein is mainly expressed (Jeong et al. 2000). This staining disappears in the pericentral region after YH439 treatment (Jeong et al. 2000). However, the level of the 37-kDa protein adduct positively correlates with the hepatic content of CYP2E1 (Jeong et al. 2000). The 37-kDa adduct could be produced by CYP2E1-mediated ethanol metabolism in addition to ADH-dependent formation (Jeong et al. 2000).

CYP2E1 Degradation: A Rapid Process

Ethanol-inducible CYP2E1 activity is measured using the enzyme markers N-nitrosodimethylamine demethylase (NDMAd), p-nitrophenol hydroxylase (PNPH) and aniline hydroxylase (AH) in rat liver microsomes (Roberts et al. 1995). Activities are found to be induced significantly after chronic ethanol feeding using all three assays (Roberts et al. 1995). Upon ethanol withdrawal, all three activities drop markedly, with NDMAd and PNPH at control values at 24 h and all subsequent time points (Roberts et al. 1995). AH activity remains threefold higher than controls at 24–72 h (Roberts et al. 1995). Immunoreactive CYP2E1 returns to control at 24 h, consonant with NDMAd and PNPH activities (Roberts et al. 1995). The prolonged induction of AH activity following ethanol withdrawal indicates that it is not a specific marker of CYP2E1-catalyzed reactions (Roberts et al. 1995). Collectively, these data are suggestive of a rapid mechanism of CYP2E1 degradation in the rat liver (Roberts et al. 1995).

Alcohol Inducible CYP2E1 Mediated Oxidative Protein Damage: A Proteomics Approach

A targeted proteomics approach utilizing biotin-N-maleimide (biotin-NM) as a specific probe to label oxidized cysteinyl residues has been employed to investigate which mitochondrial proteins are modified during and after alcohol exposure (Kim et al. 2006). Human hepatoma HepG2 cells with transduced CYP2E1 (E47 cells) are used as a model to generate ROS through CYP2E1-mediated ethanol metabolism (Kim et al. 2006). Heat shock protein 60, protein disulfide isomerase, mitochondrial aldehyde dehydrogenases, prohibitin, and other proteins are oxidized after alcohol exposure (Kim et al. 2006). This method is also used to identify oxidized mitochondrial proteins in the alcohol-fed mouse liver (Kim et al. 2006). Exposure to ethanol causes oxidation of various mitochondrial proteins that may negatively affect their function and contribute to alcohol-induced mitochondrial dysfunction and cellular injury (Kim et al. 2006).

Fasting Mediated Pretranslational Induction of CYP2E1 in Blood Lymphocytes: Its Implications for Development of Biomarkers for Pathophysiological Conditions

CYP2E1 in fresh lymphocytes is elevated about fivefold by fasting, comparable to the induction observed in cultured lymphocytes (Soh et al. 1996). This induction is accompanied by increased level of CYP2E1 mRNA (Soh et al. 1996). CYP2E1 in fresh lymphocytes is pretranslationally induced by fasting, in parallel to the hepatic enzyme, and thus the measurement of CYP2E1 in the lymphocyte homogenate may be useful to estimate the hepatic CYP2E1 level in a relatively non-invasive manner (Soh et al. 1996).

Acetaminophen Induced Cytotoxicity: Mechanisms Involved and the Role of CYP2E1

Neurotoxic Actions of the Drug Acetaminophen: Involvement of CYP2E1 Mediated JNK Activation

Acetaminophen (AAP, 4-hydroxyacetanilide), a widely used analgesic drug, causes time- and concentration-dependent apoptosis and DNA fragmentation of C6 glioma cells through activation of c-Jun N-terminal protein kinase (JNK) cell signaling pathway (Bae et al. 2001). Pretreatment with YH439, an inhibitor of CYP2E1 gene transcription, markedly reduces CYP2E1 mRNA, protein content, and activity, as well as the rate of AAP-induced JNK activation and cell death (Bae et al. 2001), suggesting the crucial role of CYP2E1 in acetaminophen mediated cytotoxicity.

Acetaminophen Induced Neurotoxicity and Decreased Pro-apoptotic Proteins p53 and p21: Role of CYP2E1

Acetaminophen or its reactive metabolite(s) can directly reduce the pro-apoptotic p53 content through mdm2-mediated ubiquitin conjugation, despite phosphorylation of p53 at its N terminus (Lee et al. 2006a). The inhibition of CYP2E1 significantly lowers the CYP2E1 enzyme activity and the rate of APAP-induced cell death while it prevents the reduction of p53 and p21 in C6 glioma cells (Lee et al. 2006a).

The Essential Role of CYP2E1 in 3-NT Adduct Formation and Protein Degradation, Independent of NOSin Acetaminophen Mediated Cytotoxicity

CYP2E1 plays a key role in nitrotyrosine protein adducts formation, ubiquitin-mediated protein degradation, and liver damage, which is independent of NOS, and decreased levels of many proteins such as cytosolic superoxide dismutase (SOD1), in the wild-type mice when compared with Cyp2e1-null mice which likely contribute to APAP-related toxicity (Abdelmegeed et al. 2010).

Induction of CYP2E1 in PPAR Alpha Null Mice: A Possible Role in NASH Development

Increased steatosis, oxidative stress, inflammation, hepatocyte apoptosis accompanied by elevated levels of ethanol-inducible CYP2E1 and TNFα observed in PPAR alpha-null mice fed a HFD, suggests that inhibition of PPARα functions may increase susceptibility to high fat-induced NASH and CYP2E1 may be associated with the process (Abdelmegeed et al. 2011).

III

M.J. Ronis

Increased CYP2E1 Mediated Oxidative Stress due to Synergistic Hepatotoxicity of Alcohol and Undernutrition

A combination of undernutrition and intragastric administration of ethanol to rats increases the induction of hepatic CYP2E1 and CYP4A1 mRNA, apoprotein, and activities. This is accompanied by increased oxidative stress (Baumgardner et al. 2007). The development of alcohol-induced liver pathology at 154 kcal·kg(−3/4)·day(−1) is accompanied by decreased expression of fatty acid synthesis genes and increased expression of PPAR-alpha-regulated fatty acid degradation pathways and greater

hepatocyte proliferation (Baumgardner et al. 2007). Undernutrition does not exacerbate alcoholic steatohepatitis despite additional oxidative stress produced by an increased induction of CYP2E1 and CYP4A1 (Baumgardner et al. 2007). However, enhanced ethanol-induced cellular proliferation, perhaps as a result of enhanced PPAR-alpha signaling, may contribute to an increased risk of hepatocellular carcinoma in undernourished alcoholics (Baumgardner et al. 2007). Thus, undernutrition and ethanol consumption both can aggravate CYP2E1 mediated oxidative stress.

Induction of CYP2E1 in Total Enteral Nutrition Based NASH Model
Total enteral nutrition (TEN) has been used to moderately over-feed rats with high-polyunsaturated fat diets to develop a model NASH (Baumgardner et al. 2008). The development of steatosis as indicated through increased expression of CD36 and l-fabp mRNA may be associated with increased fatty acid transport and the intragastric infusion of a high-polyunsaturated fat diet at a caloric level of 17% excess total calories results in pathology similar to clinical NASH which is accompanied by induction in hepatic CYP2E1 (Baumgardner et al. 2008).

Ethanol Mediated Dysregulation of Vitamin D Homeostasis: A Key Role of Ethanol Inducible CYP2E1 Mediated Oxidative Stress
Bone loss resulting from chronic ethanol abuse is frequently accompanied by altered vitamin D3 homeostasis (Shankar et al. 2008). Intragastric administration of ethanol to rats and ethanol exposure of *in vitro* in primary cultures of rat renal proximal tubule cells (RPTCs) and in NRK-52E cells reduces circulating 1,25-dihydroxycholecalciferol (1,25 (OH)2 D3) concentrations as the result of CYP24A1(1,25 (OH)2 D3-24-hydroxylase) induction (Shankar et al. 2008). CYP24A1 induction is mediated *via* mitogen activated protein kinase (MAPK) activation resulting from renal oxidative stress produced by local metabolism of EtOH *via* CYP2E1 and antidiuretic hormone-1 (Shankar et al. 2008).

L.A. Videla
NAFLD and CYP2E1: A Close Association
Oxidative stress is developed in the liver of NAFLD patients with steatosis and is exacerbated further in patients with steatohepatitis, which is associated with CYP2E1 induction (Videla et al. 2004). Oxidative stress leads to substantial protein oxidation followed by proteolysis of the modified proteins, which may explain the co-existence of a diminished antioxidant capacity and protein oxidation in the liver of patients with steatohepatitis (Videla et al. 2004). Thus, hepatic CYP2E1 induction is associated with oxidative stress in NAFLD patients.

Pesticide or Hormone Mediated Regulation of CYP2E1
The pesticide gamma-hexachlorocyclohexane (HCCH) or the hormone L-3,3,5-triiodothyronine (T3) enhance the expression and activity of CYP2E1 and that of NADPH-cytochrome P450 reductase in rat liver, regardless of the changes in total cytochrome P450 content, representing major contributory mechanisms to microsomal NADPH-dependent O2.-generation (Fernández et al. 2003). Further, the hepatic activity of the antioxidant enzyme SOD is decreased, reflecting the increased oxidative stress in the cellular environment (Fernández et al. 2003).

Induction of CYP2E1 in NAFLD Patients

The hepatic CYP2E1 content and the CLZ hydroxylation of obese patients with steatosis and particularly, with steatohepatitis are significantly higher than controls and correlates positively with both the severity of the liver damage (Orellana et al. 2006). Thus, CYP2E1 is involved in the mechanism of liver injury found in obese NAFLD patients (Orellana et al. 2006). Also, the correlation between liver CYP2E1 content and *in vivo* CLZ hydroxylation would validate the latter as a reliable indicator of liver injury in NAFLD, thus providing a simple and non-invasive method to study these patients (Orellana et al. 2006).

Higher Incidence of RsallPstl Polymorphisms in NASH Patients: Genetic Aspects of CYP2E1 Linked to Liver Injury

The c2 rare allele of RsallPstl polymorphisms but no C allele of Dral polymorphism is positively associated with chlorzoxazone hydroxylation, which in turn is correlated with liver CYP2E1 content in female obese NASH patients (Varela et al. 2008). Further, c2 allele is positively associated with liver injury in NASH (Varela et al. 2008). This allele may determine a higher transcriptional activity of the gene, with consequent enhancement in pro-oxidant activity of CYP2E1 thus leading to liver toxicity (Varela et al. 2008).

J.L. Raucy

Acetone Mediated Increased *De Novo* Synthesis of Hepatic CYP2E1 Protein: A Mechanism for Xenobiotic Mediated Regulation of CYP2E1

Acetone increases CYP2E1 protein levels in cultured rabbit hepatocytes by stimulating its rate of *de novo* synthesis (Kraner et al. 1993). Since this increase in CYP2E1 synthesis stems, at least in part, from the acetone-mediated enhancement of hepatocyte CYP2E1 mRNA content and is inhibited by the transcriptional inhibitor, alpha-amanitin, transcriptional activation of the rabbit CYP2E1 gene is apparently involved in the induction of CYP2E1 protein by acetone (Kraner et al. 1993).

Presence of CYP2E1 in Fetal Liver and Its Inducibility by Ethanol and Clofibrate: Its Implications for the Deleterious Effects of Xenobiotics on the Development of Foetus

CYP2E1 protein which exhibits a slightly lower molecular weight than that found in adult liver samples has been detected in liver samples from fetuses ranging in gestational age from 16 to 24 weeks (Carpenter et al. 1996). Embryonic CYP2E1 expression is further confirmed by the reverse transcriptase reaction with RNA from a 19-week gestational fetal liver used as template (Carpenter et al. 1996). The rate of ethanol oxidation to acetaldehyde, in human fetal microsomes is 12–27% of those exhibited by adult liver microsomes (Carpenter et al. 1996). Immunoinhibition studies with CYP2E1 antibodies reveal that the corresponding antigen is the major catalyst of this reaction in both fetal and adult tissues (Carpenter et al. 1996). Treatment of primary fetal hepatocyte cultures with either ethanol or clofibrate demonstrates a twofold increase in CYP2E1 levels compared with untreated cells (Carpenter et al. 1996). Thus, CYP2E1 is present in human fetal liver, that the enzyme is functionally similar to CYP2E1 from adults, and that fetal hepatocyte CYP2E1 is inducible in culture by xenobiotics, including ethanol (Carpenter et al. 1996).

Fetal Hepatic CYP2E1: Analogies with Adult Liver CYP2E1

The presence of CYP2E1 protein and gene in fetal liver, placenta and maternal brainof rat dams fed a liquid diet throughout gestation containing 5% ethanol has been shown (Carpenter et al. 1997). Maternal and fetal liver of dams fed ethanol display a 1.4- and 2.4-fold increase in CYP2E1 protein, respectively, compared with microsomes from pair-fed controls (Carpenter et al. 1997). The rate of chlorzoxazone metabolism by maternal hepatic microsomes from ethanol fed dams is 2.6-fold greater than that of controls (Carpenter et al. 1997). Conversely, a negligible increase is observed in the rate of metabolism by hepatic microsomes from ethanol-exposed fetuses compared with pair-fed animals (Carpenter et al. 1997). These same fetal samples exhibit greater rates of nitrosodimethylamine demethylation activity (1.5-fold) compared with microsomes from control animals (Carpenter et al. 1997). However, this increase is not as great as expected considering the 2.4-fold increase in CYP2E1 protein. Collectively, fetuses exposed to a 5% ethanol diet throughout gestation exhibit transplacental induction of a hepatic CYP2E1 that may possess different catalytic properties from the analogous adult enzyme (Carpenter et al. 1997).

Ethanol Mediated Induction of Human Lymphocyte CYP2E1

The *in vivo* chlorzoxazone metabolism and pharmacokinetic parameters with CYP2E1 expression in blood has been investigated (Raucy et al. 1997). Human subjects, who consume alcohol more frequently, exhibit higher rates of chlorzoxazone metabolism (Raucy et al. 1997). Indeed, a correlation is obtained when scores are compared with the pharmacokinetic parameter AUC for chlorzoxazone (Raucy et al. 1997). Lymphocyte microsomes reveal the presence of CYP2E1 mRNA (Raucy et al. 1997). CYP2E1 protein is 2.3-fold higher in alcoholics than in control subjects (Raucy et al. 1997). This increase in lymphocyte CYP2E1 content in alcoholic subjects coincides with a 2.1-fold increase in chlorzoxazone clearance and a twofold decrease in the AUC for chlorzoxazone (Raucy et al. 1997). Importantly, a correlation is observed between CYP2E1 content in lymphocytes and chlorzoxazone clearance rates (Raucy et al. 1997). Thus, monitoring lymphocyte CYP2E1 expression may provide a substitute for estimating hepatic activity of this P450 (Raucy et al. 1997).

Transcriptional Regulation of CYP2E1 in Human Liver by Xenobiotics (Ethanol), Drugs (Clofibrate) and Fatty Acids (Palmitate)

Primary cultures of human hepatocytes have been used to determine if certain xenochemicals could regulate CYP2E1 and CYP4A11 (Raucy et al. 2004). Ethanol significantly increases expression of CYP2E1 mRNA, but does not alter CYP4A11 mRNA accumulation (Raucy et al. 2004). In contrast, hepatocytes exposed to ethanol exhibit only a slight elevation in CYP2E1 protein and a negligible effect on CYP4A11 protein (Raucy et al. 2004). Clofibrate significantly enhances both CYP4A11 mRNA and protein, but does not increase CYP2E1 (Raucy et al. 2004). Palmitic acid significantly increases CYP2E1 mRNA (Raucy et al. 2004). The agents that enhance CYP2E1 and CYP4A11 at the transcription level have been identified and suggest that fatty acids may represent a similar mode of regulation for these P450 enzymes (Raucy et al. 2004). The lack of induction of CYP2E1 protein by ethanol in human hepatocytes indicates that for certain CYP enzymes, isolated hepatocytes may not be an adequate tool for predicting *in vivo* responses (Raucy et al. 2004).

L. Corcos

Presence and Xenobiotic Mediated Inducibility of CYP2E1 in Rat Liver Epithelial Cells

Rat liver epithelial cells (RLECs) isolated from the livers of normal 10 days old rats are largely used in co-culture with primary hepatocytes (Lerche et al. 1996). All of the different preparations of RLECs express a high level of CYP2E1 mRNA (Lerche et al. 1996). The presence of the CYP2E1 apoprotein in microsomes of RLECs, together with chlorzoxazone 6-hydroxylation, has been observed (Lerche et al. 1996). In addition, acetone treatment of these cells results in an increase in both CYP2E1 apoprotein and chlorzoxazone 6-hydroxylation activity (Lerche et al. 1996). Finally, RLECs are susceptible to N-methyl formamide- and diethylnitrosamine-induced toxicity, suggesting metabolic activation by CYP2E1 (Lerche et al. 1996). Thus, RLECs may cooperate with hepatocytes to CYP2E1-mediated metabolism in the co-culture model (Lerche et al. 1996). In addition, transfection experiments with a CYP2E1 promoter construct, in which the proximal 539 bp containing the binding site for hepatocyte nuclear factor 1 (HNF1) alpha are inserted upstream of the chloramphenicol acetyl transferase gene, demonstrates a strong induction upon co-transfection with an HNF1alpha expression plasmid (Lerche et al. 1996). Thus, RLECs provide a useful tool for studying metabolism and cytotoxicity of CYP2E1 substrates in the absence of other expressed CYPs, and for analyzing CYP2E1 promoter function (Lerche et al. 1996).

Regulation of Human Liver CYP2E1 by Interleukin 4: Crosstalk Between CYP2E1 and Immune System

Interleukin 4 (IL-4) coordinately induces CYP2E1 transcription, mRNA and apoprotein levels in human hepatoma B16A2 cellsin a PKC-dependent manner, potentially through the activity of the PKCzeta isoform as PKC inhibitors (H7 and calphostin C) strongly block any induction of the gene, as well as the IL-4-dependent translocation of PKCS (Lagadic-Gossmann et al. 2000). The study suggests a close relationship between CYP2E1 and interleukins.

Y. Funae

CYP1A2, a Co-player with CYP2E1 in Human MEOS

The ethanol oxidization activity in human hepatic microsomes and multiple forms of human hepatic CYPs expressed in B-lymphoblastoid cells has been examined to assess the contribution of CYP to the MEOS in humans (Asai et al. 1996). Although CYP2E1 is a major contributor to the MEOS in humans; CYP1A2 also plays an important role in the MEOS (Asai et al. 1996).

Acetaldehyde Oxidation Activity of CYP2E1 in Liver Microsomes: Complementary Roles for CYP2E1 and Acetaldehyde Dehydrogenase in Alcohol Metabolism

Acetaldehyde is oxidized by rat and human hepatic microsomes in the presence of NADPH, and a NADPH-dependent oxidation system MAOS (microsomal acetaldehyde-oxidizing system) has been developed to distinguish it from the

NAD-dependent acetaldehyde oxidation system of acetaldehyde dehydrogenase in mitochondria and cytosol (Kunitoh et al. 1997). Hepatic CYP2E1 mainly contributes to MAOS in rats and humans, the pathway of which may play an alternative role against acetaldehyde in the liver after alcohol consumption together with acetaldehyde dehydrogenase in the metabolism of acetaldehyde (Kunitoh et al. 1997).

Metabolism of the Anaesthetic Sevoflurane in Human Blood by CYP2E1
Since the amount of inorganic fluoride released after anesthesia with sevoflurane depends on the dose of administered sevoflurane and CYP2E1 activity in the liver, a reliable and noninvasive probe for CYP2E1 for predicting plasma inorganic fluoride levels after anesthesia has been developed (Hase et al. 2000). In patients (American Society of Anesthesiologists physical status I), aged 20–68 year undergoing body surface surgery with general anesthesia with sevoflurane, there is a significant correlation between level of CYP2E1 mRNA in mononuclear cells and the area under the plasma concentration-time curve of plasma inorganic fluoride from the beginning of sevoflurane administration to infinity in uninduced and uninhibited patients (Hase et al. 2000).

Presence of CYP2E1 mRNA in Blood Mononuclear Cells of ALD Patients: Role as a Biomarker of Liver Injury
The presence of CYP2E1 mRNA levels of mononuclear cells obtained from healthy individuals who did and did not drink habitually and patients with ALD has been investigated (Yano et al. 2001). The CYP2E1 mRNA level in mononuclear cells increases during drinking and decreases in abstinence for a short period of 3–4 days (Yano et al. 2001). Thus, CYP2E1 mRNA level may be used as an effective marker for alcoholic intake (Yano et al. 2001).

IV

A. Guillouzo
Cytokine Mediated Regulation of Hepatic CYP2E1
The cytokine IFN-gamma suppresses CYP2E1 mRNA levels in primary human hepatocyte cultures (Abdel-Razzak et al. 1993). In addition, IL-4 has the opposite effect, compared with other cytokines-IL-1 beta, IL-6 and TNF alpha, on CYP2E1 mRNA, which is increased up to fivefold (Abdel-Razzak et al. 1993). Thus, various cytokines act directly on human hepatocytes to affect expression of CYP2E1 gene (Abdel-Razzak et al. 1993).

Interactions Between the Anaesthetic Propofol and CYP2E1
While almost anesthetics are metabolized by the CYP3A4, some major volatile ones such as halothane and sevoflurane are metabolized by CYP2E1 in humans (Lejus et al. 2002). A widely used intravenous anesthetic agent, 2,6-diisopropylphenol (propofol), known to inhibit CYP3A4 and CYP1A2, inhibits CYP2E1 as detected through 6-OH hydroxylation of chlorzoxazone in human and porcine microsomes (Lejus et al. 2002). Thus, propofol could have a protective effect on toxic metabolite activation of compounds catalyzed by CYP2E1.

E. Arinc

Increased Incidence of Childhood Acute Lymphoblastic Leukemia (ALL) Is Associated with Polymorphisms in CYP2E1

The CYP2E1 gene possesses several polymorphisms in humans, and among them, CYP2E1*5B and *6 have been shown to be associated with increased risks of several chemical-induced diseases (Ulusoy et al. 2007). The possible association of CYP2E1*5B, *6 and *7B alleles, alone or in combination, with the risk of incidence of childhood acute lymphoblastic leukemia (ALL) in a Turkish population has been investigated (Ulusoy et al. 2007). When both CYP2E1*5B and *6 alleles are considered together, the risk of childhood ALL increases significantly (2.9-fold) (Ulusoy et al. 2007). Moreover, the presence of at least two variant alleles of any combination increases the risk significantly 3.9 times, suggesting a combined effect (Ulusoy et al. 2007). Individuals carrying combinations of CYP2E1*5B, *6 and *7B variants together are likely associated with the risk of developing childhood ALL (Ulusoy et al. 2007).

Association Between CYP2E1 Variant Alleles and Impaired DNA Repair Capacity: Increased Incidence of Childhood ALL

The co-presence of X-ray repair cross-complimenting group 1 (XRCC1) Arg399Gln variant and CYP2E1*5B and *6 polymorphisms in the same individuals considerably increases the risk for childhood ALL to 3.7-fold with borderline significance in a segment of Turkish population under study (Tumer et al. 2010). The observed combined effect is considerably more prominent among females (Tumer et al. 2010). Thus, the combined associations of XRCC1 399Gln, CYP2E1*5B and *6 alleles is associated with the risk of development of childhood ALL (Tumer et al. 2010).

O. Adali

Diabetes Mediated Induction of Hepatic and Extrahepatic CYP2E1

A significant induction of liver CYP2E1 protein and catalytic activities is observed in alloxan-induced rabbits and the CYP2E1 content of diabetic microsomes is highly correlated with the activities of liver aniline 4-hydroxylase and p-nitrophenol hydroxylase (Arinç et al. 2005). Similarly, induction of CYP2E1 protein and markedly increased aniline 4-hydroxylase and p-nitrophenol hydroxylase activities are also observed in alloxan induced diabetic rabbit lung and kidney (Arinç et al. 2007). Further, the procarcinogen and food contaminant, NDMA is converted to its carcinogenic form after it is activated with NDMA N-demethylase and a significant increase of liver, kidney and lung NDMA N-demethylase activity associated with CYP2E1 is shown in diabetic rabbit, suggesting the risk of nitrosamine induced carcinogenesis will be greater in liver, kidney and lung of the diabetic subjects (Arinç et al. 2007).

B.I. Ghanayem

CYP2E1 and Chemical Carcinogenesis

Methacrylonitrile

Methacrylonitrile (MAN) is a widely used aliphatic nitrile and is structurally similar to the known rat carcinogen and suspected human carcinogen acrylonitrile (AN)

(Ghanayem et al. 1999). In rats, MAN is metabolized by CYP2E1 to acetone, which is eliminated along with parent MAN in breath (Wang et al. 2002a). Gavage administration of MAN to rats causes olfactory epithelial damage and liver enlargement (Wang et al. 2002a). Further, administration of MAN to rats causes increased expression of CYP2E1 in lung, liver, and nasal tissues (Wang et al. 2002a). Also, acetone induces the expression of CYP2E1 at both the mRNA and protein levels in rat nasal and lung tissues (Wang et al. 2002a). MAN increases the expression of CYP2E1, and this effect varies as a function of time, length of exposure, and tissue examined (Wang et al. 2002a). Thus, treatment of rats with MAN results in differential expression of CYP2E1 and possibly other CYPs in tissues leading to tissue-specific toxicity via increased *in situ* formation of cytotoxic MAN metabolites (Wang et al. 2002a).

MAN and AN are metabolized *via* GSH conjugation or epoxide formation. CYP2E1 is essential for AN epoxidation and subsequent cyanide liberation (El Hadri et al. 2005). While significant reduction in blood cyanide levels occurs in MAN-treated CYP2E1-null *vs.* WT mice, AN metabolism to cyanide is largely abolished in CYP2E1-null mice (El Hadri et al. 2005). Pretreatment of mice with 1-aminobenzotriazole (ABT, CYP inhibitor) demonstrates that CYPs other than CYP2E1 also contribute to MAN metabolism to cyanide (El Hadri et al. 2005). Thus, while CYP2E1 is the only enzyme responsible for AN metabolism to cyanide, other CYPs also contribute to MAN metabolism (El Hadri et al. 2005).

Acrylonitrile and Acrylamide

Acrylonitrile (AN) and acrylamide (AM) are commonly used in the synthesis of plastics and polymers (Sumner et al. 1999). In rodents, AM and AN are metabolized to epoxides (Sumner et al. 1999). Wild-type (WT) mice excrete metabolites derived from the epoxides and from direct GSH conjugation with AM or AN. Only metabolites derived from direct GSH conjugation with AM or AN are observed in the urine from ABT-pretreated WT mice and mice devoid of CYP2E1 (P450 2E1-null). The evaluation of urinary metabolites at these doses, suggests that CYP2E1 is possibly the only CYP enzyme involved in the metabolism of AM and AN in mice, that inhibiting total CYP activity does not result in new pathways of non-P450 metabolism of AM, and that mice devoid of CYP2E1 (P450 2E1-null mice) do not excrete metabolites of AM or AN that would be produced by oxidation by other cytochrome P450s (Sumner et al. 1999).

Using CYP2E1-null mice treated with AN, it has been shown that CYP2E1-mediated oxidation is a prerequisite for AN metabolism to cyanide (Wang et al. 2002b). Since earlier studies have shown that CYP2E1 is the only enzyme responsible for AN epoxidation, it is concluded that AN metabolism to cyanoethylene oxide (CEO) is a prerequisite for cyanide formation which plays an essential role in the causation of the acute toxicity/mortality of AN, and this pathway is exclusively catalyzed by CYP2E1 (Wang et al. 2002b).

A key oxidative metabolite of acrylamide is the epoxide glycidamide, generated by CYP2E1 (Ghanayem et al. 2005a). Dose-related increases in resorption moles (chromosomally aberrant embryos) and decreases in the numbers of pregnant females and the proportion of living fetuses are seen in females mated to acrylamide-treated

wild-type mice (Ghanayem et al. 2005a). No changes in any fertility parameters are seen in females mated to acrylamide-treated CYP2E1-null mice (Ghanayem et al. 2005a). Thus, acrylamide-induced germ cell mutations in male mice require CYP2E1-mediated epoxidation of acrylamide (Ghanayem et al. 2005a).

In male germ cells, significant dose-related increases in micronucleated erythrocytes and DNA damage in somatic cells are induced in acrylamide-treated wild-type but not in the CYP2E1-null mice (Ghanayem et al. 2005b). Thus, genetic damage in somatic and germ cells of mice-treated with acrylamide is dependent upon metabolism of the parent compound by CYP2E1 (Ghanayem et al. 2005b). This dependency on metabolism has implications for the assessment of human risks resulting from occupational or dietary exposure to acrylamide (Ghanayem et al. 2005b).

Acrylamide, besides being an animal carcinogen is also, a neurotoxin, and reproductive toxin (Ghanayem et al. 2005c). The role of CYP2E1 in the epoxidation of acrylamide to glycidamide (GA) and the formation of DNA and hemoglobin (HGB) adducts has been assessed (Ghanayem et al. 2005c). Administration of acrylamide to wild-type mice causes a large increase in N7-GA-Gua and N3-GA-Ade adducts in the liver, lung, and testes (Ghanayem et al. 2005c). While traces of N7-GA-Gua adducts are measured in the tissues of acrylamide-treated CYP2E1-null mice, these levels are several fold lower than in wild-type mice (Ghanayem et al. 2005c). Significant elevation of both acrylamide- and GA-HGB adducts is detected in acrylamide-treated wild-type mice (Ghanayem et al. 2005c). In acrylamide-treated CYP2E1-null mice, levels of acrylamide-HGB adducts are roughly twice as high as those in wild-type mice (Ghanayem et al. 2005c). Thus, CYP2E1 is the primary enzyme responsible for the epoxidation of acrylamide to GA, which leads to the formation of GA-DNA and HGB adducts (Ghanayem et al. 2005c).

Urethane

Urethane ([carbonyl-(14)C]ethyl carbamate) is a fermentation by-product in alcoholic beverages and foods and is classified as reasonably anticipated to be a human carcinogen (Hoffler et al. 2003). CO(2) has been confirmed as the main metabolite of urethane (Hoffler et al. 2003). Using CYP2E1-null (KO) mice, CYP2E1's contribution to urethane metabolism has been elucidated (Hoffler et al. 2003) and it has been observed that CYP2E1, not esterase, is the principal enzyme responsible for urethane metabolism (Hoffler et al. 2003).

The relationships between CYP2E1-mediated metabolism and urethane-induced genotoxicity and cell proliferation as determined by induction of micronucleated erythrocytes (MN) and expression of Ki-67, respectively, using CYP2E1-null and wild-type mice have been investigated (Hoffler et al. 2005). Thus, CYP2E1-mediated metabolism of urethane, presumably *via* epoxide formation, is necessary for the induction of genotoxicity, and cell proliferation in the liver and lung of wild-type mice (Hoffler et al. 2005).

Multiple dosing (14)C-ethyl-labeled urethane leads to considerable bioaccumulation of urethane in CYP2E1−/− and CYP2E1+/+ mice; however, greater retention occurs in CYP2E1−/− versus CYP2E1+/+ mice (Hoffler and Ghanayem 2005). Furthermore, greater bioaccumulation of (14)C-ethyl-labeled than [(14)C]carbonyl-labeled urethane

is observed in mice (Hoffler and Ghanayem 2005). Comparison of the metabolism of ethyl-versus carbonyl-labeled urethane is necessary for tracing the source of CO(2) and therefore C-hydroxylation is a likely pathway of urethane metabolism (Hoffler and Ghanayem 2005).

Using Cyp2e1−/− mice, the relationships between urethane metabolism and carcinogenicity has been assessed (Ghanayem 2007). A significant reduction in the incidences of liver hemangiomas and hemangiosarcomas occurs in Cyp2e1−/− compared to Cyp2e+/+ mice (Ghanayem 2007). Lung nodules increase in a dose-dependent manner and are less prevalent in Cyp2e1−/− compared to Cyp2e+/+ mice (Ghanayem 2007). Bronchoalveolar adenomas are observed, and in one Cyp2e1+/+ mouse treated with 100 mg/kg urethane, a bronchoalveolar carcinoma is diagnosed (Ghanayem 2007). Significant reduction in the incidence of adenomas and the number of adenomas/lung are observed in Cyp2e1−/− compared to Cyp2e1+/+ mice (Ghanayem 2007). In the Harderian gland, the incidences of hyperplasia and adenomas are significantly lower in Cyp2e1−/− compared to Cyp2e+/+ mice at the 10 mg/kg dose, with no significant differences observed at the high or low doses (Ghanayem 2007). Thus, a significant reduction of urethane-induced carcinogenicity occurs in Cyp2e1−/− compared to Cyp2e1+/+ mice and CYP2E1-mediated oxidation plays an essential role in urethane-induced carcinogenicity (Ghanayem 2007).

1-Bromopropane

1-bromopropane (1-BrP) induces dose- and time-dependent reproductive organ toxicity and reduced sperm motility in rodents (Garner et al. 2007). Metabolites produced through CYP2E1-mediated oxidation could lead to 1-BrP-induced sperm toxicity (Garner et al. 2007). Both 1-BrP and 2OHBrP inhibit the motility of sperm obtained from WT mice *in vitro* (Garner et al. 2007). However, only 2OHBrP reduces the motility of sperm obtained from Cyp2e1−/− mice in vitro, suggesting that conversion of parent compound to 2OHBrP within the spermatozoa may contribute, at least in part, to reduced motility (Garner et al. 2007). Thus, metabolism of 1-BrP is mediated in part by CYP2E1, and activation of 1BrP via this enzyme may contribute to the male reproductive toxicity of this chemical (Garner et al. 2007).

S.A. Weinman

Synergistic Interactions Between Hepatitis C Virus Core Protein and CYP2E1: Its Implications for Enhanced Alcohol Mediated Liver Injury in Chronic Hepatitis

Huh-7 cells expressing Hepatitis C virus core protein, CYP2E1, or both are exposed to tertiary butyl hydroperoxide, TNF alpha, and/or ethanol (Otani et al. 2005). Expression of core/CYP2E1 synergistically enhances cell death induced by either tertiary butyl hydroperoxide or TNF alpha (Otani et al. 2005). After tertiary butyl hydroperoxide treatment, total ROS production increases more than threefold compared with cells that do not express core and CYP2E1 (Otani et al. 2005). Mitochondrial depolarization and reduced GSH depletion occurs as well, and cell death is prevented by inhibition of mitochondrial permeability transition or caspase activity (Otani et al. 2005). Confocal microscopy shows

that the mitochondria themselves are the origin of the ROS. In the absence of core/CYP2E1 expression, mitochondrial changes and cell death does not occur (Otani et al. 2005). Ethanol treatment further decreases mitochondrial reduced GSH content and exacerbates mitochondrial ROS production, depolarization, and cell death (Otani et al. 2005). Thus, mitochondrial ROS production is induced by hepatitis C virus core and CYP2E1, resulting in a reduction of mitochondrial antioxidant capacity and sensitivity to oxidants and TNF alpha and alcohol further depletes mitochondrial reduced GSH, which exacerbates depolarization and cell death (Otani et al. 2005).

T.R. Morgan

Development of CYP2E1 Over-Expressing Transgenic Mice and Increased Alcohol Mediated Liver Injury

Transgenic mice that express human CYP2E1 cDNA under the control of mouse albumin enhancer-promoter in the liver, when fed a nutritionally complete alcohol diet, develop more liver damage which includes higher serum ALT levels, higher histologic scores and ballooning hepatocytes than nontransgenic mice (Morgan et al. 2002).

Downregultion of Insulin Signaling due to CYP2E1 Mediated Oxidative Stress in a Rodent NAFLD Model

Hepatocyte-specific overexpression of CYP2E1 in transgenic mice increases hepatic oxidative stress in the liver, fasting insulin, and histological liver damage (Kathirvel et al. 2009). CYP2E1 over-expression reduces hepatic insulin signaling and glycogen storage and increases glucose synthesis (Kathirvel et al. 2009). Thus, an association exists between hepatic CYP2E1 and increased oxidative stress, increased systemic insulin resistance, decreased insulin signaling in the liver and increased hepatic fat accumulation (Kathirvel et al. 2009).

CYP2E1 and Nitrosative Stress: A Close Association in NAFL Model

Hepatocyte-specific CYP2E1 over-expression in transgenic mice exhibiting greater histological liver injury results in increased oxidative stress and nitrosative stress (Kathirvel et al. 2010). Gene expression of antioxidant enzymes Nrf2, CAT, GPx, HO-1 are significantly upregulated (Kathirvel et al. 2010). iNOS activity and nitrosylation of CAT and SOD is greater in liver of CYP2E1 over-expressing transgenic mice (Kathirvel et al. 2010). Failure of corresponding increase in total protein and activity of anti-oxidant enzymes suggests modification/degradation, possibly by nitrosylation, due to increased iNOS activity in a CYP2E1 overexpressing NAFL mouse model (Kathirvel et al. 2010).

C.H. Halsted

Regulation of CYP2E1 by Sex Steroids

To assess possible links between ethanol-induced oxidant stress, expression of hepatic CYP enzymes, and sex steroid status, the generation of protein adducts of acetaldehyde (AA), malondialdehyde (MDA), and 4-HNE with the amounts of CYP2E1, CYP2A, and CYP3A in the livers of castrated and noncastrated male micropigs fed ethanol for 12 months have been compared (Niemelä et al. 1999).

In castrated micropigs, ethanol feeding results in accumulation of fat, hepatocellular necrosis, inflammation, and centrilobular fibrosis, whereas only minimal histopathology is observed in their noncastrated counterparts (Niemelä et al. 1999). Ethanol feeding increases the hepatic content of CYP2E1 and CYP3A in the noncastrated animals and in CYP2E1 and CYP2A in the castrated animals most significantly (Niemelä et al. 1999). Ethanol-fed castrated animals also show the greatest abundance of perivenular adducts of AA, MDA, and HNE (Niemelä et al. 1999). In the noncastrated ethanol-fed micropigs, a low expression of each CYP form is associated with scant evidence of aldehyde-protein adducts (Niemelä et al. 1999). Significant correlations emerge between the levels of different CYP forms, protein adducts, and plasma levels of sex steroids (Niemelä et al. 1999). Thus, the generation of protein-aldehyde adducts is associated with the induction of several cytochrome enzymes including CYP2E1 in a sex steroid-dependent manner (Niemelä et al. 1999). It appears that the premature, juvenile, metabolic phenotype, as induced by castration, favors liver damage (Niemelä et al. 1999). It implicates the role of the gender differences on the adverse effects of ethanol in the liver (Niemelä et al. 1999).

Folate Deficiency Exacerbates Alcohol Induced Liver Injury: Its Association with CYP2E1

Feeding micropigs ethanol with a folate-deficient diet promotes the development of hepatic injury while increasing hepatic levels of homocysteine and S-adenosylhomocysteine (SAH) and reducing the level of S-adenosylmethionine (SAM) and the SAM-to-SAH ratio (Esfandiari et al. 2005). The induction of abnormal hepatic methionine metabolism through the combination of ethanol feeding with folate deficiency is associated with the activation of CYP2E1 and enhances endoplasmic reticulum stress signals that promote steatosis and apoptosis (Esfandiari et al. 2005).

C.K. Roberts

Insulin Mediated Downregulation of CYP2E1

The expression of major CYP isozymes in STZ-induced diabetes with concomitant insulin therapy has been studied (Sindhu et al. 2006). CYP2E1 protein is markedly induced in the STZ-induced diabetic group (Sindhu et al. 2006). Insulin therapy results in complete amelioration of CYP2E1 whereas CYP2B1 protein is partially ameliorated (Sindhu et al. 2006). By contrast, CYP2C11 protein is decreased over 99% in the diabetic group and is partially ameliorated by insulin therapy (Sindhu et al. 2006). Thus, widespread alterations exist in the expression of CYP isozymes in diabetic rats that are ameliorated by insulin therapy (Sindhu et al. 2006).

E.T. Morgan

Endotoxin Mediated Regulation of Hepatic CYP2E1

Growth hormone (GH) is an important regulator of CYP gene expression in the rat and the effects of bacterial endotoxin injection in hypophysectormized rats are compared to those in normal animals (Morgan 1993). In intact females, endotoxin suppresses total CYP and hepatic expression of CYP2C6, CYP2C7, CYP2C12, and

CYP2E1 mRNAs, as well as CYP2C12 and CYP2E1 proteins with the greatest decreases being observed with CYP2C7 and CYP2E1 mRNAs (Morgan 1993). Endotoxin treatment also induces the mRNA for the hepatic acute phase protein, haptoglobin in female rats (Morgan 1993). In hypophysectomized females supplemented with GH infusion, endotoxin causes the same or greater effects on expression of the CYP and haptoglobin gene products than are observed in the intact animals (Morgan 1993). It is concluded that the CYP suppression observed after endotoxin administration can occur independently of an effect on pituitary hormone secretion (Morgan 1993). Thus, endotoxin suppresses hepatic CYP2E1 protein and mRNA levels (Morgan 1993).

Interleukin Mediated Downregulation of Hepatic CYP2E1

CYP2E1 mRNA and protein are significantly suppressed only by the combination of IL1 and dexamethasone in rat hepatocytes cultured on Matrigel in the presence of growth hormone (Morgan et al. 1994). Further, IL6 treatment of male rats downregulates CYP2E1 mRNA at a dose of 4.5 μg/kg, which is lower than that required to induce haptoglobin mRNA, a prototype acute phase gene product (Morgan et al. 1994). Thus, hepatic CYP2E1 is regulated by cytokines, implicatimg a cross talk between immune system and CYPs.

Endotoxin Mediated Downregulation of Hepatic CYP2E1 Occurs Independently of NO

The role of NO in regulating the decreases in both gene expression and activity of hepatic CYP2E1 in endotoxemic parental and inducible nitric oxide synthase (NOS2) knockout mice has been examined (Sewer et al. 1998). Microsomal CYP2E1 protein level is decreased in both strains of mouse (Sewer et al. 1998). Similar results are obtained in parental strain endotoxemic mice co-administered the NOS inhibitor aminoguanidine (Sewer et al. 1998). Thus, the down-regulation of CYP2E1 protein and mRNA in the endotoxemic mouse can occur independently of NO production (Sewer et al. 1998).

Endotoxin (LPS) treatment suppresses both mRNA and protein expression of CYP2E1 in rats (Sewer and Morgan 1998). Coadministration of the NOS inhibitor aminoguanidine to bacterial endotoxin lipopolysaccharide (LPS)-treated rats completely inhibits the release of NO into the plasma but does not reverse the down-regulation of expression of CYP2E1 (Sewer and Morgan 1998). Thus, NO is not required for endotoxin-evoked down-regulation of CYP2E1 mRNA or protein expression as observed in rats and NOS2 knock out mice.

Endotoxin Mediated Downregulation of CYP2E1 Involves Transcriptional Suppression

The rate of transcription of the CYP2E1 gene is reduced to 10% of control levels, respectively, in rat liver within 1–2 h of injection of LPS (1 mg/kg) and injection of curcumin significantly inhibits the rapid transcriptional suppression of CYP2E1 (Cheng et al. 2003). The magnitude and rapidity of these effects indicate that transcriptional suppression is a primary reason for the decline in CYP2E1 mRNA (Cheng et al. 2003).

V

G. Robertson

Leptin Mediated Regulation of CYP2E1

In a genetic model of obesity and non-insulin dependent (type II) diabetes, the leptin-deficient ob/ob mouse, hepatic CYP2E1 levels are decreased compared to lean littermates (Leclercq et al. 2000a). Treatment with leptin increases hepatic CYP2E1 in obese mice to the levels observed in lean animals, but fails to alter CYP2E1 expression in lean animals (Leclercq et al. 2000a). Leptin also reduces food intake in treated mice compared to saline-treated controls (Leclercq et al. 2000a). In obese mice pair-fed the reduced amount of food, there is a significant increase in CYP2E1 mRNA unaccompanied by increases in CYP2E1 protein or enzyme activity (Leclercq et al. 2000a). Fasting and administration of acetone and 4-MP increases CYP2E1 mRNA as well as protein and activity in both obese and lean mice (Leclercq et al. 2000a). While CYP2E1 is still inducible in obese mice by xenobiotics and fasting, full constitutive expression of CYP2E1 requires leptin to be present (Leclercq et al. 2000a). This effect of leptin appears to be at least partly independent of the hypothalamic control of food intake (Leclercq et al. 2000a).

CYP2E1 Mediated Oxidative Stress in a NASH Model

In a dietary model of NASH-mice fed a methionine- and choline-deficient (MCD) diet, liver injury is associated with both induction of CYP2E1 and a 100-fold increase in hepatic content of lipid peroxides (Leclercq et al. 2000b). Further, microsomal NADPH-dependent lipid oxidases contribute to the formation of these lipid peroxides, and *in vitro* inhibition studies demonstrate that CYP2E1 is the major catalyst (Leclercq et al. 2000b). Thus, the induction of CYP2E1 and subsequent formation of lipid peroxides by CYP2E1 may be one of the major mechanisms for promoting NASH mediated liver injury.

T.M. Badger

Carbohydrate Deficiency Exacerbates CYP2E1 Mediated Alcohol Induced Liver Injury

In rats fed with the carbohydrate-deficient diet and ethanol, a strong positive association between low dietary carbohydrate, enhanced hepatic microsomal CYP2E1 apoprotein induction and hepatic necrosis occurs (Korourian et al. 1999). Thus, in the presence of low carbohydrate intake, ethanol induction of CYP2E1 is enhanced to levels sufficient to cause necrosis, possibly through ROS and other free radicals generated by CYP2E1 mediated metabolism of ethanol and unsaturated fatty acids (Korourian et al. 1999).

Hormonal Regulation of CYP2E1

Depletion of pituitary hormones by hypophysectomy (Hx) results in 12–14-fold increases in renal CYP2E1 compared with sixfold increases in CYP2E1 apoprotein in the liver (Chen et al. 1999). The increase in hepatic CYP2E1 is associated with increased gene transcription (Chen et al. 1999). Restoration of renal CYP2E1 to control levels by hormone treatment requires both growth hormone and an

intact testis, whereas partial restoration of CYP2E1 apoprotein levels in liver is accomplished by growth hormone, but not testosterone (Chen et al. 1999). Thus, CYP2E1 appears to be under complex endocrine regulation by pituitary and testicular hormones in a tissue specific manner (Chen et al. 1999).

Acute Alcohol Plus Glucose Mediated Downregulation of CYP2E1

FGC-4 rat hepatoma cells have been used to test the hypothesis that carbohydrates could down-regulate ethanol-induced CYP2E1 induction *in vitro* (Rowlands et al. 2003). FGC-4 cells grown in a glucose-free media and treated with 1–100 mM ethanol for 24 h exhibit a dose-dependent increase in CYP2E1, with maximum mRNA steady-state or protein levels measured at 30 or 100 mM ethanol, respectively (Rowlands et al. 2003). In cells treated with 30 mM ethanol, a glucose concentration-dependent inhibition of CYP2E1 mRNA is observed between 2.5 and 10 mM glucose (Rowlands et al. 2003). Induction by 30 mM ethanol of CYP2E1 protein is reduced in cells co-treated with 1 mM or greater glucose concentration and complete inhibition is measured with 5 mM glucose co-treatment (Rowlands et al. 2003). Under culture conditions of extremely low carbohydrate concentrations, ethanol treatment of FGC-4 cells results in elevated steady-state levels of CYP2E1 mRNA and protein; and glucose inhibits this increase (Rowlands et al. 2003).

M. J.Ronis/T. M.Badger

CYP2E1 Mediated Ethanol Metabolism: Triglyceride Accumulation, Induction of TNF-Alpha, and Chemokine Production

The effects of selective inhibition of CYP2E1 have been compared with the inhibition of overall ethanol metabolism on the development of alcoholic steatohepatitis (Ronis et al. 2010). Liver pathology scores and levels of apoptosis are elevated by total ethanol through enteral nutrition but do not differ significantly on co-treatment with CYP2E1 inhibitor DAS or ADH inhibitor 4-MP (Ronis et al. 2010). However, liver triglycerides are lower when ethanol-fed rats are treated with DAS or 4-MP (Ronis et al. 2010). Serum ALT values are significantly lower in ethanol-fed 4-MP-treated rats indicating reduced necrosis (Ronis et al. 2010). Hepatic oxidative stress and the ER stress marker tribbles-related protein 3 are increased after ethanol; further increased by DAS but partly attenuated by 4-MP. Both DAS and 4-MP reverse ethanol increases in the cytokine, TNF-alpha, and the chemokine CXCL-2 (Ronis et al. 2010). Ethanol and DAS additively induce hepatic hyperplasia (Ronis et al. 2010). Thus a significant proportion of hepatic injury after ethanol exposure is independent of alcohol metabolism (Ronis et al. 2010). Ethanol metabolism by CYP2E1 may be linked in part to triglyceride accumulation, to induction of TNF-alpha, and to chemokine production (Ronis et al. 2010). Ethanol metabolism by ADH may be linked in part to oxidative and ER stress and necrotic injury (Ronis et al. 2010).

C.P. Day

Presence of CYP2E1 c2 Allele and ADH3 Genotype Increases Risk for Alcoholic Liver Damage

Since most of the deleterious effects of alcohol are caused by its metabolism, attention has focused upon genes encoding ethanol metabolizing enzymes (Grove et al.

1998). Caucasians are polymorphic at only two of the gene loci – CYP2E1 and ADH3 (Grove et al. 1998). Although rare in Caucasians, possession of the mutant c2 allele of CYP2E1 increases the risk of alcoholic liver disease at a given level of cumulative alcohol consumption (Grove et al. 1998). This risk appears to be particularly manifest in individuals carrying the ADH3*2 allele, presumably reflecting increased metabolism of ethanol by CYP2E1 (Grove et al. 1998). In the absence of the c2 allele of CYP2E1, ADH3 genotype does not influence the risk of advanced alcoholic liver disease but, in males at least, may increase the susceptibility to alcoholism (Grove et al. 1998).

B. Fromenty

Induction of Mitochondrial Hepatic CYP2E1 due to Binge Alcohol

The effect of repeated alcohol binges on hepatic mtDNA in mice has been studied (Demeilliers et al. 2002). Levels of mtDNA are decreased for 48 h after the last dose in mice administered ethanol (Demeilliers et al. 2002). Two and 24 h after the fourth dose, DNA lesions are observed that block the progress of the polymerases and organellomtDNA synthesis is decreased (Demeilliers et al. 2002). Mitochondria exhibit ultrastructural abnormalities, and respiration is impaired 2 and 24 h after the fourth binge. CYP2E1, mitochondrial generation of peroxides, thiobarbituric acid reactants, and ethane exhalation are increased (Demeilliers et al. 2002). After repeated doses of ethanol, the accumulation of unrepaired mtDNA lesions (possibly involving lipid peroxidation-induced adducts) blocks the progress of polymerase gamma on mtDNA and prevents adaptive mtDNA resynthesis, causing prolonged hepatic mtDNA depletion (Demeilliers et al. 2002). Thus, mitochondrial structural and functional abnormalities occur along with induction of CYP2E1 due to repeated binge alcohol drinking.

Alcohol Mediated Mitochondrial CYP2E1 Induction

Ethanol increases microsomal and mitochondrial CYP2E1 in cultured rat hepatocytes and in the liver of lean mice (Robin et al. 2005). This is associated with decreased levels of GSH, possibly reflecting increased oxidative stress (Robin et al. 2005). In contrast, in leptin-deficient obese mice, ethanol administration does not increase mitochondrial CYP2E1, nor it depletes mitochondrial GSH, suggesting that leptin deficiency hampers mitochondrial targeting of CYP2E1 (Robin et al. 2005). Thus, ethanol intoxication increases CYP2E1 not only in the endoplasmic reticulum but also in mitochondria, thus favouring oxidative stress in these compartments (Robin et al. 2005).

M.A. Robin

Mitochondrial CYP2E1 and Ethanol or Acetaminophen Mediated Toxicity

When acetaminophen or ethanol are used as CYP2E1 substrates, the exclusive localization of CYP2E1 within mitochondria is sufficient to induce ROS overproduction, depletion of GSH, increased expression of mitochondrial Hsp70, mitochondrial dysfunction and cytotoxicity (Knockaert et al. 2011). Importantly, these harmful events occur despite lower cellular level and activity of CYP2E1 when compared to monkey kidney cell line COS-7 expressing CYP2E1 in both endoplasmic

reticulum and mitochondria, and this is particularly obvious with acetaminophen (Knockaert et al. 2011). Thus, mitochondrial CYP2E1 could play a major role in drug-induced oxidative stress and cell demise (Knockaert et al. 2011).

S.C. Lu

Regulation of CYP2E1 by Chronic Hepatic S-Adenosylmethionine Deficiency

In mammals, methionine metabolism occurs mainly in the liver via methionine adenosyltransferase-catalyzed conversion to SAM (Martínez-Chantar et al. 2002). Of the two genes that encode methionine adenosyltransferase (MAT1A and MAT2A), MAT1A is mainly expressed in adult liver whereas MAT2A is expressed in all extrahepatic tissues (Martínez-Chantar et al. 2002). Mice lacking MAT1A have reduced hepatic SAM content and hyperplasia and spontaneously develop nonalcoholic steatohepatitis (Martínez-Chantar et al. 2002). An abnormal expression of genes involved in the metabolism of lipids and carbohydrates occurs in MAT1A knockout mice, a situation that is reminiscent of that found in diabetes, obesity, and other conditions associated with nonalcoholic steatohepatitis (Martínez-Chantar et al. 2002). This aberrant expression of metabolic genes in the knockout mice is associated with hyperglycemia, increased hepatic CYP2E1 and UCP2 expression and triglyceride levels, and reduced hepatic GSH content (Martínez-Chantar et al. 2002). The knockout animals have increased lipid peroxidation and enhanced sensitivity to CCl4 (a substrate of CYP2E1)-induced liver damage, which is largely due to increased CYP2E1 expression because DAS, an inhibitor of CYP2E1, prevents CCl4-induced liver injury (Martínez-Chantar et al. 2002). Hepatocellular carcinoma develops in more than half of the knockout mice (Martínez-Chantar et al. 2002). Thus, SAM plays a crucial role in maintaining normal hepatic function and tumorigenesis of the liver and SAM knock out mice are more predisposed to CCl4-induced liver injury due to induction of CYP2E1 in the absence of SAM (Martínez-Chantar et al. 2002).

