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Chronic ethanol administration decreases the ligand binding properties and the cellular content of the mannose 6-phosphate/insulin-like growth factor II receptor in rat hepatocytes

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Abstract

We have shown previously that chronic ethanol administration impairs the maturation of lysosomal enzymes in rat hepatocytes. The mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF-IIR) is a protein that facilitates the transport of lysosomal enzymes into the lysosome. Therefore, we examined whether ethanol consumption altered the ligand binding properties and the cellular content of M6P/IGF-IIR. Rats were pair-fed liquid diets containing either ethanol (36% of calories) or isocaloric maltose-dextrin for either 1 week or 5–7 weeks. Hepatocytes prepared from these animals were examined for receptor–ligand binding and receptor content. One week of ethanol feeding had no significant effect on ligand [radioiodinated pentamannose phosphate conjugated to bovine serum albumin (^{125}I-PMP-BSA)] binding to hepatocytes, but cells from rats fed ethanol for 5–7 weeks bound less ^{125}I-PMP-BSA than pair-fed controls. Scatchard plot analysis revealed that the number of ^{125}I-PMP-BSA binding sites in hepatocytes from ethanol-fed rats was 49% lower than that of controls. ^{125}I-PMP-BSA binding by perivenular (PV) and periportal (PP) hepatocytes from ethanol-fed rats was, respectively, 40 and 48% lower than their controls, but there was no significant difference between these two types of hepatocytes. Ligand blot analysis using ^{125}I-insulin-like growth factor II (^{125}I-IGF-II) also showed that the receptor in lysates of hepatocytes from ethanol-fed rats bound 26–27% less ligand than controls. Similarly, immunoblot analysis of cell lysates from ethanol-fed rats revealed 62% lower levels of immunoreactive M6P/IGF-IIR than controls. Feeding rats a low carbohydrate-ethanol diet did not exacerbate the reduction in M6P/IGF-IIR-ligand binding nor did it reduce the levels of immunoreactive receptor. Our findings indicate that chronic ethanol consumption lowers M6P/IGF-IIR activity and content in hepatocytes. This reduction may account, in part, for the impaired processing and delivery of acid hydrolases to lysosomes previously observed in ethanol-fed rats.

Keywords: Lysosome; Liver; Low carbohydrate diet; Perivenular; Periportal

1. Introduction

The M6P/IGF-IIR is a 300 kDa transmembrane glycoprotein that specifically recognizes and binds to lysosomal glycoproteins bearing M6P residues and to the peptide hormone IGF-II. The receptor facilitates the transport of lysosomal precursor enzymes into the lysosome via a sequential mechanism [1–4]. In the initial event, vesicles containing lysosomal glycoprotein precursors bud off the endoplasmic reticulum and traffic to the *cis*-Golgi complex to be phosphorylated at their mannose residues. In the *trans*-Golgi network, these phosphorylated glycoproteins are sorted for targeting, after which the M6P/IGF-IIR

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Abbreviations: M6P, mannose 6-phosphate; M6P/IGF-IIR, mannose 6-phosphate/insulin-like growth factor II receptor; NC, normal carbohydrate; LC, low carbohydrate; PP, periportal; PV, perivenular; IGF-II, insulin-like growth factor II; ^{125}I-IGF-II, radioiodinated IGF-II; PMP-BSA, pentamannose phosphate conjugated to bovine serum albumin; ^{125}I-PMP-BSA, radioiodinated PMP-BSA; ASGP, asialoglycoprotein.

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specifically recognizes, binds, and transports newly synthesized acid hydrolase precursors to the endosome [4,5]. Delivery of precursors to the lysosome is achieved by a low pH-dependent dissociation of the precursors from the M6P/IGF-IIR in the increasingly acidic environment of the endosome [6,7]. The free M6P/IGF-IIR is then either recycled to the *trans*-Golgi network or moved to the cell surface to bind and internalize circulating IGF-II or lysosomal precursor proteins that have escaped the cell through the secretory pathway [8–10].

The ligand binding sites for M6P are localized to repeats 3 and 9 of the extracytoplasmic domain, whereas the high-affinity binding sites for the peptide hormone IGF-II are localized to repeats 11 and 13 of the extracytoplasmic domain on the M6P/IGF-IIR molecule [11–13]. The M6P/IGF-IIR appears to be the predominant functional receptor for lysosome biogenesis. Another receptor, called the cation-dependent M6P receptor, has a molecular mass of 46 kDa and appears to have a minor role in lysosomal enzyme maturation compared with that of the M6P/IGF-IIR [14].

Ethanol consumption has a profound effect on protein trafficking in liver cells by altering the secretion and trafficking of plasma glycoproteins and impairing plasma membrane glycoprotein assembly [15–17]. Furthermore, ethanol administration impairs synthesis and trafficking of the hepatocyte-specific ASGP receptor [18,19]. Similarly, we have shown that chronic ethanol administration impairs the maturation of the lysosomal protease, cathepsin L, in rat hepatocytes [20]. Maturation of this enzyme and a number of other lysosomal precursors requires M6P/IGF-IIR activity. In the present study, we examined whether ethanol consumption altered the ligand binding properties and the content of the M6P/IGF-IIR in isolated rat hepatocytes and whether feeding an LC-ethanol diet exacerbated these effects. We also examined whether any impairment of M6P/IGF-IIR activity by ethanol administration was differentially affected in PP versus PV hepatocytes. Portions of this work have been reported in an abstract [21].

