DRUG METABOLIZING ENZYME, DRUG TRANSPORTER EXPRESSION AND DRUG DISPOSITION ARE ALTERED IN MODELS OF INFLAMMATORY LIVER DISEASE

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DEDICATION

I dedicate this dissertation to my parents, David and Carolyn Lickteig, It was your love and support that made this possible. You have inspired me with the confidence to continuously strive to be my best, and you have never let me accept that there is anything I cannot achieve.

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ABSTRACT

Correct dosing in pharmacotherapeutics is based on the idea that too much of a drug will cause toxicity, while too little will result in failure to elicit the desired response. A major factor in the ability of a patient to handle any dose of a drug is the capacity to metabolize and eliminate that drug from the body. For the vast majority of drugs, the liver plays a key role in determining the rate at which drugs are eliminated. First, drugs must be taken up across the cell membrane into hepatocytes by uptake transporters. Once inside the hepatocyte, biotransformation enzymes metabolize and conjugate the drug to a more water-soluble compound, the distribution of which is more easily controlled. These water-soluble metabolites are then transported out of the hepatocyte by additional drug transporters either into bile for elimination, or back into the blood.

More than 2 million severe adverse drug reactions occur in the US each year and often result from interindividual variation in the ability to metabolize and eliminate drugs. This number does not include medical errors, but rather circumstances where an individual is unable to handle the standard dose of the correctly prescribed drug. Although genetics plays an important role, the greatest source of variation comes from other environmental factors such as disease states. Nonalcoholic fatty liver disease (NAFLD) is a chronic condition that comprises a spectrum of histopathologies that range from simple steatosis to the more severe steatohepatitis. Specifically, nonalcoholic steatohepatitis (NASH) has become one of the leading causes for liver transplantation in the United

States, and thus clearly become a considerable burden to the U.S. healthcare system.

It is not known whether the capacity of the liver to metabolize and excrete drugs is altered in patients with NASH. Because the liver plays such a critical role in drug metabolism and disposition, any disease state that disrupts or modifies these functions will alter the fate of a given drug within the body. It is therefore very likely that the ability of the liver to metabolize and excrete clinically relevant drugs is compromised in NASH patients.

INTRODUCTION

Fundamental considerations regarding liver anatomy and physiology

The liver is one of the largest organs in the human body and performs numerous, vital functions, many of which are related to metabolism. It is also responsible for the excretion of endogenous waste products; synthesis of bile salts; storage of vitamins (A, B_{12}, D, E, and K) and minerals (iron and coper); and scavenging of bacteria and expired blood cells.

With regard to metabolic function, the liver fulfills its role in carbohydrate metabolism by helping maintain normal blood glucose levels. When blood glucose levels become low, glycogenolysis is activated, whereby glycogen stores in the liver are metabolized to glucose for release into the bloodstream. Conversely, when blood glucose levels become high, as typically occurs after a meal, glycogenesis is turned-on, resulting in the conversion of glucose to glycogen. The liver is also the principal organ for lipid metabolism, where the process of β-oxidation is responsible for the catabolism of fatty acids. The liver synthesizes lipoproteins, which are responsible for the import and export of fatty acids, triglycerides and cholesterol into and from the liver, respectively. Protein metabolism is also performed by the liver. In additon to synthesizing a variety of plasma proteins such as clotting factors, hepatic protein metabolism mediates conversion of the potentially toxic metabolic by-product ammonia into urea for excretion in urine. A fourth facet of liver metabolism is that of endobiotic and xenobiotic metabolism. Example endobiotics include bilirubin, which is derived

from the heme group of scavenged erythrocytes, as well as steroid hormones such as estrogen. Xenobiotic substances would include a countless number of clinical drugs, e.g. fluoxetine, pravastatin and acetaminophen and environmental chemicals. This dissertation will place some emphasis on the cytochrome P450 (CYP) reductase family of liver proteins, which play a clearly discernible and integral role in the disposition of drugs in the body.

Beyond metabolism, the liver also performs active excretion of endobiotics and xenobiotics. Following absorption from the blood, the processes of metabolism and excretion for a given compound (endobiotic or xenobiotic) are necessarily coordinated functions. This becomes clear when one notes that the products of metabolism are generally more suitable substrates for the excretory mechanisms with which the liver is equipped. Active excretion of endobiotics and xenobiotics typically results either in their secretion into bile for elimination in feces, or efflux back into blood for elimination in urine. This dissertation will also place a great deal of emphasis on transporter proteins of the liver, which function in a coordinate manner with the metabolic pathways mentioned in the preceding paragraphs.

The population of cells in the liver is diverse in type and function. Greatest in abundance are hepatocytes, followed by Kupffer cells (KCs), sinusoidal endothelial cells (SECs) and Ito cells. It is to be noted that there are more than four cell types in the liver, including natural killer cells, pit cells, and dendtritic

cells. However, only heptocytes, KCs, SECs and Ito cells will be described currently in moderate detail.

Hepatocytes, also referred as parenchymal cells, are specialized epithelial cells and the exclusive site of metabolism in the liver. It logically follows that hepatocytes are also responsible for the excretion of the metabolic products they generate. This means that a variety of active transport mechanisms native to hepatocytes are critical to overall liver function. Examples would include primary active transport, which is ATP-driven, as performed by transporter proteins of the ATP-binding Cassette (ABC) superfamily. Another example is secondary active transport, which utilizes the potential energy of a concentration gradient as a means to transport substances against a gradient, e.g. co-transport and counter-transport. These are observed in the cases of Na^+/glucose co-transport and primary active transport of Na^+ and K^+ by Na^+/K^+-ATPase.

Kupffer cells (KCs) are the resident macrophages of the liver. They are derived from bone marrow cells that have traveled to the liver as blood monocytes and undergone differentiation into macrophages. The primary function of KCs is to phagocytize and degrade particulate matter, e.g. dead erythrocytes, bacteria, and other foreign material, thereby helping maintain immune homeostasis of the hepatic microenvironment. KCs are thus also capable of releasing a variety of cytokines, eicosanoids, and reactive oxygen and nitrogen species, which contribute to immune function (Laskin, 1990).

Sinusoidal endothelial cells (SECs) are able to fulfill critical support roles for the liver in virtue of their vast surface area. This implies that blood perfusing the liver must initially come into contact with the SECs that constitute an extensive lining. However, because neighboring SECs are disconnected, or discontinuous, there exist numerous pores (or fenestrae) among SECs. These are small enough to hinder passage of large particles (>250 kDa), e.g. chylomicron remnants, yet large enough to facilitate exchange of fluids and smaller molecules, e.g. O_2, albumin, acetaminophen. SECs also serve the liver by scavenging denatured proteins from blood perfusing the liver. Lastly, SECs are capable of participating in hepatic immunity, as will be discussed later.

Ito cells, also called fat-storing or stellate cells, are responsible for synthesis of the connective and structural protein collagen. Ito cells are also the body's major site of vitamin A storage. Like hepatocytes, KCs, and SECs, Ito cells have an integral role in hepatic immune function.

In order to fully appreciate the specialized functions of hepatocytes, KCs, SECs and Ito cells, it is important to have a grasp of their anatomical organization within the liver. To this end, two concepts have been established, the lobule and the acinus. The liver lobule representation divides the liver into hexagonal units with a terminal hepatic venule (or central vein) at the center. At the corners of each unit are "portal triads" consisting of a hepatic portal vein, a hepatic arteriole, and a bile duct. The lobule has three regions: centrilobular, midzonal, and periportal. Proceeding from the portal triad to the terminal hepatic

venule are channels known as sinusoids, which are essentially large, irregular capillaries. It is within these channels that SECs, KCs and Ito cells reside. However, it is SECs, as described previously, which provide a lining for sinusoids and thus coverage for the cords of hepatocytes, which likewise proceed from the portal area to the central vein. There is a relative lack of a basement membrane between SECs and hepatocytes, making it possible for fluids and small molecules to diffuse across the space between SECs and hepatocytes, known as the space of Disse. As blood enters the liver via the portal vein and the hepatic artery (periportal region), it travels down the sinusoids toward the central vein (centrilobular region), and exits the liver via the hepatic vein.

A second concept of the functional hepatic unit is the acinus. In this representation, the region closest to the entry of blood into the liver is denoted zone 1; zone 3 borders the central vein; and, predictably, zone 2 is between zones 1 and 3. Notably, these zones approximately coincide with the three regions described by the liver lobule concept. However, instead of defining the unit in terms of the anatomical structures present, e.g. portal vein, central vein, etc., the zonation associated with the acinar unit bears functional consequence with respect to gradients in the blood and in hepatocyte function. Specifically, there are gradients with respect to oxygen concentration, metabolism, glutathione and bile salts (Jungermann and Kietzmann, 2000;Groothuis, et al., 1982).

Where blood first flows into an acinus (zone 1), two-thirds of the volume arrives via the portal vein and is relatively oxygen-depleted. The remaining one-third, arriving via the hepatic artery, is relatively oxygen-rich. As this mix of blood flows toward the terminal hepatic venule, the concentration of oxygen in the blood steadily decreases, from 9-13% in zone 1 to 4-5% in zone 3.

Appropriate to their oxygen supply, hepatocytes in acinar zone 1 are rich in mitochondria and are the predominant site of fatty acid oxidation, gluconeogenesis and ammonia detoxification in the liver. Given that free radical leakage occurs during mitochondrial metabolism, it is appropriate that hepatocyte content of glutathione (GSH), the most potent endogenous antioxidant, is highest in zone 1. Also along the acinus, there exists a gradient of those enzymes involved in the bioactivation and detoxification of drugs and other xenobiotics. In particular, levels of the drug metabolizing enzyme family of cytochrome P450 (CYP) proteins are greatest in zone 3. Thus, following overdose of certain drugs and other xenobiotics that are P450 substrates, zone 3 has been demonstrated to be at greater risk for hepatotoxicity. For example, following acetaminophen overdose, the subsequent hepatotoxicity in acinar zone 3 is due to the toxic N-acetyl-p-benzoquinoneimine (NAPQI) metabolite produced by CYP2E1. While reactive metabolites formed in zone 3 such as NAPQI can be conjugated with GSH, the GSH gradient within the acinar unit renders GSH levels low in zone 3 relative to zone 1. Lastly, there is a bile salt gradient within the acinar unit. This gradient renders zone 3 low in bile salt concentrations because they are

extracted with considerable efficiency by hepatocytes in zone 1. It is thus logical to suggest that the greater portion of bile salts is excreted by zone 1 hepatocytes.

Inflammation as a Factor in Various Diseases, Including those of the Liver

Inflammation is a response to a diverse array of stimuli, e.g. bacteria, injured cells and chemical irritants that are perceived by the body to be harmful. Inflammatory responses are generally effected by a cellular component (involves extravasation of leukocytes into inflamed tissue) and/or a humoral component (involves movement of plasma fluid into inflamed tissue). Given that cellular and humoral components are involved, the inflammatory process inherently occurs in vascularized tissues such as the liver, stomach, dermis of the skin, etc. Not only does the process function to isolate and remove the harmful constituent(s), but it also initiates the healing process of the relevant tissue.

While it is important that the cascade of events that makeup the inflammatory response be propagated swiftly and discreetly, it is equally critical that the response be tightly regulated and brought to expedient resolution. When inflammation advances unchecked, the response merely escalates damage, rather than subduing the perceived threat to the organ or system. Specifically, chronic inflammation promotes a recurrent shifting of cellular and molecular mediators that collectively drive a persistent cycle of immune-mediated tissue wounding and healing at a site. It should come as no surprise that chronic inflammation is a central factor in the pathophysiology and progression of scores of diseases, ranging from cardiovascular and neurodegenerative diseases to

diabetes and cancer (Coussens and Werb, 2002;Wellen and Hotamisligil, 2005;Willerson and Ridker, 2004;Halliday, et al., 2000).

As an example, there is evidence that inflammation is an important factor in the progressive damage that occurs during type 2 diabetes (T2D) (Sjoholm and Nystrom, 2005;Alexandraki, et al., 2006;Kempf, et al., 2006). This could be anticipated, given the multiple sources of inflammation during T2D. Among the sources is adipose tissue (AT), in which native macrophages are capable of mediating a pathophysiologic condition of “adipositis” (Frayn, et al., 2003). Hence, visceral AT that accumulates during obese states, as in T2D, is not a passive, benign tissue, as once thought. In fact, AT can function as an endocrine organ insofar as it is capable of secreting of a variety of immunity- and metabolism-regulating cytokines, known as adipokines, e.g. adiponectin, leptin, resistin (Fried, et al., 1998;Hotamisligil, et al., 1993;Meier, et al., 2002;Zhang, et al., 1994;Kershaw and Flier, 2004). Thus, adipositis promotes systemic, low-grade inflammation (lesser degree than in classic inflammatory conditions), rendering it a chronic serologic feature in obese T2D patients (Fantuzzi, 2005).

During adipositis and thus T2D-associated obesity, macrophages residing in AT can modulate product secretion by adipocytes (Weisberg, et al., 2003;Xu, et al., 2003). This results in AT release of a variety of adipokines, including tumor necrosis factor (TNF)-α and Interleukin (IL)-6, which also happen to be major proinflammatory cytokines (Fried, et al., 1998;Hotamisligil, et al., 1993). As such, TNF-α and IL-6 have been implicated in the damage that occurs with T2D

progression. Both have been demonstrated capable of promoting apoptosis and consequent decline in functional pancreatic β-cell mass (Butler, et al., 2003;Cinti, et al., 2005;Dandona, et al., 2003;Donath, et al., 2005); and both are capable of inducing activation, oxidative stress and dysfunction of vascular endothelial cells, the latter of which appears to be a rate-determining step in the development of complications in microvascular circulation (Hsueh, et al., 2004;Lyon, et al., 2003;Ronti, et al., 2006;Devaraj, et al., 2005;Aggarwal and Natarajan, 1996;Hotamisligil, et al., 1995;Pasceri, et al., 2000;Pepys and Hirschfield, 2003). The example of T2D provides sufficient evidence of the concept that dysregulated inflammation can play a critical role in the damage and dysfunction that occurs during the progression of a disease state.

Cholestasis and its Models

Cholestasis is generally described as a condtion in which the flow of bile is impaired. It can result from impairment of a specific molecular process (intrahepatic cholestasis), or as a secondary consequence of some physical obstruction of bile flow (obstructive or extrahepatic cholestasis). Consequent accumulation of bile constituents in hepatocytes can produce oxidative stress, plasma membrane disruption, severe hepatocellular injury and cell death, generally owing to the detergent-like properties of bile salts (Kasahara et al., 2002). Accordingly, maintaining bile formation and enterohepatic circulation of bile acids are among the most important functions of hepatocyte membrane transport systems. At the sinusoidal (basolateral) membrane, the Na^+-

Taurocholate Co-transporting Polypeptide (NTCP) is the secondary active transporter predominantly responsible for the uptake of bile salts from portal blood into hepatocytes. At the canalicular membrane, the ATP-dependent transporters Bile Salt Excretory Protein (BSEP) and Multidrug Resistance-associated Protein 2 (MRP2) mediate secretion of bile salts and glutathione. BSEP and MRP2 are thereby responsible for the generation of bile salt-dependent and bile salt-independent bile flow, respectively(Geier, et al., 2007).

It is unsurprising that cholestasis can result from genetic abnormalities in select transporters that secrete bile constituents. The three forms of Progressive Familial Intrahepatic Cholestasis (PFIC) illustrate this. In PFIC type 1, mutations in the PFIC1 gene (locus 18q21-22) result in a loss of function of its protein product ATP8B1, which is a putative flippase transporter of phosphatidylserine and phosphatidylethanolamine (Houwen, et al., 1994;Carlton, et al., 1995;Bull, et al., 1998). Progression to cirrhosis and liver failure usually occurs by 3 to 4 years of age, and, unfortunately, this genetic disease is not sufficiently resolved by liver transplantation. This is because PFIC type 1 results in defective bile salt transport not only in the cholangiocytes (bile duct cells) of the liver but also inadequate bile acid resorption from the intestinal ileum, leading to chronic watery diarrhea (Bull, et al., 1997;Knisely, 2000;Chen, et al., 2001).

In PFIC type 2, mutations in the ABCB11 gene (locus 2q24) result in a loss of function of its aforementioned protein product, BSEP (Strautnieks, et al., 1998). As one might anticipate, this leads to significant elevations in serum bile

acid concentrations but extremely low biliary bile acid concentrations. PFIC type 2 also results in severe pruritis, frequent jaundice (hyperbilirubinemia) and persistent, progressive cholestatic liver damage, such that usually necessitates liver transplantation within the first decade of life (Bull, et al., 1997).

In PFIC type 3, mutations in the ABCB4 gene (locus 7q21) result in loss of function of its protein product MDR3 (de Vree, et al., 1998;Jacquemin, 1999;Deleuze, et al., 1996;Jacquemin, 2001). The MDR3 protein has been localized to the hepatocyte canalicular membrane where it functions as a flippase, moving phospholipids to the outer leaflet of the canalicular membrane, facing the canalicular lumen (Ruetz and Gros, 1994;Smith, et al., 1994;van, et al., 1996).

Although not elaborated above, it is rather revealing to note that the pattern of pathologic damage to the liver differs with each type of PFIC. This axiom extends to each of the different liver diseases characterized by the condition of cholestasis, i.e. the pattern of cholestatic liver damage will vary by liver disease. Furthermore, defective transport mechanisms are rarely a primary cause of those liver diseases that are often characterized by cholestatic damage, e.g. alcoholic hepatitis, viral hepatitis, primary sclerosing cholangitis. It is instead customarily the case that a disruption of bile transport mechanisms and bile flow occurs secondary to the inflammation that accompanies many liver diseases.

At this point, it is appropriate to note that a distinction can be made between two different forms of cholestasis that is based on their etiology. When

bile flow is impaired as a direct consequence of dysregulation of the transport mechanisms that drive bile flow, the condition is termed “intrahepatic” cholestasis. Conversely, when bile flow is impaired as a direct consequence of physical obstruction of bile flow, the condition is termed obstructive or “extrahepatic” cholestasis. Intrahepatic cholestasis can occur as a consequence of sepsis, cholestasis of pregnancy, primary biliary cirrhosis, alcoholic and viral hepatitis. Extrahepatic cholestasis can result from bile duct stones, primary sclerosing cholangitis, and bile duct tumors.

Extrahepatic cholestasis has long been modeled by bile duct ligation (BDL) and has been utilized in the study described in APPENDIX B of this dissertation. In addition to impaired canalicular and common bile duct flow, BDL produces a loss of bile salt secretory polarity and the accumulation of bile salts, bilirubin, and other bile constituents in hepatocytes. This results in hepatocyte apoptosis, necrosis, inflammation, bile duct proliferation and fibrosis (Arias, 1998;Schaffner, et al., 1971).

Intrahepatic cholestasis has been modeled by a variety of chemical treatments, including ethinyl estradiol, cholic acid-feeding, and α-naphthylisothiocyanate. The model of intrahepatic cholestasis that has been utilized in the study described in APPENDIX B of this dissertation is the lipopolysaccharide (LPS) model. LPS, commonly called endotoxin, is a structural component of the outer membrane of most Gram-negative bacteria. As LPS becomes bound to LPS binding protein (LBP) and enters the liver via the portal

vein, LPS-LBP can activate Kupffer cells by binding to an “LPS receptor complex” composed of three proteins: CD14, Toll-like receptor (TLR-4), and MD2 (da Silva, et al., 2001). TLR-4, which contains a cytoplasmic TIR (cytoplasmic Toll/IL-1 receptor) domain, interacts with Myeloid differentiation factor 88 (MyD88)-adaptor-like protein (MyD88/Mal). MyD88/Mal interacts with IL-1 receptor-associated kinase, which then undergoes autophosphorylation. TNF receptor-associated factor 6 (TRAF-6) then interacts with IL-1 receptor-associated kinase. TRAF-6 interacts with IL-1 receptor-associated kinase and mitogen-activated protein kinase 1, leading to phosphorylation and degredation of the inhibitor of nuclear factor of the κ-enhancer in B cells-inhibitory protein (IκB). Nuclear factor of the κ-enhancer (NF-κB) in B cells in then released and translocated to the nucleus, where it can enhance transcriptional activation of target genes such as TNF-α, IL-1β, and IL-6 (Medzhitov et al., 1997; Hoshino et al., 1999). The proinflammatory cytokines TNF-α, IL-1β, and IL-6 each have putative roles in the regulation of transporters in the liver during cholestasis. The effect of their absence on transcriptional regulation of liver transporters during experimental extrahepatic and intrahepatic cholestasis, respectively, has been investigated in APPENDIX B of this dissertation.

Nonalcholic Fatty Liver Disease (NAFLD): Risk Factors and Histopathology

NAFLD is the most common cause of abnormal liver chemistry tests in the developed world and is a significant predisposing factor for cryptogenic cirrhosis (Skelly, et al., 2001;Neuschwander-Tetri and Caldwell, 2003;Brunt, 2005). The

disease is most accurately diagnosed by histologic analysis of a liver biopsy (Neuschwander-Tetri and Caldwell, 2003). Based on published histologic observations, NAFLD actually encompasses a spectrum of pathologies that typically first manifest as “bland” or simple hepatic steatosis (fatty liver without inflammation). Simple hepatic steatosis is defined by lipid accumulation in >5% of hepatocytes, and the lipids that accumulate are predominantly macrovesicular, such that displace the hepatocyte nucleus (Brunt, 2005;Neuschwander-Tetri and Caldwell, 2003;Burt, et al., 1998). In contrast, microvesicular steatosis is more commonly a hallmark of genetic disorders with defective β-oxidation of fatty acids and acquired disorders such as Reye’s syndrome, acute fatty liver of pregnancy and drug-induced toxicity (Brunt, 2005;Brunt and Tiniakos, 2002). The condition of hepatic steatosis of the liver is generally benign, meaning that it poses no immediate health concerns. However, for reasons not entirely understood, it appears that hepatic steatosis sensitizes to the development of inflammation (Faggioni, et al., 1999). A liver that is characterized by a pathology that is both steatotic and inflamed is designated as having ‘steatohepatitis’ (Contos, et al., 2004).

The term ‘nonalcoholic steatohepatitis’ or NASH was first used in 1980 to describe histologic observations made in obese female patients who denied use of alcohol, yet had liver biopsies consistent with alcoholic hepatitis (Ludwig, et al., 1980). NASH is characterized by macrovesicular steatosis, lobular inflammation, and hepatocellular damage, including Mallory bodies, ballooning degeneration

and bridging fibrosis (Diehl, 2001). If steps are not taken to control the progression of NASH, it can advance to cirrosis and liver failure requiring liver transplant (Reynaert, et al., 2005).

A variety of primary and secondary risk factors for the development of Nonalcoholic Fatty Liver Disease (NAFLD) are presently recognized. Examples of secondary NAFLD include cases associated with various bariatric surgeries, total parenteral nutrition, and long-term use of certain medications, including amiodarone, tamoxifen, and corticosteroids (Falck-Ytter, et al., 2001). Examples of primary NAFLD include cases associated with features of the metabolic syndrome such as atherogenic dyslipidemia, arterial hypertension, hyperglycemia, insulin resistance, a proinflammatory state, and/or a prothrombotic state (Grundy, 2004). Specifically, it has been demonstrated that individuals with the metabolic syndrome are at a 4- to 11-fold increased risk for developing NAFLD (Hamaguchi, et al., 2005). At least 90% of patients with NAFLD have one or more features of the metabolic syndrome, while approximately one-third have the complete syndrome (Marchesini and Marzocchi, 2007;Pagano, et al., 2002). When one or more features of the metabolic syndrome are present at the time of NAFLD onset, regression of the disease also becomes significantly less likely than in their absence (Hamaguchi, et al., 2005).

Obesity itself presents the greatest risk factor for the development of primary NAFLD, as cross-sectional studies have verified that 25% to 93% of

NAFLD patients are obese (Marchesini and Marzocchi, 2007;Ludwig, et al., 1980;Lee, 1989;Powell, et al., 1990;Cortez-Pinto, et al., 1999;Matteoni, et al., 1999;Angulo, et al., 1999;Chitturi, et al., 2002). It is widely accepted that the dramatic increase in the prevalence of NAFLD in industrialized countries is in fact due in large part to the ongoing surge in rates of obesity (Farrell and Larter, 2006). In the year 2004, it was estimated that 22.5% of Americans were clinically obese (body mass index > 30 kg/m^2), a percentage that is twice what it was two decades ago (Angulo, 2007). In the world population, the prevalence of NAFLD lies somewhere between 14% and 24%, and while once understood to occur only obese adults, it is now known to afflict both normal weight persons and children (Browning and Horton, 2004).

Molecular Mechanisms Underlying Pathogenesis of NASH

In this dissertation, contributing to an improved understanding of the physiologic and molecular features that promote NASH is not a primary goal. Instead the major objective will be to determine the effects of experimental NAFLD on the expression and function on drug metabolizing enzymes and transporters. Nonetheless, a brief discussion of underlying factors having a putative role in the pathogenesis of NASH will be presented. Although it continues to be understood in greater detail, it can be appreciated that a number of basic biochemical processes appear to contribute to its induction. These include (1) disruption of pathways of hepatic lipid metabolism; (2) insulin resistance; (3) chronic induction of microsomal lipoxygenases; and (4) oxidative

stress. These four processes constitute the mechanisms principally involved in the “two-hit” hypothesis first proposed nearly 10 years ago (Day and James, 1998).

First, disruption of pathways of hepatic lipid metabolism may include (a) increased hepatic uptake of fatty acids; (b) increased endogenous synthesis of fatty acids; and (c) decreased disposal of fatty acids as a result of impaired mitochondrial β-oxidation, impairment of alternative pathways of fatty acid disposal (peroxisomal β-oxidation, microsomal ω and ω-1 oxidation), or decreased export of fatty acids secreted from the hepatocyte bound to apolipoprotein B as VLDL. Notably, such disruptions of hepatic lipid metabolism “prime” the hepatocyte by allowing for the accumulation of lipids. Thus, hepatic steatosis is commonly referred as the first “hit” in the mechanism of NASH induction. Support for the concept of lipid-induced sensitization of the liver is provided by studies that demonstrate the livers of rodents that naturally develop hepatic steatosis to be extremely sensitive to the hepatotoxicant LPS, resulting in the development of significant steatohepatitis (Yang, et al., 2001;Yang, et al., 1997). Conversely, it is also possible that free fatty acid overload may itself be cytotoxic or injurious to hepatocyte mitochondria, thereby resulting in up-regulation of the aforementioned extramitochondrial pathways of fatty acid oxidation.

A second biochemical process that appears to contribute to the induction of NASH is insulin resistance. Insulin resistance, a central feature of T2D and

also frequently observed during obesity, appears to enhance hepatic steatosis indirectly by favoring peripheral lipolysis and hepatic uptake of fatty acids (Marchesini, et al., 1999). It also appears possible that hypersulinemia can result in suppression of mitochondrial β-oxidation of fatty acids. This is achieved via glycolysis- and citric acid cycle-mediated generation of excess malonyl-CoA. Malonyl-CoA can then act as an allosteric inhibitor of carnitine palmitoyltransferase, which normally transports fatty acids from cytosol into mitochondria (Fong, et al., 2000). It is therefore unsurprising that NAFLD has been referred as the hepatic manifestation of insulin resistance.

