Sex differences in hepatic gene expression in a rat model of ethanol-induced liver injury

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Tadic, Stasa D., Mary S. Elm, Ha-Sheng Li, Gijsberta J. van Londen, Vladimir M. Subbotin, David C. Whitcomb, and Patricia K. Eagon. Sex differences in hepatic gene expression in a rat model of ethanol-induced liver injury. J Appl Physiol 93: 1057–1068, 2002. First published May 24, 2002; 10.1152/japplphysiol.00568.2001.—Sex differences in susceptibility to alcohol-induced liver injury have been observed in both humans and experimental animal models. Using a standard model of alcohol-induced fatty liver injury and microarray analysis, we have identified differential expression of hepatic genes in both sexes. The genes that exhibit differential expression are of three types: those that are changed only in male rats fed alcohol, those that change in only female rats fed alcohol, and those that change in both sexes, although not always in the same manner. Certain of the differentially expressed genes have previously been identified as participants in the induction of alcohol-induced liver injury. However, this analysis has identified a number of genes that heretofore have not been implicated in alcoholic liver injury; such genes may provide new areas of investigation into the pathogenesis of this disease.

ALCOHOL-INDUCED LIVER INJURY (ALI) and disease (ALD) are major health problems affecting a broad patient population of different gender, race, and social backgrounds. The present model of pathogenesis of ALI exploits the role of different cytokines acting by induction of various transcription factors that in turn modulate expression of different genes through action on appropriate response elements and binding sites on those genes. Alcohol consumption triggers intrahepatic events such as alcohol metabolism in hepatocytes, which can lead to oxidative and metabolic stress. Extrahepatic events can also be involved, such as release of gut-derived endotoxin into the circulation, which acts on Kupffer cells in the liver. Both of these types of events can initiate and perpetuate an interplay between different functional cell groups in the liver, i.e., hepatocytes, Kupffer cells, hepatic stellate or Ito cells, mononuclear cells, and neutrophils, resulting in fatty liver, necrosis, inflammation, and fibrosis seen in ALI (reviewed in 15, 16, 30, 33, 52).

In humans, significant gender differences in susceptibility to ALI are observed (31, 38). Women appear to be at greater risk for more severe ALI, in that they develop cirrhosis more quickly and with a lesser alcohol intake than do men. Some evidence from certain rat models of alcohol ingestion, i.e., the Tsukamoto-French intragastric model, shows similar female susceptibility in degree of ALI (25), but this is not a universal finding, and only very rarely have direct side-by-side comparisons between male and female rats been performed.

One of the molecular mechanisms responsible for such a sex difference in susceptibility of liver to ethanol may be caused by differential expression of hepatic genes, especially those regulated by sex hormones. These genes encode for proteins responsible for the cellular responses to a toxic stress such as the chronic consumption of ethanol. Hence, a broad screen of hepatic gene expression in animals chronically fed ethanol is necessary to achieve a greater insight into the mechanisms of cellular adaptation or the failure thereof that leads to ethanol-induced liver injury.

Development of cDNA expression arrays (microarrays) provides a new powerful tool to perform a gene-expression screen of hundreds or thousands of genes in a single hybridization (45). The study described here was designed to profile the expression of hepatic genes in both sexes in response to a chronic ethanol diet. The model of chronic ethanol feeding used in this study (Lieber-DeCarli) induces a spectrum of biochemical changes and fatty liver with minimal or no inflammation or fibrosis after 30 or more days of exposure. Use of this model is of particular interest to obtain an insight into early changes in gene expression and sex differences as a response to ethanol under such experimental conditions, because fatty liver can proceed to more severe ethanol-induced liver injury. For our gene expression studies, we used a commercially available microarray designed to screen 465 genes that are known to be related to toxic stress.

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METHODS

Alcohol Feeding Protocol

Liver samples were obtained from animals fed using the Lieber-DeCarli model (42). Male and intact female Wistar rats (Charles River, Wilmington, MA), beginning at 35–36 days of age, were divided into an alcohol-fed group with 36% of total calories as alcohol (AF) and a control group pair-fed with an isocaloric, nonalcoholic diet (IC); both groups were fed for 30–42 days, a period sufficient to develop alcoholic fatty liver. The liquid diets were obtained from BioServe (Frenchtown, NJ) and reconstituted according to directions. Intake was ad libitum for the alcohol-fed rats, and their pair-fed controls received the exact amount that the alcohol-fed animal consumed the previous day, a protocol that eliminates differences in caloric intake. Animals were killed after fed animal consumed the previous day, a protocol that eliminates differences in caloric intake. Animals were killed after fed animal consumed the previous day, a protocol that eliminates differences in caloric intake. 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Histopathological Analysis

Livers were cut into 4-μm sections and stained with hematoxylin and eosin, after which liver injury was scored blindly. Although all hepatic tissue for any given liver showed similar morphology, different hepatic units in the same section could show different scores of pathology. To be as objective in scoring as possible, both sections in slides were examined, and an average bulk score for each slide was assigned. The scoring system for grading the hepatic pathology is as follows.