2. Materials and methods

Male Wistar rats weighing 125–150 g were purchased from Charles River Laboratories and were maintained in the animal research facility of the Omaha Veterans Affairs Medical Center. This facility is accredited by the American Association for the Accreditation of Laboratory Animal Care. Liquid diets were purchased from Dyets, Inc. LD101A diets were from Purina Mills and have the same Lieber–DeCarli formulation as those from Dyets, Inc. The latter liquid diets are the same as those originally used by Lindros and Jarvelainen [22] for formulating NC and LC liquid diets and were used here to replicate their experimental protocol. Since the original publication of the latter article, the LC diet has been more appropriately called the LC high-fat diet [23], since additional calories are added in the form of polyunsaturated fat (corn oil). The macronutrient composition of the NC-control (Lieber–DeCarli) diet as percent of total calories is 47% carbohydrate, 35% fat, and 18% protein. In the NC-ethanol diet, the composition of the fat and protein are the same as that of the NC-control diet except that it contains 11% carbohydrate and 36% ethanol. The LC-control diet contains 40% carbohydrate, 44% fat, and 16% protein, whereas the LC-ethanol diet contains 5.5% carbohydrate and 34.5% ethanol. Carrier-free Na-^{125}I from Amersham Pharmacia Biotech was used for the radioiodination of PMP-BSA and IGF-II. M6P, digitonin, collagenase, Percoll, sodium dodecyl sulfate, and protein molecular weight markers were purchased from the Sigma Chemical Co. Nitrocellulose membranes (0.45 μm) and acrylamide were from Bio-Rad. Antibody 293 is a polyclonal antibody raised in rabbits against the rat placental M6P/IGF-IIR [24].

2.1. Animal procedures

Rats were given Purina chow diets until they reached 180–200 g. They were then fed the NC liquid (Lieber–DeCarli control) diet for 3 days and acclimated to the control and ethanol liquid diets (36% as calories) as previously described [25]. In separate experiments, groups of rats were pair-fed the LC (5.5%) liquid ethanol diet for 5–7 weeks [22]. Body weights of rats were measured at least once per week.

2.2. Hepatocyte isolation

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The collagenase perfusion method of Seglen [26] as modified by Kharbanda *et al.* [20] was used to isolate hepatocytes. Following perfusion, livers were excised, and cells were dispersed in 50 mL of Krebs–Ringer Hepes buffer (KRH) containing 2.5 mM calcium chloride and 2% BSA. Hepatocytes were then filtered through polypropylene mesh (100 μm pore size) and pelleted by centrifugation at 50 *g* for 3 min at 4°. Further enrichment of viable cells was performed by centrifugation at 110 *g* for 5 min at 4° through 25 or 35% Percoll. After washing twice to remove Percoll, hepatocytes were resuspended in 20–25 mL KRH containing 2% BSA and 2.5 mM calcium chloride and assessed for viability by trypan blue exclusion. Normally, cell viability was 88–95%. In certain cases, prior to collagenase perfusion, PV or PP hepatocytes were specifically isolated by either retrograde or antegrade perfusion (7 mL/min) with digitonin (2 mg/mL) according to established methods [27,28]. Contamination of hepatocyte preparations with Kupffer cells and/or sinusoidal endothelial cells (SEC) was determined immunochemically by reacting cell suspensions with mouse anti-rat macrophages (for

Kupffer cells) or with mouse anti-rat Reca-1 antigen (for SEC). The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. Immune-stained cells were detected and quantified by flow cytometry. Hepatocytes prepared and analyzed in this manner showed no detectable contamination with SEC cells. There was a small amount of contamination (~2%) with Kupffer cells when cells were prepared with 25% Percoll. Preparation of cells with 35% Percoll showed no detectable contamination.

2.3. Iodination of ligands

BSA (15 mg/mL) was conjugated to 0.2 M pentamannose phosphate (PMP-BSA) in the presence of 160 mM $NaCNBH_3$ by the method of Braulke *et al.* [29]. This conjugate was then radioiodinated with Na-^{125}I by the method of Byrd *et al.* [11]. Briefly, 25 μg PMP-BSA was iodinated with 1.0 mCi of Na-^{125}I using pre-coated IODO-GEN tubes (Pierce) for 15 min at room temperature by incubation in 0.3 M sodium phosphate buffer (pH 7.4). Iodinated PMP-BSA was separated from free iodine on a Sephadex G-50 column by eluting with the same buffer containing 1% BSA. Similarly, IGF-II was iodinated with carrier-free Na-^{125}I to specific activities between 40 and 85 μCi/μg by Enzymobead reagent from Bio-Rad.

