

## Ethanol consumption decreases the synthesis of the mannose 6-phosphate/insulin-like growth factor II receptor but does not decrease its messenger RNA

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### Abstract

The mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF-IIR) is a protein that facilitates the transport of acid hydrolases into the lysosome. We have shown that chronic ethanol consumption lowers the M6P/IGF-IIR content in rat hepatocytes. Here, we determined the steady-state level of mRNA encoding M6P/IGF-IIR, as well as the rate of receptor synthesis, to ascertain whether the ethanol-elicited reduction in receptor protein content is related to changes in either or both of these parameters. Rats were pair-fed the normal carbohydrate (NC) or low carbohydrate high-fat (LC) liquid diets containing either ethanol or isocaloric maltose-dextrin for 7–8 weeks. RNA was isolated from hepatocytes and from whole livers of these animals and subjected to reverse transcription–polymerase chain reaction (RT–PCR) to determine the mRNA levels encoding M6P/IGF-IIR. Hepatocytes isolated from these animals were also radiolabeled with Pro-mix L-[<sup>35</sup>S] *in vitro* cell labeling mix to measure incorporation into total cellular protein and the immunoprecipitated M6P/IGF-IIR protein. The steady-state levels of M6P/IGF-IIR mRNA in both hepatocytes and whole livers from ethanol-fed rats were the same as those from their respective controls regardless of whether they were fed the NC or the LC diets. Hepatocytes from ethanol-fed rats showed a 36% lower rate of total protein synthesis and an even greater reduction (70%) in receptor synthesis. When the relative rate of receptor synthesis was calculated, hepatocytes from ethanol-fed rats had a 53% lower relative rate of receptor synthesis compared with controls. Autoradiographic analysis of the immunoprecipitated receptor protein from ethanol-fed rats also indicated a 79% decline in the total M6P/IGF-IIR protein synthetic rate compared with pair-fed controls. We conclude that the ethanol-elicited reduction of M6P/IGF-IIR content was, in part, related to a concomitant reduction of receptor protein synthesis but not to a decline in its mRNA level. Thus, the ethanol-elicited decline in receptor protein synthesis may be due to defective M6P/IGF-IIR mRNA translation.

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### 1. Introduction

The M6P/IGF-IIR is a 300-kDa transmembrane glycoprotein that specifically recognizes and binds to lysosomal glycoproteins bearing mannose 6-phosphate (Man-6-P) residues and to the peptide hormone insulin-like growth factor II (IGF-II) [1]. Lysosomal glycoprotein precursors are phosphorylated at their mannose residues in the *cis*-Golgi complex and then sorted for targeting in the *trans*-Golgi network. The M6P/IGF-IIR specifically recognizes, binds, and facilitates the transport of these newly synthesized acid hydrolase precursors into the lysosomes via endosomes [1–5].

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**Abbreviations:** M6P/IGF-IIR, mannose 6-phosphate/insulin-like growth factor II receptor; NC, normal carbohydrate; LC, low carbohydrate high-fat; RT–PCR, reverse transcription–polymerase chain reaction; dNTP, deoxynucleotide triphosphate; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; ASGPR, asialoglycoprotein receptor; LBP, lipopolysaccharide binding protein; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

We have shown that chronic ethanol consumption lowers the M6P/IGF-IIR ligand binding activity and receptor protein content in rat hepatocytes [6]. To determine whether this ethanol-elicited reduction is related to changes in M6P/IGF-IIR mRNA content, we examined the steady-state level of M6P/IGF-IIR mRNA in hepatocytes and in whole livers of rats given control and ethanol diets. We also examined the rate of receptor protein synthesis in hepatocytes from control and ethanol-fed rats to ascertain whether the ethanol-elicited reduction is also related to changes in the synthesis of the receptor protein. Portions of this work have been reported previously in abstracts [7,8].

## 2. Materials and methods

### 2.1. Chemicals

All DNA primers were purchased from the University of Nebraska Medical Center, Eppley Molecular Biology Core Laboratory. *Taq* polymerase, RNasin, dNTPs, and *Hae*III-digested  $\phi$ X174 DNA markers were purchased from Promega. Collagenase, Percoll, methionine-free Minimum Essential Medium (MEM), protease inhibitor cocktail (Cat. No. #P2714), and  $^{14}\text{C}$ -labeled standard marker proteins were purchased from the Sigma Chemical Co. RNase-away was purchased from Molecular Bioproducts, Inc. Pro-mix L- $^{35}\text{S}$  *in vitro* cell labeling mix (1000 Ci/mmol) containing L- $^{35}\text{S}$  methionine and L- $^{35}\text{S}$  cysteine was purchased from Amersham.

### 2.2. Animal procedures

All animal procedures were performed in accordance with Public Health Service guidelines for the care and use of laboratory animals. 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. The LD101A liquid diet was from Purina Mills and has the same formulation as the Lieber–DeCarli diet. The diet was also modified to prepare the LC liquid diets as described previously [9]. The two forms of the liquid diets were used in order to determine whether the LC diet exacerbated the effects of ethanol exposure compared with those obtained using the normal carbohydrate formulation.

Rats were fed Purina chow diets until they reached 180–200 g. They were then divided into two groups; one group was fed the NC-control diet and the other was fed the LC-control diet, each for 3 days. The animals were then matched by weight and paired. Ethanol-fed rats were acclimated to their diets over a 3-day period, as previously described [10] before receiving full-strength ethanol diets. Pair feeding was conducted for 5–7 weeks.

Separate groups of rats were also pair-fed NC or LC control and ethanol-liquid diets for 6–8 weeks. Each animal was anesthetized with Nembutal (50 mg/kg body wt), and the liver was perfused with ice-cold 10 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose. Whole livers were removed from these animals. The livers were snap-frozen in liquid nitrogen and stored at  $-70^\circ$ . Frozen liver tissue was later used for RNA isolation.

### 2.3. Hepatocyte isolation

Rats were anesthetized by intraperitoneal injection of Nembutal (50 mg/kg body wt). Hepatocytes were isolated by the collagenase perfusion method of Seglen [11], as modified by Kharbanda *et al.* [12]. 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  $\mu\text{m}$  pore size) and pelleted by centrifugation at 50 g for 3 min at  $4^\circ$ . Further enrichment of viable cells was performed by centrifugation at 110 g for 5 min at  $4^\circ$  through 25 or 35% Percoll. Hepatocytes were washed twice, then resuspended in 20–25 mL KRH containing 2% BSA and 2.5 mM calcium chloride, and microscopically assessed for viability by trypan blue exclusion. Normally, cell viability was 88–95%. Contamination of hepatocyte preparations with Kupffer cells and/or sinusoidal endothelial cells (SEC) was determined immunochemically as recently described [6].

### 2.4. Partial hepatectomy

Partial hepatectomy was performed following the method of Higgins and Anderson [13]. Briefly, 6- to 20-week-old Wistar rats (220–540 g body wt) were anesthetized by intraperitoneal injection of Nembutal (50 mg/kg body wt), followed by either 70% partial hepatectomy or sham-surgery. Prior to closing the abdominal wall, the peritoneum was filled with saline solution (0.5% of body wt). Rats were placed on a 25–30° soft heating pad where they regained consciousness prior to their transfer to regular cages. Animals were then fed a chow diet and water *ad lib.* until they were killed 48 hr after surgery.