**Hepatic steatosis.** Hepatic steatosis was defined in hematoxylin- and eosin-stained sections as the presence in hepatocyte cytoplasm of colorless vesicles/vacuoles with very sharp borders. Because there is a lack of uniform agreement in the literature for morphological grading of hepatic steatosis (23), we have adopted with minimal modifications several scoring systems, from both clinical (17, 23, 32, 44) and experimental (7, 24) studies, as follows. Score 0: normal liver morphology, no fat drops. Vacularization of hepatocyte cytoplasm, if present, does not show a sharp border of vacuoles. Score 1: fat vesicles, mostly macrovesicles, present in scattered hepatocytes, with no identified pattern, probably tending to zones 3–2 of hepatic unit. Score 2: fat vesicles, mostly macrovesicles, present in scattered hepatocytes predominantly in zone 3 of hepatic unit, affecting 10–30% hepatocytes of zone 3. Score 3: fat vesicles, macro- and microvesicles, present in 50% or more hepatocytes in zone 3 of hepatic unit, forming areas of affected parenchyma. Score 4: fat vesicles, macro- and microvesicles, present in most of hepatocytes in zones 3 and 2 of hepatic unit, forming areas of affected parenchyma and sometimes forming bridging connection between hepatic lobules. Score 5: fat vesicles, macro- and microvesicles, present in hepatocytes of all zones of hepatic unit, forming areas of affected parenchyma that always forms bridging connection between hepatic lobules.

**Hepatic necroinflammatory damage.** The scoring system for grading hepatic necroinflammatory damage was modified from several reports on clinical (2, 22) and experimental (10, 35, 43) studies. The main difference in our model was an insignificant portal inflammation and damage to periportal hepatocytes. The extent of hepatic inflammation in our study was not significant; therefore the highest score reported still corresponds to mild/moderate inflammation. Score 0: normal liver morphology or presence of single inflammatory cells or rare small cell collection without consistent parenchymal damage. Score 1: presence of scattered inflammatory cell collections associated with hepatocyte damage/dropout. Score 2: presence of scattered small inflammatory cell collections associated with hepatocyte damage/dropout; small portion of hepatic units affected. Score 3: presence of small inflammatory cell collection in majority of hepatic units associated with hepatocytes damage/dropout. Score 4: presence of more than one inflammatory cell collection in majority of hepatic units associated with hepatocyte damage/dropout.

**Hepatic fibrosis.** The extent of hepatic fibrosis in our study was minimal and was scored as following. Score 0: normal liver morphology with presence of single collagen fibers around portal areas and big hepatic veins but not invading hepatic parenchyma. Score 1: single collagen fibers are present around majority of small-medium hepatic veins but do not invade into hepatic parenchyma. Score 2: single collagen fibers are present around majority of small-medium hepatic veins sometimes invading hepatic parenchyma and forming bridges between approximated central veins.

Serum Testosterone and Estradiol

Methods for the radioimmunoassay for serum testosterone have been previously published (11–13). Serum estradiol determinations were performed by the Radioimmunoassay Core, Center for Research in Reproductive Physiology, University of Pittsburgh School of Medicine.

RNA Isolation

Total hepatic RNA was extracted from frozen liver by using a standard guanidium thiocyanate phenol-chloroform extraction method (8) and was treated with the RNase-free DNase I provided in the microarray kit (Clontech, Palo Alto, CA) to reduce DNA contamination. Total RNA concentration and purity were determined spectrophotometrically, and the integrity of RNA samples was ascertained by appearance of distinct 28S and 18S bands of ribosomal RNAs on 1.2% agarose gel electrophoresis.

Microarray Analysis

Microarray analysis was performed according to manufacturer’s instructions (www.clontech.com) by using the Clontech Atlas Rat Toxicology II array. The 465 genes on the array represent various cellular functions that may be changed by toxic substances such as ethanol and include those for transcription factors, enzymes of common metabolic pathways, xenobiotic metabolism, receptors, cytokines, signaling molecules, immunomodulators, acute-phase proteins, antioxidant enzymes, and modifiers of protein synthesis and secretion, among others. The gene sequences were applied to a nylon membrane, with two separate spots for each gene. Each array also includes a set of housekeeping genes, together with negative control and genomic DNA. A complete list of the genes on this array is provided by the manufacturer (www.clontech.com). Each gene on the list is accompanied by the National Center for Biotechnology Information (NCBI) GenBank accession number of a published sequence of the gene and the sequence of related protein.

A total amount of 4 μg of purified RNA was used for the hybridization of each single microarray membrane. To reduce variability of changes, probes were made by pooling RNA samples from two to four animals from control or ethanol-fed group of animals and repeating the same microarray analysis two to three times. In brief, purified RNA
was converted to $^{32}$P-labeled cDNA probe by using the Moloney murine leukemia virus reverse transcriptase, master mix, and specific primer mix provided in the microarray kit (Atlas Rat Toxicology II). The cDNA probe was then purified by using G-50 Sephadex columns (Eppendorf 5-Prime, Boulder, CO), and intensity of radioactive labeling was measured by using a beta scintillation spectrometer. Microarray membranes were prehybridized with the kit’s ExpressHyb solution containing heat-denatured salmon testes DNA (100 $\mu$g/ml of buffer) for >1 h, followed by a hybridization overnight at 68°C with the cDNA probes. After hybridization, membranes were washed with 2 different washing solutions [2X sodium chloride-sodium citrate (SSC), 1% SDS, 3 × 30 min, and 0.1X SSC, 0.5% SDS, 1 × 30 min] at 68°C, then soaked in 2X SSC solution for 5 min at room temperature, wrapped in plastic wrap, and exposed to a phosphoimaging screen. The image obtained by using the phosphoimager was then converted to a PDF file, and hybridization intensity was analyzed by using Clontech AtlasImage 1.5 software.