A third biochemical process that appears to participate in the development of NASH is the chronic induction of microsomal lipoxygenases and thus the generation of lipid peroxides in the liver. The most pertinent microsomal lipid peroxidases in this case are the CYP2E1 and CYP4A enzymes. A role for CYP2E1 in NASH has been postulated based on the observation that it is induced in human patients with obesity and/or NASH, as well as following studies in rodent dietary models (Fiatarone, et al., 1991;Weltman, et al., 1996;Weltman, et al., 1998;Leclercq, et al., 2000). For example, CYP2E1 has been demonstrated to be the major microsomal catalyst in the 100-fold elevation of hepatic lipid peroxides in methionine choline-deficient (MCD) diet-induced experimental steatohepatitis in mice (Leclercq, et al., 2000). CYP4A10 and CYP4A14 may also play a role, as their expression and activity are greatly increased in wild type as well as in CYP2E1-deficient mice also during the MCD

model (Leclercq, et al., 2000). Intrahepatic elevation of lipid peroxide levels is a reliable indicator that the cellular redox balance is being disrupted, as will be further explained in the paragraph to follow. Notably, it is unlikely that CYP2E1 induction is the sole contributing factor in the development of NASH in humans, as CYP2E1 induction is a common phenomenon during treatment with certain drugs, e.g. isoniazid, that do not cause NASH.

As alluded by mention of lipid peroxide levels and cellular redox balance in the preceding paragraph, a fourth biochemical factor that may contribute to the development of NASH is oxidative stress. In spite of being continuously exposed to reactive oxygen species, hepatocytes and other aerobic cells are normally protected from oxidative injury by antioxidant pathways and endogenous antioxidants, e.g. GSH. Example pro-oxidants were nominally implied in the previous three paragraphs. Specifically, CYP2E1 or CYP4A can be a source of lipid peroxides, and induction of mitochondrial or peroxisomal activity can promote excess hydrogen peroxide formation (Leclercq, et al., 2000). During obesity, oxidative stress appears to result in compensatory up-regulation of extramitochondrial pathways of fatty acid oxidation (Yang, et al., 2000). It appears that production of oxidative stress can stimulate recruitment of inflammatory mediators by inducing expression of chemokines and cytokines, e.g. TNF-α and cellular adhesion molecules (Hotamisligil and Spiegelman, 1994). Thus, oxidative stress would seem capable of binary roles in the etiopathogenesis of NASH: (1) directly, by participating in liver injury and (2)

indirectly, by providing the stimulus for an inflammatory response that exacerbates injury and mediates fibrosis.

It is now clear that the pathogenesis of NASH is generally the result of a combination of these four factors that constitute the widely accepted “two-hit” hypothesis. Specifically, hepatic steatosis (enhanced by insulin resistance) constitutes hit number one and produces suppression and up-regulatiton of mitochondrial and extramitochondrial pathways of fatty acid oxidation, respectively. Hit number two involves the generation of oxidative stress via chronic induction of microsomal lipid peroxidation (CYP2E1 and/or CYP4A) and generation of ROS by extramitochondrial fatty acid oxidation. While ROS can directly actuate damage, they can also activate the inflammatory response, which exacerbates injury and mediates fibrosis (Day, 2002).

Animal Models of NAFLD

It is essential to keep in mind that, when studies are designed to investigate mechanisms of disease pathogenesis and function during the disease state, their findings are typically only of value when the animal model being applied is one that accurately reflects what is observed clinically in humans. With this being stated, animal models of NAFLD can be divided into two broad categories: those caused by genetic mutation and those in which the NAFLD phenotype is induced by treatment with a modifed diet. While each generally continues to be useful in terms of providing insight into the pathogenesis of steatosis and steatohepatitis, none provide the full NAFLD phenotype that is

observed in the clinical setting. Thus the maxim that “All models are wrong. Some are useful,” applies. Rather than attempt to provide an all-inclusive list of animal models of NAFLD, this section will focus on the handful of models on which the bulk of published NAFLD research has been based. These include the leptin-deficient (ob/ob) mouse, the leptin-resistant (fa/fa) Zucker rat, and the dietary methionine-, choline-deficient (MCD) model.

The ob/ob mouse strain carries a mutation on chromosome 6 in the ‘ob’ gene that prevents synthesis of leptin (Friedman, et al., 1991;Zhang, et al., 1994;Campfield, et al., 1996). This 16-kDa adipokine, produced predominantly by visceral adipocytes of white adipose tissue (WAT), stimulates satiety by acting on neurons within the ventral median nucleus of the hypothalamus (Ahima and Flier, 2000). When levels of leptin are low or entirely absent (as in ob/ob mice), the sensation of extreme hunger for food is stimulated (Pelleymounter, et al., 1995;Ahima and Flier, 2000;Fantuzzi and Faggioni, 2000). Accordingly, the ob/ob mouse phenotype exhibits hyperphagia, hyperglycemia, obesity, lethargy, and severe diabetes. When allowed standard chow ad libitum, young ob/ob mice overeat, become obese with expanded WAT stores, display increased synthesis and storage of lipids in the liver and therefore develop hepatic steatosis (Pelleymounter, et al., 1995;Hotamisligil, et al., 1996). Increased WAT mass in ob/ob mice lends to increased adipose tissue lipolysis and consequent release of long-chain fatty acids that are subsequently take up by the liver. The increased synthesis and storage of lipids in the liver is likely due in part to increased levels

of Sterol Regulatory Element Binding Protein-1c (SREBP-1c) in ob/ob hepatocyte nuclei, resulting in up-regulation of fatty acid synthase activity and de novo synthesis of fatty acids (Shimomura, et al., 1999).

Also for the study of NAFLD, the fa/fa (often referred “fatty”) Zucker rat (ZR) has proven useful. Whereas the ob/ob mouse is leptin-deficient, the fa/fa ZR has a defective leptin receptor. Specifically, the fa/fa ZR strain carries a spontaneous point mutation in a codon near the region of the leptin receptor gene that codes for the ligand-binding domain (Phillips, et al., 1996). This results in dysfunctional intracellular trafficking and/or signal transduction via the leptin receptor. Heterozygosity of the fa gene (fa/+) does not affect the ZR’s normally lean phenotype, but homozygosity (fa/fa) results in minimal responsiveness to exogenous leptin and a phenotype similar to that of the ob/ob mouse, including hepatic steatosis, hyperlipidemia, hyperphagia, obesity, and insulin-resistant diabetes. The mechanism of hepatic steatosis may be due in part to insulin-induced SREBP-1c expression and lipogenesis in hepatocytes. Consequently, as in ob/ob mice, mRNA levels of SREBP-1c, various lipogenic genes, and hepatic triglyceride content are significantly increased in the fa/fa ZR liver (Kakuma, et al., 2000).

With regard to application of the ob/ob and fa/fa models for the study of the various stages of human NAFLD, each possess inherent characteristics that make them useful in certain ways. For example, the two models display insulin-resistant diabetes and hyperglycemia, both of which are often present with

human hepatic steatosis (Enriquez, et al., 1999;Koteish and Diehl, 2001;Youssef and McCullough, 2002). The fact that simple hepatic steatosis is a naturally occurring pathology in each model facilitates the study and comparison of each to the human disease (Koteish and Diehl, 2001). Notably, ob/ob mice and fa/fa ZRs fail to spontaneously progress from hepatic steatosis to steatohepatitis. This feature has been demonstrated to render each model manipulable for investigating the mechanisms underlying the transition from steatosis to steatohepatitis (Anstee and Goldin, 2006). Specifically, the livers of these rodents have been observed to be extremely sensitive to the hepatotoxicant LPS and consequently develop significant steatohepatitis (Yang, et al., 1997;Yang, et al., 2001;Yang, et al., 1997;Yang, et al., 2000).

There are a number of shortcomings in the ob/ob and fa/fa models. The first problem is that a relationship between the animal model mutations and leptin gene polymorphisms in the population of obese persons at various stages of NAFLD has not been demonstrated (Poordad, 2004). Secondly, studies fail to demonstrate the negative relationship between leptin levels and NAFLD prevalence that these rodent models suggest. Whereas ob/ob mice are leptin-deficient, serum leptin levels in biopsy-proven NASH patients have been reported to be no different than or even elevated versus control patients (Thomas, et al., 2000;Silha, et al., 2003;Chalasani, et al., 2003;Liangpunsakul and Chalasani, 2004). Thirdly, it is known that the physiology of these rodent models is rather skewed in a number of ways, including marked suppression of thyroid, growth

hormone, adrenal, and reproductive endocrine axes (Ahima and Flier, 2000). These are not typical of human NAFLD or NASH (Poordad, 2004). A fourth limitation stems from the fact that leptin deficiency greatly limits hepatic fibrogenesis. This is because TNF-α release and expression of TGF-β-dependent genes, e.g. procollagen type 1, require leptin activity (Honda, et al., 2002;Leclercq, et al., 2002;Leclercq, et al., 2003;Saxena, et al., 2002). Coincidentally, the major profibrogenic cytokine in wound healing responses of the liver happens to be TGF-β (Bissell, et al., 2001;Nakatsukasa, et al., 1990;George, et al., 1999;George, et al., 2000). This would render both the ob/ob and fa/fa models unsuccessful in naturally developing the fibrotic phenotype appropriate for a model of NASH.

The MCD dietary model is another well-characterized model for the study of NAFLD. It has largely been investigated as a means to understand the mechanisms of the injury that occur at the steatohepatitis stage of the NAFLD spectrum. Obviously, the biochemical basis of the pathology that manifests in this model is fundamentally derived from deficiencies of the essential nutrient choline and essential amino acid methionine. Choline is critical for cell membrane integrity, transmembrane signaling, phosphatidylcholine synthesis, neurotransmission, and methyl metabolism. Methionine is a major source of dietary sulfur and an essential amino acid.

The pathways of choline and methionine metabolism are closely tied, as it appears that a pivotal role of these two biologic compounds is to serve as methyl

donors (Newberne and Rogers, 1986). Whereas methionine contains one methyl group, choline contains three and is a synthetic precursor to methionine. Specifically, mitochondrial choline dehydrogenase converts choline to betaine-aldehyde, which then undergoes oxidation in mitochondria or cytoplasm to form betaine. Then, using betaine as a methyl source, betaine:homocysteine methyltransferase catalyzes methylation of homocysteine to form methioinine. Methionine adenosyltransferase then converts methionine (direct dietary methionine or choline-derived methionine) to S-adenosylmethionine (SAM). SAM serves as a critical cofactor for many enzymatic methylation reactions, including DNA methylation, creatine biosynthesis, and phosphatidylcholine biosynthesis (Ridgway and Vance, 1988).

Under normal dietary conditions, triacylglycerol produced by the liver is exported from the organ in the form of very low-density lipoprotein (VLDL). Choline is also an essential precuror for the synthesis of phosphatidylcholine, a required component of the VLDL particle (Yao and Vance, 1988; Yao and Vance, 1989). However, during choline deficiency, the incapacity of hepatocytes to synthesize phosphatidylcholine molecules allows for intracellular accumulation of triglycerides as their synthesis continues. Methionine can serve as a methyl donor-substitute in place of choline but only until phosphatidylethanolamine-N-methyl transferase activity undergoes feedback repression by synthetic precursors of methionine formation (Hoffman, et al., 1981;Ridgway and Vance, 1988;Ridgway, et al., 1989).

In humans, mice, and rats (within hours to days), mere choline deficiency results in liver dysfunction likely owing to massive accumulation of triacylglycerol within hepatocytes (Blusztajn and Wurtman, 1983;Lombardi, 1971;Lombardi, et al., 1968;Yao and Vance, 1988;Yao and Vance, 1989;Zeisel, et al., 1991;Da Costa, et al., 1993). Inflammation, a pathologic hallmark of steatohepatitis in humans is, however, minimal and fibrosis highly variable following choline deficiency (Murray, et al., 1986). Having stated this, compounding the choline deficiency with methionine deficiency results in hepatic steatosis, inflammation and progressive fibrosis (Murray, et al., 1986;Weltman, et al., 1996). The precise molecular mechanisms that underlie the phenomena of the MCD dietary model have remained the focus of much intensive research.

Now that the disordered liver biochemistry following acute deficiency of methionine and choline has been described, the chronology and putative mechanisms of pathologic damage that occur during chronic feeding of the MCD diet to rats will be elaborated. As the two previous paragraphs explained, triacylglycerol accumulation within rat hepatocytes occurs within hours to days of methionine, choline-deficiency. The hepatic steatosis is itself histologically observable in MCD diet-fed rats after 2 weeks (George, et al., 2003;Starkel, et al., 2003). Lipid peroxidation (LPO), as measured by thiobarbituric acid-reactive substances (TBARS), are also observed to be significantly elevated (16-fold) in whole rat liver following week 2 of the treatment, which happens to be three weeks prior to any indication of hepatic fibrosis in this model (Starkel, et al.,

2003;George, et al., 2003). On that note, an indicator of stellate cell activiation [α-smooth muscle actin (α-SMA) immunostaining] in rat liver sections and mRNA expression of collagen α_1 (1) and TGFβ in whole rat liver are not changed after 2 weeks of MCD diet treatment. The aforementioned increase in hepatic LPO is accompanied by a 51% decrease in GSH levels (Starkel, et al., 2003). Cyp2E1 and Cyp4A are not likely to be contributors to the LPO at this stage, as their protein and activity levels are significantly decreased (Starkel, et al., 2003). Plasma ALT levels, an indicator of hepatocyte necrosis, are not altered at this stage either (George, et al., 2003).

By week 5, macrovesicular steatosis most strongly affects zones 2 and 3 with relative “sparing” of zone 1. The pattern of lipid accumulation at this juncture is accompanied by Kupffer cell and neutrophil infiltration and hepatocyte necrosis throughout the liver lobule (Weltman, et al., 1996;George, et al., 2003;Kirsch, et al., 2003). It is not surprising, then, that plasma ALT levels are significantly elevated (3-fold) (George, et al., 2003). The roughly 20-fold elevation in whole liver TBARS is accompanied by a 33% reduction in hepatic GSH content. At this stage, whole liver collagen α_1 (1) and TGFβ1 mRNA expression are significantly elevated, indicating initiation of hepatic fibrosis. By week 6, protein and activity of levels of Cyp2E1 and Cyp4A are decreased even further than at week 2 (Starkel, et al., 2003).

Following week 8 of MCD diet treatment, the Nathan Cherrington research group at the University of Arizona, assisted with the expertise of a veterinary

rodent pathologist, has successfully applied a histologic scoring method developed by the National Institutes of Health-sponsored NASH Clinical Research Network (Kleiner, et al., 2005). Results of the analysis, which include parameters of steatosis, lobular inflammation, and ballooning degeneration, are indicative of a pathology that corresponds with early stage NASH in human patients (Lickteig, et al., 2007). At this stage, plasma ALP and ALT levels are also significantly increased (Fisher et al., manuscript in preparation). Importantly, the data described in Appendices A, C, and D of this dissertation is derived from tissues harvested from rats following 8 weeks of MCD diet treatment.

By week 12, the pathologic damage has advanced to become most conspicuous in zone 3, with a 'chicken wire' pattern of fibrotic threads surrounding lipid-laden hepatocytes. This is accompanied by bridging fibrosis from portal vein-to-portal vein and from central vein-to-portal vein. As one might anticipate, hepatic fibrosis as indicated by stellate cell activation (α-SMA immunostaining), which is not distinct from control rat liver at weeks 2 and 5, is increased >20 fold compared to controls. Additional indicators of fibrosis, including mRNA levels of Tissue Inhibitor of Metalloproteinases (TIMP)-1 and TIMP-2 and collagen α_1 (1) become significantly increased in whole rat liver by week 12. There is also a significant increase in the previously mentioned indicator of initiation of hepatic fibrosis, TGFβ1 mRNA levels. However, TGFβ1 mRNA expression is not increased in stellate cells, Kupffer cells or endothelial cells but only in hepatocytes. Levels of TBARS also remain elevated at 12 weeks

(~40-fold) in whole rat liver, with hepatocytes being the only cell population in which TBARS are significantly increased. In spite of this apparent elevation of oxidative stress, GSH levels are only 20% lower than the control group, compared to 51% and 33% reductions at 2 and 5 weeks, respectively. Elevated serum ALT levels indicate that hepatocellular death continues at this stage (Kleiner, et al., 2005;George, et al., 2003;Starkel, et al., 2003).

Taking a step back from all of these time course data, it can be deduced that the pathologic damage that occurs in the liver during methionine and choline deficiency, including fibrosis and steatosis, begins in zone 3 (Weltman, et al., 1996). Additionally, the temporal sequence of hepatocyte steatosis and lipid peroxidation, followed by hepatocyte TGFβ1 induction and fibrosis is consistent with the proposal that lipid peroxidation may be mechanistically involved in the development of fibrosis in this model. Increased α-SMA immunostaining indicates that stellate cell activation occurs during the time course of the MCD diet treatment and promotes a 'fibrosing steatohepatitis.' The overall histologic picture, including hepatocellular injury, inflammatory recruitment and pericellular zone 3 hepatic fibrosis mimics that seen in human fibrotic disorders associated with hepatic lipid accumulation (George, et al., 2003;Starkel, et al., 2003).

Shortcomings of the MCD model of NASH have been identified. The first is with respect to body weight. Rats fed the MCD diet for 8 weeks weigh about 40% less than their control counterparts and appear cachexic. Second, hepatic steatosis is vastly acinar (zone 3) rather than periportal (zone 1) as in human

NASH (Koteish and Diehl, 2001). Third, whereas hypertriglyceridemia is a common feature in NASH patients, MCD diet-fed rats display significantly decreased serum triglyceride levels compared to control rats (Weltman, et al., 1996;Leclercq, et al., 2000). Fourth, the elevation in rat serum ALT levels is also greatly exaggerated beyond that observed in human patients with NASH (Rinella and Green, 2004). Fifth, the diabetes, insulin resistance and/or hyperglycemia observed in humans do not occur in rats in the MCD model (Rinella and Green, 2004). In fact, unpublished observations from the Nathan Cherrington lab reveal that rats fed the MCD diet are hypoglycemic after 8 weeks.

Strengths of the MCD model have also been uncovered over the years of study. Firstly, the sequence of events in the MCD model is analogous to that which occurs in NASH: hepatic steatosis, inflammatory recruitment, and hepatocyte injury precede activation of stellate cells and fibrosis by several weeks (George, et al., 2003). Secondly, the MCD model is associated with pericellular hepatic fibrosis around lipid-laden hepatocytes, which is found in all causes of steatohepatitis in humans. Thirdly, the MCD model also underscores the relevance of oxidative stress in the progression from hepatic steatosis to steatohepatitis, as oxidative damage is also prominent in the liver of humans with NASH (Clark, et al., 2002;Angulo, 2002;Reid, 2001;Tilg and Diehl, 2000). Fourth, 8 weeks' treatment of rats with the MCD diet results in a pathology that corresponds to early stage NASH in human patients (Lickteig, et al., 2007).

Drug Metabolism during Inflammation

Inflammation is a common factor in a number of disease states and is demonstrated as responsible for variability in drug metabolism (Forrest, et al., 1977;Farrell, et al., 1979;Farrell, et al., 1978;Narang, et al., 1981;Narang, et al., 1982;Narang, et al., 1985;Figg, et al., 1995;Westphal and Brogard, 1997;Kubitz, et al., 1999;Nadai, et al., 2001;Congiu, et al., 2002). LPS administration to rats, a basic model of septic inflammation, results in marked suppression of mRNA and protein levels of CYP1A2, 2B, 2E, 2C11, 2D1, and 3A2 (Renton and Nicholson, 2000). This loss of expression is presumed to be a result of increased levels of proinflammatory cytokines (e.g. TNF-α and IL-1β). Specifically, administration of TNF-α to rats results in transcriptional suppression of CYP2C11 and CYP3A2 mRNA levels (Nadin, et al., 1995).

Due to its stark increase over the past two decades, increasingly greater emphasis continues to be placed on the study of nonalcoholic fatty liver disease (NAFLD). Hepatocellular inflammation and increased levels of plasma and liver TNF-α levels mark the later stages of this disease. Elevated levels of the proinflammatory cytokines TNF-α and IL-6 have been detected in the plasma of patients with biopsy-proven NASH but not in patients with simple hepatic steatosis (Kugelmas, et al., 2003;Bahcecioglu, et al., 2005;Abiru, et al., 2006). Due to inherent inflammation associated with this disease, there is concern that drug disposition and metabolism may also be altered in patients suffering from NAFLD.

Weltmann et al. were the first to describe changes in drug metabolizing enzymes when they measured CYP2E1 and CYP3A levels in liver biopsy sections from human NASH patients. Immunohistochemistry demonstrated CYP2E1 expression to be increased in a pattern that closely followed the distribution of steatosis, often extending beyond zone 3 into zones 2 and 1. Conversely, expression of CYP3A was decreased but not quantified in comparision to control (Weltman, et al., 1998). Chalasani et al. (2003) subsequently extended the characterization of CYP2E1 in NASH when they demonstrated enhanced activity in vivo by measuring oral clearance of chlorzoxazone (Chalasani, et al., 2003).

Statement of the Problem: Clinical Significance of Liver Inflammation

Correct dosing in pharmacotherapeutics is based on the idea that too much of a drug will cause toxicity, while too little will result in failure to elicit the desired response. A major factor in the ability of a patient to handle any dose of a drug is the capacity to metabolize and eliminate that drug from the body. For the vast majority of drugs, the liver plays a key role in determining the rate at which drugs are eliminated. Several processes are required for efficient hepatic elimination. First, drugs must be taken up across the cell membrane into hepatocytes by uptake transporters. Once inside the hepatocyte, Phase I and II biotransformation enzymes act to metabolize and conjugate the drug to a more water-soluble compound, the distribution of which is more easily controlled.

These water-soluble metabolites are then transported out of the hepatocyte by additional drug transporters either into bile for elimination, or back into the blood.

More than 2 million severe adverse drug reactions occur in the US each year and often result from interindividual variation in the ability to metabolize and eliminate drugs. This number does not include medical errors, but rather circumstances where an individual is unable to handle the standard dose of the correctly prescribed drug. Although genetics plays an important role, the greatest source of variation comes from other environmental factors such as disease states. Nonalcoholic fatty liver disease (NAFLD) is a chronic condition that comprises a spectrum of histopathologies that range from simple steatosis to the more severe steatohepatitis. The U.S. Centers for Disease Control and Prevention estimates that ~60 million Americans have some form of NAFLD. While most cases of NAFLD are reversible or remain benign, 1 out of every 10 will progress to an inflammatory, fibrosing condition known as nonalcoholic steatohepatitis (NASH). Specifically, it is estimated that 6 million Americans currently suffer from NASH, which is known to be capable of progressing to cirrhosis, eventual liver failure and death. It has become one of the leading causes for liver transplantation in the United States, and thus clearly become a considerable burden to the U.S. healthcare system.

There is substantial evidence to suggest that the capacity of the liver to metabolize and excrete many drugs is greatly diminished in patients with NASH. Because the liver plays such a critical role in drug metabolism and disposition,

any disease state that disrupts or modifies these functions will alter the fate of a given drug within the body. It is therefore very likely that the ability of the liver to metabolize and excrete clinically relevant drugs is compromised in NASH patients.

PRESENT STUDY

The objective of this section is to provide a summary of Appendices A through D. The core topics to be addressed include, in APPENDIX A, efflux transporter-mediated drug disposition in models of NAFLD; in B, transcriptional regulation of liver transporters in models of inflammatory and non-inflammatory cholestasis; in C, the mRNA expression of genes of the antioxidant response during experimental NAFLD; and in D, the mRNA and protein expression of drug metabolizing enzymes during experimental NAFLD. By the end of this section, it will be clear to the reader that experimental inflammatory liver disease has a variety of consequences to the expression of drug metabolizing enzymes, drug transporters, and drug disposition.

The liver is the major organ of drug metabolism and elimination, which is encompassed by three steps of drug management. The first step is uptake transport, as performed by uptake transporters, such as the OATPs. The second step is drug metabolism, which is performed by phase I and phase II enzymes, including members of the Cytochrome P450 (CYP) family and the UGT family, respectively. The third step is efflux transport. This function is performed by MRPs and other efflux transporters that excrete their substrates into blood or bile for elimination in urine or feces. In this dissertation the processes of drug metabolism and efflux transport have been described in terms of their regulation during inflammatory. The inflammatory states specifically studied include

experimental intrahepatic cholestasis and experimental nonalcoholic steatohepatitis (NASH).

It has been demonstrated that inflammation can alter drug metabolism in both humans and in experimental models in rodents. Because NASH is also an inflammatory disease of the liver, the hypothesis that experimental NAFLD alters expression of drug metabolizing enzymes was tested. Notably, induction of the NASH model, which is characterized by inflammation and fibrosis, did not exert a global effect on drug metabolizing enzymes. Rather, a selective and dramatic suppression of Cyp2c11 mRNA and protein was observed. It is possible that additional studies would demonstrate that this suppression of Cyp2c11 expression is reflective of decreased functional capacity of the liver during experimental NASH. This is an important phenomenon to consider in this disease state because altered metabolism could render a greater potential for drug toxicity and/or adverse drug reactions.

Inflammatory liver diseases are also known to be capable of altering drug pharmacokinetics. This led to testing the hypothesis that experimental NASH, as induced by the MCD diet, can alter the pharmacokinetics of the drug acetaminophen (APAP). The parent drug acetaminophen is metabolized in the liver to three different metabolic products. Roughly half of a normal dose of APAP undergoes sulfonation by sulfotransferase enzymes to form APAP-sulfate (APAP-SULF). The second most abundant metabolite following a normal dose is APAP-glucuronide (APAP-GLUC), which is formed following glucuronidation by

UDP-glucuronosyltransferases. A much smaller portion of a dose of acetaminophen undergoes conjugation with glutathione (GSH) to form APAP-GSH. Each of these metabolites are substrates for efflux transporters, making acetaminophen ideal for the study of efflux transporter-mediated drug disposition. While the majority of a normal dose of acetaminophen is excreted into bile for elimination in the feces, it is also possible for metabolites to undergo sinusoidal efflux from the liver back into the blood for elimination in the urine.

In these studies, the APAP-GLUC metabolite was excreted into the bile at a significantly lower concentration in rats with experimental NASH than in controls. However, the concentration of APAP-GLUC in the blood and urine underwent a proportionate increase in the MCD rats. These data support the hypothesis that the experimental disease state of NASH can indeed alter the pharmacokinetics of drugs, e.g. acetaminophen. Because APAP-GLUC is known to be a substrate of the canalicular efflux transporter Mrp2 and the sinusoidal efflux transporter Mrp3, protein expression of these two transporters was also examined. It was subsequently discovered that protein expression levels of Mrp3 were dramatically increased, correlating strongly with the increased plasma levels of APAP-GLUC. Although protein expression of Mrp2 was likewise increased, it is likely that Mrp3's higher affinity for the APAP-GLUC metabolite resulted in its efflux back into the blood. While this shift in the route of elimination of APAP does not alter the therapeutic efficacy or safety of acetaminophen, there are some clinical drugs for which the glucuronide conjugate is the active

metabolite. Specifically, more recent studies also indicate that the therapeutic efficacy of drugs is likewise integrally linked to the function of drug transporters, which can distribute pharmacologically active concentrations of the drug to its target site, e.g., ezetimibe glucuronide and morphine 6-glucuronide. Therefore, future studies should place greater emphasis on determining the role of drug transporters in the pharmacokinetics and pharmacodynamics of drug therapy administered during NAFLD.

While focused was initially placed on the changes in expression that occur during experimental inflammatory liver disease, potential mechanisms of regulation were not examined. Importantly, proinflammatory cytokines have been implicated by previous studies as being involved in transporter regulation when the liver is inflamed. Specifically, in clinical cases of NASH in humans as well as in models of experimental NASH, induction of TNF-α and IL-6 has been detected. This pattern of induction of proinflammatory cytokines is not limited to NASH as it is likewise observed in clinical cases of cholestatic liver disease and experimental cholestasis in rodents.