### 2.5. RNA isolation

Total RNA was isolated from either rat hepatocytes ( $2 \times 10^6$  cells) or from frozen rat liver tissue (50–100 mg) using the Tri-Reagent from Molecular Research Center Inc. The concentration of total RNA was estimated by its absorbance at 260 nm, where one absorbance unit equals 40  $\mu\text{g}$  of RNA. Purity of the RNA was determined by the ratio of its absorbance at 260 nm to that at 280 nm. Intactness of total RNA was assessed by the appearance of 18 S and 28 S ribosomal RNA bands following agarose gel

electrophoresis in the presence of formaldehyde. To test for possible DNA primer contamination, RNA was digested for 71 min with RNase A (25 units) prior to use in RT-PCR. The absence of amplified cDNA bands in the RNase-pretreated preparations (as well as in samples devoid of RNA) indicated no DNA primer contamination.

## 2.6. Analysis of mRNA by RT-PCR

The amounts of specific mRNA species were determined by semi-quantitative RT-PCR using a kit from Clontech Lab, Inc. Rat G3PDH cDNA and human  $\beta$ -actin cDNA control primers were used as internal standards. Total RNA (2.5  $\mu$ g) was reverse-transcribed to cDNA for 1.10 hr at 45°. RT was carried out in a total volume of 25  $\mu$ L containing 1 mM dNTPs, 20 units of RNasin, 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT), 50 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 2.5  $\mu$ g RNA, and 50 pmol of the respective primer. The RT-product was denatured at 94° for 30 sec, annealed at 60° for 1 min, 15 sec and extended at 72° for 2 min for 30 cycles in a thermal cycler from Whatman Biometra, Model-Tpersonal. PCR was carried out in a total volume of 50  $\mu$ L containing 1 mM Tris-HCl (pH 9.0), 5 mM KCl, 0.01% Triton X-100, 1 mM dNTPs, 2.5 mM MgCl<sub>2</sub>, template DNA, 5 units of *Taq* polymerase, and 50 pmol of the respective primer. The final RT-PCR product (cDNA) was then separated by electrophoresis in 1% agarose in 40 mM Tris (pH 8.5) containing 40 mM acetic acid and 1 mM EDTA. The separated products were stained with ethidium bromide and quantified with a Fluor-S<sup>TM</sup> multi-Imager from Bio-Rad.

G3PDH primers were sense (5'-TGAAGGTCGGTGTCAACGGATTTGGC-3') and antisense (5'-CATGTAGGCCATGAGGTCCACCAC-3'). The predicted amplified PCR product size was 983 bp.  $\beta$ -Actin primers were sense (5'-TTGTAACCAACTGGGACGATATGG-3') and antisense (5'-GATCTTGATCTTCATGGTGCTAGG-3'), resulting in an amplified PCR product of 764 bp. Using G3PDH and  $\beta$ -actin as internal standards, the relative amounts of mRNA for M6P/IGF-IIR, ASGPR, TGF- $\beta$ 1, and LBP were analyzed. The sequences of the oligonucleotide primers used were: M6P/IGF-IIR sense (5'-CTCCCTAAGTGGCAAAGCTG-3'), antisense (5'-GTCGTCTCACTCTCATCG-3'); TGF- $\beta$ 1 sense (5'-CTTCAGCTCCACAGAGAAGAACTGC-3'), antisense (5'-CACGATCATGTTGGACAAGTCTCC-3'); LBP sense (5'-GAGGCCTGAGTCTCTCCATCT-3'), antisense (5'-TCTGAGATGGCAAAGTAGACC-3'); and ASGPR sense (5'-CAAGGCTCTCATGTGCAGAA-3'), antisense (5'-AGCGTGGAGTGATCTGCTTT-3'). The predicted amplified PCR product sizes were 580 bp for M6P/IGF-IIR, 426 bp for TGF- $\beta$ 1, 552 bp for LBP, and 350 bp for ASGPR. Results were expressed as a ratio of the fluorescent band intensities of each sample's amplified cDNA to that of the internal standard cDNA.

## 2.7. Immunoaffinity resin coupling to antibody 293

Specific anti-receptor antibody was raised in rabbits using purified M6P/IGF-IIR from rat placental plasma membranes [14–16]. This antibody or the non-immune serum from the same rabbit was covalently coupled to Affi-Gel 10 (Bio-Rad) following the method of MacDonald and Czech [17]. In brief, Affi-Gel 10 was washed three times with ice-cold water, and then three times with ice-cold 50 mM HEPES buffer (pH 7.4) containing 0.15 M NaCl (HBS). The Affi-Gel 10 was mixed at a ratio of 2 mg protein/mL of the packed Affi-Gel 10 on an end-over-end mixer at 4° for 16 hr with either anti-M6P/IGF-IIR or with non-immune serum, each of which had been dialyzed previously against HBS. These mixtures were then centrifuged at 18,000 *g* for 10 min at 4°, and the packed Affi-Gel 10 was resuspended in 100 mM ethanolamine-HCl (pH 8) and incubated for 2 hr at 4° to block the remaining functional groups of the resin. The resin-antibody or the resin-non-immune conjugates were washed three times with 50 mM HBS containing 0.05% Triton X-100 and stored at 4° in the same buffer containing 0.2% Triton X-100 and 0.02% sodium azide.

## 2.8. Radiolabeling

Hepatocytes isolated from LC ethanol-fed rats (96  $\pm$  2% viable) or their pair-fed controls (98  $\pm$  2% viable) were washed twice with methionine- and cysteine-free MEM. Hepatocytes, at a density of 5 million cells/mL, were incubated with gentle shaking at 60 rpm for 1 hr at 37° with 200  $\mu$ Ci of Pro-mix L-[<sup>35</sup>S] *in vitro* cell labeling mix/mL in a total volume of 10 mL MEM. Incubation in the labeling medium resulted in a decline in viability of 6% in hepatocytes from ethanol-fed rats and of 4% in the pair-fed controls. Radiolabeled hepatocytes were washed once with ice-cold PBS (pH 7.4), and then were pelleted and solubilized for 1 hr on ice with 2 mL of RIPA buffer [25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 4 mM EDTA, 0.1% SDS, 0.55% deoxycholate, and 1% Triton X-100 containing a protease inhibitor cocktail consisting of 4 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 2 mM EDTA, 260  $\mu$ M Bestatin, 2.8  $\mu$ M *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane (E-64), 20  $\mu$ M leupeptin, and 0.6  $\mu$ M aprotinin]. A small fraction of each solubilized cell pellet was used for DNA assay and for the determination of total incorporation of radioactivity into cellular proteins. The remainder of each lysate was centrifuged at 105,000 *g* for 30 min at 4°, and the supernatant was used for immunoprecipitation of M6P/IGF-IIR. The incorporation of sulfur-35 into total cellular proteins was determined in an aliquot (50  $\mu$ L, diluted to 10-fold) of the cell suspension using the filter disc method of Mans and Novelli [18].

Rat hepatoma cells (H4-II-E) derived from the Reuber H-35 cell lines were obtained from the American Type

Culture Collection (Cat. No. #CRL-1548) and were used to obtain a positive marker of M6P/IGF-IIR protein synthesis since these cells synthesize high levels of the receptor [17]. These cells were cultured as monolayers in 150 cm<sup>2</sup> cell culture flasks using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 µg gentamicin/mL, 100 units penicillin/mL, and 85 µg streptomycin/mL. Monolayers of hepatoma cells 75–80% confluent (approximately 30 million cells/flask) were washed once with PBS (pH 7.4) and washed twice with MEM. Cells were radiolabeled with 200 µCi of Pro-mix L-[<sup>35</sup>S] *in vitro* cell labeling mix/mL for 1 hr at 37°. Radiolabeled hepatoma cells were washed with ice-cold PBS (pH 7.4), pelleted, and solubilized on ice for 1 hr in 1 mL of RIPA buffer (pH 7.4), containing protease inhibitor cocktail.

