Decreased Binding of Asialoglycoproteins to Hepatocytes from Ethanol-fed Rats

CONSEQUENCE OF BOTH IMPAIRED SYNTHESIS AND INACTIVATION OF THE ASIALOGLYCOPROTEIN RECEPTOR*

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Chronic ethanol administration alters the process of receptor-mediated endocytosis in isolated rat hepatocytes. Using the asialoglycoprotein receptor (ASGP-R) as a model, we have previously shown decreased binding of asialoglycoproteins to this receptor after as early as 1 week of ethanol administration. In the present study, we further analyzed the mechanism(s) responsible for this impairment by determining the ligand and antibody binding characteristics of the ASGP-R in rats fed ethanol over a 5-week time course. The results presented here demonstrate that ethanol treatment for 4 days significantly impaired total ligand binding without affecting antibody binding. Ethanol administration for a longer period of 1–2 weeks resulted in intermediate impairments in both ligand and antibody binding. After 5 weeks of ethanol exposure, ligand and antibody binding were equally lowered. In contrast to total cellular receptor binding, surface binding of both ligand and antibody were decreased over the entire time course of ethanol administration. Our data indicate that the ASGP-R is initially inactivated during the time course of ethanol exposure and that a redistribution of surface receptors to intracellular compartments occurs. Northern blot analysis showed that there was a significant decrease in receptor mRNA content in the 5-week chronically fed animals but not in the animals fed for 1 week. In addition, after 5 weeks of ethanol feeding, biosynthetic labeling of the ASGP-R was decreased in the ethanol cells, indicating impaired synthesis of the ASGP-R. In summary, an early inactivation of the ASGP-R occurs during ethanol exposure followed by an actual decrease in protein and mRNA content for the receptor.

Receptor-mediated endocytosis (RME)† is a process common to many cell types including hepatocytes and has been shown to be affected by ethanol administration (1–3). During RME, molecules in the extracellular fluid bind to cell surface receptors and are internalized as receptor-ligand complexes via a clathrin-coated pit/coated vesicle pathway. Within this pathway, the vesicles lose the clathrin coats, form endosomes, and are sorted in the acidic compartment of uncoupling receptor and ligand. Once separated, ligands and receptors can be routed to the lysosomes for degradation or they can be recycled back to the cell surface (4). The hepatic asialoglycoprotein receptor (ASGP-R) is one of the well-characterized receptors that undergoes efficient recycling (5). The ASGP-R recognizes glycoproteins with terminal galactose or N-acetylgalactosamine residues and removes these potentially harmful desialylated glycoproteins from the circulation (6).

We have previously reported ethanol-induced impairments in the RME process of asialoorosomucoid (ASOR), a representative ligand for the hepatocyte-specific ASGP-R. Impairments in ligand binding, internalization, and degradation were demonstrated in hepatocytes isolated from animals fed ethanol for 5–7 weeks (1). Decreased binding of ASOR by the hepatocytes of ethanol-treated rats appeared to be a major defect identified in the multi-step process of RME. The reduced binding was shown to be due primarily to a decrease in the number of functional cell surface receptors rather than to alterations of receptor affinity. Scatchard analysis of the binding data from animals fed for 5–7 weeks showed the presence of two classes of receptors, a high and a low affinity receptor. Ethanol administration decreased the number of both classes equally and did not alter the affinity of the receptor for ASOR in either class. In later studies we showed that decreased ligand binding occurred after as early as 1 week of ethanol administration (3).