The bile duct ligation and LPS models were utilized to model the two general clinical forms of cholestasis, i.e. extrahepatic and intrahepatic cholestasis. Extrahepatic cholestasis occurs as a result of physical obstruction of bile flow, leading to an accumulation of biliary constituents and altered regulation of transporters. In contrast, intrahepatic cholestasis results from direct dysregulation of liver transporters, leading to impairment of bile flow. The

proinflammatory cytokines TNF-α, IL-1β, IL-6 and the transcription factor NF-κB have each been implicated in the transcriptional regulation of drug transporters in various models of cholestasis. Therefore, mice deficient in the activity of TNF-α, IL-1β, IL-6 or NF-κB were utilized to test the hypothesis that transcriptional regulation of liver transporters during cholestasis is dependent on cytokines.

mRNA expression analysis revealed multiple alterations in transcriptional regulation among the 21 liver transporters examined. First of all, the regulatory response following bile duct ligation was not as dramatic as that observed following LPS treatment. This is likely the consequence of essential differences between the initiating events that lead to cholestasis in these models, rather than individual activity of TNF-α, IL-1β, IL-6 or NF-κB. This also reflects the possibility that activity by these proinflammatory factors is more essential in the LPS model, as it is known that bile duct ligation does not produce the same magnitude of inflammation as LPS treatment. Notably, there were cases in which the mRNA expression of some transporters was not entirely dependent on the signaling activity of TNF-α, IL-1β or IL-6, e.g. Mrp2 following bile duct ligation and Ntcp, Bsep and Oatp1 following LPS treatment. These findings reflect the likelihood that the regulatory response to bile acids is also distinct between these two models.

To summarize the novel findings of the current dissertation, it has been discovered that experimental NASH results in selective suppression of the drug metabolizing enzyme Cyp2c11. Experimental NASH, including up-regulation of the sinusoidal efflux transporter Mrp3, likewise selectively induces efflux

transporters. Up-regulation of Mrp3 also correlated with the increase in plasma levels of APAP-GLUC, indicating the NASH disease state may result in alterations in therapeutic efficacy and safety of drugs. Finally, based on the studies performed in the LPS model of cholestasis, the proinflammatory cytokines TNF-α, IL-1β, and IL-6 released during inflammation are likely responsible for the altered regulation of drug transporters that was observed in each of these studies. However, it is unlikely that transporter regulation is entirely dependent on any individual cytokine.

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APPENDIX A: EFFLUX TRANSPORTER EXPRESSION AND ACETAMINOPHEN METABOLITE EXCRETION ARE ALTERED IN RODENT MODELS OF NONALCOHOLIC FATTY LIVER DISEASE

Abstract

Efflux transporters are responsible for the excretion of numerous xenobiotics and endobiotics and thus play an essential role in proper liver and kidney function. Nonalcoholic fatty liver diseases (NAFLDs) comprise a spectrum of disorders that range from simple fatty liver (SFL) to nonalcoholic steatohepatitis (NASH). Although the precise events leading to NAFLD are unclear, even less is known about the effects on efflux transporter expression and drug disposition. The purpose of this study was to determine the effect of NAFLD on efflux transporter expression in rat liver as well as on acetaminophen (APAP) metabolite excretion. To simulate SFL and NASH, rats were fed either a high-fat (HF) or a methionine- and choline-deficient (MCD) diet for 8 weeks. In the livers of MCD rats, there were striking increases in both mRNA and protein levels of multidrug resistance-associated protein (Mrp) 3, Mrp4, and breast cancer resistance protein, as well as increased Mrp2 protein. After administration of a nontoxic dose of APAP, biliary concentrations of APAP-sulfate, APAP-glucuronide (APAP-GLUC), and APAP-glutathione were reduced in MCD rats. The effects of the HF diet on both transporter expression and APAP disposition were by comparison far less dramatic than the MCD diet-induced alterations. Whereas APAP-sulfate levels were also decreased in MCD rat plasma, the levels

of the Mrp3 substrate APAP-GLUC were elevated. Urinary elimination of APAP metabolites was identical between groups, except for APAP-GLUC, the concentration of which was 80% higher in MCD rats. These studies correlate increased hepatic Mrp3 protein in the MCD model of NASH with increased urinary elimination of APAP-GLUC. Furthermore, the proportional shift in elimination of APAP metabolites from bile to urine indicates that MCD-induced alterations in efflux transporter expression can affect the route of drug elimination.

Abbreviations

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SFL, simple fatty liver; HF, high-fat; MCD, methionine- and choline-deficient; Mrp, multidrug resistance-associated protein; Bsep, bile salt excretory protein; Bcrp, breast cancer resistance protein; APAP, acetaminophen; APAP-SULF acetaminophen-sulfate; APAP-GLUC, acetaminophen-glucuronide; GSH, glutathione; APAP-GSH, acetaminophen-glutathione; HPLC, high-performance liquid chromatography; H&E, hematoxylin and eosin; APAP-CG/CYS, acetaminophen-cysteinylglycine/cysteine; APAP-NAC, acetaminophen-mercapturate (N-acetyl-L-cysteine); TNF-α, tumor necrosis factor-α; IL, interleukin.

Introduction

Nonalcoholic fatty liver disease (NAFLD) comprises a spectrum of pathologic lesions that ranges from hepatic steatosis to a form of fatty liver

hepatitis known as nonalcoholic steatohepatitis (NASH). Histologic features independently associated with the diagnosis of NASH in human biopsies include hepatic steatosis, hepatocyte ballooning, lobular inflammation, Mallory's hyaline, and perisinusoidal fibrosis. Moreover, NASH has the potential to advance to cirrhosis requiring liver transplant (Powell et al., 1990; Abdelmalek et al., 1995; Charlton et al., 2001; Caldwell and Hespenheide, 2002). A recent overview of clinical data estimated that NAFLD and NASH affect 17 to 33% and 5.7 to 17% of American adults, respectively. Although the etiology of NASH is not completely understood, most investigators agree that a critical baseline of steatosis requires a second "hit" of oxidative stress that promotes the subsequent inflammation and fibrosis of NASH. NAFLD is primarily associated with features of the metabolic syndrome, including obesity, diabetes, dyslipidemia, and insulin resistance (McCullough, 2006). Importantly, these NAFLD-associated features invariably require pharmacologic intervention with one or more therapeutic agents. It therefore follows that a significant portion of the NAFLD demographic group is already using prescription medication.

In the present study, NAFLD was recapitulated in two of its prominent forms, simple fatty liver (SFL) and NASH, respectively. A high-fat diet was used for the SFL model, while a methionine- and choline-deficient (MCD) diet was used for the NASH model. The histologic changes that occur in the MCD model have been thoroughly characterized and determined to be remarkably similar to those seen in human NASH (George et al., 2003). Histologic features of NASH

necessarily include hepatic steatosis, mild, lobular inflammatory infiltration, and hepatocellular ballooning degeneration (Brunt and Tiniakos, 2005).

Because the vast majority of drug metabolism occurs in the liver, it is particularly important to understand the transport processes involved in the elimination of drug metabolites formed in this organ, especially a diseased one. In the liver, there are two basic types of transporters. These include the uptake transporters, e.g., organic anion-transporting polypeptides and organic cation transporters, which facilitate the extraction of drugs from portal blood into hepatocytes, and the efflux transporters, e.g., multidrug resistance-associated proteins (Mrps), bile salt excretory protein (Bsep), and breast cancer resistance protein (Bcrp), which extrude their substrates out of hepatocytes into bile or blood. Although not all drugs require uptake transport to cross the sinusoidal membrane into hepatocytes, virtually all of their metabolites require efflux transporters to be excreted into bile and blood for biliary and urinary elimination, respectively.

Acetaminophen (APAP) provides a fine example of the integral role played by efflux transporters in drug metabolite excretion from liver. After passive diffusion into hepatocytes, APAP is mainly conjugated with sulfate (SULF) or glucuronic acid (GLUC) to form APAP-SULF and APAP-GLUC, respectively (Moldéus, 1978; Grafström et al., 1979). A small portion of a dose of APAP will undergo cytochrome P450 metabolism to the reactive metabolite N-acetyl-p-benzoquinoneimine, which is subsequently conjugated with glutathione (GSH) to

form APAP-GSH. In rats, excretion of APAP-GLUC and APAP-GSH metabolites from the liver occurs predominantly via the biliary route, whereas the APAP-SULF metabolite predominantly undergoes sinusoidal efflux and subsequent elimination in the urine (Gregus et al., 1988). Importantly, all APAP metabolites require efflux transport to be excreted from the liver, and each can be detected in both bile and urine (Gregus et al., 1988; Xiong et al., 2002; Chen et al., 2003; Manautou et al., 2005; Zamek-Gliszczynski et al., 2006a).

In vivo disposition studies and in vitro functional transport experiments indicate that Mrp2, Mrp3, Mrp4, and Bcrp each have the ability to transport a variety of unconjugated and conjugated drugs, including APAP metabolites (Büchler et al., 1996; Keppler et al., 1997; Xiong et al., 2000; Nakanishi et al., 2003; Zamek-Gliszczynski et al., 2005, 2006b). It is important to emphasize the distinctive location of these four transporters in hepatocytes, as well as their respective APAP metabolite substrates. Mrp2 and Bcrp are localized to the canalicular (apical) membrane of hepatocytes from which they excrete their substrates into the bile canaliculi. Accordingly, in a healthy liver, biliary excretion of the SULF, GLUC, and GSH conjugates of APAP is predominantly mediated by Mrp2, whereas Bcrp appears also to contribute to excretion of APAP-SULF conjugates (Büchler et al., 1996; Keppler and König, 1997, 2000; Borst et al., 2000; Keppler et al., 2000; Chen et al., 2003; Zamek-Gliszczynski et al., 2005). Mrp3 and Mrp4 are expressed at the sinusoidal (basolateral) membrane of hepatocytes and cholangiocytes from which they expel their substrates into the

blood (König et al., 1999; Donner and Keppler, 2001; Soroka et al., 2001). Sinusoidal excretion of the APAP-GLUC metabolite from hepatocytes is predominantly mediated by Mrp3, whereas Mrp4 appears to mediate excretion of APAP-SULF metabolites (Chen et al., 2001; Manautou et al., 2005; Zamek-Gliszczynski et al., 2006a). Recent studies indicate that Mrp3 and Mrp4 have an equal role in the efflux of APAP-SULF (Zamek-Gliszczynski et al., 2006b).

During other liver disease states, such as septic cholestasis, obstructive cholestasis, and alcoholic and viral hepatitis, human and rodent studies have demonstrated alterations in cytochrome P450-mediated drug metabolism, transport, and pharmacokinetics (Forrest et al., 1977; Farrell et al., 1978, 1979; Narang et al., 1981, 1982, 1985; Figg et al., 1995; Westphal and Brogard, 1997; Kubitz et al., 1999; Nadai et al., 2001; Congiu et al., 2002). However, there is little information on the effect of NAFLD on these same parameters. Therefore, the purpose of the present study was to determine the effect of NAFLD on efflux transporter expression in liver and disposition of APAP metabolites.

Materials and methods

Materials. APAP and urethane were purchased from Sigma-Aldrich (St. Louis, MO). The analytical column and guard cartridges for high performance liquid chromatography (HPLC) analysis were purchased from Bodman (Aston, PA). HPLC-grade water, HPLC-grade methanol, Tris base, sodium dodecyl sulfate, sodium chloride, and sucrose were purchased from Fisher Scientific (Pittsburgh, PA), and all other chemicals used were of reagent grade or better.

Treatment of animals. Male Sprague-Dawley rats weighing 200 to 250 g were purchased from Harlan Laboratories Inc. (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment in hanging cages with hardwood chips. Rats (n = 5–9/group) were randomly placed into one of three diet treatment groups: control group to simulate healthy liver (Harlan Teklad 4% Mouse/Rat Diet; Madison, WI), high-fat group to simulate SFL [18% butter (w/w) diet] and MCD group to simulate NASH (methionine-, choline-deficient diet) (Dyets Incorporated; Bethlehem, PA) ad libitum for 8 weeks. The Institutional Animal Care and Use Committee of the University of Arizona approved the animal studies described below.

Histology. Sections of liver from control, SFL and NASH rats were embedded in Tissue-Tek® OCT compound (Electron Microscopy Sciences; Hartfield, PA), rapidly frozen in ethanol cooled with dry ice and stored at -80º C until use. Using a Microm HM 550 cryostat (Richard Allen Scientific, Kalamazoo, MI), 5-μm slices from each diet group were generated and mounted onto Superfrost Plus slides (Fisher Scientific, Houston, TX). The mounted tissues were stained with hematoxylin and eosin (H & E) or Masson's trichrome stain. Histologic analyses were conducted under light microscopy (x40 objective) using coded slides to avoid observer bias. Parameters of the histologic analyses included degree of steatosis, inflammatory infiltration and fibrosis.

APAP excretion experiments. Under urethane-induced anesthesia (1.1 g/kg, 5 ml/kg, intraperitoneally), the left femoral artery and right femoral vein were

cannulated with PE 50 polyethylene tubing. The bile duct was subsequently cannulated with PE 10 polyethylene tubing distal to the bile duct bifurcation. Before injection of APAP, the femoral artery and vein cannulas were flushed with approximately 0.5 ml of saline-5% mannitol solution containing 50 units of heparin. APAP, dissolved in saline containing 5% mannitol, was injected into the femoral vein cannula at a dosage of 1 mmol/kg (10 ml/kg) using a 3-cc syringe with a 23-gauge needle. After APAP injection, the femoral vein cannula was flushed with a 0.5-ml injection of 0.5% saline-5% mannitol solution. At 0, 2, 10, 20, 40, 60, and 90 minutes after APAP administration, approximately 250-μL blood samples were collected from the femoral artery in heparinized tubes. Bile was collected at 15, 30, 45, 60, 75, and 90 minutes after APAP administration. To maintain urine flow, 1 ml/kg saline-5% mannitol solution was injected through the femoral vein cannula every 15 minutes after APAP administration. Urine was collected from the bladder at 90 minutes after APAP administration by ligation of the bladder and removal with a 22-gauge needle and 3-cc syringe. Livers and kidneys were also harvested at the end of each APAP disposition experiment and snap frozen in liquid nitrogen. All samples were stored at -80°C until analysis.

Detection of APAP and its metabolites in bile, plasma and urine samples. APAP and its metabolites in bile, plasma, and urine samples were quantified by high performance liquid chromatography analysis based on previously described methods (Howie, et al., 1977a;Chen, et al., 2000;Slitt, et al., 2003c). APAP and

its metabolites were resolved using a Zorbmax SB-C_{18} reverse-phase 4.6-mm x 25-cm column with a Phenomenex Security Guard Column Guard and eluted using a mobile phase composed of 12.5% HPLC-grade methanol, 1% acetic acid, and 86.5% water, run isocratically at a flow rate of 1.2 ml/minute. The elution of metabolites was monitored at a wavelength of 254 nm. Retention times of APAP and its metabolites were determined by comparison with that of authentic standards. Since this HPLC method does not separate the cysteinylglycine and cysteine conjugates of APAP, they were quantified together as APAP-CG/CYS.

Samples were analyzed using a Beckman System Gold HPLC system (Beckman Coulter, Inc., Fullerton, CA) equipped with a 128-nm solvent module and a 166-nm detector. Quantitation was based on integrated peak areas. The concentrations of APAP and its metabolites were calculated using an APAP standard curve since the molar extinction coefficients of APAP and its conjugated metabolites are approximately the same (Howie, et al., 1977b). To precipitate proteins in bile, plasma, and urine, samples were diluted 1:2, 1:2, and 1:3, respectively, with ice-cold methanol and centrifuged at 4,000 x g for 30 minutes at 4°C. The resulting supernatants were collected and diluted in mobile phase 1:3 (bile and urine) or 1:2 (plasma) prior to HPLC analysis. Liver samples were homogenized (1:9 w/v) in ice-cold HPLC grade methanol. Homogenates were centrifuged at 4,000 x g for 20 minutes at 4°C, and the supernatants were filtered

through a 0.45 μm filter. Samples were further diluted 1:3 with mobile phase prior to HPLC analysis.

RNA extraction. Total RNA from liver and kidney tissue was extracted using RNAzol B reagent (Tel-Test Incorporated; Friendswood, TX) according to the manufacturer's protocol. The quality of RNA samples was judged by the integrity and relative ratio of 28S and 18S rRNA bands following agarose gel electrophoresis.

Messenger RNA expression analysis. Rat Mrp1-Mrp7, Bsep and Bcrp mRNA levels were measured using the branched DNA signal amplification assay. To measure the levels of Mrp1-Mrp7, Bsep and Bcrp mRNA, respectively, oligonucleotide probe sets containing multiple mRNA transcript-specific capture, label, and blocker probes were designed using ProbeDesigner software v1.0 (Bayer Corporation-Diagnostics Division). The probe sets used in this study have been described previously, including Mrp1-3 (Cherrington, et al., 2002), Mrp4-6, Bsep, Mrp7 and Bcrp (Tanaka, et al., 2005). Probes were designed with an annealing temperature of approximately 63°C, which enabled the hybridization conditions to be held constant (i.e., 53°C) during each hybridization step and for each probe set. Every probe developed in ProbeDesigner was submitted to the National Center for Biotechnological Information for nucleotide comparison by the basic logarithmic alignment search tool (BLASTn), to ensure minimal cross-reactivity with other known rat sequences and expressed sequence tags.

Oligonucleotides with a high degree of similarity (≥80%) to other rat gene transcripts were excluded from the design.

Total RNA (1 µg/µL; 10 µL/well) was added to each well of a 96-well plate containing capture hybridization buffer and 50 µL of each diluted probe set. For each gene, total RNA was allowed to hybridize to the probe set overnight at 53°C. Subsequent hybridization steps were performed according to the manufacturer's protocol (QuantiGene Reagent System, Panomics; Fremont, CA). Luminescence was measured with a Quantiplex 320 bDNA Luminometer interfaced with Quantiplex Data Management Software Version 5.02 for analysis of luminescence from 96-well plates. The luminescence for each well was reported as relative light units (RLU) per 10 µg of total RNA.

Western Blot Analysis of Mrp2, Mrp3, Mrp4 and Bcrp Protein Expression. Liver and kidney crude membrane preparations were made as described previously (Slitt, et al., 2003b). Liver and kidney protein concentrations were determined using the BCA™ Protein Assay Kit (Pierce Biotechnology; Rockford, IL). Prior to analysis, samples were not boiled or treated with loading buffers containing β-mercaptoethanol. Protein sample aliquots were re-suspended in a Laemmli buffer-to-sample volume ratio of ≥ 2:1 and loaded into gel wells. Liver (40 µg/lane) and kidney (60 µg/lane) membrane proteins were electrophoretically resolved on tris-glycine, SDS-polyacrylamide gels (10% resolving gel, 4% stacking gel) by applying a 20-mA current for 1.5 hours. Proteins were transblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA) by applying

a 350-mA current for 75 minutes in a transfer buffer medium [7.2% glycine (w/v), 10% methanol (w/v), 0.5% SDS (w/v), 6% tris base (w/v)] at 4°C. The membranes were then blocked for 1 hour in 5% nonfat dry milk in TBS-Tween buffer (15 mM Tris base, 154 mM sodium chloride, 0.05% Tween 20, pH 7.4) before incubation with antibodies. Primary antibody clones, membrane incubation times and dilutions in 5% nonfat dry milk were as follows. For Mrp2, conditions included a 1:2000 dilution of mouse anti-human M_2III-6 monoclonal antibodies (ID Labs Inc.; London, ON, Canada) for 1 hour at room temperature (RT). For Mrp3, conditions included a 1:5000 dilution of affinity-purified antibodies against rat Mrp3 protein (Slitt, et al., 2003a) for 3.5 hours at RT. For Mrp4, conditions included a 1:500 dilution of goat anti-human polyclonal antibodies (Abcam, Inc.; Cambridge, MA) for 1 hour at RT. For Bcrp, conditions included a 1:500 dilution of mouse anti-human bxp-21 monoclonal antibodies (Kamiya Biomedical Company; Seattle, WA) for 1 hour at RT. Following incubation with the primary antibody, the blots were subjected to four 15-minute washes in TBS-Tween buffer. The blots were then incubated for 1 hour at RT with a horseradish peroxidase (HRP)-conjugated secondary antibody, either horse anti-mouse IgG (Cell Signaling Technology, Danvers, MA) or rabbit anti-goat IgG (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA). The secondary antibody dilutions included 1:2000 (Mrp2), 1:5000 (Mrp3 and Mrp4) and 1:3000 (Bcrp). Membranes were subjected to four additional 15-minute washes in TBS-Tween buffer. Protein-antibody complexes were detected using ECL Western blotting reagents

(Amersham Biosciences Inc.; Piscataway, NJ) and Blue Lite Autoradiography Film (ISC BioExpress; Kaysville, UT). Resulting autoradiographs were scanned with a Linotype-Hell Saphire Scanner (Linotype-Hell Corp.; Bad Homburg, Hesse, Germany) to create Tagged Image File Format (TIFF) files. Individual transporter protein bands contained in the TIFF files were quantified using ImageJ software (U. S. National Institutes of Health, http://rsb.info.nih.gov/ij/; Bethesda, MD).

Statistics. For excretion studies, statistical differences between diet groups at each time point were determined using a one-way analysis of variance followed by a Duncan's Multiple Range post hoc test. For mRNA and protein expression analyses, statistical differences between diet groups were also determined using a one-way analysis of variance followed by Duncan's Multiple Range post hoc test.

Results

Liver Histology**.** Figure 1 demonstrates the effect of the respective high-fat and MCD diets on liver histology after 8 weeks. Hepatic steatosis was apparent at 2 weeks in rats fed the MCD diet but not in rats fed the control standard diet. Notably, inflammation and fibrosis were plainly absent in the MCD group at this early time point (data not shown). However, after 8 weeks of consuming the diet, histologic analyses of H&E (A-C) and trichrome-stained sections (D-F) were indicative of two distinct NAFLD pathologies that corresponded satisfactorily with simple hepatic steatosis and early-stage NASH in human patients (Brunt and

Tiniakos, 2005). Histology of H&E-stained liver sections from the control group was unremarkable, with minimal periportal inflammatory infiltrates (A) and minimal fibrosis (D). Sections from the high-fat diet group were characterized by predominantly periportal, microvesicular steatosis (B) and minimal fibrosis (E). Sections from the MCD diet group revealed diffuse, predominantly macrovesicular steatosis (C) with mild biliary oval cell hyperplasia, mild periportal inflammatory infiltrates, and fibrosis (F).

Efflux Transporter mRNA Levels in the Liver After 8 Weeks of Feeding on the Respective High-Fat and MCD Diets. After 2 weeks of feeding on the control and MCD diets, there were no discernible differences in mRNA levels of Mrp2, Mrp3 Mrp4, or Bcrp (data not shown). This finding suggests that acute dietary deficiency of methionine and choline does not alter transcriptional regulation of these four major efflux transporters. Figure 2 illustrates the effect of 8 weeks of feeding on the respective high-fat and MCD diets on efflux transporter mRNA levels in liver. In high-fat rats, Mrp5 mRNA levels were increased 350%, whereas Mrp6 and Bsep were each decreased by 50%. Mrp1-4 and Bcrp mRNA levels were unchanged in livers of high-fat rats. In MCD rats, Mrp3, Mrp4, and Bcrp mRNA expression levels were increased 2990, 405, and 59%, respectively. Mrp1 and Mrp2 mRNA levels were not altered by the high-fat or MCD diet.

Efflux Transporter Protein Levels in Liver After 8 Weeks of Feeding on the Respective High-Fat and MCD diets. Figure 3 shows the effect of the respective high-fat and MCD diets on Mrp2, Mrp3, Mrp4, and Bcrp protein levels in liver. In

MCD rats, Mrp2, Mrp3, Mrp4, and Bcrp protein expression levels were increased 185, 253, 246, and 71%, respectively. Notably, no changes in protein levels of the major APAP metabolite transporters were observed in the livers of high-fat rats.

Effect of High-Fat and MCD Diets on Bile Flow. Figure 4 shows the cumulative amount of bile collected during the 90 min after APAP administration for control, high-fat, and MCD rats. The cumulative bile volume in high-fat rats was significantly greater than the control by 19% at 60 min and 26% at 90 min. MCD rat bile flow did not differ from the control.

Effect of High-Fat and MCD Diets on Biliary Excretion of APAP and APAP Metabolites. Figure 5, top left, shows the concentrations of APAP and its major metabolites in bile over 90 min. The concentration of APAP in the bile of MCD rats was significantly elevated compared with the control (20% higher) at 15 min. It is important to note that parent APAP that does not undergo metabolism within a hepatocyte can passively diffuse across the canalicular or basolateral membrane. Thus, any intergroup differences in the biliary or urinary concentration of APAP would not be reflective of alterations in function by efflux transporters.

The biliary concentration of each major metabolite of APAP was recurrently observed to be significantly decreased in MCD rats relative to control and high-fat rats. In contrast with APAP parent, each of these metabolites, i.e., APAP-

GLUC, APAP-GSH, and APAP-SULF, does require active transport to be excreted from hepatocytes.

Figure 5, top right, illustrates the concentration of APAP-GLUC in bile of control, high-fat, and MCD groups over the 90-min collection period. APAP-GLUC was significantly lower in MCD rats than in control rats throughout the 90 min, with the maximal difference (73% lower) being observed by 45 min. The biliary APAP-GLUC levels in MCD rats became significantly lower than those in the high-fat group by 30 min. Of the three major metabolites measured in the bile, APAP-GLUC was the most abundant metabolite detected throughout the time course in each group.

Figure 5, bottom left, illustrates the concentration of APAP-SULF in bile of control, high-fat, and MCD rats over the 90-min collection period. APAP-SULF was significantly lower in MCD rats than in control rats throughout the 90 min, with the maximal difference (67% lower) being observed by 30 min. In addition, the biliary APAP-SULF levels in MCD rats also became significantly lower than those in the high-fat group by 15 min. The biliary concentration of APAP-SULF in high-fat rats was lower than that for the control rats (by 23%) only at 15 min.

Figure 5, bottom right, illustrates the concentration of APAP-GSH in bile of control, high-fat, and MCD groups over the 90-min collection period. In the MCD group, APAP-GSH levels in bile were significantly lower than those in the control group throughout the 90 min. The maximal difference from the control group (93% lower) was observed by 30 min.

Effect of High-Fat and MCD Diets on APAP and APAP-GLUC Metabolite Concentrations in Liver. Figure 6 shows the concentrations of APAP and its APAP-GLUC metabolite in the livers of control, high-fat, and MCD rats, respectively, at the completion of the study. The APAP parent concentration in the livers of high-fat rats was 85% lower than that of the control rats. Notably, the concentration of APAP-GLUC in the livers of MCD rats was 79% lower than that of the control rats. The APAP-GSH, APAP-SULF, and APAP-CG/CYS metabolites were not detectable in livers in any group.

Effect of High-Fat and MCD Diets on Plasma Concentration of APAP and APAP Metabolites. Figure 7 shows the concentrations of APAP, APAP-GLUC, and APAP-SULF in plasma over 90 min. APAP-GSH was not detectable in the plasma of rats in these studies. For APAP (Fig. 7, top), there were no plasma level differences between the control, high-fat, and MCD groups throughout the time course. The APAP-GLUC metabolite (Fig. 7, center) was detected in the plasma of the control and high-fat groups by 10 min after APAP administration. In contrast, in the MCD group, the APAP-GLUC metabolite was present in the plasma within 2 min of APAP administration. It is interesting that by 10 min, the concentration of the APAP-GLUC metabolite in the MCD animals was 4.8-fold higher than in controls. Plasma levels of APAP-GLUC in MCD rats remained significantly elevated above those of the control group throughout the remainder of the time course by an average of 3.3-fold. APAP-SULF was present in the plasma of control, high-fat, and MCD rats within 10 min of administration of the

APAP (Fig. 7, bottom). Within 40 through 90 min, the plasma concentration of APAP-SULF in MCD rats was significantly lower than the control group by an average of 50%.

Effect of High-Fat and MCD Diets on Urinary Excretion of APAP and APAP Metabolites. Figure 8 shows the concentration of APAP and its metabolites in the urine of control, high-fat, and MCD rats at 90 min after APAP administration. There were no group differences for APAP, APAP-SULF, APAP-CG/CYS, and APAP-NAC, nor were there differences in the net (i.e., total) concentration of metabolites between control, high-fat, and MCD rats. However, the concentration of the APAP-GLUC metabolite was 80% higher in the urine of MCD rats compared with control rats. Importantly, this same metabolite was elevated in the plasma of MCD rats throughout the 90-min time course. APAP-GSH was not detected in the urine of any group.