Real-time PCR

To verify and quantify the changes in expression of some of the genes obtained by microarray method, we used real-time PCR (TaqMan) as a complementary method. We chose three genes from each membrane [tyrosine aminotransferase (TAT), lipopolysaccharide-binding protein (LPS-BP), and calreticulin precursor (CALR)] for verification. Gene-specific primers were designed by using the Primer Express 1.0 software (PE Applied Biosystems, Foster City, CA) according to the NCBI accession numbers for published sequences from GenBank, provided by the genes list in the Clontech manual. Primer sequences for TAT were 5’-TGG AGT TCA CAG AGC GGT TG-3’ (sense) and 5’-GTT ACT CGA AGC ACG TTG CTG-3’ (antisense). Primer sequences for LPS-BP were 5’-TCA TCT ACC CAC GAG GCA GTA T-3’ (sense) and 5’-CCC CAC CAA TGT AGG AAG CA-3’ (antisense). Primer sequences for CALR were 5’-CCA GAC AAC ACC TAC GAG GTG A-3’ (sense) and 5’-GTC CCA ATC ATC CCA GGA A-3’ (antisense). All primer pairs were purchased from GIBCO BRL Custom Primers, Life Technologies (Rockville, MD). Total liver RNA was used from the same control and ethanol-fed animals used in the microarray hybridization experiments. The primers used for the quantification of rat 18S rRNA (GenBank accession no. M11188) as a standard internal control were purchased from Integrated DNA Technologies (Coralville, IA); the rat 18S1 sequence was 5’-GAG GCC CTG TAA TTG GAA TGA GTC-3’ and rat 18S2 sequence was 5’-TCC CAA GAT CCA ACT ACG AGC TT-3’. SYBR green PCR reagents (PE Applied Biosystems) were used for real-time PCR, and PCR reaction was run by using the ABI PRISM 7700 sequence-detection system (PE Applied Biosystems). A more detailed description of the TaqMan protocol and conditions has been published (29, 41). Results were analyzed by using ABI PRISM 7700 Sequence Detection version 1.6.3 to obtain $C_T$ values, where $C_T$ values are threshold cycles at which a statistical significant increase in detection of SYBR green emission intensity occurs. Then, $C_T$ values are normalized to 18S endogenous control to account for variability in RNA concentrations between samples to obtain $\Delta C_T$ values. To obtain $\Delta \Delta C_T$ values, the $\Delta C_T$ value for the control rat is subtracted from the $\Delta C_T$ value of the alcohol-fed rat. Finally, the relative quantitation ($r.q.$) value is calculated as $2^{-\Delta \Delta C_T}$.

Statistics

Microarray hybridizations were performed at least two times on separate arrays with samples derived from animals fed the same protocol. For normalization of individual hybrid intensities, we used the polyubiquitin housekeeping gene because it has shown to have the most consistent expression pattern compared with other housekeeping genes on the array. Hybridization intensity of repeated array experiments were expressed as means ± SE. Student’s $t$-test was used to analyze the differences between control and ethanol-fed animals and between male AF and female AF animals, and a $P$ value of <0.05 was considered to be significant.

RESULTS AND DISCUSSION

As shown in Table 1, there was no difference in the body weights of either male or female AF rats compared with their same-sex controls. However, the livers of both male and female AF rats weighed significantly more than those of their IC controls and consequently demonstrated significantly increased liver weight-to-body weight ratios (increased 30% in males and 29% in the females, both $P < 0.05$), indicating the hyperplasia typical of alcohol-fed animals.

The results of the histopathological examination, shown in Table 2, indicate that both male and female AF rats have fatty liver. However, in this study, male AF rats also demonstrated inflammation and fibrosis

### Table 2. Liver pathology in AF male and AF female rats

<table>
<thead>
<tr>
<th>Sex</th>
<th>Fat Drops</th>
<th>Inflammation</th>
<th>Fibrosis</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF male</td>
<td>3.33 ± 0.88</td>
<td>3.33 ± 0.33</td>
<td>1.67 ± 0.33</td>
<td>8.33 ± 0.88</td>
</tr>
<tr>
<td>AF female</td>
<td>2.00 ± 0.00</td>
<td>1.00 ± 1.00*</td>
<td>0.50 ± 0.50</td>
<td>3.50 ± 0.50*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 samples of male and 2 samples of female rats. See METHODS for scoring of liver pathology for categories of fat drops, inflammation, and fibrosis. Overall value is the combined score of fat drops, inflammation, and fibrosis. *$P < 0.05$, AF male vs. AF female. Scores for IC male and female livers were zero in all categories.