Surface and intracellular ASGP-R activity can be modulated by a variety of agents (7, 8), and the receptors have been shown to undergo an intracellular inactivation-reactivation cycle that is necessary for proper receptor recycling and receptor-ligand dissociation (9). The purpose of the present study was to clarify whether the decreased ligand binding we reported after ethanol administration was attributed to an actual reduction in the number of receptors (decreased receptor content) or to an inability of the receptors to recognize ligand due to receptor inactivation. For these experiments, ligand (ASOR) and anti-ASGP-R antibody binding were performed using both intact and permeabilized hepatocytes isolated from animals fed alcohol over a time course. Ligand binding provides a measure of receptor “activity” while antibody binding is more reflective of receptor “content,” since the antibody recognizes more than the ligand binding domain. We chose to examine feeding periods of a short duration of 4 days, an intermediate period of 1–2 weeks, and a longer feeding time of 5–7 weeks. In addition, analysis of

†This work was supported by Grants AA04961 and AA07846 from the National Institute on Alcohol Abuse and Alcoholism and by the Veterans Administration. Preliminary reports of this work were presented at the Research Society on Alcohol and Alcoholism, 16, 1994, Maui, HI (Abstr. 559) and at the American Association for Liver Disease Meetings, Chicago, IL, November, 1994 (Abstr. 860). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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*The abbreviations used are: RME, receptor-mediated endocytosis; BSA, bovine serum albumin; ASOR, asialoorosomucoid; ASGP-R, asialoglycoprotein receptor; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinosopropanesulfonic acid.
messenger RNA levels after 1 and 5 weeks of feeding and in vitro biosynthesis of the ASGP-R was determined in these cells.

MATERIALS AND METHODS

Animals and Hepatocyte Preparation—Male Sprague-Dawley rats were purchased from Small Animal Supply Co. (Omaha, NE) in the weight range of 140–160 g. The animals were pair-fed control and ethanol (36% of calories) Lieber-DeCarli liquid diet (10) for periods of 4 days and 1–2 and 5 weeks. Purina chow-fed animals that were housed for the same feeding periods were also included in the study. This project was approved by the Animal Studies Subcommittee of the Omaha Department of Veterans Affairs Medical Center. Animals were handled in accordance with applicable local and federal regulations concerning laboratory animals.

Hepatocytes were obtained from the livers of the treated animals by a modified collagenase perfusion technique as described previously by our laboratory (1). The isolated hepatocytes were equilibrated in Krebs-Ringer buffer for 30 min at 37 °C and then incubated at 4 °C for the subsequent binding experiments and 37 °C for metabolic labeling studies. Additional cell aliquots were washed in cold phosphate-buffered saline, and the pellets were stored at −70 °C for future Western blot analysis and mRNA isolation.

Preparation and Labeling of Asialoglycoprotein and Protein A—Human Hepatitis B surface antigen (HBsAg) was desialylated by the neuraminidase procedure as described by Oka and Weigel (11). [125I]-ASOR and [125I]-protein A were prepared by the procedure described by Weigel and Oka (12) and in a previous paper from our laboratory (1).

Preparation of Anti-ASGP-R Antibody—The ASGP-R was purified from whole liver homogenates as described by Hudgin et al. (13) with the modifications described by Trehela et al. (14). Hepatocytes were perfused with a Krebs-Ringer buffer containing 2.5 mM CaCl2 homogenticized to a 20% mixture, and combined with an equal volume of extraction buffer (20 mM CaCl2, 400 mM KCl, 1% Triton X-100, 10 mM Tris-Cl, pH 7.5) for 20 min at 4 °C. The Triton extract was centrifuged for 30 min at 27,000 × g, and the supernatant was filtered successively through 0.8- and 0.22-μm filters before the solution was subjected to affinity chromatography on lactose-agarose columns. For this procedure, a 10-ml resin bed of α-lactose, 6% agarose (Sigma) was equilibrated with a buffer containing 50 mM CaCl2, 500 mM KCl, 0.5% Triton X-100, and 10 mM Tris-Cl, pH 7.5. After equilibration, the Triton X-100 liver extract was placed over the column, washed with over 200 ml of the equilibration buffer, and the ASGP-R was eluted in 2-ml fractions in a 40 mM ammonium acetate buffer (pH 5.0) containing 400 mM KCl and 0.5% Triton X-100. The eluted fractions were neutralized with 1 M Tris, pH 7.8, protein concentration was determined by the BCA protein assay (Pierce), and an aliquot was resolved on 10% SDS-PAGE followed by silver stain analysis to determine the purity of the obtained ASGP-R. Polyclonal antibodies against the rat ASGP-R were obtained by immunization with an initial concentration of 125 μg of the lactose affinity-purified ASGP-R in Freund's complete adjuvant. Subsequent boost injections were given every 4–6 weeks containing 75–100 μg of receptor protein in Freund's incomplete adjuvant. Antibody titers were performed by incubating isolated hepatocytes with various dilutions of antibody. Antibody binding was then detected by subsequent [3H]-protein A binding to the cells as described below.