N.G. Avadhani

Biochemical Characterization of Mitochondrial CYP2E1

Hepatic mitochondria contain an inducible cytochrome P450, referred to as P450 MT5, which cross-reacts with antibodies to microsomal CYP2E1 and it has been purified, partially sequenced, and the enzymatic properties of the rat liver mitochondrial form have been determined (Robin et al. 2001). The N terminus of purified mitochondrial CYP2E1 protein is identical to that of the microsomal CYP2E1 (Robin et al. 2001). The mitochondrial CYP2E1 displays the same catalytic activity as the microsomal counterpart, although the activity of the mitochondrial enzyme is supported exclusively by adrenodoxin and adrenodoxin reductase which may be due to a different conformational state of the mitochondrial targeted CYP2E1 (Robin et al. 2001). Further, the mitochondrial CYP2E1 is phosphorylated at a higher level compared with the microsomal counterpart (Robin et al. 2001). Thus, hepatic mitochondrial CYP2E1 possesses distinct biochemical characteristics (Robin et al. 2001).

Diabetes Mediated Induction of Mitochondrial CYP2E1

A five- to eightfold increase of CYP2E1 and glutathione S-transferase (GST) A4-4 levels occurs in mitochondria from STZ-treated rat tissues compared with those in

nondiabetic rat tissues, suggesting their possible roles in the disease process (Raza et al. 2004). Transient transfection of COS cells with CYP2E1 cDNA causes a similar accumulation of CYP2E1 and GST A4-4 in mitochondria and increased production of mitochondrial ROS (Raza et al. 2004). Thus, a marked increase in mitochondrial oxidative stress in target tissues of STZ-treated rats and implicates a direct role for mitochondrial CYP2E1 in the generation of intramitochondrial ROS.

Alcohol Inducible Mitochondrial CYP2E1: A Potential Source for Oxidative Stress

CYP2E1 is bimodally targeted to both the endoplasmic reticulum (microsomes) (mc CYP2E1) and mitochondria (mt CYP2E1) (Bansal et al. 2010). The role of mtCYP2E1 in ethanol-mediated oxidative stress in stable cell lines expressing predominantly mt CYP2E1 or mc CYP2E1 has been studied (Bansal et al. 2010). The ER+ mutation (A2L, A9L), which increases the affinity of the nascent protein for binding to the signal recognition particle, preferentially targets CYP2E1 to the endoplasmic reticulum (Bansal et al. 2010). The Mt+ (L17G) and Mt++ (I8R, L11R, L17R) mutant proteins, showing progressively lower affinity for signal recognition particle binding are targeted to mitochondria at correspondingly higher levels (Bansal et al. 2010). The rate of GSH depletion, used as a measure of oxidative stress, is higher in cells expressing Mt++ and Mt+ proteins as compared with cells expressing ER+ protein (Bansal et al. 2010). In addition, the cellular level of F(2)-isoprostanes, a direct indicator of oxidative stress, is increased markedly in Mt++ cells after ethanol treatment (Bansal et al. 2010). Notably, expression of Mt++ CYP2E1 protein in yeast cells causes more severe mitochondrial DNA damage and respiratory deficiency than the wild type or ER+ proteins as tested by the inability of cells to grow on glycerol or ethanol (Bansal et al. 2010). Additionally, liver mitochondria from ethanol-fed rats containing high mt CYP2E1 show higher levels of F(2)-isoprostane production (Bansal et al. 2010). Thus, mt CYP2E1 induces oxidative stress and augments alcohol-mediated cell/tissue injury.

G.U. Corsini

A Protective Role of CYP2E1 Against MPTP Induced Neurotoxicity

Elucidation of the biochemical steps leading to the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced degeneration of the nigrostriatal dopamine (DA) pathway has provided new clues to the pathophysiology of Parkinson's disease (Vaglini et al. 2004). In line with the enhancement of MPTP toxicity by diethyldithiocarbamate (DDC), the potentiation of the selective DA neuronal degeneration in mice by CYP2E1 inhibitors, such as DAS and PIC has been investigated (Vaglini et al. 2004). In addition, CYP2E1 mRNA and protein are present in the brain and in the basal ganglia of C57/bl mice (Vaglini et al. 2004). A kinetic analysis of MPTP and its metabolites indicates that no detoxification metabolic pathway is affected by any of these inhibitors (Vaglini et al. 2004). This does not rule out, however, that an undetected detoxification pathway involving CYP2E1 is operating (Vaglini et al. 2004). In order to provide direct evidence for this isozyme involvement, CYP2E1 knockout mice are challenged with MPTP or the combined treatment (Vaglini et al. 2004). These transgenic mice have a low sensitivity to MPTP alone, similar to their

wild-type counterparts, suggesting that it is likely that transgenic mice compensate for the missing enzyme (Vaglini et al. 2004). However, DDC pretreatment completely fails to enhance MPTP toxicity in CYP2E1 knockout mice, whereas this enhancement is regularly present in wild-type animals (Vaglini et al. 2004). Thus, the occurrence of CYP2E1 in C57/bl mouse brain is relevant to MPTP toxicity, and suggests that this isozyme may have a detoxificant role related to the efflux transporter of the toxin (Vaglini et al. 2004).

It has been shown that DDC potentiates MPTP toxicity in mice as a result of increased levels of 1-methyl-4-phenylpyridinium ion (MPP(+)) in the striatum (Pardini et al. 2008). Brain CYP2E1 inhibition by DDC in C57Bl mice is responsible for increased toxicity and striatal MPP(+) accumulation (Pardini et al. 2008). However, CYP2E1-null mice do not show any enhanced sensitivity to MPTP or any MPP(+) accumulation (Pardini et al. 2008). This unexpected finding suggests that the CYP2E1-null mice compensate with other isozymes as already described for acetaminophen-induced liver damage (Pardini et al. 2008). MPP(+) intoxication of mesencephalic cell cultures from CYP2E1-null mice indicates a reduced sensitivity of dopaminergic (DA) neurons from knockout animals (Pardini et al. 2008). Surprisingly, MPP(+) cell distribution under these conditions indicates that the toxin accumulates more intracellularly in knockout cultures, suggesting further that CYP2E1 has a role in MPP(+) storage and efflux (Pardini et al. 2008).

The sensitivity of Cyp2e1(–/–) mice to the acute administration of MPTP in comparison with their wild-type counterparts has been investigated (Viaggi et al. 2009). In Cyp2e1(–/–) mice, the reduction of striatal DA content is less pronounced 7 days after MPTP treatment compared to treated wild-type mice (Viaggi et al. 2009). Similarly, tyrosine hydroxylase immunoreactivity analysis of the substantia nigra of Cyp2e1(–/–) mice does not show any neuronal lesions after MPTP treatment (Viaggi et al. 2009). In contrast to this, wild-type animals show a minimal but significant lesioning by the toxin in this brain area (Viaggi et al. 2009). Striatal levels of DA metabolites after 7 days are variably affected by the toxin, but consistent differences between the two animal strains are not observed (Viaggi et al. 2009). Striatal MPP(+) is cleared more rapidly in Cyp2e1(–/–) mice than in wild-type animals and, consistently, striatal DA content decreases faster in Cyp2e1(–/–) mice than in wild-type animals, and 3-methoxytyramine and homovanillic acid levels show an early and sharp rise (Viaggi et al. 2009). Cyp2e1(–/–) mice are weakly sensitive to MPTP-induced brain lesions, markedly in contrast with a protective role of the enzyme as suggested previously (Viaggi et al. 2009). The differences observed between the knockout mice and their wild-type counterparts are modest and may be due to an efficient compensatory mechanism or genetic drift in the colonies (Viaggi et al. 2009).

Involvement of CYP2E1 in Acute and Chronic Behavioral Effects of Ethanol

It has been shown that acetaldehyde is an active metabolite of ethanol with central actions that modulate behavior (Correa et al. 2009). Catalase has been proposed as the main enzyme responsible for the synthesis of acetaldehyde from ethanol in the brain (Correa et al. 2009). Recent studies, however, suggest that CYP2E1 can also contribute

to the central metabolism of ethanol (Correa et al. 2009). Spontaneous locomotion in CYP2E1 knockout (KO) mice is lower than that seen in the wild type (WT) mice (Correa et al. 2009). Acute administration of ethanol increases locomotion to a similar extent in both strains of mice (Correa et al. 2009). Repeated intermittent administration of ethanol produces sensitization in both strains, but it is very subtle in the KO mice compared with the effect in the WT mice (Correa et al. 2009). KO mice show a reduction in preference for ethanol intake at low concentrations (4–8% v/v) (Correa et al. 2009). Interestingly, KO mice have higher levels of catalase protein expression in the brain and liver compared with WT mice (Correa et al. 2009). Thus, some impact of the mutation on ethanol-induced sensitization and on voluntary ethanol preference is observed (Correa et al. 2009). The lack of a substantial impact of the mutation can be explained by the fact that the CYP2E1 KO animals have a compensatory increase in catalase expression compared with WT mice, therefore possibly showing alterations in the formation of acetaldehyde after ethanol administration (Correa et al. 2009).

R.F. Tyndale

Synergistic Induction of CYP2E1 by Ethanol and Nicotine

The use of ethanol and nicotine is strongly linked; 80–95% of heavy alcohol users are also smokers (Howard et al. 2001). In humans, cigarette smoking significantly enhances CYP2E1 activity, as measured by increased metabolism of chlorzoxazone *in vivo* (Howard et al. 2001). CYP2E1 also bioactivates tobacco smoke and other procarcinogens and several hepatotoxins (Howard et al. 2001). It has been investigated whether nicotine increases CYP2E1 activity like ethanol (Howard et al. 2001). After ethanol or nicotine administration, immunostaining for CYP2E1 is increased in the centrilobular regions of rat liver (Howard et al. 2001). Hepatic CYP2E1 protein levels are increased by ethanol and nicotine (Howard et al. 2001). *In vitro* chlorzoxazone 6-hydroxylation analyses demonstrate elevated Vmax values by using hepatic microsomes from high-dose ethanol or nicotine-treated rats, with no change in affinity (Howard et al. 2001). The magnitude of enhanced chlorzoxazone metabolism by microsomes from drug-treated animals is consistent with the observed increase in CYP2E1 protein (Howard et al. 2001). Thus, nicotine may increase CYP2E1-induced toxicity and contribute to cross-tolerance in smokers and people treated with nicotine (e.g., smokers, patients with Alzheimer's disease, ulcerative colitis, neuropsychiatric motor disorders) (Howard et al. 2001).

Nicotine increases CYP2E1 protein and activity in the rat liver (Micu et al. 2003). The induction peaks at 4 h postnicotine treatment and recovers within 24 h (Micu et al. 2003). No induction is observed after a single injection, and 18 days of treatment does not increase the levels beyond that found at 7 days (Micu et al. 2003). CYP2E1 is induced by very low doses of chronic (×7 days) nicotine with an ED50 value of 0.01 mg/kg s.c.; 0.01 mg/kg in a rat model results in peak cotinine levels (nicotine metabolite) similar to those found in people exposed to environmental tobacco smoke (passive smokers; 2–7 ng/ml) (Micu et al. 2003). Thus, nicotine does not regulate CYP2E1 expression by protein stabilization (Micu et al. 2003). Nicotine increases CYP2E1 at very low doses and may enhance CYP2E1-related toxicity in smokers, passive smokers, and people treated with nicotine (Micu et al. 2003).

In monkeys, nicotine induction of *in vivo* CZN disposition is related to the rates of *in vitro* CZN metabolism and hepatic microsomal CYP2E1 protein levels (Lee et al. 2006b). Nicotine is one component in cigarette smoke that can increase *in vivo* CZN metabolism *via* induction of hepatic CYP2E1 levels (Lee et al. 2006b). Thus, nicotine exposure may affect the metabolism of CYP2E1 substrates such as acetaminophen, ethanol, and benzene (Lee et al. 2006b).

Ethanol self-administration and nicotine treatment, alone and in combination to monkeys, significantly increases *in vivo* CZN disposition compared to control animals (Ferguson et al. 2011). The effect of ethanol is only observed at higher levels of intake (Ferguson et al. 2011). Ethanol and nicotine increase CYP2E1 protein levels and *in vitro* CZN metabolism, with combined exposure to both drugs resulting in the greatest increase (Ferguson et al. 2011). Chronic exposure to ethanol and nicotine induces hepatic CYP2E1 activity and protein levels, particularly when both drugs are used in combination and when ethanol intake is high (Ferguson et al. 2011).

Nicotine pretreatment of rats increases voluntary ethanol intake on day 10 compared to saline pretreatment (Yue et al. 2009). CYP2E1 is increased 1.7, 1.8, and 1.4 fold by the three doses of nicotine alone; CYP2E1 levels are increased by voluntary ethanol intake alone and a further 2.4, 2.2, and 1.8 fold by 0.4, 0.8, and 1.2 mg/kg nicotine respectively versus saline pretreatment (Yue et al. 2009). CYP2E1 level correlates with alcohol consumption on day 10 (Yue et al. 2009). Chronic nicotine increases voluntary ethanol intake thereby enhancing CYP2E1 level (Yue et al. 2009). Thus CYP2E1 is regulated not only directly by nicotine and ethanol, but also indirectly *via* an increase in the ethanol consumption in the presence of nicotine pretreatment (Yue et al. 2009). Together this may contribute to the co-abuse of these drugs and alter the metabolism of clinical drugs and endogenous substrates (Yue et al. 2009).

Induction of Brain CYP2E1 by Nicotine and Its Underlying Mechanisms

Ethanol treatment significantly increases CYP2E1 in olfactory bulbs, frontal cortex, hippocampus and cerebellum, while nicotine induces CYP2E1 in olfactory bulbs, frontal cortex, olfactory tubercle, cerebellum and brainstem and the induction is cell-type specific as assessed through immunocytochemical analysis (Howard et al. 2003a). Consistent with the increased CYP2E1 found in rat brain following drug treatments, brains from alcoholics and alcoholic smokers show greater staining of granular cells of the dentate gyrus and the pyramidal cells of CA2 and CA3 hippocampal regions as well as of cerebellar Purkinje cells compared to nonalcoholic nonsmokers (Howard et al. 2003a). Moreover, greater CYP2E1 immunoreactivity is observed in the frontal cortices in the alcoholic smokers in comparison to nonalcoholic nonsmokers and alcoholic nonsmokers (Howard et al. 2003a). Significantly higher CYP2E1 immunostaining is observed in nicotine-treated human neuroblastoma IMR-32 cells in culture (0.1–10 nM), suggesting that nicotine could contribute to the increased CYP2E1 observed in alcoholic smokers (Howard et al. 2003a). CYP2E1 induction in the brain, by ethanol or nicotine, may influence the central effects of ethanol and the development of nervous tissue pathologies observed in alcoholics and smokers (Howard et al. 2003a).

CYP2E1 levels varies among saline-treated African green monkeybrain regions and expression is cell-type specific (Joshi and Tyndale 2006a). Chronic nicotine treatment induces CYP2E1 expression in the frontal cortex and cerebellum, specifically in cortical pyramidal neurons and cerebellar Purkinje cells but no change is seen in temporal cortex, hippocampus, putamen and thalamus (Joshi and Tyndale 2006a). CYP2E1 expression pattern in monkey brain following chronic nicotine treatment is similar to that in smokers, suggesting that nicotine may be the primary component in cigarette smoke that induces CYP2E1(Joshi and Tyndale 2006a). Increased CYP2E1 in brain may contribute to oxidative stress and alter localized metabolism, and resulting pharmacology, of centrally acting drugs metabolized by CYP2E1 (Joshi and Tyndale 2006a).

Chronic nicotine treatment increases CYP2E1 in rat liver and brain (Joshi and Tyndale 2006b). Chronic 7-day nicotine treatment shows the highest levels of CYP2E1 12 h after the last injection in frontal cortex (1.4-fold) versus 8 h in hippocampus (1.8-fold) and cerebellum (1.4-fold), returning to basal levels by 24 h (Joshi and Tyndale 2006b). In contrast, acute nicotine treatment does not induce CYP2E1 in frontal cortex and hippocampus but increases CYP2E1 in cerebellum 8 h after treatment (1.6-fold) (Joshi and Tyndale 2006b). Brain CYP2E1 mRNA levels does not increase after chronic nicotine treatment, suggesting nontranscriptional regulation (Joshi and Tyndale 2006b). Thus, humans exposed to nicotine may have altered CYP2E1-mediated metabolism of centrally acting drugs and toxins as well as altered toxicity because of oxidative stress caused by CYP2E1 (Joshi and Tyndale 2006b). Those affected may include current and passive smokers and people that may be treated with nicotine such as smokers and, potentially, patients with Alzheimer's, Parkinson's disease, or ulcerative colitis (Joshi and Tyndale 2006b).

Association of CYP2E1 Variant Allele with Alcohol and Nicotine Dependence

The CYP2E1*1D polymorphism has been associated with greater CYP2E1 inducibility (Howard et al. 2003b). The frequency of the variant allele in eight ethnic groups has been determined (Howard et al. 2003b). Further, the Canadian Native Indian, South-east Asian Canadian and Caucasian Canadian groups are stratified by alcohol and nicotine dependence (as measured by DSM-IV criteria) to examine the potential association of CYP2E1*1D with drug dependence (Howard et al. 2003b). A significantly greater frequency of the CYP2E1*1D allele occurs among Indo-Asian Canadians (0.31), Chinese Canadians (0.19), Taiwanese (0.20), Japanese Canadians (0.18), African Americans (0.13), African Canadians (0.10) and Canadian Native Indians (0.09) compared to Caucasian Canadians (0.02)(Howard et al. 2003b). Although the power of the association study is low among some subgroups, the CYP2E1*1D genotype (subjects with at least one variant allele) is associated with alcohol as well as nicotine dependence (Howard et al. 2003b). Specifically, Canadian Native Indians dependent on nicotine alone or alcohol alone exhibit significantly greater CYP2E1*1D frequencies compared to non-drug dependent controls, while the variant frequency among Southeast Asians dependent on nicotine is greater than their non-drug dependent counterparts (Howard et al. 2003b). CYP2E1*1D genotype is associated with significantly greater 3-hydroxycotinine per cigarette in

African Americans (Howard et al. 2003b). The variable frequency of CYP2E1*1D among ethnic groups suggests a greater risk for diseases putatively related to CYP2E1 in some non-Caucasian ethnic groups (Howard et al. 2003b). The association of CYP2E1*1D with alcohol and nicotine dependence suggests that CYP2E1 may contribute to the development of these dependencies (Howard et al. 2003b).

Phenobarbital Mediated Brain CYP2E1 Induction

The effect of chronic phenobarbital treatment on *in vivo* chlorzoxazone disposition, *in vitro* chlorzoxazone metabolism, and hepatic and brain CYP2E1 protein levels in African Green monkeys has been examined (Lee et al. 2006c). Phenobarbital does not induce *in vivo* chlorzoxazone disposition, *in vitro* chlorzoxazone metabolism or hepatic CYP2E1 protein levels in monkeys (Lee et al. 2006c). However, phenobarbital induces brain CYP2E1 protein levels by 1.26-fold in the cerebellum and 1.46-fold in the putamen (Lee et al. 2006c). Phenobarbital also increases cell-specific CYP2E1 expression, for example in the frontal cortical pyramidal neurons and cerebellar Purkinje cells (Lee et al. 2006c). Phenobarbital does not alter hepatic metabolism, but may alter metabolism of CYP2E1 substrates within the brain (Lee et al. 2006c).

J.M. Maher/C.D. Klaasen

Regulation of CYP2E1 by HNF 1 Alpha – A Key Regulator of Glucose Metabolism

The transcription factor HNF1alpha is involved in regulation of glucose metabolism and transport, and in the expression of several drug and bile acid metabolizing enzymes (Maher et al. 2006). Targeted disruption of the HNF1alpha gene results in decreased Cyp1a2, and Cyp2e1 expression, and increased Cyp4a1 and Cyp7a1 expression, suggesting these enzymes are HNF1alpha target genes (Maher et al. 2006).

Y. Horsmans

Down-Regulation of CYP2E1 During the Early Phase of Liver Regeneration

The modification of cytochrome P450 expression in the regenerating liver has been studied (Starkel et al. 2000). Ligation of branches of the portal vein (PBL) perfusing 70% of the liver parenchyma, which produces regeneration and atrophy within the same liver, constitutes an ideal model to study the relative specificity of the early events in the regenerating liver and their relationship to the loss of liver mass (Starkel et al. 2000). In this PBL model and in sham models, the expression and the metabolic activity of CYP2E1 have been studied (Starkel et al. 2000). The metabolic activity of CYP2E1 is transiently and simultaneously down-regulated in the regenerating and atrophying lobes during the first 2–5 h after PBL (Starkel et al. 2000). No significant modification is observed at the protein level. In contrast, iNOS protein is significantly induced in both lobes (Starkel et al. 2000). Similar results are observed after sham operation (Starkel et al. 2000). The reduction of these CYP activities in both lobes after PBL and in sham livers suggests that other mechanisms than the regenerating process itself or the reduction of the liver mass might account for such down-regulation during the early phase of liver regeneration (Starkel et al. 2000). The activation of nitric oxide (NO) and/or pro-inflammatory cytokine

production provides clues to pathways liable to affect the CYP activities in the regenerating liver (Starkel et al. 2000).

R.A. Deitrich

Regulation of CYP2E1: Role of Kinases

Phosphorylation of pure CYP2E1 is achieved *in vitro* using Ca2+/calmodulin-dependent protein kinase II (CaM kinase II), protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) (Menez et al. 1993). CaM kinase II is the most efficient enzyme capable of catalyzing this phosphorylation reaction: the maximum incorporation of 32PO4 is 0.8 mol/mol CYP2E1 in 20 min. PKA phosphorylates a maximum of 0.7 mol of 32PO4/mol of cytochrome within 60 min (Menez et al. 1993). The phosphorylation by PKC reaches a maximum of 0.19 mol of 32PO4/mol of cytochrome and this occurs within a few minutes of incubation (Menez et al. 1993). Limited digestion by *S. aureus* V8 protease (SAP) of CYP2E1, which has been phosphorylated by either PKA and PKC, yields a single major phosphopeptide with an M(r) of approximately 18,000 (Menez et al. 1993). Limited digestion of CYP2E1 that has been phosphorylated by CaM kinase II, yields phosphorylated polypeptides with M(r) of approximately 18,000 and 15,000 (Menez et al. 1993). Thus, the three kinases may be involved in the regulation of CYP2E1 (Menez et al. 1993).

Increased Ethanol Sensitivity in Brain: CYP2E1, A Key Player

The contribution of the different enzymatic pathways to ethanol oxidation in brain homogenates from mice and rats has been investigated (Zimatkin et al. 2006). The catalase inhibitors sodium azide and aminotriazole as well as CYP2E1 inhibitors DAS and beta-PIC significantly lower the accumulation of the ethanol-derived acetaldehyde (AC) and acetate in brain homogenates (Zimatkin et al. 2006). The ADH inhibitor 4-MP significantly decreases the acetate but not the AC accumulation (Zimatkin et al. 2006). Ethanol-derived AC accumulation in brain homogenates of acatalasemic mice is 47% of the control value, 91% in CYP2E1-null mice, and 24% in double mutants (with deficiency of both catalase and CYP2E1) (Zimatkin et al. 2006). The highest levels of ethanol oxidation are found in microsomal and peroxisomal subcellular brain fractions, where CYP2E1 and catalase are located, respectively (Zimatkin et al. 2006).

VI

M. Ingelman-Sundberg

Region Specific Expression of Liver CYP2E1

The acinar distribution of ethanol-inducible cytochrome P450 has been studied by immunohistochemistry using anti-rat CYP2E1 serum (Bühler et al. 1991). In all 17 human liver specimens staining is confined to perivenous hepatocytes (Bühler et al. 1991). Staining is stronger in livers of alcoholics than in non-alcoholics (Bühler et al. 1991). A similar selective perivenous staining is observed in rat liver (Bühler et al. 1991). This pattern is exacerbated by chronic ethanol treatment, with staining appearing especially intense in hepatocytes surrounding the terminal hepatic veins (Bühler et al. 1991). Thus, chronic ethanol intake causes induction of CYP2E1 in

the perivenous region and this regiospecific expression and induction may contribute to the perivenous damage caused by alcohol and several other hepatotoxins known to be metabolized by this enzyme (Bühler et al. 1991).

The regional expression of six different CYP forms in rat liver under constitutive and induced conditions has been compared (Bühler et al. 1992). Immunostaining of consecutive thin sections from control liver reveal that the same hepatocytes, forming a 6–8 cells thick layer surrounding the terminal hepatic venules, are stained for CYP2B1/2, CYP2E1 and CYP3A1 (Bühler et al. 1992). Staining of CYP2A1 extends further into the midzonal region, whereas all cells of the acinus stain for CYPEtOH2 (Bühler et al. 1992). CYPEtOH2 denotes an ethanol-inducible P450 with regulation distinct from CYP2E1 and sequence determination indicates that the protein belongs to the CYP2C family (Bühler et al. 1992). Three distinct patterns of constitutive P450 protein expression: perivenous-restricted (CYP2B1/2, CYP2E1 and CYP3A1); perivenous-dominated (CYP2A1) and panacinar (CYPEtOH2) exist (Bühler et al. 1992). Chronic exposure to ethanol causes induction of CYP2E1 in the same cells already being constitutively expressed, whereas CYPEtOH2 is more induced in the periportal area. Acetone induces CYP2E1, CYP2C11 and CYP3A1 selectively in the perivenous area (Bühler et al. 1992). A particular P450 isozyme is generally induced in the same cells where it is constitutively expressed, and that this regional selectivity is independent of the kind of inducer (Bühler et al. 1992). Thus, during maturation, the hepatocytes acquire various phenotypes in the periportal and perivenous region, to respond differently to endogenous and exogenous signals in the control of P450 expression (Bühler et al. 1992).

Dietary Fat Plus Ethanol Mediated Induction of CYP2E1

Ethanol-induction of apoCYP2B1 (2×) and paranitrophenol hydroxylase (6–8×) is the same for rats fed corn oil and rats fed tallow as the fat component (2×); but not for apoCYP2E1 (21 and 8×, respectively) and MEOS activity (8 and 2.6×, respectively) (Takahashi et al. 1992).

Chlormethiazole: An Effective and Specific Inhibitor of CYP2E1

The effects of a drug used for treatment of ethanol withdrawal states; CMZ on CYP2E1 expression in rat liver has been evaluated (Hu et al. 1994). A 4-fold induction of CYP2E1 is observed after 3 days of starvation, accompanied by a similar increase in the level of the corresponding mRNA (Hu et al. 1994). CMZ specifically inhibits the elevation of CYP2E1 mRNA and protein, but does not prevent CYP2B1 and CYP3A1 or CYP1A1 induction caused by treatment with phenobarbital or beta-naphthoflavone, respectively (Hu et al. 1994). The rate of the CYP2E1 gene transcription is inhibited greatly by CMZ treatment (Hu et al. 1994). Rats treated with ethanol in a total enteral nutrition model have higher CYP2E1-dependent hepatic microsomal activities of p-nitrophenol hydroxylase and carbon tetrachloride-induced lipid peroxidation than controls, and simultaneous CMZ treatment abolishes the ethanol-dependent induction (Hu et al. 1994). *In vitro* experiments with rat liver microsomes show that CMZ does not act as an inhibitor of CYP2E1-dependent catalytic activities or as an inhibitor of microsomal

NADPH and CYP2E1-dependent lipid peroxidation (Hu et al. 1994). Thus, CMZ might constitute an efficient and specific inhibitor of CYP2E1 expression suitable for *in vivo* experiments (Hu et al. 1994).

Modes of Regulation of CYP2E1

CYP2E1 gene has been shown to be transcriptionally activated after birth, but the expression of ethanol-inducible CYP2E1 protein, hereafter, is regulated by post-transcriptional mechanisms (Johansson et al. 1990). The constitutive expression of CYP2E1 protein is restricted to the perivenous region of the liver lobule (Johansson et al. 1990). Studies with in situ hybridization and run off experiments indicate that-this regioselectivity is caused by a higher rate of gene transcription in the perivenous hepatocytes (Johansson et al. 1990). The transcription of the CYP2E1 gene is activated by starvation, indicating that also this P450 gene is under transcriptional control under certain physiological conditions (Johansson et al. 1990).

The regulation of CYP2E1 has been studied by following mRNA levels, catalytic activities and the subcellular distribution of the apoprotein in rat liver at different time points after a single intragastric dose of acetone (Ronis et al. 1991). No changes are observed in hepatic CYP2E1 mRNA levels at any time after acetone treatment, whereas rapid rises are observed in the microsomal amount of CYP2E1 protein and CYP2E1-catalyzed 4-nitrophenol hydroxylase and carbon-tetrachloride-initiated lipid-peroxidation activities (Ronis et al. 1991). However, CYP2E1-dependent catalytic activities decline much faster than the immunodetectable CYP2E1 protein, suggesting that this cytochrome P-450 is inactivated prior to degradation (Ronis et al. 1991). Similar results are seen in primary hepatocyte cultures (Ronis et al. 1991). CYP2E1 is acutely inactivated in the endoplasmic reticulum and that degradation of this isozyme occurs, at least in part, by the lysosomal route (Ronis et al. 1991).

CYP2E1 Overexpression Upregulates Stress Related Genes in Alcohol Induced Liver Injury

Transgenic mice expressing human CYP2E1 treated with ethanol exhibit increased liver injury (Butura et al. 2009). Increased expression of glutathione transferases, monooxidases (several cytochrome P450's included, including Cyp2b9 and Cyp2c55), hydrolases, membrane proteins such as receptors (e.g. prolactin receptor), proteins involved in DNA processing, oxido-reductases and apoptosis-related genes occurs and the expression of structural genes, particularly cytokeratin 8 and 18, is highly related to pathology, suggesting that CYP2E1 overexpression aggravates injury and aids the progression of alcoholic liver disease (Butura et al. 2009).

Mitochondrial Targeting of CYP2E1

The role of the NH(2)-terminus of CYP2E1 in intracellular targeting has been investigated (Neve and Ingelman-Sundberg 1999). In the absence of the hydrophobic NH(2)-terminal sequence, a putative mitochondrial import signal is exposed which targets CYP2E1 to this organelle where it is further processed (Neve and Ingelman-Sundberg 1999).

CYP2E1 lacking the hydrophobic NH(2)-terminal hydrophobic transmembrane domain is specifically targeted to mitochondria, where it is processed to a soluble and catalytically active form (Delta2E1) with a mass of about 40 kDa (Neve and Ingelman-Sundberg 2001). Small amounts of Delta2E1 have been also observed in mitochondria isolated from rat liver, indicating that this form of CYP2E1 is also present *in vivo* (Neve and Ingelman-Sundberg 2001). The mitochondrial targeting signal has been identified and characterized by the use of several NH(2)-terminally truncated and mutated forms of CYP2E1 that are expressed in the mouse H2.35 hepatoma cell line (Neve and Ingelman-Sundberg 2001). Mitochondrial targeting of CYP2E1 is mediated through a sequence located between amino acid residues 74 and 95 and that positively charged residues as well as a hydrophobic stretch present in the beginning of this sequence are essential for this process (Neve and Ingelman-Sundberg 2001).

Transport of CYP2E1 to the Plasma Membrane

The molecular basis for the transport of rat ethanol-inducible CYP2E1 to the plasma membrane has been investigated by transfection of several different mutant cDNAs into mouse H2.35 hepatoma cells (Neve and Ingelman-Sundberg 2000). The NH(2)-terminal transmembrane domain of CYP2E1 plays a crucial role in directing the protein to the cell surface and that topological inversion of a small fraction of CYP2E1 in the endoplasmic reticulum directs the protein to the plasma membrane (Neve and Ingelman-Sundberg 2000).

Polymorphisms in CYP2E1 Gene

In almost 200 individuals belonging to either a Chinese, an Italian, or a Swedish population, two new CYP2E1 gene variants are found with functional mutations: one (CYP2E1*2) in which a G1168A point mutation in exon 2 caused an R76H amino acid substitution, and the other (CYP2E1*3) in which a G10059A base substitution in exon 8 yielded a V3891 amino acid exchange (Hu et al. 1997). The corresponding CYP2E1 cDNAs are constructed, subcloned into the pCMV4 expression vector, and expressed in COS-1 cells (Hu et al. 1997). The CYP2E1*3 cDNA variant is indistinguishable from the wild-type cDNA on all variables investigated, whereas CYP2E1*2 cDNA, although yielding similar amounts of mRNA, only causes 37% of the protein expression and 36% of the catalytic activity compared with the wild-type cDNA (Hu et al. 1997). Complete screening by single-stranded conformation polymorphism of the three populations studied reveals that these variant alleles are rare (Hu et al. 1997). Thus, the human CYP2E1 gene is functionally surprisingly well conserved compared with other cytochrome P450 enzymes active in drug metabolism, which suggests an important endogenous function in humans (Hu et al. 1997).

The 5′-flanking region of the human and rat CYP2E1 genes have been sequenced and characterized (Hu et al. 1999). The identity between the human and rat sequences (−3.8 to +1 kb) is generally between 35 and 60%, and the most similar regions are found in the proximal part of the sequence (Hu et al. 1999). Two more distant regions at −1.6 to −2.0 kb and −2.5 to −2. 8 kb in the human sequence are also found to have high identity to the rat sequence (Hu et al. 1999). A polymorphic repeat

sequence in the human gene is found between −2,178 and −1,945 bp (Hu et al. 1999). The common allele (CYP2E1*1C) contains 6 repeats (each 42–60 bp long) and the rare allele (CYP2E1*1D) have 8 repeats with an allele frequency of 1% among Caucasians and 23% among Chinese (Hu et al. 1999). The CYP2E1 5′-flanking regions of the human (−3,712 to +10 bp) and rat (−3,685 to +28 bp) genes are ligated in front of a luciferase reporter gene and transfected into rat hepatoma Fao and human hepatoma B16A2 cells (Hu et al. 1999). Important species specificity is noted in the control of gene expression and regions of negative and positive cis-acting elements are localized (Hu et al. 1999). No difference is seen in the constitutive expression between the two polymorphic forms (Hu et al. 1999).

Short Half Life of CYP2E1: Mechanism Involved

Ethanol-inducible CYP2E1 involved in the metabolism of gluconeogenetic precursors and some cytotoxins is distinguished from other cytochrome P450 enzymes by its rapid turnover (*in vivo* half-life of 4–7 h), with ligands to the haem iron, both substrates and inhibitors, stabilizing the protein (Zhukov and Ingelman-Sundberg 1999). CYP2E1 is also known to have a high oxidase activity in the absence of substrate, resulting in the production of ROS (Zhukov and Ingelman-Sundberg 1999). The rapid intracellular turnover of the enzyme may be partly due to covalent modifications by such radicals or to other changes during catalytic cycling, in which case the inhibition of electron supply from NADPH-cytochrome P450 reductase would be expected to stabilize the protein (Zhukov and Ingelman-Sundberg 1999). Fao hepatoma cells, where CYP2E1 shows a half-life of 4 h upon serum withdrawal, are treated for 1 h with 0.3 μM diphenylene iodonium (DPI), a suicide inhibitor of flavoenzymes, which results in approximately 90% inhibition of the microsomal NADPH-cytochrome P450 reductase and CYP2E1-dependent chlorzoxazone hydroxylase activities (Zhukov and Ingelman-Sundberg 1999). Subsequent cycloheximide chase reveals that the CYP2E1 half-life increases to 26 h (Zhukov and Ingelman-Sundberg 1999). The short half-life of CYP2E1 *in vivo* may be largely due to the rapid destabilization of the enzyme during catalytic cycling rather than to the intrinsic instability of the protein molecule (Zhukov and Ingelman-Sundberg 1999).

CYP2E1 Mediated Acetaldehyde Metabolism

Liver microsomes from starved and acetone-treated rats catalyze NADPH-support metabolism of acetaldehyde at a rate eightfold higher than corresponding control microsomes (Terelius et al. 1991). CYP2E1 is an aldehyde oxidase and thus metabolizes both ethanol and its primary oxidation product and this might have implications *in vivo* for acetaldehyde metabolism in liver and brain (Terelius et al. 1991).

CYP2E1 Mediated Alkyl Hydrazine Oxidation in Rat Liver Microsomes

CYP2E1, as compared to other rat liver cytochromes P-450-CYP2B1, CYP1A2, CYP2B4 and CYP2C4 is an efficient catalyst of NADPH-dependent oxidation of alkylhydrazines-1-methyl-,1-ethyl-, and 1-isopropylhydrazine to free radical intermediates, in rat liver microsomes and in reconstituted enzyme systems, a finding

that might be of importance in the development of the toxicity of these compounds (Albano et al. 1995).

CYP2E1 Mediated Benzene Oxidation in Rat Liver Microsomes

CYP2B1 and 2E1 oxidize toluene, aniline and monochlorobenzene (MCB) to water-soluble metabolites and to products covalently binding to microsomal proteins from rats at high efficiency (Gut et al. 1996). Oxidation of benzene to covalently binding metabolites is catalysed by CYP2B1 and 2E1 more effectively than the formation of water-soluble metabolites, especially at low benzene levels (Gut et al. 1996). CYP2B1 and 2E1 in rats appears essential for metabolic activation of benzene derivatives to potentially genotoxic products; 1,4-Benzoquinone dominates the covalent binding of benzene to proteins, whereas DNA adducts are largely due to benzene oxide (Gut et al. 1996).

Benzene Oxidation and CYP2E1 Levels in Human Liver Microsomes

In human liver microsomes, the oxidation of benzene, chlorzoxazone, aniline, dimethylformamide, and 4-nitrophenol are significantly correlated with each other and with the level of CYP2E1 protein (Nedelcheva et al. 1999). Moreover, benzene oxidation to water-soluble metabolites is suppressed by 0.1 mM diethyldithiocarbamate, supposedly a specific inhibitor of CYP2E1 at this level (Nedelcheva et al. 1999). None of these metabolic rates correlate with immunochemically determined levels of CYP1A2, 2C9, and 3A4 nor oxidation of 7-ethoxyresorufin, tolbutamide, and nifedipine (Nedelcheva et al. 1999). Benzene oxidation to water-soluble metabolites is characterized by typical Michaelis-Menten kinetics (Nedelcheva et al. 1999). The different benzene K(m) values seen in individual human microsomal samples are not correlated with the level or activity of CYP1A2, 2C9, 2E1, and 3A4 but could be due to CYP2E1 microheterogeneity (Nedelcheva et al. 1999). The lowest K(m) for benzene oxidation could be related to C/D and/or c1/c2 polymorphism of CYP2E1 gene (Nedelcheva et al. 1999). Covalent binding of benzene reactive metabolites to microsomal proteins is also correlated with the CYP2E1 metabolic rates and immunochemical levels (Nedelcheva et al. 1999).

CMZ Mediated Inhibition of CYP2E1: Mechanisms

Ethanol treatment of the Fao rat hepatoma cells causes a twofold induction of CYP2E1 protein levels, which is inhibited by CMZ (Simi and Ingelman-Sundberg 1999). Change of medium unexpectedly causes an increase in CYP2E1 gene transcription (CYP2E1 hnRNA) 4 h later (Simi and Ingelman-Sundberg 1999). However, CMZ fails to influence the expression of CYP2E1 hnRNA or mRNA both constitutively and after medium change, indicating no effect on gene transcription or mRNA synthesis/stability (Simi and Ingelman-Sundberg 1999). Cycloheximide treatment of the cells does not abolish the inhibitory action of CMZ, further indicating an action at the post-translational level (Simi and Ingelman-Sundberg 1999). In addition, CMZ inhibits CYP2E1 expression in V79 cells with stably expressed CYP2E1 under the control of the SV40 promoter (Simi and Ingelman-Sundberg 1999). The CYP2E1 gene is transcriptionally activated in response to medium

change and that CMZ, apart from being a transcriptional inhibitor of CYP2E1 expression, acts in addition as an efficient high-affinity post-translational inhibitor of CYP2E1, probably due to an allosteric destabilization of the enzyme (Simi and Ingelman-Sundberg 1999). This indicates a very rapid and effective CMZ-mediated inhibition of CYP2E1 *in vivo* (Simi and Ingelman-Sundberg 1999).

Presence of Hydroxyethyl Radical-CYP2E1 Adducts in Liver due to Alcohol Consumption

Antibodies from alcoholic cirrhotics specifically recognize hydroxyethyl radical-CYP2E1 adducts, suggesting the possible implication of these antigens in the development of autoimmune reactions in alcoholic liver disease (Clot et al. 1996).

Presence of CYP2E1 Autoantibodies in Liver due to Alcohol Consumption

Among alcoholics, antibodies against ethanol-derived radical protein adducts are observed that are dependent on CYP2E1 for their formation (Lytton et al. 1999). A time-dependent appearance of IgG against rat CYP3A1 and CYP2E1 is evident during chronic ethanol feeding of rats (Lytton et al. 1999). Anti-CYP2E1 reactivity shows positive correlation with the levels of hepatic CYP2E1 and is inhibited by the CYP2E1 transcriptional inhibitor chlormethiazole (Lytton et al. 1999). Screening of the human sera by enzyme-linked immunosorbent assay reveals reactivity against CYP3A4 and CYP2E1 in about 20–30% and 10–20% of the alcoholic sera, respectively (Lytton et al. 1999). Anti-human CYP2E1 reactivity in 8 of 85 alcoholic sera and 3 of 58 control sera is observed (Lytton et al. 1999). Therefore, alcoholics develop autoantibodies against CYP2E1 (Lytton et al. 1999).

Ethanol Mediated Induction of CYP2E1 Is Correlated with Increased Cytokine Expression in Liver

The zonal differences of cytokine expression in rat liver and how these are affected by alcohol exposure and by CMZ, which is a transcriptional and posttranslational inhibitor of hepatic CYP2E1 has been investigated (Fang et al. 1998). Chronic ethanol treatment significantly increases the expression of CYP2E1, microsomal p-nitrophenol hydroxylase activity (indicative for CYP2E1 enzyme activity), and the expression of TGF-beta1, TNF-alpha, and interleukin (IL)-1beta (Fang et al. 1998). CMZ treatment causes a reduction in hepatic CYP2E1 expression and in the ethanol-induced cytokine expression by 40–60% (Fang et al. 1998). Expression of IL-6, IL-2, and IL-4 mRNA occurs preferentially in the periportal region, whereas ethanol causes a pronounced increase in the perivenous expression of TGF-beta1, which is inhibited by CMZ as monitored both on the mRNA and protein levels (Fang et al. 1998). Thus, a link exists between increased CYP2E1 expression and enhanced cytokine expression as important events in the development of ALD.

Degradation of CYP2E1: Mechanisms Involved

Addition of adrenalin to primary rat hepatocytes causes a threefold increase in [32P]-incorporation into CYP2E1 (Johansson et al. 1991). Adrenalin also increases the rate of CYP2E1 degradation at similar concentrations as needed for phosphorylation of the protein ($r = 0.93$) (Johansson et al. 1991). Ethanol (75 mM) completely protects from adrenalin dependent phosphorylation and degradation of CYP2E1

(Johansson et al. 1991). Examination of para-nitrophenol hydroxylase reveals that ethanol stabilizes the catalytically active form of CYP2E1 (Johansson et al. 1991). Insulin treatment causes a stabilization of CYP2E1 (Johansson et al. 1991). Thus, degradation of CYP2E1 is the subject of hormonal control (Johansson et al. 1991).

The differences in post-translational regulation between rat liver ethanol-inducible CYP2E1 and phenobarbital-inducible CYP2B1 using hepatocyte cultures and subcellular fractions, prepared from starved and acetone-treated rats have been investigated (Eliasson et al. 1992). The intracellular degradation of CYP2E1 is rapid (approximate t1/2 = 9 h) and increased by glucagon treatment of the cells in an isozyme-specific manner, whereas CYP2B1 degradation in the same cells, is slower (t1/2 = 21 h) (Eliasson et al. 1992). The glucagon effect on CYP2E1 degradation is abolished by either cycloheximide treatment of cells, indicating the involvement of protein components with rapid turnover, or by lowering of the culture temperature to 23°C (Eliasson et al. 1992). The rapid phase of CYP2E1 degradation is not influenced by inhibitors of the autophagosomal/lysosomal pathway (Eliasson et al. 1992). *In vitro* experiments with isolated liver microsomes reveal the presence of a Mg(2+)-ATP-activated proteolytic system active on CYP2E1, previously modified by phosphorylation on Ser-129 or denatured by reactive metabolites formed from carbon tetrachloride (Eliasson et al. 1992). Imidazole, a CYP2E1 substrate, specifically inhibits the rapid intracellular degradation of CYP2E1 and also prevents phosphorylation and subsequent proteolysis in isolated microsomes (Eliasson et al. 1992). In contrast, no proteolysis of CYP2B1 occurs under the conditions used (Eliasson et al. 1992). The microsomal Mg(2+)-ATP-dependent CYP2E1 proteolysis could not be solubilized with high salt and 0.05% sodium cholate, indicating the action of membrane-integrated protease(s) (Eliasson et al. 1992). The Mg(2+)-ATP-dependent proteolytic system active on CYP2E1 is present in both rough and smooth endoplasmic reticulum (Eliasson et al. 1992). Thus, hepatic cytochromes P450 may be degraded both in a bulk process, according to the autophagosomal/lysosomal pathway and more rapidly, in a hormone- and substrate-regulated fashion, by a specific proteolytic system in the endoplasmic reticulum, active on physiologically or exogenously modified molecules (Eliasson et al. 1992).

Two serine proteinases capable of digesting CYP2E1 have been purified from sodium cholate solubilized rat liver microsomal membranes (Zhukov et al. 1993). The CYP2E1-degrading activity is illustrated through two peaks, and the two proteinases purified have a M(r) of 32,000 on SDS-PAGE, are optimally active at pH 8, and show a susceptibility to inhibitors typical of serine proteinases (Zhukov et al. 1993). CYP2E1 degradation patterns exhibited by the proteinases are identical to each other and similar to that observed during the proteolysis of endogenous CYP2E1 in the microsomal membranes, which indicates that the proteinases can degrade CYP2E1 in its native environment (Zhukov et al. 1993). Thus, a role of these proteinases in the rapid phase of cytochrome P450 degradation in the endoplasmic reticulum has been suggested (Zhukov et al. 1993).

In the Fao hepatoma cell line, CYP2E1 is found to be fairly stable (half-life of 26 h), but serum withdrawal results in its rapid disappearance from the microsomal fraction (half-life of about 7 h) as evaluated using cycloheximide

chase (Zhukov and Ingelman-Sundberg 1997). The effect of serum withdrawal could be partially reversed by the addition of albumin to the culture medium, whereas insulin and the insulin-like growth factor IGF-I have no additional effect (Zhukov and Ingelman-Sundberg 1997). The effect of serum withdrawal is specific for CYP2E1 since (a) no concomitant fast degradation of CYP2B1 and NADPH-cytochrome P-450 reductase is observed and (b) the CYP2E1 ligands ethanol and imidazole prevent the fast degradation of the enzyme (Zhukov and Ingelman-Sundberg 1997). The lysosomotropic agent ammonium chloride and the inhibitor of autophagocytosis 3-methyladenine slows down CYP2E1 degradation by about 30%, while leupeptin has no effect (Zhukov and Ingelman-Sundberg 1997). Under the same conditions, the degradation of total long-lived cell protein shows the same sensitivity to ammonium chloride, but is significantly less sensitive to 3-methyladenine and serum and not sensitive to ethanol and imidazole (Zhukov and Ingelman-Sundberg 1997). CYP2E1 degradation is inhibited by combined treatment with brefeldin A and nocodazole, which blocks both anterograde and retrograde vesicular transport between endoplasmic reticulum and the Golgi apparatus (Zhukov and Ingelman-Sundberg 1997). Thus, a selective mechanism for the degradation of membrane proteins in serum-deprived cells exists in addition to non selective autophagocytosis (Zhukov and Ingelman-Sundberg 1997). The selective degradation of CYP2E1 may be attained by means of its selective vesicular transport to an acidic post-endoplasmic reticulum compartment (Zhukov and Ingelman-Sundberg 1997).

CYP2E1 Expression in Central Nervous System

CYP2E1 and its mRNA are found to be expressed in the rat hippocampus, where the enzyme is localized mainly to the microsomal fraction (Tindberg and Ingelman-Sundberg 1996). Chlorzoxazone (CZN) is 6-hydroxylated in hippocampal homogenates (Tindberg and Ingelman-Sundberg 1996). CYP2E1 is also expressed *in vitro* in cortical glial cultures where CYP2E1 mRNA levels are found to be 1,000-fold lower than in rat liver (Tindberg and Ingelman-Sundberg 1996). Exposure of cortical glial cultures to 25 or 100 mM ethanol for 24 h causes a fourfold and sixfold increase, respectively, in the rate of CYP2E1-dependent 6-hydroxylation of CZN (Tindberg and Ingelman-Sundberg 1996). After a continuous exposure to 100 mM ethanol for 48 or 72 h, however, the hydroxylation rate is down-regulated (Tindberg and Ingelman-Sundberg 1996). CMZ inhibits the ethanol-dependent induction of CYP2E1 by 50% (Tindberg and Ingelman-Sundberg 1996). *In vivo*, acute ethanol treatment of rats results in a 1.8-fold increase in the rate of CZN 6-hydroxylation in hippocampal homogenates (Tindberg and Ingelman-Sundberg 1996). Thus, CYP2E1 is expressed and catalytically active in the rat CNS, and that CYP2E1 can be induced by a relatively low concentration of ethanol in cortical glial cultures (Tindberg and Ingelman-Sundberg 1996).

Inflammatory Factors Upregulate Brain CYP2E1

Lipopolysaccharide and interleukin-1 beta stimulate the expression of catalytically active CYP2E1 (but not CYP1A1 or CYP2B) up tosevenfold in rat brain primary cortical glial cultures (Tindberg et al. 1996). The induction reaches a maximum after

24 h and is accompanied by an increase in CYP2E1 mRNA (Tindberg et al. 1996). CMZ completely inhibits the induction of CYP2E1 at the mRNA and enzyme levels (Tindberg et al. 1996). Immunofluorescence studies show CYP2E1 to be expressed in a subset of astrocytes in the lipopolysaccharide-stimulated cortical glial cultures (Tindberg et al. 1996). In a model of global ischemic injury in the gerbil, CYP2E1 is induced *in vivo* in astrocytes in the inflammatory phase, 1–3 weeks after the lesion (Tindberg et al. 1996). Likewise, CYP2E1 is induced in the rat cortex 1 week after a focal ischemic injury (Tindberg et al. 1996). Thus, tissue-specific regulation of CYP2E1 by inflammatory factors occurs and that CYP2E1 may play a role in astrocytes during inflammation in the brain (Tindberg et al. 1996).

Alcohol Induced Liver Fibrosis Is Associated with the Induction of CYP2E1
A fatty liver with focal necrosis and fibrosis is observed in rats fed ethanol and a high fat diet by continuous intragastric tube feeding for 72 days, maintaining the blood alcohol levels above 200 mg/dl (French et al. 1993). This pathology is associated with an increased total cytochrome P450, an increased CYP2E1 isoenzyme, a decrease in the NADPH-cytochrome P450 reductase activity, an increased rate of NADPH oxidation and an increased NADPH-dependent lipid peroxidation in liver microsomes compared to controls (French et al. 1993). Serum protein adducts with MDA and 4-HNE are significantly increased. Thus, the alcohol-induced liver pathology is associated with the induction of CYP2EI, lipid peroxidation, and protein adduct formation (French et al. 1993). When isoniazid (INH) in therapeutic doses is fed to rats with ethanol, these parameters are changed in that central-central bridging fibrosis is increased, as is lipid peroxidation, whereas INH reduces the ethanol-induced decrease in the reductase, the increase in total P450 and CYP2EI, as well as the NADPH oxidation rate and the elevation of serum transaminase levels (French et al. 1993). Thus a link may exist between central-central bridging fibrosis with increased lipid peroxidation and aldehyde-protein adduct formation caused by ethanol (French et al. 1993).

VII

S.M. Bailey
Induction of CYP2E1 in a NASH Rodent Model
Feeding a HFD to mice for 16 weeks induces NASH-like pathology accompanied by elevated triacylglycerols, increased CYP2E1 and iNOS protein, and significantly enhances hypoxia in the pericentral region of the liver (Mantena et al. 2009). Chronic exposure to a HFD negatively affects the bioenergetics of liver mitochondria and this probably contributes to hypoxic stress and deleterious NO-dependent modification of mitochondrial proteins (Mantena et al. 2009).

Regulation of CYP2E1 in a Hypercholesterolemic Condition Through Combined Exposure to Alcohol and Environmental Tobacco Smoke
It has been investigated whether combined exposure to alcohol and environmental tobacco smoke (ETS) on a hypercholesterolemic background increases liver injury through oxidative/nitrative stress, hypoxia, and mitochondrial damage (Bailey et al. 2009).

Exposure to ethanol+ETS induces the largest increase in CYP2E1 and iNOS protein, as well as increased 3-nitrotyrosine, mtDNA damage, and decreased cytochrome c oxidase protein in male apoprotein E(−/−) mice compared to mice subjected to an ethanol-containing diet or ETS alone (Bailey et al. 2009). Similarly, the largest increase in HIF1alpha expression is observed in the ethanol+ETS group, indicating enhanced hypoxia (Bailey et al. 2009). Thus, ETS increases alcohol-dependent steatosis and hypoxic stress (Bailey et al. 2009). Therefore, ETS may be a key environmental "hit" that accelerates and exacerbates alcoholic liver disease in hypercholesterolemic apoprotein E(−/−) mice (Bailey et al. 2009).