### 2.9. Immunoprecipitation of M6P/IGF-IIR

High speed supernatant samples (0.5 mL each) from rat hepatocytes or from rat hepatoma cells that were radiolabeled previously were incubated with a previously optimized volume of Affi-Gel 10-antibody or the resin-non-immune conjugates (10 µL) overnight at 4° with a rotating mixer. Each mixture was centrifuged at 18,000 *g* for 10 min at 4°. The supernatant was discarded and, with intervening centrifugations, the resin-immune complex was washed

three times with 50 mM HEPES (pH 7.4) containing 0.2% Triton X-100. Each successive wash contained decreasing concentrations of NaCl of 1, 0.15, and 0 M. Each resin-containing bound immune complex was then boiled at 100° for 5 min in 30 µL of SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, containing 50 mM dithiothreitol, 1.0% SDS, and 5% sucrose) and centrifuged at 18,000 *g* for 5 min at 4°. Total radioactivity in each immunoprecipitate was determined by counting a 3-µL aliquot from the solubilized sample. The remainder of each sample was reconstituted to 40 µL with the same buffer containing 0.2% bromophenol blue. The entire sample was loaded onto a 1.5-mm thick 6% polyacrylamide gel. <sup>14</sup>C-Methylated proteins from Sigma (M9832) were used as molecular weight markers, while the immunoprecipitated receptor from the H4-II-E hepatoma cells was used as a positive marker to identify M6P/IGF-IIR. Each gel was run for 45 min at 160 V under reducing conditions. The gel containing the radiolabeled immunoprecipitated receptor proteins was fixed using the gel prep NOVEX system from Invitrogen consisting of 30% EtOH and 10% glycerol in water, followed by air-drying the gel overnight at room temperature. Each gel was then exposed to a PhosphorImager screen for 16 hr at 24°. Radioactive bands were detected and quantified densitometrically as arbitrary volume integration units (VIU) using Molecular Dynamics ImageQuant software.

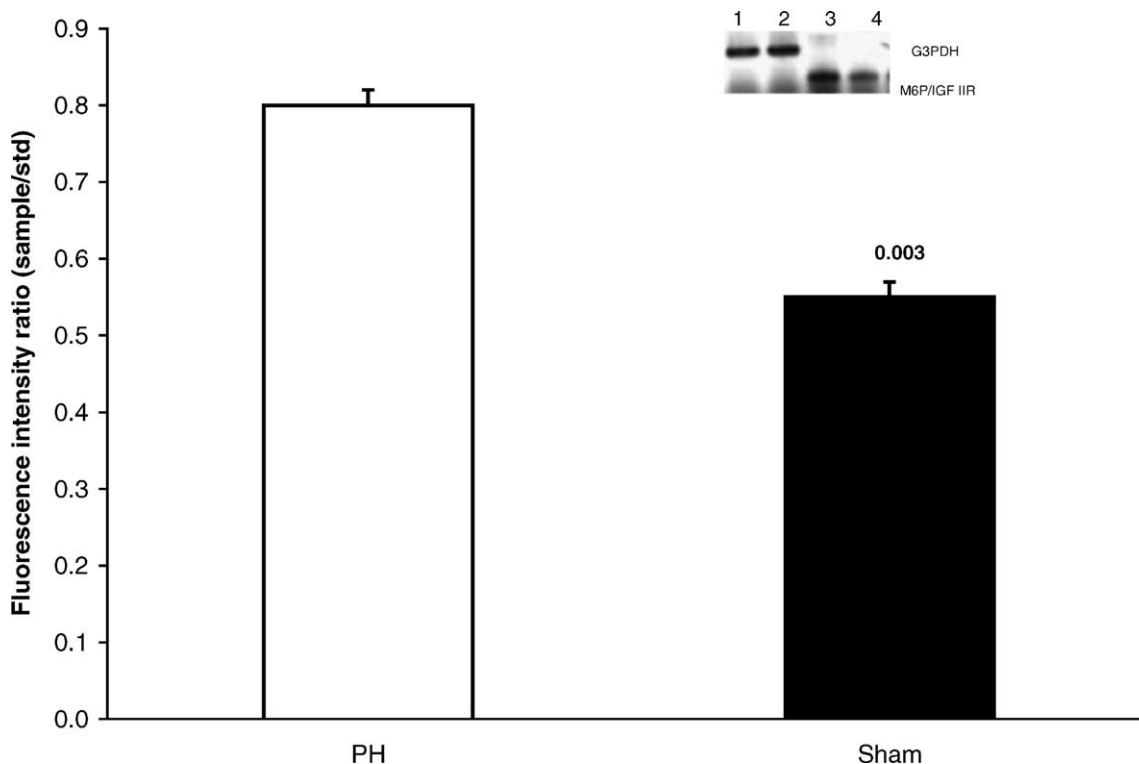


Fig. 1. RT-PCR analysis of M6P/IGF-IIR mRNA in hepatocytes from 6- to 20-week-old male Wistar rats 48 hr after surgery. Data are expressed as the mean ratio ( $\pm$ SEM) of band intensities of each amplified M6P/IGF-IIR cDNA (sample) to that of the G3PDH internal standard cDNA (std) from replicate runs of four pairs of rats. The number above the sham bar indicates a significant difference relative to partially hepatectomized (PH) rats. Inset: representative RT-PCR bands from hepatocytes of these animals. Lanes 1 and 2 are G3PDH cDNA products, and lanes 3 and 4 are M6P/IGF-IIR cDNA products from hepatocytes of PH and sham animals, respectively.

### 2.10. Statistical analysis

ANOVA models were used to compare average results among the treatment groups. A mixed-effects ANOVA model was fit to account for the paired structure of the data, in which ethanol administration, diet treatment, and/or partial hepatectomy were modeled as fixed effects, and feeding pair was modeled as a random effect. The feeding pair factor was nested within the diet group when both ethanol and diet varied. The possible interaction between ethanol exposure and diet treatment was modeled in cases where both factors were manipulated. Non-significant interactions were dropped from the model. Diagnostic plots of the residuals were created to assess the adequacy of the ANOVA model assumptions. To determine whether differences in the mean ratios from RT-PCR analyses were generated due to differences in the values of actual gene

products or to differences in the values of G3PDH (the internal standard), we used an analysis of covariance model. Again, ethanol administration was modeled as a fixed effect, feeding pair was modeled as a random effect, and the corrected values of G3PDH were modeled as a continuous covariate with the corrected values of the gene product of interest as the outcome variable. We used a Wilcoxon signed rank test to compare the results of the two animal groups in the receptor protein synthesis experiments. The averages of repeated measurements on the same animal were analyzed.

### 3. Results

Hepatocytes, as well as whole livers from rats fed either the NC or LC ethanol-liquid diets yielded equal amounts of