Ligand and Antibody Binding Assays—Binding of the representative ligand ASOR to the asialoglycoprotein ASGP-R was done as described previously by our laboratory (1–3). Briefly, after preincubation at 37 °C for 30 min, cell suspensions (2.0 × 106 cells/ml) were chilled and then incubated with [125I]-labeled ASOR (25 nmol/liter) at 0–4°C in the presence or absence of 100-fold excess of ligand. Hepatocytes treated in this manner provide a quantitative measure of specific ligand bound to surface receptors. Total ligand binding was determined by first treating the hepatocytes with 0.55 mg/ml digitonin to permeabilize the cells and then proceeding with the same concentrations and conditions used for the free ligand binding assays.

Polyclonal anti-ASGP-R antibody binding to isolated hepatocytes was assessed with the intact and permeabilized cells. For these assays, rabbit polyclonal serum to the ASGP-R at a dilution of 1:200 was added to the cells and incubated at 4 °C for 60 min. After the binding period, the cells were washed twice with Krebs-Ringer buffer, 1.5% BSA to remove unbound antibody, after which fresh Krebs-Ringer buffer media containing [125I]-protein A (6 μg/ml) was added; the incubation then continued for another 60 min. Nonspecific surface and total binding were determined by the amount of radioactivity measured in the presence of nonimmune serum in the incubation media. Nonspecific binding was routinely <10% of specific binding for both ligand and antibody binding assays.

Western Blot Analysis—Frozen hepatocyte pellets (1.0 × 106 cells) were solubilized in Laemmli denaturing sample buffer (16), and aliquots of the suspension were resolved on a 10% SDS-polyacrylamide gel using the Mini-Protein II Cell (Bio-Rad). After electrophoresis, the proteins were transferred at 10 V for 30 min onto 0.45-μm nitrocellulose using the TransBlot SD Semi-Dry Transfer Cell. After electrophoretic transfer, immunodetection of the ASGP-R content was performed as described by Collins et al. (17). Briefly, the nitrocellulose blots were incubated overnight (4°C) or for 1 h (23°C) in blocking buffer containing 0.15 M NaCl, 50 mM Tris-HCl, and 0.35% BSA (pH 7.6). The blots were then exposed to the same Tris buffer with an increased concentration of 0.5% (v/v) including either the specific antigen, the ASGP-R (1:200) or non-immune rabbit serum for 1 h at room temperature. After washing in 1% Tween 20, 50 mM Tris-HCl buffer, the blots were incubated with 1.0 × 105 cpm/ml [125I]-protein A, subsequently washed, then dried and exposed to KODAK X-OMAT film for 24 h at −70°C. Quantification of the autoradiograms was performed using the Bio-Rad model 620 video densitometer.

cDNA Hybridization Probes—The full-length cDNA clone encoding the major (prfH-4) portion of the rat liver ASGP-R was graciously provided by Dr. Kurt Drickamer (Columbia University, New York). The cDNA was inserted into the Escherichia coli strain HB101, and a 0.9-kilobase fragment was released by a double restriction nuclease digestion using BglII and BamHI. The probe was then labeled with [α-32P]dCTP (3000 Ci/mmol) (Amersham) using the Random Primer DNA labeling kit (Ambion). Normalization of the Northern blots was obtained using a common "housekeeping" gene, glyceraldehyde-3-phosphate dehydrogenase, which was kindly provided by Dr. Raul Urrutia (Mayo Foundation, Rochester, MN). The 250-base pair cDNA fragment was transformed into DH5α bacterial cells and released by digestion with EcoRI before labeling using the random primer method as above.