### Table 1. Body weight, liver weight, and liver weight/body weight of AF and their IC male and female rats

<table>
<thead>
<tr>
<th>Sex</th>
<th>Body Weight, g</th>
<th>Liver Weight, g</th>
<th>Liver Weight/Body Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC male</td>
<td>295.3 ± 13.6 (n = 11)</td>
<td>10.31 ± 1.46 (n = 11)</td>
<td>0.036 ± 0.003 (n = 11)</td>
</tr>
<tr>
<td>AF male</td>
<td>273.1 ± 10.0 (n = 9)</td>
<td>12.43 ± 1.53* (n = 9)</td>
<td>0.045 ± 0.005* (n = 9)</td>
</tr>
<tr>
<td>IC female</td>
<td>232.5 ± 9.5  (n = 4)</td>
<td>9.40 ± 0.22  (n = 4)</td>
<td>0.041 ± 0.003 (n = 4)</td>
</tr>
<tr>
<td>AF female</td>
<td>224.5 ± 13.5 (n = 2)</td>
<td>12.07 ± 0.20* (n = 2)</td>
<td>0.054 ± 0.002* (n = 2)</td>
</tr>
</tbody>
</table>

Values are means ± SE. *$P < 0.05$, alcohol-fed (AF) male vs. iscaloric pair-fed control (IC) male or AF female vs. IC female within each parameter.
and overall liver injury greater than the female AF rats (inflammation and overall injury, P < 0.05).

Table 3 shows changes in serum sex hormones in both the male and female AF groups compared with their controls. The male AF rats have a significant increase in serum estradiol levels (increased 63%, P < 0.05) and decreased serum testosterone levels (decreased 80%, P < 0.05). Additionally, the male AF rats demonstrated testicular atrophy, with testes weight/ body weight decreased 15% compared with their controls (AF mean ± SD, 0.0096 ± 0.0026 vs. IC 0.0114 ± 0.0025, P < 0.05). The chronic alcohol feeding regimen resulted in a significant decrease in serum estradiol levels in female AF rats compared with their IC rats (decrease 30%, P < 0.05). The altered levels of sex hormones in the alcohol fed rats of both sexes may reflect a delay in puberty in these animals, because the rats were started on the alcohol diets at ~35 days, before puberty.

Figure 1 displays representative images of microarray membranes hybridized to radiolabeled cDNA probes derived from RNA of control and ethanol-fed male rats. Using such a gene expression screen tool that is geared toward detecting toxicological changes, we found a number of genes whose expression is altered in rats of both sexes fed ethanol chronically. Protein products encoded by these genes belong to a broad group of various functional proteins that may be related to cellular functions altered by ethanol as a toxin, such as xenobiotic metabolism and metabolism of carbohydrates, proteins and lipids, as well as function of transcription factors, antioxidant enzymes, and modulators of protein trafficking and secretion, among others. Although some of the changed genes and their related protein products have been previously identified as involved in the pathogenesis of ethanol-induced liver injury, most of the genes, to date, have not been clearly related to ethanol effects.

Because the specific intent of this study was to investigate sex differences in response to chronic ethanol exposure, we addressed changes in gene expression in young male or female rats, with particular emphasis on genes that may be regulated by sex hormones. Not only do the sexes differ with respect to hormonal levels, but chronic alcohol exposure is known to alter serum sex hormone levels, as shown in Table 3 and Refs. 13 and 14. We have sorted gene expression data into three distinct groups: first, genes changed only in male rats fed ethanol (Table 4, Fig. 2); second, genes changed only in female rats fed ethanol (Table 5, Fig. 3); and third, genes changed in rats of both sexes (Fig. 4), although not always in the same direction.

**Gene Expression Changes in Male Rats**

The first group of genes represented in Table 4 is the one that changed in male rats fed ethanol compared with their pair-fed isocaloric controls. There were 38 genes changed in male rats fed ethanol, with a range of change from 1.77-fold to more than 25-fold. The profile of the whole group of genes outlines the complexity of changes in liver induced by ethanol. The genes in this group encode for proteins that belong to various functional groups such as transcription factors (hepatocyte nuclear factor-4α), xenobiotic metabolism (glutathione S-transferase Yc1 subunit, catechol-O-methyltransferase, cytochrome P-450 2B1, alcohol-sulfotransferase, cytochrome P-450 IIC9, heat shock 90-kDa protein-β, UDP-glucuronosyltransferase 2B); metabolism of lipids (long-chain-specific acyl-CoA dehydrogenase precursor, apolipoprotein-A1 precursor), proteins (cytoplasmic aspartate aminotransferase, tyrosine aminotransferase), and carbohydrates (phospho(enol)pyruvate carboxykinase, IGF-1B precursor); protein synthesis and secretion modifiers [protein disulfide isomerase, 40S ribosomal protein S30, ubiquitin-like protein (NEDD8), tissue inhibitor of metalloproteinase-1 precursor gene (TIMP-1), NADPH-cytochrome P-450 reductase], acute-phase pro-

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**Table 3. Serum estradiol and testosterone levels of AF and their IC control male and female rats**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Serum Estradiol, pg/ml</th>
<th>Serum Testosterone, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC male</td>
<td>5.99 ± 1.26 (n=10)</td>
<td>2.70 ± 0.56 (n=11)</td>
</tr>
<tr>
<td>AF male</td>
<td>9.78 ± 1.65* (n=6)</td>
<td>0.54 ± 0.18* (n=9)</td>
</tr>
<tr>
<td>IC female</td>
<td>36.41 ± 8.99 (n=4)</td>
<td></td>
</tr>
<tr>
<td>AF female</td>
<td>25.39 ± 15.75* (n=2)</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