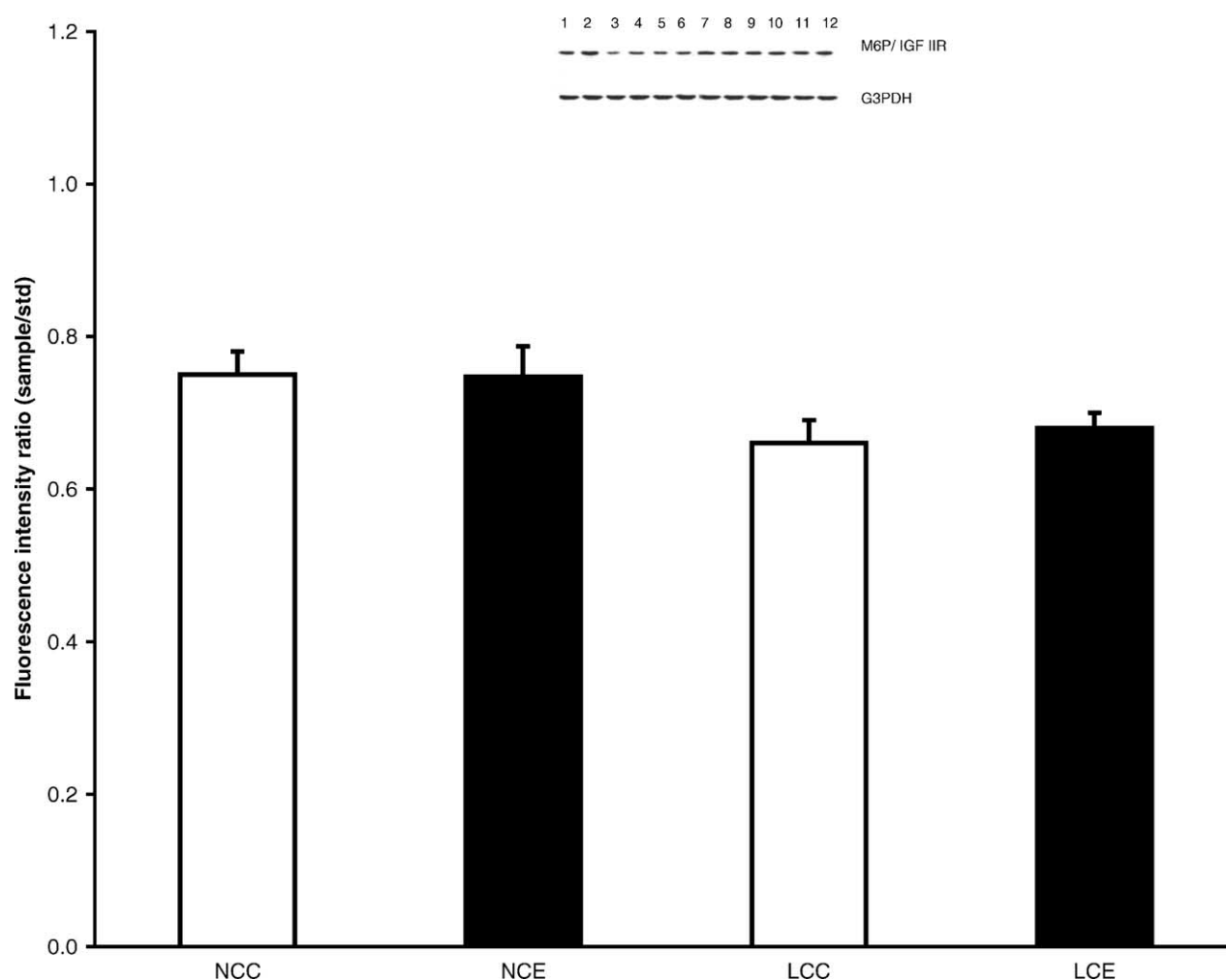


Fig. 2. RT-PCR analysis of M6P/IGF-IIR mRNA in hepatocytes from control and ethanol-fed rats fed the NC and LC diets for 5–7 weeks. Results are expressed as the mean ratio ( $\pm$ SEM) of band intensities of each amplified M6P/IGF-IIR cDNA to that of the G3PDH internal standard cDNA (std) from 12 pairs of rats in each diet group. No significant differences were observed between the ethanol-exposed and control animals ( $P = 0.71$ ). NCC = normal carbohydrate control diet, NCE = normal carbohydrate ethanol diet, LCC = low carbohydrate control diet; and LCE = low carbohydrate ethanol diet. Inset: representative RT-PCR bands of M6P/IGF-IIR cDNA (upper bands) and G3PDH cDNA (lower bands) from rat hepatocytes. Upper and lower bands have the same sampling orientation. Even numbered lanes are from animals fed ethanol diets, and odd numbered lanes are from corresponding pair-fed controls. Lanes 1–6 are cDNA products from rats fed the NC diets, and lanes 7–12 are cDNA products from rats fed the LC diets, each three pairs.



Table 1

Mean volume integration units of PCR-generated G3PDH cDNA bands from hepatocytes and from whole livers of rats pair-fed the NC or LC liquid diets

Specimen	NCC (VIU)	NCE (VIU)	LCC (VIU)	LCE (VIU)	<i>P</i> values NCC + LCC vs. NCE + LCE
Hepatocytes	28,500 ± 700	28,200 ± 800	25,300 ± 600	25,700 ± 600	0.93
Whole livers	29,500 ± 800	29,100 ± 1100	35,000 ± 1700	35,900 ± 1400	0.66

Results are the mean volume integration units (VIU ± SEM) of the cDNA bands obtained from G3PDH primers using RNA samples from hepatocytes (N = 12 pairs) and from whole livers (N = 8 pairs) of rats pair-fed the NC or LC liquid diets. NCC = normal carbohydrate control diet, NCE = normal carbohydrate ethanol diet, LCC = low carbohydrate control diet, and LCE = low carbohydrate ethanol diet. The variations in the mean intensities between the dietary groups reflect variations in gel exposure times.

total RNA as those of their respective pair-fed controls (data not shown). In these studies, RNA samples isolated from hepatocytes were taken from the same groups of animals previously used for the receptor protein content studies [6]. RNA (2.5 µg) was subjected to RT-PCR followed by electrophoresis of the amplified products in 1% agarose. Ethanol consumption had no effect on the mRNA levels for either β-actin (data not shown) or for G3PDH in hepatocytes (Table 1).

To test the validity of the RT-PCR method used here, we determined the level of M6P/IGF-IIR mRNA in hepatocytes from both partially hepatectomized (PH) rats and their sham-operated controls. M6P/IGF-IIR mRNA level is elevated as much as 4-fold in regenerating livers of PH rats [19,20]. Our analyses revealed that hepatocytes from PH

rats had a 31% higher level of M6P/IGF-IIR mRNA ( $P = 0.003$ ) than sham-operated animals (Fig. 1). While this elevation of M6P/IGF-IIR mRNA level in hepatocytes from PH rats was not the same magnitude as that reported previously [19], the results indicated that the RT-PCR method was sensitive enough to detect even subtle changes in this mRNA level.

The level of mRNA for M6P/IGF-IIR in rat hepatocytes from either NC or LC ethanol-fed rats was the same as that of their pair-fed controls (Fig. 2). However, there was a slight increase (11%) in hepatocytes in the average ratio for NC relative to LC fed rats ( $P = 0.03$ ). Similar results were obtained from whole livers of NC and LC control and ethanol-fed animals. In whole livers there was a significant difference in the average ratio between the NC and LC fed