2.4. Ligand binding assay

Freshly isolated hepatocytes were suspended in KRH containing 2% BSA and incubated for 1 hr at 37° with gentle agitation. Cells were then incubated at 4° for 30 min in the presence of 5 mM M6P, which was included to remove endogenous ligands from the M6P/IGF-IIR. Preliminary experiments indicated that M6P pretreatment elevated subsequent binding of PMP-BSA to whole cells by nearly 2-fold compared with untreated hepatocytes (data not shown). These same mixtures were also used in the presence or absence of 0.06% digitonin to permeabilize the cells and to allow ligand interaction with intracellular receptor. The digitonin concentration was essentially the same as that used by Weigel *et al.* [30] and by Casey *et al.* [31] and was confirmed here in preliminary experiments using various digitonin levels (data not shown). Following pretreatment, the cells were washed twice to remove M6P, recounted, and one million cells were added to individual tubes either with or without 5 mM M6P or 0.06% digitonin in a final volume of 0.5 mL. Following the method of Byrd *et al.* [11], reaction mixtures were incubated with either a fixed quantity or various amounts of ^{125}I-PMP-BSA (7.5×10^6 cpm/μg of PMP-BSA) for 2 hr at 4° with constant mixing. ^{125}I-PMP-BSA cell surface binding assays were performed with intact cells. In total ligand-binding assays (i.e. intracellular and surface receptors), hepatocytes that were permeabilized with 0.06% digitonin were used. The same concentration of digitonin was also included in the incubation mixture. Unbound ^{125}I-PMP-BSA was removed by pelleting and washing the cells twice with KRH. Radioactivity bound to the cell pellet was assessed in a Packard auto-gamma counter (Downers Grove). Previous studies using the hepatocyte-specific ASGP receptor have revealed that greater than 90% of that receptor–ligand complex remains associated with digitonin-permeabilized hepatocytes [31,32]. Since the M6P/IGF-IIR protein is larger than the ASGP receptor protein and since it is localized mostly intracellularly, release of this receptor or the receptor–ligand complexes into the supernatant would be expected to be at least comparable to that of the ASGP receptor. Specific ligand binding to the M6P/IGF-IIR was quantified by subtracting the average radioactivity detected in the presence of M6P (non-specific binding) from that detected without M6P. Binding was expressed as either counts per minute or femtomoles of ligand bound per million cells. The kinetics of ^{125}I-PMP-BSA binding to digitonin-permeabilized hepatocytes showed that maximum M6P/IGF-IIR binding was achieved after 2 hr of incubation at 4° (Fig. 1). Hence, 2 hr was used as the standard incubation time in all experiments. Ligand binding was linear as a function of cell number ranging from 0.25 to three million cells per reaction tube (data not shown).

2.5. Ligand blot assay

The method of Byrd *et al.* [11] was used to measure IGF-II binding to M6P/IGF-IIRs from hepatocyte lysates. Briefly, 200 μg lysate protein was loaded onto 1.5 mm thick 6% polyacrylamide gels for SDS–PAGE performed for 15–16 hr at 12 mA. Proteins from the gels were transblotted onto nitrocellulose membranes (0.45 μm pore size) at 0.35 A for 3 hr. After blocking the membrane for 30 min in 10 mM Tris–HCl (pH 7.4) containing 3% Nonidet P-40, the bound proteins were renatured for 2 hr in 10 mM Tris–HCl containing 1% BSA (both done at 4°). Next, the transblotted membrane was incubated with 2 nM ^{125}I-IGF-II (2.5×10^6 cpm/μg of IGF-II) for 15 hr at 4° in the same buffer. Unbound ^{125}I-IGF-II was removed by washing the blot three times for 10 min each with 15 mL of 10 mM Tris–HCl buffer containing 0.1% Tween-20. The membrane was then exposed to X-ray film (Kodak) or a PhosphorImager screen. Radioactive bands were detected and quantified densitometrically as arbitrary volume integration units (V.I.U.) using Molecular Dynamics ImageQuant software. Our preliminary tests with ligand blot as well as immunoblot assays showed that the densitometry arbitrary volume integration units were directly proportional to the receptor protein concentrations (data not shown).

2.6. Immunoblot assay

Hepatocyte lysate proteins were subjected to SDS–PAGE under non-reducing conditions and then transferred

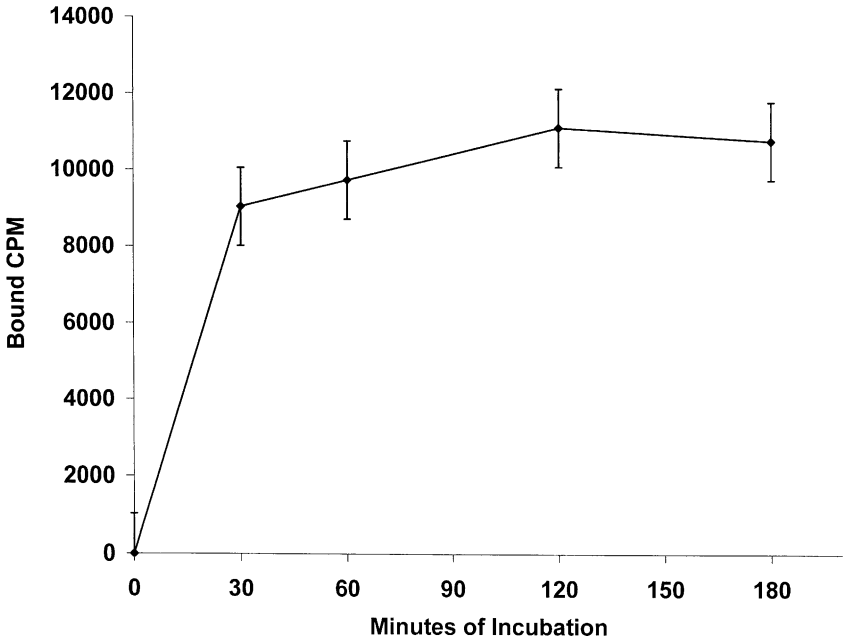

Fig. 1. Kinetics of ^{125}I-PMP-BSA (7.5×10^6 cpm/μg of PMP-BSA) binding to digitonin-treated rat hepatocytes. Reaction mixtures for total binding were prepared as described in "Materials and methods". Tracer (10^6 cpm) was added to each reaction mixture. At 120 min the amount of radiolabeled ligand bound to cells was 11,100 cpm or 1.1% of the total amount added. Values are means ± SD for four experiments.

onto 0.45 μm nitrocellulose membranes. Blots were blocked with 4% nonfat dry milk in 15 mM Tris–HCl buffer, pH 7.4, containing 0.1% Tween-20 (TBST) at 24° for 1 hr. Then blots were incubated with antibody 293 (1:200 dilution) in 4% nonfat dry milk in TBST for 1 hr at 24°, followed by three successive washes with TBST alone for 5 min each. The blots were incubated with ^{125}I-Protein A for 1 hr at room temperature followed by three washes as above, and then exposed to X-ray film at −80°. The immunoreactive radiolabeled bands were detected by autoradiography and quantified by densitometry.

