Downregulation of nuclear sex steroid receptor activity correlates with severity of alcoholic liver injury

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Eagon, Patricia K., Mary S. Elm, Stasa D. Tadic, and Amin A. Nanji. Downregulation of nuclear sex steroid receptor activity correlates with severity of alcoholic liver injury. Am J Physiol Gastrointest Liver Physiol 281: G342–G349, 2001.—Chronic ethanol ingestion in rats and humans results in significant alterations in sex steroid levels and expression of sex hormone-dependent phenotype. In this study, we used the intragastric feeding model in male rats to determine hepatic sex hormone receptor activity under circumstances of chronic ethanol exposure and differing degrees of liver injury induced by type of dietary fat. Pathological analysis and quantitation of hepatic androgen receptor (AR) and estrogen receptor (ER) activity, serum sex hormones, and sex hormone-responsive protein and mRNA expression were performed. The activity of the physiologically relevant nuclear form of both AR and ER was significantly decreased with ethanol and correlated inversely with the severity of liver injury. Serum testosterone levels, as well as expression of an androgen-dependent hepatic mRNA, were decreased by ethanol and progressive liver injury. Serum estradiol increased with liver injury. We postulate that these changes in receptor activity may be due to the oxidative stress, reduced cellular energy, and/or altered cytokine milieu known to occur in this model.

ethanol; estrogen; androgen

MAMMALIAN LIVER OF BOTH SEXES is responsive to sex hormones; receptors for both estrogen and androgen have been characterized in human and rodent liver (9–14, 26–28). In addition, sex steroids appear to influence the development and course of several liver diseases, including benign diseases such as hepatic adenoma (HA) and focal nodular hyperplasia, as well as hepatocellular carcinoma (12, 27). Alterations in activity and subcellular distribution of hepatic estrogen receptor (ER) and androgen receptor (AR) have been demonstrated to occur in at least some of these diseases. An increase in either total ER activity or in the activity of the biologically relevant nuclear form of the ER has been demonstrated in HA and focal nodular hyperplasia specimens compared with either the histologically normal portion of the liver surrounding the lesion or to normal nondiseased liver (27). Androgens and anabolic steroids have also been associated with HA (1, 9, 12), and in liver specimens from patients with HA, there is a higher nuclear AR activity in the adenomatous areas (9). In a male rat model of peroxisome proliferator-induced hepatic hyperplasia and carcinoma, an increase in AR activity occurs during hyperplasia and in the resultant tumors; however, ER activity increases early during hyperplasia but then decreases significantly in late stages of hyperplasia and in neoplastic liver (8).

Chronic ethanol ingestion results in both liver injury and in significant alterations in sex hormone levels and sex hormone-responsive phenotype. Men who drink chronically are often hypogonadal, with low serum testosterone levels, and these men may also display elevations of serum estrogens and a feminization of breast, liver, and skin tissues (11). In humans, chronic ethanol abuse has been associated with increases in cytosolic ER activity (39). In animal studies, male rats fed a standard Lieber/DeCarli ethanol-containing diet demonstrate reductions in serum testosterone and elevations in serum estradiol (15). The livers of these rats undergo demasculinization, with loss of AR and androgen-dependent estrogen metabolizing enzyme activity (15, 34), and feminization, as evidenced by an increase in serum ceruloplasmin, an estrogen-responsive protein synthesized by the liver (34). The Lieber/DeCarli ethanol model used in those studies, however, results in mainly fatty liver with little necrosis or inflammation (6). The aim of the current study was to determine the activity of hepatic sex hormone receptors, serum sex hormone levels, and the expression of sex hormone-responsive genes in another rat model in which the degree of alcohol-induced liver injury can be modulated by using different fat sources in the diet (17, 22, 24, 36). This well-documented model uses intragastric indwelling catheters to maintain a constant infusion of an ethanol-containing or control diet (17, 36). Ethanol in the presence of medium-chain triglycerides (MCT) produces no liver injury, and palm oil (PO) fed with ethanol results in only fatty liver. Corn oil (CO), containing mainly polyunsaturated fatty acids, results