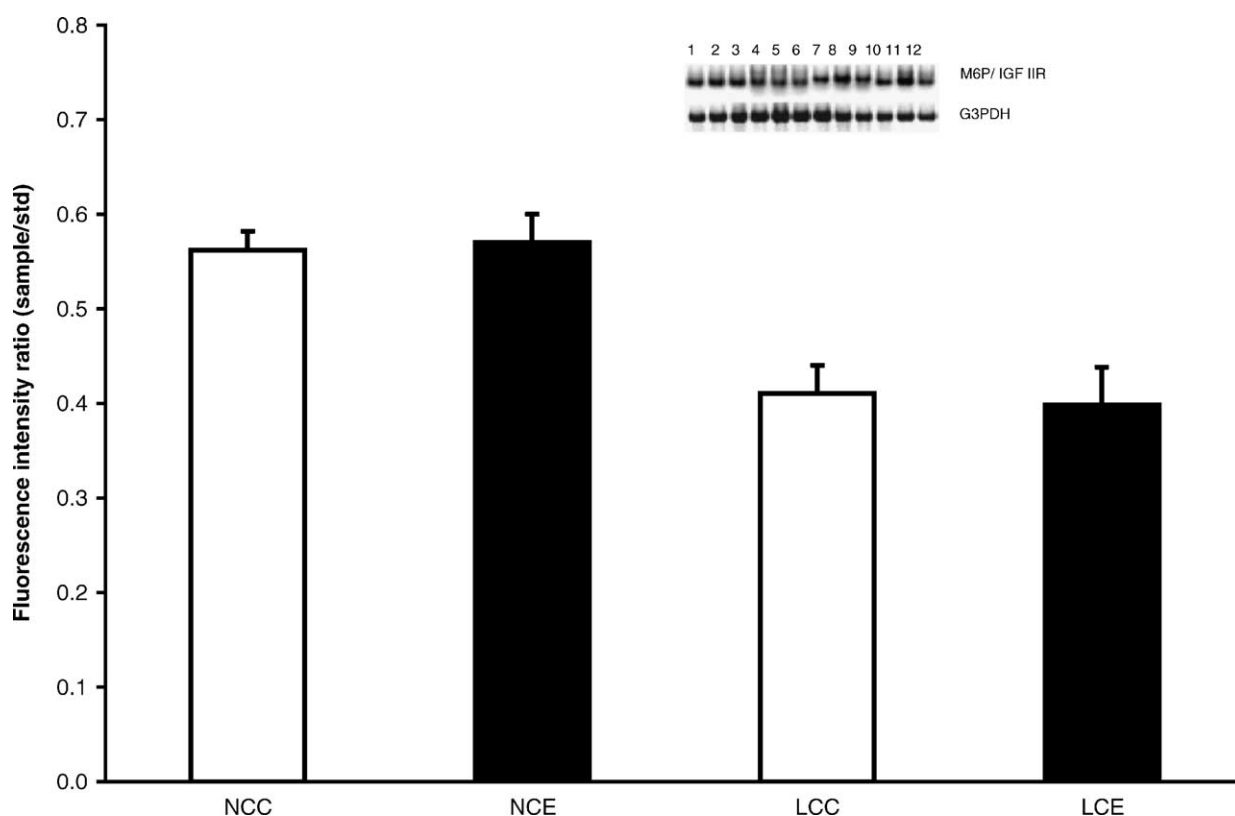


Fig. 3. RT-PCR analysis of M6P/IGF-IIR mRNA in whole livers from control and ethanol-fed rats fed the NC and LC diets for 7–8 weeks. Results are expressed as the mean ratio (±SEM) of band intensities of each amplified M6P/IGF-IIR cDNA (sample) to that of the G3PDH internal standard cDNA (std) from eight pairs each. Inset: representative RT-PCR bands of M6P/IGF-IIR cDNA (upper bands) and G3PDH cDNA (lower bands) from whole livers of rats. Upper and lower bands have the same sampling orientation. Even numbered lanes are from animals fed ethanol diets, and odd numbered lanes are from their pair-fed controls. Lanes 1–6 are cDNA products from rats fed the NC diet, while lanes 7–12 are from rats fed the LC diet, each three pairs.

rats ( $P < 0.001$ ) (Fig. 3). However, this difference was due, in large part, to the correction of G3PDH. Here, the value of the internal standard from whole livers of LC diet-fed rats was numerically larger than that of the NC diet-fed rats when the absolute densitometric values of the G3PDH bands in these animals were compared (see footnote to Table 1). In both hepatocytes and in whole livers, there was no evidence of an interaction between ethanol administration and diet consumption, meaning the effect of ethanol exposure did not differ by diet treatment ( $P = 0.7$  and  $P = 0.6$ , respectively). Thus, the remainder of our RNA analyses used samples derived only from rats fed the NC control and ethanol-liquid diets.

Hepatocytes from NC ethanol-fed rats had 33% lower levels of ASGPR mRNA than their pair-fed controls ( $P = 0.004$ ), confirming previous findings determined by northern blotting [21]. Whole livers from NC ethanol-fed rats showed a 19% ( $P = 0.008$ ) decline of ASGPR mRNA compared with livers from pair-fed control rats (Fig. 4). The level of TGF- $\beta$ 1 mRNA in hepatocytes from rats

subjected to NC-ethanol administration was 40% higher than controls ( $P < 0.009$ ). In whole livers this difference was reduced to 16% but was still significant ( $P = 0.001$ ) (Fig. 5). Similarly, hepatocytes from NC ethanol-fed rats showed 40% higher level of LBP mRNA than their pair-fed controls ( $P = 0.009$ ), while in whole livers of ethanol-fed rats the mRNA level was 16% higher ( $P = 0.006$ ) than controls (Fig. 6).

It was apparent from the latter analyses that, while there were significant differences in certain mRNAs between control and ethanol-fed rats, the magnitude of this difference changed between hepatocytes and whole livers. The reason for this is not immediately clear. However, it may be related to the relative expression of these mRNAs in isolated hepatocytes versus whole livers. Hepatocytes constitute 70% of the cellular content of rat liver, while the remaining 30% are non-parenchymal cells [22]. Thus, for example, the hepatocyte-specific ASGPR mRNA would be more enriched in hepatocytes than the whole liver, and the data presented here may reflect such differences [23].