RNA Isolation and Hybridizations—Total RNA was processed from the isolated hepatocytes using the guanidinium thiocyanate extraction procedure described by Chomczynski and Sacchi (18). Polyadenylated RNA was obtained according to the manufacturer's instructions using either the MiniRiboSep Ultra mRNA isolation kit (Becton Dickinson Labware) or the Micro-FastTrack mRNA isolation kit from Invitrogen. The typical yield from both commercial kits was 1–5 μg of RNA per million cells. Total RNA (30 μg), mRNA (1.5 μg), and 3 μg of a 0.24–9.5-kilobase RNA Ladder (Life Technologies, Inc.) were denatured in 50% formamide, 16% formaldehyde in 1 × MOPS buffer at 65 °C for 5 min, chilled on ice, and then fractionated in a 1% agarose/1% formaldehyde gel. After separation, the RNA was transferred to Hybond membrane (Amersham) by capillary action overnight and then incubated at 80°C for 2 h. The blot was prehybridized for 2 h at 65 °C in 5 × SSPE (0.15 M NaCl, 9 mM NaH2PO4, 1.25 mM EDTA, pH 7.4) containing 5 × Denhardt's with 0.5% SDS. The filter was hybridized in the same solution for 48 h at 65°C. After hybridization, the blots were washed in 1 × SSPE/SDS buffers as described by Maniatis et al. (19), followed by exposure to Fuji RX film for 2–4 days or overnight incubation in the PhosphorImager (Molecular Dynamics).

Hybridization Labeling—The incorporation of radiolabeled amino acids into the total protein pool was determined by incubating hepatocytes (5.0 × 106 cells/ml) in methionine or leucine-free Dulbecco's modified Eagle's medium + 0.5% BSA with either trace amounts of [35S]methionine (200 μCi/ml, Amersham, 1000 Ci/mmol) or 2 μCi [3H]leucine (Amersham, 40–75 Ci/mmol) for various periods at 37°C. At 0, 30, 60, and 90 min, 0.1-ml aliquots were removed, and the incorporation of methionine or leucine was measured by the recovered trichloroacetic acid-precipitable radioactivity. No difference in the labeling efficiency was observed between control and ethanol-treated cells using the above label or the 2 μCi [3H]leucine label. The higher concentration of leucine was used to adjust the pool size of the amino acid so that the specific activity of the intracellular leucine was maintained the same as that of extracellular leucine (20).

The incorporation of [35S]methionine into newly synthesized ASGP-R was performed as described by Schwartz and Rup (21). Briefly, hepatocytes were isolated and incubated with [35S]methionine (100 μCi/ml, Amersham, 1000 Ci/mmol) or 2 μCi [3H]leucine (Amersham, 40–75 Ci/mmol) for periods of 0–90 min, and 0.1-ml aliquots were removed at each time point. The washed cell pellets were initially solubilized and placed over a lactose column, and the eluted receptor fraction was then subjected to immunoprecipitation as described below.

Immunoprecipitation of the ASGP-R—Immunoprecipitation of the ASGP-R protein was performed according to the procedure of McPhaul and Berg (22) with the following modifications. Cell aliquots that had been labeled as described above were first solubilized in 0.05 M Tris (pH...
RESULTS

In the first series of experiments, we determined the ability of $^{125}$I-ASOR and a rabbit antibody generated against the rat ASGP-R to bind to total cellular receptors (comprised of surface plus intracellular receptors). By using both of these methods, we could determine ethanol-induced alterations in receptor activity (reflected by changes in ligand binding) versus alterations in receptor content (reflected by alterations in antibody binding). Hepatocytes were treated with low levels of digitonin (0.055% final concentration) to permeabilize cells and make intracellular receptors available for ligand and antibody binding. Use of this procedure to quantitate cellular binding of $^{125}$I-ASOR has been described previously by our laboratory (2).

