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EFFECT OF CHRONIC ETHANOL ADMINISTRATION ON THE UPTAKE AND DEGRADATION OF ASIALOGLYCOPROTEINS BY THE PERFUSED RAT LIVER

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Abstract—We have shown previously reduced binding, internalization, degradation and receptor–ligand dissociation during receptor-mediated endocytosis (RME) of ^{125}I-asialoorosomucoid (ASOR) by hepatocytes isolated from rats fed ethanol for 4–6 weeks. In the present study, we investigated the effect of ethanol feeding on RME by using the intact perfused liver as a model. Male, Sprague–Dawley rats were fed a liquid diet containing either ethanol (36% of calories) or isocaloric carbohydrate. Receptor-mediated endocytosis of ^{125}I-ASOR was then examined over a time course of perfusion. In all cases, clearance of the labeled glycoprotein was followed by a slower but steady appearance of acid-soluble products in the medium. Ethanol-fed animals had a significantly ($P < 0.01$) slower rate of clearance of the labeled ligand from the circulating perfusate than did control animals. Impairment of ASOR surface binding and degradation in ethanol-fed animals was also demonstrated in this model. When we examined the subcellular distribution of labeled ligand after various times of perfusion, we found that in control livers, a shift of radiolabeled ligand from the subcellular fractions containing endosomes and plasma membranes to fractions containing lysosomes occurred, while significantly less ligand was shifted to the lysosomes of ethanol-treated rats. These results show that ethanol administration inhibits RME of ASOR in the isolated perfused liver model, thus confirming our earlier reported defects in isolated hepatocytes. In addition, transport of ligand along the intracellular RME pathway was also shown to be altered by ethanol treatment as indicated by the impaired movement of ASOR from the endosomal to the lysosomal compartment.

The uptake and intracellular transport of many proteins in cells begin with the binding of a ligand to cell surface receptors followed by internalization via coated pits and vesicles. This general process is known as receptor-mediated endocytosis (RME†) and provides the cell with intracellular pathways by which proteins are targeted along specific routes [1–4]. RME is now well-recognized as a general mechanism by which many cells take up biologically important molecules, including hormones (insulin and epidermal growth factor), transport proteins (low density lipoproteins), lysosomal enzymes and desialylated glycoproteins.

We are studying RME because of our previous findings, indicating altered glycoprotein trafficking in livers of ethanol-treated rats [5–7]. We are especially interested in the mammalian receptor that mediates the rapid uptake and lysosomal degradation of galactose-terminated desialylated glycoproteins by RME. This receptor has been well studied and characterized in hepatocellular systems including perfused livers, regenerating livers and isolated hepatocytes [8–10]. We have shown previously impaired RME of a representative asialoglycoprotein, asialoorosomucoid (ASOR), in hepatocytes isolated from chronically ethanol-fed rats [11–13]. In the present study, we wanted to further investigate and confirm these ethanol-induced impairments in intact liver, a model which is more physiological than isolated hepatocytes. We also wanted to use a system that would provide enough sample tissue to examine subcellular location of internalized ligand during intracellular processing. For these experiments we chose an *in situ* model, the isolated perfused liver, which alleviates potential problems associated with the isolation of hepatocytes using collagenase digestion. With this model, we studied the effect of ethanol feeding for 4–5 weeks on binding, internalization, degradation and intracellular processing of ^{125}I-ASOR.

MATERIALS AND METHODS

Materials. Human orosomucoid (α_1-acid glycoprotein), Cm-cellulose, phosphotungstic acid, EDTA, BSA (fraction V), neuraminidase (type X), Hepes and 1,3,4,6-tetrachloro-3,5-diphenylglycouril were from the Sigma Chemical Co. (St Louis, MO). Male Sprague–Dawley rats (100–125 g) were obtained from the Small Animal Supply Co (Omaha, NE). Na^{125}-I (10–20 mCi/μg iodine) was from the Amersham Corp. (Arlington Heights, IL). All other chemicals were reagent grade.

Nutritionally adequate liquid diets were formulated according to Lieber and DeCarli [14] and

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† Abbreviations: RME, receptor-mediated endocytosis; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; TCA, trichloroacetic acid; and PTA, phosphotungstic acid.

purchased from Bio-Serv, Inc. (Frenchtown, NJ). The ethanol diet contained 18% of total calories as protein, 35% as fat, 11% as carbohydrate, and 36% as ethanol. In the control diet, ethanol was replaced isocalorically with carbohydrate.