*Values are means ± SE. *P < 0.05, AF male vs. IC male or AF female vs. IC female within each parameter.*
Table 4. Differentially expressed genes in livers of male rats fed an alcohol-containing diet

<table>
<thead>
<tr>
<th>Protein/Gene Accession No.</th>
<th>Mean Ratio</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocyte nuclear factor 4-α (HNF4-α)</td>
<td>D10554</td>
<td>9.04</td>
</tr>
<tr>
<td>Immune system proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement component 4-binding protein-α precursor (C4BPA)</td>
<td>Z50051</td>
<td>2.47</td>
</tr>
<tr>
<td>Lipopolysaccharide-binding protein (LPS-BP)</td>
<td>L32132</td>
<td>6.48</td>
</tr>
<tr>
<td>Transporters/carrier proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 1 precursor (IGFBP-1)</td>
<td>M89791</td>
<td>5.77</td>
</tr>
</tbody>
</table>

Relative intensities of hybridization to microarray membranes were compared between samples derived from AF and paired IC male rats. Ratios expressed in the table represent the mean ratios ± SE of the AF rats from 2–4 separate microarray analyses compared to those of the control animals. A ratio >1 indicates upregulation of the gene expression in the alcohol-fed rats, whereas a ratio <1 indicates a downregulation of the stated gene. Genes in bold type are those that are also differentially expressed in liver of female rats fed alcohol.

Some of those genes and related proteins have been already known as associated or changed by ethanol effects (IGF-1B and IGF-1 binding protein, cytoplasmic aspartate aminotransferase, retinoid X receptor α) or involved in pathogenesis of ALI (TIMP-1, LPS-BP).
All other genes encode for functional proteins involved in processes that may be related to early biochemical and metabolic changes induced by ethanol that further may proceed to later morphological damage.

Furthermore, some of the genes changed only in male rats fed ethanol are regulated by androgens (C4-BPα, carbonic anhydrase 3, CYP2B1, Apo-A1, MB-COMT, NADPH-CYP reductase, IGF-1B, TIMP-1), further suggesting that a modifying or regulatory role of sex hormones (androgen or estrogen) on hepatic functions may change in response to chronic ethanol exposure, and this change may create sex-related difference in susceptibility to ethanol. For example, the gene that encodes for carbonic-anhydrase III is downregulated in AF male rats and is testosterone dependent (3). The reduction of expression may represent a loss of a potential “hepatoprotective” function of androgen, because the product of this gene has been suggested recently as a potential protective factor against oxidative stress. In particular, this protein protects against oxidative stress-induced apoptosis (39), and an increase is associated with decreased aldehydic protein adducts and minimal histopathology in male micropigs fed ethanol (40).

Gene expression of another androgen-regulated gene, apolipoprotein-A1 precursor (28, 51), was upregulated in

Table 5. Differentially expressed genes in livers of female rats fed an alcohol-containing diet

<table>
<thead>
<tr>
<th>Protein/Gene</th>
<th>Accession No.</th>
<th>Mean Ratio</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin-like growth factor binding protein 1 precursor (IGFBP-1; IBP-1)</td>
<td>M89791</td>
<td>1.77</td>
<td>0.00</td>
</tr>
<tr>
<td>Xenobiotic metabolism; carbohydrate, lipid, and protein metabolism</td>
<td>U94585</td>
<td>0.49</td>
<td>0.17</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase (PEPC)</td>
<td>K03248</td>
<td>2.49</td>
<td>1.09</td>
</tr>
<tr>
<td>Cytochrome P-450 2E1 (CYP2E1)</td>
<td>J02627</td>
<td>0.41</td>
<td>0.18</td>
</tr>
<tr>
<td>UDP-glucuronosyltransferase 2B</td>
<td>U06273</td>
<td>0.58</td>
<td>0.08</td>
</tr>
<tr>
<td>Tyrosine aminotransferase (TAT)</td>
<td>M18340</td>
<td>3.60</td>
<td>1.76</td>
</tr>
<tr>
<td>Alcohol sulfotransferase A (AD-ST)</td>
<td>X63410</td>
<td>0.50</td>
<td>0.22</td>
</tr>
<tr>
<td>Copper-zine-containing superoxide dismutase 1 (Cu-Zn-SOD-1)</td>
<td>Y00404</td>
<td>3.97</td>
<td>1.80</td>
</tr>
<tr>
<td>Liver catalase (CAT)</td>
<td>M11670</td>
<td>0.35</td>
<td>0.22</td>
</tr>
<tr>
<td>Enzymes/proteins involved in protein turnover: post-translational modification proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein E precursor (APOE)</td>
<td>J02582</td>
<td>0.28</td>
<td>0.24</td>
</tr>
<tr>
<td>Endoplasmic reticulum stress protein 72 precursor (ERP72); calcium-binding protein 2 (CABP2)</td>
<td>M86870</td>
<td>0.27</td>
<td>0.23</td>
</tr>
<tr>
<td>Immune system proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipopolysaccharide-binding protein (LPS-BP)</td>
<td>L32132</td>
<td>0.47</td>
<td>0.29</td>
</tr>
<tr>
<td>C-reactive protein (CRP)</td>
<td>M83176</td>
<td>0.60</td>
<td>0.38</td>
</tr>
<tr>
<td>Kinases; heat shock proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dual-specificity mitogen-activated protein kinase kinase 2 (MAPKK2)</td>
<td>D14592</td>
<td>0.14</td>
<td>0.06</td>
</tr>
<tr>
<td>Calreticulin precursor (CALR); calcium-binding protein 3 (CABP3)</td>
<td>X53363</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Relative intensities of hybridization to microarray membranes were compared between samples derived from AF and paired IC female rats. Ratios expressed in the table represent the mean ratios ± SE of the AF rats from 2–4 separate microarray analyses compared to those of the control animals. A ratio >1 indicates an upregulation of the gene expression in the alcohol-fed rats, whereas a ratio <1 indicates a downregulation of the stated gene. Genes in bold type are those that are also differentially expressed in liver of male rats fed alcohol.
our study of male rats, suggesting that change of the lipoprotein profile in chronic male alcoholics may be attributed to an ethanol-induced alteration of hormonal control of lipoprotein metabolism and/or synthesis. Another example is the finding of an overexpression (10-fold) of CYP2B1 gene induced by ethanol. This gene is normally expressed in very low levels in both sexes but is also highly induced by phenobarbital only in male rats and under a strong regulatory role of androgens (4). Overexpression of this particular xenobiotic pathway may be a part of an overall induction of xenobiotic metabolism induced by ethanol that serves as a detoxification pathway but one that could also create potentially harmful effects or metabolites.