F.J. Gonzalez

Regulation of CYP2E1 by HNFalpha – A Key Player in the Development of Type 3 Diabetes

HNF1alpha is a liver enriched homeodomain-containing transcription factor that has been shown to transactivate the promoters of several CYP genes, including CYP2E1, CYP1A2, CYP7A1, and CYP27, *in vitro* (Cheung et al. 2003). In humans, mutations in HNF1alpha are linked to the occurrence of maturity onset diabetes of the young type 3, an autosomal dominant form of non-insulin-dependent diabetes mellitus in which afflicted subjects generally develop hyperglycemia before 25 years of age (Cheung et al. 2003). Mice lacking HNF1alpha also develop similar phenotypes reminiscent of non-insulin-dependent diabetes mellitus (Cheung et al. 2003). A marked reduction in expression of CYP2E1 gene is observed in the livers of mice lacking HNF1alpha, suggesting a crucial role for HNF1alpha in the regulation of CYP2E1 *in vivo* (Cheung et al. 2003).

Development of CYP2E1-humanized Mouse and Increased Acetaminophen Mediated Hepatotoxicity

A transgenic mouse line expressing the human CYP2E1 gene has been established (Cheung et al. 2005). Human CYP2E1 protein expression and enzymatic activity have been observed in the liver of CYP2E1-humanized mice (Cheung et al. 2005). Treatment of mice with the CYP2E1 inducer acetone demonstrates that human CYP2E1 is inducible in this transgenic model (Cheung et al. 2005). Hepatotoxicity resulting from the CYP2E1-mediated activation of the CYP2E1 substrate acetaminophen has been observed in the livers of CYP2E1-humanized mice (Cheung et al. 2005). Thus, in this humanized mouse model, human CYP2E1 is functional and can metabolize and activate different CYP2E1 substrates such as chlorzoxazone, p-nitrophenol, acetaminophen, and acetone (Cheung et al. 2005).

Development of CYP2E1 Null Mice and Feedback Regulation of CYP2E1 by Acetaminophen

The resistance of Cyp2e1-null mice to APAP treatment has been studied in wild-type and Cyp2e1-null mice (Chen et al. 2008). However, unexpectedly, profiling of major known APAP metabolites in urine and serum reveals that the contribution of CYP2E1 to APAP metabolism decreases with increasing APAP doses administered (Chen et al. 2008). Measurement of hepatic GSH and hydrogen peroxide levels exposes the importance of oxidative stress in determining the consequence of APAP

overdose (Chen et al. 2008). Urinary ions high in wild-type mice treated with 400 mg/kg APAP have been elucidated as 3-methoxy-APAP glucuronide (VII) and three novel APAP metabolites, including S-(5-acetylamino-2-hydroxyphenyl)mercaptopyruvic acid (VI), 3,3′-biacetaminophen (VIII), and a benzothiazine compound (IX) (Chen et al. 2008). Dose-, time-, and genotype-dependent appearance of these minor APAP metabolites implicate their association with the APAP-induced toxicity and potential biomarker application (Chen et al. 2008). Overall, the oxidative stress elicited by CYP2E1-mediated APAP metabolism might significantly contribute to APAP-induced toxicity (Chen et al. 2008).

Acetaminophen: Both a Substrate and Inducer for CYP2E1
Serum metabolomics of APAP-induced hepatotoxicity in control and APAP-treated wild-type and Cyp2e1-null mice reveals that the CYP2E1-mediated metabolic activation and oxidative stress following APAP treatment can cause irreversible inhibition of fatty acid oxidation, potentially through suppression of PPARalpha-regulated pathways (Chen et al. 2009).

M.P. Waalkes
The Nitric Oxide Donor Pro Drug V-PROLI/NO Protects Against Arsenic Induced Hepatotoxicity as an Inducer and Substrate of CYP2E1
O(2)-Vinyl 1-[2-(Carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (V-PROLI/NO) is a nitric oxide (NO) donor prodrug that is metabolized by liver cytochromes P450 to release NO (Qu et al. 2009). V-PROLI/NO increases CYP2E1 transcriptional expression in a dose-dependent manner and CYP2E1 expression is directly related to the level of NO produced and the reduction in arsenic cytotoxicity in HepG2 cells (Qu et al. 2009). The prodrug, V-PROLI/NO, protects against arsenic toxicity in cultured human liver cells, reducing cytolethality, apoptosis and dysregulation of mitogen-activated protein kinases, through generation of NO formed after metabolism by liver cell enzymes, possibly including CYP2E1 (Qu et al. 2009).

N. Chalasani
CYP2E1 Is Induced and Is Associated with Several Metabolic Disarrangements in Non-diabetic Patients with NASH
The hepatic CYP2E1 activity and its correlates in a cohort of nondiabetic patients with NASH (NDN) and controls have been determined to explore its role in the pathogenesis of human NASH (Chalasani et al. 2003). Hepatic CYP2E1 activity assessed through the oral clearance (CL(PO)) of chlorzoxazone (CHZ) in 20 NDN and 17 age, gender, and body mass index (BMI)-matched controls reveals that the CL(PO) of CHZ is significantly greater in NDN compared with controls (Chalasani et al. 2003). Lymphocyte CYP2E1 mRNA is significantly higher in NDN compared with controls (Chalasani et al. 2003). The BMI, respiratory quotient, high-density lipoprotein, triglycerides, insulin, insulin resistance, hypoxemia, and beta-OH butyrate significantly correlate with hepatic CYP2E1 activity (Chalasani et al. 2003). In conclusion, hepatic CYP2E1 activity and lymphocyte CYP2E1 expression are enhanced in NDN (Chalasani et al. 2003). The significant correlations noted between CYP2E1 and hypoxemia and beta-OH butyrate suggest that these factors

play a role in increased CYP2E1 activity that is seen in patients with NASH (Chalasani et al. 2003).

C.J. Omiecinski

Region-Specific CYP2E1 Expression in Brain

The regional and cellular expression patterns of CYP2E1 in human brain tissue have been identified (Farin and Omiecinski 1993). The mRNA expression pattern of CYP2E1 although detected in all brain regions examined, the red nucleus (RN), and substantia nigra (SN) exhibit lower levels of CYP2E1 mRNA expression compared to other regions (Farin and Omiecinski 1993). Thus, localized biotransformation events within the certain central nervous system may account for toxicities initiated by exposure to certain environmental chemicals (Farin and Omiecinski 1993).

Presence of CYP2E1 in Human Umbilical Vein Endothelial Cells

In view of the potential role of CYP2E1 – a biotransformation enzyme in the metabolism of protoxicants in the circulatory system, the CYP2E1 expression has been determined in several primary cultures of human umbilical vein endothelial cells (HUVEC), each established from a different individual (Farin et al. 1994). Constitutive level of CYP2E1 gene expression in HUVEC cultures is evident (Farin et al. 1994). Constitutive CYP2E1 transcript levels are present in all HUVEC cultures examined and vary among individuals (Farin et al. 1994). Thus, human endothelial cells express CYP2E1 gene products and CYP2E1 may play important roles in determining metabolic fates for circulating protoxicants (Farin et al. 1994).

Presence of CYP2E1 in HPV-Expressing Oral and Cervical Epithelial Cells and Its Inducibility by Polycyclic Aromatic Hydrocarbons: *In Vitro* Indicators for Exposure to Tobacco Smoke

Established primary and human papillomaviruses (HPV)-immortalized oral and cervical epithelial cell lines have been analyzed for morphology, mRNA and protein expression patterns of CYP2E1 to study the oral and cervical epithelial-specific expression of CYP2E1 (Farin et al. 1995). Primary human oral and cervical epithelial cells have been immortalized using retroviral infection with HPV-16 E6/E7 genes (Farin et al. 1995). Primary human keratinocyte cells have been immortalized by transfection of HPV-18 and made tumorigenic with nitrosomethylurea treatment (Farin et al. 1995). Expression profile for CYP2E1 has been evaluated in these cultures in the presence or absence of a polycyclic aromatic hydrocarbons (PAH) inducer. CYP2E1 mRNA expression is greatest in the oral epithelial cultures and detectable in all other epithelial cultures except for the HPV-18 immortalized keratinocyte cell line (Farin et al. 1995). CYP2E1 protein is detectable in primary and HPV-immortalized oral and cervical epithelial cultures (Farin et al. 1995). Thus, both primary and HPV immortalized cells appear to express CYP2E1 necessary for the activation of tobacco-specific nitrosamines and PAHs and an *in vitro* system has been characterized which should prove useful in examining interactive mechanisms of HPV with xenobiotic activation in the etiology of squamous cell carcinomas (Farin et al. 1995).

Development of a Quantitative Competitive Reverse Transcriptase-Polymerase Chain Reaction Assay to Determine Hepatic CYP2E1 mRNA

A quantitative competitive RT-PCR (QC RT-PCR) assay has been developed to measure mRNA levels of seven human CYP genes and microsomal epoxide hydrolase (EH) simultaneously (Andersen et al. 1998). This assay employs an exogenous recombinant RNA (rcRNA) molecule as an internal standard that shares PCR primer and hybridization probe sequences with CYP1A1, CYP1A2, CYP2A6/7, CYP2D6, CYP2E1, CYP2F1, CYP3A4/5/7, and EH mRNA (Andersen et al. 1998). Because each rcRNA molecule contains several primer sequences, an entire battery of genes that exhibit differential responsiveness to various classes of xenobiotics may be measured simultaneously from one population of cDNA molecules (Andersen et al. 1998). The quantitative profiles of CYP and EH mRNA abundance in eight human livers has been demonstrated. CYP2E1 mRNA maintains the highest abundance and least variation in all livers examined (Andersen et al. 1998). CYP1A2, CYP2A6/7, CYP2D6, CYP3A4/5, and EH mRNAs are approximately one order of magnitude less abundant than CYP2E1 transcripts, with CYP2D6 levels exhibiting the greatest variation between individuals (Andersen et al. 1998). This QC RT-PCR assay may prove valuable for measuring basal and induced mRNAs in different cell types in vitro, as well as in biomonitoring applications where individuals are exposed or hypersusceptible to certain xenobiotic-initiated toxicities (Andersen et al. 1998).

Phosphatidylinositol 3′ Kinase and Hepatic CYP2E1

Insulin-associated signaling pathways are crucial in the regulation of hepatic physiology (Sidhu et al. 2001). Recent inhibitor-based studies have implicated a mechanistic role for phosphatidylinositol 3′ kinase (PI3K) in the insulin-mediated suppression of CYP2E1 mRNA levels in hepatocytes (Sidhu et al. 2001). The dose dependence for this response and for the effects of insulin and extracellular matrix on PI3K signaling and CYP2E1 mRNA expression levels has been determined in a highly defined rat primary hepatocyte culture system (Sidhu et al. 2001). Although PI3K inhibitors wortmannin and LY294002 reverse the suppressive effects of insulin on CYP2E1 expression, these effects only occur at concentrations well in excess of those required to achieve complete inhibition of protein kinase B (PKB) phosphorylation (Sidhu et al. 2001). These same concentrations produce cytotoxic responses as evidenced by perturbed cellular morphology and elevated release of lactate dehydrogenase (Sidhu et al. 2001). Thus, the suppression of CYP2E1 mRNA expression by insulin is not directly associated with PI3K-dependent pathway activation, but rather is linked to a cytotoxic response stemming from acute challenge with PI3K inhibitors (Sidhu et al. 2001).

D.J. Petersen

Ethanol Mediated Aldehyde Protein Adduct Formation Is Associated with Induction of CYP2E1 in Liver

The hepatocellular formation of aldehyde-protein adducts during early stages of alcohol-induced liver injury has been characterized (Sampey et al. 2003). After 36 days of treatment, rats receiving the intragastric administration of a total enteral nutrition diet containing ethanol display hepatic histopathologies characterized by marked micro- and

macrosteatosis associated with only minor inflammation and necrosis (Sampey et al. 2003). Alcohol administration results in a threefold elevation of plasma alanine aminotransferase activity and threefold increases in hepatic CYP2E1 apoprotein and activity (Sampey et al. 2003). Significant fivefold increases in MDA- and 4-HNE modified proteins are detected in liver sections prepared from rats treated with alcohol (Sampey et al. 2003). The MDA- or 4-HNE modified proteins are contained in hepatocytes displaying intact morphology and are colocalized primarily with microvesicular deposits of lipid (Sampey et al. 2003). Alcohol-induced lipid peroxidation is an early event during alcohol-mediated liver injury and may be a sensitizing event resulting in the production of bioactive aldehydes that have the potential to initiate or propagate ensuing proinflammatory or profibrogenic cellular events (Sampey et al. 2003).

Induction of Hepatic CYP2E1 by Ethanol Is Associated with Decreased Peroxiredoxin 6 Expression

Periredoxins constitute a family of antioxidant enzymes. The effect of chronic ethanol treatment on inducing hepatic oxidative stress and peroxiredoxin 6 (PRX6) expression has been investigated (Roede et al. 2008). After 9 weeks of treatment with an ethanol-containing diet, significant increases in serum ALT activity, liver to body weight ratio, liver triglycerides, CYP2E1 protein expression, and CYP2E1 activity are observed (Roede et al. 2008). Chronic ethanol feeding results in oxidative stress as evidenced by decreases in hepatic GSH content and increased deposition of 4-hydroxynonenal and 4-oxononenal protein adducts (Roede et al. 2008). In addition, novel findings of decreased PRX6 protein and mRNA and increased levels of carbonylated PRX6 protein are observed in the ethanol-treated animals compared to the pair-fed controls (Roede et al. 2008). Lastly, NF-kappaB activity is found to be significantly increased in the ethanol-treated animals (Roede et al. 2008). Chronic ethanol treatment results in oxidative stress, implicating NF-kappaB activation as an integral mechanism in the negative regulation of PRX6 gene expression in the mouse liver (Roede et al. 2008). Thus, ethanol mediated hepatic CYP2E1 induction may be accompanied by downregulation of PRX6 gene expression.

M.R. Juchau

Presence of CYP2E1 in Prenatal Human Cephalic Tissues

RT-PCR with oligonucleotide primers designed to target cDNA nucleotides 1,241–1,357 corresponding to exons 8 (3′ end) and 9 (5′ end) in human genomic CYP2E1 detect consistently strong signals in 9 of 10 prenatal human brains (Boutelet-Bochan et al. 1997). Cephalic tissues analyzed are between 54 and 78 days of gestation (Boutelet-Bochan et al. 1997). RT-PCR signals for expression of CYP2E1 in corresponding human hepatic or adrenal tissues are weaker or, with only two exceptions, undetectable (Boutelet-Bochan et al. 1997). CYP2E1 mRNA levels in human prenatal whole brain tissues tend to increase as a function of gestational age but, at the early stages investigated, are far lower than the constitutive levels in hepatic tissues of adult humans or male rats (Boutelet-Bochan et al. 1997). Localized CYP2E1-dependent cephalic bioactivation of ethanol, with associated generation of several reactive chemical species, could contribute significantly to the etiology of neuroembryotoxic effects of prenatal ethanol exposure (Boutelet-Bochan et al. 1997).

Inhibition of Human Prenatal Hepatic All-Trans-Retinoic Acid Formation by Ethanol Mediated Lipid Peroxidation Products Could Be Possibly Linked to Increased Generation of Lipid Peroxides by Ethanol-Inducible CYP2E1

Biotransformation of all-trans-retinol (t-ROH) and all-trans-retinal (t-RAL) to all-trans-retinoic acid (t-RA) in human prenatal hepatic tissues (53–84 gestational days) has been investigated using human adult hepatic tissues as positive controls (Khalighi et al. 1999). Catalysis of the biotransformation of t-ROH by prenatal human cytosolic fractions results in accumulation of t-RAL with minimal t-RA (Khalighi et al. 1999). Oxidations of t-ROH catalyzed by prenatal cytosol are supported by both NAD+ and NADP+, although NAD+ is a much better cofactor (Khalighi et al. 1999). In contrast, catalysis of the oxidation of t-RAL to t-RA appears to be solely NAD+ dependent (Khalighi et al. 1999). Substrate Km values for conversions of t-ROH to t-RAL and of t-RAL to t-RA are 82.4 and 65.8 μM, respectively (Khalighi et al. 1999). At concentrations of 10 and 90 mM, ethanol inhibits the conversion of t-ROH to t-RAL by 25 and 43%, respectively, but does not inhibit the conversion of t-RAL to t-RA significantly (Khalighi et al. 1999). In contrast, acetaldehyde reduces the conversion of t-RAL to t-RA by 25 and 87% at 0.1 and 10 mM respective concentrations (Khalighi et al. 1999). Several alcohols and aldehydes known to be generated from lipid peroxides also exhibit significant inhibition of t-RA biosynthesis in human prenatal hepatic tissues (Khalighi et al. 1999). Among the compounds tested, 4-HNE is highly effective in inhibiting the conversion of t-RAL to t-RA (Khalighi et al. 1999). A 20% inhibition is observed at a concentration of only 0.001 mM, and nearly complete inhibition is produced at 0.1 mM (Khalighi et al. 1999). Human fetal and embryonic hepatic tissues each exhibit significant CYP2E1 protein and mRNA expression and chlorzoxazone 6-hydroxylation suggesting that lipid peroxidation can be initiated via CYP2E1-catalyzed ethanol oxidation in human embryonic hepatic tissues (Khalighi et al. 1999). Thus, ethanol may affect the biosynthesis of t-RA in human prenatal hepatic tissues directly and indirectly (Khalighi et al. 1999). Ethanol and its major oxidative metabolite, acetaldehyde, both inhibit the generation of t-RA (Khalighi et al. 1999). Concurrently, the CYP2E1-catalyzed oxidation of ethanol can initiate lipid peroxidation via generation of a variety of free radicals (Khalighi et al. 1999). The lipid peroxides thereby generated could then be further converted *via* CYP2E1-catalyzed reactions to alcohols and aldehydes, including 4-HNE, that act as potent inhibitors of t-RA synthesis (Khalighi et al. 1999).

Quantitative Assessment of CYP2E1 in Human Cephalic Tissues

The proposed role of CYP2E1 in the etiology of alcohol teratogenesis ie. its significant contribution to ethanol metabolism and the formation of the highly reactive metabolite acetaldehyde and the leaky property of this enzyme which results in the generation of ROS that can induce oxidative stress and cytotoxic conditions deleterious to development has been investigated (Brzezinski et al. 1999). CYP2E1 has been quantified in prenatal human brain, a tissue that is highly vulnerable to the damaging effects of ethanol throughout gestation (Brzezinski et al. 1999). In microsomal samples prepared from pools of brain tissues, both immunoreactive and functional proteins have been detected (Brzezinski et al. 1999). CYP2E1 transcript

is consistently detected in RNA samples prepared from individual brain tissues (Brzezinski et al. 1999). There is a dramatic increase in human brain CYP2E1 content around gestational day 50 and a fairly constant level is maintained throughout the early fetal period, until at least day 113 (Brzezinski et al. 1999). The relatively low levels of the CYP isoform present in conceptal brain may be sufficient to generate reactive intermediates that elicit neuroembryotoxicity following maternal alcohol consumption (Brzezinski et al. 1999).

R.G. Thurman
Formation of Aldehyde Protein Adducts Is Associated with Induction of Hepatic CYP2E1
Expression of both CYP2E1 and CYP3A correlates with the amount of acetaldehyde, MDA, and 4-HNE adducts in the liver of rats fed alcohol with a high-fat diet for 2–4 weeks according to the Tsukamoto-French procedure (Niemelä et al. 1998). Distinct CYP2E1-positive immunohistochemistry is seen in 3 of 7 of the ethanol-fed animals. In 5 of 7 of the ethanol-fed animals, the staining intensities for CYP3A markedly exceed those obtained from the controls (Niemelä et al. 1998). Thus, acetaldehyde and lipid peroxidation-derived adducts are generated in the early phase of alcohol-induced liver disease (Niemelä et al. 1998). The formation of protein adducts appears to be accompanied by induction of both CYP2E1 and CYP3A (Niemelä et al. 1998).

M. Murray
Differential Regulation of CYP2E1 in Obesity and Diabetes
In obese male Zucker rats, a significant decrease in the catalytic activities of hepatic NDMA demethylase (CYP2E1/P-450j) and aniline p-hydroxylase occurs (Zaluzny et al. 1990). In streptozotocin-induced diabetic male Wistar rats, significant increases in the rates of hepatic N-nitrosodimethylamine demethylase and aniline p-hydroxylase occur (Zaluzny et al. 1990). Further, significant correlation is found between serum concentrations of insulin and catalytic activity of P-450j (Zaluzny et al. 1990).

L.H. Lash
Renal CYP2E1 Expression
CYP2E1 protein is expressed in rat kidney microsomes at approximately 10% of hepatic levels (Cummings et al. 1999). Microsomes from renal cortical, proximal tubular (PT) and distal tubular (DT) cells all express CYP2E1, with DT microsomes expressing slightly higher levels than PT microsomes (Cummings et al. 1999). In contrast, chlorzoxazone hydroxylation activity is markedly higher in microsomes from PT cells than in those from DT cells (Cummings et al. 1999). A pattern of CYP2E1 mRNA distribution similar to that of CYP2E1 protein is observed in PT and DT cells (Cummings et al. 1999).

Renal and Hepatic CYP Mediated Metabolism of Trichloroethylene
Pretreatment of rats with pyridine increases trichloroethylene (Tri) metabolite chloral hydrate (CH) formation in both liver and kidney microsomes, whereas pretreatment of rats with clofibrate increases CH formation only in kidney microsomes (Cummings et al. 2001). Pyridine increases CYP2E1 expression in both rat liver and

kidney microsomes, whereas clofibrate has no effect on hepatic but increases renal CYP2E1 protein level (Cummings et al. 2001). Studies with the general P450 inhibitor SKF-525A and the CYP2E1 competitive substrate chlorzoxazone provide additional support for the role of CYP2E1 in both tissues (Cummings et al. 2001). However, pretreatment of rats with either pyridine or clofibrate has no effect on CYP2E1 or CYP2C11 protein levels or on CH formation in isolated cells (Cummings et al. 2001). Thus, Tri can be metabolized to at least one of its CYP metabolites in the kidneys (Cummings et al. 2001).

CYP2E1 Mediated Tri- and Perchloroethylene Metabolism

Modulation of P450 status in hepatocytes produces larger changes in Tri- and perchloroethylene (Perc)-induced cytotoxicity than in kidney cells, with non-selective P450 inhibitors increasing toxicity (Lash et al. 2007). Induction of CYP2E1 with pyridine also markedly increases sensitivity of hepatocytes to Tri but has little effect on Perc-induced cytotoxicity (Lash et al. 2007).

I. Rusyn

Crucial Role of Hepatic CYP2E1 in Oxidative DNA Damage

In rats and wild-type mice, Tsukamoto-French model of intragastric ethanol infusion treatment for 4 weeks leads to an increase in oxidative DNA damage and induction of expression of the base excision DNA repair genes that are known to remove oxidative DNA lesions (Bradford et al. 2005). No increase in either of the endpoints is observed in ethanol-treated Cyp2e1-null mice, whereas the magnitude of response in p47(phox)-null mice and transgenic hCyp2e1 is identical to that in wild types (Bradford et al. 2005). The increase in expression of DNA repair genes is completely abolished by treatment with the P450 inhibitor 1-aminobenzotriazole (Bradford et al. 2005). In conclusion, oxidative stress to DNA is induced in liver by ethanol (Bradford et al. 2005). Furthermore, although it has been shown that nicotinamide adenine dinucleotide phosphate oxidase-derived oxidants are crucial for the development of ethanol-induced liver injury, CYP2E1 is required for the induction of oxidative stress to DNA, and thus may play a key role in ethanol-associated hepatocarcinogenesis (Bradford et al. 2005).

M.A. Correia

Ubiquitination of CYP2E1 – Mechanisms Involved

CYP2E1 substrate complexation converts it into a stable slow-turnover species degraded largely *via* autophagic lysosomal degradation (Wang et al. 2011). Substrate decomplexation/withdrawal results in a fast turnover CYP2E1 species, putatively generated through its futile oxidative cycling, that incurs endoplasmic reticulum-associated ubiquitin-dependent proteasomal degradation (UPD). CYP2E1 thus exhibits biphasic turnover in the mammalian liver (Wang et al. 2011). The heterologous expression of human CYP2E1 in *Saccharomyces cerevisiae* shows that its autophagic lysosomal degradation and UPD pathways are evolutionarily conserved, even though its potential for futile catalytic cycling is low due to its sluggish catalytic activity in yeast (Wang et al. 2011). This suggests that other factors (i.e. post-translational modifications or "degrons") contribute to its UPD (Wang et al. 2011).

Indeed, in cultured human hepatocytes, CYP2E1 is detectably ubiquitinated, and this is enhanced on its mechanism-based inactivation (Wang et al. 2011). Studies in Ubc7p and Ubc5p genetically deficient yeast strains versus corresponding isogenic wild types identify these ubiquitin-conjugating E2 enzymes as relevant to CYP2E1 UPD (Wang et al. 2011). Consistent with this, *in vitro* functional reconstitution analyses reveal that mammalian UBC7/gp78 and UbcH5a/CHIP E2-E3 ubiquitin ligases are capable of ubiquitinating CYP2E1, a process enhanced by protein kinase (PK) A and/or PKC inclusion (Wang et al. 2011). Inhibition of PKA or PKC blocks intracellular CYP2E1 ubiquitination and turnover (Wang et al. 2011). Through mass spectrometric analyses, some CYP2E1 phosphorylation/ubiquitination sites in spatially associated clusters have been identified (Wang et al. 2011). Thus, these CYP2E1 phosphorylation clusters may serve to engage each E2-E3 ubiquitination complex *in vitro* and intracellularly (Wang et al. 2011).

P. Saenger

Type 1 Diabetes Mediated Regulation of Lymphocyte CYP2E1

CYP2E1 is imeasured in peripheral lymphocytes of 14 patients with uncontrolled insulin-dependent diabetes mellitus (Song et al. 1990). Only one major form (mol wt, 48,000 Da) of CYP2E1 is detected with a specific polyclonal antibody against CYP2E1 (Song et al. 1990). Levels of CYP2E1 are very low to undetectable in human lymphocytes from seven normal subjects (Song et al. 1990). However, levels of CYP2E1 are elevated in lymphocytes from patients with insulin-dependent diabetes mellitus (Song et al. 1990). Elevated levels of CYP2E1 protein correlate positively with the levels of hemoglobin A1, a metabolic indicator in diabetic subjects (Song et al. 1990). In one study subject in whom diabetic control is improved, the drop in hemoglobin A1C levels is accompanied by normalization of CYP2E1 levels (Song et al. 1990).

M.J. Czaja

TNF Alpha and CYP2E1 Mediated Hepatotoxicity: Close Partners

TNF-alpha-treatment of rat hepatocyte cell line RALA255–10G transfected with pCI-neo expression vectors (containing the human CYP2E1 cDNA in either a sense or antisense orientation resulting in differential CYP2E1 expression) demonstrate that overexpression of CYP2E1 converts the hepatocyte TNF-alpha response from proliferation to apoptotic and necrotic cell death (Liu et al. 2002). Death occurs despite the presence of increased levels of NF-kappaB transcriptional activity and is associated with increased lipid peroxidation and GSH depletion (Liu et al. 2002). CYP2E1-overexpressing hepatocytes have increased basal and TNF-alpha-induced levels of c-Jun NH(2)-terminal kinase (JNK) activity, as well as prolonged JNK activation after TNF-alpha stimulation (Liu et al. 2002). Sensitization to TNF-alpha-induced cell death by CYP2E1 overexpression is inhibited by antioxidants or adenoviral expression of a dominant-negative c-Jun. Increased CYP2E1 expression sensitizes hepatocytes to TNF-alpha toxicity mediated by c-Jun and overwhelming oxidative stress (Liu et al. 2002). Thus, the chronic increase in intracellular oxidant stress created by CYP2E1 overexpression may serve as a mechanism by which hepatocytes are sensitized to TNF-alpha toxicity in liver disease (Liu et al. 2002).

Hepatic CYP2E1 and Oxidative Stress Mediated Injury: *In Vitro* Evidence

To determine the effect of CYP2E1 expression on the hepatocellular response to injury, stably transfected hepatocytes expressing increased (S-CYP15) and decreased (AN-CYP10) levels of CYP2E1 are generated from the rat hepatocyte line RALA255-10G (Jones et al. 2002). S-CYP15 cells have increased levels of CYP2E1 protein and mRNA, catalytic activity, and increased cell sensitivity to death from acetaminophen (Jones et al. 2002). Death in S-CYP15 cells is significantly decreased relative to that in AN-CYP10 cells following treatment with hydrogen peroxide and the superoxide generator menadione (Jones et al. 2002). S-CYP15 cells undergo apoptosis in response to these ROS, whereas AN-CYP10 cells die by necrosis (Jones et al. 2002). This differential sensitivity to ROS-induced cell death is partly explained by markedly decreased levels of GSH in AN-CYP10 cells (Jones et al. 2002). However, chemically induced GSH depletion triggers cell death in S-CYP15 but not AN-CYP10 cells (Jones et al. 2002). Increased expression of CYP2E1 confers hepatocyte resistance to ROS-induced cytotoxicity, which was mediated in part by GSH (Jones et al. 2002). However, CYP2E1 over-expression leaves cells vulnerable to death from GSH depletion (Jones et al. 2002).

Induced Hepatic CYP2E1 and ERK Activation

Chronic CYP2E1 overexpression leads to sustained extracellular signal-regulated kinase 1/2 (ERK1/2) activation mediated by epidermal growth factor receptor (EGFR)/c-Raf signaling in CYP2E1 over-expressing hepatocytes (Schattenberg et al. 2004). This adaptive response in hepatocytes exposed to chronic oxidative stress confers differential effects on cellular survival, protecting against menadione-induced apoptosis, but sensitizing to necrotic death from PUFA (Schattenberg et al. 2004).

Induction of Hepatic CYP2E1 and Downregulation of Insulin Signaling

The effects of *in vitro* and *in vivo* CYP2E1 overexpression on hepatocyte insulin signaling have been examined (Schattenberg et al. 2005). CYP2E1 overexpression in a hepatocyte cell line decreases tyrosine phosphorylation of insulin receptor substrate IRS-1 and IRS-2 in response to insulin (Schattenberg et al. 2005). CYP2E1 overexpression is also associated with increased inhibitory serine 307 and 636/639 IRS-1 phosphorylation (Schattenberg et al. 2005). In parallel, the effects of insulin on Akt activation, glycogen synthase kinase 3, and FoxO1a phosphorylation, and glucose secretion are all significantly decreased in CYP2E1 overexpressing cells (Schattenberg et al. 2005). This inhibition of insulin signaling by CYP2E1 overexpression is partially c-Jun N-terminal kinase dependent (Schattenberg et al. 2005). In the methionine- and choline-deficient diet mouse model of steatohepatitis with CYP2E1 overexpression, insulin-induced IRS-1, IRS-2, and Akt phosphorylation are similarly decreased (Schattenberg et al. 2005). Thus increased hepatocyte CYP2E1 expression and the presence of steatohepatitis result in the down-regulation of insulin signaling, potentially contributing to the insulin resistance associated with NAFLD (Schattenberg et al. 2005).

Hydroxynonenal Mediated Hepatic Toxicity and CYP2E1

The effect of HNE on hepatocyte injury and JNK activation has been examined in cells under chronic oxidant stress from overexpression of the prooxidant enzyme

CYP2E1, which occurs in NAFLD (Singh et al. 2009). CYP2E1-generated oxidant stress sensitizes a rat hepatocyte cell line to death from normally nontoxic concentrations of HNE (Singh et al. 2009). CYP2E1-overexpressing cells undergo a more profound depletion of GSH in response to HNE secondary to decreased gamma-glutamylcysteine synthetase activity (Singh et al. 2009). GSH depletion leads to overactivation of JNK/c-Jun signaling at the level of mitogen-activated protein kinase kinase 4 that induces cell death (Singh et al. 2009). Oxidant stress and the lipid peroxidation product HNE cause synergistic overactivation of the JNK/c-Jun signaling pathway in hepatocytes, demonstrating that HNE may not be just a passive biomarker of hepatic oxidant stress but rather an active mediator of hepatocellular injury through effects on JNK signaling (Singh et al. 2009).

H.M. Mehendale

Thioacetamide Mediated Hepatotoxicity and Diabetes: Crucial Role of CYP2E1

Thioacetamide (TA)-induced hepatotoxicity is potentiated in STZ-induced diabetic rats (Wang et al. 2000). Hepatic CYP2E1 appears to be primarily involved in bioactivation of TA (Wang et al. 2000). In the STZ-induced diabetic rat, diabetes-induced CYP2E1 appears to be responsible for the potentiated liver injury; even though hepatic flavin-containing monooxygenase (FMO1) is induced in the diabetic rat, it is unlikely to mediate the potentiated TA hepatotoxicity (Wang et al. 2000).

Thioacetamide Mediated Hepatotoxicity and Diet Restriction: Crucial Role of CYP2E1

A 4.6-fold increase in CYP2E1 protein, which corresponds with a threefold increase in CYP2E1 activity as measured by chlorzoxazone hydroxylation is observed in rats maintained on dietrestriction (DR, 35% of ad libitum fed rats, 21 days) (Ramaiah et al. 2001). A single administration of 50 mg of TA/kg is given to rats 24 and 18 h after pretreatment with pyridine (PYR) and isoniazid (INZ), specific inducers of CYP2E1 (Ramaiah et al. 2001). TA liver injury is >2.5- and >3-fold higher at 24 h in PYR + TA and INZ + TA groups, respectively, compared with the rats receiving TA alone (Ramaiah et al. 2001). Pyridine pretreatment results in significantly increased total CYP450 content accompanied by a 2.2-fold increase in CYP2E1 protein and twofold increase in enzyme activity concordant with increased liver injury of TA, suggesting mechanism-based bioactivation of TA by CYP2E1 (Ramaiah et al. 2001). Hepatic injury of TA in DR rats pretreated with DAS is significantly decreased (60%) at 24 h. CCl(4) (4 ml/kg i.p.), a known substrate of CYP2E1, causes lower liver injury and higher animal survival confirming inhibition of CYP2E1 by DAS pretreatment (Ramaiah et al. 2001). Thus, induction of CYP2E1 may account as the primary mechanism of increased bioactivation-based liver injury of TA in DR rats (Ramaiah et al. 2001).

Thioacetamide Bioactivation and CYP2E1: Conclusive Evidences

The role of CYP2E1 using cyp2e1 knockout mice (KO) in TA mediated liver injury has also been investigated (Chilakapati et al. 2007). Injury assessed over time (0–48 h) in wild type (WT) and KO mice after LD(100) dose (500 mg/kg) in WT

reveals that while WT mice exhibit robust injury which progress to death, KO mice exhibit neither initiation nor progression of injury (Chilakapati et al. 2007). Thus, CYP2E1 is responsible for TA bioactivation (Chilakapati et al. 2007).

VIII

P.K. Seth

Lymphocyte CYP2E1 Mediated Lipid Peroxidation

Rat blood lymphocytes catalyse NADPH dependent (basal) lipid peroxidation and demethylation of NDMA (Dey et al. 2002). Treatment of rats with ethanol or pyrazole or acetone results in significant increase in the NADPH dependent lipid peroxidation and the activity of NDMA-d in blood lymphocytes (Dey et al. 2002). *In vitro* addition of CCl(4) to the blood lymphocytes isolated from control or ethanol pretreated rats results in an increase in the NADPH dependent lipid peroxidation (Dey et al. 2002). Significant inhibition of the basal and CCl(4) supported NADPH dependent lipid peroxidation and NDMA-d activity in blood lymphocytes isolated from control or ethanol pretreated rats by dimethyl formamide or dimethyl sulfoxide or hexane, solvents known to inhibit CYP2E1 catalysed reactions in liver and anti-P450 2E1, indicate the role of CYP2E1 in the NADPH dependent lipid peroxidation in rat blood lymphocytes (Dey et al. 2002). Similarities in the NADPH dependent lipid peroxidation and NDMA-d activity in blood lymphocyte with the liver microsomes suggest that blood lymphocyte CYP 2E1 could be used as a surrogate to monitor and predict hepatic levels of the enzyme (Dey et al. 2002).

D. Parmar/P.K. Seth

Lymphocyte CYP2E1: A Suitable Marker for the Hepatic Enzyme

Freshly isolated rat blood lymphocytes are characterized by the presence of significant mRNA of CYP2E1 in control rats (Dey et al. 2005). Lymphocyte CYP2E1 demonstrates significant immunoreactivity, comigrating with the liver isoenzyme, in freshly isolated control rat blood lymphocytes (Dey et al. 2005). Similar to that observed in liver, ethanol treated rat blood lymphocytes exhibit increased CYP2E1 isoenzyme (Dey et al. 2005). Blood lymphocytes are also found to catalyze the CYP2E1 dependent N-demethylation of NDMA which increases with known CYP2E1 inducers (Dey et al. 2005). Significant increase in the apparent Vmax and the affinity towards the substrate in rat blood lymphocytes occurs indicating that as observed in liver, the increase in mRNA and protein expression following exposure to CYP2E1 inducers is associated with the increased catalytic activity of CYP2E1 in freshly isolated rat blood lymphocytes (Dey et al. 2005). Thus, similarities of the blood lymphocyte CYP2E1 with the liver enzyme suggest that lymphocyte CYP2E1 levels in freshly isolated rat blood lymphocytes could be used to monitor tissue enzyme levels (Dey et al. 2005).

D. Parmar

Brain CYP2E1 Expression: Region Specific Phenomenon

Rat olfactory lobes exhibit the highest CYP2E1 expression and catalytic activity in control rats (Yadav et al. 2006). Furthermore, several fold increase in the mRNA expression and activity of CYP2E1 in cerebellum and hippocampus while

a relatively small increase in the olfactory lobes and no significant change in other brain regions following ethanol pretreatment indicate that CYP2E1 induction may be involved in selective sensitivity of these brain areas to ethanol induced free radical damage and neuronal degeneration (Yadav et al. 2006).

***In Vitro* Evidence for Brain CYP2E1**

The expression and catalytic activity of the constitutive and inducible forms of CYP2E1 in cultured rat brain neuronal and glial cells has been characterized (Kapoor et al. 2006). These cells exhibit relatively twofold higher activity of NDMA-d when compared with the liver enzyme (Kapoor et al. 2006). Pretreatment with ethanol reveals a significant time and concentration dependent induction in NDMA-d activity in both cell types (Kapoor et al. 2006). Significant induction of CYP2E1 protein and mRNA occurs in the cultured brain cells (Kapoor et al. 2006). Interestingly, the neuronal cells exhibit greater magnitude of induction than the glial cells (Kapoor et al. 2006). The relatively higher degree of induction in cultures of neurons indicates enhanced sensitivity of neurons to the inductive effects of ethanol (Kapoor et al. 2006). This enhanced induction of CYP2E1 in neuronal cells indicates that like regional specificity, cell specificity also exists in the induction of CYP2E1 (Kapoor et al. 2006).

Induction of Fetal Brain CYP2E1 with the Pesticide Lindane

Low dose prenatal exposure to the pesticide lindane has the potential to produce overexpression of xenobiotic metabolizing CYP1A, 2B and 2E1 isoenzymes in brain and liver of the rat offsprings which may account for the behavioral changes observed in the rat offsprings (Johri et al. 2007).

Parkinson's Disease Is Associated with CYP2E1*5B Allele

The presence of four combinations of glutathione S-transferase T1 (GSTT1) null and manganese-superoxide dismutase MnSOD(−9Val)/GST null and monoamine oxidase-B(MAO-B) variant allele G (MAOB-G)/CYP2E1*5B (RsaI) and MAO-B-AG/CYP2E1*5B and dopamine receptor-D2 (DRD2) (Taq1A-het) genotypes in the patients exhibit several fold higher and significant association with risk to Parkinson's disease (PD) (Singh et al. 2008). Polymorphism in the genes involved in detoxification and dopamine regulation may modulate the susceptibility to PD and could be important risk factors in the pathogenesis of PD (Singh et al. 2008).

Head and Neck Squamous Cell Carcinoma and CYP2E1 Polymorphism

A significant increase in Head and Neck Squamous Cell Carcinoma (HNSCC) risk occurs in cases with variant genotypes of CYP2E1*5B (RsaI) and CYP2E1*6 (DraI) (Ruwali et al. 2009). Haplotype T-A is associated with a greater than tenfold increase in risk for HNSCC (Ruwali et al. 2009). A several fold increase in HNSCC risk in cases carrying a combination of variant genotypes of CYP2E1 with the null genotype of glutathione-S-Transferase M1 (GSTM1) or X-Ray Repair Cross Complementing Group I (XRCC1) variant genotypes occurs (Ruwali et al. 2009). Alcohol or tobacco use (both smoking and chewing) are also found to interact with variant genotypes of CYP2E1 in significantly enhancing HNSCC risk (Ruwali et al. 2009). This increase in risk associated with an interaction of CYP2E1

genotypes with GSTM1 or XRCC1 or with tobacco and alcohol use demonstrates the importance of gene-gene and gene-environment interactions in the development of HNSCC (Ruwali et al. 2009).

Alcoholic Cirrhosis and CYP2E1 Polymorphism

The variant genotypes of CYP2E1 *5B exhibit significant association with the alcoholic liver cirrhosis when compared to non-alcoholic controls or non-alcoholic cirrhosis patients or alcoholic controls (Khan et al. 2009). Haplotype T-A-T is found to be associated with more than fivefold increase in risk for alcoholic cirrhosis (Khan et al. 2009). Likewise, combination of variant genotype of CYP2E1 *5B with null genotype of GSTM1, a phase II detoxification enzyme, results in several fold increase in risk in alcoholic cirrhotic patients when compared with non-alcoholic controls or non-alcoholic cirrhotic patients (Khan et al. 2009). Further, the combination of variant genotype of CYP2E1 *5B with gamma-aminobutyric acid receptor gamma2 (GABRG2), significantly increases the risk upto 6.5-fold in alcoholic cirrhotic patients when compared with non-alcoholic controls thereby suggesting the role of gene-gene interaction in alcoholic cirrhosis (Khan et al. 2009).

A much higher risk to alcoholic liver cirrhosis is observed in patients carrying a combination of wild genotypes of alcohol dehydrogenase ADH1C (ADH1C*1/*1) and variant genotype of ADH1B (ADH1B*2/*2) or CYP2E1 (CYP2E1*5B) or null genotype of GSTM1 (Khan et al. 2010). Thus, an interaction occurs amongst the genes involved in metabolizing alcohol and in generating and detoxifying free radicals with susceptibility to alcoholic liver cirrhosis (Khan et al. 2010).

Induction of Lymphocyte CYP2E1 in Alcoholic Liver Cirrhosis

Significant increases in CYP2E1 mRNA and protein expression are observed in freshly prepared blood lymphocytes isolated from alcoholic controls (ACs) and alcoholic liver cirrhotic (ACP) patients as compared with respective nonalcoholic controls (NACs) or nonalcoholic cirrhotic patients (NACP) patients (Khan et al. 2011). A concomitant increase in NDMA demethylase activity is evident in the blood lymphocytes of ACs and ACP patients (Khan et al. 2011). Interestingly, the comparative increase observed in CYP2E1 expression is of greater magnitude in the blood lymphocytes isolated from ACP patients, although they abstained from alcohol drinking (Khan et al. 2011). Thus, significant increase in the CYP2E1 mRNA and protein expression in the blood lymphocytes, isolated from early stage ACP patients, can be used to predict alcohol-induced toxicity (Khan et al. 2011).

V. Ravindranath

Ethanol Mediated Induction of Brain CYP2E1

The presence of CYP2E1 and associated mono-oxygenase activities in brain and the effect of chronic ethanol treatment on brain CYP has been studied (Anandatheerthavarada et al. 1993). Aniline hydroxylase, NDMA N-demethylase and p-nitrophenol hydroxylase activities are detectable in brain microsomes from untreated rats and are about 5, 125 and 8.3%, respectively, of the corresponding hepatic levels (Anandatheerthavarada et al. 1993). Chronic ethanol treatment results in induction of the above enzyme activities in brain microsomes by 243, 496 and 155%, respectively (Anandatheerthavarada

et al. 1993). Addition of the antisera raised against rat liver CYP2E1 markedly inhibits brain microsomal p-nitrophenol hydroxylase activity (Anandatheerthavarada et al. 1993). The induction of brain CYP2E1 occurs following chronic ethanol administration (Anandatheerthavarada et al. 1993). The preferential localization of the enzyme occurs in the neuronal cell bodies in the cortex, hippocampus, basal ganglia, hypothalamic nuclei and reticular nuclei in the brainstem of rats treated chronically with ethanol (Anandatheerthavarada et al. 1993). Thus, chronic alcohol ingestion could enhance the sensitivity of certain regions of the brain to environmental chemicals that are metabolized to more toxic derivatives by the CYP system (Anandatheerthavarada et al. 1993).

Characterization of Rat Brain Mitochondrial CYP2E1

The ability of brain mitochondria to metabolize the potent carcinogen NDMA is more than twofold that of the corresponding microsomal activity, while the 7-ethoxycoumarin-O-deethylase activity is significantly lower in mitochondria (Bhagwat et al. 1995). The immunoreactive bands for CYP (2B1/2B2), CYP1A1, and CYP2E1 are present in isolated brain mitochondria (Bhagwat et al. 1995). Various antibodies to CYP (2B1/2B2), CYP1A1, and CYP2E1 inhibit the brain mitochondrial monooxygenase activities to significant, though varying extent (Bhagwat et al. 1995). Chronic ethanol administration results in twofold induction of total CYP content and the monooxygenase activities known to be mediated by CYP2E1, such as NDMA-N-demethylase and p-nitrophenol hydroxylase in brain mitochondria (Bhagwat et al. 1995). The study demonstrates the presence of multiple forms of P450 in the rat brain mitochondria, their inducibility, and their capability to metabolize xenobiotics (Bhagwat et al. 1995).

Region Specific Induction of Brain CYP2E1

Chlorzoxazone hydroxylation in brains from the chronic ethanol treated rats is induced in hippocampus and cortex, downregulated in brainstem, and unchanged in cerebellum, striatum, and thalamus (Upadhya et al. 2000). The presence of functionally active CYP2E1 is also seen in human brain regions obtained at autopsy from traffic accident victims (Upadhya et al. 2000). The constitutive presence of a corresponding CYP2E1 transcript in rat and human brain is observed (Upadhya et al. 2000). The constitutive expression of CYP2E1 preferentially in the neuronal cells in rat and human brain is observed (Upadhya et al. 2000). CYP2E1 expression is seen in neurons within the cerebral cortex, Purkinje and granule cell layers of cerebellum, granule cell layer of dentate gyrus, and pyramidal neurons of CA1, CA2, and CA3 subfields of hippocampus in both rat and human brain (Upadhya et al. 2000). Thus, the constitutive expression of CYP2E1 in brain, its differential induction in rat brain regions by chronic ethanol treatment, and its topographic distribution in rat and human brain has been demonstrated (Upadhya et al. 2000).

Aparajita Dey

Ethanol/High Glucose Inducibility of CYP2E1: *In Vitro* Evidence and Potential Implications for Liver Injury due to Alcohol Consumption in Diabetics

Using the recombinant human hepatoma cell line VL-17A that over-expresses the alcohol metabolizing enzymes – alcohol dehydrogenase (ADH) and CYP2E1,

the mechanism and mode of cell death due to chronic ethanol exposure have been studied (Chandrasekaran et al. 2011). Chronic alcohol exposure causes a significant decrease in viability in VL-17A cells (Chandrasekaran et al. 2011). Chronic ethanol mediated cell death in VL-17A cells is predominantly apoptotic, with increased oxidative stress as the underlying mechanism (Chandrasekaran et al. 2011). Interestingly, the level of the antioxidant GSH is found to be upregulated in VL-17A cells treated with ethanol, which may be a metabolic adaptation to the persistent and overwhelming oxidative stress (Chandrasekaran et al. 2011).

Oxidative stress parameters are greatly increased and apoptotic cell death is observed in high glucose exposed VL-17A cells (Chandrasekaran et al. 2012a). Inhibition of CYP2E1 or caspase 3 or addition of the antioxidant trolox leads to significant decreases in high glucose mediated oxidative stress and toxicity (Chandrasekaran et al. 2012a). Thus, the over-expression of ADH and CYP2E1 in liver cells is associated with increased high glucose mediated oxidative stress and toxicity (Chandrasekaran et al. 2012a).

The toxicity due to chronic alcohol plus high glucose has been studied in VL-17A cells (Chandrasekaran et al. 2012b). When present together, ethanol plus high glucose treated VL-17A cells exhibit greater oxidative stress and toxicity than other groups (Chandrasekaran et al. 2012b). Apoptosis is observed in the ethanol plus high glucose treated VL-17A cells accompanied by increased CYP2E1 protein expression (Chandrasekaran et al. 2012b). The combined oxidative insult due to alcohol plus high glucose leads to greater liver injury, which may prove to be a timely warning for the injurious effects of alcohol consumption in diabetics (Chandrasekaran et al. 2012b).

1.3 Conclusions

As illustrated through the above sections, CYP2E1 appears to play a crucial role in drug metabolism and disease development. CYP2E1 exerts its multifarious activities through several mechanisms, oxidative stress being the major one. The occurrence of CYP2E1 in the body is apparently ubiquitous i.e. it exhibits a wide tissue specific expression. CYP2E1, apart from its role as a xenobiotic metabolizing enzyme, is also involved in modulating several physiological processes. Several drugs acts as inducers and substrates for CYP2E1, thus the therapeutic indices for the drugs can be altered due to the actions of CYP2E1. CYP2E1 is induced in several pathophysiological conditions and CYP2E1 is actively involved or associated with progression of diseases or chemical induced carcinogenesis. Certain pathophysiological conditions can exacerbate alcohol mediated cellular injury due to synergistic induction of CYP2E1, while some agents can act as co-inducers for CYP2E1. Besides, polymorphisms in CYP2E1 are linked with incidences of several diseases. Thus, the role of CYP2E1 as a key player in health and disease cannot be understated.