Discussion

The current study presents novel data that demonstrate the effects of experimental NAFLD on transporter expression and drug disposition. The clinical relevance of these topics is underscored by the fact that the NAFLD patient population, a demographic that uses a great quantity and variety of prescription medications, is expected to continue growing at an alarming rate (Kopelman, 2000; Brunt and Tiniakos, 2005). To expand our comprehension and/or potentially develop pharmacologic treatment for this disorder, extensive studies of accurate animal models will serve as an invaluable tool. It is thus pertinent to

note that the current models of NAFLD (i.e., high-fat and MCD diets) provided accurate histologic representations of SFL and NASH, respectively.

It is important to note that there were no alterations in bile flow between the control and modified diet groups. The cumulative bile flow was significantly elevated in high-fat rats but only at the later time points. Each of these groups is a contrast to the obese Zucker rat (a model of insulin resistance and obesity) in which bile secretory function in fatty liver is impaired, nonetheless in the absence of cholestatic injury (Pizarro et al., 2004; Geier et al., 2005).

This is the first study to examine liver efflux transporter expression in high-fat and MCD dietary models of simple hepatic steatosis and NASH in rats. Both models resulted in multiple alterations in liver expression of efflux transporters. It is interesting to note that when the mRNA levels of a given efflux transporter were altered with the high-fat diet, the MCD model generally generated the same effect (i.e., increased Mrp5 in liver and decreased Mrp6 in liver). In contrast, only MCD rats were characterized by up-regulation of mRNA and protein levels of the hepatic efflux transporters Mrp3, Mrp4, and Bcrp.

The obvious alterations in transporter expression and drug disposition in the current study encourage further investigation into mechanisms of transcriptional regulation during NAFLD that would be likely to provide greater insight into necessary differences at the molecular level between simple fatty liver and steatohepatitis in rats. It is useful to note that, at the histologic level, the major principal difference between the high-fat and MCD rats was the presence

of inflammatory infiltrates and fibrosis in MCD rat livers. Thus, one important factor to consider with regard to efflux transporter regulation during NAFLD is the role of proinflammatory cytokines. Elevated levels of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 have been detected in the plasma of patients with biopsy-proven NASH but not in patients with simple hepatic steatosis (Kugelmas et al., 2003; Bahcecioglu et al., 2005; Abiru et al., 2006). Consistent with the current model, the early stages of MCD-induced NASH are characterized by up-regulation of hepatic IL-6 mRNA and elevated plasma levels of TNF-α (Chawla et al., 1998; Stärkel et al., 2003). Although there is little information on the role of IL-6 in the regulation of hepatic transporters in rats, the role of TNF-α in this case has been well studied by using the endotoxin-induced model of sepsis (Whiting et al., 1995; Green et al., 1996; Geier et al., 2003; Cherrington et al., 2004). Specifically, down-regulation of Mrp2 mRNA during endotoxemia has been demonstrated to be dependent on TNF-α (Geier et al., 2003). In contrast, in obese Zucker rats, which develop elevated plasma levels of TNF-α, abrogation of TNF-α activity with etanercept has no effect on Mrp2 protein levels (Liu et al., 2002; Pizarro et al., 2004). Although there are limited data on the hepatic and serum levels of proinflammatory cytokines in rat models of NAFLD, it seems reasonable to propose that cytokines may be important in transporter regulation in the MCD model of NASH.

Importantly, the biochemical mechanisms by which the high-fat and MCD diets induce their respective pathologies in the liver are markedly distinct. Thus,

direct comparison of the effects, e.g., gene expression, between the high-fat and MCD groups was deemed inappropriate for statistical analysis in these studies. In the high-fat dietary model, induction of hepatic steatosis is most likely a consequence of increased net flux of lipids through the liver. In contrast, the MCD model achieves its histologic recapitulation of NASH via a series of events consistent with lipid peroxidation as a pathogenic mechanism for initiation and perpetuation of liver injury, inflammatory recruitment, stellate cell activation, and fibrogenesis (George et al., 2003). Although the initial steatosis of the MCD model is not associated with the peripheral insulin resistance that is frequently observed in human NASH, the consequent damage, including hepatocellular injury, inflammatory recruitment, and zone 3 fibrosis does resemble that observed in human NASH (George et al., 2003).

It is pertinent to note that the difference between the "control" and MCD diets used in the current study was not exclusive to the presence or absence of methionine and choline. An arguably more appropriate dietary control would be achieved by merely resupplementing the MCD diet with methionine and choline. Nonetheless, both diets applied in the current study are, in fact, fully defined in terms of their amino acid content, and it is thus known that the control diet (Teklad 7001 4% Mouse/Rat Diet) is methionine- and choline-sufficient, resulting in a histology reflective of a healthy liver (Fig. 1). At the same time, there is no question that the methionine and choline deficiency is responsible for the pathology observed (Fig. 1F). However, it is not entirely clear whether the

alterations in transporter expression (and thus APAP-GLUC metabolite excretion) are the consequence of a chronic dietary deficiency in methionine and choline, or, conversely, a regulatory response to the progressive damage and/or steatosis that occur as a result of the deficiency. According to the mRNA and histologic analysis of control and MCD rat livers after 2 weeks of the respective diets, acute biochemical deficiency of methionine and choline is clearly not responsible for inducing alterations in transporter expression.

The latter part of the current study examined the disposition of acetaminophen metabolites during NAFLD. In high-fat rats, there were no significant differences in the concentration of APAP metabolites in the plasma or urine and relatively few differences in the biliary excretion of metabolites. It is therefore apparent that simple hepatic steatosis has no discernible effect on the disposition of APAP metabolites. In contrast, there were obvious alterations in the disposition of the APAP-GLUC, APAP-GSH, and APAP-SULF metabolites in MCD rats.

One of the most important observations in the current study was the altered disposition of APAP-GLUC metabolites in the rats with MCD-induced NASH. The marked reduction in the biliary concentration of APAP-GLUC was accompanied by a concurrent increase in its plasma and urine concentrations. These were in the range of those observed after pretreatment of the same strain of rats with various Mrp3-inducing compounds, including phenobarbital (Brouwer and Jones, 1990; Xiong et al., 2002; Slitt et al., 2003). In hepatocytes of normal

rat liver, Mrp3 protein is expressed at much lower levels than Mrp2. Thus, although Mrp3 has a much higher affinity for APAP-GLUC, this metabolite is normally excreted predominantly into bile in rats (Brouwer and Jones, 1990; Gregus et al., 1990). However, in the livers of rats with MCD-induced NASH, the expression levels of Mrp2 and Mrp3 appear to have become relatively equal, probably resulting in competition for the APAP-GLUC substrate. It therefore follows that the apparent shift from biliary to urinary excretion is most likely due to 1) the increased Mrp3 protein levels in liver and 2) the higher affinity of Mrp3 for the APAP-GLUC metabolite.

The concentration of APAP-SULF in bile was significantly lower in MCD rats compared with the control rats throughout the entire time course. This result was unanticipated, given the substantial up-regulation of both Mrp2 and Bcrp, which are responsible for the canalicular excretion of APAP-SULF. However, the plasma concentration of APAP-SULF was also consistently lower in MCD rats, and although the decrease of APAP-SULF in MCD rat urine did not reach statistical significance, it too appears to be slightly lower than the control. Therefore, it would seem that the differences in disposition may have resulted from a decrease in sulfotransferase expression, sulfotransferase activity, and/or the sulfotransferase cofactor 3'-phosphoadenosine-5'-phosphosulfate.

The concentration of APAP-GSH in bile was also decreased in MCD rats throughout the time course. This result was also unforeseen, given the pronounced induction of Mrp2 protein levels. In contrast, the concentrations of

APAP-GSH derivatives APAP-CG/CYS and APAP-NAC in urine were not altered in MCD rats. These data indicate that the sinusoidal efflux transporters responsible for the hepatovascular excretion of APAP-GSH and its derivatives were able to maintain this function in MCD rat livers. However, it is also clear that fewer APAP-GSH metabolites and derivatives were formed in MCD rat livers. This effect may be partly due to the reduction ex vivo in cytochrome P450-mediated bioactivation of APAP to the reactive N-acetyl-p-benzoquinoneimine (data not shown), which undergoes a spontaneous electrophilic reaction with GSH to form APAP-GSH. Hepatic GSH levels were not altered in either model of NAFLD (data not shown).

The metabolic capacity of the kidneys to form derivatives of the APAP-GSH conjugate is also an important consideration in the case of APAP disposition. After efflux of APAP-GSH conjugates from the liver into the sinusoidal blood, they are rapidly degraded into APAP-CG, APAP-CYS, and APAP-NAC by the kidneys (Moldéus, 1978). Thus, only the degradation products of APAP-GSH, including APAP-CG, APAP-CYS, and APAP-NAC, are normally detectable in the urine (Gregus et al., 1988). Mrp2, which is expressed in the brush border membrane of rat proximal tubule cells, is presumably responsible for the tubular secretion of these APAP-GSH derivatives (Schaub et al., 1997). There were no alterations in the urinary excretion of the APAP-GSH derivatives. Bcrp and Mrp4 are also present at high levels in rat kidneys and appear to be important in the secretion of sulfate conjugates from proximal tubule cells (Jonker

et al., 2002; Mizuno et al., 2004; Tanaka et al., 2005). In contrast, Mrp3 is expressed at the basolateral membrane of renal proximal tubule cells and may participate in efflux of APAP-GLUC into blood (Kuroda et al., 2004). Although Mrp3, Mrp4, and Bcrp are each expressed to a considerable extent in the kidney, their contribution to renal excretion of APAP-GLUC (by Mrp2) and APAP-SULF (by Mrp4 and Bcrp) is probably very limited. This is because, in rats and humans, the vast majority of APAP biotransformation, including sulfation, glucuronidation, and glutathione conjugation, occurs in the liver, where sulfotransferase, UDP-glucuronosyl transferase, and glutathione S-transferase activities make their greatest contribution to drug metabolism (Levy and Yamada, 1971; Slattery and Levy, 1979; Galinsky and Levy, 1981; Vendemiale et al., 1996; Lohr et al., 1998).

In summary, we report that diet-induced models of simple hepatic steatosis and NASH produce multiple changes in efflux transporter expression in rats. Importantly, the MCD dietary model of NASH resulted in decreased biliary excretion of APAP-GLUC, APAP-SULF, and APAP-GSH. Furthermore, the MCD model of NASH altered the vectorial excretion of APAP-GLUC, shifting excretion from bile to blood instead of blood to bile. This shift in vectorial excretion of APAP-GLUC correlated with induction of the basolateral efflux transporters Mrp3 and Mrp4. Thus, the current study provides proof of principle that changes in drug transporter expression during liver disease, i.e., NASH, can result in profound alterations in the drug disposition process. More recent studies also indicate that the therapeutic efficacy of drugs is likewise integrally linked to the

function of drug transporters, which can distribute pharmacologically active concentrations of the drug to its target site, e.g., ezetimibe glucuronide and morphine 6-glucuronide (Zelcer et al., 2005; Oswald et al., 2006a,b). Therefore, future studies should place greater emphasis on determining the role of drug transporters in the pharmacokinetics and pharmacodynamics of drug therapy administered during NAFLD.

Footnotes

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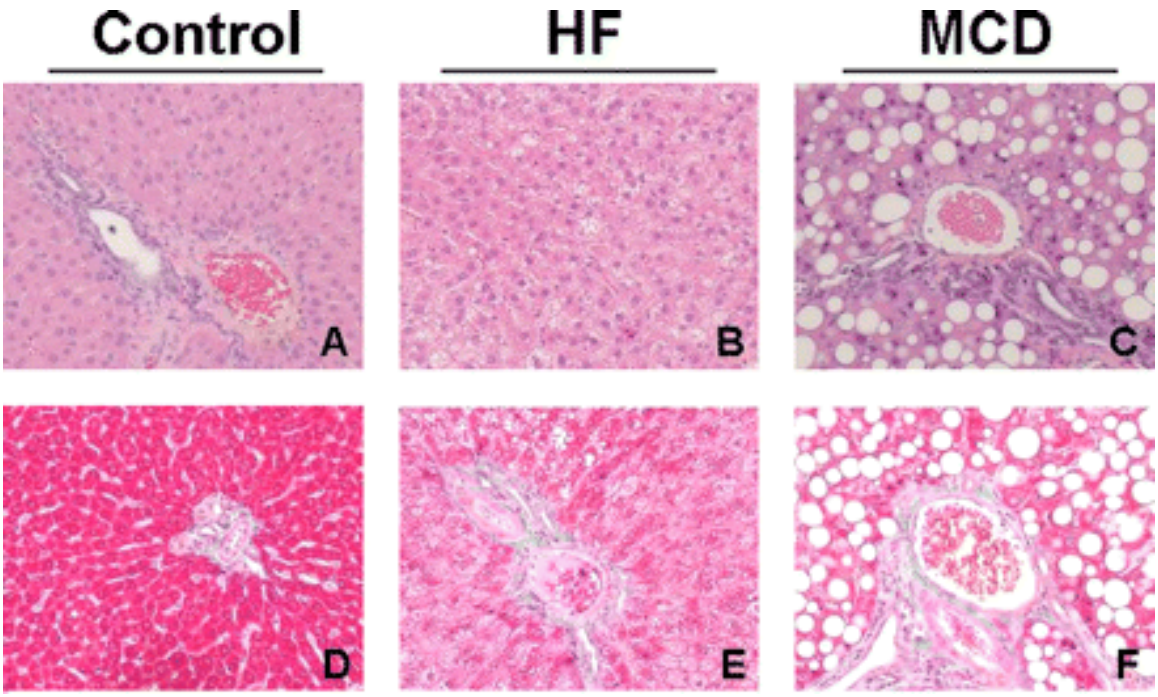

Figure 1. H&E- and trichrome-stained sections of rat liver. After 8 weeks of feeding of a control, high-fat, or MCD diet, 5-μm liver cryosections from each group were stained with hematoxylin and eosin (A-C) or Masson's trichrome (D-F). Histologic analyses were conducted using light microscopy at 40x magnification.

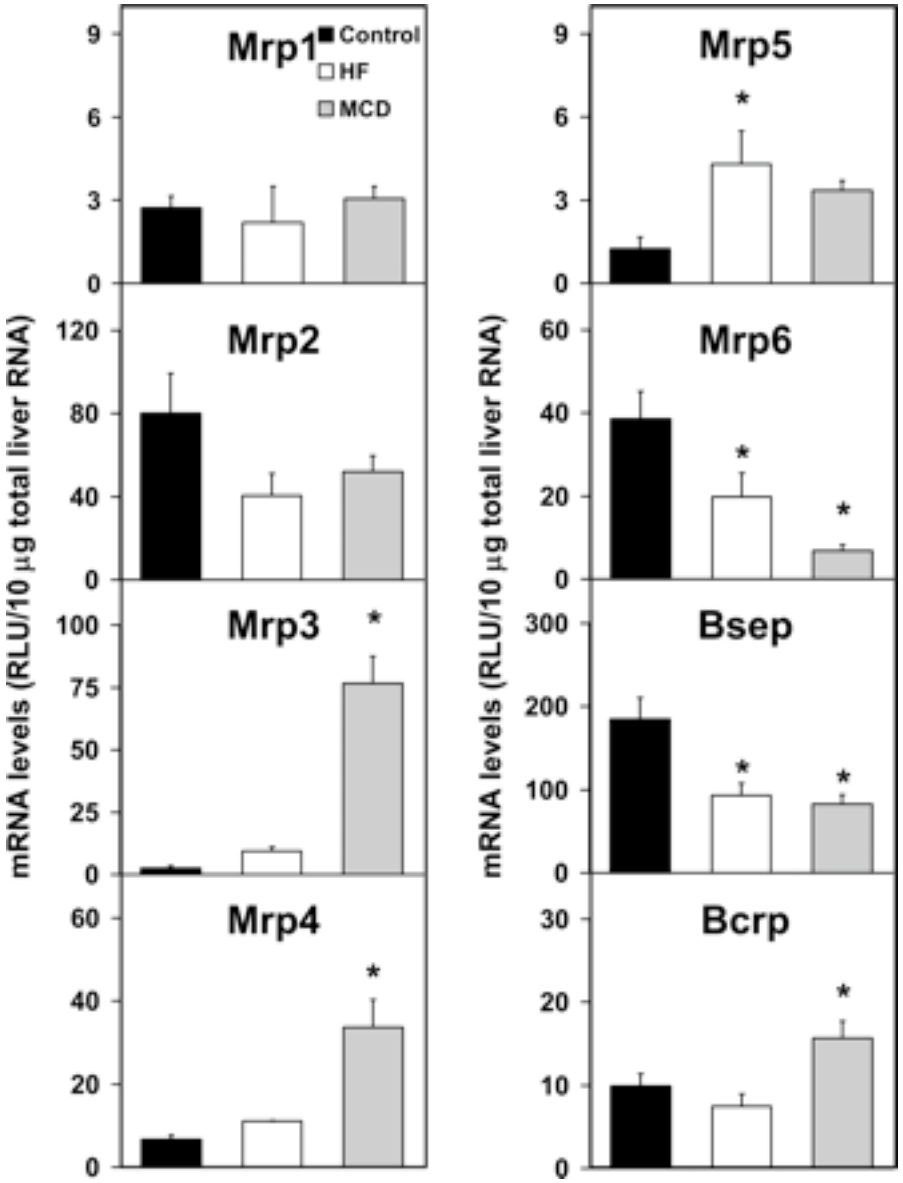

Figure 2. Efflux transporter mRNA levels in the liver. After 8 weeks of feeding of a control, high-fat, or MCD diet, total RNA was isolated from the livers of male Sprague-Dawley rats. Levels of mRNA were analyzed by the branched DNA (bDNA) assay and expressed as relative light units (RLU) ± S.E.M. (n = 5). *, a significant difference from the control group ($p \leq 0.05$).

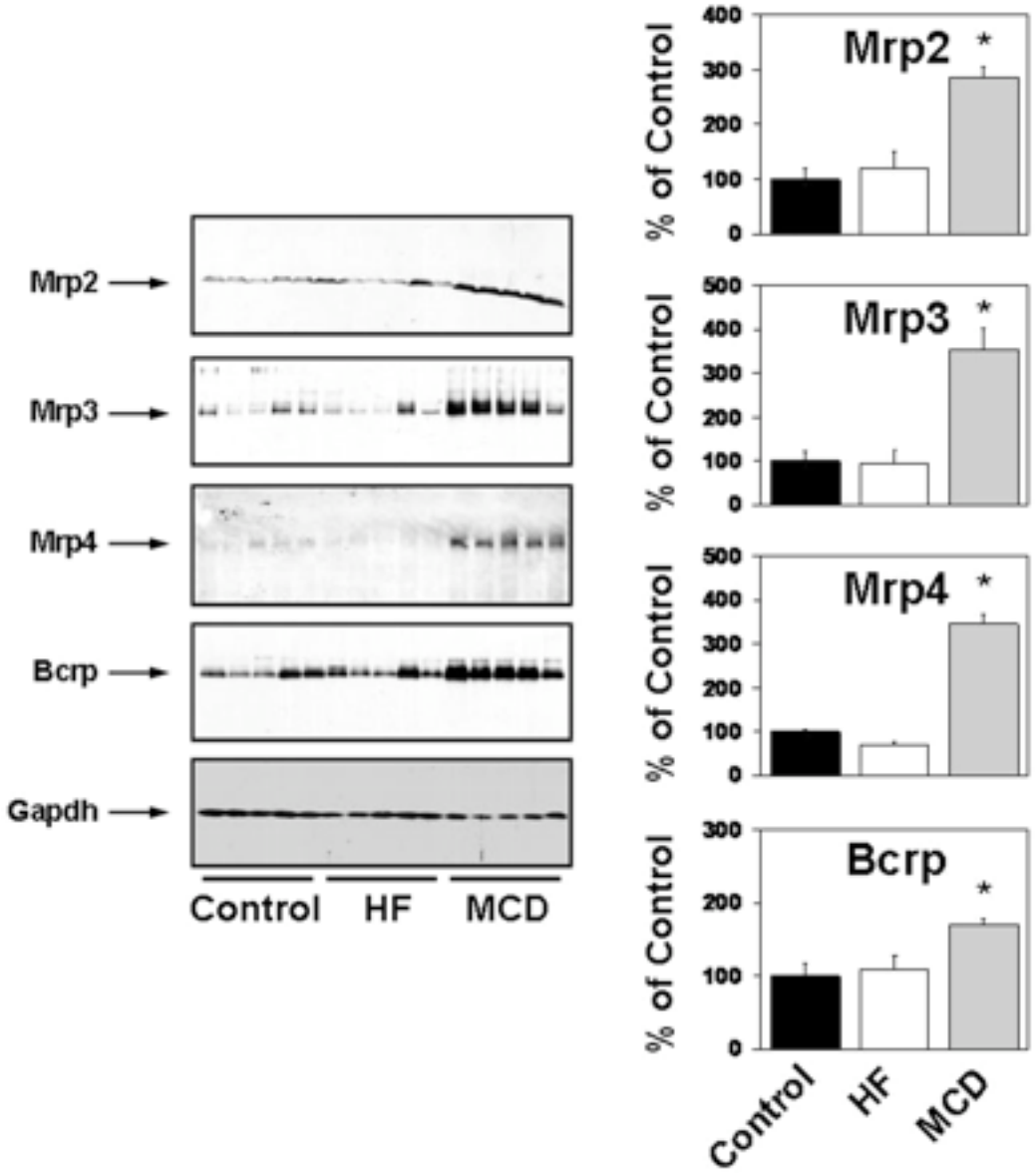

Figure 3. Efflux transporter protein levels in liver. After 8 weeks of feeding of a control, high-fat, or MCD diet, liver crude membrane fractions from male Sprague-Dawley rats were analyzed by Western blot (40 µg of protein/lane). Transporter protein bands were quantified using ImageJ software and are expressed as a percentage of the control group ± S.E.M. (n = 5). *, significant difference from the control group ($p \leq 0.05$). Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

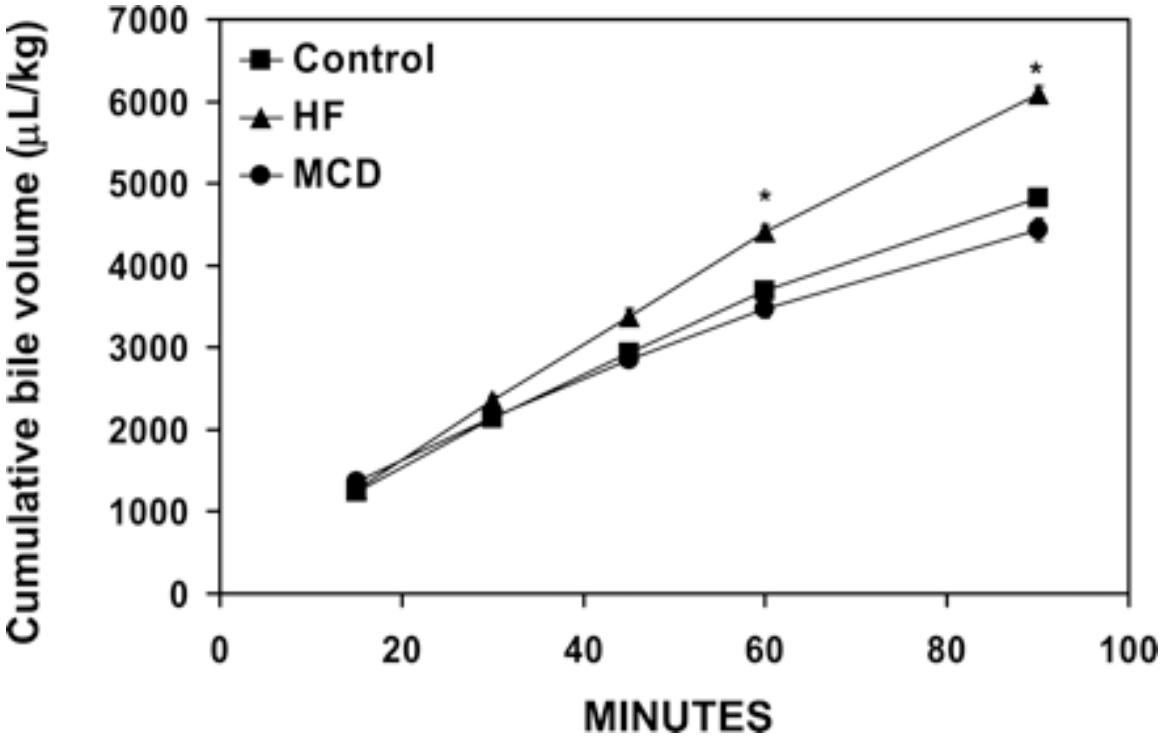

Figure 4. Effect of high-fat and MCD diets on bile flow. After 8 weeks of feeding of a control, high-fat, or MCD diet, APAP excretion experiments were conducted. The left carotid artery and bile duct were cannulated, and APAP was administered. Immediately after the APAP dose, bile was collected in 15-min segments over 90 min. The data are represented as the mean ± S.E.M. *, significant difference from the control group ($p \leq 0.05$).

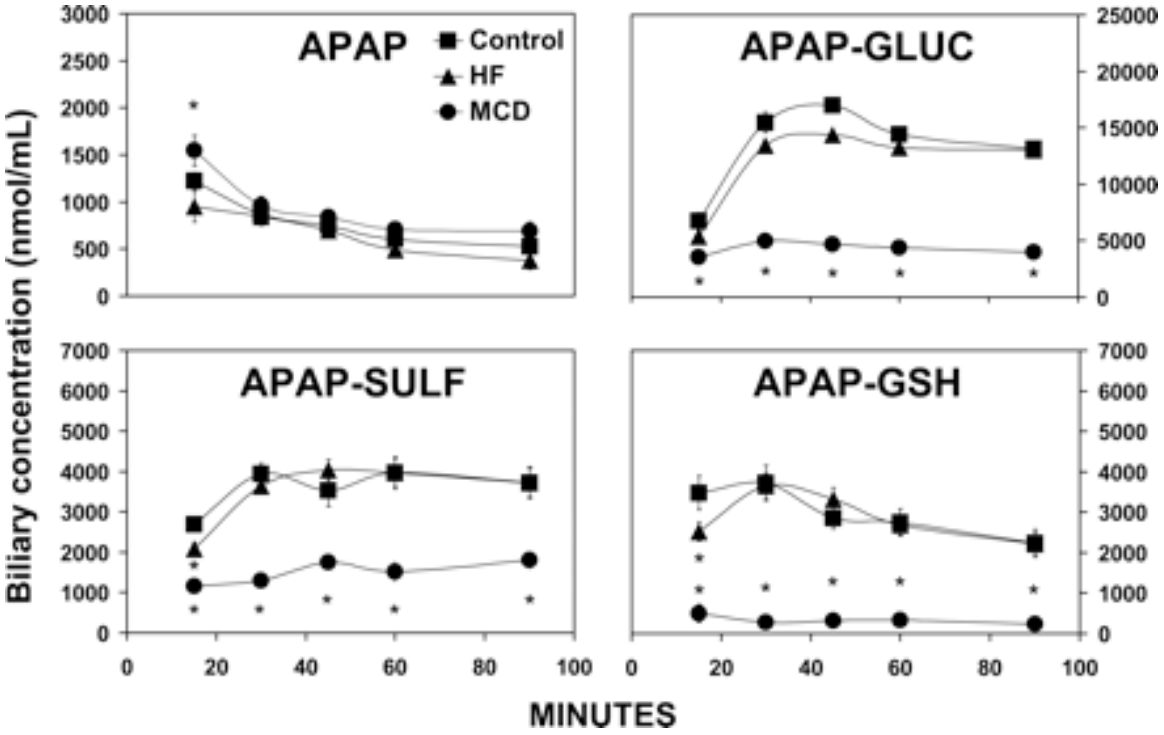

Figure 5. Effect of high-fat and MCD diets on biliary excretion of APAP and APAP metabolites. Experimental conditions were the same as those for Fig. 4. The data are represented as the mean concentration ± S.E.M. *, significant difference from the control group ($p \leq 0.05$).