Preparation and labeling of ASOR. Human orosomucoid was desialylated with *Clostridium perfringes* neuraminidase following the procedure of Oka and Weigel [15] as previously described [11].

Ethanol treatment of rats. Animals were initially maintained on a Purina rat chow diet until they reached 150–160 g and were then divided into two groups. The rats were housed in individual cages and acclimated to the Lieber–DeCarli control diet for 3 days. These rats were then weight-matched and paired so that one rat received the liquid diet containing ethanol as 36% of total calories, while the second animal was pair-fed the isocaloric control diet. Chow-fed rats were included as an additional control group to establish that any differences were a result of ethanol consumption and not due to other dietary factors. Twenty-four hours prior to liver perfusion, the animals were meal-fed as previously described [11] to minimize variations in feeding patterns between the ethanol-fed rats and their pair-fed controls.

Perfusion of rat liver. Rats that were fed either ethanol or control liquid diet or Purina rat chow for 4–6 weeks were anesthetized by injection of sodium pentobarbital (5 mg/100 g body wt). Upon opening the abdominal cavity, loose ties were placed around the hepatic portal vein and the inferior vena cava. The bile duct was cannulated with PE intramedic tubing. The hepatic portal vein and inferior vena cava were then cannulated and tied in place. The liver was perfused with Krebs–Ringer buffer (37°) at a flow rate of 25–30 mL/min to clear the liver of trapped erythrocytes and equilibrated with this perfusate for 30 min prior to the addition of ^{125}I-ASOR. For studies involving uptake and degradation of saturating levels of ASOR, the liver was cyclically perfused with 600 μg ASOR (20×10^6 cpm) in a total volume of 100 mL. At various times, 0.5-mL aliquots of perfusate were removed and added to 10% TCA/1% PTA to precipitate intact ligand. Degradation of ligand was assessed by amounts of acid-soluble radioactivity in the perfusate. Each experiment was terminated by excising the liver, and the tissue was then homogenized in 0.25 M sucrose, pH 7.4. The resulting homogenate was tested for the presence of TCA-precipitable and acid-soluble radioactivity.

Subcellular fractionation. For experiments where ligand processing was examined, the labeled ASOR (25 μg, 5×10^6 cpm) was perfused through the liver in 1 min (total volume of 25 mL). Perfusion medium was then switched to Krebs–Ringer buffer without ligand, and after an additional 1 min the unbound and surface-bound ASOR were removed by the addition of 20 mM EDTA to the buffer [16]. At the indicated times after EDTA treatment, subcellular fractions were prepared according to the method of de Duve *et al.* [17] except that the homogenates were centrifuged initially at 30 *g* for 10 min to remove unbroken cells and debris. Further fractionation yielded the following four fractions which were analyzed: (1) a nuclear pellet, (2) a heavy-and-light mitochondrial pellet which is the lysosome-rich fraction, (3) a microsomal pellet which includes pinocytic vesicles (endosomes) and plasma membrane fragments [18, 19], and (4) a final supernatant fraction. These four fractions were assayed for enzyme activity and content of radiolabeled ligand.

Viability parameters. Assessment of liver viability was conducted during each perfusion [20] as further described here. Initially, the liver was grossly evaluated by observation for general appearance using color, lack of any frank anomalies (cysts, lesions, necrosis, etc.) and size. During the perfusion procedure, oxygen consumption by the liver was evaluated every 30 min by use of a YSI model 53 oxygen monitor to determine whether hypoxia was occurring. The presence of lactate dehydrogenase activity in the perfusion medium was also determined as a measure of the extent of enzyme leakage due to cell injury resulting from prolonged perfusion.

Determination of surface binding of ASOR. Surface receptor number was determined in the perfused livers by the specific binding of ^{125}I-ASOR at 0–4° according to the basic method described by Dunn and Hubbard [21]. Briefly, livers were cyclically perfused *in situ* with saturating levels of ^{125}I-ASOR (1 μg/mL) at 0–4° for 90 min to allow binding to cell surface receptors. Clearance of the ligand was monitored by periodically sampling the perfusate. At the end of the binding period, livers were washed free of unbound ligand by switching to a single-pass perfusion mode by perfusing with cold (0–4°) Krebs–Ringer buffer for 15 min. At this time, a small lobe (< 0.2 g) was tied off and excised; this piece of liver was homogenized in 0.25 M sucrose, and its acid-precipitable radioactive content was determined as a measure of total (specific plus nonspecific) binding activity. Surface-bound ASOR was then displaced in the intact liver by adding EDTA (final concentration of 20 mM) to the perfusate. The total volume of the recirculating perfusate was around 100 mL. Aliquots of the perfusate were removed at the indicated times to monitor receptor–ligand dissociation, which was usually complete by 10 min. After EDTA treatment, the liver was weighed and homogenized, and the acid-precipitable radioactive content (a measure of nonspecific binding) was determined as described above. ASGP binding capacity was expressed as femtomoles ASOR bound per gram wet weight of liver and converted to receptors per hepatocyte using 124, 122, and 112×10^8 hepatocytes per gram wet weight liver for chow-fed, pair-fed and ethanol-fed animals respectively [11, 22] and assuming a 1:1 interaction for the ligand–receptor complex.