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in fatty liver, necrosis, and inflammation when fed with ethanol, while fish oil (FO) fed with ethanol produced the most severe liver injury (22, 24). Use of such a model that results in differing severities of liver pathology allows us to evaluate the relationship between the biochemical and pathological changes. Thus it was of interest to extend the observations on hormone receptors in models showing no or very little liver injury to a model where changes in receptor activity could be related to severity of ethanol-induced pathological changes in the liver.

**MATERIALS AND METHODS**

**Animal model.** Male Wistar rats weighing between 275 and 300 g were fed a liquid diet by continuous infusion through permanently implanted gastric tubes as described previously (22, 24). The rats were given their total nutrient intake by intragastric infusion. The percentage of calories derived from fat was 35% of total calories. Vitamins and minerals were given as described previously (22, 24). The amount of ethanol was modified to maintain high levels of blood ethanol (150–300 mg/dl) throughout the day. The amount of ethanol was increased up to 16 g/kg day as tolerance developed.

Eight groups of rats (4–6 rats per group) were fed different dietary fats to produce increasing degrees of liver injury: MCT plus dextrose or ethanol, PO plus dextrose or ethanol, CO plus dextrose or ethanol, and FO plus dextrose or ethanol. All control animals were pair-fed the same diet as ethanol-fed rats except that ethanol was isocalorically replaced by dextrose. The progressive liver injury using this dietary protocol is consistent and well documented (22, 24). All diets were prepared fresh daily. The FO diet was stored in airtight containers under nitrogen in a cold room at 4°C. Lipid peroxidation was measured to exclude the possibility of autoxidation. All animals were killed after 1 mo of treatment with the experimental diets. A sample of liver was obtained for histopathologic analysis, and the remainder of the liver was cut into small pieces, which were transferred to plastic vials and placed in liquid nitrogen. The vials were stored at −80°C. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals.

**Histopathological analysis.** A small sample of the liver was obtained and formalin fixed when the rats were killed. Hematoxylin and eosin stain was used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat) was scored 1+ with <25% of cells containing fat; 2+ with 25–50% of the cells containing fat; 3+ with 51–75% of the cells containing fat; and 4+ with >75% of the cells containing fat. Necrosis and inflammation were quantified as the number of necrotic or inflammatory foci per low power field. At least three different sections were examined per sample of liver; all assessments were performed by a pathologist blinded to the specific experimental protocol. The total pathology score was calculated for use in linear regression analysis (17, 24).

**Measurements of blood alcohol.** Blood was collected from tail vein, and alcohol concentration was measured using an alcohol dehydrogenase kit from Sigma Chemical (St. Louis, MO).

**AR and ER assays.** Cytosolic and nuclear fractions were prepared, and single point triplicate receptor binding assays were performed on these fractions as previously described (7, 9, 10, 14). The validity of these single point assays has been established (10). Cytosolic ER and AR were quantitated by incubating separate aliquots (200 μl) of cytosol overnight at 4°C with 3.75 nM [2,4,6,7-3H]estradiol ([3H]E2) for ER or 5 nM [3H]R1881 (methyltrienolone) for AR in the absence (total binding) and presence (nonspecific binding) of a 100-fold excess of unlabeled diethylstilbestrol or R1881. All cytosolic receptor assays were performed in the presence of 10 mM sodium molybdate, an agent protective of receptor activity and a phosphatase inhibitor (14). The difference between the total and nonspecific values was considered to be specific binding. All assay tubes for cytosolic ER also contained a 500-fold excess of 2-methoxyestradiol (1.88 μM) to block the estrogen binding activity of a cytosolic estrogen sulfotransferase under these conditions, and for AR, 50 nM triamcinolone acetonide to block any contribution of the glucocorticoid receptor to total binding. Bound steroid was separated from free at the end of the incubation by using cold dextran-coated charcoal as previously described (14). Supernatants were removed and placed in Biosafe II scintillation fluid (RPI, Mt. Prospect, IL) to determine radioactivity.