An intriguing finding may be an upregulation of NADPH-cytochrome P-450 reductase gene in ethanol-fed male rats, also influenced by androgens (5). It has been shown recently that this enzyme is implicated in the short cellular half-life of CYP2E1 and thus may limit the activity of this enzyme as a known pathway of reactive oxygen species generation in ALI (58).

Two genes related to IGF-1, IGF-1B precursor and IGF-binding protein-1 precursor gene, were upregulated in ethanol-fed male rats in our study. It has been shown that IGF-1 mRNA and protein levels are reduced in ethanol-fed animals, whereas mRNA levels for IGF-binding proteins are increased (27). Testosterone may increase the IGF-1 circulatory levels, contrary to effects of ethanol on IGF-1 (59), suggesting an interesting link to a mechanism of greater susceptibility of male sex to alcohol-induced myopathy, because skeletal muscle is a target tissue to positive effects of IGF and serum testosterone levels are decreased in ethanol fed male rats and humans. However, without an anal-

Fig. 3. Alcohol-induced alteration of hepatic gene expression in AF female rats compared with their pair-fed female IC controls. Hybridization intensities as determined by phosphoimaging were compared by using AtlasImage 1.5 software for each of microarray membranes. Each individual array represents a mix of RNA from 2 rats from the stated groups, and each RNA pool was subjected to microarray analysis 2–3 times. Bars represent means of 2–3 determinations ± SE. *Statistical differences between AF female rats and their IC female rats for each gene (P < 0.05). See Table 5 for definitions of gene abbreviations.

Fig. 4. Sex differences and similarities in hepatic gene expression in AF rats compared with their pair-fed IC controls. Hybridization intensities as determined by phosphoimaging were compared using AtlasImage 1.5 software for each of microarray membranes. Each individual array represents a mix of RNA from 2–3 rats from the stated groups, and each RNA pool was subjected to microarray analysis 2–3 times. Bars represent means of 2–3 determinations ± SE. *Statistical differences between AF male rats and their IC male rats and between AF female rats and their female IC rats for each gene (P < 0.05).
ysis of the protein products, it is impossible to determine whether the induction of these two genes impacts on the bioavailability of IGF-1.

Although epidemiological studies have shown greater overall susceptibility of female sex to ethanol-induced liver injury (38), there may be specific sex differences for certain of the effects of ethanol, so that each sex might have susceptibility to a specific type of injury. For example, the finding of an upregulation of TIMP-1 gene in AF male rats may suggest their potentially greater susceptibility to liver fibrosis and progression to cirrhosis, because this TIMP-1 inhibits cellular breakdown of collagen. This theory is supported by a recent study showing an anti-inflammatory effect of estrogen-induced downregulation of TIMP-1 (57).