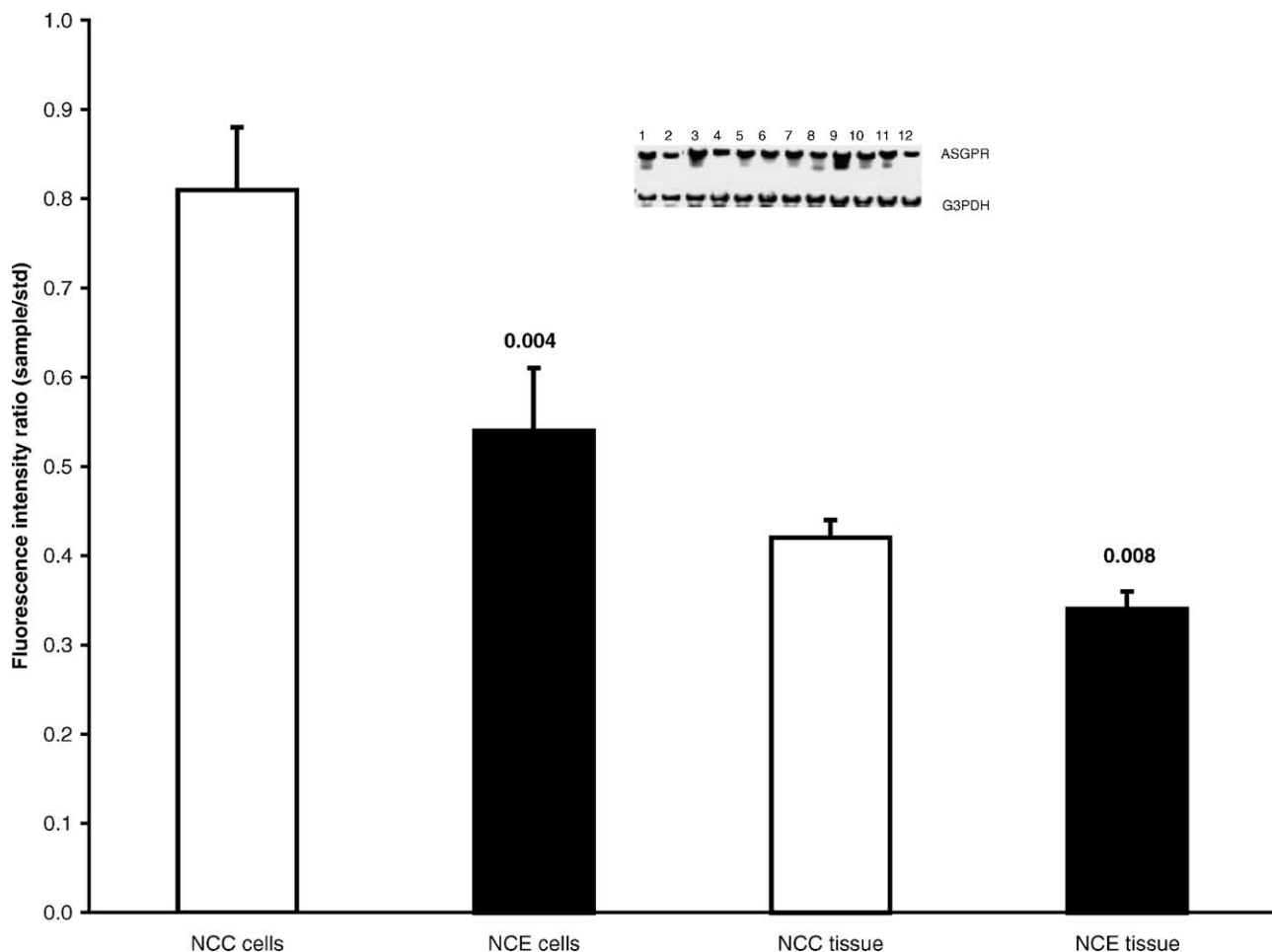


Fig. 4. RT-PCR analysis of ASGPR mRNA from hepatocytes (cells) and from whole livers (tissue) of rats fed the NC control and ethanol diets for 5–8 weeks. Results are expressed as the mean ratio ( $\pm$ SEM) of band intensities of each amplified ASGPR cDNA to that of the G3PDH cDNA from nine pairs each for hepatocytes and eight pairs each for whole livers. Numbers above the NCE bars indicate a significant difference relative to the NCC bars. Inset: representative RT-PCR bands of ASGPR cDNA (upper bands) and G3PDH cDNA (lower bands) from hepatocytes from six pairs of rats. Upper and lower bands have the same sampling orientation. Even numbered lanes are from rats fed the ethanol diet, and odd numbered lanes are from their pair-fed controls.

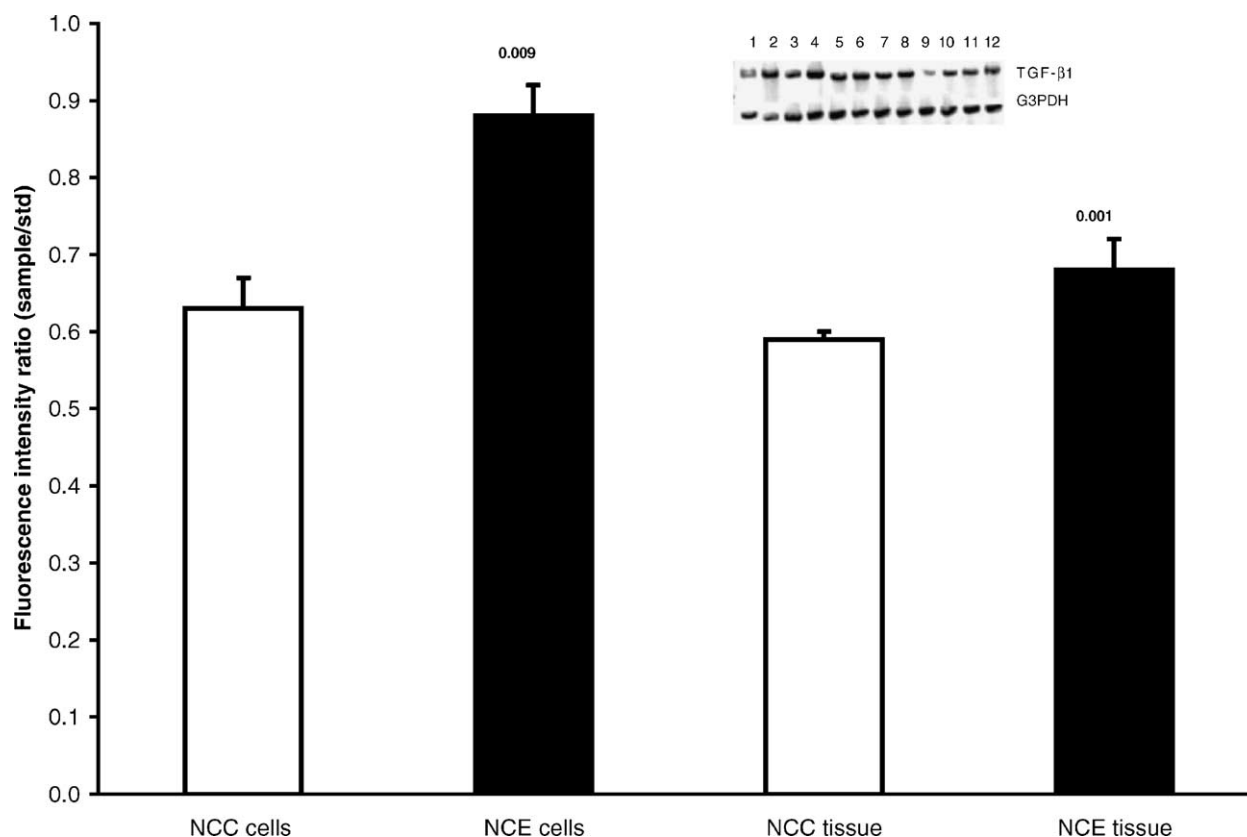


Fig. 5. RT-PCR analysis of TGF- $\beta$ 1 mRNA from hepatocytes (cells) and from whole livers (tissue) of rats fed the NC control and ethanol diets for 5–8 weeks. Results are expressed as mean ratio ( $\pm$ SEM) of band intensities of each amplified TGF- $\beta$ 1 cDNA to that of the G3PDH cDNA from nine pairs each for hepatocytes and eight pairs each for whole livers. Numbers above the NCE bars indicate a significant difference relative to the NCC bars. Inset: representative RT-PCR bands of TGF- $\beta$ 1 cDNA (upper bands) and G3PDH cDNA (lower bands) from hepatocytes from six pairs of rats. Upper and lower bands have the same sampling orientation. Even numbered lanes are from rats fed the ethanol diet, and odd numbered lanes are from their pair-fed controls.

Incorporation of Pro-mix L-[ $^{35}$ S] *in vitro* cell labeling mix into total cellular proteins was determined in hepatocytes from rats pair-fed the LC diet. Both NC- and LC-ethanol diets decrease the M6P/IGF-IIR content in rat hepatocytes to the same extent after chronic ethanol consumption [6]. Thus, changes in the rate of M6P/IGF-IIR protein synthesis in hepatocytes from rats pair-fed the LC-ethanol diet should also reflect a situation similar to that in animals pair-fed the NC diets. Compared with controls, there was a 36% reduction of total protein synthesis in hepatocytes from LC ethanol-fed rats. However, these cells

exhibited a 70% decline in receptor protein synthesis compared with their pair-fed controls (Table 2). Thus, the rate of receptor protein synthesis relative to the synthesis of total cellular proteins was 53% lower in hepatocytes from ethanol-fed rats compared with their pair-fed controls (Fig. 7). Autoradiographic analysis of the immunoprecipitated receptor protein from ethanol-fed rats indicated a 79% decline in the M6P/IGF-IIR synthetic rate in these cells compared with pair-fed controls. Hepatocytes from a limited number of chow-fed rats showed similar rates of synthesis to those of pair-fed controls (data not shown).