Nuclear ER activity was quantitated by an exchange assay using separate aliquots (200 μl) of nuclear suspension incubated at 30°C for 1.5 h with 3.75 nM [3H]E2 and a 500-fold excess of 2-methoxyestradiol in the absence (total binding) and presence (nonspecific binding) of a 200-fold excess of unlabeled diethylstilbestrol. Nuclear AR was quantitated by an exchange assay at 4°C for 16 h in the presence of 3.3 nM [3H]R1881 and 50 nM triamcinolone acetonide and in the absence and presence of a 200-fold excess of unlabeled R1881. Bound steroid was separated from free at the end of the incubation period by repeated washing of nuclei (7). Total receptor activity was calculated by adding together the cytosolic and nuclear activity values based on steroid binding per gram of liver.

**Other assay methods.** All methods for the radioimmunoassay for serum testosterone and serum ceruloplasmin activity have been previously published (8, 34). Serum estradiol determinations were performed by the Radioimmunoassay Core, Center for Research in Reproductive Physiology, University of Pittsburgh School of Medicine.

**Northern blot analysis.** Our Northern blot analysis protocol has been described (29, 34). The probe used was a full-length cDNA estrogen sulfotransferase probe (1.2 kb) developed in our laboratory and generated by digesting the purified pCR-II plasmid with Xho I and labeling with [α-32P]dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) and the multiprime DNA labeling system (Amersham Pharmacia, Piscataway, NJ). The blots were subsequently stripped and reprobed with a cDNA for rat gliceraldehyde-3-phosphate dehydrogenase (GAPDH) to standardize blots for RNA loading. Northern blots were analyzed by quantitative densitometry using a BioRad Video Densitometer. The results from two Northern blot analyses were averaged.

**Other reagents and materials.** Radioactive [3H]E2, 97 Ci/mmol, and [3H]R1881, 82 Ci/mmol, were purchased from New England Nuclear (Boston, MA). The sources of other materials have been described (7).

**Statistical methods.** All experimental groups were compared with their age-matched treatment control group or other indicated groups, using either a paired or independent Student’s *t*-test as appropriate for the samples of tissue or serum available. Results were expressed as means ± SE, and *P* ≤ 0.05 was considered to be significant. Linear regression analysis was performed by the SlideWrite Plus program for Windows, Version 4.0 (Advanced Graphics Software, Carls-
bad CA), and a t-test was used to determine the statistical significance of the correlation coefficients.

RESULTS

Histopathological analysis. As shown in Table 1, the type of fat present in the diet altered the severity of ethanol-induced liver injury. The MCT-containing diet, even in the presence of ethanol, resulted in essentially no injury. All other fat types induced fatty liver to varying degrees. Significant necrosis and inflammation was present only in the groups fed ethanol-containing diets with CO and FO, with the latter resulting in the greatest overall degree of injury.

Serum sex steroids. Figure 1 demonstrates changes in serum sex hormones in the different experimental groups. In each of the dietary groups, ethanol exposure reduced testosterone values (Fig. 1A) compared with the control animals fed the same fat source. Serum estradiol (Fig. 1B) was not significantly altered by ethanol in any of the groups showing an absence or only moderate liver injury (MCT, PO, CO). However, the serum estradiol was significantly increased in the group with greatest liver injury (FO-ethanol). It also appears that the fat source could have an impact on testosterone and estradiol levels in the control dextrose-fed groups as well. Testosterone levels in the PO-dextrose and the CO-dextrose groups tended to be higher than in the MCT- or FO-dextrose control groups, although only the PO-dextrose is significantly different from either of the latter (P < 0.05). Estradiol levels were higher in the MCT-dextrose group than in the PO- and FO-dextrose groups (P < 0.03).