Our results show that after 4 days of feeding, binding of ASOR to permeabilized cells was decreased significantly (22%) in ethanol-fed animals when compared to controls (Fig. 1A). In contrast, no difference in binding of anti-ASGP-R antibody between cells from control and ethanol-fed animals was observed (Fig. 1B). These results indicated an initial inactivation of the ASGP-R during the early 4-day feeding period. After the chronic 5–7-week feeding period, both ligand and antibody binding were equally decreased by an average of 50%, reflecting a decrease in receptor content after long-term ethanol feeding. The intermediate period of 1–2 weeks of feeding showed that total ligand binding was decreased by 40%, but antibody binding was reduced by only 25% in ethanol animals when compared to controls (Fig. 1A and B). Thus, on the basis of the ligand and antibody binding assays, it appears that during the early stages of chronic ethanol consumption the ASGP-R is inactivated, and after long-term ethanol feeding the actual content of the receptor has been lower.

In agreement with the data for antibody binding, Western blot analysis confirmed these changes in receptor protein content. For these experiments hepatocytes were lysed, subjected to SDS-PAGE, and probed with anti-rat ASGP-R antibody and $^{125}$I-protein A. After 4 days of ethanol feeding, no change in the amount of major 42-kDa subunit of the ASGP-R was observed, while after 5 weeks of feeding, levels of the receptor were significantly lowered (Fig. 2A and C). Intermediate decreases in protein content for the receptor were seen after the 1–2-week feeding period (Fig. 2B), similar to data obtained with permeabilized cells. These data confirm that receptor content is decreased after the later period of ethanol administration.

We next examined surface ligand and antibody binding in intact isolated hepatocytes at 4 °C to determine if alterations in surface receptor content followed a similar pattern as was observed for total receptor content. Results of these studies showed that both ASOR and antibody binding to surface receptors were significantly impaired after all three periods of ethanol exposure (Fig. 3). The impairments in ASOR binding were similar after all three time periods, while antibody binding was decreased to a greater extent after the longer 5-week period.

These results indicated that after the early 4-day period, surface receptors were internalized but may have been unable to recycle back to the plasma membrane, since surface antibody binding was decreased without a corresponding decrease in binding to the total receptor population. To more closely examine a possible difference in receptor distribution between cells of control and ethanol-fed animals, we expressed the antibody binding data as a ratio of surface binding to total binding after the three periods of ethanol feeding (Table I). Surface receptors accounted for 53% of the total population of receptors in control animals for the 4-day and 1–2-week feeding periods. In contrast, in the ethanol animals, only 39% of the total receptor number was found on the surface of the cells. These results indicate an intracellular accumulation of receptors in the ethanol animals after the early and intermediate times of feeding. After the chronic feeding period of 5 weeks, no differences in receptor distribution were observed between control and ethanol animals (Table I).

We wanted to gain more information about the mechanism(s) responsible for these alterations in receptor activity and content that had occurred after ethanol feeding. For these studies, we chose to study an early feeding period of 6–8 days and a longer chronic period of alcohol administration of 5–7 weeks since these were the two periods that distinguished changes in receptor content and activity. Similar to the first series of experiments described above, ligand binding was significantly...
Ethanol-impaired Asialoglycoprotein Receptor Function

**Fig. 2.** Immunochemical detection of the total ASGP-R content. Hepatocytes were obtained from ethanol and control pair-fed rats after 4 days (A), 1–2 weeks (B), and 5 weeks (C) of ethanol administration. A 30-μl aliquot of a 1.0 × 10⁶ cell/ml suspension of control (C) and ethanol (E) cells was loaded into the gel well, and the proteins were resolved by SDS-PAGE followed by immunoblot analysis as described under "Materials and Methods." The transferred proteins were probed with anti-ASGP-R antibody and detected with 125I-protein A. Autoradiograms of the samples are shown on the left with the corresponding densitometric scans on the right.