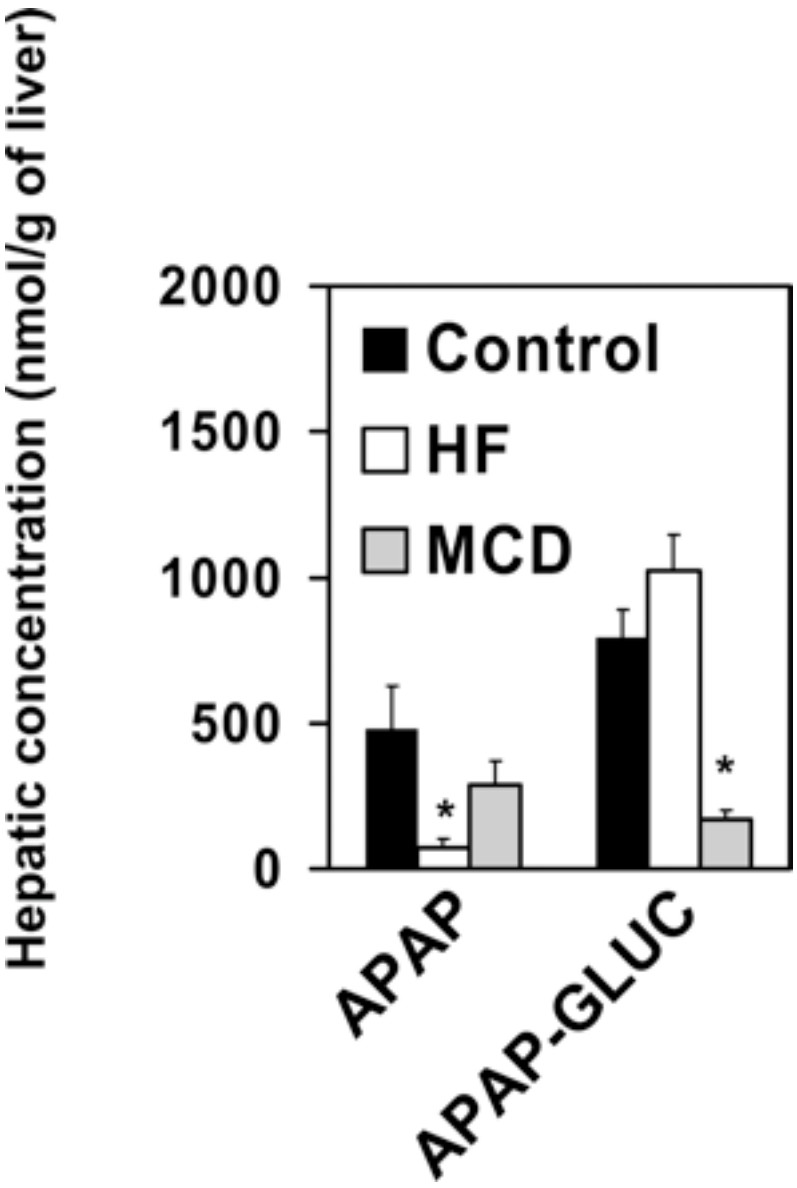

Figure 6. Effect of high-fat and MCD diets on APAP and APAP-GLUC metabolite concentrations in liver. Liver samples were collected at the end of the APAP excretion experiment and were stored at -80°C before analysis by HPLC as described under Materials and Methods. The APAP-GSH, APAP-SULF, and APAP-CG/CYS metabolites were not detectable in livers from all three groups of rats. The data are represented as the mean concentration ± S.E.M. *, significant difference from the control group ($p \leq 0.05$).

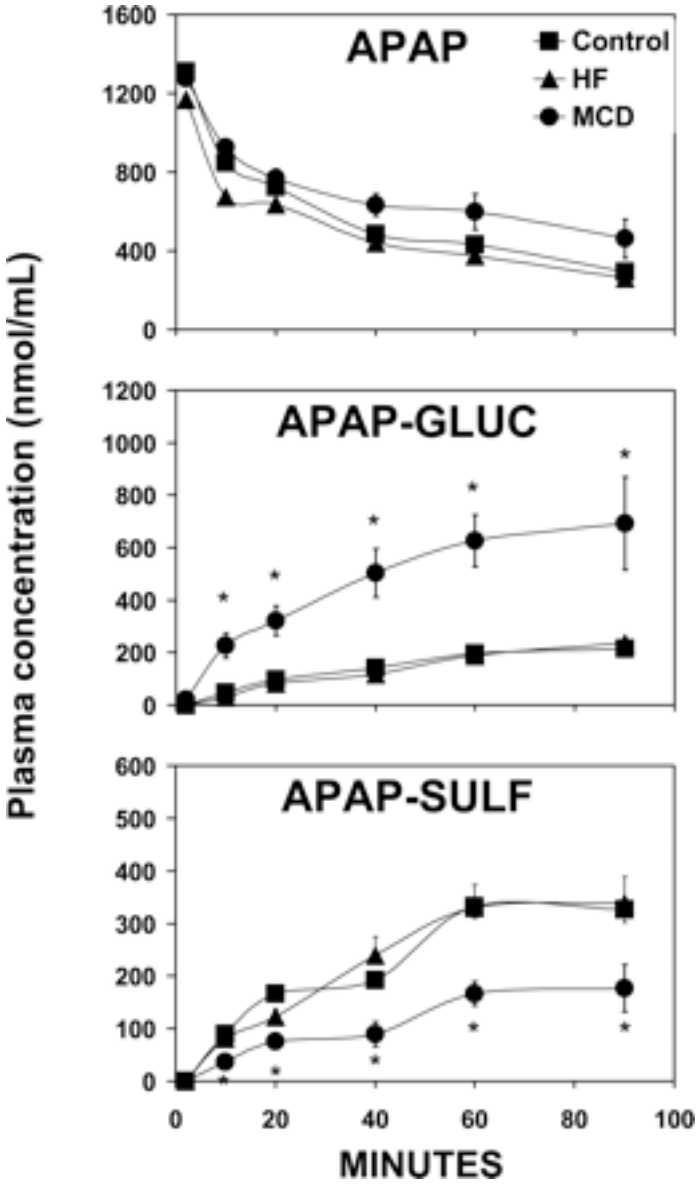

Figure 7. Effect of high-fat and MCD diets on plasma concentrations of APAP and APAP metabolites. Experimental conditions were the same as those for Fig. 4.The APAP-NAC and APAP-CG/CYS metabolites were not detectable in the plasma of all three groups of rats. The data are represented as the mean concentration ± S.E.M. *, significant difference from the control group ($p \leq 0.05$).

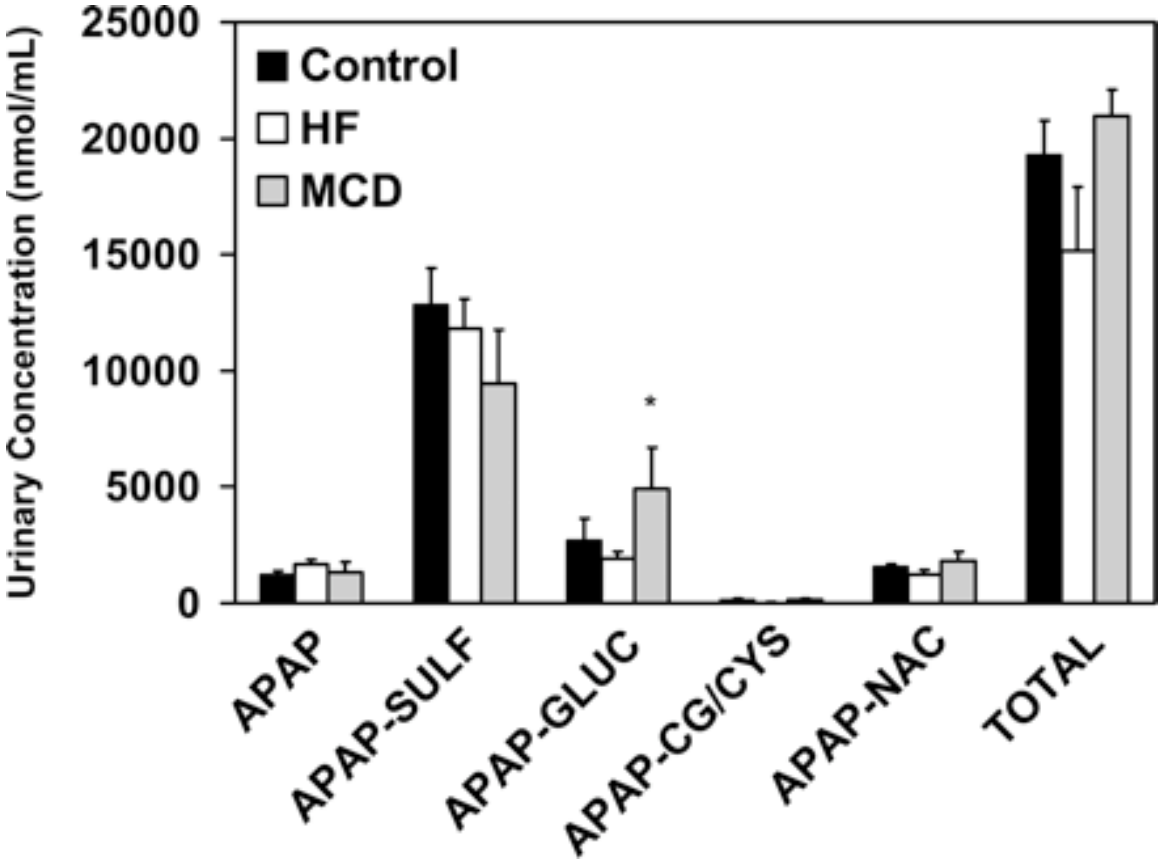

Figure 8. Effect of high-fat and MCD diets on urinary excretion of APAP and APAP metabolites. Experimental conditions were the same as those for Fig. 4. This figure shows the cumulative concentrations of APAP and its respective metabolites in the urine 90 min after APAP administration. The data are represented as the mean concentration ± S.E.M. *, significant difference from the control group ($p \leq 0.05$).

APPENDIX B: DIFFERENTIAL REGULATION OF HEPATIC TRANSPORTERS IN THE ABSENCE OF TUMOR NECROSIS FACTOR-α, INTERLEUKIN-1β, INTERLEUKIN-6, AND NUCLEAR FACTOR-κB IN TWO MODELS OF CHOLESTASIS

Abstract

Hepatic transporters are responsible for uptake and efflux of bile acids and xenobiotics as an essential aspect of liver function. When normal vectorial transport of bile acids by the apical uptake and canalicular excretion transporters is disrupted, cholestasis ensues, leading to accumulation of toxic bile constituents and considerable hepatocellular damage. The purpose of this study was to assess the role of cytokines and nuclear factor-κB (NF-κB) in the transcriptional regulation of transporters in two models of cholestasis, lipopolysaccharide (LPS) administration and bile duct ligation (BDL). In wild-type (WT) and knockout mouse strains lacking tumor necrosis factor (TNF) receptor-1, interleukin (IL)-1 receptor I, IL-6, or inhibitor of κB (IκB) kinase β, transporter mRNA levels in liver were determined using branched DNA signal amplification 16 h after LPS administration or 3 days after BDL. In WT mice, LPS administration tended to decrease mRNA levels of organic anion-transporting polypeptide (Oatp) 2, Na+-taurocholate cotransporting polypeptide (Ntcp), Oatp1, Oatp4, bile salt excretory protein (Bsep), multidrug resistance-associated protein (Mrp) 2, and Mrp6 compared with saline treatment, whereas it increased Mrp1, 3, and 5 levels. Similar changes were observed in each knockout strain after LPS

administration. Conversely, BDL decreased only Oatp1 expression in WT mice, meanwhile increasing expression of Mrp1, 3, and 5 and Oatp2 expression in both WT and knockout strains. Because the transcriptional effects of BDL- and LPS-induced cholestasis reflect dissimilarity in hepatic transporter regulation, we conclude that these disparities are not due to the individual activity of TNF-α, IL-1, IL-6, or NF-κB but to the differences in the mechanism of cholestasis.

Abbreviations

Ntcp, Na+-taurocholate cotransporting polypeptide; Bsep, bile salt excretory protein; MRP, multidrug resistance-associated protein, Mrp; Oatp, organic anion transporting polypeptide; BDL, bile duct ligation; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; NF-κB, nuclear factor-B; WT, wild type; TNFR-1, tumor necrosis factor receptor 1; RI, receptor I; IKK-βΔhep, liver-specific Inhibitor of κB kinase β-deficient; IKK-β(f/f), inhibitor of κB kinase β-floxed; bDNA, branched DNA; Oat, organic anion transporter; Mdr, Multidrug-resistant; TNFR1(–/–), tumor necrosis factor receptor-1 knockout; IL-1R(–/–), interleukin-1 receptor knockout; IL-6(–/–), interleukin-6 knockout.

Introduction

Cholestasis is generally defined as a condition in which bile flow is impaired. Consequent accumulation of bile constituents in hepatocytes produces oxidative stress, plasma membrane disruption, severe hepatocellular injury, and necrotic and/or apoptotic cell death, probably due to the detergent-like properties of bile salts (Kasahara et al., 2002). Accordingly, maintaining bile flow and

enterohepatic circulation of bile acids are among the most important functions of hepatocyte membrane transport systems. At the sinusoidal membrane, Na+-taurocholate cotransporting polypeptide (Ntcp) is a secondary active transporter that is predominantly responsible for the uptake of bile salts from portal blood into hepatocytes. At the canalicular membrane, the ATP-dependent transporters bile salt excretory protein (Bsep) and multidrug resistance-associated protein2 (Mrp2) mediate secretion of bile salts and glutathione. Bsep and Mrp2 are thereby responsible for the generation of bile salt-dependent and bile salt-independent bile flow, respectively.

Besides maintaining bile flow, a number of other hepatic transporters aid in uptake and biliary excretion of a wide variety of both endogenous and exogenous compounds. Many such transporters are constitutively expressed in the liver, including secondary active transporters such as the organic anion-transporting polypeptides 1, 2, and 4 (Oatp1, 2, and 4). These transporters are responsible for the uptake of a wide variety of organic anions from sinusoidal blood into hepatocytes and contribute to the first-pass effect. Aside from vectorial disposition of substrates into bile, several members of the Mrp family of transporters (Mrp1, Mrp3, and Mrp4) have been identified at the sinusoidal membrane where, conversely, they appear to mediate the sinusoidal efflux of a variety of substrates (Rius et al., 2003; Slitt et al., 2003).

Bile duct ligation (BDL) is used as a model to study extrahepatic, obstructive cholestasis. In addition to impaired canalicular and common bile duct

flow, BDL produces a loss of bile salt secretory polarity and the accumulation of bile salt, bilirubin, and other bile constituents in hepatocytes. To study intrahepatic cholestasis, LPS administration is a useful model. Upon entering the liver in portal blood, LPS, a component of the outer cell wall of Gram-negative bacteria, stimulates Kupffer cells to release proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Busam et al., 1990). Previous studies have supported the notion that these three cytokines are essential in the regulation of transporter expression in models of intrahepatic cholestasis (Whiting et al., 1995; Green et al., 1996; Geier et al., 2003; Siewert et al., 2004). However, data regarding the activation and/or influence of cytokines in hepatic transporter regulation during obstructive cholestasis are not entirely clear (Bohan et al., 2003; Geier et al., 2005). In vivo studies have shown that bile acids are capable of inducing Kupffer cells to release proinflammatory cytokines and subsequently effect transcriptional alterations in the neighboring parenchymal cells (Miyake et al., 2000).

Whereas alterations in transporter regulation following extrahepatic obstruction are the result of toxic stress from accumulating bile, LPS induces alterations in transporter expression and function that appear to be the direct cause of cholestasis. The LPS model thus makes it possible to study transporter regulation directly, rather than the secondary response to the accumulation of bile constituents that results from biliary obstruction.

Among the molecular mediators in cholestasis that may possess key regulatory roles are the proinflammatory cytokines TNF-α, IL-1β, and IL-6. Three

lines of evidence have suggested that expression of hepatic transporters during cholestasis is dependent on intact cytokine signaling. First, it has been demonstrated that LPS-induced reduction in bile flow can be prevented by administration of TNF-α antibodies (Whiting et al., 1995). Second, recombinant TNF-α produces a down-regulation of Ntcp and Mrp2 mRNA levels (Green et al., 1996; Kim et al., 2000). Third, administration of dexamethasone, an anti-inflammatory steroid, blocks release of cytokines and prevents down-regulation of transporters by LPS (Roelofsen et al., 1995; Kubitz et al., 1999; Cherrington et al., 2004).

Currently, the understanding of the critical molecular mediators that regulate transporter expression during intrahepatic and extrahepatic cholestasis remains incomplete. The purpose of this study was to perform a comprehensive assessment of the role of the TNF-α, IL-1β, IL-6, and nuclear factor-κB (NF-κB) in the transcriptional regulation of transporters in two models of cholestasis, LPS administration and BDL.

Material and Methods

Chemicals. LPS (from Escherichia coli serotype 011:B4) and all other chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO).

Animals and Treatments. Male C57BL6/J [wild type (WT)] mice, as well as mice homozygous for the targeted mutation of tumor necrosis factor receptor-1 (TNFR1), IL-1 receptor I (RI), and IL-6, were purchased from The Jackson

Laboratory (Bar Harbor, ME). Liver-specific IκB kinase β (IKK-β)-deficient [IKK-βΔhep] male and IKK-β-floxed [IKK-β(f/f)] mice were generated as described previously (Maeda et al., 2003). All animals were allowed water and standard chow ad libitum. Housing and experimental procedures were in accordance with the U.S. National Institutes of Health and the American Association for Laboratory Animal Science guidelines.

In each model of cholestasis, six groups of age-matched male mice (25–35 g) underwent treatment. BDL surgery was performed under pentobarbital (Associated Medical Supply, Scottsdale, AZ) anesthesia (75 mg/kg i.p.) and sterile surgical conditions, with the common bile duct being ligated at two locations. Sham controls underwent laparotomy, without ligation of the bile duct. For the LPS model, LPS (4 mg/kg, 5 ml/kg) or saline vehicle was administered to mice by i.p. injection. Livers were collected and snap-frozen in liquid nitrogen (16 h after LPS administration or 72 h after BDL surgery) and stored at –80°C until RNA isolation was performed.

RNA Isolation. Total RNA was isolated from liver using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. The integrity of each RNA sample was confirmed by gel electrophoresis before analysis.

Development of Specific Oligonucleotide Probe Sets for bDNA Analysis. Probe sets for mouse Oatp1, 2, 4, 5, 9, 11, 12, and 14; Oat2 and 3; Ntcp; Bsep;

and Mrp1–7 and 9 were used as described previously (Buist and Klaassen, 2004; Aleksunes et al., 2005; Cheng et al., 2005; Maher et al., 2005). The probe set for mouse Mdr1b is described in Table 1. These target sequences were analyzed by ProbeDesigner Software (version 1.0; Genospectra, Fremont, CA). Oligonucleotide probes designed were specific to a single mRNA transcript (e.g., Mrp1). All oligonucleotide probes were designed with a Tm of 63°C enabling hybridization conditions to be held constant (i.e., 53°C) during each hybridization step and for each oligonucleotide probe set. Each probe developed in ProbeDesigner was submitted to the National Center for Biotechnological Information for nucleotide comparison by the basic logarithmic alignment search tool (BLASTn), to ensure minimal cross-reactivity with other known mouse sequences and expressed sequenced tags.

Branched DNA Assay. All reagents for analysis (i.e., lysis buffer, capture hybridization buffer, amplifier/label probe buffer, and substrate solution) were supplied in the Quantigene Discovery Kit. Specific oligonucleotide probes were diluted in lysis buffer supplied in the Quantigene HV Signal Amplification Kit (Genospectra, Fremont, CA). Total RNA (1 µg/µl, 10 µl) was added to each well of a 96-well plate containing capture hybridization buffer and 50 µl of each diluted probe set. Total RNA was allowed to hybridize to each probe set overnight at 53°C. Subsequent hybridization steps were carried out as per the manufacturer's protocol, and luminescence was measured with a Quantiplex 320 bDNA luminometer (Bayer Diagnostics, East Walpole, MA) interfaced with Quantiplex

Data Management Software (version 5.02; Bayer Diagnostics) for analysis of luminescence from 96-well plates.

Statistics. Statistical differences were determined by two-way analysis of variance followed by Duncan's multiple range post hoc test [$p \leq 0.05$, n = 3–5 for WT, TNFR1(–/–), IL-1RI(–/–), and IL-6(–/–) mice]. Data obtained from experiments performed in IKK-βΔhep and IKK-β(f/f) mice (n = 2–3) were subjected to Student's t test.

Results

Effects of BDL Surgery and LPS Administration on Hepatic Transporter Expression in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) Mice. In Figs. 1, 2, 3, 4, 5, 6, 7, 8, significant down-regulation in mRNA levels will be described as a percent decrease compared with the control, whereas significant up-regulation will be described as a fold increase. Figure 1 illustrates the effect of BDL surgery and LPS administration on Oat2, Oat3, and Ntcp mRNA levels in livers of WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice. In WT and TNFR1(–/–) mice, BDL decreased Oat2 mRNA levels in liver by 66 and 80% relative to sham-operated controls. Although not statistically significant, BDL reduced Oat2 levels in livers of IL-1R(–/–) mice by 72%. BDL significantly decreased Ntcp levels by 70% in livers of TNFR1(–/–) but had no significant effect on Ntcp mRNA levels in livers of WT, IL-1R(–/–), or IL-6(–/–) mice. BDL did not significantly affect Oat3 mRNA levels in livers of any of the four mouse strains. In sham-operated TNFR1(–/–) mice, the

mRNA expression levels of Ntcp were significantly elevated (2.5-fold) above the sham-operated WT mice.

Figure 1 also shows that, in WT mice, mRNA expression of the Oat family members Oat2 and Oat3 did not undergo significant changes after LPS administration, whereas Ntcp was down-regulated significantly by 58% compared with the saline control. The Oat2 mRNA levels in each of the cytokine knockouts, TNFR1(–/–), IL-1RI (–/–), and IL-6(–/–), were significantly decreased by 68, 74, and 66% of the saline control, respectively. In contrast, LPS increased Oat3 mRNA in the IL-6(–/–) mice (2-fold). Messenger RNA levels of Ntcp in livers of WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice after LPS administration were decreased by 73, 93, 86, and 90%, respectively. In saline-treated TNFR1(–/–) mice, the mRNA expression levels of Ntcp were significantly elevated (2.6-fold) above the saline-treated WT group.

Figure 2 shows the consequence of BDL surgery to Oatp1, 2, 4, 5, 9, 11, 12, and 14 mRNA levels in livers of WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice. BDL resulted in a significant decrease in Oatp1 mRNA expression in each of the four strains, with 84, 88, 81, and 74% reductions in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice, respectively. In contrast, Oatp2 mRNA levels were increased in all four strains, with significant increases being detected in the IL-1R(–/–) (5.4-fold) and IL-6(–/–) (3.6-fold). Similar to Oatp1, Oatp4 mRNA expression was decreased by BDL in all four strains, but this decrease was significant in only the TNFR1(–/–) strain (57%). Bile duct ligation did not have any

significant effect upon the mRNA levels of Oatp5 or 9. The overall effect of BDL on Oatp11 mRNA expression was similar to that observed for Oatp2, i.e., increased in all four strains but to a significant extent in only the WT (3.9-fold) and TNFR1(–/–) mice (2.3-fold). Interestingly, it was only in the IL-6(–/–) mice that Oatp12 and 14 mRNA expression levels were significantly up-regulated by 2.3- and 12-fold, respectively.

In sham-operated mice, the mRNA expression levels of Oatp4 in TNFR1(–/–) and Oatp11 in IL-6(–/–) mice were significantly elevated by 1.7- and 2.7-fold compared with WT mice. Conversely, mRNA expression levels of Oatp14 in sham-operated mice were significantly lower than WT in the TNFR1(–/–) (by 94%), IL-1R(–/–) (by 94%), and IL-6(–/–) mice (by 86%).

Figure 2 further demonstrates that LPS administration results in overall decreases in Oatp1 and 4 mRNA levels in each of the four mouse strains. Importantly, these are the three most highly expressed Oatp isoforms found in mouse liver (Cheng et al., 2005). Specifically, Oatp1 mRNA levels were decreased by 90, 89, 88, and 87% in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice, respectively. Oatp2 expression underwent 28, 76, 59, and 50% reductions in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice, respectively, which were significant in the TNFR1(–/–) and IL-6(–/–) mice. Oatp4 mRNA underwent significant decreases of 65, 79, 59, and 83% in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice, respectively. Although there was significant up-regulation of Oatp5 mRNA expression (1.8-fold) in WT mice in response to LPS

administration, Oatp9 mRNA levels were significantly decreased in TNFR1(–/–) mice (37%). Conversely, mRNA expression of Oatp11 and 14 was significantly up-regulated in all four strains after LPS administration. Namely, Oatp11 mRNA levels were increased 2.8-, 4.1-, 2.8-, and 16-fold, respectively, and Oatp14 mRNA expression was increased 8-, 10-, 12-, and 12-fold, respectively, in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice. Oatp12 mRNA expression was significantly up-regulated (2.2-fold) in only the IL-6(–/–) mice. In saline-treated TNFR1(–/–) mice, mRNA expression levels of Oatp9 were significantly increased (1.6-fold) relative to WT.

Figure 3 illustrates the effect of BDL surgery and LPS administration on Mdr1b and Bsep mRNA levels in livers of WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice. Although BDL appeared to increase Mdr1b mRNA levels in all four strains, the up-regulation was significant in only the IL-6(–/–) mice (10-fold). Interestingly, BDL resulted in a significant reduction (46%) in Bsep mRNA expression in only the TNFR1(–/–) mice. Although LPS had no significant effect on Mdr1b gene expression, Bsep mRNA levels were significantly decreased in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice by 59, 88, 70, and 91%, respectively.

Figure 4 illustrates the effect of BDL surgery on Mrp1–7 and 9 mRNA levels in livers of WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice. In general, the mRNA expressions of Mrp1, 3, 4, 5, and 7 were increased in each of the four mouse strains after bile duct ligation. For Mrp1, this up-regulation of mRNA

expression was significant in only the IL-6(–/–) mice (10-fold). Significant up-regulation of both Mrp3 and Mrp5 mRNA levels was observed after BDL of all four strains. Mrp3 mRNA levels were up-regulated 4.5-, 4.8-, 3.4-, and 3.4-fold in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice, respectively. Mrp5 mRNA levels were up-regulated 4.5-, 4.8-, 3.4-, and 3.4-fold in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice, respectively. Generally, Mrp4 and Mrp7 mRNA expressions were also increased in response to BDL, but the increases were significant only in the case of Mrp4 expression in IL-1R(–/–) (8-fold) and IL-6(–/–) (2.2-fold) mice. Mrp6 expression, in contrast, was significantly reduced in TNFR1(–/–) (79%) and IL-1R(–/–) mice (70%). In sham-operated TNFR1(–/–) mice, there was a significant elevation in Mrp2 (1.7-fold) and Mrp6 (1.5-fold) mRNA expression levels compared with sham-operated WT mice. Also in sham-operated TNFR1(–/–) mice, mRNA expression levels of Mrp9 were significantly decreased (by 72%) compared with levels in the sham-operated WT mice. In sham-operated IL-1R(–/–) mice, Mrp4 (by 88%) and Mrp9 (by 68%) mRNA levels were significantly decreased relative to those in sham-operated WT mice. Finally, in IL-6(–/–) sham-operated mice, mRNA expression levels of Mrp4 were significantly decreased (by 30%) relative to those in sham-operated WT mice.