AR activity. Figure 2A demonstrates a decrease in total AR activity in all ethanol-treated groups, but the decrease is significant and most pronounced in the FO-ethanol group. There appears to be little effect of fat source alone on total receptor activity, since the receptor activity in the control rats from each group did not differ. The activity of nuclear AR (Fig. 2B) is significantly decreased only in the CO- and FO-ethanol groups; these are also the groups showing necrosis and inflammation. The nuclear AR activity in the ethanol-treated groups correlated inversely with severity of liver injury as measured by total pathology score (r = −0.55, P < 0.05; Fig. 2C). Activity of nuclear receptor in the control groups appears to be somewhat altered by fat source alone; the activity in the CO- and FO-dextrose groups is higher than in either of the other control groups (P < 0.05). Cytosolic receptor activity was not altered significantly by ethanol treatment in any group (Table 2). Hepatic mRNA levels for the androgen-dependent liver-specific estrogen sulfotransferase also decreased as a result of ethanol exposure and liver injury; mRNA expression, as determined by

Table 1. Histopathologic analysis of livers of rats fed ethanol-containing diets with different fat sources

<table>
<thead>
<tr>
<th>Diet</th>
<th>Fatty Liver (0–4)</th>
<th>Necrosis (0–2)</th>
<th>Inflammation (0–2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT</td>
<td>0</td>
<td>0.2 ± 0.4</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>PO</td>
<td>2.0 ± 0.7</td>
<td>0.4 ± 0.4</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>CO</td>
<td>3.4 ± 0.5*</td>
<td>1.5 ± 0.4†</td>
<td>1.8 ± 0.4†</td>
</tr>
<tr>
<td>FO</td>
<td>4.0 ± 0.0*</td>
<td>2.0 ± 0.0*†</td>
<td>2.0 ± 0.0*†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Liver specimens were stained with hematoxylin and eosin and scored as described in MATERIALS AND METHODS. None of dextrose-fed groups showed any pathological changes except for occasional foci of inflammation. FO and CO, fish and corn oil, respectively. *P < 0.01 vs. medium-chain triglycerides (MCT) + ethanol and †P < 0.01 vs. palm oil (PO) + ethanol.
Northern blot analysis, was reduced 64, 89, and 77% in the MCT-, CO-, and FO-ethanol groups, respectively. The decrease in EST expression may reflect the reduction in serum androgen levels in these groups.

ER activity. Ethanol feeding did not significantly affect total ER in the different dietary groups, although there was a tendency toward lower values in the CO- and FO-ethanol groups (Fig. 3A). Again, the fat source alone had no influence on total ER activity. The activity of the nuclear ER (Fig. 3B) was not affected by ethanol in either the MCT or the PO groups but was numerically decreased (P < 0.12) in the CO-ethanol group and significantly decreased in the FO-ethanol group (P < 0.05). As seen with the nuclear AR, the nuclear ER activity correlated inversely with severity of liver injury (Fig. 3C; r = -0.72, P < 0.01). Cytosolic ER activity was not affected by ethanol treatment in any of the groups studied (Table 2).

Serum ceruloplasmin activity. Serum ceruloplasmin activity was measured in the different groups as an indicator of the estrogen response of the liver. No change in ceruloplasmin activity was seen in any of the groups except for the FO-ethanol group, where there was a numerical but not significant (P = 0.13) reduction in ceruloplasmin activity (Fig. 4). This observation likely reflects the pronounced decrease in nuclear ER activity in this group. In none of the groups was there a change in serum levels of either total serum protein or albumin.