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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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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*,
Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_2,

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

A. Suggested causes for alcohol toxicity
Redox state changes (↓NAD^+/NADH)
Acetaldehyde formation
Membrane effects
Immune response
Hypoxia
Kupffer cell activation
Cytokine formation
Mitochondrial damage
Oxidative stress
B. Suggested mechanisms for alcohol-induced oxidative stress
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, ↓$\Delta\psi$, ↑permeability transition
Hypoxia
Formation of 1-hydroxyethyl radical
Induction of CYP2E1

Table 2.2 Characterization of cytochrome P450 2E1

Cytochrome P4502E1 (CYP2E1)
A. A minor pathway of ethanol metabolism
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_2O_2
B. Induced by
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 TNFα-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

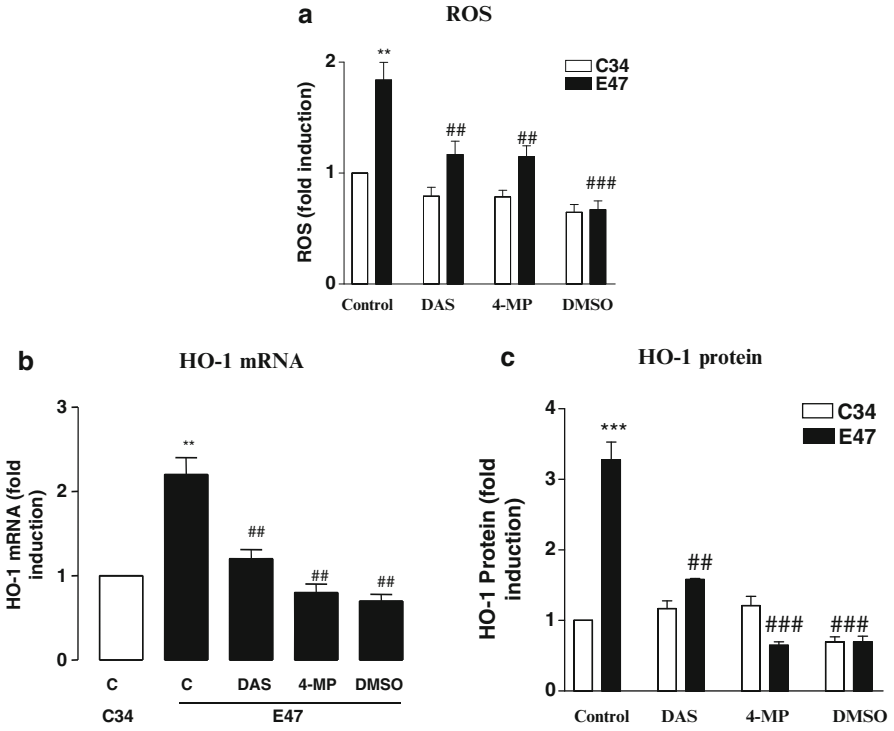

Fig. 2.1 Effect of inhibitors of CYP2E1 on (**a**) ROS production in E47 and C34 cells and (**b**, **c**) the induction of HO-1 mRNA and protein. E47 and C34 cells were incubated with 0.2 mM diallyldisulfide (*DAS*), 1 mM 4-methylpyrazole (*4-MP*) and 200 mM dimethylsulfoxide (*DMSO*) and ROS production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

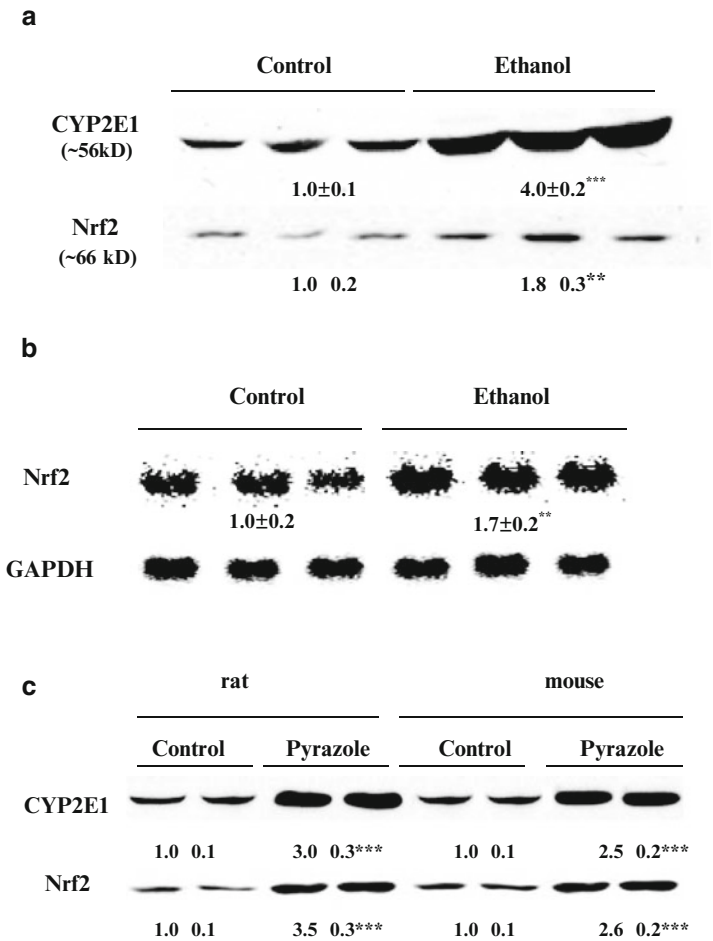

Fig. 2.2 Nrf2 protein (**a**, **c**) and mRNA (**b**) are elevated by chronic ethanol feeding to mice (**a**, **b**) or by acute pyrazole injection in rats or mice (**c**), conditions which elevate CYP2E1 protein levels (**a**, **c**). Numbers under the blots refer to (**a**) CYP2E1 or Nrf2/β-actin ratio;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

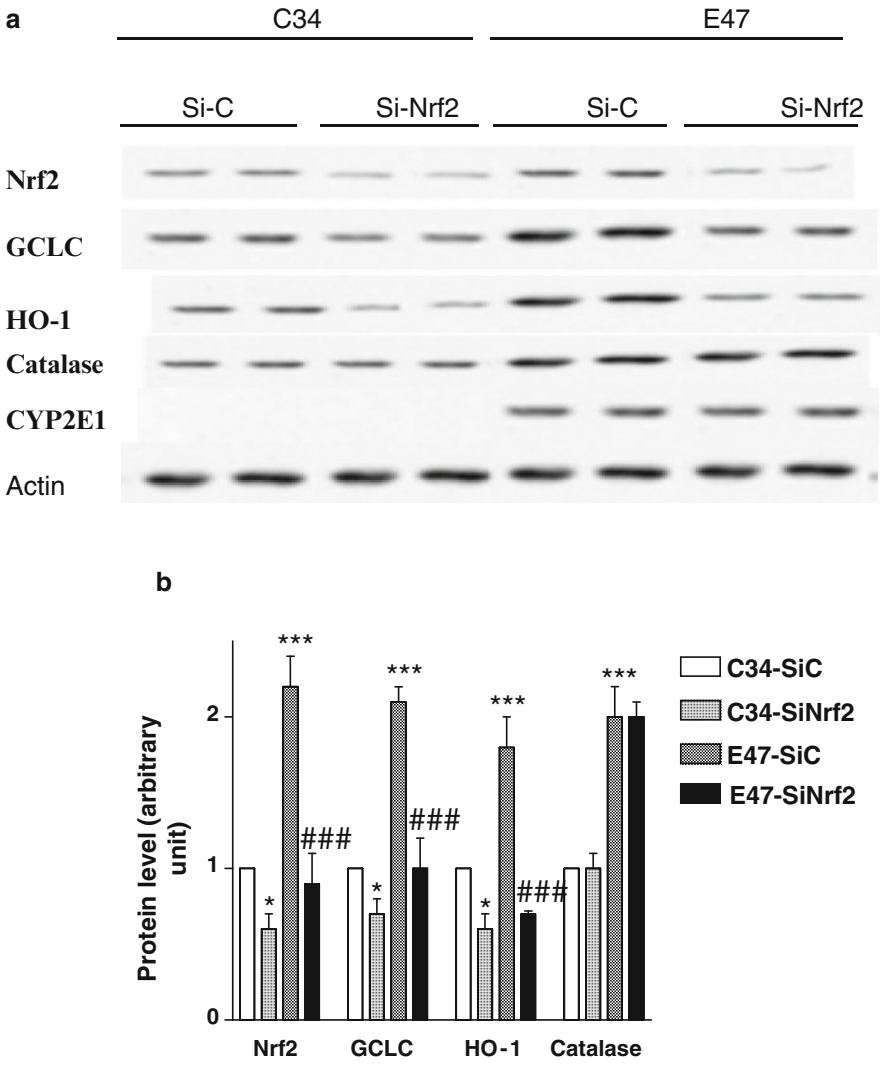

Fig. 2.3 SiRNA against Nrf2 decreases the elevated protein levels of Nrf2, GCS (*GCLC*) and HO-1 but not catalase in E47 cells. The SiRNA against Nrf2 had no effect on CYP2E1 protein levels in the E47 cells. 2.5 million E47 or C34 cells per well were transfected with either Si-C or Si-Nrf2. After 30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

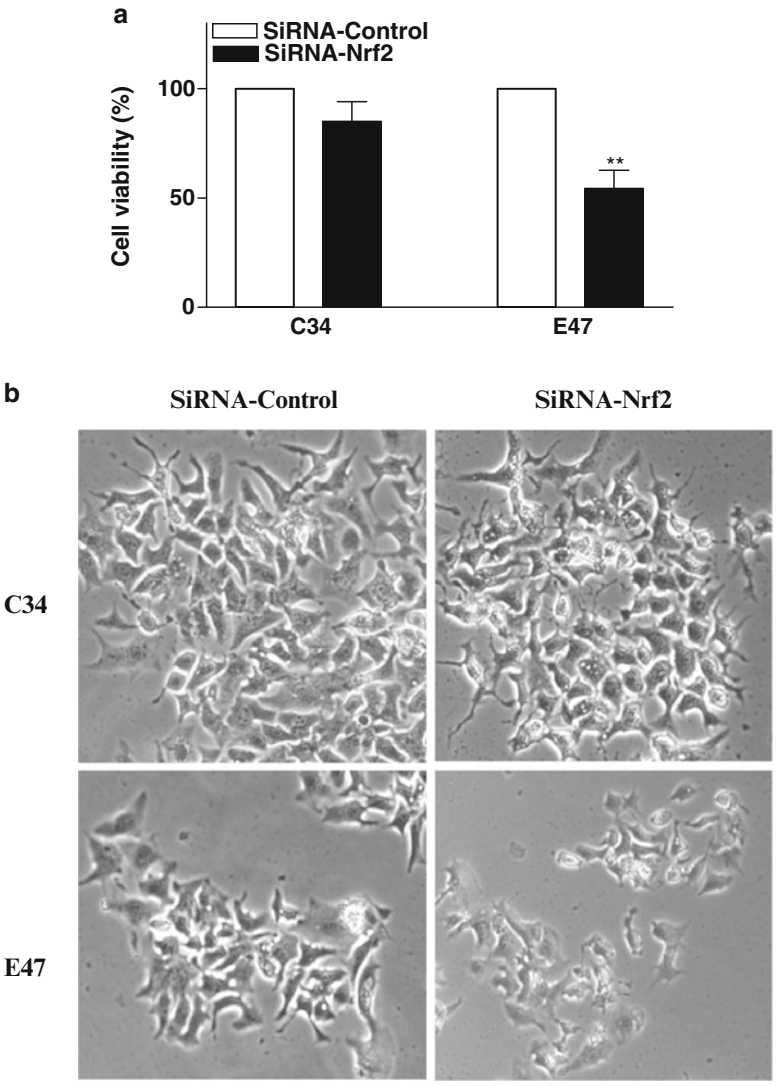

Fig. 2.4 SiRNA against Nrf2 causes toxicity in E47 cells to a greater extent than in C34 cells as shown by a MTT reduction assay (**a**) or morphology (**b**)

toxicity in C34 cells but did cause a mild toxicity in E47 cells (cell viability 80 ± 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 ± 8.5% to 10.7 ± 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 μM-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-kB 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-kB element; treatment with ethanol increased NF-kB 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-kB. Whether NF-kB 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 CYP2E1in 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. CCl_4), 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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Chapter 3
The Role of Cytochrome P450 2E1 in Ethanol-Mediated Carcinogenesis

Helmut K. Seitz and Xiang-Dong Wang

Abstract We and others have shown that chronic alcohol consumption results in the induction of CYP2E1 in the liver. We have also detected for the first time such an induction in the mucosa of the small intestine and the colon. The overall induction of CYP2E1 shows interindividual variations and occurs already following a daily ingestion of 40 g of ethanol after 1 week. CYP2E1 induction is associated with an increased metabolism of ethanol resulting in the generation of reactive oxygen species (ROS) with direct and indirect carcinogenic action. ROS generated by CYP2E1 may lead to lipid peroxidation and lipid peroxidation products such as 4-hydroxynonenal bind to DNA forming highly carcinogenic exocyclic etheno DNA-adducts. The generation of these adducts has been shown in cell cultures in animal experiments as well as in human liver biopsies. CYP2E1 also metabolizes various procarcinogens present in diets and in tobacco smoke to their carcinogenic metabolites. Among these, nitrosamines seem to be the most important carcinogens. CYP2E1 also degrades retinoic acid and retinol to polar metabolites. Metabolism of retinoic acid not only results in the loss of retinoic acid promoting carcinogenesis through an increase in cell proliferation and dedifferentiation but also in generation of polar metabolites with apoptotic properties. We have shown that chlormethiazole is a specific CYP2E1 inhibitor in humans. Chlormethiazole inhibits CYP2E1 activity and thus blocks the formation of DNA adducts in cell cultures, restores retinoic acids in alcohol fed animals and

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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*,
Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_3,

inhibits chemical induced ethanol mediated hepatocarcinogenesis. Thus, there is increasing evidence that CYP2E1 induced by chronic alcohol consumption plays an important role in alcohol mediated carcinogenesis.

Keywords CYP2E1 • Ethanol • Liver • Carcinogenesis

3.1 Introduction

Chronic ethanol consumption is a risk factor for the development of various cancers including cancer of the upper alimentary tract, the colorectum, the liver and the breast (Seitz and Stickel 2007). The mechanisms by which alcohol stimulates carcinogenesis are still unclear and they may differ between tissues. Most recently, the International Agency for Research on Cancer (IARC) in Lyon, France, has classified ethanol containing beverages as carcinogenic (Baan et al. 2007) and acetaldehyde (AA) its first metabolite, as a carcinogen (Salaspuro 2009). *In vitro* and *in vivo* studies as well as data from human studies clearly show the mutagenic and carcinogenic potency of AA especially for the upper alimentary tract (oropharynx, larynx and esophagus) (Seitz and Stickel 2010). However, in addition to the action of AA, other mechanism may also be involved in ethanol mediated carcinogenesis, predominantly in the liver. Such mechanisms may include oxidative stress (Seitz and Stickel 2006), epigenetic modifications due to a disturbed methyl transfer (Stickel et al. 2006), loss of retinol and retinoic acid (Wang and Seitz 2004) as well as severe changes in intracellular signal transduction (Aroor and Shukla 2004), to name only a few.

Since our laboratory has focused in the last 30 years among others intensively on the role of cytochrome P450 2E1 (CYP2E1) in ethanol mediated carcinogenesis, we will discuss here principally the role of CYP2E1 in ethanol mediated hepatocarcinogenesis. This includes its role in the generation of reactive oxygen species (ROS) with consecutive lipid peroxidation and DNA damage as well as its role in the activation of various procarcinogens and in the alteration of retinoid metabolism. We also will briefly address CYP2E1 in extrahepatic tissues. The involvement of CYP2E1 in the pathogenesis of alcoholic liver disease (ALD) will be discussed preliminarily as linked to carcinogenesis.

3.2 Induction and Inhibition of CYP2E1

Lieber and co-workers were the first who have described a significant induction of hepatic CYP2E1 following chronic ethanol consumption (Lieber and DeCarli 1970). This induction was associated with an enhanced metabolism of ethanol due to an enhanced activity of the microsomal ethanol oxidizing system (MEOS) with CYP2E1 as an important constituent. It was also noted that the increase of CYP2E1

observed after alcohol intake is due to stabilization of CYP2E1 rather than to a *de novo* synthesis (Perez and Cederbaum 2003). However, neither the dose of ethanol nor the time range of its induction following ethanol ingestion was well known. Therefore, we measured CYP2E1 activity using the chlorzoxazone test in ten healthy volunteers who received 40 g of ethanol as red wine every day for 4 weeks (Oneta et al. 2002). Three major findings were noted: (1) CYP2E1 activity was found to be significantly increased with an alcohol dose of 40 g daily, (2) this increase was already observed after 1 week of drinking and was further enhanced after 4 weeks of drinking and (3) not every volunteer reacted with a CYP2E1 induction. While in the majority of individuals CYP2E1 increased, some individuals did not react to chronic alcohol consumption demonstrating an interindividual response. The reason why individuals react differently with respect to CYP2E1 induction following chronic alcohol ingestion is not known. Since CYP2E1 is associated with various negative effects (see below), these results may be of importance to explain, at least in part, the fact that only 40% of heavy drinkers develop alcoholic hepatitis and only 10–20% develop cirrhosis of the liver (Teli et al. 1995).

Most of CYP2E1 is located in the liver. However, also other tissues contain CYP2E1 and chronic ethanol ingestion is capable to induce CYP2E1. We found a 2–3 fold increase in CYP2E1 after alcohol intake in the mucosa cells of the small intestine (Seitz et al. 1979; Seitz and Lieber 1980) as well as in the large intestine (Seitz et al. 1982) and in the lungs (Seitz and Lieber 1980; Pikkarainen et al. 1980, 1981) of rats. Yet, other tissues such as the pancreas (Norton et al. 1998) and the brain (Upadhya et al. 2000) also contain CYP2E1.

In men chronic alcohol consumption results in a striking induction of CYP2E1 in the esophageal mucosa which correlated with the amount of alcohol consumed (see below) (Millonig et al. 2011). A similar induction was observed in human biopsies from the oropharynx and from the large intestine (Seitz 2012, personal communication).

Various factors may affect the ethanol mediated induction of CYP2E1. For example, we found a significant gender effect in hepatic microsomes. Microsomes from female rats revealed a 42% increase of microsomal arylhydrocarbon hydroxylase activity associated with an enhanced capacity to activate benzo(a)pyrene (BP) to mutagen in the Ames test (Seitz et al. 1981b).

Another factor is age. It is well established that cellular functions decrease with age including microsomal and mitochondrial function. Therefore, it is not surprising that the induction of CYP2E1 in 36 months old Fischer rats was less pronounced as compared to 12 months old or even young animals (Seitz et al. 1989). It has to be pointed out that CYP2E1 is not only induced by chronic ethanol consumption but it was also found elevated in patients with non-alcoholic fatty liver disease (NAFLD) (Weltman et al. 1998). Both free fatty acids as well acetone (accumulated in the liver of diabetics) induce CYP2E1 (Lieber 2004).

In a recent unpublished study, a comparison of hepatic CYP2E1 between alcoholic steatohepatitis (ASH) and NAFLD was performed in 100 patients. Liver biopsies from these patients showed a significant greater CYP2E1 induction in patients with

ASH as compared to patients with NAFL or NASH. In addition, we also observed a CYP2E1 induction in liver biopsies from 20 children with NASH (Seitz and Mueller, personal communication). In animal studies, the prevention of high fat diet induced CYP2E1 by tomato carotenoids greatly inhibited NASH-promoted hepatocarcinogenesis mainly as a result of reduced oxidative stress (Wang et al. 2010b).

To study the role of CYP2E1 in alcohol associated diseases, it seems necessary to modulate the level of CYP2E1 and to evaluate alcohol effects in the presence and absence of CYP2E1. One approach is the use of a CYP2E1 knock out mouse model or of mice overexpressing CYP2E1. Another approach would be to use a specific CYP2E1 inhibitor. Chlormethiazole (CMZ) is a central acting sedative which is used in some European countries for alcohol detoxification therapy. It has been shown in preliminary animal experiments to inhibit CYP2E1 (Hu et al. 1994).

We investigated the *in vivo* and *in vitro* effect of CMZ on CYP2E1 in humans (Gebhardt et al. 1997). The activity of CYP2E1 was assessed using chlorzoxazone as a probe. The 6-hydroxychlorzoxazone/chlorzoxazone blood concentration ratio, reflecting the CYP2E1 activity, was determined in ten controls and in 24 alcoholic patients who had entered the hospital for detoxification. Alcoholic patients were administered either CMZ (1.3–2.3 g/day) or chlorazepate (100–300 mg/day) as a sedative. CYP2E1 was found to be significantly increased in patients treated with chlorazepate, while in patients treated with CMZ the hydroxylation of chlorzoxazone was almost completely inhibited. After 7–14 days of ethanol withdrawal, alcoholic patients treated with chlorazepate had ratio values similar to those of controls, whereas values from alcoholic patients treated with CMZ remained low even though CMZ doses were gradually decreased. Pharmacological studies in controls showed that CMZ mediated inhibition was present even when CMZ was not detectable in the blood. In addition, the effect of CMZ on CYP2E1 was studied *in vitro* using human liver microsomes. Dixon plot analysis showed a noncompetitive inhibition with a Ki of 12 μmol/L (Gebhardt et al. 1997). Since CYP2E1 induction after chronic ethanol consumption has detrimental effects on the liver through free radical formation, treatment of alcohol detoxification with CMZ may be beneficial.

3.3 Activation of Procarcinogens *via* CYP2E1

Since CYP2E1 not only catalyzes ethanol oxidation through MEOS to AA but also the metabolism of various drugs (e.g. acetaminophen, isoniazid), xenobiotics and procarcinogens, we wondered whether its induction by ethanol may also lead to an enhanced metabolism of certain procarcinogens which actually may result in an activation of the carcinogen to its ultimate metabolite. The first studies on carcinogen activation were performed in the laboratory of Dr. Charles Lieber. In these pioneer studies we could show an increased activation of various procarcinogens including dimethylnitrosamine (DMN), benzo(α)pyrene (BP) and pyrolyzates of tryptophan and tobacco by hepatic and intestinal microsomes following chronic ethanol ingestion (Lieber et al. 1979; Seitz et al. 1978, 1980; 1981a, b; Garro et al. 1981). To study the

effect of chronic ethanol consumption on the mutagenicity of various procarcinogens, the Ames test was used. Microsomes from chronically ethanol fed animals and from control animals were incubated with a procarcinogen and Salmonella typhimurium TA1530. The mutation of S. typhimurium counted as colony formed units on Histidine deficient medium has been used as a measure for the mutagenicity of the carcinogen which has been generated by the microsomal activation.

DMN is metabolized by various isozymes which need CYP2E1 for its activation to the ultimate carcinogen. The ability of ethanol to induce DMN demethylase activity is of particular interest because it is detectable over a DMN concentration range of 0.3–100 mM (Garro et al. 1981). This is in contrast to other microsomal enzyme inducers such as phenobarbital, 3-methylchloranthrene, and polychlorinated biphenyls which increase the activity of DMN demethylase isozymes whose activity is detectable only at relatively high DMN concentration (>40 mM) and repress the activity of low K_m DMN demethylases (Argus et al. 1975; Venkatesan et al. 1970). This effect of ethanol appears to be due to the induction of CYP2E1 and a selective affinity for DMN has been demonstrated with the ethanol-induced CYP2E1 (Garro et al. 1981). The DMN concentrations are sufficiently low to suggest that chronic ethanol ingestion may enhance the *in vivo* metabolic activation of DMN at DMN concentrations that may be pathologically relevant. This enhanced activity of DMN demethylase was associated with an enhanced capacity to activated DMN to a mutagen in the Ames test at these low DMN concentrations (Garro et al. 1981). An increased activation of DMN by human liver microsomes from alcoholics has also been reported (Amelizad et al. 1989).

On the other hand, ethanol is an effective competitive inhibitor of DMN demethylase activity. This capacity to act both as an inducer and inhibitor may explain the conflicting results of ethanol influence on DMN-mediated carcinogenicity, particularly when the route of exposure and the presence or absence of ethanol at the time exposure are taken into account (Seitz and Osswald 1992).

As pointed out in the introduction, enhanced microsomal conversion of many structurally diverse carcinogens has been observed after an inductive pretreatment with ethanol. This includes compounds and mixtures found in tobacco smoke such as BP; various nitrosamines as already discussed and tobacco pyrolyzates; models of dietary carcinogens including DMN and tryptophan pyrolyzate and other hepatotoxins and carcinogens such as carbon tetrachloride, 2-aminofluorene, 2-acetylaminofluorene, benzene, 4-aminobiphenyl, cyclophosphamide, isoniazid and methylazoxymethanol. In some instances these inductive effects exhibit tissue, substrate, gender and species specificities. For example, in the intestine, ethanol increased microsomal activation of BP and tryptophan pyrolyzate but not tobacco pyrolyzate whereas lung microsomes from ethanol-fed rats exhibited an enhanced capacity to activate the promutagens in tobacco pyrolyzate but did not exhibit any increased activity toward BP or tryptophan pyrolyzate (Seitz and Osswald 1992). Although the mutagens being activated in tobacco pyrolyzate are not known it is of interest that lung microsomes from alcohol-fed rats also exhibit an enhanced capacity to activate the tobacco mutagen N-Nitrosopyrrolidine (NPyr) (Farinati et al. 1985). In addition, intestinal microsomes from rats following ethanol consumption were more active to

activate 2-aminofluorene (Seitz et al. 1980) while hepatic microsomes were not (Neis et al. 1985). The enhanced intestinal activation of procarcinogens after alcohol can increase the bio-availability of these compounds and thus, result in elevated concentrations of carcinogens in the portal vein and in the systemic circulation. It is noteworthy that one important component of tobacco pyrolyzate is nitrosopyrolidine which has also been shown to be activated by esophageal microsomes much stronger as compared to microsomes from control animals (Farinati et al. 1985).

These data may be of clinical relevance, since (1) heavy drinkers also smoke heavily. Both factors work synergistic in upper alimentary tract cancer. Thus, the carcinogens present in tobacco smoke (nitrosamines, BP) are enhanced activated by ethanol induced CYP2E1. This activation does occur not only in the liver but also in the oropharyngeal and esophageal mucosa, at the site where the cancer develops. (2) Some trace amounts of nitrosamines are ubiquitous and it has been reported that they also occur in some alcoholic beverages.

3.4 Generation of ROS Through Ethanol Metabolism via CYP2E1

As pointed out ethanol metabolism through CYP2E1 not only produces acetaldehyde but also, especially when induced, generates various reactive oxygen species including H_2O_2, OH- and carbon centered OH-. These ROS may be neutralized by a potent antioxidative defense system (Seitz and Stickel 2006). However, chronic alcohol consumption injures this system mostly due to acetaldehyde and leads among others to a significant decrease of glutathione. Thus, ROS increase and this has dramatic consequences. ROS not only activates JNK with consecutive expression of the activator protein 1 (AP-1) gene leading to cellular hyperregeneration, a procarcinogenic state but also causes lipid peroxidation. Lipid peroxidation products such as malondialdehyde and 4-hydroxynonenal (4-HNE) are generated. It has been shown that 4-HNE binds to adenosine and cytosine forming highly carcinogenic exocyclic etheno DNA adducts (Moriya et al. 1994). These adducts have been identified in the liver of patients with ALD and other types of liver disease associated with inflammation and oxidative stress (Frank et al. 2004) as well as in the urine of patients with viral hepatitis (Nair et al. 2010).

In a series of experiments using CYP2E1 overexpressing HepG2 cells- E47 cells we could show that these etheno-DNA adducts correlate significantly with CYP2E1 and 4HNE (Wang et al. 2009). Adduct formation could be blocked by the use of the specific CYP2E1 inhibitor CMZ (Moriya et al. 1994). Furthermore, CYP2E1, 4HNE and etheno-DNA adducts also correlated significantly in liver biopsy samples from patients with ALD (Wang et al. 2009). Such a significant correlation has also been observed in the esophagus (Millonig et al. 2011). Endoscopically appearing normal esophageal mucosa of patients with alcohol induced esophageal cancer were biopsied and CYP2E1, 4HNE and etheno-DNA adducts were determined immunohistologically. A significant correlation between these parameters was found

demonstrating that CYP2E1 is a causal factor for the generation of these highly carcinogenic DNA lesions. Most interestingly in this study the amount of alcohol consumed correlated significantly with CYP2E1 (Millonig et al. 2011).

Most recently, we determined CYP2E1, etheno-DNA adducts and histomorphology in liver biopsies from 60 patients with various severities of ALD and 39 patients with NAFLD (29 with NASH and 19 with NAFL). A significant correlation was observed between CYP2E1 and hepatic steatosis, hepatic fibrosis and etheno-DNA adducts demonstrating the important role of CYP2E1 in the progression of metabolic liver disease and hepatic carcinogenesis (Seitz 2012, personal communication).

Etheno-DNA adducts were also measured in leptin deficient, insulin resistant Zucker rats which develop obesity and NASH. Etheno-DNA adducts were found to correlate significantly with hepatic fat and with CYP2E1 (Wang et al. 2009). However, more interesting was the observation that when alcohol was administered to these animals, CYP2E1 expression as well as adduct formation was further enhanced emphasizing that additional alcohol consumption even at moderate levels in NASH may be deleterious (Wang et al. 2009, 2010a).

3.5 The Role of CYP2E1 in the Metabolism of Retinoids and Their Importance in Carcinogenesis

Retinoic acid (RA), one of the most active forms of retinoids, is an important factor in the regulation of cell growth, apoptosis and cell differentiation. The ability of RA to elicit these effects lies in its ability to regulate gene expression at specific target sites within the body. Both retinoic acid receptors (RARα, RARβ, and RARγ) and retinoid X receptors (RXRα, RXRβ, and RXRγ) function as transcription factors, regulating gene expression by binding as dimeric complexes to the retinoic acid response element (RARE) and the retinoid X response element (RXRE) located in the 5′ promoter region of susceptible genes. RA binding causes a conformational change in the retinoid receptors, allowing for the dissociation of co-repressors (e.g. NCoR, SMRT, histone deacetylase-containing complexes) and recruitment of co-activators (e.g. CBP/p300, ACTR, DRIP/TRAP), some of which have histone acetyltransferase activity for chromatin decondensation and others which establish contact with the basal transcriptional machinery. Thus, reduction of RAmay lead to uncontrolled cellular proliferation, loss of cell differentiation and dysregulated apoptosis, which can act to promote the process of carcinogenesis.

It has been reported that chronic ethanol consumption results in a significant depletion of retinol in the liver of patients with ALD (Leo and Lieber 1982). To study the mechanisms of the loss of retinol and RA in the livers of animals following the administration of an alcohol containing diet, we isolated microsomes and incubated them with RA. We demonstrated that rat hepatic microsomes from ethanol fed rats when incubated with RA showed an enhanced degradation of RA resulting in the generation of polar RA metabolites such as 18-OH-RA and 4-oxo-RA as compared to microsomes from control animals (Liu et al. 2001). It has also been

shown that this *in vitro* metabolism of RA can be inhibited by CMZ and by CYP2E1 antibodies demonstrating the involvement of CYP2E1 in the metabolism of RA. In addition, rats which received ethanol chronically showed decreased levels of hepatic RA associated with an increase in CYP2E1, which were almost completely restored when CMZ was added to the diet. The data suggest that CMZ can restore both hepatic retinol and retinyl ester concentrations to normal levels in ethanol-fed rats through blocking enhanced both degradation of vitamin A and mobilization of vitamin A from the liver into the circulation (Liu et al. 2002), indicating that CYP2E1 is the major enzyme responsible for the alcohol-enhanced catabolism of retinoids in hepatic tissue after exposure to alcohol. Furthermore, not only hepatic concentrations of RA normalized with CMZ but also changes seen in cell proliferation and cell cycle behavior observed after ethanol administration (Chung et al. 2001).

It is possible that CYP2E1 enzyme induction in chronic intermittent drinking could continue to be a factor destroying retinol and retinoic acid even after alcohol is cleared. This may explain why chronic and excessive alcohol intake is a risk not only for hepatic, but also for extrahepatic cell proliferation and carcinogenesis, because CYP2E1 is also present and inducible by alcohol in the esophagus, forestomach, and surface epithelium of the proximal colon. Moreover, the impaired retinoid homeostasis leads to aberrant retinoid receptor signaling through up-regulation of the c-Jun N-terminal kinase (JNK) signaling pathways.

We showed that ethanol mediated CYP2E1 induction not only results in reduced RA levels but also in increased oxidative stress. Both increased oxidative stress as well as low RA (via decreased mitogen-activated protein kinase phosphatase-1 (MKP-1)) results in an activation of the JNK pathway. Since there is crosstalk between JNK pathway and RXR/RAR receptors, the activated JNK pathway activates AP-1 gene resulting in an increase in c-fos and c-jun (which are 14-fold increased in the livers of ethanol fed rats as compared to controls) (Wang et al. 1998).

The overall effect of excessive alcohol ingestion is dysregulated apoptosis, cellular proliferation, immune function and inflammation, which can act to promote the process of carcinogenesis. Nutritional interventions that serve to inhibit CYP2E1 induction and restore normal retinoid signaling and functioning may offer protection at the cellular level and represent a means to modify alcohol-related cancer risk in high-risk human populations. However, special attention should be paid to the potential detrimental effects of polar metabolites of retinoids generated during alcohol drinking. Unidentified polar metabolites of RA metabolism observed when microsomes from ethanol fed rats were incubated with RA had clear apoptotic properties, since these metabolites resulted in a change of the hepatic mitochondrial membrane potential, a release of cytochrome C and an activation of the caspase cascade (Dan et al. 2005).

Taking this observation into consideration the intake of vitamin A or β-carotene, a precursor of vitamin A together with ethanol may lead to increased hepatic apoptosis and cellular injury which has been described in the baboon (Leo and Lieber 1983). In animal studies, high dose lycopene significantly increased alcohol induced CYP2E1 protein and increased hepatic TNF-α mRNA and inflammation (Veeramachaneni et al. 2008). These data indicate an interaction between chronic

alcohol consumption and dietary supplementation, and increased CYP2E1 and TNF-α especially in rats fed alcohol and high dose lycopene suggests a need for caution among individuals consuming high amounts of alcohol and lycopene.

In considering the efficacy and complex biological functions of retinoids and provitaminA carotenoids in human cancer prevention, nutritional intervention using CYP2E1 inhibitors that target different signaling pathways could provide complementary or synergistic protective effects against alcohol-related cancer risk. In summary, CYP2E1 is primarily involved in the degradation of retinol and RA resulting in loss of RA and in the generation of apoptotic unidentified polar metabolites. Chronic ethanol consumption induces CYP2E1 with a consecutive enhancement of these consequences. The loss of RA may be at least one factor in ethanol mediated hepatocarcinogenesis (Seitz 2000).

3.6 The Role of CYP2E1 in Chemically Induced Hepatocarcinogenesis

As discussed above the induction of CYP2E1 by chronic ethanol consumption may stimulate carcinogenesis through various mechanisms including (1) activation of carcinogens (2) ROS production and (3) reduction of retinoic acid. In this context it is interesting that the generation of carcinogenic exocyclic etheno-DNA adducts is enhanced in CYP2E1overexpressing HepG2 cells, that hepatic CYP2E1 correlated significantly with these adducts in animals as well as in human liver biopsies and that finally transgenic mice over-expressing CYP2E1 showed an enhancement of hepatic injury following chronic ethanol administration (Morgan et al. 2002). On the other hand, CYP2E1 knockout mice showed a significant reduction of their liver injury following chronic ethanol ingestion (Lu et al. 2008) and revealed less oxidized DNA products as compared to wild type animals when they received ethanol (Bradford et al. 2005). Furthermore, chlormethiazole (CMZ) not only strongly reduced ALD in rats receiving the Tsukamoto French diets (Gouillon et al. 2000), but also normalized retinoic acid levels in chronically ethanol fed animals.

To study the effect of CYP2E1 inhibition on chemically induced hepatocarcinogenesis, we used diethylnitrosamine (DEN) for tumor induction (Chavez et al. 2011). After initiation of hepatocarcinogenesis by 20 mg DEN/kg body weight, rats received ethanol containing and control LieberDeCarli diets for 6 and 10 months. We found that chronic ethanol consumption over a 10 month period led to the development of hepatocellular adenoma in the DEN-initiated rats, not in non-ethanol fed rats given the same dose of DEN (Chavez et al. 2011). Our study clearly shows that alcohol can act as a promoter in hepatic carcinogenesis, independently of any role in carcinogen activation or tumor initiation and uncomplicated by viral infection, inadequate diet, or PH of the liver. In addition, chronic ethanol consumption led to impaired proliferation of normal hepatocytes in this animal model (Chavez et al. 2011).

Recently, we found that the alcohol increased the expression of TNFα and NFκB as well as the occurrence of preneoplastic hepatic foci and adenoma were significantly reduced when CMZ was concomitantly applied demonstrating the important role of

CYP2E1 in hepatocarcinogenesis (Ye et al. 2012). In addition, after 10 months most animals in the alcohol fed group developed hepatic adenomas while this was not the case in the control group and CMZ had a significant inhibitory effect on the generation of adenomas (Ye et al. 2012).

3.7 Summary and Conclusion

Chronic ethanol consumption results in the induction of CYP2E1 in the liver but also in other tissues. This induction shows interindividual variation. CYP2E1 induction is associated with an increased metabolism of ethanol resulting in the generation of reactive oxygen species with direct and indirect carcinogenic properties. CYP2E1 also metabolizes various procarcinogens present in diets and in tobacco smoke to their carcinogenic metabolites and degrades retinoic acid. The metabolism of RA not only results in a loss of RA promoting carcinogenesis through an increase in cell proliferation and dedifferentiation but also in the generation of metabolites with apoptotic properties. Chlormethiazole, a specific CYP2E1 inhibitor inhibits DNA lesions in cell culture, restores RA in alcohol fed animals and inhibits chemically induced ethanol mediated hepatocarcinogenesis. Thus, there is increasing evidence that CYP2E1 induced by chronic ethanol consumption plays an important role in ethanol mediated carcinogenesis.

Acknowledgements Original studies were supported by the Deutsche Forschungsgemeinschaft (DFG), by a Gerhard Katsch and an Aschefellowship of the German Society of Gastroenterology, by a Biomedical and Health Research Grant of the European Community, by grants of the Manfred Lautenschläger and DietmarHopp Foundations, by the Volkswagen Foundation, by the Gottlieb Daimler and Carl Benz Foundation, and by a Heinz Götze Memorial Grant. Original work was further supported by NIH/NIAAA grant R01AA12682. Any opinions, findings, conclusion or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the National Institute of Health.

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Chapter 4
The Importance of CYP2E1 in the Pathogenesis of Alcoholic Liver Disease and Drug Toxicity and the Role of the Proteasome

Samuel W. French

Abstract The chapter discusses about the critical role of CYP2E1 in ethanol mediated liver injury and its association with NASH. Ethanol metabolism by CYP2E1 generates hydroxyethyl radicals which promote ethanol hepatotoxicity. Greater induction of CYP2E1 and hence greater liver injury occurs with co-administration of ethanol and drugs. Induction of CYP2E1 leads to prominent epigenetic effects and CYP2E1 polymorphism may be associated with alcoholic liver disease. These are some aspects of CYP2E1, amongst many others which account for its importance in the context of drug metabolism and disease development and have been reviewed in the chapter.

Keywords CYP2E1 • Drug toxicity • Liver

4.1 Introduction

Cytochrome P450 2E1 (CYP2E1) plays a role in the pathogenesis of alcoholic and non-alcoholic steatohepatitis as well as in certain types of drug toxicity. The regulation of CYP2E1 levels and the mechanisms of intoxication that results in the liver when CYP2E1 is involved are diverse. The various mechanisms involved include: oxidative stress, lipid peroxidation, fatty acid hydroxylation, hydroxyethyl formation, autoimmunity, and epigenetic and metabolic changes. The involvement of CYP2E1 in liver injury is confirmed by inhibitors such as DAS, PIC and CMZ and by utilizing cell lines and knockout mice.

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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*,
Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_4,

4.2 The Role of CYP2E1 in Liver Damage Caused by Ethanol

CYP2E1 plays a role in the oxidant damage caused by ethanol ingestion by generating lipid peroxide diene conjugates, malondialdehyde (MDA), hydroxynonenal (HNE) and protein carbonyls, while the antioxidants such as glutathione and vitamin E are depleted (Nordman et al. 1992; Clot et al. 1994; Nordmann 1994).

Rouach et al. (1997) found that rats fed ethanol by intragastric tube for 1 month had increased levels of CYP2E1, which correlated with lipid peroxidation, protein carbonyl formation and pathology score and correlated inversely with unadenylated GS (glutathione synthetase) activity, and levels of glutathione, cytosolic protein thiols and α-tocopherol. Glutathione peroxidase (GPx) activity was decreased, which would reduce the elimination of H_2O_2. GPx is inactivated by free radicals and peroxidases. The reduction of α-tocopherol, the main chain-breaking antioxidant, requires free or loosely bound iron. Non-heme iron was reduced in the liver of the ethanol fed rats, possibly due to its consumption by increased synthesis of hemoproteins such as CYP2E1. Lipid peroxidation was a result of (1) a high level of CYP2E1 induction, (2) a decrease in glutathione and α-tocopherol, (3) a decrease in GPx activity and (4) a lack of adaptive increase in GST activity. GS activity and CYP2E1 are both located centrilobular so CYP2E1 could inactivate GS which is especially sensitive to free radical attack. CYP2E1 is known as a universal "enzyme killer" due to its production of active oxygen. The results supported the conclusion that the hepatic injury as determined by the pathology score and protein carbonyl formation was significantly correlated with CYP2E1 levels indicating that the free radical attack generated by CYP2E1 caused the liver injury. CYP2E1 catalyzes the production of superoxide and H_2O_2 and chelated iron generates the hydroxyl radical, which further produces the reactive hydroxyl radical (Caro and Cederbaum 2004).

Originally when the intragastric fed ethanol rat model was used to determine the role of CYP2E1 in alcoholic liver pathogenesis, adducts to serum proteins were formed by products of lipid peroxidation, namely MDA and 4-HNE (French et al. 1993). The livers were fatty and showed inflammation, necrosis and fibrosis. CYP2E1 levels increased tenfold, microsomal NADPH-dependent lipid peroxidation was increased to 40 fold, NADPH oxidation increased 2.5 fold. MDA increased fourfold. Using the same rat model but adding diallyl sulfide to the diet (a suicide-inhibitor and competitive inhibitor of CYP2E1) reduced the pathology score, the steatosis of the liver and CYP2E1 levels to 1/5, indicating that the increased level of CYP2E1 was responsible for the increase in lipid peroxidation products and liver pathology induced by ethanol feeding (Morimoto et al. 1993).

In rats fed ethanol intragastrically CYP2E1 also generates hydroxyethyl radicals which is associated with the stimulation of lipid peroxidation and the development of liver damage (Albano et al. 1999). These hydroxyl free radicals are capable of inducing specific antibodies which can be observed in ethanol fed animals as well as in patients who abuse alcohol. These antibodies formed against CYP2E1 can cause liver damage by cell-mediated immunotoxic reaction towards liver cells (Albano et al. 1999).

4.2.1 *Hydroxyethyl Radicals Generated by CYP2E1/Ethanol Metabolism: Significance in Ethanol Hepatotoxicity*

Following the serum product lead Albano et al. (1996) to demonstrate that hydroxyethyl free radicals developed in the intragastric fed rat model of alcoholic liver disease (ALD) in a CYP2E1 dependent manner. These studies were motivated by the fact that (1) hydroxyethyl radicals are formed during ethanol oxidation and that CYP2E1 is involved in the free radical generation (Albano et al. 1998, 1991). (2) The treatment of rats with diallyl sulfide or phenylethyl isothiocyanate (PIC), which inhibits the CYP2E1 level increases due to ethanol feeding, reduces lipid peroxidation measured *in vitro* in liver microsomes and *in vivo* in the plasma of alcohol fed animals (Morimoto et al. 1995a). In these studies, the inhibitors prevented the increase in CYP2E1 protein and mRNA induction as well as changing the distribution and intensity of staining of CYP2E1 in the liver lobule. They showed that CYP2E1 levels correlated positively with blood alcohol levels and the severity of the pathology score.

It had been shown that hydroxyl radicals covalently bind to microsomal proteins (Albano et al. 1993) and that these adducts have immunologic properties leading to antibody formation. These antibodies specifically react with hydroxyethyl-derived epitopes of proteins (Moncada et al. 1994). Similar antibodies were found in the blood of patients with alcoholic cirrhosis leading to autoimmune reactions (Clot et al. 1995; Dupont et al. 1998; Albano et al. 1998; Lytton et al. 1999; Albano 2002).

CYP2E1 inhibitors DAS and PIC significantly decreased hydroxyethyl radicals in the liver microsomes incubated *in vitro* with ethanol (Albano et al. 1996). These inhibitors also reduced the formation of hydroxyethyl radical-derived epitopes that were detectable *in vivo* in the livers of ethanol-fed rats. The formation of lipid peroxidation products such as liver MDA and plasma hydrogen peroxide levels and the titers of antibodies against MDA adducts to proteins were reduced in the same proportion that DAS and PIC inhibited the epitopes. These results linked induction of CYP2E1 by ethanol to the stimulation of lipid peroxidation, to the severity of the pathologic scores and the levels of hydroxyethyl radical-derived epitopes. Rats fed ethanol developed antibodies that reacted with hydroxyethyl radical-modified proteins. The formation of these antibodies was reduced by feeding DAS or PIC with ethanol.

The activity of the CYP2E1 measured in human alcoholics by using the chlorzoxazone oxidation assay, was correlated with the levels of antibodies that had formed against hydroxyl ethyl radicals or MDA protein adducts. When CYP2E1 was activated in these patients, 78% of them showed IgG that reacted with hydroxyethyl free radical-protein adducts. The 22% of patients that showed no IgG reaction also showed no CYP2E1 induction. IgG against MDA products were increased in 100% of the patients. Chlorzoxazone oxidation activity was higher in the alcoholics with clinical and biomedical signs of liver disease implying that the liver pathology was linked mainly to hydroxyethyl radical-protein adducts rather than MDA protein adduct formation (Dupont et al. 1998).

Hydroxyethyl free radicals are produced by CYP2E1 non- enzymatically (Albano et al. 1999) by the interaction of ethanol with hydroxyl radicals (OH) originating from iron-catalyzed degradation of H_2O_2 through the Fenton reaction as follows:

$$H_2O + Fe^{2+} \rightarrow OH + OH^- + Fe^{3+} CH_2CH_2OH + OH \rightarrow CH_3C(^{\bullet})\left(HOH + H_2O\right)$$

Superoxide dismutase (SOD), catalase and hydroxyl radical scavengers benzoate and mannitol, prevent hydroxyethyl radical generation by liver microsomes. CYP2E1 has a high NADPH oxidase activity which leads to the production of O_2^- and H_2O_2. Thus the interaction of iron with O_2^- and H_2O_2 released by CYP2E1 might generate oxidizing species able to form hydroxyethyl radicals by abstracting one electron from the ethanol molecule (Albano et al. 1999). However, hydroxyethyl radicals could also originate as a side product during CYP2E1-catalyzed oxidation of ethanol to acetaldehyde and the ferric cytochrome P-450-oxygen complex might be responsible for the one electron oxidation of ethanol as follows: $CYP2E1 - Fe_3^{\bullet}O_2 + CH_3CH_2OH \rightarrow CH_3C(^{\bullet})HOH + H_2O_2 + CYP2E1 - Fe^{3+}$. This represents a minor pathway in the process of ethanol oxidation by microsomal enzymes (Albano et al. 1999).

CYP2E1-hydroxyethyl radical adducts are present in liver microsomes from ethanol fed rats as demonstrated by Western blot. Liver microsomes from rats fed the CYP2E1 inhibitors DAS or PIC reduced the hydroxyethyl radical adducts by one half, indicating that CYP2E1 was responsible for the adduct formation (Albano et al. 1999). Rat liver microsomes with NADPH and radioactive ethanol contain hydroxyethyl radical residues which are covalently bound to the microsomal proteins (Albano et al. 1993).

Human liver microsomes incubated with ethanol form CYP2E1-hydroxyethyl radical adducts (50 KD) which were identified using an anti-hydroxyethyl radical antibody by Western blot and immunoprecipitation techniques (Clot et al. 1996). CYP2E1-hydroxyethyl radical adducts are also detectable by Western blot in human liver plasma membrane proteins (Clot et al. 1997). These adducts were detected using confocal microscopy immunofluorescence detection of anti CYP2E1 and anti-hydroxyethyl radical antibodies (Clot et al. 1997). There was a positive correlation between the extent of liver pathology and the levels of hydroxyethyl radical-derived epitopes in liver microsomes from rats fed ethanol intragastrically (Clot et al. 1997). The most compelling evidence for a role of hydroxyethyl radical mediated immune liver injury is the *in vitro* observation that isolated rat hepatocytes exposed to ethanol can be killed by an antibody-dependent cell-mediated cytotoxic reaction when sera from patients with ALD and normal human blood mononuclear cells are added (Albano et al. 1999).

The level of auto antibodies against CYP2E1 in sera of patients and the liver CYP2E1 levels have been found to be positively correlated (Lytton et al. 1999). Rats fed ethanol intragastrically for 1 month developed an increase in IgG reactivity to CYP2E1 compared to baseline levels (2–3 fold). This effect was specific for anti-CYP2E1 autoantibodies. Chlormethiazole (CMZ) administration reduced the level of autoantibodies formed against CYP2E1. The level of CYP2E1 in the livers and

the sera correlated positively in the individual rats. There was a positive correlation of the antibodies found in the sera and the pathology score in the rats fed ethanol intragastrically for 2 months.

Autoantibodies against CYP2E1 are increased in frequency in the sera of alcoholics (Lytton et al. 1999). This is especially true in patients with HCV who drink alcohol where anti-CYP2E1 auto reactivity correlated with the severity of periportal interface hepatitis (Vidali et al. 2002). When the induction of CYP2E1 activity, measured by chlorzoxasone oxidation, was present in alcoholic patients, IgG reacting with hydroxyethyl free radical-protein adducts were also found (Dupont et al. 1998). The chlorzoxazone oxidation levels correlated with the presence of clinical and biochemical signs of alcoholic liver disease (ALD). ALD patients also have high titers of antibodies directed against protein adducts formed with different products of lipid peroxidation and antigens derived from the combination of malondialdehyde and acetaldehyde. They also have antiphospholipid antibodies which recognize cardiolipin with B_2-glycoprotein 1. These observations suggest that ethanol induced oxidative stress is stimulating immune reactions towards both liver cells and self-antigens (Albano 2002).

4.3 The Pathogenesis of Liver Pathology due to Alcohol-Induced CYP2E1

Much of the deleterious effects of ethanol oxidation by the liver were first attributed to the microsomal ethanol oxidation system (MEOS) (French 1989; Lieber 1999). We had reviewed the early history regarding CYP2E1 and the essential role of NADH in the oxidation of ethanol in the microsomal fraction of hepatocytes.

$$CH_3CH_2OH + NADPH + O_2 MEOS \rightarrow \left(H_3CHO + NADP^+ + 2H_2O\right).$$

Compared to ADH, MEOS has a higher Km for alcohol. It therefore contributes to the oxidation of alcohol at higher blood alcohol levels (BAL) and plays a larger role in the elimination rate of ethanol after induction by chronic ethanol ingestion. The question is how might MEOS contribute to the liver injury caused in chronic ethanol ingestion? The higher the BAL is, the steeper the O_2 gradient from portal to central venous blood is observed. This suggests that MEOS may account for the increase in the rate of ethanol oxidation and O_2 uptake by the liver at higher BAL. In this way it would account for the centrilobular hypoxia that results during ethanol metabolism. At peak alcohol levels NADH levels are high, NAD+ is low, ATP levels are reduced and O_2 levels are reduced (Li et al. 2004; Bardag-Gorce et al. 2002a, b).

Oxidation of ethanol by MEOS does not generate ATP, which is the explanation for the energy wasting which occurs when ethanol is oxidized, thus accounting for the depletion of energy stores. A diet high in fat increases the ethanol elimination rate by MEOS (Kanayama et al. 1984) and accentuates the liver pathology caused by feeding ethanol intragastrically in rats. MEOS induction by ethanol ingestion increased the potential for liver injury caused by the acceleration of xenobiotics or

by free radical production and the hydroxyl free radicals generated by MEOS where ethanol is oxidized nonenzymatically. MEOS accelerates the metabolism of chemicals such as acetaminophen, paracetamol, halothane and carbon tetrachloride to toxic metabolites when ethanol induces an increase of CYP2E1.

MEOS induction by ethanol accelerates the oxidation of ethanol to acetaldehyde causing increased levels of acetaldehyde in the blood and tissues after chronic ethanol ingestion. Formation of adducts with glutathione and precursors deplete the free radical scavenger capacity of the hepatocytes, making the liver cell more susceptible to oxidative stress and lipid peroxidation. Generation of NADPH by MEOS favors free radical formation which depletes free radical scavengers. Acetaldehyde stimulates collagen synthesis by stellate cells to induce centrilobular pericellular fibrosis.

There is evidence that the amount of fat (Tsukamoto et al. 1986) and type of fat fed with ethanol affects the degree of liver pathology and this correlates with the level of induction of MEOS and CYP2E1. When corn oil was fed with ethanol intragastrically severe fatty liver, inflammation and necrosis resulted and MEOS was increased eightfold. CYP2E1 was elevated 20 fold. When tallow was fed with ethanol, no liver pathology developed, MEOS was increased 1.8 fold and CYP2E1 was increased eightfold (Takahashi et al. 1992).

Unlike dietary tallow, which prevents alcohol liver disease, fish oil as a source of dietary fat increases the liver injury over that seen with corn oil when fed with ethanol (Morimoto et al. 1994). The level of CYP2E1 induction was the same in both corn oil fed and fish oil fed models (13 vs. 10 fold). The increase in the levels of lipid peroxidation was the same. NADPH oxidation was increased by feeding fish oil with ethanol as was the reductase. The pathology score was equal. However, there was more inflammation, necrosis and fibrosis in the livers from rats fed fish oil with ethanol. When diallyl sulfide (DAS), an inhibitor of CYP2E1, was fed with ethanol to rats intragastrically, DAS reduced CYP2E1 levels both by competitive inhibition and by suicide-inhibitor action. DAS fed to rats reduced the level of CYP2E1 in the ethanol fed rats to 1/5 of the level achieved by feeding ethanol alone. In controls fed DAS, the CYP2E1 levels were reduced 13 fold. NADH oxidase activity was unaffected. Lipid peroxidation was increased 40 fold by ethanol feeding and DAS reduced this level 70 fold. DAS feeding normalized the ALT levels and reduced the fatty liver and pathology score. The results indicated that induction of CYP2E1 plays a role in the oxidative stress and the liver pathology that results from feeding ethanol. When phenyl isothiocyanate (PIC), another CY2E1 inhibitor, when fed with ethanol intragastrically did not prevent the elevation of ALT, fatty liver, NADPH oxidation or CYP2E1 protein levels but did prevent increased lipid peroxidation by ethanol. CYP2E1 immuno-localized to the centrilobular hepatocytes in ethanol fed and pair fed control rats but DAS and PIC fed with or without ethanol caused the location of CYP2E1 to shift to the periportal hepatocytes with focal loss of staining by hepatocytes. DAS and PIC cause the fat laden hepatocytes to shift to a periportal location in the rats fed ethanol. The significance of this observation is that the location of steatosis and CYP2E1 shifted to the periportal location is that CYP2E1 over expression and fatty change caused by ethanol are causally related.

The levels of CYP2E1 mRNA correlated with the levels of BAL when ethanol was fed but not when either DAS or PIC was fed with ethanol.

When an attempt to establish a relationship between lipid peroxidation and the presence of liver disease in ethanol fed rats was made, the lipid peroxidation could account for only part of the disease. For instance, feeding CYP2E1 inhibitors decreased liver microsomal lipid peroxidation to below the dextrose control levels (Morimoto et al. 1993, 1995a). Yet when the liver pathology was semi-quantitated in the livers of these groups of rats, the pathology was only partially ameliorated by these inhibitors (Morimoto et al. 1993, 1995a). Likewise, the CYP2E1 inhibition was only partially effective in reducing ethanol induction of CYP2E1 (Morimoto et al. 1993, 1995a). The role of lipid peroxidation in the reduction of arachidonic acid 20:4 (n-6) was studied in the livers of the same ethanol-fed rats (Morimoto et al. 1993, 1995a, b) used to study the fatty acid composition of liver lipids (French et al. 1997). Ethanol feeding reduced the hepatic levels of arachidonic acid 20:4 (n-6) (French et al. 1970). Ethanol feeding by intragastric tube again markedly reduced the percentage of arachidonic acid 20:4 (n-6). CYP2E1 inhibitors DAS and PIC only partially ameliorated this effect (French et al. 1997; Morimoto et al. 1995b). Lipid peroxidation by CYP2E1 could not totally account for the reduction in 20:4 (n-6). Microsomal metabolism was measured using radio labeled lauric acid and arachidonic acid 20:4. Ethanol feeding caused an increase in ω-1 hydroxylation of lauric acid, which was partially reduced by DAS and PIC feeding with ethanol. This correlated with changes in chloroxazone hydroxylation (CYP2E1 activity assay) but CYP2E1 does not catalyze ω-1 hydroxylation of lauric acid. When arachidonic acid 20:4 (n-6) metabolites were totaled, an increase was found in the ethanol fed rats but DAS or PIC did not inhibit this change, indicating that CYP2E1 was not responsible for it. This indicated that other isoforms of P450 were involved but not CYP2E1. The reduction in arachidonic acid 20:4 was negatively correlated with the formation of epoxide metabolites such as 14, 15-Epoxyeicosatrienoic acid (EET), 11, 12-EET and 8, 9-ETT, which could have contributed to the decrease in 20:4 observed in the ethanol fed rats (French et al. 1997).