**Fig. 3.** Determination of surface ASGP-R content and activity over a time course of ethanol treatment. Animals were fed liquid diets containing ethanol (■) as 36% of calories or an isocaloric control diet (○) for 4 days, 1–2 weeks, and 5 weeks. The binding of [125I]-ASOR (A) and anti-ASGP-R antibody and [125I]-protein A (B) to intact isolated hepatocytes at 4 °C was performed as described under "Materials and Methods." Results are expressed as femtomoles bound per million cells and are means ± S.E. for six determinations. Values that are significantly different from controls are indicated (*, p < 0.05).

**Fig. 4.** Northern blot analysis of ASGP-R mRNA in isolated hepatocytes after 1 and 5 weeks of ethanol administration. mRNA was obtained from animals fed a control diet or ethanol as 36% of calories for 1 and 5 weeks. The extracted mRNA was fractionated on agarose gels, transferred to nylon membranes, and hybridized with the random-labeled cDNA probes as described under "Materials and Methods." The expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is also shown, indicating uniform loading of the RNA samples onto the gel. The results shown are one representative Northern blot from six independent experiments.

**Table 1**

| Cell type | 4 days | 1-2 weeks | 5 Weeks |
|-----------|--------|-----------|---------|
| Control   | 0.53   | 0.53      | 0.46    |
| Ethanol   | 0.39*  | 0.39*     | 0.46    |

decreased after both 6–8 days and 5 weeks of feeding in this new series of feeding experiments. The decreased ligand binding after the early 6–8-day feeding period was again due to receptor inactivation, since antibody binding was not impaired after this period (data not shown). Antibody binding was, however, significantly decreased in the ethanol cells after the chronic 5-week feeding period. We then used this series of animals to examine the expression of the asialoglycoprotein receptor gene encoding the major component of the rat receptor (RHL-1) by Northern blot analysis. Fig. 4 shows a typical Northern blot for the ASGP-R RNA that was extracted from the cells of animals fed for the 6–8-day and 5-week periods. The same blots were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase, and the results normalized to this internal standard. When results of six to eight pairs of animals were analyzed, the mRNA level for the RHL-1 subunit of the ASGP-R was significantly decreased by 30–50% (p < 0.05) after 5 weeks of ethanol exposure; whereas after early treatment, no difference was seen in the ethanol-fed animals as compared to controls.

To correlate the observed decreases in ASGP-R mRNA levels with protein synthesis, biosynthetic labeling experiments were performed. Hepatocytes from the 5-week chronically treated animals were incubated with either 2 mM [3H]leucine or trace amounts of [35S]methionine for up to 90 min at 37 °C in methionine or leucine-free media. The higher concentration of leucine was used to expand the extracellular leucine so that the specific activity of intracellular leucine was maintained at the same level as that of extracellular leucine. Labeling with [35S]methionine provided higher specific activity for labeling. As shown in Fig. 5, the incorporation of either amino acid into the total protein pool was not altered after the chronic administration of ethanol. Larger aliquots were used to determine the incorporation of [35S]methionine into the newly synthesized ASGP-R. Data from a representative labeling experiment examining radioactivity in purified ASGP-R is shown in Fig. 6 and shows that ASGP-R production increased over the 90-min time course of labeling in hepatocytes isolated from both the control and ethanol-treated animals and that the bands are noticeably less intense at each time point in the ethanol samples. When data from six to eight pairs of animals were analyzed, the amount of [35S]methionine incorporated into the ASGP-R was significantly decreased by an average of 50% at all time points during the incubation period in the ethanol-fed animals as compared to controls. These results indicate that the reduced ASGP-R transcript levels after prolonged ethanol ingestion lead to a reduction in the synthesis of the receptor.
We have previously shown that chronic ethanol administration alters multiple aspects of the hepatic RME pathway (1–3). One major finding that consistently occurs is a decrease in ligand binding after as early as 1 week of ethanol administration, which persists through at least 6–8 weeks of feeding. The decreased ligand binding appears to be due to different reasons during these early (1–2 weeks) and later (5–8 weeks) periods of feeding. After more chronic ethanol administration (greater than 5 weeks), decreased binding appears to be due to an actual decrease in receptor content, which is characterized by decreases in both ligand and antibody binding. In contrast, after the earlier times of feeding, decreased ligand binding is not associated with decreased protein content for the ASGP-R, showing receptor inactivation. Along with this early inactivation of the receptor, an intracellular accumulation of receptors occurs in the ethanol cells. After the prolonged 5-week feeding period, however, the decreased receptor content in ethanol cells is reflected by equal decreases in surface and intracellular receptor number.