2.7. Statistical analysis

Statistical analysis of the results between the control and ethanol-fed groups was performed by Student's *t*-test. To determine the interaction of diet and ethanol, a two-factor analysis of variance was applied to compare results from rats fed the NC and LC diets. Post-hoc analysis of significant interactions was conducted by multiple *t*-tests using Bonferroni's method.

3. Results

Hepatocytes isolated from rats pair-fed the Lieber–DeCarli control and ethanol diets (Dyets, Inc.), for 1 week or for 5–7 weeks, were used to assess surface and total receptor binding of 2 nM ^{125}I-PMP-BSA. Hepatocytes from control and ethanol-fed rats showed no difference in the amount of ^{125}I-PMP-BSA bound to the cell surface after either 1 week or after 5–7 weeks of feeding (Table 1). The average amount of ligand bound on the cell surface as a percentage of total cellular receptor binding (intracellular + surface) was 4.4% for controls and 3.4% for ethanol-fed rats. After 1 week of pair-feeding, there was no significant difference in ligand binding to total receptors in cells from ethanol-fed rats compared with those from pair-fed controls. However, after 5–7 weeks of pair-feeding, binding to total receptors in cells of ethanol-fed rats was 27% lower than that of controls (Fig. 2).

Scatchard plot analysis [33] was performed using various concentrations of ^{125}I-PMP-BSA to determine the number of available ligand binding sites in hepatocytes

Table 1
Surface binding ^{125}I-PMP-BSA to hepatocytes from control and ethanol-fed rats for 1 week and 5 weeks

Experiment	fmol bound/million cells	*P* value	% Surface bound	*P* value
1 week-control	0.29 ± 0.3	–	3.87 ± 2	–
1 week-EtOH	0.14 ± 0.1	0.1	2.26 ± 2	0.29
5 week-control	0.37 ± 0.3	–	4.89 ± 3	–
5 week-EtOH	0.23 ± 0.1	0.18	4.50 ± 2	0.85

Results are the means ± SD from five experiments. Percent surface bound ligand is calculated from the cpm bound to the cell surface divided by the cpm bound to total receptor. *P* values indicate comparisons between the 1-week control and the 1-week ethanol-fed rats and the 5-week control and the 5-week ethanol-fed rats, respectively.

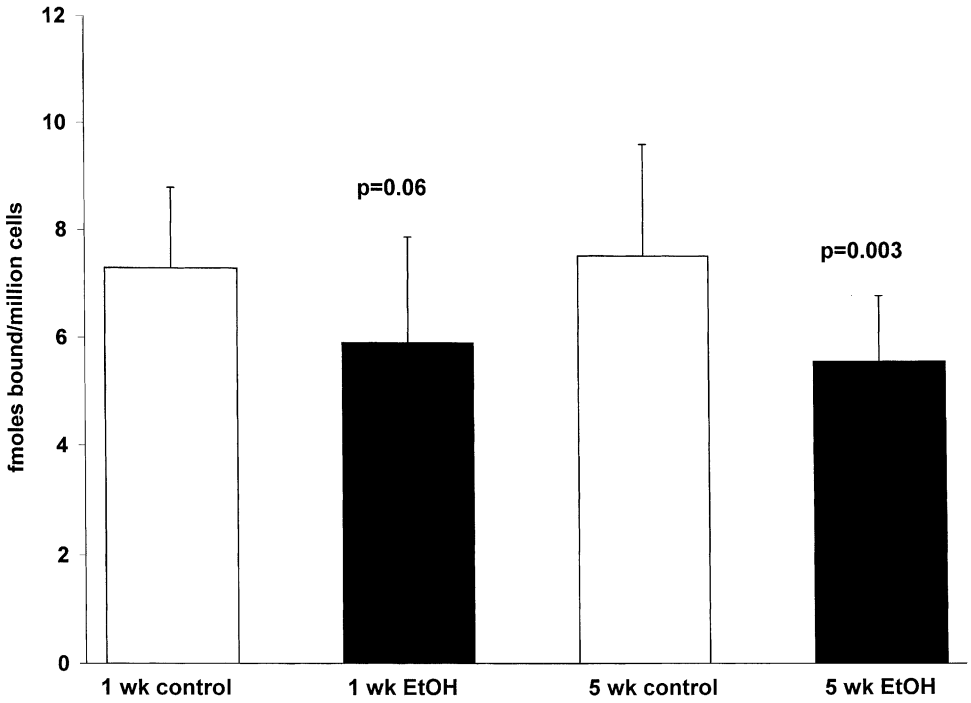

Fig. 2. Total receptor binding by ^{125}I-PMP-BSA using hepatocytes isolated from rats fed the Lieber–DeCarli control and ethanol (EtOH) diets for 1 week and for 5 weeks. Values are means ± SD for five pairs of animals. Cells were incubated in the presence of 0.06% digitonin.

from 5-week ethanol-fed rats and their pair-fed controls (Fig. 3). This analysis indicated that hepatocytes from ethanol-fed rats had 49% fewer available ^{125}I-PMP-BSA binding sites than cells from pair-fed controls (Table 2), obtained from Scatchard plots (Fig. 3). There was no significant alteration in the M6P/IGF-IIR binding affinity.