When lauric acid(omega-1)-hydroxylation by liver microsomes from ethanol fed rats was studied in the presence of immunoinhibitors specific for CYP2E1, the increase in hydroxylation caused by ethanol was inhibited (Amet et al. 1998). Lauric acid hydroxylation induced by ethanol correlated positively with the level of induction of CYP2E1 by ethanol feeding (Amet et al. 1998). The ω-1 hydroxylation of oleic and linoleic acid was increased threefold in liver microsomes from rats fed ethanol 1 month intragastrically (Amet et al. 2000). However, there was no effect on ω w-hydroxylation of these fatty acids (Amet et al. 2000).

Chlormethiazole (CMZ) feeding was used to inhibit CYP2E1 activity *in vivo*. Rats fed ethanol by intragastric tube were fed CMZ with ethanol to determine the role that CYP2E1 plays in the pathogenesis of experimental ALD. CMZ has been used to treat alcohol withdrawal symptoms. CMZ has been shown to inhibit CYP2E1 activity *in vivo* and *in vitro* in alcoholic patients (Gebhardt et al. 1997). Documentation of the inhibition of CYP2E1 activity *in vivo* in the rats fed ethanol was achieved using the chlorzoxazone 6-hydroxylation assay. CMZ did not affect the induction of

CYP2E1 by ethanol. But it decreased the activity of CYP2E1 to the control levels (Gouillon et al. 2000). After feeding ethanol for 2 months, with or without CMZ, it was shown that the pathology score was significantly reduced by feeding CMZ with ethanol. There was less necrosis, less inflammation and absence of fibrosis when CMZ was fed with ethanol for 2 months. When ethanol was fed alone, centrilobular fibrosis and bridging fibrosis developed. Centrilobular immunohistochemical staining for CYP2E1 was markedly diminished by CMZ feeding in the liver of control rats compared to the livers of rats fed ethanol plus CMZ.

To relate these changes to CYP2E1-induction by ethanol CYP2E1 overexpressing transgenic mice were fed ethanol for 16 weeks (Morgan et al. 2002). CYP2E1 enzyme activity in liver microsomes was up regulated fivefold compared to the transgenic mice pair fed the control diet. CYP2E1 activity (p-nitrophenol hydroxylation) and protein concentration (Western blot) changes were highly correlated. ALT levels were elevated in the transgenic mice fed ethanol, fourfold higher than the controls. The total pathology was higher in the Tg mice fed ethanol for 16 weeks as was the macro vesicular fat. Tg mice fed ethanol showed ballooning of hepatocytes located in the centrilobular region where intense expression of CYP2E1 staining was located as demonstrated by immunohistologic staining of hepatocytes.

Feeding micropigs ethanol with or without a folate deficient diet, which induced abnormal methionine metabolism for 14 weeks, activated CYP2E1 and enhanced endoplasmic reticulum stress signals and promoted steatosis and apoptosis (Esfandiari et al. 2005).

4.4 Drug Hepatitis Involving Drugs That Are Metabolized by CYP2E1 When CYP2E1 Has Been Induced by Ethanol Ingestion

Drug hepatitis is enhanced when CYP2E1 is induced by chronic ethanol feeding, e.g. acetaminophen (Lee et al. 1996). To test this concept, rats fed intragastrically for 1 month had a non-toxic dose of INH (3.6 mg/kg/day) added to their diet with or without ethanol (French et al. 1993) for 72 days. Ethanol increased liver necrosis, fibrosis and the total pathology score when compared to alcohol feeding alone. The fibrosis was extensive with bridging fibrosis in the INH fed rats fed ethanol. Serum AST levels were higher when INH was fed but not in the pair-fed control rats. Electron microscopy showed the formation of mega mitochondria in the hepatocytes from the rats fed ethanol + INH. The stellate cells were activated in the areas of scarring. Lipid peroxidation formation was significantly greater in the livers of the rats fed ethanol + INH compared with the rats fed ethanol alone. The levels of CYP2E1 in the liver of the rats fed ethanol plus INH were double the levels of the rats fed ethanol alone. It is clear from this comparison that CYP2E1 was responsible for the increase in liver oxidative stress, pathology and fibrosis induced by ethanol. INH alone did not induce CYP2E1 or any liver abnormalities compared to the control.

4.5 CYP2E1 Induction: Effect on Epigenetic Expression

The transgenic CYP2E1 over-expressing mouse model of chronic intragastric ethanol feeding was utilized to determine the effect of CYP2E1 over expression on gene expression changes induced by ethanol. The results were compared with wild type mice fed ethanol for 4 weeks (Butura et al. 2008). The livers were subjected to microarray analysis (Affymetrix). The clinical chemistry results indicated a relationship between increased CYP2E1 with an increased liver pathology score and selective induction of genes of possible importance to the genesis of the pathology observed. MDA levels, para-nitrophenol hydroxylation and total pathology score were increased in the Tg ethanol fed group compared with wild type mice fed ethanol. Liver necrosis and fat accumulation were prominent in the Tg ethanol fed animals. To determine how the variables not included in the derivation of the total pathology score were causally related to the pathology score, a principal component analysis (PCA) was performed on those variables. A plot of the first principal component (PCI) that corresponded to the direction in the data set in which the variation was the largest *versus* the total pathology score showed that there was a relationship between the two. PCI was accomplished for 16.1% of the total variation within the data set. The pathology score was related to the experimental group and the highest score was obtained by the Tg mice fed ethanol. The same was true for PCA. Further studies showed that the pathology score was most highly influenced by the para-nitrophenol-activity measured in the liver microsome fraction, reflecting the metabolism by CYP2E1.

The scaled expression of about 22,000 probe sets were analyzed in order to identify possible biomarker genes which correlated with CYP2E1 induction and the pathology observed. 5,400 genes were investigated. The most significant changes in gene expression were between the wild type mice fed ethanol compared with wild type controls. This involved 250 genes. The Tg mice fed ethanol compared with the wild type fed ethanol involved 65 genes. The Tg controls didn't differ from the wild type control mice. The largest group of genes up regulated in the ethanol fed Tg mice compared with wild type mice fed ethanol were the glutathione transferases, i.e., GST, followed by mono-oxidases, i.e., cytochrome P450s including CYP2b9, CYP2C55, and hydrolases. Down regulated genes included genes involved in intermediary metabolism, in biosynthesis, iron transport, antiapoptotic genes and genes involved in fatty acid biosynthesis and lipid metabolism. When the ethanol fed Tg mice were compared with Tg controls, the expression of 70 genes was changed. Notably those genes involving cell structure such as cytoskeletal organization were up regulated. Down regulated genes included cholesterol metabolism. The influence of CYP2E1 induced through ethanol feeding of Tg mice included an increase in the expression of TGFβ4 and growth arrest specific 5, 30 genes total. Correlations (Spearmans) were performed between CYP2E1 and TNFα (–0.5) and Jun D (0.54) using RT-PCR. Correlation between gene changes and the pathology scores were also done. The highest correlations were for cellular structural proteins like CK8 and CK18 (0.95 $p = 0.0001$ and 0.74 respectively), succinic dehydrogenase (0.73).

Gene expression changes correlating with CYP2E1 activity (Spearmans), (the 30 genes most correlated with the pathology score) included ubiquitin-like (0.86) and uncoupling protein 2 in mitochondria (0.84).

An important revelation of this study was the observation that out of 22,000 probe sets studied, cytokeratin 8 was the number one gene correlating to the total pathology score. Cytokeratin 8 is one of two keratins in Mallory Denk bodies that form in hepatocytes in alcoholic steatohepatitis (ASH). Using the HepG2 hepatoblastoma cell line that over expresses CYP2E1 (E47 cells), CK8 was induced by ethanol *in vitro* and overtime Mallory-Denk like bodies formed (Bardag-Gorce et al. 2006a, b). The proposed sequence of events of Mallory-Denk body formation is that oxidative stress due to CYP2E1 oxidation of alcohol to free radical intermediates induces inhibition of the turn over of cytokeratin 8 and 18 leading to their accumulation and aggregation due to the inhibition of the 26s proteasome by-products of oxidative stress. The fact that CK8 and CK18 are the number one genes to be overexpressed in the Tg mice *in vivo* adds to the connection of CYP2E1 over expression and Mallory-Denk body formation, which occurs only in mice and humans. The correlations were at a highly significant level (CK8 $p < 0.0001$ and CK18 $p < 0.003$). A cytokeratin fragment of CK18 is found in human MDBs in liver biopsies (Amidi et al. 2007) as well as in the serum of patients with ASH (Gonzalez-Quintela et al. 2006) and is used as a biomarker of disease progression.

Early studies on gene expression changes at the peaks and troughs of the urinary alcohol cycle (UAL) of the intragastric feeding model of experimental alcoholic liver disease using global microarray analysis, showed that fatty acid synthetase, ADH-1, CYP2E1, MAPK3, DNA-damage-inducible protein 45, CYP12A1, catechol-o-methyltransferase, NADH-CYP450 and b-5 reductase, were up regulated at the peaks. Ubiquitin, IGF-1, retinoid receptor alpha, fibrinogen beta, CYP7, c-Jun and beta actin were up regulated at the troughs. By RT-PCR, CYP2E1 was up regulated at the peaks and iNOS was decreased at the troughs (French 2001). Cox2 was down regulated at peaks and up regulated at troughs. VEGF was up regulated at both troughs and peaks. Erythropoietin was up regulated at the peaks. Heat shock protein 70 and CTGF were up regulated at both peaks and troughs. The only experimental variable between peaks and troughs was the BAL. This means that the mechanisms of liver injury at the peaks and troughs differed and the changes during the peaks were especially related to the up regulation of CYP2E1, although protein levels of CYP2E1 were the same at peaks and troughs in this model (Bardag-Gorce et al. 2002a, b). Genes changed by hypoxia and an increase in the NADH/NAD ratio occur at the peaks of the cycle. Genes changed at the troughs were related to growth, apoptosis and mitochondrial antioxidant activity (French 2002).

To better relate *in vitro* and *in vivo* changes in gene expression to high levels of blood ethanol (BAL), the gene microarrays from the liver of rats fed ethanol intragastrically and the HepG2 cell line transduced to overexpress human CYP2E1 (E47 cells) were exposed to 100 mM ethanol and were compared (Bardag-Gorce et al. 2006a, b). The gene microarrays performed on the livers from rats fed ethanol intragastrically showed clearly that changes in the number of genes were much greater and different at the peaks compared to the controls. In fact there were only a few changes in gene

expression at the troughs compared to the controls. A large number of genes in most functional pathways, especially kinases, were up regulated at the peaks at ~100 mM ethanol concentration. The *in vivo* arrays showed that the expression of 1,300 genes were changed at the peaks of the UAL cycle compared to controls fed isocaloric dextrose. The expression of 400 genes was changed when the peaks were compared to the troughs of the UAL cycle. The expression of 230 genes was changed when the controls were compared with the troughs of the cycle. The expression of CYP2E1 was not changed either at the peaks or the troughs of the cycle. PPARγ expression was up regulated at the peaks, Bcl2 was down regulated. The fatty acid biosynthesis genes were up regulated at the peaks compared to the controls. Forty-two kinases were up regulated at the peaks compared to the controls.

The *in vitro* microarrays were done using the Cederbaum model of the HepG2 cell line over expressing human CYP2E1 (E47 cells). The control HepG2 cells, not expressing CYP2E1 contained only the vehicle used in the CYP2E1 gene transfection (C34 cells). The cell lines were exposed first to arachidonic acid, then Fe/NTA, then 100 mM ethanol comparable to the *in vivo* BAL. They were then incubated for 24 h (Caro and Cederbaum 2001). Microarray analysis was done after 24 h incubation of the cultures of hepatocytes using 30,000 probe sets. Most of the genes in the different functional pathways were down regulated by ethanol treated Tg over expressing CYP2E1 cells compared to the control HepG2 ethanol treated cells. However, 14 genes in the TGFβ signaling pathway were up regulated, and 15 genes in the insulin signaling pathway were up regulated by ethanol in the Tg CYP2E1 cells compared to the control cells treated with ethanol. Catalase and SOD2 were up regulated in the Tg ethanol treated cells compared to the treated controls.

When the changes in gene expression in the rats at the peaks *vs.* control *in vivo* were compared with changes in the Tg treated *vs.* treated controls *in vitro* IL-1α was up regulated both *in vivo* and *in vitro*. Likewise, MAPKK8, Ctgf and glycerol3 phosphate dehydrogenase were up regulated in both models. Many genes common to both *in vivo* and *in vitro* were down regulated. Discrepancies also existed. For example PPAR was up regulated *in vivo* and down regulated *in vitro.*

4.6 Role of the Proteasome in CYP2E1 Stabilization

Using the intragastric tube feeding model of chronic alcoholic liver disease in the rat, a loss of activity of 26s proteasome was observed (Donohue et al. 1998). The proteasome is a key regulatory agent activating NFκB, β-catenin, and Nrf-2 preventing HIF-1α activation, degrading signaling proteins, cytoskeleton proteins and regulating the check points of the cell cycle. TheCYP2E1 protein is stabilized when the 26s proteasome is inhibited by ethanol, accounting for its induction where no increase in synthesis is involved in its increase by ethanol. Intragastric tube feeding of ethanol caused a 35–40% decrease in chymotrypsin-like (chI/L) and trypsin-like (T-L) activity. Proteasome subunits were not diminished. Free radical products on the subunits appear to contribute to the inhibition of proteolysis of CYP2E1 because

PIC, a CYP2E1 inhibitor, reduced the inhibition of the ChT-L activity. Lipid peroxidation levels were inversely correlated when compared to the ChT-L activity (Fataccioli et al. 1999). In these studies, both ChT-L and peptidyl glutanyl peptide hydrolase (PGPH) activities were decreased when ethanol was fed intragastrically, but not when fed *ad libitum.* Only the alcohol treatment paradigm associated with overt oxidative stress produced a significant inhibition of the proteasome activity. The loss of proteasome activity was associated with an increased formation of MDA, cytosolic protein thiols, protein carbonyls, and glutamine synthetase. The reduced turnover of CYP2E1 may have been related to the inhibition of the catalytic core of the 26s proteasome where subsequent high levels of CYP2E1 are achieved in the intragastric tube feeding rat model (Takahashi et al. 1992). In this model, CYP2E1 is a substrate for ubiquitin and proteasome-mediated proteolysis (Robert et al. 1995; Robert 1997; Wu and Cederbaum 1996; Yang and Cederbaum 1996).

In another study using the intragastric feeding model, CYP2E1 protein levels were increased 14 fold whereas the mRNA expression levels remained unchanged (Gouillon et al. 1999). Ubiquitin conjugates measured by Western blot were reduced by 60%. Ubiquitin mRNA levels were reduced by tenfold when ethanol was fed. The activity of ChT-L and T-L were significantly reduced by ethanol. The net effect was to consistently reduce turnover and stabilization of CYP2E1 which lead to the increased levels of CYP2E1.

In order to determine the mechanisms of CYP2E1 inhibition of the proteasome induced by ethanol, CYP2E1 knockout black C57 mice were utilized (Bardag-Gorce et al. 2000). The proteasomal ChT-L activity was decreased in the ethanol fed wild type mice but not in the KO mice. The 26s proteasome activity was decreased more by ethanol feeding (40% inhibition) than was the 20S proteasome (20% inhibition) signaling that the regulatory 19S component of the proteasome was more affected by CYP2E1 oxidation of ethanol than was the 20S component. Individual hepatocytes lost immunostaining of the proteasome in the centrilobular region in the livers of both ethanol-fed wild type mice and the knockout mice. CYP2E1 was increased 5.75 fold in the wild type mice fed ethanol. No CYP2E1 was detected in the KO mice livers. There was an increase in the products of protein oxidation (carbonyl proteins) in the wild type mice fed ethanol but not in controls or KO mice. This is a clear indication of the role played by the increased CYP2E1 oxidation of ethanol leading to the accumulation of oxidized proteins (Bardag-Gorce et al. 2000). CMZ inhibition of CYP2E1 activity *in vivo* also significantly inhibited the reduction in the activity of the proteasome (ChT-L and T-L) caused by ethanol (Gouillon et al. 2000).

CYP2E1 activity is also stabilized by substrate binding (Banerjee et al. 2000). Substrate binding inhibits the intracellular degradation of proteins by ubiquitin at the catalytic site of CYP2E1 (French et al. 2001). CYP2E1 is a terminal oxidase located in the microsomal membrane by the N-terminus and the rest of the molecule is located within the cytosol (De Lemos-Chiarandini et al. 1987; Sakaguchi et al. 1987). CYP2E1 contains two major cytoplasmic domains (CD1 and CD2) (Banerjee et al. 2000). The cytosolic domains are accessible for degradation by the 26S proteasome. The concentration of CYP2E1 is regulated by a variety of substrates such

as ethanol. The increase in CYP2E1 induced by ethanol is due in part to stabilization of ethanol as a substrate of the enzyme by binding to CYP2E1 which blocks the access of the proteasome to degrade it. In this way, ethanol prolongs the life of CYP2E1 (Song et al. 1989). As long as the substrate binds to CYP2E1, it cannot be degraded by the proteasome because the ubiquitin binding site is at the substrate-binding site (Banerjee et al. 2000).

Previous studies have shown that CYP2E1 degradation involves the ubiquitin proteasome pathway (Wang et al. 1999; Goasduff and Cederbaum 2000). The CYP2E1 protein is degraded by the 26S pathway after ubiquitination (Wang et al. 1999; Korsmeyer et al. 1999). CYP2E1 ubiquitination occurs on the CD2 domain (Banerjee et al. 2000) which contains two lysines, Lys 317 and 324. All previous studies published involved the expression of CYP2E1 *in vitro* in tissue culture. To study this phenomenon *in vivo*, the reversible proteasome inhibitor PS341, a boron based chemical was used to inhibit the activity of the proteasome on CYP2E1 turnover in ethanol fed rats (Bardag-Gorce et al. 2001). PS341 acts on the catalytic N-terminal threonine residue of the proteasome catalytic ChT-L subunit to inhibit its activity. Male rats were given ethanol intragastrically for 30 days. Ethanol feeding was stopped at the same time that PS341 was administered intraperitoneally and the rats were sacrificed 24 h later. Administration of the proteasome inhibitor should prevent the decrease in CYP2E1 that occurs after ethanol withdrawal by preventing CYP2E1 degradation. Inhibition of ChT-L activity by PS341 was documented. The CYP2E1 increase in the ethanol fed rats persisted after ethanol withdrawal and after PS341 treatment but not in the untreated rats 24 h after ethanol withdrawal. The results clearly showed that CYP2E1 was stabilized *in vivo* when ethanol caused inhibition of the proteasome. CYP2E1 was degraded by the proteasome during ethanol withdrawal due to reconstitution of proteasome activity and degradation was prevented when the proteasomal ChT-L was inhibited by the specific inhibitor PS341. *In vitro* studies have shown that CYP2E1 was degraded by the proteasome using the cell line HepG2 transfected to overexpress CYP2E1 (Yang and Cederbaum 1996).

Constitutive CYP2E1 has a short half-life. The protein turns over in 6–7 h (Song, et al. 1989). However, induced CYP2E1 has a prolonged turnover as shown in these studies where the activity of the proteasome was inhibited by PS341. However, when no PS341 was injected, the ethanol induced CYP2E1 returned to constituent levels in 24 h after ethanol withdrawal. Hsp 90, a chaperone protein, may facilitate proteasomal degradation of CYP2E1 since the inhibition of Hsp 90 by geldanamcyin prevented the digestion of CYP2E1 (Goasduff and Cederbaum 2000). The blood ethanol level does not affect the degree of inhibition of proteasomal ChT-L activity by ethanol treatment (Bardag-Gorce et al. 2002a, b).

The mechanism of CYP2E1 stabilization by proteasomal activity inhibition due to ethanol treatment has been investigated by correlating the decreased proteasomal activity 1, 3, 7, 15 and 30 days after ethanol feeding intragastrically. The maximum induction of CYP2E1 was reached after 15 days of ethanol feeding (Bardag-Gorce et al. 2005). It took 1 month of ethanol feeding, however, before ethanol inhibition of the proteasome ChT-1 activity was detected. The same was observed when T-L

activity was assayed. Likewise, it took 1 month of ethanol feeding to induce 4HNE (4-hydroxynonenal) to significant levels (Bardag-Gorce et al. 2005). Regression analysis showed a positive correlation between CYP2E1 and 4HNE levels over a 30 day period. There was a negative correlation between 4HNE levels and the T-L activity when the 26S proteasome fraction was purified from the liver cytosol using fractionation on glycerol density gradient centrifugation. The peak fraction was used to detect 4HNE adduct formation in the proteasomal subunits. 4HNE was localized at the Rpt4 subunit band by Western blot. The ATPase Rpt 4 is a subunit of the 19S regulatory part of the proteasome that functions in the binding of the 20S proteasome to the regulatory 19S proteasome. This adduct could reduce the functions of the 19S proteasome by impeding its binding to the 20S proteasome.

There are many other things that could modify the rate of protein turnover by the proteasome, such as modifications of the chaperone system of delivery of altered proteasomes, changes in the ubiquitination pathway components and the deubiquitination enzyme components. Chronic ethanol ingestion using the rat intragastric feeding model modifies many aspects of the proteasome-interacting protein (Bousquet-Dubouch et al. 2009). Using a non-denaturing multiple centrifugation procedure to preserve proteasome-interacting proteins (PIPS), ICAT and MSMS spectral counting and further confirmed by Western blot, showed that the levels of several PIPs were decreased in the isolated proteasome fractions. This included PA28 alpha/beta proteasome activator subunits, and 3 proteasome-associated deubiquitins, Rpn 11, ubiquitin C-terminal hydrolase 14 and ubiquitin carboxyl-terminal hydrolase L5. The C-terminal of Rpn13 was missing in the ethanol proteasome fraction, which probably altered the linking of the ubiquitin carboxyl-terminal hydrolase L5 to the proteasome. Ecm29, a protein known to stabilize the interactions between the 20S proteasome and its activators, was decreased in the isolated ethanol proteasome fractions. These changes support the concept that ethanol inhibits the proteasome by several different mechanisms including altering PIPs proteasome regulatory complexes which bind to the proteasome.

4.7 Role of CYP2E1 in Drug Cholestatic Hepatitis (DDC) Plus Ethanol

DDC (Diethyl-1,4-dehydro-2,4,6-trimethyl-3,5-pyridine decarboxylate) induces a chronic drug hepatitis over a 5 month feeding period in mice. When ethanol was added to the diet for 7 days Mallory Denk bodies (MDB) formed and CK18 protein, a constituent of MDBs, was quantitated. Both these protein levels were increased (Zhang-Gouillon et al. 1998). This suggested that inhibition of the proteasome was the reason for the increased protein levels which resulted in an excess amount of protein for aggresome formation. CYP2E1 was increased probably by the same mechanism, since CYP2E1 mRNA was not increased. However, without the presence of ethanol and adding CMZ to inhibit CYP2E1, MDB formation was enhanced

in the DDC model (Bardag-Gorce et al. 2001, 2005). Likewise, when CYP2E1 KO mice were fed DDC, this too markedly increased the formation of MDBs (Bardag-Gorce et al. 2001) compared to wild type mice. The opposite effect was obtained when Tg Mice over expressing CYP2E1 formed fewer MDBs than did the control mice fed DDC (Bardag-Gorce et al. 2005). DDC fed wild type mice had reduced levels of CYP2E1 compared to controls and this was reversed by withdrawal of DDC. DDC CMZ fed mice had even lower levels of CYP2E1 and DDC fed CYP2E1 KO mice had only a trace of CYP2E1 by Western blot. CYP2E1 overexpressing Tg mice fed DDC had the highest levels of CYP2E1. It was clear that the CYP2E1 levels correlated inversely with MDB formation. When ubiquitin conjugates were measured by Western blot in the different groups of CYP2E1 expressing mice fed DDC, a negative correlation was also found between CYP2E1 levels and accumulation of ubiquitinated proteins implying that loss of CYP2E1 activity was associated with failure of the 26s proteasome to remove damaged proteins destined for proteolysis by the proteasome (Bardag-Gorce et al. 2005).

4.8 Role of CYP2E1 in Non-alcoholic Hepatitis (NASH)

CYP2E1 expression and activity is increased in the livers of human and animal models of NAFLD/NASH raising the question as to whether CYP2E1-generated ROS could contribute to insulin resistance (Weltman et al. 1996, 1999). Transduction of CYP2E1 promoted liver injury in a mouse model of NASH (ob/ob mice) (Dey and Cederbaum 2007). Additional evidence has been obtained using the HepG2 tissue culture model which over expressed CYP2E1 and the rodent model of NASH fed a methionine-choline deficient diet (Schattenberg et al. 2005).

When Tg mice over expressing CYP2E1 were fed a 20% calorie derived fat diet for 8 months CYP2E1 levels were increased compared with wild type mice as were the ALT levels, liver triglyceride levels, MDA, and carbonyl protein levels, indicating that oxidative stress had developed (Kathirvel et al. 2009). The fasting plasma levels were fourfold higher than the controls. Correlations between CYP2E1 activity and oxidative stress markers, phosphorylated proteins in the insulin signaling cascade and phosphorylated c-jun were made by regression analysis. Strong correlations were found with MDA, protein carbonyls, the level of phosphorylated proteins in the insulin signaling pathway (IRSI and 2, Akt/PkB, GSK3α and FOXO1α) and phosphorylated c-jun. It was concluded that the data supported a potential role for CYP2E1 in hepatic insulin resistance *in vivo* (Kathirvel et al. 2009). Further studies using the same model showed the up regulation of Nrf2, SOD1-2, catalase, GPx, HO-1 and iNOS (Kathirvel et al. 2010). The data indicated that the over expression of CYP2E1 causes oxidative and nitrosative stress.

A high fat diet (up to 85% of control levels) was fed using the intragastric rat model of force feeding, to mice deficient in TNFR1 and wild type controls for 9 weeks. The mice developed obesity hyperglycemia, hyperinsulinemia, and hyperlipidemia, glucose intolerance and insulin resistance. Mice (40%) developed

steatohepatitis (increased TNFα, leptin and reduced adiponectin expression). PPAR expression was reduced. CYP2E1 was reduced but CYP4a was increased. TNFR1 deficiency did not prevent NASH (Deng et al. 2005).

4.9 CYP2E1 Polymorphism and Genetic Links to Alcoholic Liver Disease

Many groups have searched the possible association between CYP2E1 genetic polymorphism and the development of ALD. CYP2E1 Rsa1 allele polymorphism is located in the 5′-transcript Ticon regulatory region. This allele is associated with altered gene expression which has functional consequences. The more common allele, C1 has lower expression rates compared to the mutant allele (C2). Thus the C2/C2 allele which lacks the Rsa1 restriction site, is associated with a higher transcriptional activity (tenfold) compared to the more common C1/C1 allele (Hayashi et al. 1991; Tsutsumi et al. 1994). C2 is much more common in Asia (~25%). In a study of Mexican-American men 18% were found to have the c2allele. Many of these men were frequent and heavy drinkers (Caetano and Clark 1998). In one study, 101 Mexican-American alcoholics were recruited (age 37 ± 11.8 years old) that consumed more than 80 g alcohol daily for more than 5 years (Konishi et al. 2003). The results were compared with 104 nonalcoholic Mexican-American men. Polymorphism genotype was determined by PCR and restriction fragment length. An association was found between CYP2E1 Rsa1-c2. in c2 and alcohol abuse. The % of subjects who carried the 1c2 allele was significantly higher in alcoholics (34%) compared to non-alcoholics (22.1%). The subjects whose alcohol drinking onset age was younger than 25 years had much higher CYP2E1 C2 allele frequency than the control nonalcoholic (22.1% vs. 15.7%) (Konishi et al. 2003).

4.10 Conclusions

Thus, CYP2E1 has an essential role in liver damage caused by ethanol and is associated with NASH. Hydroxyethyl radicals generated by CYP2E1/ethanol metabolism have a vital role in ethanol hepatotoxicity. Ethanol ingestion and drug administration leads to greater induction of CYP2E1 and hence greater liver injury. CYP2E1 induction has prominent epigenetic effects and CYP2E1 polymorphism may be associated with alcoholic liver disease. These are some aspects of CYP2E1, amongst many others which underlie its importance in drug metabolism and disease development.

Acknowledgement The author thanks Adriana Flores for typing the manuscript and Barbara French for editing it.

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Chapter 5
Relevance of CYP2E1 to Non-alcoholic Fatty Liver Disease

Ann K. Daly

Abstract Non-alcoholic fatty liver disease (NAFLD) and its progression to steatohepatitis (NASH) and cirrhosis is a growing problem in most developed countries. Increased hepatic expression of CYP2E1, which carries out omega hydroxylation of fatty acids, was first shown in a mouse model of NASH and this was later also reported for human NASH, though not all studies agree with this finding and further larger studies are still needed. In view of its role in fatty acid metabolism which leads to increased levels of toxic lipid peroxides and its possible increased expression in NASH, CYP2E1 is an attractive candidate for a role as a genetic risk factor for both NAFLD generally including progression to NASH. Two studies have focused on the variant allele *CYP2E1*5*, which may be associated with increased CYP2E1 expression. Both reported increased frequencies of this allele in NASH patients, though statistical significance was not achieved because of small sample sizes. Some more indirect data also suggests a relationship between high CYP2E1 activity and progression to NASH. However, three recent genome-wide association studies on NAFLD have failed to find any evidence that single nucleotide polymorphisms in or adjacent to the *CYP2E1* gene contribute to susceptibility. Further studies are needed to investigate a possible role in disease progression in addition to susceptibility and the possibility that statistical power in the existing studies was insufficient to detect a relatively small contribution to disease susceptibility.

Keywords CYP2E1 • Gene polymorphism • NASH • NAFLD

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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*,
Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_5,

5.1 Introduction

As reviewed in other chapters, the relevance of CYP2E1 to ethanol metabolism and to alcoholic liver disease is well established and has been studied for the last 40 years approximately (Lieber and DeCarli 1970). In addition to induction of CYP2E1 by ethanol, it has been known for some time that CYP2E1 is induced in diabetic and obese humans and rodents (Hong et al. 1987; Song et al. 1986, 1987). The increased expression of CYP2E1 in type II diabetes and obesity raised the possibility that CYP2E1 expression might be relevant to the pathogenesis of nonalcoholic fatty liver disease (NAFLD). NAFLD covers a disease spectrum ranging from hepatic steatosis alone though non-alcoholic steatohepatitis (NASH) to cirrhosis and is an increasingly common cause of liver dysfunction in developed countries (for review see Anstee et al. (2011a, b); de Alwis and Day (2008)). NAFLD is strongly associated with obesity, insulin resistance, type 2 diabetes and dyslipidaemia. Though most patients with these conditions develop steatosis alone, a minority progress to more advanced liver disease characterized by inflammation, fibrosis, cirrhosis and, in some cases, hepatocellular carcinoma.

Increased expression of CYP2E1 was first described in hepatocytes from rats fed a methionine-choline diet, which is an established model for human steatohepatitis (Weltman et al. 1996), followed by a study demonstrating a similar effect by immunohistochemical analysis of liver biopsies from steatohepatitis patients (Weltman et al. 1998). Both the general role of CYP2E1 in the NAFLD disease process and the possibility that either interindividual variability in levels of CYP2E1 or in ability to induce expression of this enzyme could affect susceptibility to and severity of this disease is of considerable interest. In particular, whether CYP2E1 could be a target for novel treatments and whether *CYP2E1* genotype contributes to individual risk for disease development are both questions that are of importance to understanding the pathogenesis of NAFLD.

5.2 CYP2E1 and NAFLD

Though ethanol is the best established inducer of CYP2E1 (Lieber and DeCarli 1970; Song et al. 1986), it has been known since the 1980s that CYP2E1 induction can also occur in diabetic rats (Song et al. 1987) and during starvation (Hong et al. 1987). It was subsequently demonstrated that CYP2E1 catalyzes omega hydroxylation of fatty acids (Laethem et al. 1993). The demonstration that hepatic CYP2E1 levels are elevated in a rat model of NASH (Weltman et al. 1996) and in patients with NASH (Weltman et al. 1998) suggests that fatty acids are also CYP2E1 inducers. Whether CYP2E1 induction is of direct relevance to disease pathogenesis in terms of either human NAFLD generally or NASH development remains unclear. Studies in animal models suggest it could be a factor but studies in humans have generally involved small numbers and given contradictory results.

Several different mouse models exist for both NAFLD and NASH though none of these is a true model for progression of NAFLD to NASH (for review see De Minicis and Svegliati-Baroni (2011); Hebbard and George (2011)). The most widely used models are the high fat diet (HFD) where mice given a high fat diet show steatosis, weight gain and insulin resistance and the methionine and choline-deficient (MCA) diet model where mice show some characteristics of NASH. Using the MCA diet model, a large increase in lipid peroxide formation in the liver was detected in parallel with CYP2E1 induction (Leclercq et al. 2000) but importantly, in mice lacking the CYP2E1 gene, lipid peroxides were still formed via CYP4A, which was also upregulated by the MCA diet. Lipid peroxide formation is likely to be an important contributor to NASH pathophysiology since it may result in serious toxicity within cells but CYP2E1 may have additional effects that could also contribute to NAFLD more generally. In particular, CYP2E1 overexpression in a hepatocyte cell line was demonstrated to impair insulin signalling with decreased insulin receptor substrate (IRS)-1 and IRS-2 phosphorylation and other effects including decreased glycogen synthase kinase 3 activity and glucose secretion detected in response to insulin (Schattenberg et al. 2005). Insulin resistance is an important feature of NAFLD and a possible contribution from CYP2E1 to this resistance is an interesting finding. In a more recent study using transgenic mice with CYP2E1 overexpressed specifically in hepatocytes and fed the HFD, higher fasting insulin levels and decreased insulin signalling was seen, which is consistent with the *in vitro* findings (Kathirvel et al. 2009). The converse was also demonstrated in a separate study involving CYP2E1 knockout mice fed the HFD (Zong et al. 2012). In this case, the mice did not gain weight and show insulin resistance. A further study involving the transgenic CYP2E1 overexpressing mice showed increased levels of both lipid peroxidation and protein carbonylation together with decreased activity for certain enzymes that protect against oxidative stress, possibly due to their inactivation by nitrosylation (Kathirvel et al. 2010).

Since the original observations showing increased CYP2E1 levels in NASH in human liver by immunohistochemistry (Weltman et al. 1998), the number of investigations in humans has been limited. One follow-up study compared CYP2E1 expression and activity in liver biopsies from patients with either steatosis alone or NASH (Chtioui et al. 2007) but found no difference between the two patient groups for either parameter. However, CYP2E1 activity correlated positively with both body mass index and steatosis score. Evidence for increased CYP2E1 protein levels in both steatosis and NASH was seen in a study on liver biopsies which also measured CYP2E1 enzyme activity *in vivo* using chlorzoxazone phenotyping. The phenotyping analysis showed significantly higher activity in NASH cases compared with both steatosis and controls (Varela et al. 2008). A study on adult explanted livers involving immunohistochemistry and measurement of protein and mRNA levels found that CYP2E1 levels appeared to fall with progression to NASH (Fisher et al. 2009). In a recent study on children with NAFLD where CYP2E1 protein levels in liver biopsies were determined, no increase in protein levels for either NASH cases only or NAFLD as a whole compared with samples showing normal histology was observed (Bell et al. 2011). In addition, levels of lipid peroxidation did not differ

between biopsies with and without NASH, though body mass index did correlate with levels of lipid peroxidation. It therefore appears that there is considerable inconsistency between studies on human CYP2E1 expression in NAFLD. This possibly reflects the fact that all the studies discussed above involved relatively small numbers of samples and larger studies are clearly needed.

5.3 Genetics of NAFLD Including Possible Role of CYP2E1 Polymorphisms

5.3.1 Background

There is evidence for a role for genetic factors in NAFLD from both family and inter-ethnic variation studies. In a recent study on families which included overweight children with NAFLD (Schwimmer et al. 2009), fatty liver was significantly more common in the siblings and parents of the children with NAFLD. Another study on monozygotic and dizygotic twins showed that serum alanine aminotransferase (ALT) and fasting serum insulin intrapair correlations were significantly higher in the monozygotic compared to the dizygotic twins (Makkonen et al. 2009). In a study on the offspring of participants in the Framingham Heart Study, early-onset paternal obesity was associated with elevated ALT levels in offspring, suggesting a genetic predisposition to developing elevated ALT levels and possibly NAFLD (Loomba et al. 2008). For NAFLD in adults, Struben et al. (2000) described the co-existence of NASH and/or cryptogenic cirrhosis in 7 out of 8 kindreds studied and another study (Willner et al. 2001) found that 18% of 90 patients with NASH had an affected first-degree relative. This clustering could simply be due to heritability of obesity and insulin resistance, the main risk factors for NAFLD. However, other studies examining ethnic differences in the prevalence of NAFLD suggest that susceptibility may have a specific genetic component (Browning et al. 2004a, b). It appears that African-Americans, though as prone to obesity as Americans of European or Hispanic origin, show a lower incidence of both steatosis and cryptogenic cirrhosis. This may be due to different patterns of fat accumulation and a lower incidence of insulin resistance among obese African-Americans (Guerrero et al. 2009). US Hispanics also appear more susceptible to NAFLD than US individuals of European ethnic origin (Browning et al. 2004a; Williams et al. 2010).

5.3.2 Candidate Gene Studies on NAFLD

A large number of candidate gene association studies together with three genome-wide association studies (GWAS) on susceptibility to NAFLD have now been reported (Anstee et al. 2011a; Daly et al. 2011). The candidate gene studies have focussed particularly on genes relevant to lipid metabolism and oxidative stress and have

reported a limited number of positive associations (Daly et al. 2011). The most consistent positive message from both candidate gene and genome-wide association studies is that genotype for *PNPLA3*, which codes for the enzyme patatin-like phospholipase domain-containing 3, also known as adiponutrin, modulates risk of developing NAFLD. This enzyme is a serine protease whose function is still somewhat unclear but appears to contribute to triacylglycerol hydrolysis (Huang et al. 2011). Evidence from a large number of studies including two separate GWAS suggests that for development of steatosis, there is an increased risk in those carrying one or two copies of a *PNPLA3* variant allele associated with a nonsynonymous mutation (Romeo et al. 2008; Sookoian and Pirola 2011; Speliotes et al. 2011). Further studies using candidate gene approaches indicated that *PNPLA3* genotype also predicts fibrosis severity in NAFLD (Sookoian and Pirola 2011; Valenti et al. 2010). The odds ratio for developing severe steatosis or fibrosis associated with the possession of the *PNPLA3* variant allele has been found to be approximately two in most studies. The *PNPLA3* association with NAFLD susceptibility and severity has been observed in several different ethnic groups (Li et al. 2012; Sookoian and Pirola 2011).

A number of other genes have also been reported to be associated with susceptibility to NAFLD and severity of several phenotypic features but with the exception of an association of a polymorphism in the manganese-dependent superoxide dismutase gene (*SOD2*) with development of severe fibrosis and NASH (Al-Serri et al. 2012; Namikawa et al. 2004), generally these associations are generally inconsistent with some studies showing associations but others failing to confirm them (see Daly et al. (2011) for detailed review).

5.3.3 *Candidate Gene Studies on CYP2E1 as a Genetic Risk Factor in NAFLD*

Genetic polymorphism in CYP2E1 is generally a well studied area. There is evidence of approx. 20 fold interindividual variation in expression of CYP2E1 in human livers though phenotyping studies using the muscle relaxant chlorzoxazone as probe in European populations have demonstrated only two to threefold variation in levels of activity (Kim and O'Shea 1995). A number of genetic polymorphisms in *CYP2E1* have been reported with the majority occurring in either upstream sequences or introns and mostly appearing to lack functional significance. Polymorphisms affecting coding sequences are rare. One of these, R76H encoded by *CYP2E1*2*, is associated with decreased catalytic activity and occurs at a low frequency in a Chinese population but has not been detected in other ethnic groups (Hu et al. 1997). It has been suggested that a polymorphism in the 5′-flanking region within a putative HNF-1 binding site may be of functional significance with *in vitro* studies suggesting that this allele shows approximately tenfold higher transcriptional activity than the wild-type (Hayashi et al. 1991). This variant allele (*CYP2E1*5*) occurs at a frequency of 0.27 in Japanese but only 0.02 in Europeans (Kato et al. 1992). Our overall understanding of the molecular basis of interindividual variation in CYP2E1 expression is still unclear. There is also a possibility that there is interindividual

variability in ability to induce this enzyme, as reported in a study on ethanol induction of CYP2E1 (Dupont et al. 1998), but again this is not well understood.

The possibility that CYP2E1 genotype could be a risk factor for development of NAFLD or determine disease progression has been investigated in only two small studies to date. The first of these concerned NAFLD only and found that the *CYP2E1*5* allele was more common in a group of 28 Chinese patients with "obese or diabetic" fatty liver compared with 40 controls (Piao et al. 2003). The second study concerned female steatosis (n = 18) and NASH (n = 17) patients without diabetes (Varela et al. 2008) and found that there was an apparent increase in the frequency of *CYP2E1*5* in the NASH cases compared with both healthy controls and steatosis cases but the genotype frequency differences were not statistically significant. However, when CYP2E1 protein levels in liver biopsies and enzyme activity levels *in vivo* were compared, CYP2E1 protein levels were higher in both NASH and steatosis liver biopsies and NASH patients showed higher levels of activity *in vivo* compared with steasosis cases and controls. Carriage of the *CYP2E1*5* also correlated significantly with higher *in vivo* CYP2E1activity, which is in general agreement with previous *in vitro* data suggesting that this allele shows approximately tenfold higher transcriptional activity than the wild-type (Hayashi et al. 1991). Together the two studies on CYP2E1 genotypes in NAFLD provide a suggestion that carriage of at least one *CYP2E1*5* allele might be a risk factor in NAFLD but their small size and failure to see statistical significance for actual genotypes due to small numbers is a serious limitation. As well as performing larger studies, investigating a wider range of polymorphisms and areas such as interindividual variation in ability to induce CYP2E1 in NAFLD cases would be worthwhile. *CYP2E1*5* has also been found to be a risk factor for development of alcoholic liver disease which has common features to NAFLD (Grove et al. 1998; Pirmohamed et al. 1995), though more recent studies on associations of this allele with the disease are less positive (for review see Anstee et al. (2011b)). However, the possible association with susceptibility to alcoholic liver disease is likely to relate more to the role for CYP2E1 in ethanol metabolism than fatty acid metabolism as in NAFLD. A recent report suggesting that the *CYP2E1* gene contributes to interindividual variability in alcohol response (Webb et al. 2011) is an added complication to seeking parallels with NAFLD.

5.3.4 Genome-Wide Association Studies on NAFLD

In the last 5 years, several genome-wide association studies (GWAS) on NAFLD have been performed (Chalasani et al. 2010; Romeo et al. 2008; Speliotes et al. 2011). These studies involve genotyping cases and controls for single nucleotide polymorphisms (SNPs) throughout the genome. Performing a GWAS means that a large number of possible genetic associations for a disease are being examined, not simply those that are biologically obvious, and also enables the possibility that genetic polymorphism in *CYP2E1* contributes to NAFLD susceptibility to be investigated. Table 5.1 summarizes the main features of all three studies and their findings.

Table 5.1 Genome-wide association studies on NAFLD

Reference	No. of cases and controls	No. of SNPs typed	Main findings
Romeo et al. (2008)	2,111 American individuals in cohort study who underwent hepatic fat content analysis by proton nuclear magnetic resonance analysis	12,138 nonsynonymous variant SNPs genotyped with 9,229 passing QC	Single variant in PNPLA3 (rs738409) strongly associated with hepatic fat content ($P = 5.9 \times 10^{-10}$). No other SNP showed $P < 5.4 \times 10^{-6}$ (threshold for significance based on Bonferroni correction)
Chalasani et al. (2010)	239 American females with biopsy-proven NAFLD	373,397 SNPs with 324,623 used in the final analysis	Most significant association with quantitative histologic activity score (NAS) was for rs2645424 in farnesyl diphosphate farnesyl transferase 1 (*FDFT1*) gene ($P = 8.0 \times 10^{-7}$)
Speliotes et al. (2011)	7,176 Americans with extent of hepatic steatosis measured using computed tomography studied in GWAS. Replication of findings in 592 American biopsy-proven NAFLD cases and 1,405 population controls	300,000–500,000 (meta analysis using data obtained for several cohorts)	Associations ($p < 10^{-4}$) seen for *PNPLA3*, *NCAN*, *GCKR* and *LYPLAL1* with respect to both extent of steatosis and for biopsy-proven NAFLD compared with controls

Though each of these studies suffers from a range of limitations and there is a need for further studies where the severity of NAFLD with respect to genotype in a larger group of cases with liver biopsy is analyzed, no evidence for any signal either within the *CYP2E1* gene or adjacent to it has so far emerged. It remains possible that there is insufficient statistical power in the studies described so far to detect a signal from the *CYP2E1* gene or that a genetic regulator of CYP2E1 expression but not *CYP2E1* itself affects susceptibility.

5.4 Concluding Remarks

The relevance of CYP2E1 to NAFLD and NASH is a slightly neglected area, particularly recently. Though data suggesting a role for CYP2E1 in both NAFLD and NASH from animal models has appeared generally promising, attempts to establish whether CYP2E1 expression changes in human disease during the progression of human liver to NAFLD and then further progression to NASH have been limited, probably because of the difficulties in obtaining suitable samples for analysis, especially in large numbers. Better animal and cellular models for both NAFLD and NASH would facilitate progress. In terms of CYP2E1 as a genetic risk factor for NAFLD, it is increasingly likely that as for other complex diseases with a genetic component, many different variants contribute with the overall contribution from each being quite small (Hirschhorn and Gajdos 2011). For example, in the case of type II diabetes, one of the most extensively studied complex diseases, polymorphisms in more than 30 different genes have been demonstrated to contribute to susceptibility but odds ratios for some of these genes are as low as 1.1 (Voight et al. 2010). Detecting such small effects requires in the order of 10,000 cases for statistical significance and, though NAFLD is now a very common disease worldwide, it is unlikely that DNA collections of this size are yet available. Building up such a large DNA collection from NAFLD cases is particularly challenging because of the importance of including only cases where histology has been definitively established by liver biopsy (Anstee et al. 2011c). However, given the increasing frequency of NAFLD in developed countries and the current lack of any effective treatment, performing such large studies should be given a high priority since they may lead to development of new treatments and design of more effective strategies for preventing disease progression.

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Chapter 6
CYP2E1-Catalyzed Alcohol Metabolism: Role of Oxidant Generation in Interferon Signaling, Antigen Presentation and Autophagy

Natalia A. Osna and Terrence M. Donohue Jr.

Abstract Cytochrome P450 2E1 (CYP2E1) is one of two major enzymes that catalyze ethanol oxidation in the liver. CYP2E1 is also unique because it is inducible, as its hepatic content rises after continuous (chronic) ethanol administration, thereby accelerating the rate of ethanol metabolism and affording greater tolerance to heavy alcohol consumption. However, the broad substrate specificity of CYP2E1 and its capacity to generate free radicals from alcohol and other hepatotoxins, places CYP2E1 as a central focus of not only liver toxicity, but also as an enzyme that regulates cytokine signaling, antigen presentation, and macromolecular degradation, all of which are crucial to liver cell function and viability. Here, we describe our own and other published work relevant to the importance of CYP2E1-catalyzed ethanol oxidation and how this catalysis affects the aforementioned cellular processes to produce liver injury.

Keywords CYP2E1 • Antigen • Degradation • Ethanol • Liver

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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*, Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_6,

6.1 CYP2E1 Regulation by Ethanol Exposure

6.1.1 Hepatic Ethanol Oxidation in the Liver

Excessive alcohol consumption affects virtually all organs in the body but, because the liver is the principal site of ethanol oxidation, liver disease is the most prevalent clinical complication of problem drinking. At low to moderate alcohol levels in the bloodstream (1–2 mM; 4.6–9.2 mg/dL), ethanol is oxidized principally by the cytosolic class I alcohol dehydrogenase (ADH-I), with a Michaelis constant (K_m) of 1–2 mM ethanol. Moderate alcohol doses quickly saturate ADH-I, the activity of which is not appreciably altered by ethanol administration *in vivo* (Donohue et al. 2006). However, there is a report to indicate that binge or chronic drinking increases the class III ADH activity, which has a higher K_m for ethanol than the class I enzyme (Haseba and Ohno 2010). The other ethanol-oxidizing enzyme, cytochrome P450 2E1 (CYP2E1), in the smooth endoplasmic reticulum (ER) has a ~tenfold higher K_m for ethanol (10–17 mM; 46–78 mg/dL) than ADH-I, but is also highly inducible, so that ethanol clearance is increased in heavy drinkers, both by accelerated CYP2E1 catalysis and an enhancement of the CYP2E1 V_{max} that results from an increase in its content (Norberg et al. 2003). Thus, at and above legally intoxicating blood alcohol levels of 17.4 mM (80 mg/dL) ADH and CYP2E1, both oxidize ethanol. Both enzymes generate reactive metabolites in the form of acetaldehyde, but CYP2E1 has a much slower catalytic turnover than ADH in this conversion. Others have reported that, *in vitro*, recombinant CYP2E1 catalyzes the subsequent oxidation of acetaldehyde to acetate (Bell-Parikh and Guengerich 1999). Also, because of its broad substrate specificity, CYP2E1 metabolizes other structurally diverse substrates ranging from ethanol, the industrial solvent, CCl_4, the anaesthetic, halothane and the analgesic, acetaminophen. The metabolic products generated by CYP2E1 from the metabolism of these compounds are reactive aldehydes, free radicals, halogenated free radicals and quinones, respectively. The latter molecules are capable of nonenzymatically reacting with proteins and lipids, creating adducted proteins and lipid peroxides, which are potentially or genuinely poisonous. The catalytic diversity of CYP2E1 and its ability to generate oxidants has prompted numerous investigations of its role as a major cause of ethanol-induced injury, not only in liver but also in other organs such as the brain, where peroxisomal catalase and ER-associated CYP2E1 are the two major alcohol-oxidizing enzymes (Zimatkin et al. 2006).

6.1.2 Induction of CYP2E1 by Ethanol

Lieber et al. made the initial discovery of CYP2E1 as a component of liver microsomes and called it the microsomal ethanol oxidizing system or MEOS. It went by this and other names (e.g. 450-ALC; P450j) until the P450 family of genes was systematically reclassified (Nebert et al. 1987). MEOS (CYP2E1) is induced by

chronic ethanol consumption, based upon the frequent finding of its elevated catalytic activity (compared with untreated controls) of microsomal (ER) fractions isolated by ultracentrifugation of liver extracts (Lieber and DeCarli 1968; Lieber 1970; Lieber et al. 1970).

The mechanism of CYP2E1 induction by ethanol, pyrazole, 4-methylpyrazole (both the latter two compounds actually inhibit CYP2E1 catalysis) and acetone is rather unique among the variety of compounds that increase CYP2E1 levels by enhancing the levels of its messenger RNA (Song et al. 1986). Ethanol induces the enzyme principally by stabilizing CYP2E1 from degradation by the ubiquitin-proteasome system. Roberts et al. (1995) reported a fivefold increase in the *in vivo* half life of CYP2E1 in livers of ethanol-fed rats, to indicate that CYP2E1 is stabilized by ethanol administration. They demonstrated that polyubiquitylation of CYP2E1 is increased in livers of ethanol-fed animals. This reflected an accumulation of undegraded CYP2E1, which the authors showed to be a substrate (ethanol)-level stabilization of CYP2E1. Both ubiquitylation and degradation of CYP2E1 are blocked by inclusion of 60 mM ethanol in liver cytosols (Roberts et al. 1995), indicating that ethanol itself, provides substrate protection of CYP2E1 from proteolysis. These findings were confirmed by others, using chronic intragastric ethanol feeding (Bardag-Gorce et al. 2002). More recent work has revealed a possible reciprocal relationship between CYP2E1 catalysis and proteasome activity (Wu et al. 2005; Bardag-Gorce et al. 2006). This is described in greater detail (by French) in another chapter of this volume.

6.2 Oxidant Generation and Interferon Signaling

There is evidence that ethanol oxidation in the liver inhibits the function of a number of cellular signaling pathways by forming aldehyde- and other oxidant-derived adducts with proteins, causing modifications of their structures and their signaling properties (Stadtman and Berlett 1998; Rubbo et al. 1996). The major alcohol-metabolizing enzymes, ADH and CYP2E1 are highly expressed in hepatocytes. In experimental conditions, when isolated hepatocytes are placed into culture for extended periods, the expression of ADH and CYP2E1 diminishes, which confounds interpretation of experimental results (Wan et al. 2005; Osna et al. 2005). As an alternate experimental model, recombinant hepatoma cells that constitutively express either or both ADH and/or CYP2E1 have been successfully used for ethanol studies. Examples of these cell lines are transfected HepG2-based cells: E-47 ($CYP2E1^+$) cells, VA-13 (ADH^+) cells, and VL-17A ($CYP2E1^+$/ADH+) cells (Nieto et al. 2002; Osna et al. 2011a, b) transfected Huh7-based cells (Huh7CYP) (Osna et al. 2010) or untransfected WIF-B cells that naturally express both enzymes (Schaffert et al. 2004). Studies from many laboratories demonstrated the involvement of CYP2E1 and ADH in the formation of protein adducts in liver cells, contributing to the development of alcoholic liver disease (ALD) and Mallory-Denk Body formation (Wu et al. 2005, 2010; Wu and Cederbaum 2008; French et al. 2001).