Figure 4 also displays the effect of LPS administration on Mrp1–7 and 9 mRNA levels. As occurred after BDL, LPS administration generally induced up-regulation of Mrp1, 3, 5, and 7 mRNA levels in all four strains of mice. Up-regulation of Mrp1 mRNA expression was significant in WT (3.6-fold), TNFR1(–/–

) (2.4-fold), IL-1R(–/–) (5.7-fold), and IL-6(–/–) mice (5.8-fold); Mrp3 up-regulation was significant in only the IL-1R(–/–) mice (3.4-fold); Mrp5 up-regulation was significant in IL-1R(–/–) (2-fold) and IL-6(–/–) mice (2.1-fold); and Mrp7 up-regulation was significant in only the IL-1R(–/–) mice (1.9-fold). In contrast, mRNA levels of Mrp2 and Mrp6 were generally decreased by LPS administration. Mrp2 down-regulation was significant in TNFR1(–/–) (80%), IL-1R(–/–) (59%), and IL-6(–/–) (69%) mice. Mrp6 down-regulation was significant in WT (65%), TNFR1(–/–) (79%), IL-1R(–/–) (70%), and IL-6(–/–) (56%) mice. In saline-treated TNFR1(–/–) mice, Mrp3 mRNA expression levels were significantly increased (2.3-fold), whereas Mrp5 levels were significantly decreased (by 51%). In saline-treated IL-1R(–/–) mice, Mrp5 mRNA expression levels were significantly decreased (by 45%) below those in saline-treated WT mice. Finally in saline-treated IL-6(–/–) mice, mRNA levels of Mrp3 were elevated (2.5-fold) over those in WT mice, whereas Mrp5 (by 42%) and Mrp6 (by 41%) levels were decreased below those in saline-treated WT mice.

Effect of BDL Surgery and LPS on Hepatic Transporter Expression in IκB Kinase β-deficient, IKK-βΔhep, and IκB Kinase β-floxed, IKK-β(f/f) Mice. Figure 5 shows that BDL surgery did not significantly alter Oat2 or Oat3 mRNA levels. Although BDL surgery did not affect Ntcp mRNA levels in IKK-β(f/f) mouse liver, it did produce significant down-regulation of Ntcp in the IKK-β⊗hep mice (35%). Generally, LPS administration resulted in down-regulation of Oat2, Oat3, and Ntcp mRNA levels in both IKK-β(f/f) and IKK-βΔhep mouse liver. The down-

regulation of Oat2 mRNA expression was significant in IKK-β(f/f) mouse liver (85%), Oat3 down-regulation was significant in both IKK-β(f/f) (63%) and IKK-βΔhep (58%) mouse liver, and Ntcp down-regulation was likewise significant in both strains (66 and 73%, respectively). In sham-operated IKK-βΔhep mice, Oat3 mRNA expression levels were significantly decreased (by 84%) below those in the sham-operated IKK-β(f/f) group.

Figure 6 illustrates the effects of BDL and LPS administration on mRNA levels of Oatp family members. BDL produced up-regulation of Oatp2 (2-fold) and Oatp5 (2.1-fold). Importantly, this effect was observed in only IKK-β(f/f) mice. There was no significant change in the levels of Oatp1, 2, 4, 9, 12, and 14 mRNA after BDL. As was observed in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice (Fig. 2), LPS administration generally resulted in down-regulation of Oatp1, 2, 4, and 5 mRNA levels in both IKK-β(f/f) and IKK-βΔhep mice. Thus, after LPS administration, Oatp1 underwent significant reductions of 57 and 60% in mRNA expression in IKK-β(f/f) and IKK-βΔhep mice, respectively; Oatp2 underwent 96 and 80% reductions in IKK-β(f/f) and IKK-βΔhep mice, respectively; and Oatp4 underwent 75 and 41% reductions in IKK-β(f/f) and IKK-βΔhep mice, respectively. In IKK-βΔhep mice, Oatp5 (48%), Oatp12 (17%), and Oatp14 (58%) mRNA each underwent significant decreases. Conversely, Oatp11 was significantly induced by LPS in both IKK-β(f/f) (4.6-fold) and IKK-βΔhep mice (6-fold), an effect also observed in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice

(Fig. 2). In sham-operated IKK-βΔhep mice, Oatp2 and Oatp5 mRNA levels were significantly increased above those in sham-operated IKK-β(f/f) mice by 2.2- and 2.1-fold. In saline-treated IKK-βΔhep mice, Oatp14 mRNA levels were also significantly elevated (2.2-fold) above those in saline-treated IKK-β(f/f) mice.

Figure 7 demonstrates the effect of BDL surgery and LPS administration on Mdr1b and Bsep mRNA expression. BDL had no significant effect on Mdr1b mRNA, whereas Bsep mRNA was significantly decreased only in IKK-βΔhep mice (48%). After LPS administration, Mdr1b mRNA levels were significantly decreased only in IKK-βΔhep mice (77%). As was observed in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice (Fig. 3), LPS administration decreased Bsep mRNA expression in IKK-β(f/f) (70%) and IKK-βΔhep mice (82%).

Figure 8 illustrates the effect of BDL surgery and LPS administration on Mrp1–7 and 9 mRNA levels in livers of IKK-β(f/f) and IKK-βΔhep mice. BDL induced up-regulation of Mrp3 mRNA expression in IKK-β(f/f) (2-fold) but not IKK-βΔhep mouse liver. In IKK-βΔhep mice, Mrp5 mRNA expression was significantly decreased (67%) by BDL. Also in IKK-βΔhep mice, Mrp7 was increased (2.5-fold) by BDL. After LPS administration, Mrp1 mRNA expression was increased in both IKK-β(f/f) (16-fold) and IKK-βΔhep mice (2.3-fold), as seen also in WT, TNFR1(–/–), IL-1R(–/–), and IL-6(–/–) mice (Fig. 4). LPS also induced Mrp7 up-regulation in IKK-βΔhep mice (2.7-fold), whereas it decreased Mrp2 (49%) and Mrp5 (59%) mRNA levels in IKK-β(f/f) and IKK-βΔhep mice, respectively. In sham-operated

IKK-βΔhep mice, mRNA levels of Mrp1 were increased (7.0-fold), whereas Mrp4 levels were decreased (52%) below those in the IKK-β(f/f) sham-operated mice. In saline-treated IKK-β⊗hep mice, Mrp1 mRNA levels were similarly increased (7.3-fold) above those in the saline-treated IKK-βΔhep mice. Conversely in saline-treated IKK-β⊗hep mice, Mrp5 mRNA levels were decreased (28%) relative to those in the saline-treated IKK-β(f/f) mice.

Discussion

In the current study we investigated the liver expression of 21 transporter genes after BDL and LPS administration, models of extrahepatic and intrahepatic cholestasis, respectively. The present data confirm previous reports that demonstrated the ability of LPS to down-regulate mRNA levels of the sinusoidal uptake transporters Oatp1, Oatp2, Ntcp, and Oatp4 in mouse and rat liver (Trauner et al., 1998; Geier et al., 2003; Cherrington et al., 2004; Siewert et al., 2004). Furthermore, the present study illustrates the fact that Oat2 mRNA levels in liver tend to decrease after LPS administration, which is similar to the observation in rats (Cherrington et al., 2004). Our data also demonstrated that LPS decreased mRNA levels of Bsep and Mrp2 in mouse liver. This finding is consistent with other reports in which down-regulation of Bsep and Mrp2 mRNA expression was observed after LPS administration (Hartmann et al., 2002; Siewert et al., 2004). Together, these findings have important implications for understanding the pathogenesis of cholestasis because the protein products of these two transcripts are largely responsible for bile formation. Their down-

regulation is probably a direct cause of cholestasis after LPS exposure or during sepsis. The finding that expression of the efflux transporters Mrp1, 3, 5, and 7 tended to increase in livers of WT mice after LPS administration is novel. However, these data are not consistent with earlier studies that demonstrated the lack of changes in Mrp1 and down-regulation of Mrp3 in mice (Hartmann et al., 2002; Siewert et al., 2004). In rats, however, up-regulation of Mrp1, Mrp3, and Mrp5 has likewise been reported (Vos et al., 1998; Donner and Keppler, 2001; Cherrington et al., 2004). Importantly, the Mrp transporters that were induced by LPS function to export organic anions back into the blood from the hepatocyte. Up-regulation of these transporters could play a hepatoprotective role during cholestasis.

As with LPS, BDL down-regulated expression of the uptake transporters Oat2 and Oatp1 in livers of WT mice. This is consistent with other studies that demonstrated down-regulation of Oatp1 and Oatp4 in rats and Oatp4 in mice following BDL (Ogawa et al., 2000). In contrast, BDL induced up-regulation of Mrp3 and Mrp5 in livers of WT mice. Mrp5 up-regulation is a new finding among mice and rats (Soroka et al., 2001; Bohan et al., 2003; Cherrington et al., 2004; Denk et al., 2004). The lack of alteration in liver Mrp2 mRNA expression has been a common observation in rats although decreased expression has also been reported (Ogawa et al., 2000; Paulusma et al., 2000; Hyogo et al., 2001).

The second component of this study was to determine whether the effects of BDL surgery and LPS administration on mRNA levels are dependent on TNF-

α, IL-1β, IL-6, or NF-κB activity. In forms of extrahepatic cholestasis (primary sclerosing cholangitis, bile duct carcinoma, and gallstones), the initial insult results from physical obstruction of the flow of bile, causing an accumulation of bile constituents in the liver, consequent hepatocellular damage, and recruitment of inflammatory mediators. By contrast, various forms of intrahepatic cholestasis (primary biliary cirrhosis, cholestasis of sepsis, and alcoholic hepatitis), are initiated by and remain characterized by inflammation that produces disruption of hepatic transporter-mediated bile flow and consequent cholestasis. Importantly, each of the above clinical diagnoses of intrahepatic cholestasis has been documented as having elevated serum levels of TNF-α, IL-1β, or IL-6 (Bird et al., 1990; Khoruts et al., 1991; O'Donohue and Williams, 1996; Simpson et al., 1997; Neuman et al., 2002). Additionally, NF-κB is a primary regulator of inflammatory responses and accordingly plays a central role in cholestatic liver diseases such as viral hepatitis and alcoholic liver disease (Barnes and Karin, 1997; Heyninck and Beyaert, 2001). These and other findings discussed in our introduction suggest that the cytokines TNF-α, IL-1β, and IL-6 and the transcription factor NF-κB are integral components of signal transduction and gene regulation during cholestasis. Therefore, to determine the requirement of TNF-α, IL-1β, IL-6, and NF-κB for transcriptional regulation of hepatic transporters during cholestasis, mice homozygous for the corresponding targeted mutations [TNFR1(–/–), IL-1RI(–/–), IL-6(–/–), and IKK-βΔhep] were used.

There were a number of perplexing discrepancies in basal mRNA expression levels of transporters in the six genotypes used in this study. It is unclear how to account for such differences. Additionally, BDL and LPS treatment, respectively, appear to have had opposite effects on levels of a given mRNA transcript between the six genotypes studied. For example, BDL effected a significant change (increase) in Oatp5 mRNA levels in only IKK-β(f/f) mice. Conversely, LPS induced Oatp5 only in WT, meanwhile signaling down-regulation in the IKK-β(–/–) mice. The mechanism of transcriptional regulation of Oatp5 during obstructive or LPS-induced cholestasis is not well studied. Similar discrepancies were observed with Oatp11, Mrp5, and Mrp6. In each case, the mechanisms that underlie the differential regulation of the various genotypes are unclear. Thus, speculation with regard to mechanism will not be issued at this time. Studies including electrophoretic mobility shift assay and supershift assay will be necessary to fully assess the role of NF-κB in the transcriptional regulation of these transporters.

Statistical analyses indicate that mRNA levels of Oat3, Oatp9, Mrp2, and Mrp9 after BDL are not dependent on the individual activity of TNF-α, IL-1β, IL-6, or NF-κB. In contrast to the lack of Mrp2 mRNA alterations shown currently, a previous report found Mrp2 mRNA expression to be down-regulated in both WT and TNFR1(–/–) after 14 days of BDL (Bohan et al., 2003), as opposed to 3 days (current study). Both findings nonetheless support the hypothesis that transcriptional regulation of Mrp2 during obstructive cholestasis is TNF-α-

independent. Additionally, it is evident that bile duct ligation had a nearly equivalent effect on Oat2, Oatp1, and Mrp4 in all six strains. Oat2 and Oatp1 mRNA levels were generally down-regulated after BDL, regardless of the absence of TNF-α, IL-1β, IL-6, or NF-κB activity.

As was observed for some genes during extrahepatic cholestasis, transcriptional control of a number of liver transporters during intrahepatic cholestasis clearly does not have an individual requirement for TNF-α, IL-1β, IL-6, or NF-κB activity. These include Ntcp, Bsep, Oatp1, Oatp11, Mrp1, and Mrp9. The ability of LPS to down-regulate Ntcp, Oatp1, and Bsep mRNA expression has been demonstrated previously in vivo in rats and mice (Trauner et al., 1998; Hartmann et al., 2002; Geier et al., 2003; Cherrington et al., 2004; Siewert et al., 2004; Geier et al., 2005). The current results in IL-6(–/–) mice also confirm a previous report in which LPS administration resulted in transcriptional down-regulation of Ntcp, Bsep, and Oatp1 that was equally robust in WT and IL-6(–/–) mice (Siewert et al., 2004). In addition to these transporters, it is evident that mRNA levels of Oat2, Oatp2, Oatp4, and Mrp2 were also generally down-regulated by LPS, although not all significantly, in all six strains of mice. Down-regulation of Oatp2 and Mrp2 has been reported previously in both rats and mice after LPS administration (Vos et al., 1998; Donner et al., 2001; Hartmann et al., 2002; Geier et al., 2003; Cherrington et al., 2004; Siewert et al., 2004). Furthermore, recombinant TNF-α, IL-1β, and IL-6, like LPS, are each capable of producing down-regulation of Ntcp, Bsep, Oatp1, Oatp2, and Mrp2 mRNA

expression in mice (Hartmann et al., 2002; Siewert et al., 2004; Geier et al., 2005). Given that both TNF-α and IL-1β have the ability to signal activation of NF-κB-mediated transcription, each is likely to constitute at least one facet of compensatory signaling in the acute-phase response to LPS in TNFR1(–/–) and IL-1RI(–/–) mice. Given the redundant nature of cytokine signaling, it is reasonable to suggest that the remaining cytokines in these knockout mice are capable of mediating compensatory signaling within the acute-phase response to LPS (Taga and Kishimoto, 1992).

The current study indicates that the extrahepatic and intrahepatic models of cholestasis produce a variety of effects on transcriptional regulation of hepatic transporters in mice. We conclude that these effects reflect important differences in initiating events leading to cholestasis, rather than individual activity of TNF-α, IL-1β, IL-6, or NF-κB. Overall, the changes that occurred in the LPS model included decreased expression of uptake (Ntcp and Oatps) and excretion transporters (Bsep and Mrp2), thereby reducing further accumulation of organic anions in the liver while failing to eliminate them into the bile. The current findings thus indicate that LPS is capable of inducing intrahepatic cholestasis. Importantly, simultaneous up-regulation of sinusoidal efflux transporters (Mrps) was observed in both the LPS and BDL models. Both models therefore indicate a hepatoprotective response to cholestasis via up-regulation of sinusoidal efflux transporters that would enable the efflux of organic anions and thus minimize the organ's exposure to the toxic compounds.

Footnotes

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This work was presented in part previously: Lickteig AJ, Slitt AL, and Cherrington NJ (2004) Regulation of hepatic transporters during cholestasis is independent of TNF-α, IL-1 and IL-6 activity, in Proceedings of the 43rd Annual Meeting of the Society of Toxicology; 2004 Mar 21–25; Baltimore, MD. Society of Toxicology, Reston, VA.

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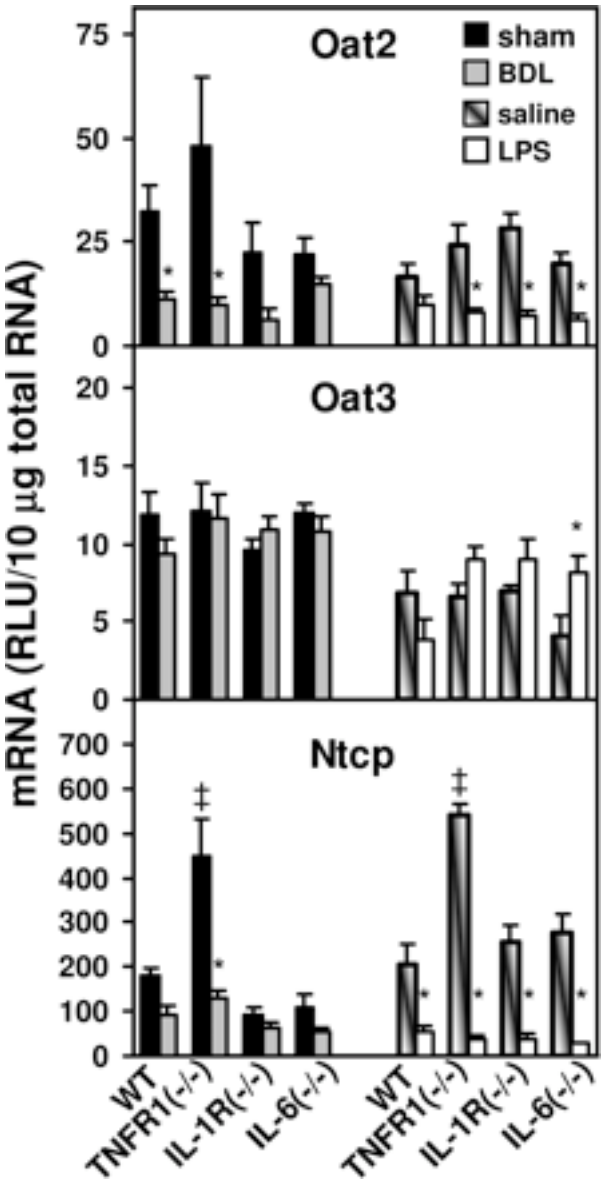

Figure 1. Hepatic Oat2, Oat3, and Ntcp mRNA expression in WT, TNFR1(–/–), IL1-RI(–/–), and IL-6(–/–) mice after BDL and LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data are expressed as relative light units (RLU) ± S.E.M. *, significance from sham operation/saline treatment ($p \leq 0.05$). ‡, significantly different from sham operation/saline treatment ($p \leq 0.05$).

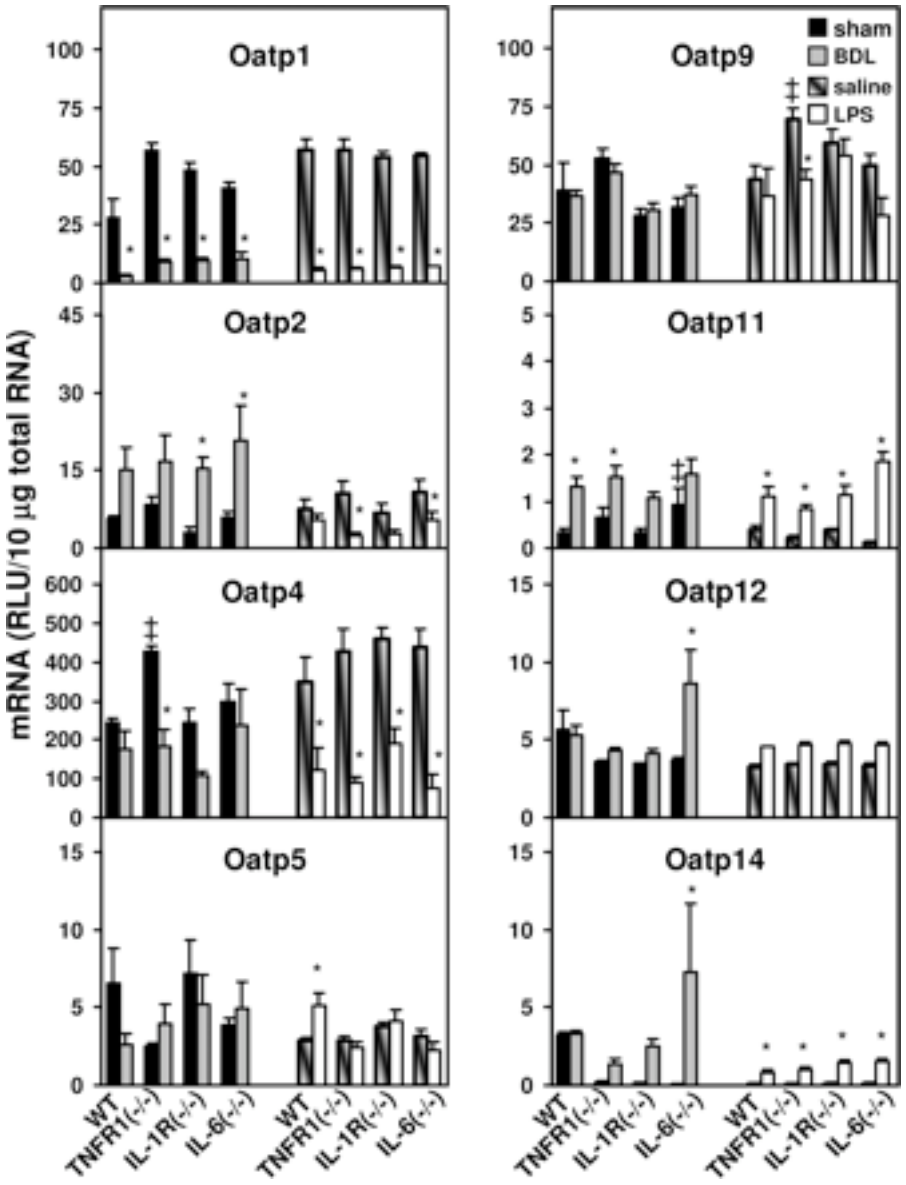

Figure 2. Hepatic Oatp family mRNA expression in WT, TNFR1(–/–), IL1-RI(–/–), and IL-6(–/–) mice after BDL and LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data are expressed as relative light units (RLU) ± S.E.M. *, significance from sham operation/saline treatment ($p \leq 0.05$). ‡, significantly different from sham operation/saline treatment ($p \leq 0.05$).

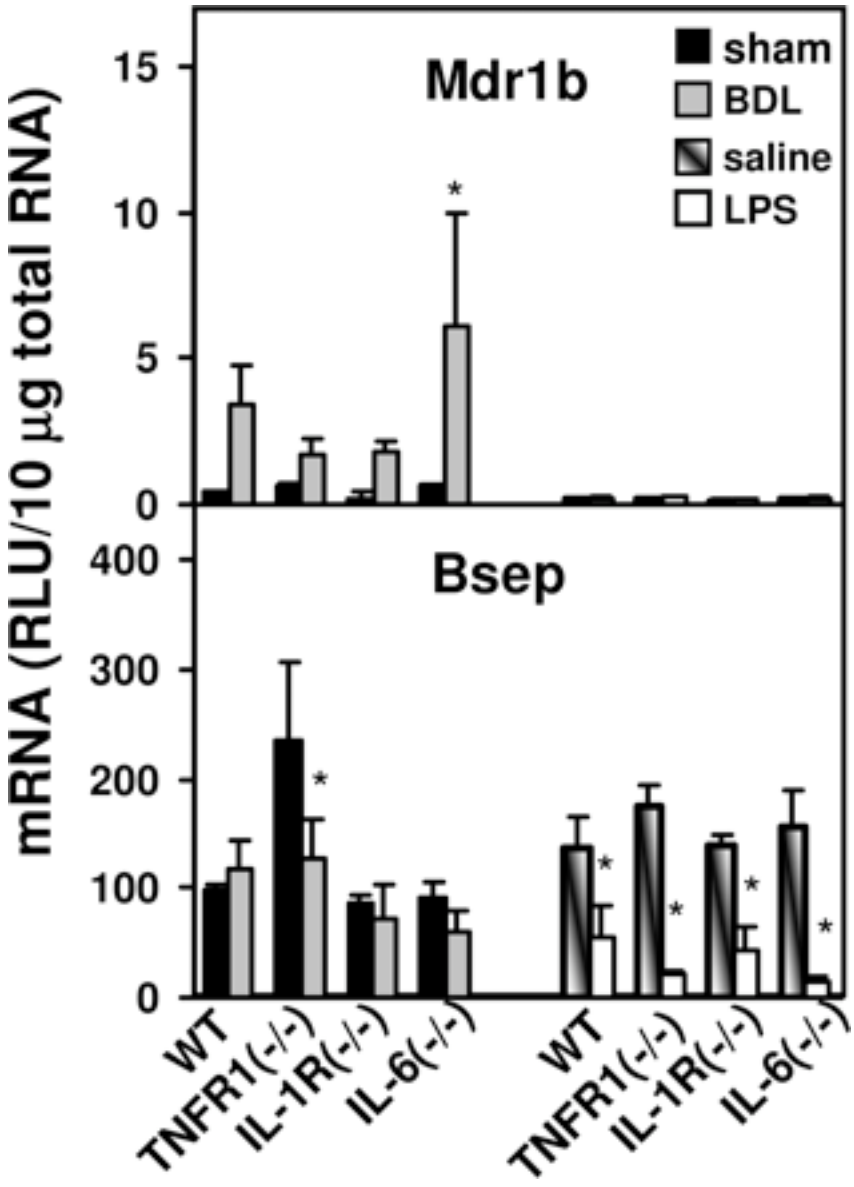

Figure 3. Hepatic Mdr1b and Bsep mRNA expression in WT, TNFR1(–/–), IL1-RI(–/–), and IL-6(–/–) mice after BDL and LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data are expressed as relative light units (RLU) ± S.E.M. *, significance from sham operation/saline treatment ($p \leq 0.05$).

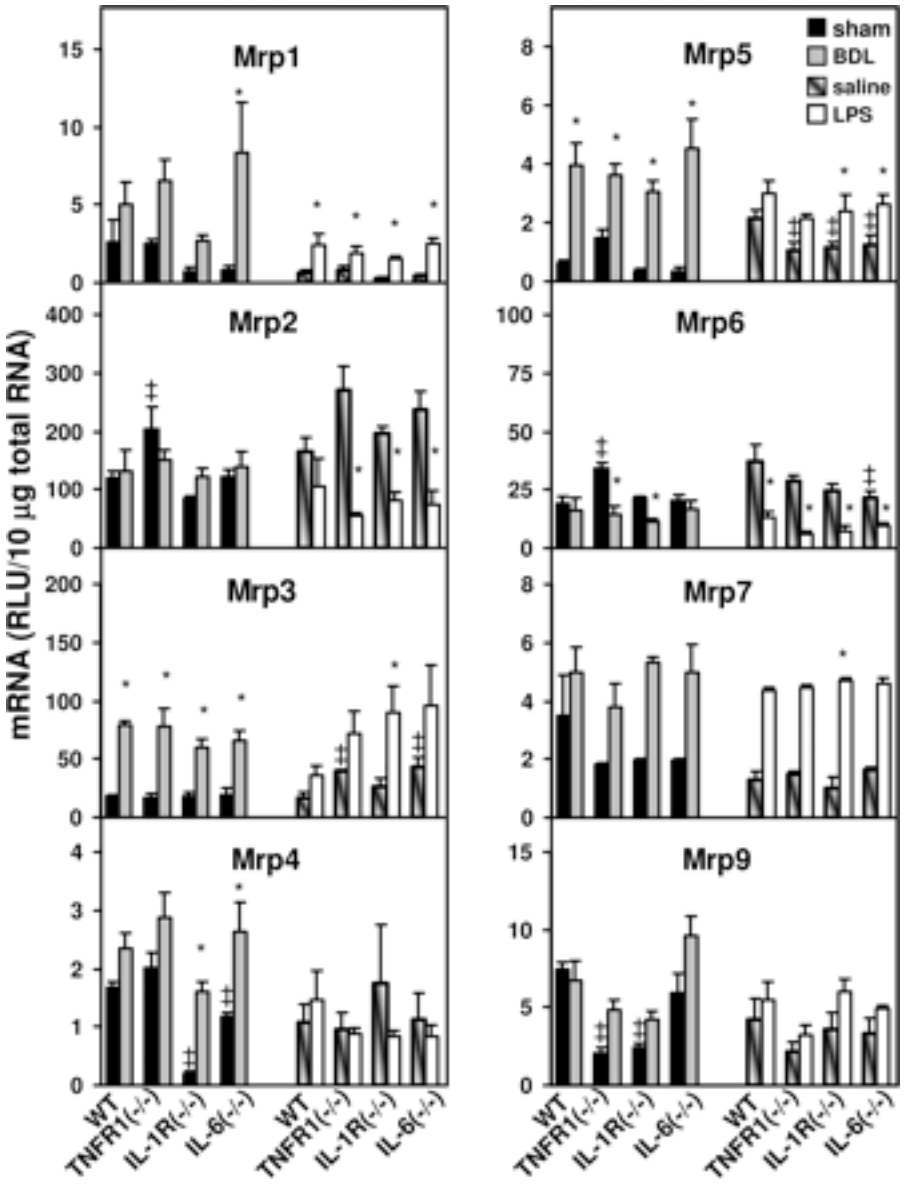

Figure 4. Hepatic Mrp family mRNA expression in WT, TNFR1(–/–), IL1-RI(–/–), and IL-6(–/–) mice after BDL and LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data are expressed as relative light units (RLU) ± S.E.M. *, significance from sham operation/saline treatment ($p \leq 0.05$). ‡, significantly different from sham operation/saline treatment ($p \leq 0.05$).