DISCUSSION

This study shows that in male rats, serum sex hormone levels and hepatic sex hormone receptors may be
profoundly altered by chronic ethanol ingestion and that the activity of these receptors correlates inversely with the extent of liver injury. In particular, the activity of the most physiologically relevant form of both AR and ER, the nuclear form, decreased with increased severity of liver injury. It is also noteworthy that the influence of liver injury is a more significant factor than the levels of circulating hormone with respect to activity of nuclear receptors. The type of fat in the diet alone did not influence total receptor activity because all control groups demonstrated similar receptor activity. Thus the demonstrated loss of nuclear receptor activity in the ethanol-treated rats was a result of the combination of ethanol and degree of liver injury.

The ethanol-induced decrease in serum testosterone demonstrated in the current study is consistent with our previous observations (34) and with the well-documented testicular toxicity of ethanol (11, 13, 37, 38). The results in this study also suggest that, even though the MCT-ethanol diet causes no liver injury, there still appears to be an effect of ethanol on the testes. However, altered hepatic metabolism may also contribute to the low level of serum testosterone (31). The effect of ethanol feeding on estrogen levels in this study appears to be less dramatic than that shown with the Lieber/DeCarli model (15, 34).

Fig. 3. Hepatic estrogen receptor activity (ER) in male rats fed different diets. Activity of total receptor (A) and nuclear form (B) of hepatic ER was measured in isocaloric control (solid bars) and ethanol-fed (hatched bars) rats at 30 days (see MATERIALS AND METHODS). Rats were fed different diets of different composition as noted in Fig. 1 legend. No. of rats in each group: 6 rats in PO-E; 4 rats each in all other groups.

Fig. 4. Serum ceruloplasmin activity in male rats fed different diets. Serum oxidase activity of ceruloplasmin, an estrogen-responsive protein synthesized by the liver, was measured in isocaloric-fed (solid bars) and ethanol-fed (hatched bars) rats after death using a standard colorimetric assay (see MATERIALS AND METHODS). Rats were fed different diets of different composition as noted in Fig. 1 legend. No. of rats in each group: 5 rats each in MCT-D, CO-D, PO-D; 4 rats each in MCT-E, PO-D, FO-D; 3 rats in PO-E.
account for this discrepancy is that animals in the Lieber/DeCarli model (15, 34) were treated for a much longer period. Also, in that study, hepatic estrogen clearance was shown to be impaired by virtue of significant reductions in both the 2-hydroxylation and the sulfation pathways of estrogen metabolism (34). Our current study also showed a decrease in the steady-state mRNA level for the estrogen sulfotransferase enzyme, suggesting that the sulfation pathway in these rats may also be impaired.

A decrease in AR activity, along with a decrease in serum testosterone, has also been shown previously (15, 34) and appears to be influenced by duration of ethanol exposure (34). The results in this current work demonstrate, in addition, a significant inverse correlation between the decrease in nuclear AR activity and severity of liver injury. The groups in which ethanol was fed in the MCT and PO diets had essentially unchanged nuclear AR activity; in contrast, there was a significant loss of nuclear AR activity in the ethanol groups with more severe injury (CO and FO) (Fig. 2C). These reductions in nuclear AR were also accompanied by a decrease in the mRNA expression of the hepatic androgen-dependent enzyme estrogen sulfotransferase; this decrease was particularly marked in the CO and FO groups.

We also show that ethanol-induced liver injury was associated with a decrease in nuclear ER activity, and a significant inverse correlation was seen between nuclear ER activity and the severity of ethanol-induced liver injury (Fig. 3C). The decrease in ER activity observed in the present study is in contrast to observations made in humans, where an increase in hepatic cytosolic ER was seen in alcoholic men (39). The alterations in ER activity may reflect time-dependent changes, because rats fed the Lieber/DeCarli diet for an extended time (90 days) demonstrated a reduction in total hepatic ER activity, whereas an increase in ER activity is noted after 30 days of exposure (34).