In our laboratory, we have focused our studies on the effects of ethanol on proteasome function, which is regulated by interferons *via* the JAK-STAT signaling pathway.

Interferon gamma (IFNγ) is a major pro-inflammatory cytokine that is naturally released by immune cells. It enhances proteasome activity, promotes antigen presentation, regulates cell proliferation, apoptosis and prevents liver fibrosis (Ramana et al. 2002; Stark et al. 1998). IFN-γ signaling is initiated *via* the Janus kinase I-signal transducer and activator of transcription1 (JAK-STAT1) pathway. JAKs can phosphorylate STAT1 on both tyrosine (Tyr) and serine (Ser) residues. Generally, IFNγ-induced STAT1 phosphorylation on Tyr residue 701 activates protective functions of viable cells (such as processing of peptides for antigen presentation), while Ser 727 phosphorylation transduces pro-apoptotic (death) signals (Stephanou and Latchman 2003). In addition to phosphorylation, IFN signaling is regulated at the level of activated STAT1 attachment to DNA to target the genes of Gamma-Interferon Activated Sites (GAS). Several factors negatively regulate IFN signaling. These include the SH2-containing phosphatases (SHP1 and SHP2), which bind their SHP domains to phosphorylated Tyr residues and dephosphorylate them (Wormald and Hilton 2004). Other factors that regulate STAT1 phosphorylation are the suppressors of cytokine signaling (SOCS 1 and SOCS 3). They both block JAKs phosphorylation of STAT1 (upstream from STAT1 phosphorylation) (Hilton 1999). Also, SOCS1 is an E3 ubiquitin ligase that ubiquitylates phosphorylated JAK2, leading to its subsequent degradation by the 26S proteasome (Ungureanu et al. 2002). A third group of negative regulators is the protein inhibitors of activated STAT1 (PIAS1), which blocks IFN signaling by preventing STAT1 attachment to DNA (Wang et al. 1998; Marques et al. 2004). These negative regulators of IFN signaling are activated by certain events/factors that interfere with IFN-regulated signal transduction.

Exposure to ethanol of liver-derived hepatoma cells suppresses subsequent IFN signaling. To characterize this suppression, we exposed VL-17A ($CYP2E1^+/ADH^+$) cells to 50–100 mM ethanol and found that, while ethanol oxidation suppresses STAT1 phosphorylation on Tyr701, Ser 727 phosphorylation is unchanged (Osna et al. 2005) (Fig. 6.1). These suppressive effects of ethanol on IFN signaling are required for its metabolism, as they are prevented by simultaneous treatment with the ethanol oxidation inhibitor, 4MP. VL-17A cells require 3 days treatment with 50–100 mM ethanol to partially block STAT1 phosphorylation. The resistance of these cells is attributed to their glutathione levels, which are three- to four-fold higher than in primary hepatocytes. However, after glutathione depletion by treatment with L-buthioninesulfoximine (BSO), a shorter duration of exposure ethanol is required to achieve the same decrease in STAT1 phosphorylation.

We investigated which ethanol-metabolizing enzyme generates the oxidants that affect STAT1 phosphorylation, by exposing E-47 ($CYP2E1^+$) and VA-13 (ADH^+) cells to 50 mM ethanol. Remarkably, ethanol-exposed VA-13 cells exhibit blocked IFNγ-induced STAT1 phosphorylation by 85%, and the latter effect is inhibited by 4MP. In E-47 cells, suppression of IFNγ signaling depends on the basal level of CYP2E1 activity. When basal CYP2E1 activity is higher (17 ± 5 units/mg protein), ethanol exposure inhibited STAT1 phosphorylation, while cells with lower basal

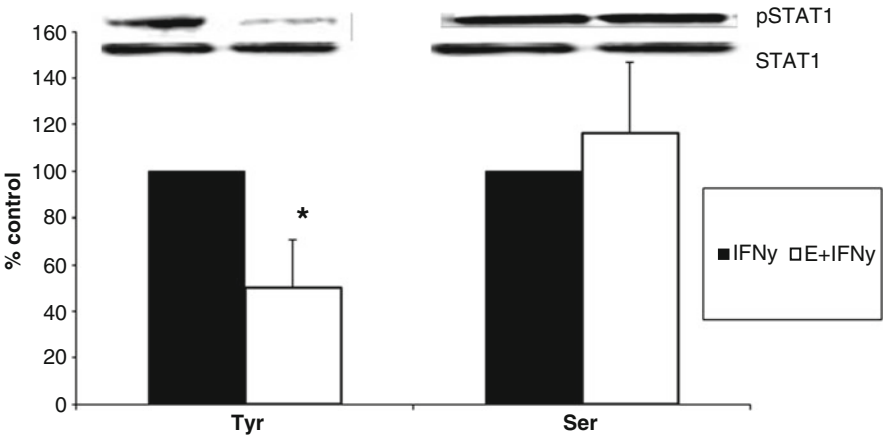

Fig. 6.1 Effects of EtOH on IFNγ-induced STAT1 phosphorylation on Tyr and Ser residues in VL-17A cells. VL-17A cells were treated or not with 100 mM EtOH for 72 h and then exposed to IFNγ for 1 h. pSTAT1 Tyr, pSTAT1 Ser and STAT1 were each determined by Western blot. * indicates a significant difference ($p<0.05$) between IFNγ-treated and IFNγ + EtOH-treated samples

CYP2E1 activity (7.3 ± 3 units/mg protein) do not exhibit this response. Furthermore, in CYP2E1$^+$/ADH$^+$ VL-17A cells, the decrease in STAT1 phosphorylation is partially blocked by diallylsulfate, a CYP2E1 inhibitor and by uric acid, an anti-oxidant, indicating that CYP2E1 plays an equally important role as ADH in regulating IFN signaling (Osna et al. 2005). Only in CYP2E1-expressing (E-47 and VL-17A cells) is there an induction of SOCS1, the negative regulator of STAT1 phosphorylation. It is noteworthy that, like many other regulatory proteins, SOCS1 is degraded by the proteasome (Greenhalgh and Hilton 2001; Kile and Alexander 2001; Vuong et al. 2004). Ethanol treatment of VL17A cells inhibits proteasome activity, which is associated with enhanced SOCS1 content in these cells, indicating that ethanol oxidation stabilizes SOCS1 from degradation in VL-17A cells (Osna et al. 2003; Donohue et al. 2006). The involvement of CYP2E1 in SOCS1 stabilization is probably related to CYP2E1-dependent generation of peroxynitrite (a reaction product of nitric oxide and supreroxide) in liver cells, which induces high oxidative stress and blocks proteasome function (Osna et al. 2004). In addition, nitration by peroxynitrite of Tyr residues on STAT1 itself may also lower its activation. However, ADH-generated products also participate in the modulation of IFN signaling, as acetaldehyde and/or elevated redox potential (i.e. NADH$^+$/NAD ratio) suppress(es) STAT1 phosphorylation. The mechanism of this latter event is not yet clear but, it is known that acetaldehyde, as well as malondialdehyde-acetaldehyde (i.e. MAA), bind to lysine residues on proteins (Tuma et al. 1991), one of which is Lys 705, in the primary sequence of STAT 1 and which may influence phosphorylation of the neighboring Tyr 701 in that protein. Additionally, acetaldehyde reacts directly with tyrosine residues in hemoglobin, which would likely block phosphorylation of this residue in STAT1 (Stevens et al. 1981).

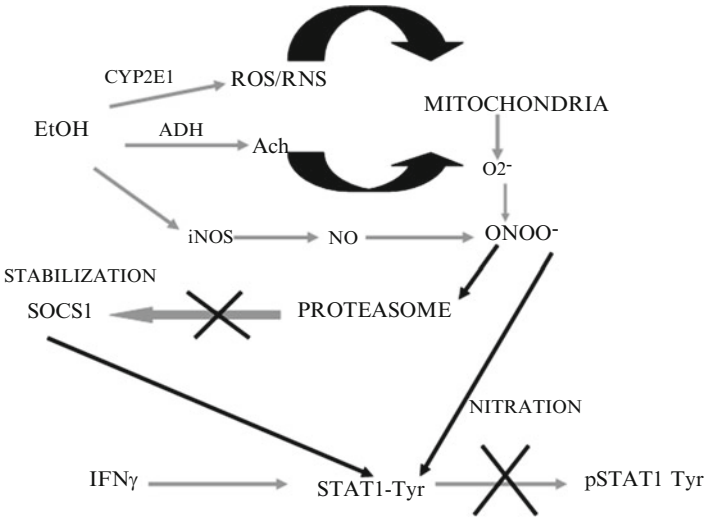

Fig. 6.2 The proposed effects of EtOH metabolism on IFNγ-induced STAT1 phosphorylation in VL-17A cells. EtOH is metabolized by ADH and CYP2E1 to Ach and ROS/RNS. These reactive intermediates damage mitochondria, thereby increasing superoxide (O_2^-) leakage. Because EtOH also induces iNOS, enhancing nitric oxide (NO) formation, there is increased formation of PN ($ONOO^-$), a reaction product of O_2^- and NO. High concentrations of PN block proteasome activity, thereby stabilizing SOCS1, which prevents IFNγ signaling. PN also reacts with STAT1 on Tyr residues, making them unavailable for phosphorylation. *Gray arrows* show positive effects, *black arrows* show negative effects

Ethanol-induced suppression of IFN-activated STAT1 phosphorylation is demonstrable in recombinant ethanol-metabolizing HepG2, HepB6 and WIF-B cells (Osna et al. 2007, 2009). In addition to the reduction of STAT1 phosphorylation in these cells, we also observed a decrease in activated STAT1 binding to DNA due to ethanol-induced PIAS1 expression (unpublished data).

In view of these, findings we propose the following mechanism of ethanol-induced regulation of STAT1 phosphorylation in liver cells (Fig. 6.2): IFNγ-induced STAT1 phosphorylation on Tyr 701 is decreased by ethanol metabolism, and both ADH- and CYP2E1-generated products contribute to that reduction. However, only CYP2E1-generated products elevate SOCS1 levels. Stabilization of SOCS1 is due to suppression of proteasome activity by ethanol-induced peroxynitrite generation, which occurs during high oxidative stress in liver cells.

6.3 Proteasome Activity and CYP2E1: Reciprocal Regulation by IFNγ and Ethanol

The proteasome is a large proteolytic enzyme particle, which degrades most intracellular proteins both in the cytosol and nucleus. It regulates normal physiological processes by degrading native short-lived proteins and some longer-lived

constitutive enzymes, including the ethanol-metabolizing enzymes, CYP2E1 and ADH (Mezey et al. 2001). The proteasome also affects signal transduction by degrading signaling proteins and their negative regulators. The proteasome plays an important role in protein quality control, as it removes aged, damaged cytotoxic proteins and degrades antigens to smaller peptides for MHC class I-restricted antigen presentation (Chen and Hochstrasser 1996; Kim and Maniatis 1996). Many of the aforementioned processes are enhanced by the cytokine, IFNγ, a potent activator of proteasome activity.

VL-17A and E-47 are hepatocyte-like hepatoma cell lines. In these cells, proteasome activity is stimulated by direct treatment with IFNγ as early as 4 h after initial exposure (Osna et al. 2003). This stimulation requires expression of CYP2E1, as parental HepG2 cells, which do not express CYP2E1 do not respond to IFNγ by proteasome activation. Because CYP2E1 activity fluctuates about twofold between various cell passages, we have correlated basal CYP2E1 activity in VL-17A cells with the stimulatory effect of IFNγ on proteasome function and showed that this response is dependent on CYP2E1 catalysis: cells with higher CYP2E1 activity exhibit higher proteasome activity after treatment with the cytokine (Pearson $r = 0.938$, $p < 0.05$). In addition, IFNγ-induced activation of proteasome is suppressed by diallylsulfide (DAS), a specific CYP2E1 inhibitor.

We investigated which product of CYP2E1 catalysis regulates proteasome activation by IFNγ. Because inducible nitric oxide synthase (iNOS) produces nitric oxide (NO) after stimulation by IFNγ (Wink and Mitchell 1998), this same mechanism is also operative when IFNγ enhances proteasome activity, due to the ability of NO and superoxide to react together and form peroxynitrite (PN). Therefore, we measured proteasome activity in IFNγ-exposed VL-17A cells in the presence of the iNOS inhibitors, N, ω Nitro-L-arginine methyl ester (L-NAME) or epigallocatechingallate (EGCG) or the peroxynitrite scavenger, FeTPPS. Each treatment blocked the IFNγ-induced rise in proteasome activity, clearly indicating that both CYP2E1 (a source of superoxide) and iNOS (a source of NO) contribute to the formation of PN. Furthermore, the effect of PN on proteasome function is biphasic and dose-dependent; direct exposure to low doses of PN (less that 2 mM) or to 0.01–0.1 mM of the PN donor, SIN-1, enhances proteasome function, while higher doses of PN (2–10 mM) suppress it (Osna et al. 2005). The involvement of PN in the regulation of proteasome function has been also demonstrated by others (Grafstrom et al. 1994; Grune et al. 1998, 2001; Amici et al. 2003).

Dose-dependent regulation of proteasome activity by PN is related to the ability of PA28, an activator of the 20S proteasome, to open "the substrate gate" of the 20S proteasome. This contact is limited to the level of proteasomal nitration: higher nitration accompanying high oxidative stress lowers proteasome activity, while low nitration, related to low oxidative stress stimulates proteasome activity (Osna et al. 2005; Pickering et al. 2002; Pickering and Davies 2012). When IFNγ treatment of VL-17A cells generates a low amount of PN and the level of proteasome nitration is also low, exposure to this cytokine enhances proteasome activity. This enhancement occurs via the JAK-STAT1 pathway it is prevented by treatment with tyrphostin AG490, an inhibitor of this pathway (Osna et al. 2003).

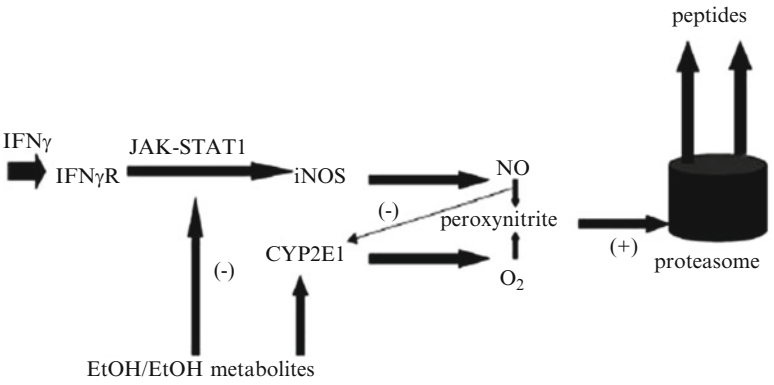

Fig. 6.3 IFNγ mediated activation of the JAK-STAT1 pathway. IFNγ initiates activation of the JAK-STAT1 pathway via IFNγR on liver cells and thereby, activates iNOS to generate NO. CYP2E1 catalyzes superoxide production. The reaction product, PN, at low, almost physiological doses activates proteasome function and enhances the effects that are down-stream from proteasome cleavage (such as generation of peptides for antigen presentation), In the presence of ethanol, CYP2E1 produces more superoxide due to generation of higher levels of oxidative stress will be. Ethanol metabolites block JAK-STAT1 signaling and prevent STAT1 phosphorylation by IFNγ, thereby decreasing (or even blocking) the response of proteasome to IFN

Ethanol treatment of VL-17A cells decreases proteasome function and blocks proteasome activation by IFNγ. The latter is related to a higher level of oxidative stress induced by ethanol metabolism, which in turn causes a higher level of proteasome nitration, disintegrating the contacts between 20S proteasome and PA28 (Osna et al. 2003, 2005). Thus, under mild oxidative stress, which is accompanied by IFN production, the proteasome may be activated by low concentrations of PN and likely enhances intracellular proteolysis. However, under more severe oxidative stress, higher amounts of PN suppress proteasome activity, thereby decreasing intracellular protein degradation.

The following scheme of proteasome regulation by peroxynitrite in liver cells is shown in Fig. 6.3.

6.4 Interferon-Dependent Antigen Processing and Presentation in Liver Cells

Both the 20S and 26S proteasome forms generate peptides for MHC class I-restricted antigen presentation (Rock et al. 2002; Qian et al. 2006). Processing of antigens is a necessary step for recognition by cytotoxic T-lymphocytes (CTLs) of virally infected cells. To be recognized by CTLs, “non-self” antigens are processed to peptides of 8–10 amino acids, which then bind to MHC class I molecules in the endoplasmic reticulum (Racanelli and Rehermann 2006), and the complex is presented on the cell surface. Initial degradation of antigenic proteins in the cytoplasm is catalyzed

by the proteasome. This enzyme is activated by the pro-inflammatory cytokine, interferon gamma (IFNγ) (Rock et al. 2002) via the JAK-STAT pathway. IFN induces formation of the immunoproteasome, into which is incorporated specific β subunits that replace constitutively expressed β-subunits. This subunit replacement allows the proteasome to cleave antigenic proteins to peptides of uniform size for presentation on the cell surface.

While dendritic cells and macrophages are "professional" antigen presenters, hepatocytes also display "foreign" antigen-MHC class I complexes. This presentation is critical for outcomes of viral hepatitis B and C, where elimination of infected hepatocytes is the major function of CTLs. Hepatocytes, in these cases, serve as the target cells that are subjected to clearance by CTLs. Thus, persistence of infected hepatocytes in the liver leads to a chronic course of viral hepatitis. Epidemiological studies have provided supporting evidence of severe and rapid progression of chronic hepatitis B and C in alcoholics (Perrien et al. 2002). Alcohol abuse strongly accelerates and prolongs the pathogenesis of HCV infection by increasing oxidative stress, fibrosis progression and the risk of death from cirrhosis. However, the exact pathogenic pattern of alcohol-aggravating effects on HCV infection is not yet clear. Impairment of antigen processing/presentation during or after exposure to alcohol is one of the likely reasons for chronic persistence of HCV and HBV infections.

We studied the effects of ethanol on antigen processing/presentation in hepatocytes and ethanol-metabolizing hepatoma cells (VL-17A, WIFB, HepB6 cells) to test the hypothesis that ethanol-induced oxidative stress impairs the function of antigen-trimming enzymes, thereby preventing their ability to process antigenic proteins (Osna et al. 2007). Because protein degradation for antigen presentation depends, in part, on proteasome function, we examined the effects of oxidative stress inducers, including ethanol, on the peptidase activities of the proteasome. For experiments we used the peptides that are presented in the context of MHC class I, which are genotype- and origin-specific for cells and are known CTL epitopes. Thus, for human VL-17A cells, we used extended HBV core peptide 18–27, **FLPSDFFPSV**.

The generation of peptides for antigen presentation requires proteasome chymotrypsin-like (Cht-L) and trypsin-like (T-L) activities, which cleave peptides on the C-termini of hydrophobic and basic residues, respectively (York et al. 1999). We therefore examined whether prior ethanol exposure to VL-17A cells affects either or both peptidase activities (Osna et al. 2007). To detect 20S and 26S proteasome activities, we incubated cytosols in the presence and absence of 0.5 mM ATP, respectively. Prior treatment of VL-17A cells with ethanol suppresses ChT-L and T-L activities of both proteasome forms. Ethanol exposure decreases the Cht-L activity of the 26S proteasome to a greater extent (77% reduction) than that of the 20S proteasome (50% reduction). The patterns of ethanol-induced suppression are similar for both forms of the enzyme. Thus the Cht-L activity exhibited greater sensitivity to ethanol than the T-L activity. Inhibition of Cht-L activity by ethanol was blocked by simultaneous incubation with 4MP or DAS, indicating that CYP2E1 affects proteasome activity. This was confirmed by suppression of proteasome activity, which correlates inversely with the ethanol-elicited elevation of CYP2E1 activity.

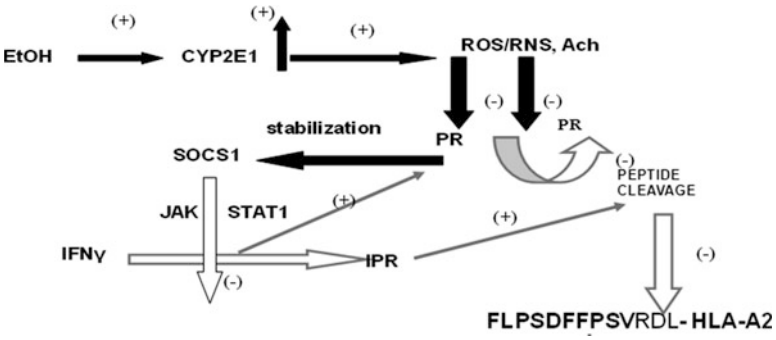

Fig. 6.4 Proposed mechanism of regulation of peptide cleavage by ethanol metabolism. Ethanol (EtOH) treatment induces CYP2E1 activity, which catalyzes production of oxidants: reactive oxygen (ROS), and nitrogen species (RNS) and acetaldehyde (Ach). These products block proteasome (Pr). Suppressed proteasome activity causes stabilization of SOCS1, a negative regulator of STAT1 phosphorylation. Elevated levels of SOCS1 disrupt IFNγ signaling by inhibiting STAT1 phosphorylation, thereby preventing activation of PA28 and formation of immunoproteasome (IPr). Finally, generation of C-extended peptides for MHC class I-restricted antigen presentation is blocked. Enhancing effects of ethanol are shown by *black arrows*

Ethanol exposure also suppresses the IFNγ-induced expression of the immunoproteasome subunit, LMP2 and the 20S proteasome activator, PA28, in these cells. As a consequence, there is a reduction in cleavage of C-extended peptide, **FLPSDFFPSV**RDL, after incubation of this peptide with proteasome-containing cytosol fractions from VL-17A cells. Treatment of VL-17A cells with IFN itself, does not affect C-extended peptide cleavage. However, when cells are exposed to both ethanol and IFNγ, peptide hydrolysis is partially accelerated, compared with fractions from cells exposed to ethanol alone. These results have been confirmed using WIF-B cells and indicate that oxidative stress blocks not only proteasome activity, but also the responsiveness of proteasome/immunoproteasome to IFNγ and its ability to catalyze peptide cleavage. Suppression of IFNγ-induced peptide cleavage is related to ethanol-elicited disruption of IFNγ signaling via the Jak-STAT1 pathway, partially due to stabilization of SOCS1, a negative signaling regulator and proteasome substrate (Fig. 6.4).

To demonstrate that ethanol-elicited decrease in cleavage by proteasome resulted in lower expression of peptide-MHC class I complexes on liver cell surface, we employed another model, the display of SIINFEKL (ovalbumin peptide recognized by CTLs) in the context of C57Bl6 mouse MHC class I, H2Kb (SIINFEKL-H2Kb). For ethanol exposure, we used HepB6 cells, a cell line obtained from immortalized hepatocytes of C57Bl6 mice and transfected with CYP2E1 and ADH. These CYP2E1+ cells also possess low ADH activity (Osna et al. 2009). This study revealed that the suppression of SIINFEKL-H2Kb presentation by ethanol metabolism correlates with the reduction of IFNγ-induced proteasome activity and decreased cleavage of C-extended peptide, SIINFEKL-TE, delivered into the cell. Effects of ethanol were blocked by 4MP and catalase, the latter a H_2O_2 scavenger, indicating the involvement

of ethanol metabolism and CYP2E1. The observed results were similar to those with hepatocytes freshly isolated from livers of C57Bl6 mice. Also, the HSP90 inhibitor, geldanamycin, which inhibits the loading of the antigenic peptide onto MHC class I and Brefeldin A, an inhibitor of trafficking via ER, both decreased SIINFEKL-H2Kb presentation, showing that complex formation is chaperoned by HSP90 and requires trafficking through the ER to be presented on the cell surface.

Since H2Kb expression is minimal on HepB6 cells, the cells were pre-exposed to IFNγ to induce H2Kb expression, and the display of SIINFEKL-H2Kb on HepB6 surface was dependent on IFNγ signaling in these cells. In contrast, hepatocytes constitutively express H2Kb. Even without prior IFNγ treatment, we observed ethanol-elicited suppression of both proteasome activity and the peptide presentation in hepatocytes, suggesting that the effects of ethanol on these parameters in mouse liver cells are not completely based on defective IFN signaling. While studying IFN signaling in HepB6 cells, we found no changes in STAT1 phosphorylation, but the attachment of STAT1 to DNA was lower after ethanol treatment (Osna et al. 2009) due to elevation of ethanol-induced PIAS1, which competes with activated STAT1 for DNA binding. This effect was prevented by 4MP treatment, indicating that it required ethanol oxidation.

From these results, we conclude that by CYP2E1-dependent impairment of proteasome function and IFN signaling, ethanol metabolism reduces the processing of precursor antigenic proteins, thereby decreasing antigen presentation on the hepatocyte surface.

6.5 Involvement of CYP2E1 in the Ethanol-Induced Regulation of Autophagy

6.5.1 Physiological Function of Autophagy

All eukaryotic cells digest parts of their cytoplasmic contents by autophagy, which literally means, “self eating”. This catabolic process was discovered long ago (De Duve and Wattiaux 1966), but it has received a great deal of recent attention, because of its vital role in maintaining cellular quality control and longevity. Newer evidence indicates the involvement of autophagy in clinically significant pathological states, including cancer, liver disorders, and neurological disease (Rabinowitz and White 2010; Rautou et al. 2010). Remarkably, specific factors that participate in autophagy are also essential for the propagation of liver pathogens including the hepatitis B and C viruses.

Three forms of autophagy, macroautophagy, microautophagy and chaperone-mediated autophagy operate inside cells. Each involves the hydrolysis of macromolecular substrates inside lysosomes but they vary in the method of substrate delivery to the lysosome. Macroautophagy, which is synonymous with autophagy, is the most predominant form, during which autophagic vacuoles (AVs or autophagosomes) sequester and envelop particulate and soluble cytoplasmic components, then deliver

the enclosed cargo to lysosomes by fusing with these organelles to degrade the AV contents. Macroautophagy is initiated by AV formation, which is a complex signaling process directed by the products of autophagy-related genes (Atgs) and is detailed in other reviews (Rautou et al. 2010; Donohue 2009; Czaja 2011). Briefly, AV formation is induced by physiological signals such as fasting, exposure to toxins, or to hormones, such as glucagon. Beginning with a membrane precursor called the phagophore, AV formation proceeds through a series of protein interactions that lead to development of the mature, double-membrane AV, which is identified by a lipidated protein biomarker named the microtubule associated light chain-3-II (LC3-II). The latter binds tightly and selectively to AV membranes, serving as a qualitative and quantitative index of AV content. Once the AV is fully formed with its cargo, it is trafficked to lysosomes, which degrade the AV contents, including LC3II. The latter degradation step is essential not only for normal macromolecular turnover but also for removal of potentially toxic biopolymers, protein aggregates and damaged organelles. The degradation products, which are amino acids, nucleotides, fatty acids and monosaccharides are transported to the cytoplasm and utilized either for re-synthesis of larger molecules or for energy generation. Maintenance of a "proper" level of proteins, larger lipids (e.g. triglycerides) and complex carbohydrates (e.g. glycogen) is essential for organ homeostasis and derives from a balance between their synthesis and degradation.

Microautophagy and chaperone-mediated autophagy contribute significantly to macromolecular degradation but are more specific in their substrate selectivity. These two processes involve the direct breakdown by lysosomes of smaller particles (microautophagy) or of specific proteins that carry specific sequences that are recognized by molecular chaperones. Both these processes are described in greater detail elsewhere (Donohue 2009; Czaja 2011; Cuervo et al. 1997).

6.5.2 Autophagy in Liver During Alcohol Consumption

Besides this chapter, other recent papers specifically address autophagy and ethanol hepatotoxicity (Osna et al. 2011a, b; Ding et al. 2011a). Problem drinkers, alcoholics, and alcohol-fed rodents commonly develop enlarged livers. Hepatomegaly is caused by intrahepatic accumulation of proteins and lipids (Baraona et al. 1977). Protein accumulation represents a slowdown of protein catabolism, particularly those intracellular proteins degraded in lysosomes by autophagy (Donohue et al. 1989; Singh et al. 2009). We postulate that ethanol-induced steatosis (fatty liver) is caused in part by alterations in autophagy as well (see below). Our previous work has shown that, compared with lysosomes isolated from control rats, lysosomes from chronically ethanol-fed rats have a reduced capacity for proteolysis, an elevated intralysosomal pH, a greater susceptibility to leak, and a lower content of cathepsin L. The cathepsin L deficiency results from an ethanol-induced delay in cathepsin L precursor trafficking to the lysosomal compartment (Donohue et al. 1994; Kharbanda et al. 1995, 1996, 1997). Ding et al. reported that acute ethanol exposure (i.e. binge drinking)

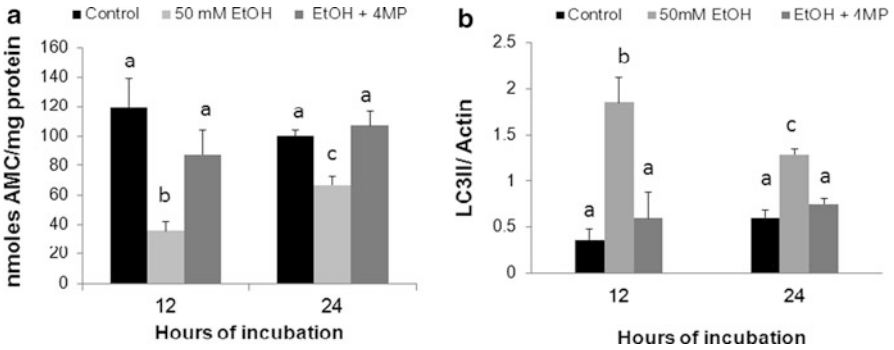

Fig. 6.5 Ethanol-elicited decline in proteasome activity (panel A) and increase in LC3II in PCLS (panel B). Data are mean values (±SEM). Microtubule associated light chain- 3-II (LC3-II) precision cut liver slices (PCLS). Note: 4MP data were similar to that of EtOH+4MP and are not shown

enhances hepatocellular AV content and that these transitory organelles rather selectively target mitochondria and lipid droplets for degradation (Ding et al. 2010). We recently confirmed these findings (Thomes et al. 2012) and demonstrated that in ethanol-exposed precision-cut slices from rat liver, ethanol oxidation generates metabolites that enhance AV content and which simultaneously inhibit the 20S proteasome (Thomes et al. 2012). Both effects are blocked when ethanol oxidation is inhibited by 4-methylpyrazole (Fig. 6.5). In addition, chronic ethanol administration to mice decreases proteasome activity but increases AV and LC3II content (Fig. 6.6), to suggest that ethanol oxidation exerts a reciprocal effect on the proteasome and AV content (Thomes et al. 2012). This occurs in part because LC3 is not only an autophagy substrate, but also a 20S proteasome substrate (Thomes et al. 2012). Thus, when proteasome activity is inhibited by ethanol oxidation, LC3 content rises because its degradation is impaired. As expected, the ethanol-induced decline in proteasome activity also causes a two- to three-fold elevation in CYP2E1 and ADH protein levels (Fig. 6.7), confirming that both ethanol-metabolizing enzymes are proteasome substrates (Roberts et al. 1995; Mezey et al. 2001). The rise in AVs (LC3II) in livers of chronically ethanol-fed mice is associated with increased levels of p62, a protein, which binds to LC3II and is a rather selective autophagy substrate. The latter findings (unpublished) lead us to suggest that, during chronic ethanol consumption, the intracellular content of AVs and p62 increase because ethanol oxidation not only enhances AV biogenesis but also blocks AV and p62 degradation. In this context, it is also noteworthy that the rise in p62 activates the cytoprotective transcription factor, Nrf2, (Komatsu et al. 2010) which is the topic of Chap. 2 in this volume.

Presently, we do not completely understand the exact mechanism(s) by which ethanol administration decreases proteasome activity and increases AV content. Data from others indicate (cf. Chap. 4 in this volume) that the induced level of CYP2E1 in chronically ethanol-fed rats generates oxidants that enhance formation of the lipid peroxide, 4-hydroxynonenal (4-HNE). This molecule rather selectively forms adducts with proteasome subunit Rpt4, an ATPase in the 19S regulatory particle

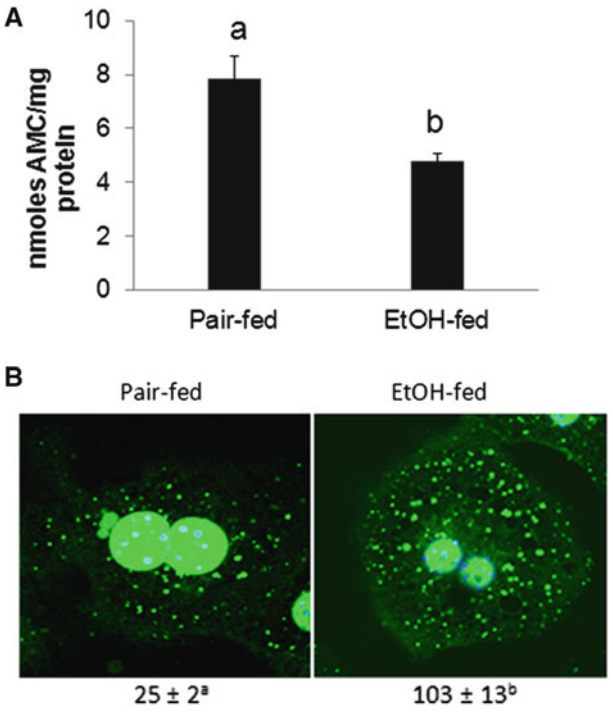

Fig. 6.6 Chronic ethanol feeding inhibits proteasome activity (panel A) and enhances AVs (panel B) in mouse hepatocytes. Mean values (±SE) from 15 randomly selected images per group are given under each micrograph. Letter symbols have same meaning as in Fig. 6.5

of the 26S proteasome and that maintains interaction between the 19S particle and the 20S catalytic core. Disruption of this interaction is associated with the reduction of proteasome peptidase activity (Wu et al. 2005). Interestingly, the rise in AV content after acute *and* chronic ethanol administration is attributed to similar phenomena, but the target(s) of the reactive species appear(s) to be different during binge vs. chronic drinking. During acute (binge) ethanol consumption, oxidants and reactive intermediates derived from a rapid increase in ethanol metabolism inhibit the mammalian target of rapamycin complex 1 (mTORC1) a large regulatory kinase that normally enhances anabolic reactions and suppresses autophagy. Inhibition of mTORC1 allows AV biogenesis to proceed, thereby enhancing cellular AV (LC3II) content. During chronic ethanol administration the metabolic picture shifts, as the generation of acetaldehyde and reactive species is more consistent, due to greater regularity and frequency of drinking. In the liver, these conditions favor acetaldehyde- and malondialdehyde-acetaldehyde- adduct formation with proteins (Tuma et al. 1996; Tuma 2002), among which tubulin, the subunit protein of microtubules is a susceptible reactant. Tubulin polymerization into microtubules is inhibited by acetaldehyde-tubulin adduct formation (Smith et al. 1989), which implies that their loss of function ultimately prevents fusion of AVs with lysosomes, resulting in their intracellular accumulation. In support of this, we have observed that in hepatocytes

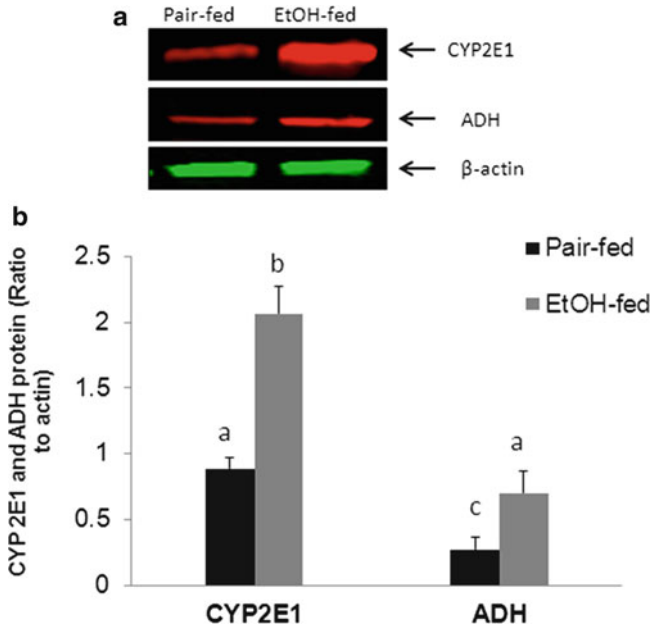

Fig. 6.7 (*Panel A*) Western blot of CYP2E1 and ADH protein levels in hepatocyte lysates. (*Panel B*) Denistometric mean (± SE) values of CYP2E1 and ADH proteins from mouse hepatocytes after chronic ethanol feeding

from chronically ethanol-fed transgenic GFP-LC3 mice, AV-lysosome co-localization is much lower than in cells from control animals (data not shown). We made similar observations in ethanol-metabolizing recombinant Hep G2 cells (unpublished data). At this time, we do not know whether the aforementioned oxidants that inhibit mTORC1 and microtubule formation are derived exclusively from CYP2E1 catalysis, ADH catalysis or enhanced mitochondrial leakage. Preliminary data from recombinant ethanol-metabolizing HepG2 cells indicate that ethanol oxidation by both ADH and CYP2E1 contribute to this process (unpublished results).

6.5.3 Alcohol-Induced Fatty Liver

Alcoholic steatosis is characterized by increased triglyceride (TG) deposition in liver cells. It is caused, in part, by accelerated fatty acid synthesis (You et al. 2002), a slowdown of fatty acid oxidation (Donohue 2007) and enhanced hepatic retention of VLDL caused by an ethanol-induced secretory defect (Kharbanda et al. 2009). Singh et al. showed that autophagy inhibition causes steatosis and that the buildup of fatty acids suppresses autophagy, presumably by feedback inhibition (Singh et al. 2009). These findings prompted Wu et al. to examine ethanol-induced steatosis and autophagy in HepG2 (E-47) cells that selectively express CYP2E1 (Wu et al. 2010). They found that ethanol-exposed E47 cells have higher TG

accumulation than in C34 cells that do not express CYP2E1. Ethanol exposure (50–150 mM) for 4–5 days enhances AV (LC3II) content in C34 cells but not in E47 cells. The authors concluded that enhanced expression of CYP2E1 through its induction by ethanol in E47 cells causes triglyceride (TG) accumulation, which, in turn, suppresses autophagy. However, these studies provided no indication of the temporal sequence of CYP2E1 induction, TG accumulation, and autophagic suppression or whether they occurred concurrently. Others recently reported that exposure of isolated hepatocytes to the saturated fatty acid, palmitate suppresses AVs in liver cells but that the unsaturated fatty acid, oleate enhances AV levels (Mei et al. 2011). Recent research has focused on selective autophagy of organelles, specifically mitophagy (autophagy of mitochondria) and lipophagy (autophagy of lipid droplets or LDs). Ding et al. showed that ethanol exposure enhances hepatocellular AVs, and that these selectively target mitochondria and LDs, (Ding et al. 2010, 2011b). The apparently selective engulfment and subsequent destruction of LDs and damaged mitochondria likely represent hepatoprotective responses, as unchecked fat accumulation and mitochondrial damage are cytotoxic. It is not clear whether ethanol-elicited steatosis actually represents stalled lipophagy. However, with enhanced fatty acid biosynthesis and slower oxidation of fatty acids it is conceivable that the autophagic system is overwhelmed by the rise in inhibitory fatty acids that interfere with the autophagic degradation of LDs.

6.5.4 Interferon-Induced Autophagy

Considerable discussion in this chapter has described the effect of IFN-γ on proteasome activity and signal transduction. Recently-published results indicate that interferon treatment of human hepatocellular carcinoma (Huh-7) cells induces autophagy in these cells, which, in turn, induces cell death in a manner that does not involve apoptosis. Other data revealed that interferon exposure enhances autophagy and triggers the disruption of lysosomes, causing necrotic cell death (Chang et al. 2011). Autophagy induction by IFN-γ requires the action of interferon regulatory factor-1 (IRF-1), as knockdown of IRF-1 blocks IFN-γ-induced autophagy (Li et al. 2012). The relationship of these findings to those that describe the aforementioned blockade by ethanol of IFN-γ signaling is speculative, as the signaling mediators of each IFN-γ effect (JAK-STAT vs IRF-1) are different and likely differentially regulated.

6.6 Summary and Future Directions

This chapter has described the regulation by ethanol administration of CYP2E1 and the impact of its catalysis on IFN signal transduction and antigen presentation. The intertwinements of these physiological processes are important, not only from the viewpoint of basic science, but also from that of clinical science. This is because alcohol enhances the refractory nature of infectious diseases, such as hepatitis

C virus, as the clinical course of this is prolonged by oxidant stress derived from ethanol metabolism. The latter process, which is, in part, catalyzed by CYP2E1, thwarts current medical interventions of viral therapy. It is the subject of vigorous research, attempting to comprehend why innate and adaptive immunity in alcoholics is compromised and why infected patients who continue to drink resist current viral therapies.

Similarly, our current understanding of autophagy and its vital role in maintaining cellular integrity is rapidly expanding with the identification of the cellular Atgs that govern the initiation of this key catabolic process. We and others have established that ethanol oxidation by alcohol dehydrogenase and cytochrome P450 2E1 significantly induces AV formation in liver cells during acute (binge) drinking. However, during longer periods of drinking (i.e. chronic ethanol administration) it is likely that the degradative steps in autophagy are blocked, These findings underscore the variables that are encountered in the same alcohol feeding model system. The outcome of each depends upon the duration of ethanol exposure. While here, we did not discuss the role of autophagy in immunity, it is important to know that autophagic degradation of microbial invaders is also a key cellular defense mechanism that is likely affected by heavy drinking.

Acknowledgement Research cited and performed by the authors and described in this chapter are results of work supported with resources and use of facilities at the Nebraska-Western Iowa Health Care System of the Department of Veterans Affairs Veterans Health Administration, Office of Research and Development. Financial support was provided by grants from the National Institute on Alcohol Abuse and Alcoholism (to T.M.D and N.A.O.) and by Biomedical Research Funds from the Department of Veterans Affairs (To T.M.D.) and by internal funding sources from the Section of Gastroenterology and Hepatology, and from a Dean's Reviewed Research Grant from the University of Nebraska Medical Center.

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Chapter 7
CYP2E1, Oxidative Stress, Post-translational Modifications and Lipid Metabolism

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Abstract Chronic alcohol-mediated down-regulation of hepatic ST6Gal1 gene leads to defective glycosylation of lipid-carrying apolipoproteins such as apo E and apo J, resulting in defective VLDL assembly and intracellular lipid and lipoprotein transport, which in turn is responsible for alcoholic hepatosteatosis and ALD. The mechanism of ethanol action involves thedepletion of a unique RNA binding protein that specifically interacts with its 3′-UTR region of ST6Gal1 mRNA resulting in its destabilization and consequent appearance of asialoconjugates as alcohol biomarkers. With respect to ETOH effects on Cardio-Vascular Diseases, we conclude that CYP2E1 and ETOH mediated oxidative stress significantly down regulates not only the hepatic PON1 gene expression, but also serum PON1 and HCTLase activities

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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*,
Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_7,

accompanied by depletion of hepatic GSH, the endogenous antioxidant. These results strongly implicate the susceptibility of PON1 to increased ROS production. In contrast, betaine seems to be both hepatoprotective and atheroprotective by reducing hepatosteatosis and restoring not only liver GSH that quenches free radicals, but also the antiatherogenic PON1 gene expression and activity.

Keywords CYP2E1 • Alcohol • PON1 • ROS • Liver

7.1 Introduction

Galβ1, 4GlcNAc α2,6-sialyltransferase (ST6Gal1) mediates the addition of α2,6-linked sialic acid to glycoproteins in the Golgi compartment. Down-regulation of its gene and consequent impaired activity of ST6Gal1 seems to be the major cause for the appearance of asialoconjugates in the blood of long-term alcoholics. Therefore, mechanism(s) involved in the regulation of ST6Gal1 gene is important and clinically relevant. ST6Gal1 is strongly expressed in liver and can be either up- or down-regulated by a number of factors. In our rat alcohol-feeding model, rat ST6Gal1 expression was reduced to as much as 59% by long-term alcohol treatment compared with the pair-fed control group in a dose dependent manner (Rao and Lakshman 1999). It will be of major importance and clinical significance if the regulation of ST6Gal1 gene can be mimicked in a human liver cell model, especially if it can be achieved within a few days of ethanol exposure in a human liver culture system rather than *in vivo* that may take prolonged period of ethanol exposure.

To define the significance and molecular mechanisms of aberrant sialylation in alcoholics, we focused our attention on sialidases and sialyltransferases (Ghosh et al. 1993), two of the key enzymes involved in the metabolism of glycoproteins and glycolipids. Our studies have shown that long-term ethanol administration decreases the hepatic activity of β-galactoside-α2,6-sialyltransferase (ST6Gal1) in the rat liver via the down-regulation of the ST6Gal1 gene (Rao and Lakshman 1997). In human beings, the vast majority of ethanol is oxidized to acetaldehyde by the hepatocytes of the liver (Crabb et al. 1987). However, whether or not ethanol metabolism by the liver is a prerequisite for its action in down-regulating ST6Gal1 gene has not been established.

On the other hand, CYP2E1-mediated oxidation of ethanol produces a state of oxidative stress by generating reactive oxygen species (ROS) within the cells that is responsible for the progression of alcoholic liver disease or cell damage (Hartley and Peterson 1997; Chen et al. 2001; Nagy 2004). Therefore, it is possible that the mechanism of action of ethanol in regulating ST6Gal1 gene expression may be mediated via ROS. One of the key metabolites generated because of oxidative stress is the α,β-unsaturated aldehyde, 4-hydroxy-2-nonenal (HNE), which may be more harmful than ROS because it has a longer half-life and can easily diffuse into cellular membranes (Dai et al. 1993). In the present report, we have taken advantage of

cultured human wild-type HepG2 liver cells that do not metabolize ethanol because they lack the key ethanol-metabolizing enzymes, namely, cytochrome P450 2E1 (CYP2E1: ethanol-inducible) and alcohol dehydrogenase (ADH). However, when these wild-type liver cells are stably transfected with either CYP2E1 gene or high alcohol dehydrogenase (HAD) gene, they are efficient in metabolizing ethanol and truly reflect ethanol metabolism by human liver *in situ*. Thus, the three HepG2 cell types, the wild-type cells, CYP2E1 cells, and HAD cells are ideal models to clearly define the true action of ethanol in regulating ST6Gal1 gene. It will be shown that ethanol causes the down-regulation of ST6Gal1 gene only in the ethanol-metabolizing liver cells, but not in the wild type. In contrast, acetaldehyde, the immediate product of ethanol oxidation caused the down-regulation of ST6Gal1 gene even in the wild type. Furthermore, we will also show that HNE strongly down-regulates ST6Gal1 gene in CYP2E1 cells. Thus, it is unequivocally demonstrated that ethanol oxidation leading to generation of acetaldehyde and/or ROS is obligatory for its regulatory action on ST6Gal1 gene in human liver.

The complete cDNA for rat ST6Gal1 is 4.2-kb, with a 2.7-kb 3′-untranslated region (UTR) region. Sequence analysis by computer software revealed several conserved sequences among different species within ST6Gal1 3′-UTR. Therefore, it is reasonable that this extremely long 3′-UTR may play important roles in the posttranscriptional regulation of rat ST6Gal1. The 3′-UTR of many eukaryotic mRNAs has been implicated in a variety of cellular processes, such as mRNA stability, processing, polyadenylation, localization, and translational regulation. In each case, functional activity appears to be mediated, in part, by a specific interaction of RNA-binding proteins, which target cellular RNAs to form RNA-protein complexes (Jain et al. 1995; Zaidi and Malter 1995; Wang et al. 1996; Joseph et al. 1998; Ostareck-Lederer et al. 1998; Sakai et al. 1999; Gilmore et al. 2001; Kufel et al. 2003). More and more reports have suggested that mRNA stability is frequently determined by RNA-protein interactions, and those interactions frequently occur within the 3′-UTR. Thus, RNA-protein interactions are crucial for maintaining proper RNA metabolism. We have undertaken to analyze the RNA-protein interactions within the 3′-UTR of ST6Gal1 to characterize the binding protein with which this RNA sequence interacts and to investigate whether chronic ethanol feeding affects the status of this binding protein and consequently that of the ST6Gal1 mRNA transcripts. It will be unequivocally demonstrated in the present investigation that a 41-kDa binding protein specifically binds to the 3′-UTR of ST6Gal1 mRNA and stabilizes the mRNA. Significantly, chronic alcohol feeding decreases this liver cytosol binding protein, leading to impaired binding and destabilization of ST6Gal1 mRNA.

On the other hand, paraoxonase 1(PON1) is a multifunctional antioxidant enzyme tightly associated with high density lipoprotein (HDL) that exhibits not only the capacity to prevent LDL oxidation and destroy oxidized low density lipoproteins (LDL) (Aviram et al. 1998; Shih et al. 1998; Mackness et al. 2003; Navab et al. 1997), but also can detoxify the homocysteine metabolite, homocysteine thiolactone (HCTL), which can pathologically cause protein damage by homocysteinylation of the lysine residues, thereby leading to atherosclerosis (Jakubowski 2000, 2007;

Aviram and Rosenblat 2004). The importance of PON1 with respect to protection against Cardio-Vascular Diseases (CVD) is supported by recent studies in Type II diabetics showing a strong correlation between decreased HCTLase activity with the severity of CVD (Lakshman et al. 2006; Jakubowski 2007). It has been previously shown that heavy alcohol down-regulates PON1 expression and activity (Sierksma et al. 2002; Rao et al. 2003). It has also been demonstrated that ROS can inhibit PON1 activity (Rao et al. 2003; Horke et al. 2007). Dietary ω-3 polyunsaturated fatty acids (ω-3 PUFA) could also adversely affect PON status presumably by generating more ROS in spite of their promising hypolipidemic effect. Therefore, any dietary supplement that can protect the depletion of intracellular glutathione (GSH) would be effective in preserving PON1 status.

Betaine (trimethyl glycine) is a substrate for the liver enzyme betaine homocysteine methyltransferase (BHMT) which transfers methyl group to convert homocysteine to methionine (Finkelstein et al. 1972). Betaine plays an important role in reducing fatty liver (Barak et al. 1997), depletes homocysteine levels in liver (Kharbanda et al. 2005), and has been reported to restore the decreased liver GSH level in lipopolysaccharide-treated rats (Balkan et al. 2005). Therefore, it is reasonable that dietary betaine may prevent the deleterious effects of heavy alcohol and ω-3 PUFA on PON1 status by altering hepatic GSH. We have undertaken this study (i) to explore the effects of feeding high and low ω-3 PUFA diets, especially in the presence of chronic heavy alcohol in the diet on PON1 expression and activity and (ii) to test whether betaine can protect these alterations caused by chronic alcohol and ω-3 PUFA by restoring hepatic GSH.

7.2 Ethical Guideline and Alcoholic Specimen Criteria

This research was approved by the institutional review board and the research and development committee of this medical center as well as those of the participating medical center from Australia. An informed consent was obtained from each patient before taking samples. Postmortem human liver specimens (12 samples in each group; all sample identities were kept anonymous) were purchased from Tissue Transformation Technologies (Edison, NJ) according to the following criteria:

1. Non–alcohol-drinking group (ND): less than 1 alcoholic beverage per day (below 14 g ethanol per day) in the past 10 years before death.
2. Moderate alcohol drinkers (MD): 1 to 3 alcoholic beverage(s) per day (14–42 g ethanol per day) in the past 10 years before death.
3. Heavy alcohol drinkers (HD): more than 6 alcoholic beverages per day (≥84 g ethanol per day).

Four explant liver samples with advanced alcoholic liver disease (ALD) (end-stage cirrhosis without viral hepatitis or other defined liver disease) and 5 normal subjects (partial donation; controls without liver disease) were also used to study the expression

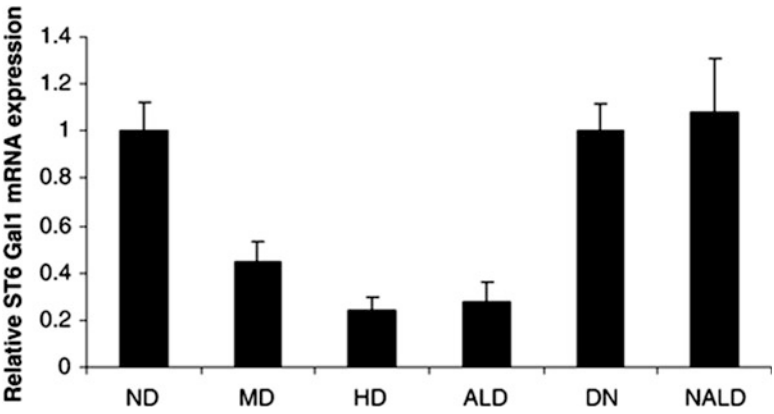


Fig. 7.1 Real-time RT-PCR analyses of ST6Gal1 mRNA in human liver specimens in the study groups. Total RNA from the ND group (n = 12), the MD group (n = 12), the HD group (n = 12), the end-stage ALD group (n = 4), the normal liver donors (n = 5), and the NALD group (n = 12) was reverse transcribed and used in the real-time PCR. The RNA levels were normalized to the level of β-actin. Each sample analysis was performed in triplicate independently; and each bar graph represents the mean ± SEM of 12 samples in each group except for the ALD and DN groups, in which *bar graph* represents the mean ± SEM of 4 and 5 samples, respectively. DN indicates normal liver donors

of ST6Gal1. Twelve liver biopsy samples taken from patients with chronic liver disease of nonalcoholic etiology were used as the nonalcoholic liver disease (NALD) group.