Table 2

Mean radioactivity incorporation into total cellular protein, immunoprecipitated M6P/IGF-IIR protein, and mean volume integration units of receptor protein bands from hepatocyte lysates of rats pair-fed the LC liquid diets

Group	Total radiolabeled (cpm/ $\mu$ g DNA)	Immunoprecipitated (cpm/ $\mu$ g DNA)	% Synthesis	Densitometric (VIU)
LCC	393,300 $\pm$ 52,700	840 $\pm$ 240	0.20 $\pm$ 0.05	316,200 $\pm$ 138,000
LCE	253,200 $\pm$ 36,600	260 $\pm$ 60	0.10 $\pm$ 0.02	67,000 $\pm$ 31,200
P values	0.03	0.03	0.03	0.03

Results are the mean incorporation of radioactivity into total cellular protein and the immunoprecipitated receptor protein; both are expressed as cpm/ $\mu$ g DNA  $\pm$  SEM; N = 6. M6P/IGF-IIR protein synthesis results are expressed as percent synthesis ( $\pm$ SEM) of the immunoprecipitated protein (cpm/ $\mu$ g DNA) to that of the total incorporation of radioactivity into cellular proteins (cpm/ $\mu$ g DNA) from six paired samples. Densitometric volume integration units (VIU  $\pm$  SEM) were obtained from autoradiographic analysis of the immunoprecipitated protein bands after 6% SDS-PAGE from six paired samples. LCC = low carbohydrate control diet, and LCE = low carbohydrate ethanol diet.



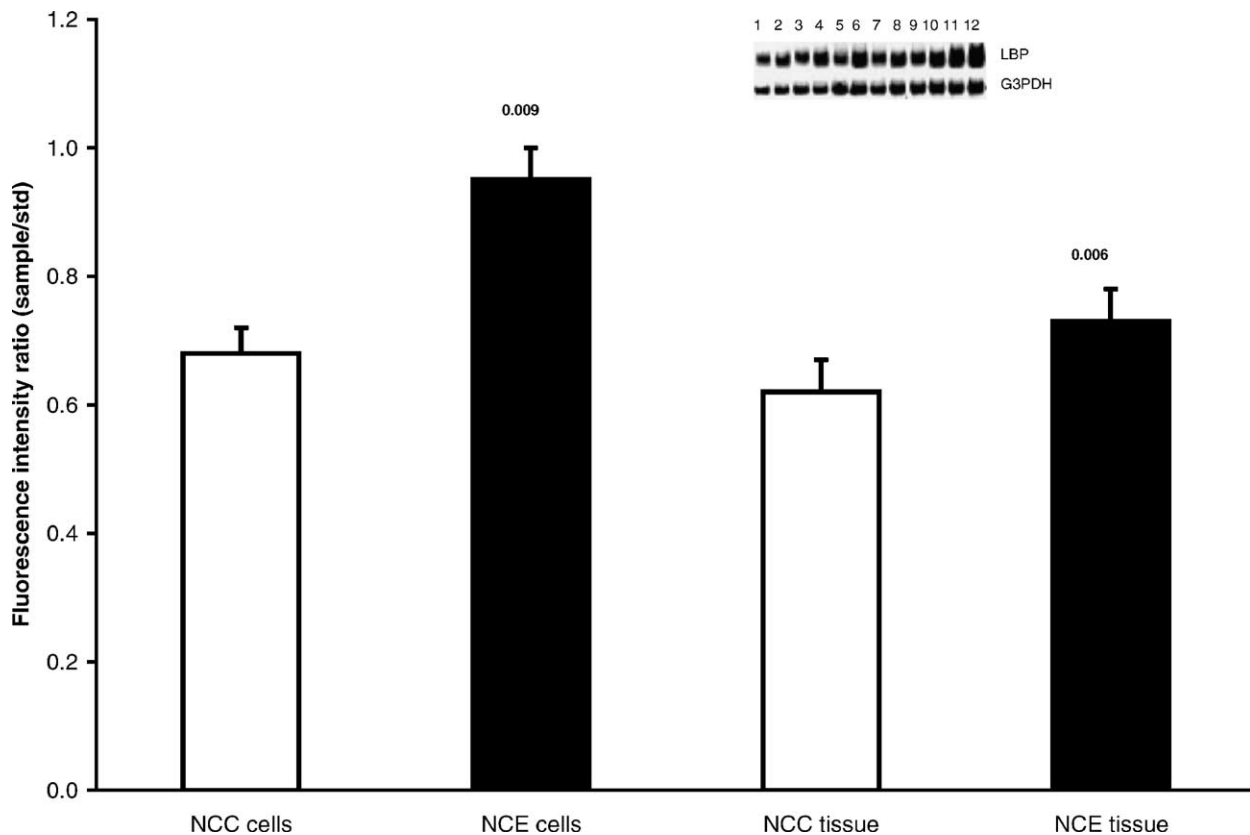


Fig. 6. RT-PCR analysis of LBP mRNA from hepatocytes (cells) and from whole livers (tissue) of rats fed the NC control and ethanol diets for 5–8 weeks. Results are expressed as the mean ratio ( $\pm$ SEM) of band intensities of each amplified LBP cDNA to that of the G3PDH internal standard cDNAs from eight pairs each. Numbers above the NCE bars indicate a significant difference relative to the NCC bars. Inset: representative RT-PCR bands of LBP cDNA (upper bands) and G3PDH cDNA (lower bands) from hepatocytes from six pairs of rats. Upper and lower bands have the same sampling orientation. Even numbered lanes are from rats fed the ethanol diet, and odd numbered lanes are from their pair-fed controls.

#### 4. Discussion

We have reported previously that chronic ethanol consumption causes a reduction of M6P/IGF-IIR content in rat hepatocytes [6]. The ethanol-elicited reduction in the amount of immunoreactive M6P/IGF-IIR indicated that ethanol administration decreases the intracellular steady-state levels of receptor protein either due to decreased receptor synthesis or enhanced receptor degradation. Accelerated receptor catabolism is unlikely, because ethanol consumption both decelerates overall hepatic protein degradation [24,25] and decreases the breakdown of specific proteins such as cytochrome P450-2E1 [26], the ligand for the ASGPR and specific cytoplasmic proteins [27,28]. The results presented here indicate that the ethanol-elicited reduction of M6P/IGF-IIR content is not related to a concomitant reduction of its mRNA, indicating that ethanol consumption affects receptor expression at a post-transcriptional step. Our results also confirmed that chronic ethanol consumption elevated the levels of mRNA for LBP and for TGF- $\beta$ 1 and simultaneously decreased ASGPR mRNA levels in hepatocytes and in livers of ethanol-fed rats, as reported by others [21,29–31]. Both the elevation and decline of the latter mRNAs by ethanol

occur with a concomitant increase or decrease in the proteins they encode [21,30–32]. Nuclear run-on assays and mRNA half-life studies showed that the ethanol-elicited increase in LBP mRNA level is due to activation of transcription factors AP-1 and C/EBP- $\beta$  as well as to ethanol-elicited LBP mRNA stabilization [33,34]. The mechanisms for the ethanol-induced reduction of ASGPR mRNA and the elevation of TGF- $\beta$ 1 mRNA are not known. We speculate that the ethanol-induced rise in the TGF- $\beta$ 1 mRNA level may be due to its post-transcriptional stabilization, similar to that of the increase in tumor necrosis factor- $\alpha$  mRNA, which is stabilized in rat Kupffer cells after ethanol exposure [35].