Concomitant with the reduced nuclear ER activity in the FO-ethanol group was a reduction in the oxidase activity of serum ceruloplasmin, an estrogen-responsive protein synthesized and secreted by the liver. This observation is similar to that seen in another liver injury model, the peroxisome proliferator model, in which rats fed diethylhexylphthalate for 16 wk demonstrate reduced hepatic ER activity and reduced serum ceruloplasmin activity, while serum estrogen levels are elevated (7).

We also noted that serum levels of estradiol and testosterone were affected by the different forms of fat, in the absence of ethanol. The few studies that have addressed the effect of dietary fat types on sex hormone levels provide conflicting data. One study, for example, showed no relationship between the dietary fat content or type and serum estradiol levels in women (2). In other studies in mice, a high fat diet with 43% of calories from CO resulted in higher mammary gland ER-binding activity (18) and serum estradiol levels (4) than in mice fed a diet containing 16% of calories from CO. These results reflected the amount rather than the type of fat. It is possible that different fat types may alter hepatic or extrahepatic metabolism of sex steroids; to date, no studies have addressed this possibility. It is of interest that, of the control groups, the MCT group had the highest estradiol level and the lowest testosterone level, suggesting that this diet may promote peripheral and/or hepatic aromatization.

On the contrary, there are a number of factors that could account for the loss of AR and ER activity observed in the present study. First, chronic ethanol exposure results in low cellular energy levels in the liver (20). Because steroid receptors must be phosphorylated to be active, especially with respect to DNA nuclear binding (35), an overall depletion of high-energy phosphates available for phosphorylation may lead to decreased activity. The binding methods used in this report measure the activity of the receptors, suggesting that a decrease in available energy to maintain the receptors in an active form may contribute to our observed loss of receptor activity. In support of this hypothesis, it is known that ATP levels are decreased in livers of rats fed ethanol using the intragastric model (16). Oxidative stress may also contribute to the loss of receptor activity. Levels of oxidative stress, as determined by measurements of plasma 8-isoprostane and microsomal conjugated dienes, are increased in rats fed unsaturated fats and ethanol (24); in the current study, these same groups have the lowest nuclear receptor activity. Indeed, studies have shown that oxidative stress reduces the ability of the ER to bind to relevant DNA sequences and to transactivate estrogen-responsive genes (19).

Other factors that could influence hepatic sex hormone receptor activity in ethanol-fed animals include cytokines involved in the pathogenesis of ethanol-induced liver injury (23) such as tumor necrosis factor-α that acts through transcription factors like nuclear factor-κB (NF-κB) (23). Increased NF-κB activity in liver has been documented in rats fed ethanol and unsaturated fatty acids (23), and this factor may contribute to the observed decrease in nuclear AR activity in our study, because NF-κB has recently been established as a negative regulator of AR activity in liver (30, 33).

The implications of a decrease in hepatic AR and ER activity as a result of ethanol-induced liver injury are, at the present time, unclear. One apparent consequence is the loss of activity of androgen-dependent enzymes such as estrogen sulfotransferase and estrogen 2-hydroxylase (15, 34), resulting in an inefficient hepatic metabolism of estrogen. Loss of receptor activity in alcohol-fed animals may also contribute to progression of liver injury. This possibility is suggested by studies that show the inverse relationship between ER expression and TNF-α gene expression (32). The ER may also have a potential role in affecting blood flow via changes in endothelial nitric oxide synthase activity and nitric oxide synthesis (5), thus altering centrilobular hepatic blood flow and influencing centrilob-
Sexts steroid receptors and alcoholic liver injury (16). We have previously shown in the intragastric model a decreased nitric oxide production by nonparenchymal liver cells in association with ethanol-induced liver injury (21). Therefore, a decreased level of liver ER is consistent with decreased nitric oxide production in rats showing liver injury (21). However, the full impact of the alcohol-induced loss of hepatic sex steroid receptor activity requires further study, because the exact roles of both estrogen and androgen in liver function are not well characterized.

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