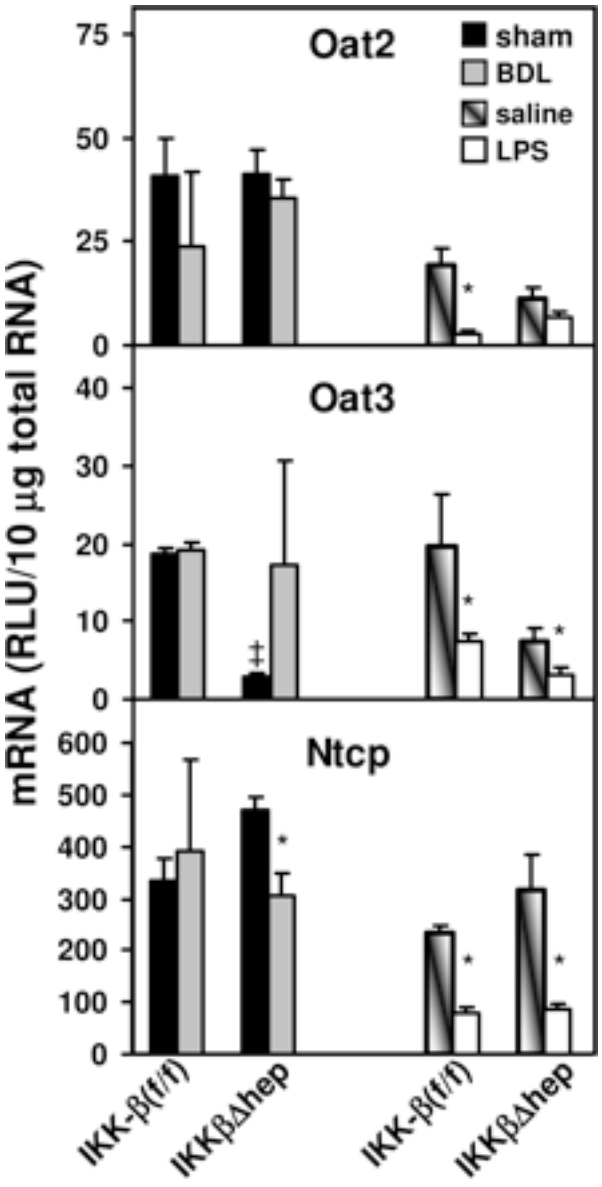

Figure 5. Hepatic Oat2, Oat3, and Ntcp mRNA expression in IKK-β(f/f) and IKK-βΔhep mice after BDL or LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data are expressed as relative light units (RLU) ± S.E.M. *, significance from sham operation/saline treatment ($p \leq 0.05$). ‡, significantly different from IKK-β(f/f) sham operation/saline treatment ($p \leq 0.05$).

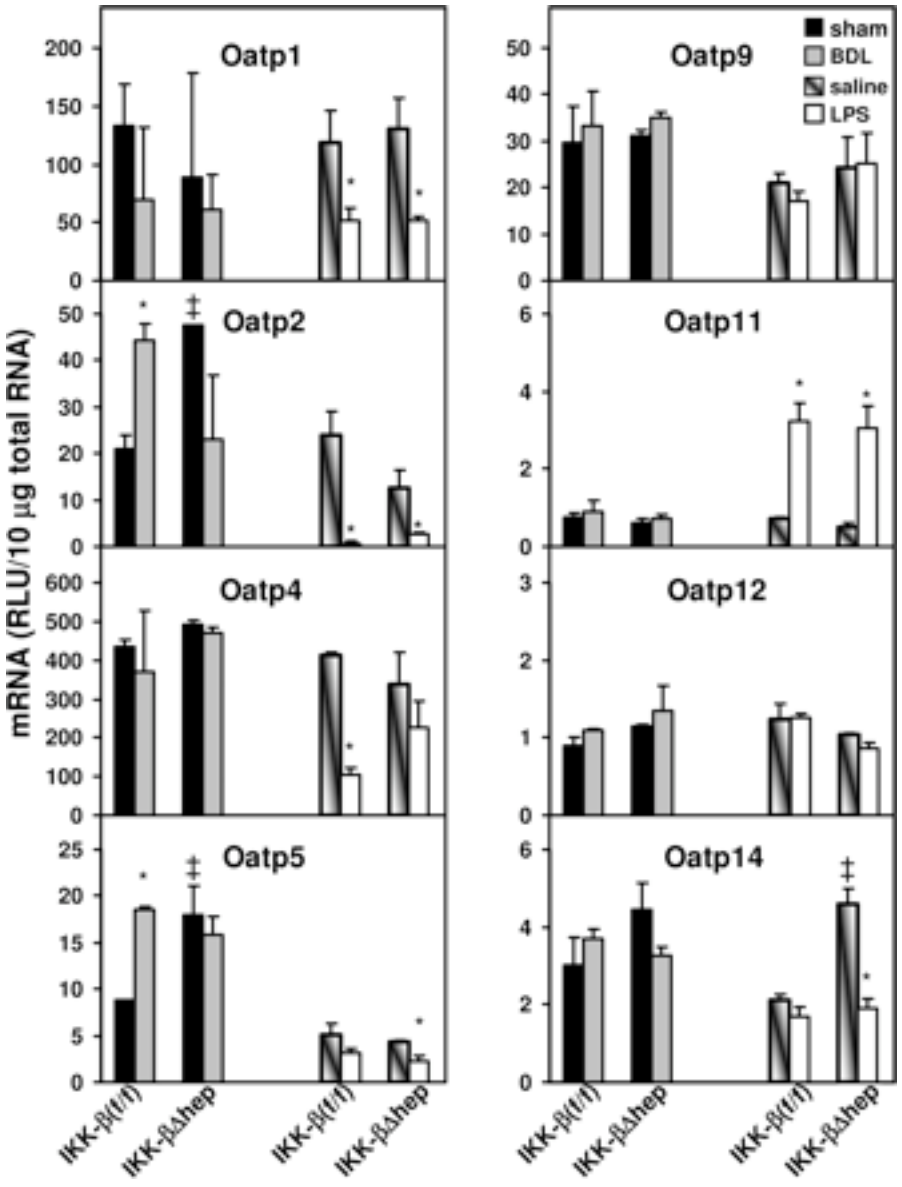

Figure 6. Hepatic Oatp family mRNA expression in IKK-β(f/f) and IKK-βΔhep mice after BDL or LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data are expressed as relative light units (RLU) ± S.E.M. *, significance from sham operation/saline treatment ($p \leq 0.05$). ‡, significantly different from IKK-β(f/f) sham operation/saline treatment ($p \leq 0.05$).

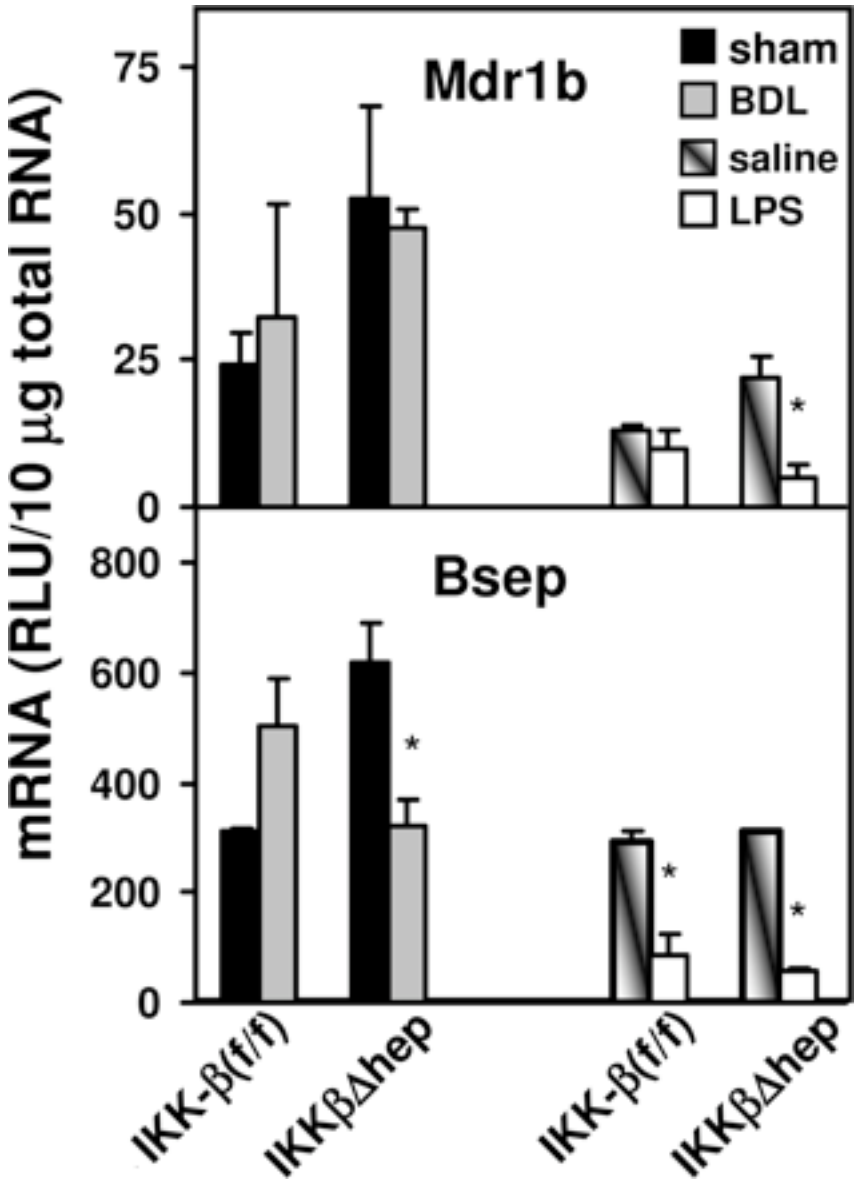

Figure 7. Hepatic Oat2, Oat3, and Ntcp mRNA expression in IKK-β(f/f) and IKK-βΔhep mice after BDL or LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data are expressed as relative light units (RLU) ± S.E.M. *, significance from IKK-β(f/f) sham operation/saline treatment ($p \leq 0.05$).

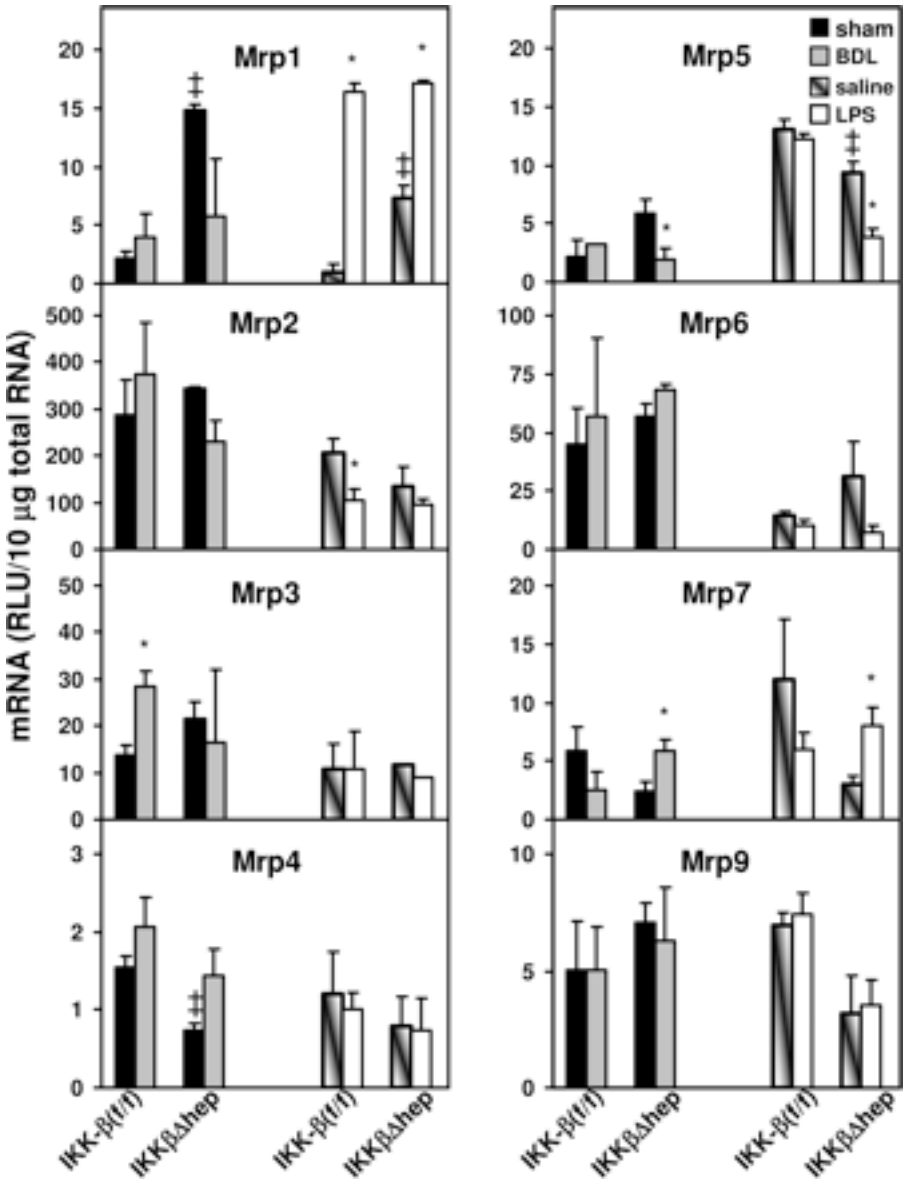

Figure 8. Hepatic Mrp family mRNA expression in IKK-β(f/f) and IKK-βΔhep mice after BDL or LPS administration. Total hepatic RNA from male C57BL6/J mice that had undergone BDL/sham operation or LPS/vehicle treatment (4 mg/kg) was analyzed by the bDNA assay. Data are expressed as relative light units (RLU) ± S.E.M. *, significance from sham operation/saline treatment ($p \leq 0.05$). ‡, significantly different from IKK-β(f/f) sham operation/saline treatment ($p \leq 0.05$).

APPENDIX C: GENES OF THE ANTIOXIDANT RESPONSE UNDERGO UPREGULATION IN A RODENT MODEL OF NONALCOHOLIC STEATOHEPATITIS.

Abstract

Nonalcoholic fatty liver disease encompasses a spectrum of hepatic pathologies ranging from simple fatty liver to an inflammatory state known as nonalcoholic steatohepatitis (NASH). NASH is also characterized by severe hepatic oxidative stress. The goal of this study was to determine whether genes of the antioxidant response are induced in rodent models of nonalcoholic fatty liver disease. To simulate simple fatty liver and NASH, respectively, male Sprague-Dawley rats were fed a high-fat (HF) or a methionine and choline-deficient (MCD) diet for 8 weeks. Key marker genes of the antioxidant response that are known to undergo upregulation via activation of Nuclear Factor Erythroid 2-Related Factor 2 were measured using the branched DNA signal amplification assay. Messenger RNA levels of the antioxidant response, including NAD(P)H:quinone oxidoreductase-1 (Nqo1), Glutamate cysteine ligase catalytic (Gclc), and Heme oxygenase-1 (Ho-1), were significantly induced in MCD rat liver but not in HF rat liver. Furthermore, Nqo1 protein expression and activity underwent significant upregulation in MCD rat liver but not in HF rat liver. These data strongly indicate that the pathology induced by the MCD dietary model of NASH results in upregulation of the antioxidant response in rats.

Abbreviations

Nonalcoholic Fatty Liver Disease (NAFLD); Nonalcoholic Steatohepatitis (NASH); Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2); NAD(P)H:quinone oxidoreductase-1 (Nqo1); Epoxide hydrolase (Eh); Heme Oxygenase-1 (Ho-1); Glutamate Cysteine Ligase Catalytic (Gclc); Methionine and Choline-Deficient (MCD).

Introduction

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of hepatic histological lesions ranging from simple hepatic steatosis to hepatic steatosis with inflammation, i.e. nonalcoholic steatohepatitis (NASH). The progression of NASH is invariably characterized by hepatocyte injury via ballooning degeneration, fibrosis, inflammatory infiltrates, and Mallory's hyaline. Recent estimates indicate that in the United States the prevalence rates are in the range of 17%–33% for NAFLD and 5.7%–17% for NASH (McCullough, 2006). NASH is a clinically relevant pathology because it may progress to end-stage liver disease (cirrhosis), requiring transplant.

Although the etiology of NASH is not completely understood, it is generally accepted that a critical baseline of steatosis requires a second "hit" of oxidative stress that promotes the subsequent inflammation and fibrosis of NASH. There is also substantial evidence that this oxidative stress persists during the progression of NASH. For example, oxidative stress parameters, including serum peroxide levels and a measure of the mean oxidative stress index of serum in

human NASH patients have been found to correlate strongly with the degree of liver fibrosis (Horoz et al., 2005). Furthermore, mRNA levels of Heme Oxygenase-1 (HO-1), the protein product of which functions to confer cytoprotection against oxidative injury, has been observed to correlate in a positive manner with the severity of the liver pathology in human patients with NASH. Importantly, HO-1 is a key marker gene for the cellular response to oxidative stress and is one among a larger battery of genes that are inducible via activation of the transcription factor Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2). In this same group of patients, hepatic GSH levels were depleted by an average of 57% (Malaguarnera et al., 2005).

Consistent with the human disease state, the methionine and choline-deficient (MCD) dietary model of NASH has been shown to result in reduced GSH levels in the whole rat liver. Moreover, the histologic changes that occur in the MCD model have been thoroughly characterized and determined to be remarkably similar to those seen in human NASH. For example, fibrogenesis progresses from the centrilobular (zone 3) location, with fibrotic strands enveloping steatotic hepatocytes. While there are likely differences between the factors that cause steatosis after feeding the MCD diet and in NASH, the sequence of steatosis, chronic hepatocyte injury and hepatic inflammation that precede stellate cell activation and fibrosis are also analogous to the human disease (George et al., 2003).

There is relatively little information regarding the redox status of the liver in models of NAFLD, including NASH, in terms of the antioxidant response. Therefore, the purpose of this study was to examine the mRNA expression levels of critical marker genes of the antioxidant response, including Epoxide hydrolase (Eh), Glutamate-cysteine ligase, catalytic subunit (Gclc), Heme oxygenase-1 (Ho-1), and NAD(P)H:quinone oxidoreductase-1 (Nqo1). Nqo1 protein expression and activity levels were also assessed.

Materials and Methods

Materials. Tris base, sodium dodecyl sulfate, sodium chloride, and sucrose were purchased from Fisher Scientific (Pittsburgh, PA). 2,6-Dichlorophenol-indophenol (DCPIP), dicumarol, sucrose, Tris-hydrochloride, and nicotinamide adenine dinucleotide phosphate in the reduced form (NADPH) were obtained from Sigma Chemical Co. (St. Louis, MO).

Treatment of Animals. Male Sprague-Dawley rats weighing 200–250 g were purchased from Harlan Laboratories Inc. (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment in hanging cages with hardwood chips. Rats were randomly placed into one of three diet treatment groups (n = 5–9/group): control group to simulate healthy liver (Harlan Teklad 4% Mouse/Rat Diet; Madison, WI), high-fat (HF) group to simulate simple fatty liver (SFL) [18% butter (w/w) diet] and MCD group to simulate NASH (methionine-, choline-deficient diet) (Dyets Incorporated, Bethlehem, PA) ad libitum for 8 weeks. The Institutional Animal Care and Use Committee of the

University of Arizona approved this animal study. Most importantly, histologic analyses of H&E-stained sections are indicative of two distinct pathologies that correspond with simple hepatic steatosis and early-stage NASH in human patients (unpublished observations).

RNA Extraction. Total RNA from liver tissue was extracted using RNAzol B reagent (Tel-Test Incorporated, Friendswood, TX) according to the manufacturer ’s protocol. The quality of RNA samples was judged by the integrity and relative ratio of 28S and 18S rRNA bands following agarose gel electrophoresis.

Messenger RNA Expression Analysis. Eh, Gclc, Ho-1, and Nqo1 mRNA levels in rat liver were measured using the branched DNA signal amplification assay. Oligonucleotide probe sets containing multiple mRNA transcript-specific capture, label, and blocker probes were designed using ProbeDesigner software v2.0 (Bayer Corporation-Diagnostics Division). The probe sets used in this study have been described previously [5,6].

Total RNA (1 µg/µL; 10 µL/well) was added to each well of a 96-well plate containing capture hybridization buffer and 50 µL of each diluted probe set. For each gene, total RNA was allowed to hybridize to the probe set overnight at 53º C. Subsequent hybridization and quantitation steps were performed according to the manufacturer ’s protocol (QuantiGene Reagent System, Panomics, Fremont, CA).

Western Blot Analysis of Nqo1 Protein Expression. Liver cytosolic protein preparations were made as described previously (Campbell et al., 1987). Protein concentrations were determined using the BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Liver cytosol proteins (30 µg/lane) were electrophoretically resolved on tris-glycine, SDS-polyacrylamide gels (10% resolving gel, 4% stacking gel). Proteins were transblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were incubated with primary antibody (rabbit anti-rat Nqo1, diluted 1:500) overnight at 4º C and secondary antibody (goat anti-rabbit horseradish peroxidase-conjugated, diluted 1:2000) for 1 h at room temperature. Protein-antibody complexes were detected using ECL Western blotting reagents (Amersham Biosciences Inc., Piscataway, NJ) and Blue Lite Autoradiography Film (ISC BioExpress, Kaysville, UT).

NQO1 Activity Assay. NQO1 activity was calculated by measuring the colorimetric oxidation of NADPH to NADP+, using 2,6-dichlorophenolindophenol (DCPIP) as substrate. The disappearance of NADPH was measured at 600 nm for over 1 min. NQO1 activity was measured in 1-ml reactions (27º C) containing liver cytosol, 200 µmol/L NADPH, 40 µmol/L DCPIP, and Tris-HCl buffer (25 mmol/L Tris-HCl [pH 7.4], 0.7 mg/ml bovine serum albumin). Parallel reactions were performed in the presence of 20 µmol/L dicumarol. The rate of dicumarol-sensitive NQO1 activity was determined as the difference between the uninhibited and dicumarol-inhibited rates and was normalized to total cytosolic protein as previously described (Aleksunes et al., 2006).

Statistics. For mRNA and protein expression and activity assays, statistical differences from the control group were determined using a one-way analysis of variance followed by Duncan's Multiple Range post hoc test. Statistical differences were determined by two-way analysis of variance followed by Duncan's multiple range post hoc test ($p \leq 0.05$).

Results

mRNA levels of antioxidant response genes in the liver following 8-week treatment with HF and MCD diets, respectively. Figure 1 shows that in HF group rats, only mRNA levels of Eh were significantly increased (1.9-fold). In contrast, in MCD diet-treated rats, all four antioxidant response genes also underwent significant upregulation, including Eh (230%), Gclc (360%), Ho-1 (260%), and Nqo1 (400%).

Nqo1 protein expression and activity levels in the liver following 8-week treatment with HF and MCD diets, respectively. Figure 2 shows that there were no changes in the protein expression levels of Nqo1 in the liver of neither HF nor MCD diet-treated rats. However, activity levels of Nqo1 in the livers of MCD diet-treated rats were significantly increased by 88%.

Discussion

In the present study, NAFLD was recapitulated in rats in two of its prominent forms, SFL and NASH, respectively. It is thus important to note that the current models of NAFLD (i.e., HF and MCD diets) provided accurate histologic representations of SFL and early-stage nonalcoholic steatohepatitis

(NASH) as it is observed in humans, respectively. While the HF diet was successful in inducing hepatic steatosis, there was an apparent lack of induction of the antioxidant response, indicating the absence of oxidative stress during hepatic steatosis. In contrast, 8-week treatment of rats with the MCD diet resulted in transcriptional upregulation of prototypical marker genes of the antioxidant response.

The term ‘oxidative stress’ comprises a broad spectrum of conditions that cause a change in the redox balance of a cell. It generally results from increased production of free radical species within the cell or following treatment with pro-oxidant xenobiotics (Nguyen et al., 2004). In line with this, genes that are typically induced by oxidative stress encode proteins that help control the cellular redox balance and defend the cell. Transcription of many of these enzymes is coordinately regulated through antioxidant response elements (AREs) in the respective gene promoters, with Nrf2 being the transcription factor that binds to AREs to activate these genes. Example genes include the four examined in the current study: Eh, Gclc, Ho-1, and Nqo1.

Eh, a microsomal enzyme that first became known for its ability to detoxify genotoxic epoxides, has also been shown to be induced under conditions of oxidative stress, including GSH depletion (Jerina et al., 1974; Kang et al., 2002). Ho-1, which performs the rate-limiting step in heme degradation, undergoes robust induction under conditions of oxidative stress, e.g. 67-fold induction of Ho-1 mRNA levels in liver following hepatic GSH depletion in rats (Bauer et al.,

1998). NQO1, a cytosolic enzyme possessing the ability to reduce a variety of endogenous quinones, is comparably induced by the oxidative stress of experimental cholestasis (Aleksunes et al., 2006; Cadenas et al., 1992). Consistent with a role for Nrf2-mediated induction, Eh, Ho-1, and Nqo1 have each also been shown to undergo upregulation in rat liver in response to compounds that activate Nrf2, such as trans-stilbene oxide (Slitt et al., 2006). Similarly, Gclc, which codes for the catalytic subunit of Glutamate-cysteine ligase (Gcl), the rate-limiting enzyme of GSH synthesis, can be activated by the pro-oxidant xenobiotic tert-butylhydroquinone, apparently in an Nrf2-dependent manner (Yang et al., 2005).

The induction of oxidative stress by the MCD diet is adequately supported by other studies that have employed this model. For example, lipid peroxidation has been observed to be increased approximately 20-fold and GSH levels reduced by 34% in the hepatocytes of rats fed the MCD diet for 5 weeks (George et al., 2003). In line with these observations, the current study has revealed that at least four genes of the oxidative stress-inducible antioxidant response undergo transcriptional upregulation during experimental NASH. Furthermore, activity levels of Nqo1 underwent significant induction, indicating that hepatocytes have retained an important cellular defense capacity against the oxidative stress during experimental NASH. Thus, a better understanding of the role of Nrf2 during experimental NASH may assist in improving our understanding of the

human disease, or possibly provide a novel target for hepatoprotection during NASH.