Enzyme assays. Prior to assay for enzyme activity, all cell fractions were subjected to brief sonication. All assays were carried out in duplicate at 37°. Spectrophotometric measurements were made with a Beckman DU 70 spectrophotometer. Lactate dehydrogenase activity was determined by the assay of Bergmeyer *et al.* [23]. Glutamate dehydrogenase was assayed as described by Olson and Anfinsen [24], while acid phosphatase was determined by the method of Walter and Schutt [25]. Glucose-6-phosphatase was assayed as described by Harper [26].

General. Protein was determined by the method of Lowry *et al.* [27], using bovine serum albumin as

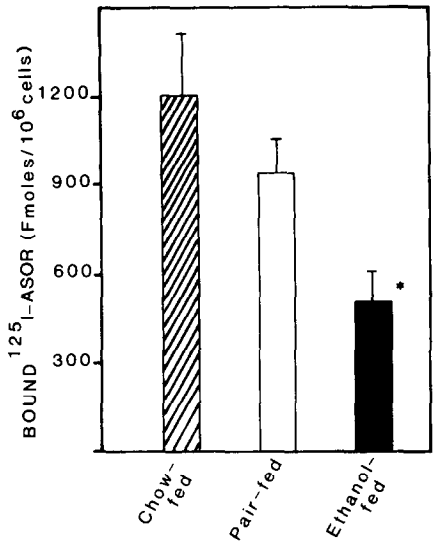

Fig. 1. Surface binding of ^{125}I-ASOR to intact livers from chow-fed, pair-fed, and ethanol-fed rats. Livers were initially perfused at 37° for 45 min to increase and equilibrate the number of cell surface receptors. Livers were then cooled to 0–4° and the number of specific receptors was assessed in the presence of 1.0 μg/mL ^{125}I-ASOR for 60 min as described under Materials and Methods. Results are shown as means ± SE for 6–8 determinations. Values significantly different from pair-fed controls are indicated: (*) $P < 0.01$.

the standard. ^{125}I-Radioactivity was determined by using an LKB automatic gamma counter. Statistical analyses were carried out using Student's *t*-test.

RESULTS

Initial characterization of perfused livers. Livers of chow-fed, pair-fed and ethanol-fed rats were characterized initially by determining rates of uptake of oxygen by the liver and amounts of lactate dehydrogenase activity released into the perfusion medium from each liver over the perfusion period. When data were analyzed based on enzyme leakage, oxygen uptake or gross appearance, no significant differences among the three groups were apparent. Overall, viability of perfused livers as determined by the above parameters was judged to be comparable between the three groups.

Determination of binding site number. Livers from all three groups were compared with respect to the amount of radioactive ASOR able to bind to the cell surface. The average binding capacities of the liver from both chow-fed and pair-fed controls were similar and corresponded to around 750,000 receptor molecules/cell (Fig. 1). These data agree well with our previously reported results in isolated liver cells [11, 12]. Livers from ethanol-fed animals bound 45–50% less ligand (an average of 390,000 molecules/cell) (Fig. 1). These data confirm results obtained in our previous studies using isolated hepatocytes and indicate that chronic ethanol administration alters the binding properties of the liver for ASOR in the isolated perfused liver model.

Kinetics of uptake and degradation of ^{125}I-ASOR in perfused livers of ethanol-fed and control rats. These experiments were conducted at 37° over a 3-hr period, and amounts of ligand present in the incubation medium were in excess of the total surface binding capacity of the cells throughout the time course of perfusion. Linear clearance of intact ligand from the perfusate was observed in both the control and ethanol-fed animals from 15 min to 3 hr although the rate of clearance of the radioactive glycoprotein was 50–60% less in the ethanol-treated animals when compared to either pair-fed or chow-fed controls (Fig. 2). Clearance of the 600 μg of radioactive glycoprotein from the perfusate was followed by a slower but steady appearance of acid-soluble (degradation) products in the medium. Acid-soluble radioactivity was released into the perfusate starting at 15 min after the addition of ^{125}I-ASOR and continued to increase over the 3-hr time course (Fig. 3). In control animals, the degradation products after 3 hr accounted for 50% of the total glycoprotein added to the liver, whereas in the ethanol-fed animals only 30% of the radioactivity taken up by the liver appeared as degradation products. Thus, ethanol administration to these animals markedly impaired degradation of ASOR by the perfused liver.