Separate groups of rats fed the Lieber–DeCarli (NC) liquid diet for 5–7 weeks were used to isolate PP or PV

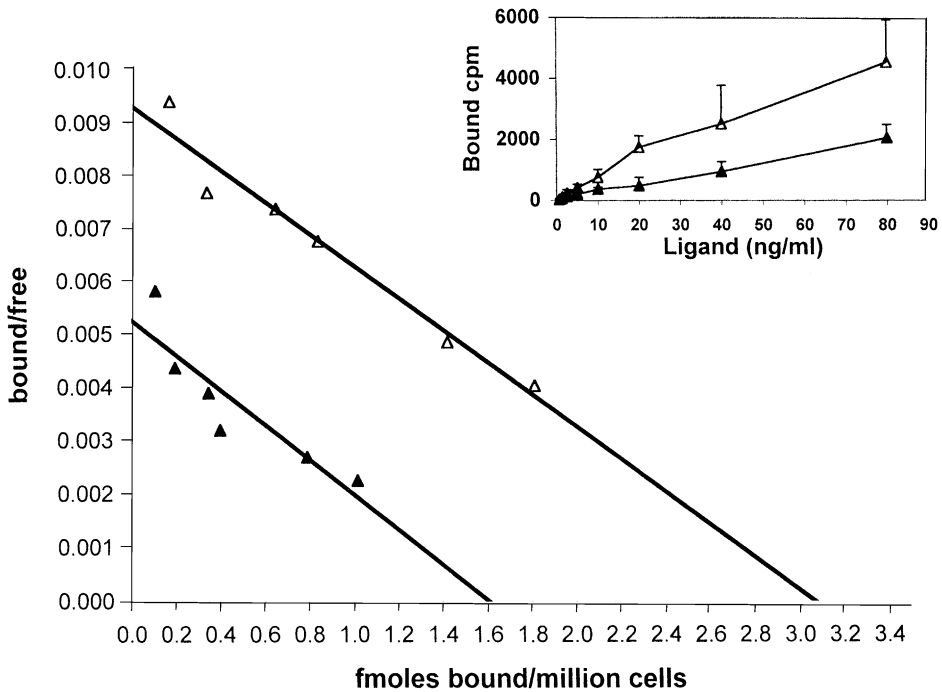

Fig. 3. Scatchard plot of ^{125}I-PMP-BSA binding to hepatocytes isolated from pair-fed controls (△) and ethanol-fed rats (▲). Triplicate reaction mixtures, each containing one million cells and 0.06% digitonin, were incubated with various concentrations of ^{125}I-PMP-BSA (0.31–200 ng/mL) at 4° for 2 hr. The plot represents binding to hepatocytes from one pair of rats from the NC group fed for 5 weeks. Inset: Concentration-dependent binding of ^{125}I-PMP-BSA by hepatocytes from rats fed the NC-control diets (△) or NC-ethanol diets (▲). Each point represents the mean ± SD, $N = 5$.

Table 2
Mean values of Scatchard parameters from hepatocytes of control and ethanol-fed rats given normal and low carbohydrate diets

Experiment	B_{max} (fmol/million cells)	P value	Binding sites (molecules/cell)	K_d (nM)	P value
NC-control	3.20 ± 0.5	–	1926 ± 309	0.74 ± 0.3	–
NC-EtOH	1.63 ± 0.7	0.0006	981 ± 406	0.52 ± 0.3	0.10
LC-control	2.60 ± 0.4	1565 ± 263	0.62 ± 0.3	–	
LC-EtOH	1.34 ± 0.5	0.002	809 ± 332	0.52 ± 0.3	0.4

Binding experiments were done as described in "Materials and methods". Only high-affinity binding site data were determined according to the procedure of Scatchard [33]. Mean B_{max} (±SD) values were calculated from the Scatchard plot of five pairs of NC-control and NC-ethanol-fed rats and from five pairs of LC-control and LC-ethanol-fed rats. Binding sites per cell were calculated from each individual B_{max}. K_d values were calculated from five pairs of each group by the slopes of their individual Scatchard plots (slope $= -1/K_d$). Statistical analyses were performed by the two-factor ANOVA test. Ethanol significantly affected B_{max}, but there were no significant interactions between the diets.

hepatocytes. Hepatocytes from the PV region are considered to be more susceptible to alcohol-induced injury than cells from the PP region [34,35]. We sought to determine whether this ethanol-induced susceptibility might also be reflected in differences in ^{125}I-PMP-BSA binding to M6P/IGF-IIRs in cells from these two regions. Ligand binding to the surface of PP cells and PV cells was not affected by chronic ethanol administration (data not shown). ^{125}I-PMP-BSA binding to total receptors in PV cells from ethanol-fed rats was 40% lower than in PV cells from controls. Similarly, ligand binding to PP hepatocytes from ethanol-fed rats was 48% lower than their corresponding PP controls. Ligand binding to control PP cells was the same as that to control PV cells (Fig. 4). Ligand binding by PP cells from ethanol-fed rats was also equal to that by PV cells from the same group of animals.

Feeding rats an LC-ethanol diet has been shown to exacerbate liver injury, leading to panlobular steatosis and enhanced inflammatory foci, compared with rats fed an NC-ethanol diet [22]. We determined whether the LC-ethanol diet exacerbated the reduction in ligand binding seen with the NC (Lieber–DeCarli control) diet. Our Scatchard analyses provided no indication that the LC diet caused any further impairment of binding (Table 2). Ligand blots of hepatocyte lysate proteins showed that binding of ^{125}I-IGF-II to proteins from hepatocytes of NC-ethanol-fed rats was 27% lower than their corresponding NC controls, while binding of ^{125}I-IGF-II to proteins from LC-ethanol-fed rats was 26% lower than their LC controls (Fig. 5 and inset). We attempted similar ligand blots using ^{125}I-PMP-BSA, but the bands detected on nitrocellulose membranes could not be quantified due to high background. Immunoblot assays