The results presented here with M6P/IGF-IIR mRNA also indicate that ethanol can differentially affect the protein and mRNA levels of different gene products. Examples of such dis-coordinate regulation of protein content and mRNA levels by ethanol and other hepatotoxins have been reported elsewhere. For example, both glycogen synthase activity (a + b) and content in rat liver are decreased significantly by chronic ethanol consumption without changing the glycogen synthase mRNA level [36]. Conversely, cytochrome P450-2E1 (CYP2E1) activity and its apoprotein are both elevated 4-fold in livers of

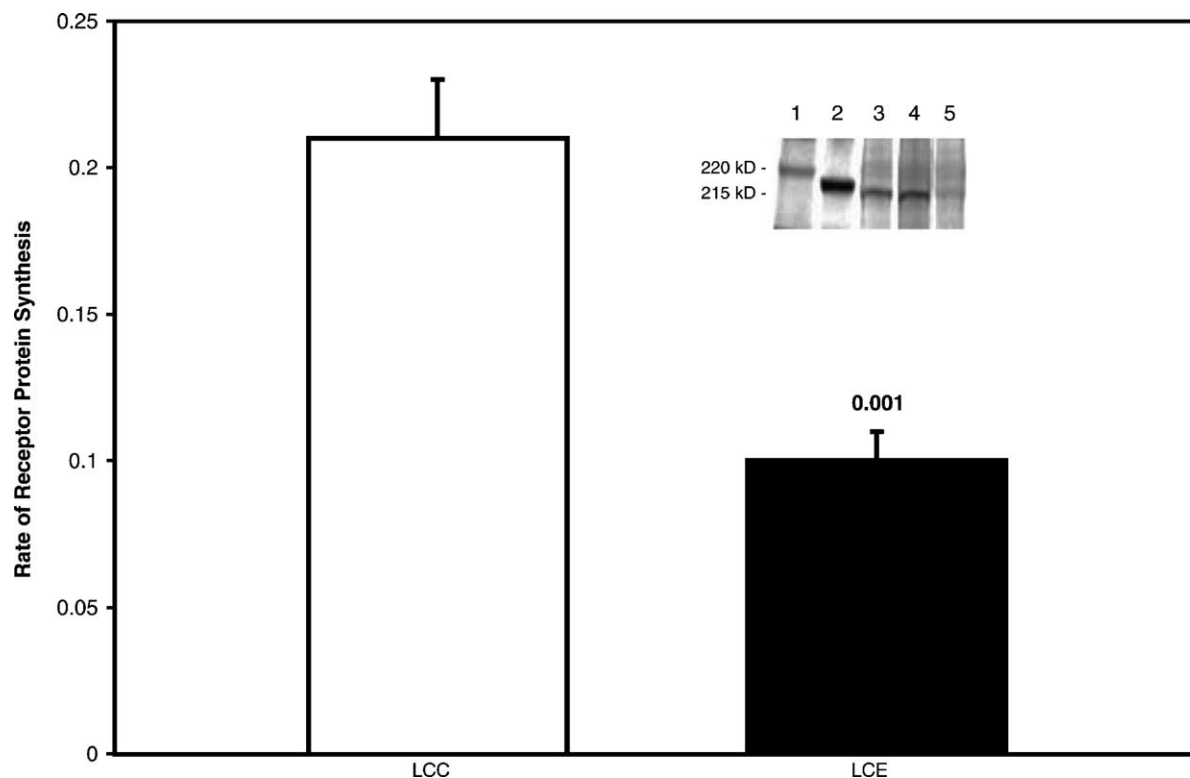


Fig. 7. M6P/IGF-IIR protein synthetic rate relative to the synthesis of total cellular proteins in hepatocytes from rats pair-fed the LC liquid diets for 5–7 weeks. The relative rates of M6P/IGF-IIR protein synthesis from Purina-fed rats were measured in the same age group to that of rats pair-fed the LC liquid diets. Percent synthesis results from six paired samples are expressed as the mean ratio ( $\pm$ SEM) of the radioactivity in the immunoprecipitated receptor protein (cpm/ $\mu$ g DNA) to that in total cellular protein (cpm/ $\mu$ g DNA). The number above the LCE bar indicates a significant difference relative to the LCC bar. Inset: representative newly synthesized M6P/IGF-IIR bands from rat hepatocytes of one pair of samples. Lane 1 =  $^{14}$ C-methylated proteins, molecular weight marker 220 kDa; lane 2 = a newly synthesized M6P/IGF-IIR band from rat hepatoma (H4-II-E) cells, molecular weight corresponding to 215 kDa under reduced conditions; lane 3 = hepatocytes from rat fed the Purina diet; lane 4 = LCC; and lane 5 = LCE. Corresponding non-immune serum controls for each sample are not shown, but they exhibited no detectable bands.

ethanol-fed rats without affecting the CYP2E1 mRNA level [37]. In the latter case, the increase in CYP2E1 occurs via a direct stabilization of the apoprotein by ethanol [26]. Phenobarbitone treatment causes a significant reduction of the epidermal growth factor receptor (EGFR) protein content, but there is no change in EGFR mRNA in mouse hepatocytes [38]. All these findings support the view that a change in the content of a specific protein (such as M6P/IGF-IIR) due to ethanol or other hepatotoxin may occur as a result of post-transcriptional events.

It is known that ethanol consumption inhibits the initiation of general protein synthesis by reducing the activity of eukaryotic initiation factor (eIF2B), thereby impairing overall hepatic mRNA translation [39]. The results presented here, showing a decline in total protein synthesis by hepatocytes (Table 2), appear to support this finding. Impaired syntheses of specific proteins, including glycogen synthase [36], and of myocardial proteins, due to inhibition of peptide chain initiation by ethanol administration, have been reported [40,41]. It has also been reported that ethanol has a rather novel effect on the translation of apolipoprotein B mRNA by a post-transcriptional editing factor. In this event, a single base, cytidine, is changed to uridine, which changes the glutamine codon

(CAA) to a translational stop codon (UAA) [42,43]. Others have reported ethanol-induced alterations in ribosomal structure and function, which reduce the fidelity of the mRNA translation machinery [44]. However, this effect appears to occur chiefly in mitochondrial ribosomes without affecting cytoplasmic ribosomes in livers of ethanol-fed animals [45,46].

Because ethanol consumption is known to inhibit the initiation of general protein synthesis, we postulate that ethanol consumption may cause impaired M6P/IGF-IIR protein synthesis due to interference with the efficiency of M6P/IGF-IIR mRNA translation, possibly at the same step. Hepatocytes from ethanol-fed rats showed a 53% decline in the rate of M6P/IGF-IIR protein synthesis relative to that of total cellular protein synthesis, indicating that M6P/IGF-IIR protein synthesis was impaired to a greater degree than general protein synthesis by ethanol administration. We also observed that the immunoprecipitated receptor protein from ethanol-fed rats was 79% lower than that from pair-fed controls, which is consistent with a 60% reduction in receptor content in hepatocytes from rats fed the ethanol diet [6]. Overall, our results demonstrate that the ethanol-elicited reduction of M6P/IGF-IIR content was, in part, related to a reduction of

receptor protein synthesis but not to a decline in its mRNA level. Thus, the ethanol-elicited reduction in the M6P/IGF-IIR content as a result of impaired M6P/IGF-IIR protein synthesis may involve an inefficient translation of the existing M6P/IGF-IIR mRNA to a functional receptor protein.

Our findings are significant because M6P/IGF-IIR is very important for trafficking mannose 6-phosphate bearing hydrolase precursors from the Golgi to lysosomes. Therefore, impaired synthesis of the receptor protein results in the reduction of receptor content causing inefficient trafficking of these precursors. Our findings may also bear clinical relevance because M6P/IGF-IIR has a putative role in the regulation of cell proliferation. Cells with lower receptor expression have a selective growth advantage over cells that express normal levels of receptor [47,48], indicating that the M6P/IGF-IIR protein may act as a tumor suppressor. The hypothesis that *M6P/IGF-IIR* acts as a tumor suppressor gene has been supported by the findings that multiple mutations occur at the active binding sites of the receptor in various tumor types and that these mutations correlate with tumor incidence or progression [49,50]; these include mutations in the *M6P/IGF-IIR* gene in human hepatocellular carcinoma [50,51]. Since heavy drinkers as well as alcoholics with liver disease are both at higher risk than nondrinkers for developing hepatocellular carcinoma [52], depletion of the M6P/IGF-IIR by alcohol consumption is one mechanism that could enhance proliferation of normally quiescent liver cells in these individuals.

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