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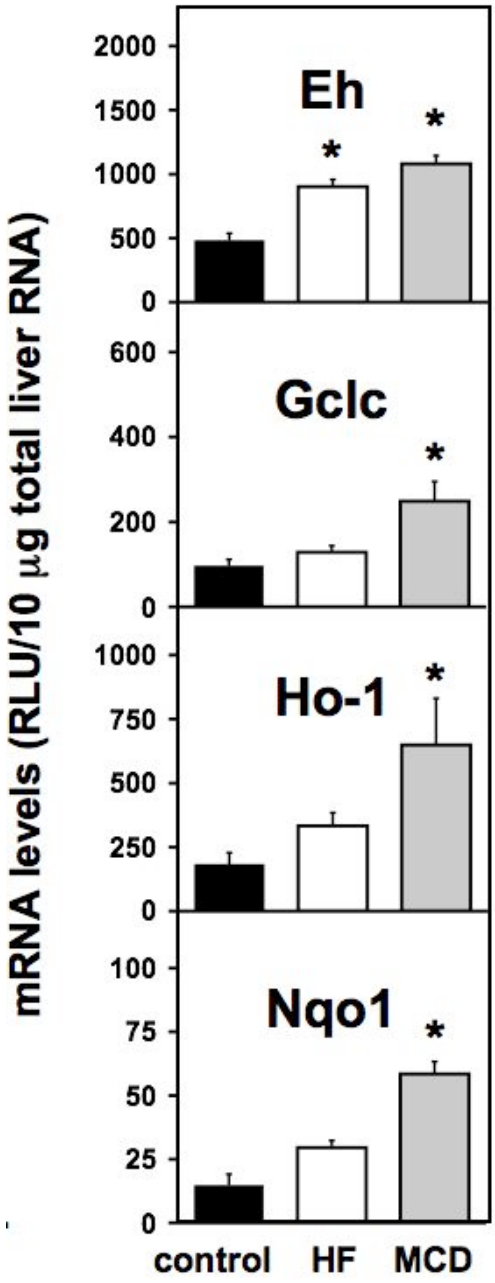

Figure 1. Messenger RNA levels of antioxidant response genes in the liver following 8-week treatment with high-fat and MCD diets, respectively. Following 8 weeks on a control, high-fat, or MCD diet, total RNA was isolated from the liver of male Sprague-Dawley rats. Levels of mRNA were analyzed by the bDNA assay and expressed as relative light units (RLU) ± standard error of the mean (SEM) (n = 5). Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

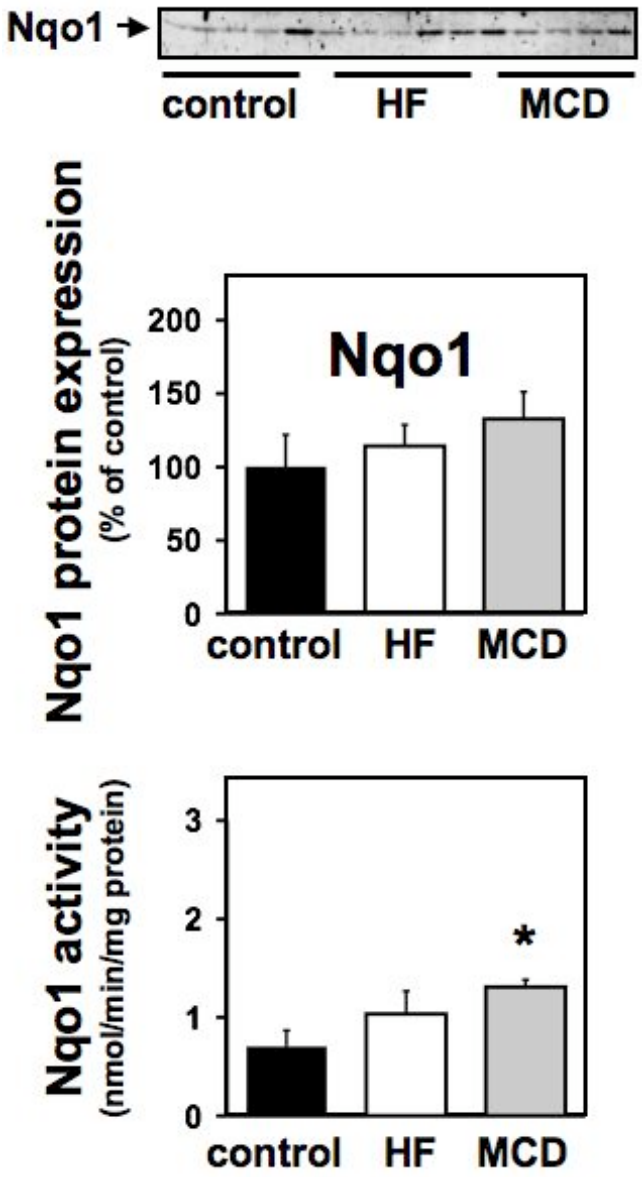

Figure 2. Nqo1 protein expression and activity levels in the liver following 8-week treatment with high-fat and MCD diets, respectively. Following 8 weeks on a control, high-fat, or MCD diet, liver cytosol fractions from male Sprague-Dawley rats were analyzed by Western blot (30 µg of protein/lane). Protein bands were quantified using ImageJ software and are expressed as percentage of the control group ± SEM (n = 5). Nqo1 activity was also analyzed in liver cytosolic fractions from all three treatment groups. The data are presented as nanomoles of DCPIP reduced per minute per milligram of protein ± SEM (n = 5). Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

APPENDIX D: EXPRESSION OF DRUG METABOLIZING ENZYMES DURING EXPERIMENTAL NONALCOHOLIC FATTY LIVER DISEASE

Abstract

Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of pathologies known to progress from simple fatty liver (SFL) to the severe disease of nonalcoholic steatohepatitis (NASH). Importantly, a number of medical conditions (e.g. type 2 diabetes, dyslipidemia, hypertension) are commonly associated with NAFLD. It is thus established that a significant portion of NAFLD patients currently takes a variety of prescription medications. The vast majority of the 2 million adverse drug reactions that occur annually in the U.S. are thought to be the result of environmental factors such as disease state and nutritional status. Phase 1 and 2 drug metabolizing enzymes are essential for the conversion of drugs into hydrophilic, more readily excretable metabolites. The purpose of this study was to determine the effect of NAFLD on the expression of a number of the major DMEs in rat liver. To simulate SFL and NASH, rats were fed either a high-fat (HF) or a methionine-, choline-deficient (MCD) diet for 8 weeks. In MCD liver, branched DNA analysis revealed a 41% increase in Cyp2e1 and a 55% decrease in Cyp2C11. Western blot analysis of HF liver revealed a 38% decrease in Cyp2E1 protein expression and an 83% decrease in Cyp2c11. In MCD liver, protein expression of Cyp2C11 was decreased by 91%. In combination with the changes in drug transporter expression that are known to occur during NASH, accompanying alterations in drug metabolism may also contribute to alterations

in the route of drug elimination, efficacy and safety of various clinically relevant drugs in NASH patients. (DK-068039, ES-07091, ES-011646)

Abbreviations

CYP, Cytochrome P450 reductase; HF, high-fat; MCD, methionine, choline-deficient; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SFL, simple fatty liver;

Introduction

NAFLD encompasses a spectrum of pathologies that range from “bland” or simple hepatic steatosis (fatty liver without inflammation) to steatohepatitis (fatty liver with inflammation). Simple hepatic steatosis is defined as macrovesicular lipid accumulation in >5% of hepatocytes (Burt, et al., 1998;Neuschwander-Tetri and Caldwell, 2003). The condition of hepatic steatosis of the liver is generally benign, meaning that it poses no immediate health concerns. However, for reasons not entirely understood, it appears that hepatic steatosis places the liver at risk for the development of inflammation (Yang, et al., 2000;Yang, et al., 2001;Yang, et al., 1997). A liver pathology that is characterized by both steatosis and inflammation is designated as having steatohepatitis (Contos and Sanyal, 2002). The term ‘nonalcoholic steatohepatitis’ or ‘NASH’ was first used in 1980 to describe histologic observations made in obese female patients who denied use of alcohol, yet had liver biopsies consistent with alcoholic hepatitis (Ludwig, et al., 1980). NASH is characterized by macrovesicular steatosis, lobular inflammation, and

hepatocellular damage, including Mallory bodies, ballooning degeneration and bridging fibrosis (Diehl, 2001).

A predominant clinical concern regarding NAFLD and especially NASH is that, if steps are not taken to control its progression, it can advance to cirrosis and liver failure requiring liver transplant (Reynaert, et al., 2005). However, an issue that is arguably of equal importance is that of drug safety in NAFLD patients. The NAFLD patient demongraphic is one that consumes a great quantitiy and variety of prescription drugs. This becomes clear when recalling the fact that many of the primary risk factors for the development of NAFLD are themselves clinical conditions that typically require pharmacologic intervention. Example clinical conditions generally include features of the metabolic syndrome, i.e. insulin resistance, dyslipidemia, and hypertension (Grundy, 2004). To place the issue in proper perspective, at least 90% of patients with NAFLD have one or more features of the metabolic syndrome, while approximately one-third have the complete syndrome (Clark and Diehl, 2003;Marchesini, et al., 2003;Pagano, et al., 2002). The fact that the effect of NAFLD on the expression of drug metabolizing enzymes has not been adequately addressed should be a cause for concern among clinicians.

The Cytochrome P450 (CYP) enzyme family is of particular importance in this case because it is responsible for the metabolic inactivation or activation of the majority of clinical drugs. It is a fact that there exists substantial interindividual variation in the activities of P450 enzymes in humans. These can result in altered

therapeutic efficacy and safety of certain medicines. However, a large component of interindivudal variability cannot be explained by genetic factors alone. In fact, only ~20% of all adverse drug reactions that occur in patients are recognized as attributable to genetic factors (Lazarou, et al., 1998). The expression of the drug metabolizing P450 enzymes is likely also influenced by a variety of environmental factors including nutrition and disease state (Renton, 2004). NAFLD and the health complications associated with it are exemplary environmental factors that may have to potential to influence drug metabolism. The current study was undertaken to determine the effect of experimental NAFLD on expression of drug metabolizing enzymes of the CYP family.

Materials and Methods

Chemicals. Tris base, sodium dodecyl sulfate, sodium chloride, and sucrose were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Treatment of Animals. Male Sprague-Dawley rats weighing 200–250 g were purchased from Harlan Laboratories Inc. (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment in hanging cages with hardwood chips. Rats were randomly placed into one of three diet treatment groups (n = 5–9/group): control group to simulate healthy liver (Harlan Teklad 4% Mouse/Rat Diet; Madison, WI), high-fat (HF) group to simulate simple fatty liver (SFL) [18% butter (w/w) diet] and MCD group to simulate NASH (methionine-, choline-deficient diet) (Dyets Incorporated, Bethlehem, PA) ad

libitum for 8 weeks. The Institutional Animal Care and Use Committee of the University of Arizona approved this animal study. Importantly, liver sections from rats treated for 8 weeks with the respective HF and MCD diets have been subjected to histologic analysis with a scoring system that was developed by the NASH Clinical Research Network (Kleiner, et al., 2005). Results of the analysis, which included parameters of steatosis, lobular inflammation, and ballooning degeneration, were previously demonstrated to be indicative of two distinct NAFLD pathologies, such that correspond satisfactorily with simple hepatic steatosis and early-stage NASH in human patients (Lickteig, et al., 2007).

RNA Extraction. Total RNA from liver tissue was extracted using RNAzol B reagent (Tel-Test Incorporated, Friendswood, TX) according to the manufacturer ’s protocol. The quality of RNA samples was judged by the integrity and relative ratio of 28S and 18S rRNA bands following agarose gel electrophoresis.

Branched DNA Analysis. Cyp1a2, Cyp2b1, Cyp2c11, Cyp2d1, and Cyp2e1 mRNA levels in rat liver were measured using the branched DNA signal amplification assay. Oligonucleotide probe sets containing multiple mRNA transcript-specific capture, label, and blocker probes were designed using ProbeDesigner software v2.0 (Bayer Corporation-Diagnostics Division). The probe sets used in this study have been described previously (Hartley and Klaassen, 2000;Buist, et al., 2003).

Total RNA (1 µg/µL; 10 µL/well) was added to each well of a 96-well plate containing capture hybridization buffer and 50 µL of each diluted probe set. For each gene, total RNA was allowed to hybridize to the probe set overnight at 53º C. Subsequent hybridization and quantitation steps were performed according to the manufacturer 's protocol (QuantiGene Reagent System, Panomics, Fremont, CA).

Western Blot Analysis of Cytochrome P450 Protein Expression. Rat liver microsomes were isolated according to the method of Lake (Lake, 1987). Protein concentrations were determined using the BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Liver microsome proteins (30 µg/lane) were electrophoretically resolved on tris-glycine, SDS-polyacrylamide gels (10% resolving gel, 4% stacking gel). Proteins were transblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Primary antibody clones, membrane incubation times and dilutions in 5% nonfat dry milk were as follows. For Cyp1a2, conditions included a 1:200 dilution of goat polyclonal antibodies (S-18; sc-9835) (Santa Cruz Biotechnology; Sant Cruz, CA, USA) for 1 h at RT. For Cyp2c11, conditions included a 1:1500 dilution of rabbit polyclonal antibodies (ab3571) (Abcam; Cambridge, MA, USA) for 1 h at RT. For Cyp2d1, conditions included a 1:5000 dilution of rabbit polyclonal antibodies (CR 3210) (Biomol International, LP; Plymouth Meeting, PA, USA) for 1 h at RT. For Cyp2e1, conditions included a 1:5000 dilution of rabbit polyclonal antibodies (ab4239) (Abcam, Inc.; Cambridge, MA, USA) for 1 h at RT. The secondary antibody dilutions included

1:20,000 (Cyp1a2, Cyp2c11, and Cyp2d1) and 1:5000 (Cyp2e1). Protein-antibody complexes were detected using ECL Western blotting reagents (Amersham Biosciences Inc.; Piscataway, NJ) and Blue Lite Autoradiography Film (ISC BioExpress; Kaysville, UT).

Statistical Analysis. For mRNA and protein expression analyses, statistical differences between diet groups were determined using a one-way analysis of variance followed by Duncan's Multiple Range post hoc test.

Results

mRNA expression levels of cytochrome P450 enzymes after 8 weeks of feeding on control, HF, and MCD diets, respectively. Figure 1 illustrates mRNA expression levels of cytochrome P450 enzymes in rat liver following 8 weeks of treatment with the respective HF and MCD diets. In the HF group, mRNA levels of Cyp1a2, Cyp2b1, Cyp2c11, Cyp2d1, and Cyp2e1 are not altered. In the MCD group, mRNA levels of Cyp2c11 are decreased by 55%, whereas Cyp2e1 mRNA levels were increased by 41%.

Protein expression levels of cytochrome P450 enzymes after 8 weeks of feeding on control, HF, and MCD Diets, respectively. Figure 2 illustrates the effect of 8 weeks of feeding on the respective HF and MCD diets on protein expression levels of cytochrome P450 enzymes in rat liver. In the HF group, protein levels of Cyp2c11 and Cyp2e1 are decreased by 83% and 38%, respectively. In the MCD group, Cyp2c11 are decreased by 91%.

Discussion

The goal of the current study was analyze the expression of a number of drug metabolizing enzymes of the CYP family during experimental NAFLD. While the expression of a select few CYP enzymes was demonstrated to be altered during experimental NAFLD, it is important to begin by placing weight on the models utilized here. In order for the current study to have been able to provide potentially useful information for clinicians, the HF and MCD models would have to be an accurate model of the expression of drug metabolizing enzymes in human NAFLD patients. The MCD model of NASH was selected for the current study because chronic methionine and choline deficiency in the rat results fibrosing steatohepatitis characterized by zone 3 steatosis and fibrosis. The histologic picture in the MCD model closely mimics that seen in human fibrotic disorders associated with hepatic lipid accumulation and the sequence of events that lead to this pathology are analogous to that which occurs in NASH (George, et al., 2003). Importantly, 8 weeks' treatment of rats with the MCD diet results in a pathology that corresponds to early stage NASH in human patients (Lickteig, et al., 2007).

While there is relatively little published information describing the effect of NAFLD on the drug metabolism capacity of the liver, one enzyme that has been studied extensively is CYP2E1. CYP2E1 up-regulation has been recurrently observed in humans to be associated with NAFLD and obesity, including an increase in hepatocellular CYP2E1 expression in patients with NASH, the advanced form of NAFLD (Weltman, et al., 1998). It has even been suggested

that steatohepatitis associated with morbid obesity is mediated, in part, by CYP2E1 induction and CYP2E1-mediated oxidative injury (Robertson, et al., 2001).

One of the more surprising results in this study is the down-regulation of Cyp2e1 expression in rats fed the HF diet. This is inconsistent with previous observations that diet-induced obesity in rats increases liver Cyp2e1 protein expression, thereby enhancing the metabolism of Cyp2e1 substrates (Raucy, et al., 1991). This could lead one to speculate that differences in dietary nutrient content are responsible for the differential effects of hepatic steatosis on Cyp2e1 protein levels in rats. Whereas the study by Raucy et al., induced hepatic steatosis via treatment with a diet composed of 60% vegetable shortening (w/w), the HF diet applied in the current study contained 18% butter (w/w). It is likely that an investigation into the types of fat present (e.g. unsaturated, monounsaturated, polyunsatured, etc.) in these rat livers would reveal markedly different contents between each steatosis-inducing diet.

The absence of altered protein expression of Cyp2e1 in the MCD diet-treated rats is also an interesting in the context of previous work. Specifically, in Wistar rats, following 2 and 6 weeks of MCD diet treatment, Cyp2e1 protein expression is decreased 18% and 63%, respectively. That particular study seemed to indicate that Cyp2e1 activity does not contribute to the advancement of the MCD rat liver pathology from steatosis to steatohepatitis (Starkel, et al., 2003). However, in the Sprague-Dawley strain (also used in the current study),

MCD diet treatment produces a significant increase in Cyp2e1 protein and activity after 4 weeks of treatment (Weltman, et al., 1996). It is possible that the effect of the MCD diet on Cyp2e1 expression is partially strain-dependent, but the expression may also vary considerably during the time-course of the treatment.

Determining the mechanisms responsible for the rat Cyp2e1 regulation following the diet treatments was not a focus of the current study. Nonetheless, it is worth noting that the mRNA levels of Cyp2e1 were not reflective of protein expression levels in each of the NAFLD models. In HF rats, Cyp2e1 protein expression underwent a decrease in the absence of any alteration in Cyp2e1 mRNA levels. Inflammation is one factor that can safely be excluded as a possibility, i.e. the histologic analysis provided no such evidence that it was present. It also remains possible that post-transcriptional and/or post-translational regulation may be responsible for the decreased protein levels. Previous studies have demonstrated that the Cyp2e1 mRNA molecule contains one or more features that prevent it from efficient engagement by the eukaryotic translation manchinery (Kocarek, et al., 2000).

It is possible that the hepatic steatosis induced by the respective HF and MCD diets interferes with the translational efficiency of Cyp2e1. In MCD rats, Cyp2e1 protein levels did not undergo an increase in spite of the increase in mRNA levels. In contrast, overt inflammation is routinely observed in MCD-treated rats, and inflammation has indeed been observed to have influence on

post-transcriptional regulation of Cyp2e1 (Starkel, et al., 2003;Bissell, et al., 2001;George, et al., 2003;Hakkola, et al., 2003;Stanley, et al., 1988;Morgan, 1993).

It is true that the rat Cyp2e1 is good ortholog of the human CYP2E1 and shares numerous substrate drugs (Lewis, 2003; Kessova and Cederbaum, 2003). However, given that the expression profile of Cyp2e1 in the HF and MCD rats is not reflective of human NAFLD, neither model is likely to serve as a useful model to test the safety and therapeutic efficacy of Cyp2e1-metabolized drugs.

In both HF and MCD diet-treated rats, expression levels of Cyp2c11 (both mRNA and protein) are significantly decreased. However, because there is not a human ortholog to rat Cyp2c11, it's not possible to extrapolate the consequences of this finding. Nonetheless, it could potentially be helpful to know which regulatory mechanisms are responsible for the Cyp2c11 down-regulation, as those pathways may be activated in human NAFLD. In vitro experiments in primary rat hepatocytes demonstrate that Cyp2c11 transcription is suppressed by glucagon through its second messenger cAMP (Iber, et al., 2001). However, the likelihood of glucagon having a role in the transcriptional suppression of Cyp2c11 in HF and MCD rats in unlikely. This is because neither model is diabetic and would therefore not be expected to display high plasma levels of glucagon.

With it being understood that mRNA and protein expression levels are not always reflective of activity, it would still seem worthwhile to discuss the observation that Cyp2b1 mRNA levels were not significantly altered. Cyp2b1 has

remained the subject of intense research arguably because it is known to be a reliable marker gene of Constitutive Androstane Receptor (CAR) activation (Wei and Moore, 2000). However, CAR is known to activate more hepatic drug metabolizing enzymes and pathways in addition to Cyp2b1 transcription (Guo, et al., 2003;Stanley, et al., 2006;Costa, et al., 2005;Dixit, et al., 2005). Knowing that CAR is apparently not activated nonetheless also adds to our understanding of the HF and MCD models of NAFLD.

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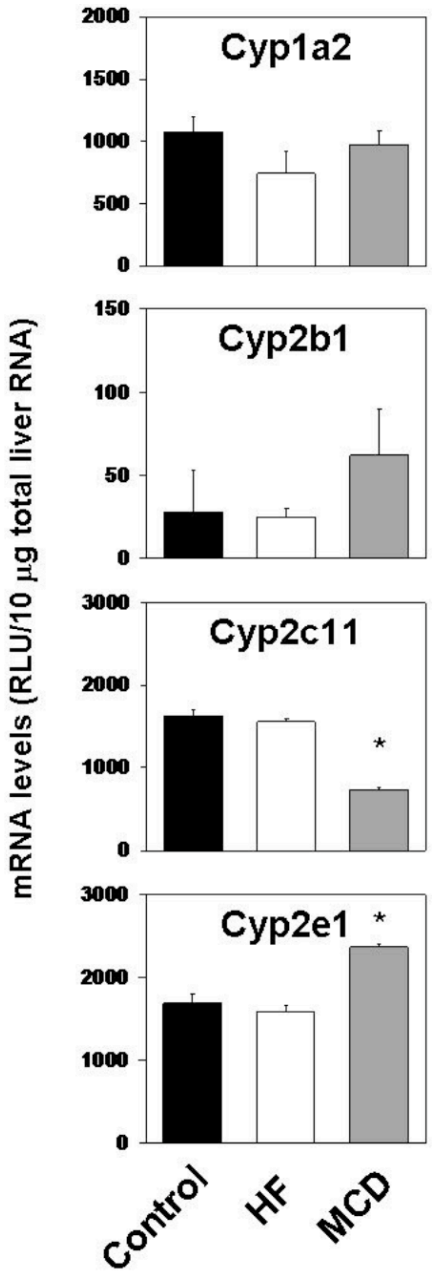

Figure 1. mRNA levels of cytochrome P450 enzymes in the liver following 8-week treatment with high-fat and MCD diets, respectively. Following 8 weeks on a control, high-fat, or MCD diet, total RNA was isolated from the liver of male Sprague-Dawley rats. Levels of mRNA were analyzed by the bDNA assay and expressed as relative light units (RLU) ± standard error of the mean (SEM) (n = 5). Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

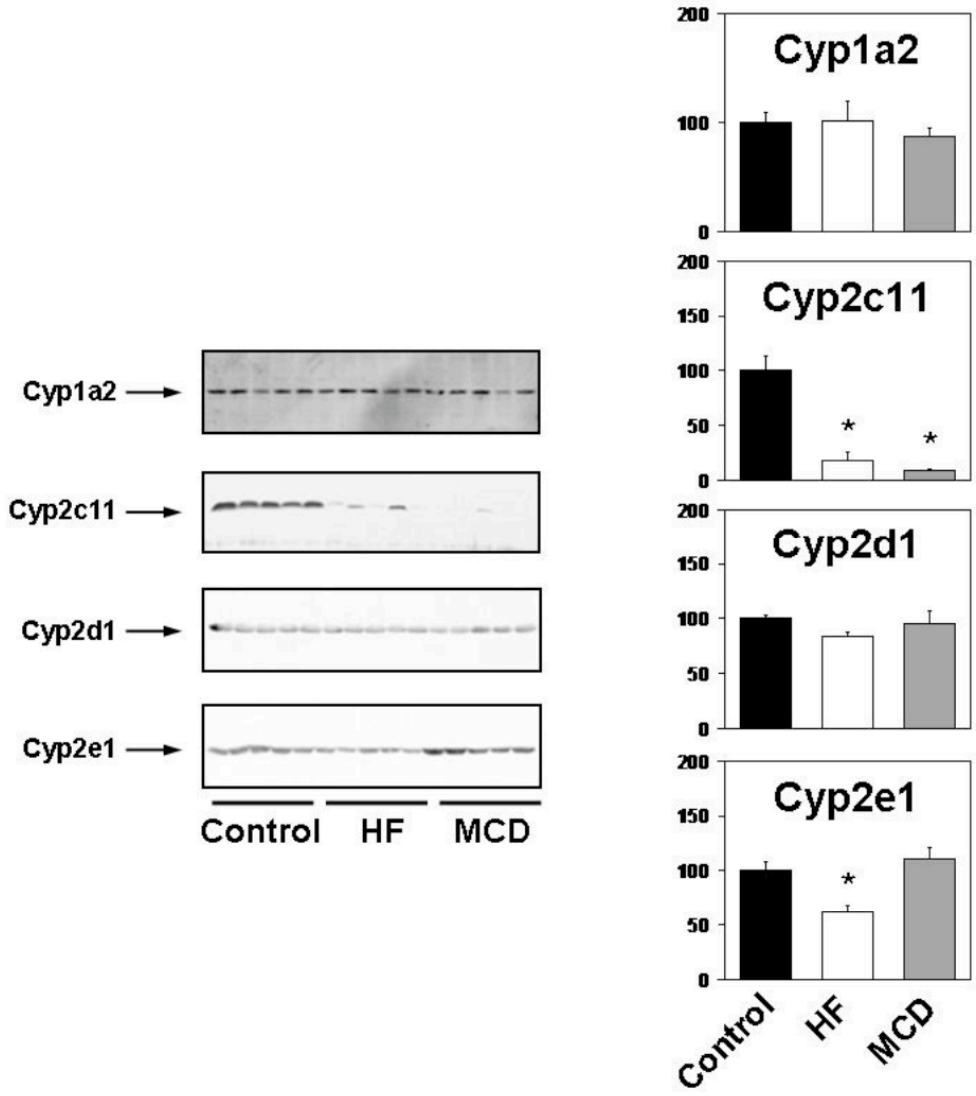

Figure 2. Protein levels of cytochrome P450 enzymes in the liver following 8-week treatment with high-fat and MCD diets, respectively. Following 8 weeks on a control, high-fat, or MCD diet, liver crude membrane fractions from male Sprague-Dawley rats were analyzed by Western blot (30 µg of protein/lane). Cytochrome P450 protein bands were quantified using ImageJ software and are expressed as a percentage of the control group ± S.E.M. (n = 5). *, significant difference from the control group ($p \leq 0.05$).

APPENDIX E HUMAN/ANIMAL SUBJECT APPROVAL

The Institutional Animal Care and Use Committee (IACUC) of the University of Arizona has approved the animal studies described in this dissertation, under the protocol entitled “Hepatobiliary Transport and Gene Expression During Non-alcoholic Steatohepatosis (NASH).”