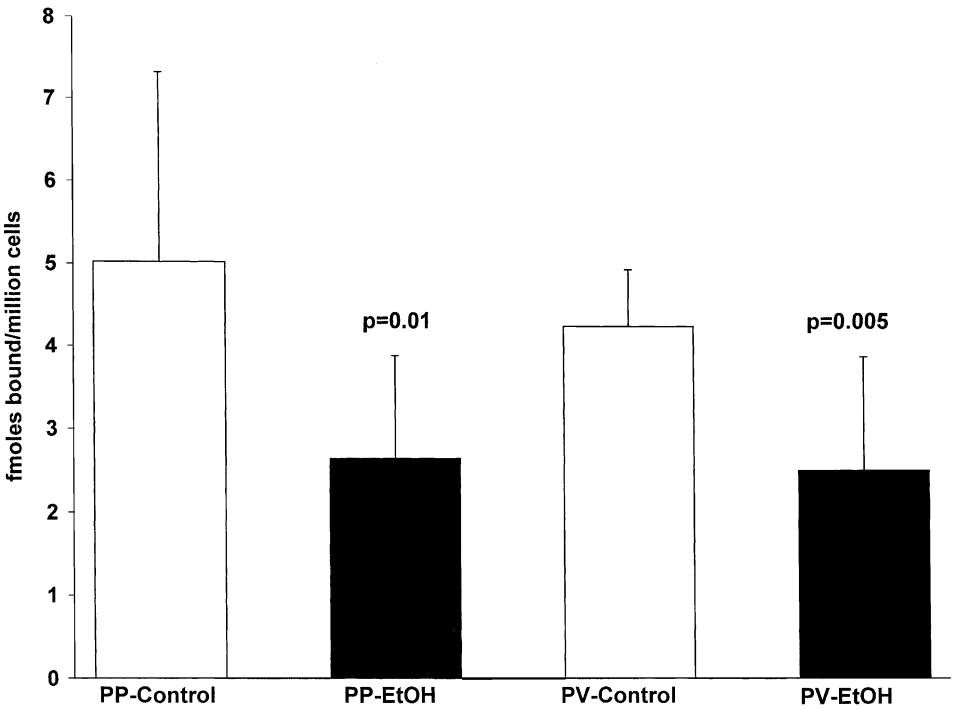

Fig. 4. Binding of ^{125}I-PMP-BSA to PP and PV hepatocytes isolated from rats fed the Lieber–DeCarli control and ethanol (EtOH) diets for 5–7 weeks. Isolation of cells and incubation mixture preparation are described in "Materials and methods". Results are means ± SD for five experiments.

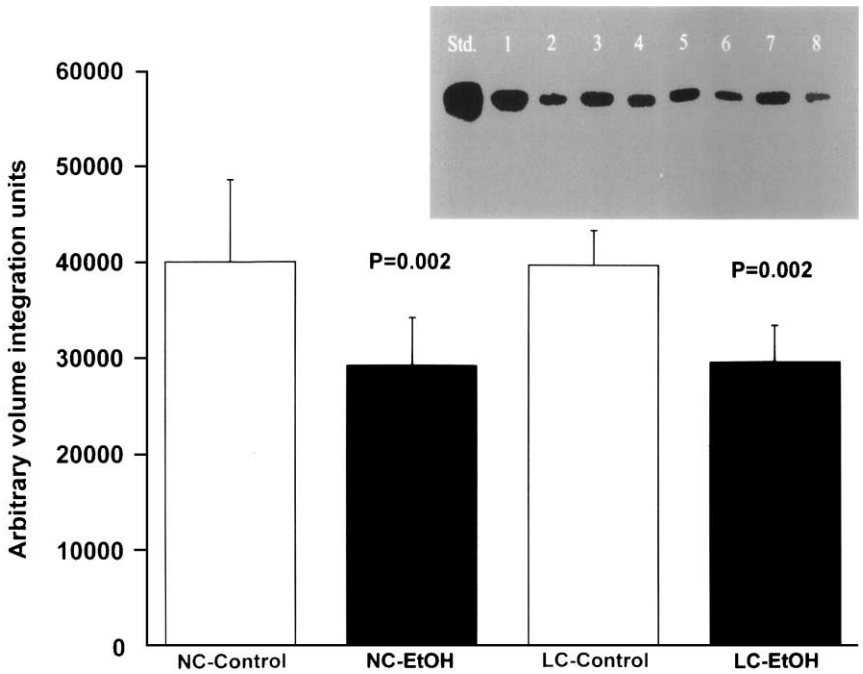

Fig. 5. Ligand blot of ^{125}I-IGF-II to lysate proteins from rats fed the NC and LC diets for 5–7 weeks. Results are mean arbitrary volume integration units (±SD) for five experiments. Inset: Representative labeled bands of hepatocyte lysate proteins probed with ^{125}I-IGF-II (2.5×10^6 cpm/μg of IGF-II). Lanes 1 and 3 are lysate proteins from rats fed the NC-control diet. Lanes 2 and 4 are lysate proteins from rats fed the NC-ethanol (NC-EtOH) diet. Lanes 5 and 7 are lysate proteins from rats fed the LC-control diet. Lanes 6 and 8 are lysate proteins from rats fed the LC-ethanol diet. M6P/IGF-II receptor, purified from plasma membrane of buffalo rat liver (BRL-3A) cells, was used as a standard.