We also examined in greater detail the mechanism(s) responsible for the decreased receptor content that was apparent after the more chronic ethanol feeding period. The levels of mRNA for the ASGP-R in the two cell types was determined after both

DISCUSSION

We have previously shown that chronic ethanol administration alters multiple aspects of the hepatic RME pathway (1–3). One major finding that consistently occurs is a decrease in ligand binding after as early as 1 week of ethanol administration, which persists through at least 6–8 weeks of feeding. The decreased ligand binding appears to be due to different reasons during these early (1–2 weeks) and later (5–8 weeks) periods of feeding. After more chronic ethanol administration (greater than 5 weeks), decreased binding appears to be due to an actual decrease in receptor content, which is characterized by decreases in both ligand and antibody binding. In contrast, after the earlier times of feeding, decreased ligand binding is not associated with decreased protein content for the ASGP-R, showing receptor inactivation. Along with this early inactivation of the receptor, an intracellular accumulation of receptors occurs in the ethanol cells. After the prolonged 5-week feeding period, however, the decreased receptor content in ethanol cells is reflected by equal decreases in surface and intracellular receptor number.

We also examined in greater detail the mechanism(s) responsible for the decreased receptor content that was apparent after the more chronic ethanol feeding period. The levels of mRNA for the ASGP-R in the two cell types was determined after both
1 and 5 weeks of feeding. Gene expression of mRNA for the receptor was decreased after 5 weeks of feeding, but not after the early 1-week feeding period. These data for ASGP-R mRNA correlated well with the levels of receptor protein that were present at the two periods. When Zern and co-workers (23) examined the effect of ethanol administration on another liver-specific gene (that for albumin synthesis), they found that chronic ethanol ingestion actually stimulated albumin mRNA content in vivo in rats. An increase in mRNA for albumin was also noted in baboons after chronic ethanol feeding (24). The results we present here show a decreased mRNA content for ASGP-R after 5 weeks of ethanol feeding. Since the ASGP-R is a protein that is synthesized in the liver, it appears that not all mRNAs of genes responsible for liver-specific protein production are equally affected after ethanol administration. Along with the decreased mRNA content for the ASGP-R, the ability of the cells from ethanol-fed animals to synthesize ASGP-R from labeled methionine was also impaired after the chronic ethanol feeding periods. In contrast to the decreased receptor synthesis, amino acid incorporation into the total cellular protein pool was not altered in cells from ethanol-fed animals. Work from other laboratories on the effect of ethanol administration on protein synthesis in vitro in hepatocytes, as well as in isolated microsomes from mice and rats, likewise did not find ethanol-induced alterations in incorporation of amino acids into the total protein pool (25, 26). We do not know at this time whether defects in post-transcriptional processing by the mRNA for the ASGP-R are present in alcohol-fed animals; however, the fact that message level was decreased to a similar extent as was receptor synthesis would indicate that the decreased receptor mRNA levels alone could explain the decreased in vitro synthesis of the receptor.