Gene Expression Changes in Female Rats

Genes changed only in female rats fed alcohol, compared with their isocalorically pair-fed controls, are shown in Table 5. Some of these genes are known to be regulated or affected by estrogen levels: apolipoprotein E precursor, cytochrome P-450 2E1, copper-zinc-containing superoxide dismutase-1, liver catalase, and C-reactive protein. A downregulation of their expression in this study may suggest that the physiological regulatory effects of estrogen on hepatic functions may be decreased after chronic ethanol consumption, and indeed estradiol levels are lower in the AF rats in this study. Some of those regulatory functions may have a protective effect, and their decrease may create greater susceptibility of female sex to ethanol-induced effects. For example, the alcohol-induced downregulation of apolipoprotein E precursor gene, which is regulated by estrogen via estrogen receptors (48), may suggest a possible reduction of antiatherogenic apolipoprotein E and a change to a more atherogenic (i.e., male) lipid profile. This is supported by recent findings that overexpression of this gene has a protective effect in development of atherosclerosis in mice (49). Another example of the changes in females is the downregulation of liver catalase gene, the protein product of which metabolizes reactive peroxides. Such a downregulation may account for a reduced hepatic antioxidant defense in female rats exposed to ethanol for a longer time period. Our findings correlate with the study in which reduced catalase activity in liver was found after decreased gonadal production of estrogen (47). Expression of the copper-zinc-containing superoxide dismutase-1 gene was upregulated in the AF female rats; this finding may reflect the decrease of serum estrogen levels or possible alteration in estrogen receptor function, because an increased activity of this enzyme was also shown after oophorectomy (21). However, the potential pathological significance of this change is less clear.

We have also found a decreased expression of the CYP2E1 gene in AF female rats. This result may suggest a diminished estrogen regulatory role or decreased activity of estrogen receptors in the liver, because it has been shown that CYP2E1 enzyme activity correlates directly with serum estrogen levels, i.e., increased by estradiol administration (1, 36). It is not clear whether this change of CYP2E1 gene expression may have an effect on CYP2E1 enzyme activity and whether a subsequent protective or negative effect may result.

Expression of the gene that encodes for the synthesis of C-reactive protein is also downregulated in AF female rats. The function of this acute-phase protein as an immunomodulator and evidence of its regulation by estrogen may suggest a greater susceptibility of female rats to ethanol-induced inflammatory liver damage (11, 37).

Gene With Changed Expression in Both Sexes of Ethanol-Fed Rats

The third group of genes is the one for which expression was changed in both sexes, male and female rats (boldface gene names in Tables 5 and 6 and Fig. 4), although the pattern of expression change was sex-different in two-thirds of the genes. Among the three genes that showed the same pattern of change (upregulation in both sexes in AF rats), two are known to be regulated by androgen [IGF binding protein-1 precursor gene and phosphoenolpyruvate carboxykinase (55), whereas the tyrosine aminotransferase gene has no sex differences in regulation of its protein product activity (42). Because phosphoenolpyruvate carboxykinase catalyses the rate-limiting step of hepatic gluconeogenesis, this may represent a molecular mechanism for a higher susceptibility of male rats to alcohol-induced alteration in glucose metabolism and the hypoglycemia associated with ethanol consumption. Alternately, this finding may suggest the ability of the alcohol-fed males to compensate metabolically to increase serum glucose concentrations.

Six genes were changed in AF rats of both sexes but with different patterns of change: increased expression in male rats and decreased expression in female rats (Tables 4 and 5, boldface gene names). Functions of the protein products of these genes suggest that there may be sex differences in susceptibility and especially a potentially greater susceptibility of female rats to ethanol diet in regard to various liver functions altered by ethanol. The genes for two enzymes involved in xenobiotic metabolism, UDP-glucuronosyltransferase gene (18) and alcohol sulfotransferase gene (46, 54), are downregulated in AF female rats, suggesting a sex difference and a potentially greater susceptibility of female rats to effects of ethanol on various enzymatic pathways involved in the process of detoxification. Alcohol sulfotransferase gene is also more highly expressed in untreated female than in male rats, and its downregulation in female rats fed ethanol may reflect ethanol-induced changes in sexually dimorphic liver function and “switching” of the normal sexual phenotype of the liver and other organs, as observed previously in chronic (male) alcoholics.

The gene for precursor for endoplasmic reticulum stress protein 72 is also diversely regulated in AF rats depending on sex (upregulated in male and downregulated in female rats). This protein has a protein disul-
possible isomerase activity and is involved in regulation of protein secretion (53), suggesting that some protein trafficking functions may be more profoundly impaired in female rats. There is also a significant downregulation of the dual-specificity mitogen-activated protein kinase kinase 2 gene in female rats, which may result in diminished ability of AF females to initiate liver regeneration (6).

One very intriguing finding in our study is the diverse expression pattern of LPS-BP gene. LPS-BP is essential for rapid induction of an inflammatory response when a small amount of LPS is present. Recent studies have generated new insight into LPS kinetics and suggest a protective role of LPS-BP (21), contrary to previous beliefs (20, 56). The significant upregulation of LPS-BP in male rats and the opposite trend in female rats may suggest greater susceptibility of female sex to inflammatory effects of chronic ethanol consumption and may help explain the previous observation that alcohol-induced liver injury in females develops more quickly and after less amount of alcohol (19, 31, 38).

Verification of Microarray Results By Real Time RT-PCR

Figure 4 depicts a logarithmic amplification plot for real-time RT-PCR quantification of mRNA levels of hepatic tyrosine amino transferase in AF and IC male rats, using gene-specific primers, as well as the plot for the normalization standard 18S rRNA. As shown in Fig. 5 and Table 6, three genes were verified in several different AF and IC rat pairs of both sexes (TAT, LPS-BP, and CALR). As noted in Table 6, this technique verifies the direction and extent of the changes observed by microarray analysis, although the values for the fold increase or decrease are not necessarily equivalent.