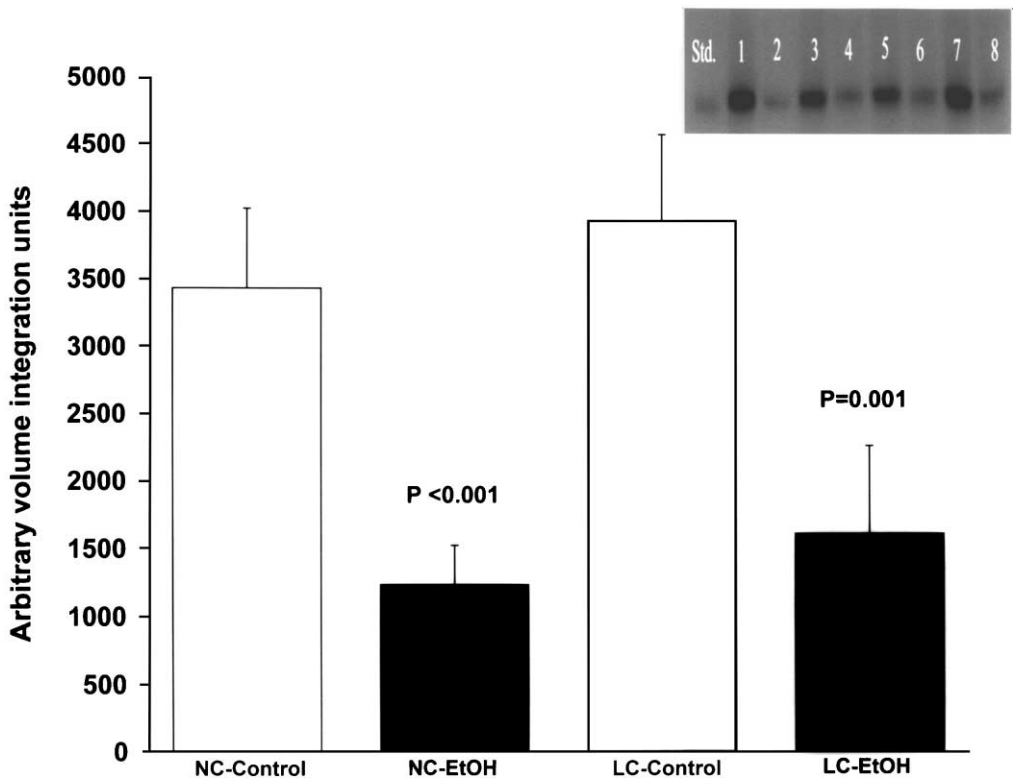

Fig. 6. Immunoblot analysis of M6P/IGF-IIR in hepatocyte lysates from rats fed the NC-ethanol (NC-EtOH) diet for 5–7 weeks and their pair-fed controls. Results are expressed as mean arbitrary volume integration units (±SD) for five experiments. Inset: Representative bands of immunoreactive receptor detected with ^{125}I-Protein A. Lanes 1 and 3 are lysate proteins from rats fed the NC-control diet. Lanes 2 and 4 are lysate proteins from rats fed the NC-ethanol diet. Lanes 5 and 7 are lysate proteins from rats fed the LC-control diet. Lanes 6 and 8 are lysate proteins from rats fed the LC-ethanol diet. M6P/IGF-IIR, purified from plasma membrane of buffalo rat liver (BRL-3A) cells, was used as a standard.

using an anti-M6P/IGF-IIR antibody demonstrated a 62% reduction of immunoreactive material in lysate proteins from hepatocytes of both NC-ethanol-fed and LC-ethanol-fed rats, when compared with their corresponding controls (Fig. 6 and inset).

4. Discussion

Using three methods of receptor quantification (ligand binding to hepatocytes, ligand blotting, and immunoblotting), we observed that chronic ethanol administration decreased the activity and the content of M6P/IGF-IIRs in rat hepatocytes. The whole cell ligand binding assay described here measures both the M6P/IGF-IIR as well as the 46 kDa receptor, known as the cation-dependent M6P receptor (*CD-MPR*) [14]. Thus, we cannot exclude the possibility that this receptor may have been affected similarly by ethanol administration. However, the M6P/IGF-IIR appears to be the predominant receptor for lysosomal enzyme trafficking in most cell types [5,36–38]. The ionic conditions (i.e. 2.5 mM $CaCl_2$) employed here for ^{125}I-PMP-BSA binding to whole cells were favorable for both types of receptors. Based on studies with the 46 kDa receptor from bovine liver, the pH of the ligand binding reaction used here (7.4) was much more favorable for binding to the larger receptor. The 46 kDa receptor shows optimal binding in the pH range from 6.0 to 6.3 [8,39]. Furthermore, the smaller receptor binds with much less affinity than the larger receptor to certain lysosomal enzymes [39]. We observed that the magnitude of the decrease of IGF-II binding to the M6P/IGF-IIR from hepatocyte lysates of ethanol-fed rats (Fig. 5) was comparable to that observed with PMP-BSA binding to whole cells (Fig. 2). These results would suggest that there was a minimal effect of ethanol on the 46 kDa receptor. On the other hand, the immunoblot results (Fig. 6) indicated a much larger decline in the level of immunoreactive receptor than that which was detected by whole cell or ligand binding. Thus, further analyses are required to determine conclusively whether ethanol consumption can similarly affect the 46 kDa M6P receptor.