7.2.1 Ethanol Per Se, Not Other Liver Pathological Conditions, Mediates the Down-Regulation of ST6Gal1 Gene

Our previous study (Gong et al. 2007) showed that ST6Gal1 mRNA was significantly down-regulated in liver samples from long-term alcoholics without liver disease. To rule out that other liver pathological conditions may also affect the expression of ST6Gal1, we repeated our real-time quantitative RT-PCR analyses on liver samples from nondrinkers, moderate drinkers, and heavy drinkers together with liver explant samples from alcoholic liver disease subjects as well as liver samples from hepatitis subjects without alcohol exposure (NALD group). The results are shown in Fig. 7.1. Thus, compared with the nonalcohol group, ST6Gal1 mRNA level was decreased on average by 50% ($p < 0.01$) and by 70% ($p < 0.01$) in moderate– and heavy–alcohol-drinking groups, respectively, just as we reported previously (Gong et al. 2007). Similar results were also found in liver explants from advanced ALD patients compared with normal subjects (ND). The ST6Gal1 mRNA level was decreased by as much as 65% ($p < 0.01$) in end-stage alcoholic liver disease subjects, whereas ST6Gal1 mRNA level was not changed or slightly increased in the NALD, compared

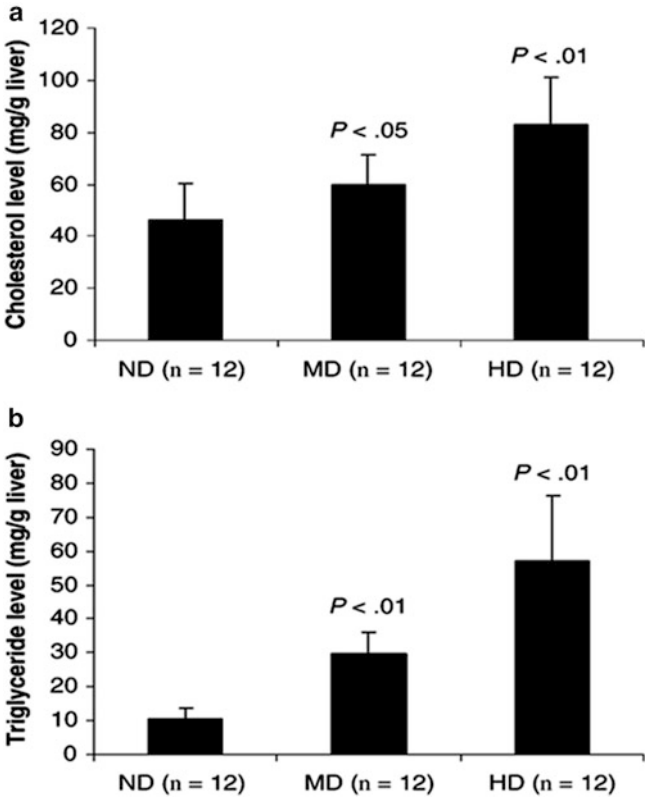
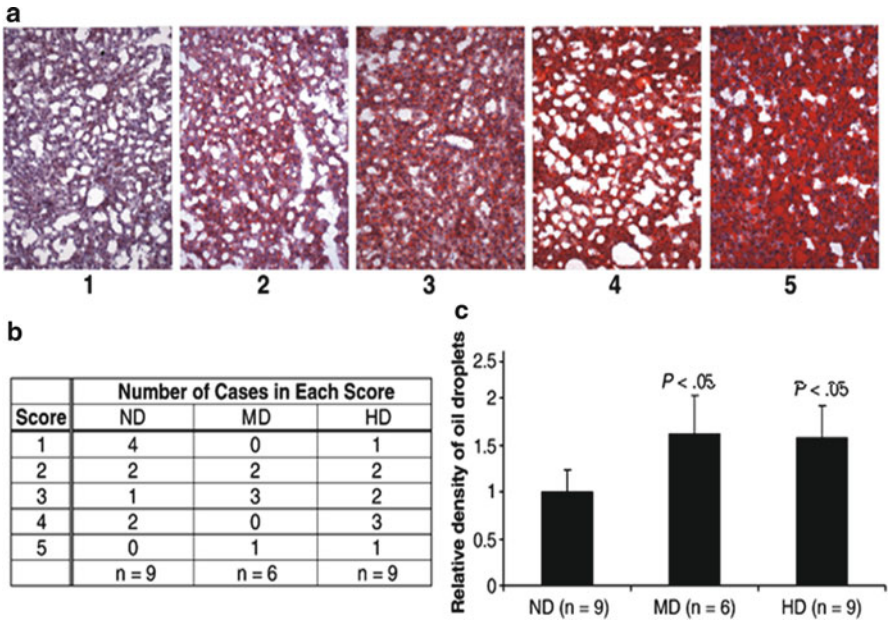


Fig. 7.2 Liver cholesterol and triglyceride levels in the study groups. Aliquots of each liver lipid extract were analyzed for (**a**) cholesterol and (**b**) triglycerides as described in the methods section. Each sample analysis was performed in duplicate and repeated at least three times on different dates. The concentration for cholesterol and triglyceride is expressed as milligram per gram of liver. *Bar graph* represents the mean ± SEM of 12 specimens in each group

with normal controls. These results indicate that ethanol per se may be the major cause for the down-regulation of ST6Gal1 gene in alcoholics.

7.2.2 Liver Lipid Deposition Directly Correlates with the Amount of Alcohol Consumed

Liver total cholesterol level was increased by more than 30% ($p<0.05$) and 75% ($p<0.01$) in the MD and HD groups, respectively, compared with the ND group (Fig. 7.2a). Triglyceride was increased by more than 100% ($p<0.01$) in the MD group and by more than 300% ($p<0.01$) in the HD group compared with the ND group (Fig. 7.2b).



	Number of Cases in Each Score		
Score	ND	MD	HD
1	4	0	1
2	2	2	2
3	1	3	2
4	2	0	3
5	0	1	1
	n = 9	n = 6	n = 9

Fig. 7.3 Histopathology of human liver specimens and lipid deposits in the study groups. Cryosections of tissues were prepared as described in the materials and methods section. Sections were stained with oil red O and counterstained with hematoxylin. All ca × 120; bar = ca 100 μm. (**a**) Representative photomicrographs from the standard set used to calibrate scoring of lipid accumulation. Numbers *below* each *image frame* represent the score for that frame, with *1* indicating the least and *5* indicating the most lipid accumulation. (**b**) Number of samples from each group that fits into each score according to the standard in Fig. 7.3a as determined subjectively by the *scoring panel*. (**c**) Quantification of oil droplets in oil red O–stained sections as determined by the Image-Pro Plus version 6.1 method. Bar graph represents the mean ± SEM of nine specimens from the ND group, six specimens from the MD group, and nine specimens from the HD group, with the ND group set at 1 for convenience

7.2.3 Alcohol Consumption Correlates Positively with Hepatic Steatosis

To investigate how alcohol consumption affects liver pathologically, we performed histopathology of liver tissues from the ND (n = 9), MD (n = 6), and HD (n = 9) groups. In hematoxylin and eosin–stained sections, discrete, blank vacuoles were uncommon in the livers from the ND and MD groups and prominent in the livers of the HD group (data not shown). In oil red O–stained liver sections, bright red–stained lipid droplets were scored as described in the methods section. One calibrator image for every score is shown in Fig. 7.3a. The number of cases put into each score as determined by the blinded scoring panel in the ND, MD, and HD groups is listed in Fig. 7.3b. As the amount of alcohol consumption increased, there was a clear shifting from lower score scale to higher score scale. The mean density of lipid accumulations determined with the Image-Pro Plus v6.1 software on the digital photomicrographs of the various experimental groups is shown in Fig. 7.3c. There

was greater than 70% more lipid deposition in the drinking groups (MD + HD combined) compared with the ND group.

7.2.4 *Strong Multivariate Relationships Exist Between Alcohol-Drinking History, ST6Gal1 Gene Expression, and Liver Lipid Deposits*

There was a strong relationship between alcohol-drinking history, ST6Gal1 mRNA expression, and liver lipid score ($F = 8.68$, $p < 0.001$). Alcohol-drinking history and ST6Gal1 mRNA expression together accounted for 56.6% of the lipid score variance, and both predictors made a significant correlation to prediction accuracy (for ST6Gal1, $p < 0.001$; for drinking history, $p < 0.01$) in a multivariate model. When both alcohol history and lipid deposit score were coded as ordinal, the Pearson correlation was 0.35 ($p = 0.09$). However, by examining the 95% confidence intervals around the mean liver lipid deposit scores for each of the 3 levels of drinking history, we found that subjects with a history of heavy or moderate drinking had significantly more lipid deposits (mean = 3.11, confidence interval = 2.26–3.96 and 2.11–3.89 for heavy and moderate drinkers, respectively) than subjects with no drinking (mean = 2.11, confidence interval = 1.26–2.96, $p < 0.05$). The univariate relationship between ST6Gal1 mRNA expression and drinking history was very strong and significant ($F = 337.09$, $p < 0.0001$, $R2 = 0.97$). Mean expression levels by drinking history are shown in Fig. 7.4.

7.2.5 *Expression of CYP2E1 and ADH in the Respective HepG2 Cell Lines*

To confirm that CYP2E1 and ADH were expressed in the stably transfected CYP2E1 and HAD cells, respectively, but not in the wild type, Western blot analysis was carried out with the extracts of the respective cells and the wild-type cells using antihuman CYP2E1 and anti-human ADH. As shown in Fig. 7.5a, a 55-kDa band corresponding to CYP2E1 was identifiable only in the extracts of CYP2E1 cells but not in wild-type cells. Furthermore, CYP2E1 activity, as measured by p-nitrophenol oxidation rate, was found to be 53.2 pmol/min/mg of protein in the CYP2E1 cells, thus agreeing with the results of Yang and Cederbaum (1997). CYP2E1 activity was undetectable in the wild-type cells. Similarly, it can be seen in Fig. 7.5b that a strong 39-kDa band corresponding to human ADH subunit was identifiable in the extracts of HAD cells but only a faint one in the wild type. Furthermore, ADH activity, as measured by ethanol oxidation was found to be 216.2 nmol/h/mg of protein similar to the results reported by Clemens et al. (1995). Negligible ADH activity was detectable in the wild-type cell extracts.

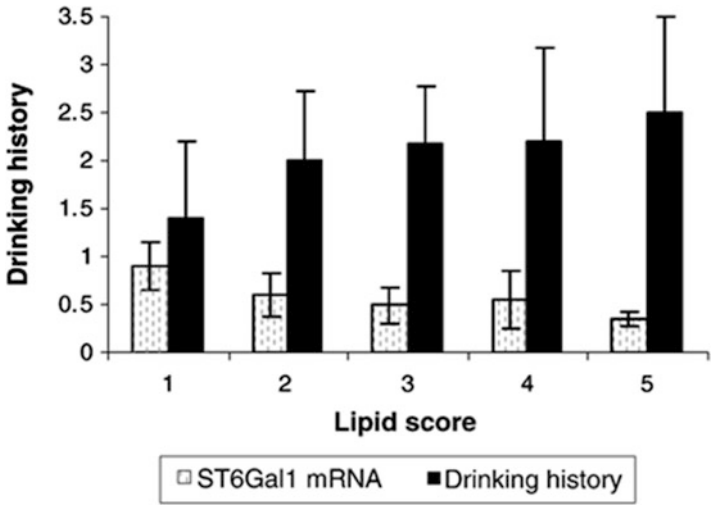


Fig. 7.4 Multivariate analyses of ST6Gal1 gene expression, drinking history, and liver lipid deposit in the study groups. The relative ST6Gal1 mRNA expression from all samples in the ND (n = 12), MD (n = 12), and HD (n = 12) groups was plotted together with drinking history against liver lipid score. Drinking history was coded as heavy = *3*, moderate = *2*, and none = *1* to obtain a mean. *Error bars* represent the 95% confidence interval around the mean

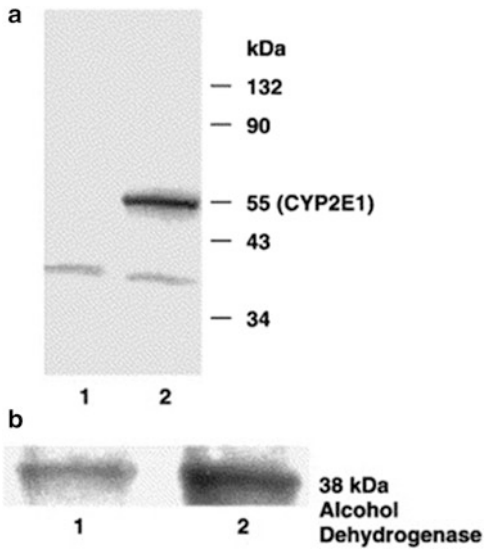


Fig. 7.5 Western blot analysis of CYP2E1 and HAD cells. Twenty micrograms equivalent of total protein extracts from CYP2E1 and HAD cells were subjected to Western blot analyses using the polyclonal CYP2E1 and ADH antibodies, respectively. (**a**) *Lane 1* indicates wild-type cell extract; *lane 2*, CYP2E1 cell extract. (**b**) *Lane 1* indicates wild-type cell extract; *lane 2*, HAD cell extract

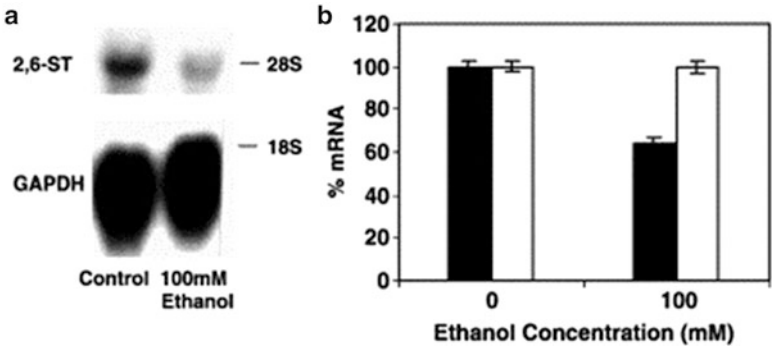


Fig. 7.6 Effect of ethanol on ST6Gal1 mRNA level in CYP2E1 cells. CYP2E1 cells were incubated without or with 100 mmol/L ethanol for 72 h and the total RNA was extracted and subjected to Northern blot analysis. (**a**) A representative Northern blot analysis of ST6Gal1 mRNA from total RNA extracts of control and 100 mmol/L ethanol-treated cell extracts. *Lane 1*, RNA from CYP2E1 cells without ethanol exposure. *Lane 2*, RNA from the same cell line after exposure to 100 mmol/L of ethanol. (**b**) Relative levels of ST6Gal1mRNA. Each value is the mean ± SEM of determinations from five independent experiments, each of which was run in duplicate cell cultures. *Black solid bars* indicate ST6Gal1mRNA; *white open bars*, GAPDH mRNA

7.2.6 Effect of Ethanol on ST6Gal1 mRNA Expression in CYP2E1 Cells

The time course effect of 100 mmol/L ethanol on ST6Gal1 mRNA level in CYP2E1 cells showed 5% (NS) decrease at 24 h, 8% (NS) at 48 h, and 39% at 72 h. Therefore, the concentration effect of ethanol was carried out at the 72-h time point. A representative Northern blot analysis of ST6Gal1 mRNA from the total RNA extracts of control and ethanol-treated cells is shown in Fig. 7.6a. Figure 7.6b shows the effect of ethanol concentration on the relative levels of ST6Gal1 mRNA in CYP2E1 cells after 72 h of incubation. It can be seen from the figure that the level of ST6Gal1 mRNA was decreased by 46% ($p < 0.05$) at 100 mmol/L ethanol compared with untreated CYP2E1 cells. The housekeeping gene GAPDH expression was not affected by ethanol treatment under identical conditions.

7.2.7 Effect of Ethanol on ST6Gal1 mRNA Expression in HAD Liver Cells

Because ethanol effect on ST6Gal1 mRNA was not evident before the 72-h point (data not shown), the concentration effect of ethanol was carried out at the 72-h time point. Figure 7.7 shows the effect of ethanol concentration on the relative levels of

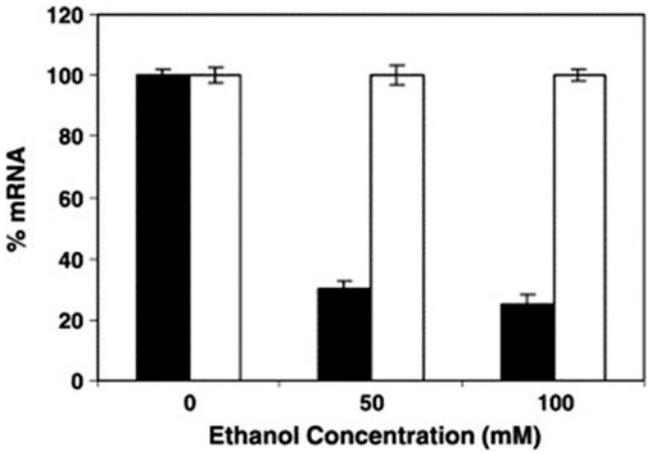


Fig. 7.7 Effect of ethanol exposure time on ST6Gal1 mRNA level in HAD cells. HAD cells were incubated without or with 50 and 100 mmol/L ethanol for 72 h and the total RNA was extracted and subjected to Northern blot analysis. *Lane 1* is RNA from untreated HAD cells, *lane 2* is RNA from HAD treated with 50 mmol/L ethanol for 72 h, and *lane 3* is RNA from same cell line treated with 100 mmol/L ethanol for 72 h. Expression of human ST6Gal1 dramatically down-regulated in treated cells. Each value is the mean ± SEM of determinations from five independent experiments, each of which was run in duplicate cell cultures. *Black solid bars* indicate ST6Gal1 mRNA; *white open bars*, GAPDH mRNA

ST6Gal1 mRNA in HAD cells after 72 h of incubation. It can be seen from the figure that the level of ST6Gal1 mRNA was decreased by 70% ($p<0.05$) at 50 mmol/L ethanol and by 76% ($p<0.05$) at 100 mmol/L ethanol as compared with untreated HAD cells. The housekeeping gene GAPDH expression was not affected by ethanol treatment under identical conditions.

7.2.8 Effect of Ethanol and Acetaldehyde on ST6Gal1 mRNA Expression in Wild-Type HepG2 Liver Cells

The concentration effects of ethanol and acetaldehyde were carried out at the 72-h time point. Figure 7.8 shows that the effect of ethanol concentration on the relative levels of ST6Gal1 mRNA in wild-type cells decreased negligibly after 72 h of incubation with 100 mmol/L ethanol. In contrast, the relative level of ST6Gal 1 mRNA was decreased by 35% ($p<0.05$) at 50 μmol/L acetaldehyde and by 69% ($p<0.05$) at 100 μmol/L acetaldehyde compared with untreated wild-type cells. The housekeeping gene GAPDH expression was not affected by ethanol or acetaldehyde exposure under identical conditions.

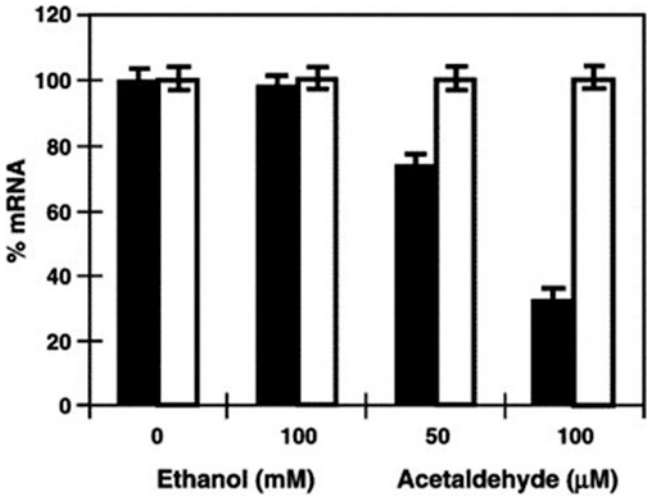


Fig. 7.8 Effect of ethanol and acetaldehyde on ST6Gal1 mRNA level in wild-type cells. Wild-type cells were incubated without or with 100 mmol/L ethanol or 50 and 100 μmol/L acetaldehyde for 72 h and the total RNA was extracted and subjected to Northern blot analysis. Each value is the mean ± SEM of determinations from five independent experiments, each of which was run in duplicate cell cultures. *Black solid bars* indicate ST6Gal1 mRNA; *white open bars*, GAPDH mRNA

7.2.9 Effect of HNE on ST6Gal1 mRNA Expression in CYP2E1 Cells

The concentration effect of HNE was carried out at the 72-h time point. Figure 7.9 shows the effect of HNE concentration on the relative levels of ST6Gal1 mRNA in CYP2E1 cells after 72 h of incubation. It can be seen from the figure that the level of ST6Gal1 mRNA was decreased by 46% ($p<0.02$) at 32 μmol/L HNE, by 49% ($p<0.02$) at 64 μmol/L HNE, and by 61% ($p<0.02$) at 96 lmol/L HNE compared with untreated CYP2E1 cells. The housekeeping gene GAPDH expression was not affected by HNE treatment under identical conditions.

7.2.10 Partial Identification of the Liver Cytosol Protein That Forms Complex with the 3′-UTR of Rat ST6Gal1 mRNA

The interaction of the cytosol fraction from control rat liver with ^{32}P-labeled ST6Gal1 mRNA probe of 2029-bp length covering nucleotides immediately downstream from the stop codon was analyzed by electrophoretic gel mobility shift assays (EMSA) on 8% native polyacrylamide gels. As can be seen in Fig. 7.10a, incubation of the ^{32}P-labeled ST6Gal1 mRNA probe with as little as 1 μg of cytosol

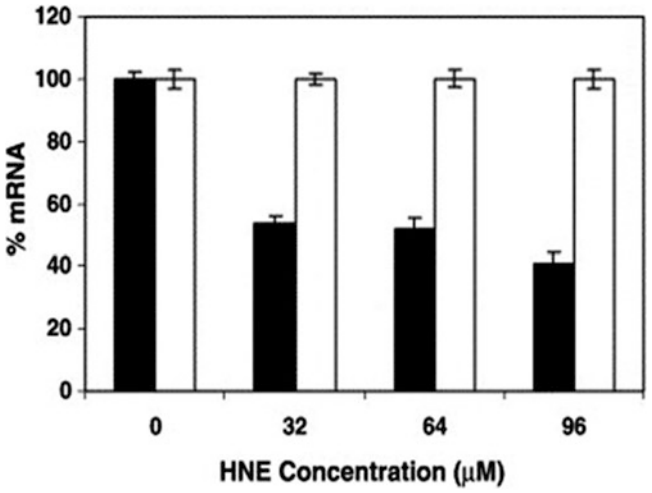


Fig. 7.9 Effect of HNE on ST6Gal1 mRNA level in CYP2E1 cells. CYP2E1 cells were incubated without or with indicated concentrations of HNE for 72 h and the total RNA was extracted and subjected to Northern blot analysis. Each value is the mean ± SEM of determinations from five independent experiments, each of which was run in duplicate cell cultures. *Black solid bars* indicate ST6Gal1 mRNA; *white open bars*, GAPDH mRNA

protein retarded the migration of the probe, leading to the appearance of a RNA-protein complex radioactive band (Fig. 7.10a, lane 1). There was an increase in the intensity of this band with increasing amount of the cytosol protein up to 50 μg, beyond which no further increase occurred (Fig. 7.10a, lanes 1–7). Prior incubation of the cytosol fraction with 100-fold molar excess of unlabeled cold RNA probe completely quenched the binding of ^{32}P-labeled RNA probe as evidenced by the loss of the intensity of the RNA-protein complex band (Fig. 7.10b, lane 4). Likewise, RNA-protein complex formation was abolished by treating the cytosol fraction with proteinase K or preheating at 56°C for 10 min (Fig. 7.10b, lanes 5 and 6).

7.2.11 Importance of Secondary Structure of RNA in RNA-Protein Complex Formation

Figure 7.10b (lanes 1–3) shows the interaction of the cytosol protein with the RNA probe that was first heat-denatured at 90 °C for 10 min and either cooled down rapidly on ice to maintain the denatured structure (Fig. 7.10b, lane 2) or cooled down gradually to room temperature toallow the stable RNA secondary structure to form (Fig. 7.10b, lane 3), compared with normal binding reaction with unheated RNA probe (Fig. 7.10b, lane 1). It is obvious from the figure that the same strong signal for the RNA-protein complex band could be observed with both denatured and renatured RNA probes.

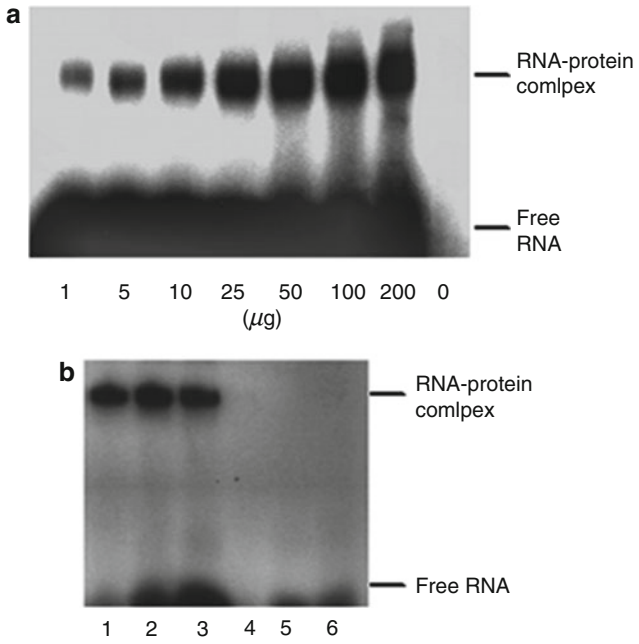


Fig. 7.10 Characterization of a cytosol protein that bind to ST6Gal1 mRNA. (**a**) Effect of the amount of cytosol protein from rat liver on 3′-UTR of ST6Gal1 mRNA-protein complex formation. Indicated amounts of cytosol fraction were incubated with 32P-labeled RNA probe (2.0nM final concentration) in the standard binding assay. (**b**) Effect of RNA secondary structure, competitive binding, and pretreatment with proteinase K or heat on RNA-protein complex formation. The standard binding assay was carried out with the cytosol fraction and the 32P-labeled RNA that was unheated (*lane 1*), preheated to 90 °C for 10 min, and then rapidly cooled to 4 °C (*lane 2*) or gradually cooled to room temperature (*lane 3*), competitive binding with 100-fold excess of cold mRNA probe (*lane 4*), cytosol fraction treated with proteinase K (*lane 5*), or prior heating (*lane 6*)

7.2.12 The RNA-Protein Interaction Protects 3′-UTR from RNase Digestion

To investigate whether the formation of protein-ST6Gal1 complex plays any functional role in ST6Gal1 mRNA stability, RNase digestion assays were performed with the longest RNA probe (probe 1) either before or after the binding reactions. As can been seen from Fig. 7.11, the specific protein binding to the 3′-UTR of ST6Gal1 completely protected it from digestion by RNases S, V1, and A and partially protected it from RNase T1. It is worthy to note that RNase S is nonspecific for digesting RNA; RNase V1 cleaves only base-paired nucleotide, whereas RNase A cleaves 3′-U and C residues. On the other hand, RNase T1 cleaves only at the 3′-G residue; therefore, it is possible that some RNA sequence may remain intact after RNase T1 digestion.

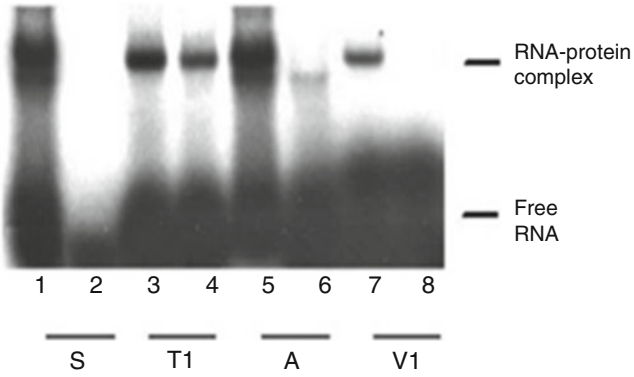


Fig. 7.11 Effects of various RNases on the binding of 3′-UTR of ST6Gal1 mRNA to the cytosol protein. The standard binding assay was carried out with same amount of ^{32}P-labeled RNA probe either before or after treatment with various RNases prior to incubation with the cytosol binding protein. *Lanes 1 and 2*, before and after treatment with RNase S; *lanes 3 and 4*, before and after treatment with RNase T1; *lanes 5 and 6*, before and after treatment with RNase A; *lanes 7 and 8*, before and after treatment with RNase V1. In all of the assays in Figs. 7.16 and 7.17, the formation of RNA-protein complex was determined by EMSA

7.2.13 Partial Characterization of Protein Interacting with 3′-UTR of ST6Gal1 mRNA by UV Cross-Linking

As a direct method to determine the molecular mass of this protein under denatured condition, the products of the binding reaction with the ^{32}P-labeled ST6Gal1 RNA probe were UV cross-linked, and the noncross-linked RNA was removed by RNase T1 digestion. The resulting ^{32}P-labeled RNA-protein complex was separated by SDS-PAGE along with a set of molecular mass standards on a separate lane. As shown in Fig. 7.12, a single band corresponding to 41 kDa was seen.

7.2.14 Thirteen-Base Pair Motif of ST6Gal1 mRNA Is Sufficient for the Specific Protein to Bind

To identify the shortest possible nucleotide sequence of 3′-UTR of ST6Gal1 with which the binding protein interacts, the 2029-bp of the RT-PCR template was digested with restriction enzymes, and the sequentially truncated templates were used for RNA probe synthesis and EMSA (Fig. 7.13a). As shown in Fig. 7.13b, all of the probes listed in Fig. 7.13a interacted with the protein, indicating that probe 5, which is only 304-bp immediate downstream from the stop codon, was clearly sufficient to facilitate the binding interaction. However, it was difficult to visualize the bands by EMSA, even with prolonged exposure with RNA probes that were shorter than probe 5, due to the limited incorporation of [^{32}P]UTP. Therefore, after narrowing the binding region

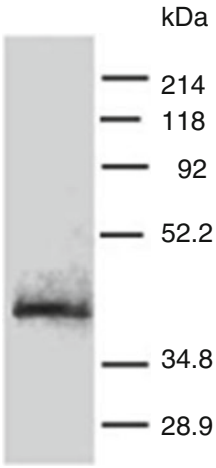


Fig. 7.12 Characterization of the binding protein by UV cross-linking. The standard binding reaction was carried out between ^{32}P-labeled probe and cytosol protein. The complex was UV cross-linked as described under Materials and Methods, and the protein samples were analyzed by SDS-PAGE gel. The positions of pre-stained Broad Range molecular mass markers (Bio-Rad) are shown in *middle*

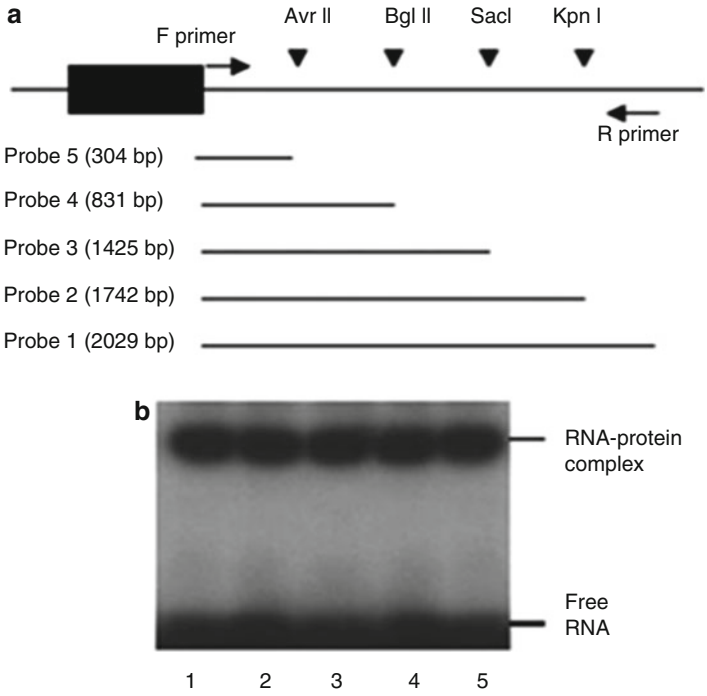


Fig. 7.13 Schematic chart for mapping of the protein-ST6Gal1 interaction. (**a**) The 2029-bp RT-PCR fragments were digested with KpnI, SacI, BglII, and AvrII. The corresponding fragments with the T7 RNA polymerase promoter sequence were gel purified and used for generating RNA probes. *Black bar*, coding region for ST6Gal1. (**b**) All five RNA probes synthesized from the templates in (**a**) were used in the binding reaction, and the RNA-protein complexes were visualized by EMSA. *Lanes 1–5* indicate the complexes for RNA probes 1–5, respectively

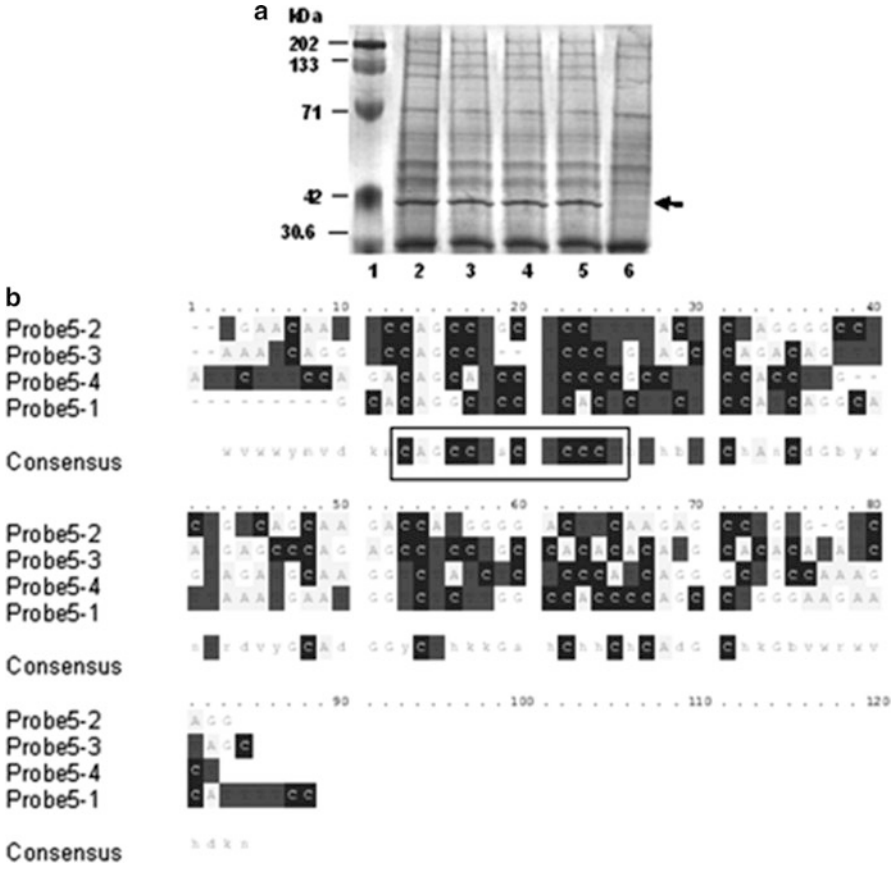


Fig. 7.14 Identification of the ST6Gal1 3′-UTR mRNA sequences that the cytosol protein(s) interact with: (**a**) Four biotin-labeled 80-bp RNA probes (5-1, 5-2, 5-3, and 5-4) that cover the 304-bp region (probe 5 in Fig. 7.19a) were incubated with liver cytosol protein and then added to SA-PMP beads. The bound proteins were eluted and run on SDS-PAGE gel and were stained. *Lane 1*, Kaleidoscope protein molecular mass marker (Bio-Rad). *Lanes 2–5*, proteins eluted from SA-PMP beads with the above biotin-labeled probes 5-1, 5-2, 5-3, and 5-4. *Lane 6* is a negative binding control. *Arrowhead* points to a 41-kDa specific protein band. (**b**) Sequence alignment analysis for the cDNA region that is complementary to the four 80-bp RNA probes covering the entire 304-bp region. A 13-bp consensus sequence is repeated four times within the 304-bp immediately downstream of the stop codon. The consensus sequence is *boxed*

to 304-bp downstream from the stop codon (probe 5), 5′-biotin-labeled RNA probes were made within this region and were used to further map the binding region. Therefore, four 5′-end biotin labeled 80-bp RNA probes covering the entire length of the 304-bp RNA probe 5 were synthesized and tested in the standard binding reaction. Surprisingly, in contrast to other nonspecific faint bands that were observed when the cytosol fraction was incubated with the magnetic beads alone (Fig. 7.14a, lane 6), a strong band corresponding to 41 kDa was observed with equal binding intensity exclusively for each of these 80-bp probes when they were incubated with the cytosol fraction and the magnetic beads (Fig. 7.14a, lanes 2–5). Further analysis revealed that

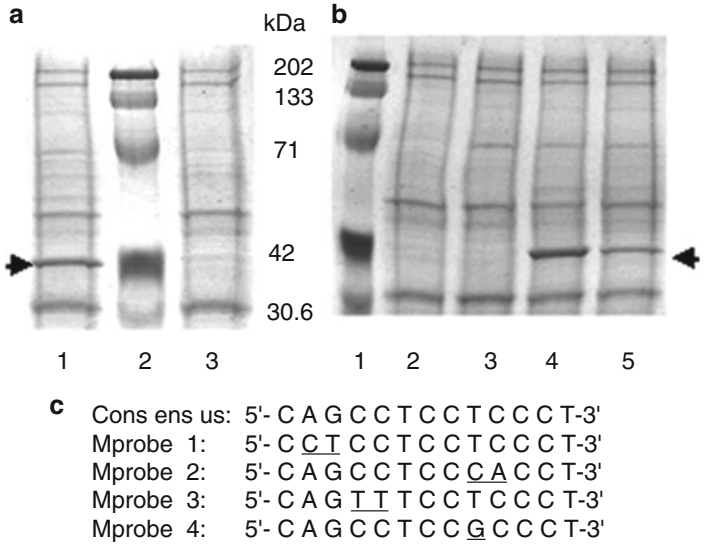


Fig. 7.15 Identification of the minimal sequence (element) that is essential for the ST6Gal1 RNA-protein interaction. (**a**) RNA probe corresponding to the 13-bp consensus sequence (*lane 1*) and nonconsensus sequence (*lane 3*) were used in the binding reaction. Kaleidoscope protein molecular mass marker (Bio-Rad) (*lane 2*). (**b**) Mutagenesis analysis. RNA probes corresponding to the 13-bp consensus sequence were mutated as indicated in (**c**). Binding reactions were performed as described under Materials and Methods. *Lane 1*, kaleidoscope marker; *lane 2*, *Mprobe 1*; *lane 3, Mprobe 2*; *lane 4*, *Mprobe 3*; *lane 5*, *Mprobe 4*. (**c**) Alignment of the consensus sequence and the mutated sequences. The mutated nucleotides are *underlined*

all of the four 80-bp probes shared the 13-bp consensus sequence as shown in Fig. 7.14b. Further mapping of the 80-bp probe showed that only the RNA probe corresponding to the 13-bp consensus sequence showed binding (Fig. 7.15a, lane 1) but not to the probe corresponding to the remaining nonconsensus sequence (Fig. 7.15a, lane 3). Significantly, when the nucleotides AG and TC were mutated to CT and CA, respectively, in the 13-bp consensus sequence, the corresponding RNA probes completely lost their binding ability (Fig. 7.15b, lanes 1 and 2). However, when only the nucleotide T was mutated to nucleotide G (Fig. 7.15b, lane 5), the corresponding RNA probe still showed partial binding ability. The mutation of other nucleotides, like CC to TT, did not affect the binding (Fig. 7.15b, lane 4).

7.2.15 The Specificity of the Cytosolic Binding Protein that Interacts with ST6Gal1 mRNA

ST8Sia1 is another member of sialyltransferase family, which links the sialic acid residues to another sialic acid residue of brain glycoconjugates through an α2,8-bond. In an attempt to test the specificity of this binding protein to ST6Gal1 mRNA, it was reasonable to test whether this binding protein interacted with ST8Sia1 mRNA

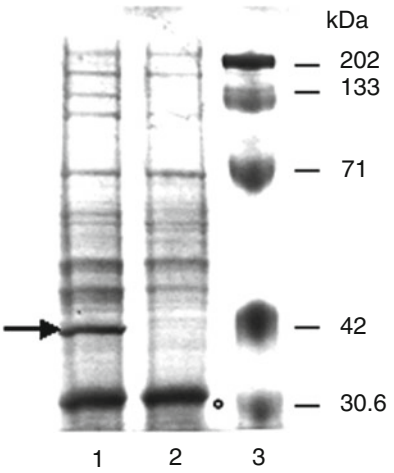


Fig. 7.16 Specificity of the protein that binds to 3′-UTR of ST6Gal1. Biotin-labeled RNA probe synthesized from ST6Gal1 mRNA (*lane 1*) and ST8Sia1-mRNA (*lane 2*) 3′-UTRs were used separately in the standard binding assay as described in Fig. 7.5 legend. *Lane 3*, kaleidoscope protein marker (Bio-Rad). *Arrowhead* points to a 41-kDa specific protein band that is present only in *lane 1* but not in *lane 2*

probe. Biotin-labeled ST8Sia1 RNA probe was used along with the labeled ST6Gal1 probe to study the specificity of their binding to the liver cytosolic fraction. As shown in Fig. 7.16, a band corresponding to 41 kDa was found only with ST6Gal1 probe (lane 1) but not with ST8Sia1 probe (lane 2) under identical conditions.

7.2.16 *Effects of Chronic Ethanol Exposure* In Vivo *on the Status of the Binding Protein*

As shown in Fig. 7.17a, there was a dietary ethanol concentration- dependent decrease in the binding intensity of the RNA-protein complex band that virtually disappeared when the cytosol fraction from the livers of rats fed with 36% of their dietary calories because ethanol was tested in the standard binding reaction. The intensity of the UV cross-linked ^{32}P-labeled RNA protein complex band also progressively decreased with increasing concentration of dietary ethanol (Fig. 7.17b).

7.2.17 *Influence of Chronic Ethanol and Betaine on Animal Body and Liver Weights*

The initial and final body weights, final liver weights and the liver weights as per cent of body weights of various experimental groups are given in Table 7.1. It can be seen that both LFO and HFO Control groups gained significant body weights. On the other

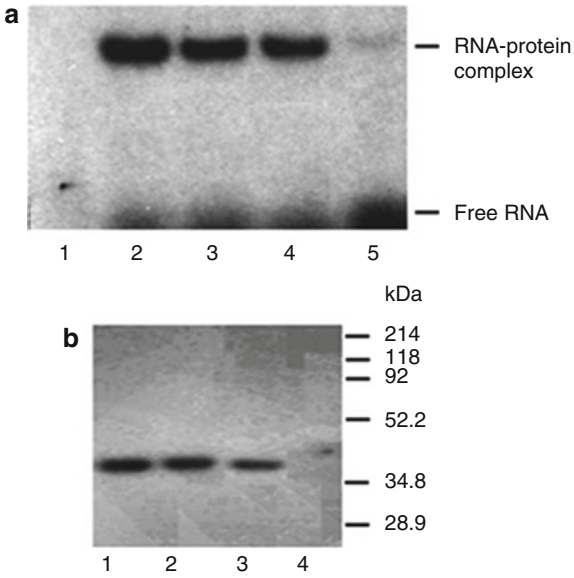


Fig. 7.17 Effects of various concentrations of dietary ethanol feeding on the expression of binding protein. The formation of ^{32}P-labeled RNA probe and cytosol protein complex was determined by EMSA (**a**) and by UV cross-linking (**b**). (**a**) RNA-protein complex with the cytosol fraction from: *lane 1*, negative control without the cytosol fraction in the reaction; *lane 2*, control liver; *lane 3*, 10.8% ethanol group; *lane 4*, 21.6% ethanol group; and *lane 5*, 36% ethanol group. (**b**) UV cross-linked RNA-protein complex with the cytosol fraction from: *lane 1*, control liver; *lane 2*, 10.8% ethanol group; *lane 3*, 21.6% ethanol group; and *lane 4*, 36% ethanol group

hand, the corresponding LFOE and HFOE Alcohol groups did not gain as much body weight compared to their controls. In contrast, the gains in weights of the LFOEB and HFOEB groups were markedly decreased compared to their corresponding LFOE and HFOE groups, respectively. Moreover, the hepatosomatic index increased by 23% ($p<0.05$) in both LFOE and HFOE Alcohol groups. The corresponding increases in hepatosomatic index values for the LFOEB and HFOEB groups were 23% ($p<0.05$) and 32% ($p<0.05$), respectively. For the sake of the ease of interpretations with regard to the influence of chronic ethanol and high ω-3 PUFA, all results described below are compared against the low ω-3 PUFA control group.

7.2.18 Influence of Chronic Ethanol and Betaine Liver PON1 mRNA Expression in Rats Fed Low and High ω-3 PUFA Diets

As shown in Fig. 7.18, liver PON1 mRNA expression was decreased by 23% ($p<0.01$) in the high ω-3 PUFA-fed group compared to the low ω-3 PUFA group. Chronic ethanol feeding decreased liver PON1 mRNA expression by 25% ($p<0.01$)

Table 7.1 Effects of ETOH and Betaine in low and high fish oil diets on body weight, liver weight, and liver to body weight ratio

Dietary groups ($n=4$)	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Final liver weight (g)	Hepatosomatic index
LFO	130.1±3.9	244.5±5.9	114.4±6.2	7.8±0.4	3.2±0.16
LFOE	160.0±4.8	233.4±4.8[a]	73.4±4.8[a]	7.5±0.3	3.2±0.20
LFOEB	157.2±3.2	185.2±5.2[b]	28.2±2.2[b]	7.2±0.4	3.9±0.24[a]
HFO	159.4±2.9	268.2±7.3	108.8±4.3	8.4±0.4	3.1±0.18
HFOE	178.3±4.2	228.3±6.7[c]	50.3±4.6[c]	8.8±0.6[a]	3.8±0.21[c]
HFOE	177.6±3.0	201.9±3.5[d]	24.3±3.0[d]	8.1±0.4	4.1±0.26[c]

Values are means±SEM. Means in a column with different superscripts differ significantly ($p<0.05$) as calculated by Tukey's test

[a] Compared to LFO group

[b] Compared to the corresponding LFOE or HFOE groups

[c] Compared to HFO group

[d] Compared to HFO or HFOE. Hepatosomatic Index = (Liver weight × 100)/body weight

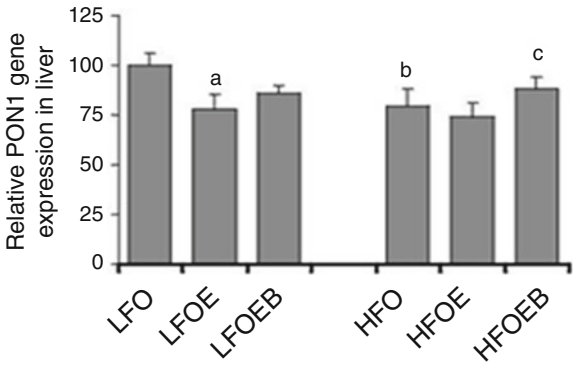


Fig. 7.18 Influence of chronic ethanol and betaine on PON1 mRNA expression in livers of rats fed low and high w-3 PUFA diets. The animals in the indicated groups (n = 4) were pair-fed their respective Lieber-DeCarli control or alcohol containing liquid diets for 8 weeks after which the animals were killed and each liver was analyzed for PON1 mRNA. The relative expression of PON1 mRNA in the HFO groups is expressed as percent of the corresponding values in the LFO groups. The data are means ± SEM. Statistical significance of variance was calculated using Tukey's test; *a*: $p < 0.01$ compared to LFO group; *b*: $p < 0.01$ compared to LFO group; *c*: $p < 0.01$ compared to HFOE group

in low ω-3 PUFA group, and 30% ($P < 0.01$) in high ω-3 PUFA group compared to the low ω-3 PUFA control group. Betaine significantly restored liver PON1 mRNA expression to 90–95% ($p < 0.01$) in high ω-3 PUFA alcohol groups compared to low ω-3 PUFA control group.

7.2.19 Influence of Chronic Ethanol and Betaine on Serum PON1 and HCTLase Activities in Rats Fed Low and High ω-3 PUFA Diets

As shown in Figs. 7.19 and 7.20, high *ω-3* PUFA significantly decreasedserum PON1 activity by 20% ($p < 0.05$) and homocysteine thiolactone (HCTL)ase activity by 28% ($p < 0.05$). Correspondingly, serum PON1 activity decreased by 23% ($p < 0.05$) and 58% ($p < 0.01$) while serum HCTLase activity decreased by 25% ($p < 0.05$) and 59% ($p < 0.01$) in the low and high *ω-3* PUFA ETOH groups, respectively. Significantly, betaine stimulated serum PON1 activity by 200% ($p < 0.01$) in the high ω-3 PUFA alcohol group to the level of low ω-3 PUFA group while betaine had marginal effect in low ω-3 PUFA alcohol group. Betaine caused similar changes in serum HCTLase activities in the high ω-3 PUFA alcohol group.

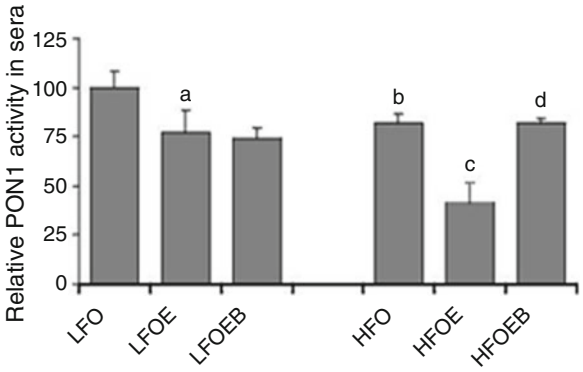


Fig. 7.19 Influence of chronic ethanol and betaine on serum PON1 activity in rats fed low and high w-3 PUFA diets. The animals in the indicated groups (n = 4) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of the betaine for 8 weeks after which the animals were killed and serum sample was analyzed for PON1 activity. The relative PON1 activity in the various experimental groups is expressed as percent of the corresponding values in the LFOE groups. The data are means ± SEM. Statistical significance of variance was calculated using Tukey's test; *a*: $p < 0.01$ compared to LFO group; *b*: $p < 0.05$ compared to LFO group; *c*: $p < 0.01$ compared HFO group; *d*: $p < 0.01$ compared to HFOE group

7.2.20 Influence of Chronic Ethanol, ω-3 PUFA and Betaine on Liver GSH Levels in Rats Fed Low and High ω-3 PUFA Diets

As shown in Fig. 7.21, GSH in the liver was very similar in both groups of rats fed low or high ω-3 PUFA diet. However, chronic alcohol significantly decreased liver GSH levels by 27% ($p < 0.05$) in low ω-3 PUFA group and by 38% ($p < 0.01$) in high ω-3 PUFA group. Betaine restored liver GSH levels in both low and high ω-3 PUFA alcohol groups nearly to the level in low ω-3 PUFA control group ($p < 0.05$).

7.2.21 Influence of Chronic Ethanol, ω-3 PUFA and Betaine on Serum ALT

As shown in Fig. 7.22, serum ALT increased moderately by 28% ($p < 0.01$) in LFOE group compared to the LFO group that was significantly restored to the level of LFO group by betaine supplementation. In contrast, serum ALT increased dramatically by 60% ($p < 0.01$) in HFOE compared to the HFO group. Betaine feeding significantly blocked the increase in serum ALT level in HFOEB group than in the HFO group ($p < 0.05$).

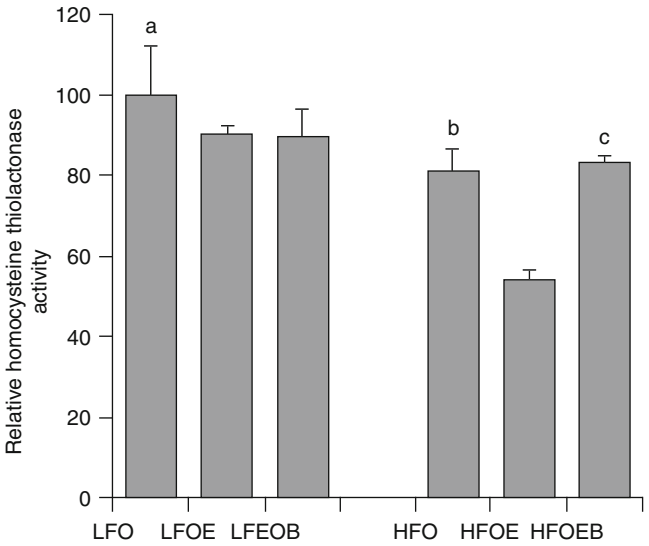


Fig. 7.20 Influence of chronic ethanol and betaine on serum homocysteine thiolactonase activity in rats fed low and high w-3 PUFA diets. The animals in the indicated groups (n = 4) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of the betaine for 8 weeks after which the animals were killed and serum sample was analyzed for homocysteine thiolactonase activity as described in the Methods section. Homocysteine thiolactonase activity expressed as percentage in sera. The data are means ± SEM. Statistical significance of variance was calculated using Tukey's test; *a*: $p < 0.01$ compared to HFO group; *b*: $p < 0.01$ compared to HFOE group; *c*: $p < 0.01$ compared to HFOE

7.2.22 Influence of Chronic Ethanol, ω-3 PUFA and Betaine on Liver Lipid Score

As seen in Fig. 7.23, oil red O stained liver sections of low ω-3 PUFA alcohol fed rats showed no significant changes of fat deposition compared to low ω-3 PUFA. Whereas betaine in low ω-3 PUFA alcohol diet significantly reduced fat deposition compared to low ω-3 PUFA alcohol and low ω-3 PUFA groups ($p < 0.05$); steatosis increased significantly in rats fed high ω-3 PUFA diet versus low ω-3 PUFA ($p < 0.05$). Ethanol significantly increased liver steatosis in rats fed high ω-3 PUFA compared to all other groups ($p < 0.01$). Betaine significantly prevented liver steatosis that occurred in both low and high ω-PUFA alcohol groups as well as high ω-PUFA group ($p < 0.01$).