The early effects of ethanol administration on receptor inactivation and redistribution were also intriguing and may be related to each other. The build-up of intracellular receptors resulting in altered distribution could be due to a variety of reasons, two of which could be very prominent. One mechanism would be an impaired ability of internalized surface receptors to recycle back to the plasma membrane for reuse. In support of this model, we have previously shown that recycling of the ASGP-R is impaired after ethanol feeding (1). Receptor recycling is dependent on successful dissociation of receptor-ligand complexes inside the cell, and a decrease in the dissociation of receptor and ligand after ethanol feeding could contribute an additional mechanism, which would lead to an intracellular accumulation of receptors. Indeed, we have previously reported that one alteration in the RME process after ethanol administration is a decreased ability of the ligand, ASOR, to dissociate from its receptor during continuous endocytosis (2, 27). During normal operation of the RME cycle, internalized receptor-ligand complexes are transported by way of endosomal vesicles, and dissociation occurs within these vesicular compartments. An acidic intravesicular pH in the endosomes is thought to play a major role in the dissociation of receptor-ligand complexes and aid in the subsequent transport and routing of both receptor and ligand. We have recently reported that there is a decreased ability of the endosomes from ethanol-fed animals to acidify in the presence of added ATP (28). This impaired acidification of endosomes after ethanol treatment could result in alterations in dissociation of receptor and ligand and contribute to decreased receptor recycling and altered receptor function.

Weigl et al. (9) have postulated that, in addition to endosomal acidification, receptor-ligand dissociation may be influenced by factors that affect functioning of the receptor itself, such as receptor inactivation. This second explanation for altered dissociation is also an attractive one and one that may be relevant to our system of examining ethanol-induced effects of ligand processing and receptor function. The ASGP-R, pool is composed of two classes, or subsets, of receptor types. One set of receptors, State 2 receptors, is responsible for the bulk of endocytosis activity by the receptor (>80%), having been shown to constitutively recycle (even in the absence of ligand) and to participate in a reversible inactivation/reactivation cycle during the recycling pathway (9). The reversible inactivation/reactivation cycle is thought to facilitate both receptor-ligand dissociation and segregation of free receptor and ligand. Activity and recycling of these State 2 receptors are susceptible to modulation by a variety of agents, including azide (a metabolic energy poison), colchicine (an agent that disrupts microtubules), and monensin and chloroquine (lysosomotropic agents). These agents can induce inactivation and/or redistribution of the State 2 receptors (7, 8). Medh and Weigel (29) have shown that the inactivation/reactivation cycle for the ASGP-R can be reconstituted in permeabilized rat hepatocytes. In those studies, treatment with ATP showed a time-dependent and dose-dependent inactivation of the receptor. The fact that ATP produced inactivation suggests that the activation or inactivation of the receptor may be linked to the phosphorylation state of the receptor. Additional work from Weigel’s laboratory also indicated that phosphorylation regulates ASGP-R function and/or receptor distribution within the cell (15). In the present report, we show that ethanol administration results in receptor inactivation and redistribution to the interior of the cell early during the feeding period. The inactivation/redistribution of the ASGP-R after early ethanol administration, which we have identified is intriguing and may indicate that ethanol has an effect on the State 2 receptor population in liver cells. It will be interesting to examine whether these changes after ethanol administration are accompanied by changes in the phosphorylation state of the receptor.

In summary, we have shown in an animal model of ethanol administration that function of a liver-specific receptor, the ASGP-R, is altered. Early during the feeding scheme (up to 2 weeks), ligand binding to the receptor is decreased without a corresponding change in protein content. Upon prolonged feeding, decreased ligand binding is reflected by an actual decrease in protein content of the receptor. The decreased protein content is accompanied by a loss of ASGP-R-specific mRNA and a decreased ability of the receptor to be synthesized from labeled amino acids. The early changes in receptor function are characterized by a loss of ligand binding without a loss of protein or mRNA content. The “inactivated” receptor we have described here may be subject to degradation and/or altered processing once inside the cell. The mechanism and consequences of this inactivation must be assessed by further studies to determine its role in alcohol liver injury and the concomitant altered protein trafficking, which results during this injury process.

Acknowledgments—We gratefully acknowledge the excellent technical assistance of Joseph Toder for some of the studies and thank Dahn Clemens for help with the molecular biology experiments.

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