Possible Mechanisms of Sex-Specific Susceptibility to Ethanol-Induced Liver Damage and Other Effects of Ethanol

The pattern of change of genes regulated by sex hormones in AF animals shows that regulatory role of sex hormones may change as a result of ethanol exposure and that such change may be protective or harmful, depending on previously “protective” or “inhibitory” role of sex hormone in a specific physiological instance. The effects of the sex steroids on various liver functions are mediated through hepatic sex hormone receptors (cytosolic and nuclear). Ethanol may affect hormonal regulation of hepatic function by three mechanisms: 1) by decreasing the gonadal production of the hormone and thus the serum levels of the hormone and overall bioavailability; 2) by affecting the function of sex hormone receptors, through the effects on protein synthesis or activity, or 3) by changing the sex hor-

Table 6. Relative expression of the genes TAT, LPS-BP, and CALR in liver of male and female rats chronically fed alcohol as compared to their same-sex controls not exposed to alcohol

<table>
<thead>
<tr>
<th>Sex</th>
<th>TAT</th>
<th>LPS-BP</th>
<th>CALR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol-fed male</td>
<td>6.0 ± 1.8</td>
<td>4.5 ± 0.8</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Alcohol-fed female</td>
<td>2.5 ± 0.9</td>
<td>0.9 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

See Tables 4 and 5 for definitions of gene abbreviations. Values are means ± SE of from 2–6 pairs of male rats and 2 pairs of female rats run in triplicate for each gene. Relative quantitation (r.q.) value is expressed as $2^{-\Delta\Delta CT}$, where $\Delta CT$ is the threshold cycle normalized to the endogenous control (rat 18S rRNA) for each alcohol and control liver sample and $\Delta\Delta CT$ is the difference between $\Delta CT$ of alcohol-fed rat and $\Delta CT$ of its pair-matched isocaloric control. The r.q. values of the control rats are equal to 1 and are not shown. The r.q. values indicate relative fold increase or decrease in number of copies of gene sequence relative to its control. Values greater than fourfold difference (r.q. > 4 and r.q. < 0.25) from control are considered significant changes in gene expression. An r.q. value > 1 indicates an upregulation whereas an r.q. < 1 indicates a downregulation of a given gene.
mone metabolism in the liver. All three proposed mechanisms may affect regulatory role of the major sex hormone, and sexual dimorphism may change or “switch” to opposite-sex phenotype. That switch in some of hepatic dimorphic functions may proceed to further morphological damage induced by ethanol. One of such examples is the change in secondary sexual characteristics in chronic alcoholics that accompanies or may precede further development of ALD. Our previous studies identified changes in all of the above parameters. In particular, males exposed chronically to alcohol develop a more female pattern of serum sex hormones and of liver gene expression. A striking example of the latter is the alcohol-induced loss of androgen-dependent estrogen metabolizing enzymes in male rats (14, 50). The changes noted in some of the genes in male rats, especially those that are under androgen control, may lead to a hypothesis that the major sex hormone, i.e., androgen in male rats and estrogen in female rats, may have an overall protective role.

Our findings of gene expression changes for certain functions controlled by sex hormones make an ultimate hypothesis of greater susceptibility of female sex seem more complex. Our results may also imply that different sexes are more susceptible to a certain alterations induced by ethanol, such are greater susceptibility of female sex to xenobiotic metabolism and oxidative/inflammatory damage and potentially greater susceptibility of male rats to liver fibrosis. This may change the concept of the overall greater susceptibility of one, i.e., female, sex to ethanol-induced liver injury.

It should be emphasized that these preliminary findings represent changes in young rats, comparable to young adolescents in humans. Because the rats were started on the alcohol regimen before puberty, the changes noted may reflect an alcohol-induced delay in puberty. The possible consequence of such a delay is seen by the hormone levels in Table 3. If the female rats had been exposed to alcohol after puberty had occurred and normal estrogen levels were established, perhaps we would have observed more liver injury, as is typical of the adult women studied by others. Further, the potential effect of estrogen as a cofactor in alcoholic liver injury cannot be ignored in the young male rats in this study, given that they have very high estrogen-to-testosterone ratios, and thus their greater liver injury may be related to their estrogen levels.

Overall Conclusion Regarding Findings of This Study and Unanswered Questions

This is a novel type of gene expression study and a novel approach to study the regulatory role of sex hormones on liver functions. Although microarray analysis represent a powerful gene expression screening method with regard to the extent of information about the potentially changed genes in AF animals, it cannot address some of very important questions. First, one cannot conclude from the gene expression change the actual impact on synthesis and function of encoded protein product, and further, the impact of the ratio of change of gene expression on protein synthesis remains to be verified by studies of the protein products of the gene. Thus further complementary studies such as real-time quantitative PCR and protein and activity quantitation are needed to address further posttranscriptional and posttranslational events for each gene chosen for a specific mechanistic study. Lastly, it is not always obvious whether a change in expression of a given gene is a cause of, or caused by, the alcohol-induced liver pathology. However, the specific value of a study such as this is that the analysis gives valuable initial information that may direct future studies of ethanol-induced liver injury. Furthermore, it draws more attention to genes that have not been previously implicated in the pathogenesis of ethanol-induced liver injury, thus opening many possibilities for study of new genes and proteins and their potential role in ALI.

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