7.3 Significance of the Results

As part of our continuous efforts to better understand how ethanol affects human liver pathologically, we have previously reported that long-term ethanol causes down-regulation of ST6Gal1 gene (Gong et al. 2007). In the current expanded study,

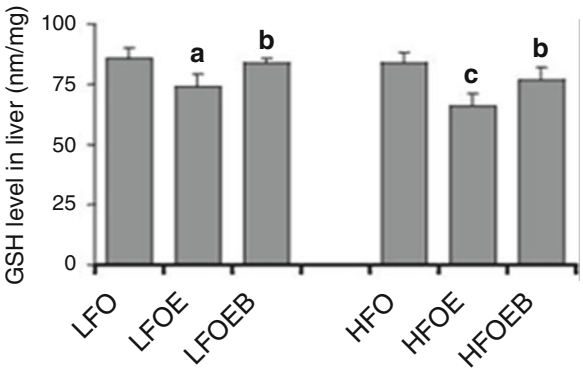


Fig. 7.21 Influence of chronic ethanol, w-3 PUFA, and betaine on liver GSH level in rats fed low and high w-3 PUFA diets. The animals in the indicated groups (n = 4) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of betaine for 8 weeks after which the animals were killed and each liver was analyzed for reduced GSH level in liver quantified as described in the Methods section. The data are means ± SEM. Statistical significance of variance was calculated using Tukey's test; *a*: $p < 0.05$ compared to LFO group; *b*: $p < 0.05$ compared to the corresponding LFOE or HFOE groups; *c*: $p < 0.01$ compared to HFO group

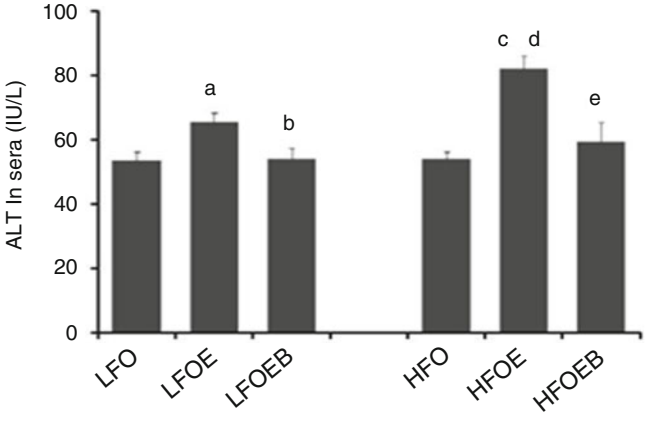


Fig. 7.22 Influence of chronic ethanol, w-3 PUFA, and betaine on serum ALT in rats fed low and high w-3 PUFA diets. The animals in the indicated groups (n = 4) were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of betaine for 8 weeks after which the animals were killed and each serum sample was analyzed for ALT level. The relative ALT activity in the various experimental groups is expressed as international units per liter. The data are means ± SEM. Statistical significance of variance was calculated using Tukey's test; *a*: $p < 0.01$ compared to LFO group; *b*: $p < 0.05$ compared to the corresponding LFOE group; *c*: $p < 0.01$ compared to HFO or LFO groups; *d*: $p < 0.01$ compared to LFOE group; *e*: $p < 0.01$ compared to HFOE group

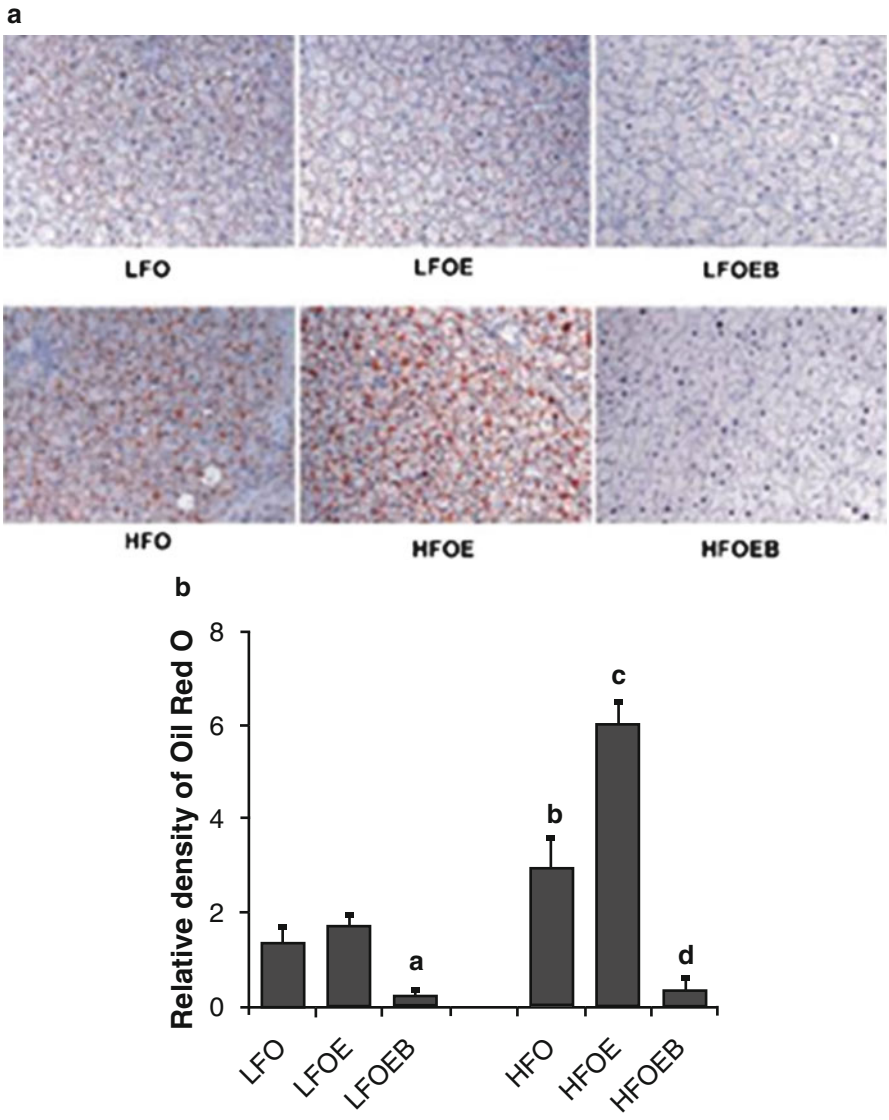


Fig. 7.23 Influence of chronic ethanol, w-3 PUFA, and betaine on liver steatosis in rats fed low and high w-3 PUFA diets. The animals in the indicated groups were pair-fed their respective Lieber-DeCarli alcohol containing liquid diets supplemented with the indicated concentration of betaine for 8 weeks after which the animals were killed and each liver section was stained with oil red O, counter stained with hematoxylin, and the extent of lipid deposition in the liver was quantified. (**a**) Representative liver histochemistry of each group. (**b**) A *bar graph* shows the rela¬tive lipid score analyses in various groups. The data are means ± SEM from three animals. Statistical significance of variance was calculated using Tukey's test; *a*: $p < 0.05$ compared to LFOE group; *b*: $p < 0.05$ compared to the corresponding LFO groups; *c*: $p < 0.01$ compared to HFO or LFO groups; *d*: $p < 0.01$ compared to HFOE or HFO or LFOE

we have not only confirmed our previous findings, but also have provided a direct evidence that the down-regulation of ST6Gal1 is most likely due to long-term ethanol exposure, but not due to pathologic conditions (Fig. 7.1).

Liver sections stained with oil red O from the various experimental groups clearly show that even moderate drinking of alcohol causes fat deposits in the liver that exacerbate after heavy drinking compared with the non-drinking group (Figs. 7.2 and 7.3). It must be pointed out that the samples belonging to metabolic and genetic obesity were excluded from the study analyses. Both histometric methods demonstrated much more lipid accumulation in the livers of the drinking groups than in the nondrinking groups and thus histologically (Fig. 7.3) support our biochemical findings (Fig. 7.2). These results, together with molecular biology evidence (Fig. 7.1), imply that the steatosis found in the drinking groups compared with the nondrinking groups may be more specific to ethanol consumption. Our results are consistent with other reports that ST6Gal1 gene expression is unaltered or up-regulated in nonalcoholic or neoplastic liver diseases (Petretti et al. 2000; Wang et al. 2003; Dall'Olio et al. 2004).

When drinking history, ST6Gal1 mRNA level, and liver lipid deposit are all considered in the multivariate statistical analysis, there is a strong correlation among drinking history, ST6Gal1 gene expression, and liver lipid deposit (Fig. 7.4).

We have also used human hepatocyte HepG2 cell lines as a model to study the effects of ethanol on the regulation of ST6Gal1 gene expression because ethanol metabolism takes place primarily in the liver. That the expression of ST6Gal1 mRNA was affected by ethanol only in ethanol-metabolizing cells but not in the wild type (Figs. 7.6, 7.7 and 7.8) clearly demonstrates the importance of ethanol metabolism for eliciting this regulatory response. The two major hepatic ethanol-metabolizing enzymes are CYP2E1 and alcohol dehydrogenase. Both of these enzymes oxidize ethanol to acetaldehyde, which is more toxic than ethanol itself. The fact that acetaldehyde, the immediate product of ethanol oxidation, is effective in down-regulating ST6Gal1 mRNA confirms that ethanol may mediate this response via acetaldehyde.

On the other hand, CYP2E1-mediated oxidation of ethanol also produces a state of oxidative stress by generating ROS within the cells that can lead to the generation of a key metabolite, the α,β-unsaturated aldehyde, HNE. HNE may be more harmful than ROS because it has a longer half-life and can easily diffuse into cellular membranes (Esterbauer et al. 1991). Under normal conditions, intracellular HNE concentration is less than 1.0 μmol/L, but it can reach a level of as high as 100 μmol/L under oxidative stress (Burczynski et al. 2001). The fact that HNE strongly down-regulates ST6Gal1 mRNA even at 32 μmol/L (Fig. 7.9) strongly supports the concept that oxidative stress caused by ethanol oxidation via CYP2E1 could also play a key role in regulating this gene.

Sialyltransferases are a family of enzymes consisting of more than 18 glycosyltransferases that catalyze the transfer of sialic acid from cytidine monophospho-N-acetylneuraminic acid to the nonreducing terminal positions on the oligosaccharide chains of glycoproteins and glycolipids (Harduin-Lepers et al. 2001). Terminal sialic acids are key determinants of carbohydrate structures involved in a variety

of biologic events, such as viral-host recognition (Gagneux et al. 2003), cell-cell adhesion (Schauer 1985; Pilatte et al. 1993; Lin et al. 2002) and tumor cell invasiveness (Zhu et al. 2001). In particular, alteration of sialic acids, generally found in the nonreducing terminus of most glycoproteins and glycolipids, has been associated with long-term alcohol exposure (Ghosh et al. 2001). A number of reports have provided evidences that long-term alcohol exposure interferes with the metabolism of the complex glycoconjugates of certain circulating as well as membrane-bound species in human and experimental animals (Lakshman et al. 1999; Stibler and Borg 1981), resulting in either the appearance of a number of carbohydrate-deficient glycoconjugates including carbohydrate-deficient transferrin (Stibler and Borg 1981), sialic acid–deficient apolipoprotein J (Ghosh et al. 2001), and α1-acid glycoprotein in the plasma of long-term alcohol consumers (Tsutsumi et al. 1994).

Ethanol exposure down-regulates the expression of ST6Gal1 mRNA in two ethanol-metabolizing human liver cell lines, the CYP2E1 cells and HAD cells but not in wild-type HepG2 cells, which do not metabolize ethanol. Thus, this study assumes major importance and clinical relevance because ST6Gal1 gene regulation in a human liver cell model is demonstrated within a few days of ethanol exposure, whereas it's *in vivo* regulation in liver generally takes prolonged period of ethanol exposure. The possible mechanism(s) of action ethanol seems to be mediated via acetaldehyde as well as via ROS. Further work is warranted to shed light as to how acetaldehyde and ROS regulate the expression of ST6Gal1 and what signaling pathways are involved.

ST6Gal1 mediates the transfer of α2,6-linked sialic acid to glycoproteins. Down-regulation of the expression of this gene and consequent impaired activity of ST6Gal1 has been associated with the increase of asialoconjugates in the blood of chronic alcoholics and retention of glycoproteins in the liver. In our previous studies (Ghosh and Lakshman 1997; Rao and Lakshman 1997, 1999), we showed that chronic ethanol feeding in rats caused a marked decrease (59% reduction compared with controls) of the ST6Gal1 activity as well as its mRNA level in liver that was due to decreased stability of ST6Gal1 mRNA. In our present work, we have further characterized the mechanism of action of ethanol in the destabilization of ST6Gal1 mRNA. We report here for the first time the identification and partial characterization of a liver cytosol protein that specifically binds to the 3′-UTR of the ST6Gal1 mRNA and plays a role in its stability. Significantly, chronic ethanol feeding decreases the intracellular concentration of this binding protein leading to the destabilization of ST6Gal1 mRNA and its down-regulation by rapid degradation.

The observed binding intensity of RNA-protein complex as a function of protein concentration (Fig. 7.10a) reveals that the binding reaction seems to be saturated at 50 μg of cytosol protein when the RNA concentration is kept at 2.0nM. Unfortunately, it is premature to carry out Scatchard analysis using the crude cytosol fraction to determine the dissociation constant for this binding protein. This has to wait until the purification of this protein to homogeneity is achieved. Nonetheless, if the stoichiometry of interaction of the RNA with the binding protein is mole per mole, our data imply that the relative abundance of this binding protein in normal

rat liver cytosol fraction is approximately 0.0035% of total cytosol protein (based on our finding that the molecular mass of the binding protein is 41 kDa). The specificity of this binding is demonstrated by virtual absence of the binding in the presence of 100-fold excess of the unlabeled probe (Fig. 7.10b, lane 4). The polypeptide nature of this binding protein is confirmed by its sensitivity to proteinase K and heat treatment (Fig. 7.10b, lanes 5 and 6). Based on the fact that the binding protein interacts equally well with both native and denatured RNA probes (Fig. 7.10b, lanes 2 and 3), it is clear that the proper secondary structure of RNA is not an absolute prerequisite for this interaction to occur. The fact that the RNA-protein complex was resistant to digestion by various types of RNases, whereas the naked RNA was totally destroyed on treatment by the same RNases, clearly shows that the binding protein specifically protects the RNA from degradation (Fig. 7.11). UV cross-linking experiments revealed a molecular mass of the binding protein to be around 41 kDa (Fig. 7.12). The fact that the 3′-UTR of ST8Sia1, another related sialyltransferase, failed to show any interaction with this binding protein (Fig. 7.16) strongly supports our finding this binding protein is highly specific for interaction with the ST6Gal1 3′-UTR.

Based on the fact that the secondary structure of ST6Gal1 3′-UTR is not critical for this specific protein-ST6Gal1 interaction (Fig. 7.10b, lanes 2 and 3), it is logical that this protein should interact with the ST6Gal1 3′-UTR in a sequence specific manner. Our data shows that the RNA probe that covers the 304 bp immediate downstream from the stop code of ST6Gal1 3′-UTR shows tight binding to this binding protein (Fig. 7.13). Surprisingly, all four RNA probes (5-1, 5-2, 5-3, and 5-4) of 80-bp length spanning the entire 304-bp probe showed equal binding intensity to the binding protein (Fig. 7.14a). Sequence alignment analysis of these four probes revealed that the corresponding cDNA sequences for the four 80-bp RNA probes share 13-bp consensus sequences (Fig. 7.14b). Further analysis confirmed that this consensus sequence is not present in other areas of the ST6Gal1 3′-UTR and coding region. The fact that only the 13-bp consensus sequence but not the remaining nonconsensus sequence on the 80-bp region showed binding activity (Fig. 7.15a) clearly establishes that this 13-bp consensus sequence serves as the specific binding site for this protein. Mutagenesis analysis conclusively proved that the conserved nucleotides AG and TC are critical for the RNA-protein interaction (Fig. 7.15b). Thus, a mutation of even a single nucleotide seems to affect the binding.

Although the identity of the 41-kDa protein is yet to be determined, its specificity and high-affinity interaction with the narrow UTR region of ST6Gal1 mRNA clearly indicates its critical role in regulating the ST6Gal1 mRNA metabolism as evidenced by the influence of chronic ethanol exposure that leads to its destabilization (Ghosh and Lakshman 1997; Rao and Lakshman 1997, 1999). The formation of the RNA-protein complex progressively decreased with increasing dietary ethanol concentration leading to its virtual disappearance in the livers of rats fed with 36% of the total calories as ethanol (Fig. 7.17a, b). A parallel 45% ($p<0.05$) decrease in plasma SIJ in the 36% ethanol group compared with the control group fully confirms our concept that chronic ethanol feeding destabilizes ST6Gal1 mRNA level by decreasing

the amount of this specific binding protein that interacts with the 3′-UTR of ST6Gal1 mRNA and leads to the generation of asialoconjugates in the blood of alcoholics.

Our present study demonstrates for the first time another unique interaction of a liver cytosolic-specific binding protein with the 3′-UTR region of ST6Gal1 mRNA that protects it from degradation in normal rat liver. We further show that chronic ethanol exposure down-regulates this mRNA by depleting this specific binding protein leading to ethanol-mediated destabilization of ST6Gal1 mRNA. It is significant to point out that the amount of ethanol consumption, when it is fed at 36% of the total dietary calories, amounts to 12–14 g/kg body weight per day, a value that is comparable with ethanol drinking by human heavy alcoholics (>100 g/day that is equivalent to ≥6 drinks/day). Thus, this specific defect caused by chronic ethanol exposure in alcoholics is the most likely cause for the blood appearance of asialoconjugates such as carbohydrate-deficient transferrin (Stibler et al. 1987), sialic acid-deficient apolipoprotein J (Ghosh et al. 2001), and asialo-α1-acid glycoprotein (Tsutsumi et al. 1994) that serve as excellent biomarkers for chronic alcohol consumption.

It is significant to point out that our data are consistent with the existing concept that dietary ω3-fatty acids in low amounts (2.8% of the total dietary calories) may reduce liver injury by increasing antioxidants such as GSH peroxidase and catalase (Nanji et al. 1995), whereas in high amounts (13.8% of the total dietary calories) ω3-fatty acids seem to be detrimental as evidenced by marked increase in serum ALT in chronic alcohol-fed high ω3-fatty acids group (Fig. 7.22). This was further confirmed by enormous increase in liver lipid score in the chronic alcohol-fed high ω3-fatty acids unlike the corresponding low ω3-fatty acids group. These results are in agreement with the previous studies using intragastric alcohol feeding rat model (French et al. 1993; Nanji et al. 1995; Tsukamoto et al. 1995). The ability of betaine in chronic alcohol-fed low and high ω3-fatty acids diets significantly ameliorated the increased serum ALT level strongly suggests that betaine prevents liver injury induced by ethanol (Fig. 7.22). This is further confirmed by our finding that betaine prevents liver steatosis as evidenced by significant decrease in liver lipid score in betaine supplemented chronic alcohol-fed low and high ω3-fatty acids groups (Fig. 7.23a, b). Presumably, the action of betaine may be mediated via increased generation of liver S-adenosylmethionine (SAM) level (Barak et al. 1997) that is required to methylate phosphotidylethaonalamine to phosphotylcholine, which may restore the impaired VLDL synthesis and secretion (Purohit et al. 2007).

Consistent with numerous alcohol feeding studies, the present study confirms that alcohol-fed animals do not seem to gain as much wait as the control animals in spite of pair-feeding lead to increased hepatosomatic index. Strikingly, betaine feeding seems to further reduce the gain in body weight that results in greater increase in the Hepatosomatic index (Table 7.1). It must be pointed out that, apart from the liver gross appearance liver histopathology (Fig. 7.23) as well as ALT (Fig. 7.22) data clearly point out that the hepatosteatosis and liver injury caused by chronic ethanol are markedly alleviated by betaine feeding in both low and high ω3-fatty acid fed animals.

With respect to chronic alcohol and Atherogenesis, our present study clearly shows that feeding high ω-3 PUFA diet for 8 weeks in rats significantly down-regulates not only the hepatic PON1 gene expression (Fig. 7.18), but also serum PON1 (Fig. 7.19) and HCTLase (Fig. 7.20) activities. Reduced PON1 activity in high ω-3 PUFA versus low ω-3 PUFA suggests that fish oil has a dose effect and implicates its susceptibility to increased ROS production. These results are consistent with a previous study showing decreased PON1 activity in fish oil-fed rats compared to the rat groups fed separately with triolein and tripalmitin (Kudchodkar et al. 2000). Significantly, chronic ethanol-mediated further decreases in liver PON1 mRNA expression (Fig. 7.18) and serum PON1 (Fig. 7.19) and HCTLase (Fig. 7.20) activities in high ω-3 PUFA fed animals imply that chronic ethanol-induced cytochrome P450 2E1 (CYP2E1) exacerbates the accelerated generation of ROS in the presence of high ω-3 PUFA that may be responsible for these deleterious effects on PON1 status. Chronic heavy ethanol is known to induce CYP2E1 that mediates the generation of ROS during ethanol oxidation (Marí and Cederbaum 2000; Morimoto et al. 1994). The greater decreases in serum PON1 (Fig. 7.19) and HCTLase (Fig. 7.20) activities relative to modest corresponding changes in hepatic PON1 mRNA levels (Fig. 7.18) caused by chronic ethanol further confirm the susceptibility of PON1/HCTLase enzymes to oxidative stress prevailing in ethanol and high ω3-fatty acid-fed conditions (Figs. 7.19 and 7.20).

Based on our present study as well as on the current literature in this area, we have summarized in Fig. 7.16, our suggested hypotheses on the possible mechanism/s of action/s of ω3-fatty acids and betaine in ameliorating chronic alcohol-mediated alterations in PON1 and HCTLase activity in relation to ROS and GSH status. Accordingly, in addition to ROS, homocysteine is a well known factor involved in vascular disease (Griendling et al. 2000; McCully 1993). High fish oil and alcohol have been reported to increase plasma homocysteine by 20% (Piolot et al. 2003) and by 62% (Stickel et al. 2000), respectively, in rats suggesting that they may lead to earlier cardiovascular and liver abnormalities by synergistically increasing tissue homocysteine level. Excess of homocysteine, in turn, is likely to increase S-adenosylhomocysteine (SAH) levels, leading to decreased SAM:SAH ratios that trigger the endoplasmic reticulum stress response and associated liver cell apoptosis (Esfandiari et al. 2005, 2007). Betaine can convert homocysteine to methionine by transmethylation and homocysteine to cysteine by transulfuration, and thereby can reduce the levels of toxic homocysteine in the blood. Our study fully supports this concept since betaine significantly increased both PON1 and HCTLase activities. To our knowledge, this is the first time high ω-3 PUFA as well as chronic heavy ethanol have been shown to markedly affect serum HCTLase activity (Fig. 7.20). These findings could explain why heavy alcoholics, especially when they are also on high ω-3 PUFA diet are susceptible to cardiovascular diseases.

Further, our findings of a significant decrease in endogenous liver GSH in chronic ethanol-fed high ω-3 PUFA group (Fig. 7.21) support the critical role of GSH in the regulation of PON1/HCTLase activities (Figs. 7.20 and 7.21). The ability of betaine to restore the decreased level of liver GSH found in chronic ethanol-fed high ω-3

PUFA group with concomitant restoration of serum PON1/HCTLase activities strongly suggests that the protective role of betaine may be mediated via increases in thiol group containing superoxide dismutase and GSH both of which help in quenching free radicals (Balkan et al. 2005). Important mechanism of betaine is to activate cystathionine pathway to synthesize cysteine and glycine that are precursors for the synthesis of GSH. Liver is the major organ to synthesize GSH and export to other organs to quench free radicals generated by excessive alcohol metabolism. Further work is necessary to delineate the molecular mechanism of action of betaine on PON1 regulation.

7.4 Conclusions

We conclude that chronic alcohol-mediated down-regulation of hepatic ST6Gal1 gene leads to defective glycosylation of lipid-carrying apolipoproteins such as apo E and apo J, resulting in defective VLDL assembly and intracellular lipid and lipoprotein transport, which in turn is responsible for alcoholic hepatosteatosis and ALD. The mechanism of ethanol action involves thedepletion of a unique RNA binding protein that specifically interacts with its 3′-UTR region of ST6Gal1 mRNA resulting in its destabilization and consequent appearance of asialoconjugates as alcohol biomarkers. With respect to ETOH effects on CVD, we conclude that CYP2E1 and ETOH mediated oxidative stress significantly down regulates not only the hepatic PON1 gene expression, but also serum PON1 and HCTLase activities accompanied by depletion of hepatic GSH, the endogenous antioxidant. These results strongly implicate the susceptibility of PON1 to increased ROS production. In contrast, betaine seems to be both hepatoprotective and atheroprotective by reducing hepatosteatosis and restoring not only liver GSH that quenches free radicals, but also the antiatherogenic PON1 gene expression and activity.

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Chapter 8
The Role of CYP2E1 in Alcohol Metabolism and Sensitivity in the Central Nervous System

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Abstract Ethanol consumption has effects on the central nervous system (CNS), manifesting as motor incoordination, sleep induction (hypnosis), anxiety, amnesia, and the reinforcement or aversion of alcohol consumption. Acetaldehyde (the direct metabolite of ethanol oxidation) contributes to many aspects of the behavioral effects of ethanol. Given acetaldehyde cannot pass through the blood brain barrier, its concentration in the CNS is primarily determined by local production from ethanol. Catalase and cytochrome P450 2E1 (CYP2E1) represent the major enzymes in the CNS that catalyze ethanol oxidation. CYP2E1 is expressed abundantly within the microsomes of certain brain cells and is localized to particular brain regions. This chapter focuses on the discussion of CYP2E1 in ethanol metabolism in the

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A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*, Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0_8,

CNS, covering topics including how it is regulated, where it is expressed and how it influences sensitivity to ethanol in the brain.

Keywords CYP2E1 • CNS • Ethanol

8.1 Introduction

Excessive alcohol consumption (alcoholism) causes morbidity and mortality in populations of varied ages, regions and ethnicities. Millions of people suffer from alcoholism and billions of dollars are spent annually on alcohol related toxicities (Mokdad et al. 2004; Ginter and Simko 2009; Brust 2010). Excessive alcohol intake leads to extensive tissue damage in organs (such as liver, heart and the respiratory system), as well as promoting disease states, including diabetes mellitus and neuropsychiatric disorders (Brust 2010). The central nervous system (CNS) is particularly susceptible to ethanol where it acts as a depressant. Exposure of the CNS to alcohol may also cause behavioral changes, such as anxiety, aggression, euphoria, poor motion coordination and compromised memory and linguistic proficiency (Vasiliou et al. 2006; Alfonso-Loeches and Guerri 2011).

Alcohol is a class of organic compounds with carbon-bound hydroxyl group(s). The most commonly ingested alcohol is ethanol (CH_3CH_2OH), an ethane backbone bound by a hydroxyl group. Due to its high affinity for water, once ingested, ethanol is rapidly absorbed from all parts of the gastrointestinal tract largely by simple diffusion into the blood. It is then quickly distributed throughout the body. The majority of ethanol metabolism occurs in the liver. Several distinct pathways contribute to the metabolism of ethanol. Alcohol dehydrogenases (ADHs), catalase and cytochrome P450 2E1 (CYP2E1) are involved in its conversion to acetaldehyde and aldehyde dehydrogenases (ALDHs) convert acetaldehyde to acetate. While ethanol metabolic pathways in the liver are relatively well characterized, considerably less is understood regarding enzymes and pathways involved in ethanol metabolism in the brain. This chapter provides a summary of current knowledge about ethanol metabolism and sensitivity in the CNS, with an emphasis on the role of CYP2E1 in these processes.

8.2 Ethanol Metabolism in the CNS

Three enzymes are responsible for oxidizing ethanol to acetaldehyde: ADH, catalase, and CYP2E1 (Fig. 8.1). Acetaldehyde is further oxidized to acetate by ALDHs (Vasiliou et al. 2006). The mammalian ADH enzyme family has five classes, each with several isoforms (Estonius et al. 1996). Class 1 consists of ADH isoforms 1, 2 and 3; these are responsible for the majority of ethanol metabolism in the liver (Edenberg 1998). The role of ADH enzymes in the brain, once thought to be nonexistent, is now evident but poorly understood. Some isoforms of ADH have been

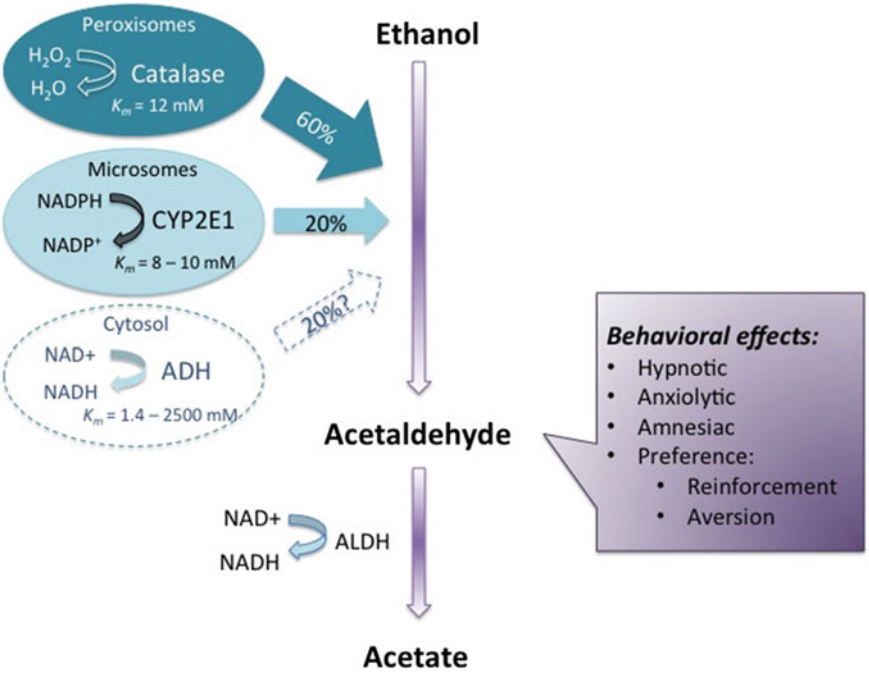

Fig. 8.1 Metabolic pathways of ethanol metabolism in the Central Nervous System (CNS). Three enzymes are responsible for oxidizing ethanol to acetaldehyde: alcohol dehydrogenase (ADH), catalase and cytochrome P450 2E1 (CYP2E1). Acetaldehyde is further oxidized to acetate by acetaldehyde dehydrogenase (ALDH). Acetaldehyde is responsible for many of the behavioral effects associated with ethanol consumption. The contribution of each enzyme to total ethanol oxidation occurring in the CNS is as follows: catalase, serving as the primary enzyme, accounts for 60%; CYP2E1 is inducible and accounts for 20%; the remaining 20% is unknown but is thought to involve ADH. Isoforms of ADH present in the CNS have a wide range of affinity for ethanol

identified in the brain in small quantities. ADH3 was found in the brain but ethanol was shown to be a poor substrate for this particular isoform, with a *Km* of greater than 2.5 M (Beisswenger et al. 1985). mRNAs for ADH1, a low-*Km* isoform (*Km* = 1.4 mM) (Zakhari 2006), has also been detected in the brain (Martinez et al. 2001). Interestingly, ADH activity was not detected in the whole brain homogenate but was found to be localized to granular and Purkinje cells of the cerebellum (Galter et al. 2003). Therefore, if ADHs have a role in the CNS, they appear to be localized to particular brain regions and their function remains to be defined.

There is growing evidence that catalase serves as the primary ethanol metabolizing enzyme in the brain, as well as in the heart and kidney (Vasiliou et al. 2006). Catalase is found primarily in the peroxisome (Fig. 8.1) where it catalyzes the conversion of hydrogen peroxide to oxygen and water. Catalase has a *Km* of 12 mM for ethanol oxidation (Thurman et al. 1972), which is much lower than that of ADH3, indicating ethanol to be a better substrate for catalase. Catalase accounts for 60% of

ethanol oxidation in the brain (Zimatkin et al. 2006) and appears to play a major role in mediating the psychopharmacological effects of ethanol (Vasiliou et al. 2006; Correa et al. 2008). Through the examination of the effect of inhibitors on acetaldehyde production and the use of transgenic mice, Zimatkin and colleagues (2006) verified the importance of catalase in ethanol oxidation in the brain.

Recent studies have also found that CYP2E1 plays a significant role in ethanol oxidation to acetaldehyde in the brain (Vasiliou et al. 2006; Zimatkin et al. 2006). CYP2E1, a membrane-bound protein, catalyzes the oxidation of a variety of endobiotics (such as retinoids and prostaglandins) and xenobiotics (such as drugs and alcohols) (Cheung et al. 2005; Dey and Kumar 2011). It has a relatively high activity towards ethanol oxidation with a *Km* of 8–10 mM (Zakhari 2006). The *CYP2E1* gene is well conserved in mammalian species and CYP2E1 protein is expressed in various tissues and cell types (Lee et al. 1996). While CYP2E1 is most abundantly expressed in hepatocytes, it has been reported in CNS where its expression has been found to be region-, cell type- and organelle- specific (Howard et al. 2003a, b). CYP2E1 has traditionally been considered to be a microsomal protein. However, recent studies indicate that it is also present in the mitochondria through a distinct mitochondrial-import mechanism (Robin et al. 2001, 2002; Knockaert et al. 2011). In this organelle, it promotes oxidative stress during the production of acetaldehyde and thereby has the potential to trigger a series of unfavorable molecular events in neuronal cells (Raza et al. 2004; Bansal et al. 2010).

8.3 Acetaldehyde in Ethanol Sensitivity of the Brain

Acetaldehyde is the primary and highly reactive metabolite of ethanol. It has been implicated as a significant contributor to a broad spectrum of adverse physiological and pathophysiological effects of ethanol (Zimatkin et al. 2006). Physiological alterations ascribed to acetaldehyde include changes in glial cell function and differentiation (Sarc et al. 2011). Behavioral alterations include euphoria (intense happiness), anxiolytic (reduced anxiety), hypnotic (also known as sleep time), amnesiac (loss of memory) and aggression, as well as reinforcement or aversion to voluntary ethanol consumption (preference). The contribution of acetaldehyde in each of these distinct ethanol-associated behaviors has been subject to experimental examination. For example, Quertemont and colleagues (2004) examined the role of acetaldehyde in the anxiolytic, hypnotic and amnesiac behavioral effects of ethanol consumption. An association between acetaldehyde and the hypnotic (at higher doses) and amnesiac (dose-dependent) behavioral effects of ethanol were observed. However, acetaldehyde was not involved in the anxiolytic effects of ethanol. These results implicate acetaldehyde in the hypnotic, amnesiac and preferential behaviors of ethanol. Interestingly, data derived from experiments conducted in transgenic animals contradict the theory that acetaldehyde contributes to ethanol's hypnotic effect. Specifically, in acatalasemic, CYP2E1-null or catalase/CYP2E1 dual-deficient mice, a decrease in blood acetaldehyde levels was accompanied by

an increase in ethanol-induced sleep time, especially at high doses of ethanol (Vasiliou et al. 2006).

Acetate is the secondary metabolite of ethanol and, unlike acetaldehyde, can freely cross the BBB. Acetate is metabolized to acetyl-CoA, which can be used in the citric acid cycle or converted into acetylcholine, a neurotransmitter. While the primary source of energy in the brain is glucose, ethanol decreases glucose uptake and potentially switches the source of energy to acetate (Zimatkin et al. 2011). Short sleep or high tolerance rats have been found to have a lower amount of acetyl-CoA in the frontal cortex than long sleep rats, indicating the inborn deficiency of acetyl-CoA is compensated for by ethanol-derived acetate (Zimatkin et al. 2011). It is likely that the tolerance in short sleep rats can be attributed to their ability to use acetate for acetyl-CoA production better than the long sleep rats. Hence, acetate may contribute to the hypnotic effects of ethanol.

In the context of voluntary consumption of ethanol (or ethanol preference), acetaldehyde in the CNS has reinforcing effects. When directly administered to the ventral tegmental area (VTA) of the brain, acetaldehyde reproduces the same activating and reinforcing behavioral effects as ethanol administration (Correa et al. 2003), implicating the role of acetaldehyde in ethanol preference. Supporting this proposal is the observation that inhibition of acetaldehyde formation (by microinjection of a catalase-inhibiting shRNA lentiviral vector directly to the VTA) resulted in a 94% reduction in voluntary ethanol consumption by high alcohol preferring rats (Karahanian et al. 2011). This treatment also eliminated the increased dopamine efflux in the nucleus accumbens normally seen after systemic ethanol administration. However, there was no interruption in normal dopamine pathways as demonstrated by a normal dopamine response to methamphetamines and tyrosine hydroxylase, the enzyme responsible for formation of L-dopa, remained active. Voluntary ethanol consumption was shown to be stimulated by administration of a lentiviral vector encoding ADH that increased acetaldehyde production (Karahanian et al. 2011). These studies strongly support the notion that acetaldehyde produced in the brain mediates the reinforcing effects of ethanol consumption.

There are multiple working theories regarding the involvement of acetaldehyde in ethanol preference (Davis and Walsh 1970; Melchior and Myers 1977; Rodd-Henricks et al. 2002; Vasiliou et al. 2006). Some think acetaldehyde alone may mediate the reinforcing effects attributed to ethanol in the CNS. Others believe acetaldehyde may act as a modulator, augmenting the actions of ethanol. It is also possible that acetaldehyde may react with an endogenous substance (e.g. dopamine) to form another biologically-active compound (e.g. salsolinol), which may serve as the active compound in the reinforcing behavior (McBride et al. 2002). Whatever the mechanism, it is clear that acetaldehyde in the CNS has a reinforcing role in voluntary ethanol consumption. Outside of the CNS, however, acetaldehyde appears to have the opposite effect. Due to metabolism by the abundance of ALDH in the blood brain barrier (BBB), acetaldehyde formed outside of the CNS (i.e., in the periphery) is unable to enter the CNS (Zimatkin 1991). Accordingly, acetaldehyde exists in two physiological compartments separated by the BBB, *viz*. in the CNS and in the periphery. The effects of acetaldehyde in the periphery are

concentration-dependent: reinforcing effects are seen at very low concentrations while mostly aversive affects at normal to high concentrations (McBride et al. 2002). An acetaldehyde "burst" (measured as a spike in blood acetaldehyde levels), occurring as a result of the higher activity of an isoform of ADH, appears to cause reduced voluntary ethanol intake (aversion). Wistar rats conditioned for high ethanol intake were genetically altered to express decreased hepatic ALDH activity (85% reduction). Predictably, this resulted in higher concentrations of circulating acetaldehyde after ethanol consumption (Quintanilla et al. 2007). These animals exhibited a 50% reduction in voluntary ethanol consumption, showing that, contrary to its reinforcing effects in the brain, acetaldehyde outside the CNS tends to have an aversive effect. Future studies quantifying ethanol metabolites, acetaldehyde and acetate, to ensure concordance with normal ethanol intoxication will be vital in investigating the behavioral effects of ethanol with respect to physiological conditions (Deitrich 2004).

8.4 Acetaldehyde Production in the Brain

Before the discovery of ethanol-oxidizing pathways in the brain, research focused on the potential of acetaldehyde to cross into the brain. Ethanol and acetate can freely cross the BBB while acetaldehyde cannot (Deitrich et al. 1989). The observation that acetaldehyde concentrations in the CNS do not correspond to levels in the blood (Tabakoff et al. 1976; Westcott et al. 1980; Deitrich 2011) is consistent with this contention and indicates that acetaldehyde measured in the CNS is produced there.

The discovery of the catalase pathway for acetaldehyde formation in the brain (Aragon et al. 1992; Gill et al. 1992) served as an important first step in our understanding about the role of acetaldehyde in the effects of ethanol in the CNS. Studies using inhibitors of catalase (e.g., aminotriazole or sodium azide) and acatalasemic mice (i.e., have a genetic catalase deficiency that results in $\approx$50% reduction in brain catalase levels) have revealed catalase to be responsible for approximately half of ethanol metabolism occurring the CNS (Zimatkin et al. 2006). This finding indicates the existence of other ethanol metabolizing pathways in the brain. A contender for an alternative pathway is CYP2E1, an isoform of cytochrome P450 which is inducible by ethanol.

8.5 CYP2E1 Expression in the Brain

Although the presence of CYP2E1 in the brain had been established, early research using brain homogenates argued against CYP2E1 participating in ethanol oxidation (Gill et al. 1992). There are several explanations for these negative data: First, the cytochrome P450 inhibitor used, metyrapone, displays specificity for the isozyme CYP2B rather than CYP2E1. Second, the agents used to inhibit catalase can, at high doses, also inhibit CYP2E1 (Zimatkin et al. 2006), leading to an overestimation of

the role of catalase in the brain. Third, the presence of ADH in the brain, specifically some isoforms of ADH such as ADH3 (Beisswenger et al. 1985; Kerr et al. 1989) may account for the discrepancy seen upon catalase inhibition rather than it being attributed to CYP2E1.

In a three part experiment by Zimatkin and colleagues (2006), ethanol oxidation was studied by measuring the concentration of acetaldehyde and acetate in perfused mouse brains and brain homogenates. Catalase was found to mediate 60% of ethanol metabolism in the brain. Inhibition of ADH decreased acetate levels, but had no effect on acetaldehyde levels, likely due to non-specific inhibition of ALDH by the ADH inhibitor used in the experiments (although this has not been confirmed). Inhibition of CYP2E1 (using dallyl sulfide (DAS) or beta-phenethyl-isothiocyanate) led to a significant decrease in both acetaldehyde and acetate (Deitrich 2004). Experiments using animal models harboring genetic deficiencies in CYP2E1 and/or catalase indicate that CYP2E1 is responsible for approximately 20% of ethanol metabolism in the brain (Zimatkin et al. 2006). After considering the combined contributions of catalase and CYP2E1 to ethanol oxidation in the brain, there remains 20% of ethanol oxidation unaccounted for; this is speculated to be attributable to ADH and/or other enzymes (Person et al. 2000; Zimatkin et al. 2006).

The metabolism of ethanol by the prenatal brain has been of great interest, particularly given the impact ethanol consumption by pregnant women may have on the developing CNS of the fetus. Prenatal brain homogenates are capable of generating acetaldehyde from ethanol (Person et al. 2000). While CYP2E1 activity was shown to be present in the prenatal brain of humans, acetaldehyde formation appeared to occur by a mechanism independent of CYP2E1, catalase or ADH (Person et al. 2000). However, at birth (and in states of starvation) CYP2E1 induction by ethanol is rapid (Abdulla et al. 2006). While experiments using whole brain homogenates to investigate CYP2E1 metabolism of ethanol have often been equivocal, examination of specific brain locations have provided evidence of oxidation of ethanol by CYP2E1. This apparent disparity may be a consequence of whole brain enzymatic systems overshadowing discrete systems of specific brain regions and/or interference by fatty tissue that is abundant in the brain. Brain region-specific metabolism of ethanol may be extremely important. It is well established that specific brain regions are responsible for different CNS functions. Accordingly, the generation of different levels of ethanol metabolites depending on the enzyme present and the region could be responsible for different effects of ethanol on CNS function. For example, the presence of catalase and CYP2E1 ensure local formation of acetaldehyde, which mediates the positive reinforcing, motor disorder and local neurotoxic effects of alcohol (Zimatkin et al. 2006). Local metabolism results in local accumulation of ethanol metabolites and its condensation products (acetaldehyde, salsolinol, acetate) that would mediate the selective disturbances in certain brain structures and their involvement inthe behavioral effects of alcohol (Zimatkin et al. 2006). Ethanol treatment of male rats induced 1.7–2-fold increases in CYP2E1 protein and activity in olfactory bulbs, the frontal cortex, the hippocampus and the cerebellum (Howard et al. 2003a, b). In this same study, nicotine administration was shown to also induce CYP2E1 in the same CNS locations. These findings offer an

explanation for the frequent co-addiction seen between ethanol and nicotine. Further, they underscore how the metabolism of ethanol is up-regulated upon co-treatment with nicotine and the corresponding CYP2E1 induction. In other animal studies, higher concentrations of CYP2E1 have been found in the hippocampus, medulla and substantia nigra (Howard et al. 2003a, b), brain regions responsible for memory and potentiation, autonomic functions, and dopamine regulation, respectively. By influencing acetaldehyde production at these sites, CYP2E1 may play a critical role in mediating the effects of ethanol.

CYP2E1 mRNA, protein, enzyme activity and induction by ethanol have been found in neural and glial cells of the CNS (Howard et al. 2003a, b). In a study of human brains obtained from smoking and non-smoking alcoholics, increased expression of CYP2E1 protein was found in granular cells of the dentate gyrus and pyramidal cells of the hippocampus (Howard et al. 2003a, b). Animal studies have found a higher concentration of CYP2E1 protein in astrocytes and glial cells following inflammatory induction (Sarc et al. 2011). The identification of CYP2E1 in dopaminergic neurons of the substantia nigra suggests that it may be involved in dopamine regulation (Howard et al. 2003a, b) and have potential implications in Parkinson disease.

Astroglial cells represent the majority of the brain cell population. They play an important role in the (i) developmental guidance of migrating neurons, (ii) regulation of neurotransmitter and ion levels, (iii) nutrition of neurons, and (iv) production of neurotrophic factors (Sarc et al. 2011). Astrocytes also represent a major site for the detoxification or bioactivation of neurotoxins and they are important contributors to the creation of the immune response in the brain (Sarc et al. 2011). Ethanol exposure changes growth, differentiation and function of neuronal and astroglial cells (Vemuri and Chetty 2005; Vangipuram and Lyman 2010; Sarc et al. 2011). For example, in rat primary cultures of astroglial cells, ethanol and acetaldehyde were both shown to be able to modulate TNF-alpha and IL6 production (Sarc et al. 2011). Therefore, alterations in acetaldehyde concentration through CYP2E1 induction have the potential to directly modulate astroglial cell function; however, more research is needed.

The intracellular processing of ethanol and its metabolites are important when considering how ethanol can influence cellular function. As noted, catalase is found primarily in the peroxisome and CYP2E1 is found primarily in the endoplasmic reticulum or microsomes of the cell (Fig. 8.1). As such, it is likely that the majority of ethanol is metabolized to acetaldehyde in these parts of the cell. Acetaldehyde must then diffuse to the mitochondria where it would be oxidized by ALDH2 to acetate (Deitrich et al. 2006). En route, acetaldehyde can interact with other molecules in the intracellular milieu and thereby alter cellular function.

Study of enzyme activities have been conducted in subcellular fractions of homogenates from mouse and rat brains (Zimatkin et al. 2006). These studies confirmed highest ethanol metabolizing activities of catalase and CYP2E1 to be present in the peroxisomal and microsomal fractions, respectively. Ethanol oxidation in the microsomal fraction increased with the addition of NADPH (a known CYP2E1 cofactor) and was decreased by CYP2E1 inhibitors, strengthening the contention that CYP2E1 is an essential enzyme in ethanol metabolism in the microsomal fraction. Ethanol oxidation was decreased in the microsomal fraction of CYP2E1-null mice (Vasiliou et al. 2006). The failure of CYP2E1 absence (in the CYP2E1-null

mice) to prevent oxidation of ethanol by the microsomal fraction suggests that other enzymatic pathways may play a role (Vasiliou et al. 2006). Given the apparent specific subcellular locations of the ethanol-metabolizing enzymes, there is the potential that acetaldehyde could cause elicit cellular effects during its passage to the mitochondria where it is converted to acetate.

8.6 Regulation of CYP2E1 in the CNS

CYP2E1 can activate small, low molecular weight compounds. Some endogenous substrates for CYP2E1 include acetone and fatty acids (which are abundant in the brain) (Lieber 1999). CYP2E1 can also activate toxic compounds and procarcinogens found in tobacco smoke, such as nitrosamine compounds (Lieber 1999). Accordingly, induction of CYP2E1 by ethanol can promote bioactivation of these compounds (Howard et al. 2003a, b). As noted, CYP2E1 can also be induced by nicotine (found in cigarette smoke), which can result in bioactivation of the above compounds and in increased ethanol oxidation. Indeed, Howard and colleagues (2003a, b) determined that CYP2E1 was induced by nicotine, which offers an explanation for the increased self-administration of ethanol after exposure of rats to nicotine (Hauser et al. 2012). Induction of CYP2E1 in the CNS by nicotine likely increases the metabolism of ethanol to acetaldehyde, a result consistent with the theory that increases in CNS acetaldehyde causes reinforcement in ethanol preference.

In addition to chemical activation, CYP2E1 is differentially regulated by inflammation and infectious conditions (Abdulla et al. 2006). In this study, peripheral and central nervous system inflammation was induced in rats by lipopolysaccharide (LPS; an endotoxin that induces an inflammatory response in most animals) via intraperitoneal and intracerebroventricular administration, respectively. Under both conditions, inflammation caused an initial increase in hepatic CYP2E1 mRNA levels following by a decrease in CYP2E1 activity. The discovery of this regulatory mechanism in the liver in response to CNS inflammation led to the hypothesis that astroglial cells may have the potential to regulate CYP2E1 under similar circumstances (Abdulla et al. 2006). In another study in rat primary cortical glial cells, LPS was shown to induce the expression of catalytically-active CYP2E1, with a maximum induction occurring at 24 h (Tindberg et al. 1996). Clearly, the effects of inflammation on CYP2E1 regulation in the CNS are complex and differ greatly from cell culture models to *in vivo* studies.

8.7 Genetic Variation of CYP2E1 in Ethanol Sensitivity

The gene encoding the CYP2E1 enzyme is highly conserved among species, indicating its evolutionary importance. Genetic factors influence sensitivity and tolerance to ethanol in laboratory animals and humans (Bhave et al. 2006). Genetic factors influence sensitivity and tolerance to ethanol in laboratory animals and humans (Bhave et al. 2006). Genetic manipulation of enzyme expression has

permitted mechanistic investigation of the roles of specific enzymes in ethanol oxidation. For example, acetaldehyde production following ethanol consumption is reduced in acatalasemic mice (Vasiliou et al. 2006). Acetaldehyde production by brain homogenates from CYP2E1-null mice was not affected by the absence of CYP2E1 (Vasiliou et al. 2006), bringing into question the importance of this enzyme in ethanol oxidation. However, acetaldehyde production in CYP2E1-null/acatalasemic mice was significantly lower than the already reduced levels observed in acatalasemic mice. The difference between the levels confirms the significance of the CYP2E1 enzyme (Vasiliou et al. 2006).

A role for genetic polymorphisms in the genes coding for the enzymes in ethanol metabolism was proposed to explain ethanol preference between inbred C57BL/6 (high preference) and DBA/2 (low preference) strains of mice (Bhave et al. 2006). There are nine known ADH-coding genes and three catalase-coding genes in the mouse (Bhave et al. 2006). The genes encoding cytochrome P450 families range from 82 to 102, depending on the source (Bhave et al. 2006). A gene array allowed the comparison of genes from the two mouse lines bred for different preferences for ethanol but naïve to ethanol exposure. As quantified by the array, the intensity for CYP2E1 was low or undetectable in ethanol naïve mice (Bhave et al. 2006). The failure to observe any increase could be due to testing the whole brain tissue rather than specific locations, cells or subcellular fractionations. The main result of this study was that ethanol preference was due to genetic differences in ALDH2, the enzyme responsible for metabolizing acetaldehyde to acetate. The low preferring DBA2 strain had higher amounts of ALDH mRNA in the CNS and the high preferring C57BL/6 had lower levels of ALDH (Bhave et al. 2006). Low levels of ALDH would potentially cause increased concentrations of acetaldehyde, providing additional evidence that acetaldehyde in the CNS is reinforcing.

There is the potential that induction of CYP2E1 by nicotine could contribute to metabolic tolerance in smoking alcoholics. This possibility led to research into the impact of polymorphisms that induce CYP2E1 on alcohol dependence. Specifically, the CYP2E1*1D polymorphism was found to occur with greater frequency in nicotine- or alcohol- dependent populations and it showed an association with nicotine- and alcohol- dependence (Howard et al. 2003a, b). The greater inducibility of CYP2E1 associated with this polymorphism is likely to contribute to the development of nicotine and ethanol co-dependencies (Howard et al. 2003a, b), because of increased metabolism of ethanol and nicotine. Other polymorphisms for CYP2E1 exist in different populations; however, correlations between genotype (polymorphism) and phenotype (ethanol preference or dependence) are yet to be conducted.

8.8 Conclusion

The discovery of ethanol metabolizing enzymes in the CNS has led to much research into the effect of ethanol and its metabolic products on behavior and addiction. Because CYP2E1 can be induced by ethanol and nicotine, it has been

the focus of major studies in addiction and co-addiction involving ethanol. Some studies investigating ethanol sensitivity focus on the primary product of ethanol oxidation, acetaldehyde, while others examine both acetaldehyde and its final product, acetate. To determine ethanol effects in response to the presence and quantity of ethanol and its metabolites, behavioral, locomotor or preferential tests are usually conducted in animals. Through the use of inhibitors or inducers of the main ethanol-metabolizing agents, as well as genetic manipulation of enzyme expression, the importance of CYP2E1 in the CNS and in regulating sensitivity to ethanol is beginning to be revealed.

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Index

A. Dey (ed.), *Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism*, Subcellular Biochemistry 67, DOI 10.1007/978-94-007-5881-0,