It has been reported that ethanol administration to pregnant dams does not influence M6P/IGF-IIR content in livers from their fetuses [40]. Similarly, we reported finding equal levels of immunoreactive M6P/IGF-IIR protein on immunoblots of postnuclear fractions of liver from adult control and ethanol-fed rats [41]. However, the latter results were expressed per total liver weight, which, in ethanol-fed rats, is 17–34% higher than that in pair-fed control animals [25,42,43]. In addition, whole liver, which includes both hepatocytes and non-parenchymal cells, was used in both the previous studies. Hepatocytes are the predominant cell population in liver, but all hepatic cell types contribute to the total mass of M6P/IGF-IIR protein, which could be differentially regulated, depending upon the cell type. For example, it was reported previously that hepatic stellate cells respond to carbon tetrachloride-induced liver injury by increasing the amount of M6P/IGF-IIR [44]. It is possible that a significant percentage of the M6P/IGF-IIR may have been similarly regulated in non-parenchymal liver cells from ethanol-fed rats. Because of this, these cells may have contained a larger proportion than the parenchymal cells of receptor protein that contributed to the total receptor content.

Casey and colleagues [45] have shown that both total and surface binding by the ASGP receptor is reduced significantly in hepatocytes from ethanol-fed rats. Their findings are analogous with those reported here, except that we observed that ethanol consumption had no effect on ligand binding to the cell surface M6P/IGF-IIR (Table 1). The differential effect of ethanol on these two receptors at the cell surface may be due, in part, to the relative amounts of each. About 10% of M6P/IGF-IIR is localized at the cell surface, and about 90% of the receptor is localized intracellularly [46]. In our hands, about 95% of the M6P/IGF-IIR was intracellular, which is also consistent with other reports [47,48]. In contrast, about equal amounts of the ASGP receptor are found on the cell surface and the cell interior [49]. The large differences in the intracellular distribution of ASGP and M6P/IGF-II receptors would imply that the trafficking mechanisms responsible for the distribution of the ASGP receptor between the plasma membrane and the cell interior would be more significantly impaired by alcohol than those of the M6P/IGF-IIR, which remains largely inside the cell.

Chronic ethanol consumption reduced ligand binding to the M6P/IGF-IIR irrespective of liver zonal differences (Fig. 4). Our findings with pair-fed control animals indicated that PP cells had the same capacity as PV cells to bind PMP-BSA, suggesting that M6P/IGF-IIR expression is regulated uniformly in hepatocytes from both the PP region (zone 1) and the PV region (zone 3). In contrast, Casey *et al.* observed that the ASGP receptor appears to be differentially regulated, with a greater number of binding sites in hepatocytes from zone 3. In their studies, alcohol consumption affected receptor activity more severely in PV hepatocytes than in PP hepatocytes [49]. Thus, the extent of receptor expression may determine whether there is a differential effect of ethanol in these two hepatic cell types.

Our M6P/IGF-IIR quantification by Scatchard plot analysis, ligand blotting, and immunoblot analysis failed in all cases to show that LC-ethanol feeding further reduced the quantity of M6P/IGF-IIRs beyond that already produced by the NC-ethanol diet. Feeding ethanol with an LC diet for 6 weeks has been shown previously to produce greater liver damage than that produced by feeding an ethanol diet containing normal amounts of carbohydrate (NC diet) [22]. We observed that LC-ethanol-fed rats showed 15% higher levels of blood alcohol and 89% higher levels of hepatic triglycerides than those of NC-ethanol-fed rats (data not shown). These results suggest that despite the enhance-

ments in these parameters, the M6P/IGF-IIR is refractory to such changes over an extended treatment period. However, since these are early effects of chronic ethanol consumption, it is not clear whether more serious liver injury would further down-regulate the receptor.

The reduction in the level of immunoreactive M6P/IGF-IIR in hepatocytes of ethanol-fed rats suggests that ethanol administration decreased the intracellular steady-state levels of receptor protein either due to decreased receptor synthesis or enhanced receptor degradation. We have no evidence to indicate which mechanism is operative, but an ethanol-induced accelerated catabolism of the receptor seems unlikely. This is based on previous findings that ethanol consumption decelerates hepatic protein degradation in general [50,51] and decreases the catabolism of specific proteins, including cytochrome P4502E1 [52], the ligand for the ASGP receptor, and specific cytoplasmic proteins [49,53]. Tworek *et al.* demonstrated that the synthesis rate of the ASGP receptor is reduced by 50% in hepatocytes of ethanol-fed rats. This reduction in ASGP synthesis is due, in part, to a corresponding reduction in the steady-state level of the mRNA that encodes the RHL-1 subunit of that receptor protein [19]. In view of these findings, we are examining whether ethanol administration causes an alteration in the synthesis of the M6P/IGF-IIR and influences the steady-state levels of its mRNA.

Previous work from our laboratory has demonstrated that ethanol administration retards lysosomal cathepsin L maturation without affecting the synthesis of the precursor enzyme [20]. Thus, an ethanol-elicited decline in the hepatocellular content of the M6P/IGF-IIR could ultimately result in an insufficient quantity of receptor available for binding the procathepsin L precursor, as well as other M6P bearing proteins destined for localization into the lysosome. Furthermore, ethanol administration impairs the acidification of lysosomal and prelysosomal vesicles in hepatocytes [41,54]. This latter impairment would compound the ethanol-elicited depletion of the receptor, because a delay in the pH-dependent dissociation of the M6P/IGF-IIR from its ligand would likely result in a longer duration of receptor recycling and further retard the processing of acid hydrolase precursors during biogenesis of the lysosome.

Our findings are also of broader interest in light of the presumed role of the M6P/IGF-IIR in the regulation of cell proliferation. Cells with reduced expression of the M6P/IGF-IIR reportedly have a selective growth advantage over cells that express normal levels of receptor because the receptor protein has a putative role as a tumor suppressor [11,47,55–57]. Alcoholics with liver disease as well as heavy drinkers are both known to be at higher risk than nondrinkers for developing hepatocellular carcinoma [58]. Depletion of the M6P/IGF-IIR from hepatocytes by alcohol consumption is one mechanism that could enhance proliferation of normally quiescent liver cells in these individuals.

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