Effect of ethanol treatment on processing of ^{125}I-ASOR in intact livers. To determine the effect of ethanol on intracellular ligand processing, liver homogenates were prepared and fractionated after selected times of perfusion with subsaturating levels of ^{125}I-ASOR in control and ethanol-fed animals. Marker enzymes, lactate dehydrogenase (soluble fraction), glutamate dehydrogenase (mitochondrial marker), β-hexosamidase (lysosomal marker) and glucose-6-phosphatase (microsomal fraction which is also the endosome-rich fraction), were used to characterize the various fractions. Maximal activity for each of the marker enzymes was found in the appropriate subcellular fraction, and there was no significant difference between results obtained with control and ethanol-treated rats (data not shown).

The subcellular distribution of radioactivity clearly showed that at early time periods (15 min) the majority of ligand present in both control and ethanol-fed animals was present in the endosome-containing fraction (Fig. 4). This material represents ligand contained within various intermediate vesicles on the way to the lysosomes. Although plasma membranes would also appear in this fraction, ligand bound to surface receptors was displaced by EDTA treatment prior to initial homogenization of the liver. By 45 min in the control livers, a decrease in the amount of total radioactivity in the endosome-containing fraction had become apparent, and by 90 min less than 35% of the total ligand present in the liver was located in this fraction. In ethanol-treated livers, on the other hand, significantly greater amounts of receptor–ligand complexes remained within the endosome-rich fraction over the time course of perfusion (up to 90 min) when compared to controls. In fact, radioactivity in the endosomal fraction in livers from the ethanol-treated animals still accounted for greater than 60% of the total labeled ligand present in the liver after 90 min of perfusion (Fig. 4).

In conjunction with the decreased radioactivity in

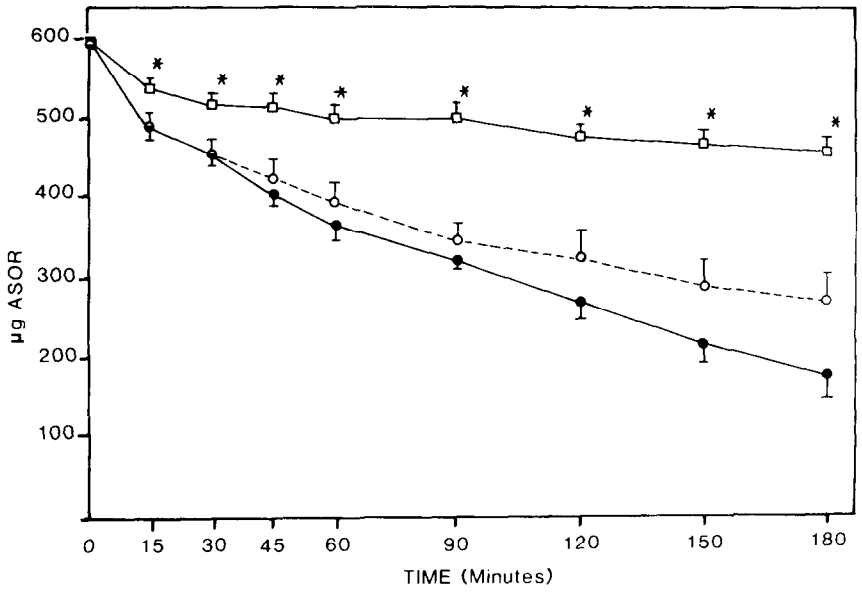

Fig. 2. Uptake of ^{125}I-ASOR by isolated perfused livers from chow-fed (○), pair-fed (●), and ethanol-fed (□) rats. The liver was cyclically perfused at 37° for 180 min after the addition of 600 μg ^{125}I-ASOR (10 μCi). Aliquots of perfusate were removed at the specified times, and intact ligand was precipitated with acid as described in Materials and Methods. Results are expressed as means ± SE for 6–10 determinations in each group. Values significantly different from controls are indicated: (*) $P < 0.01$.

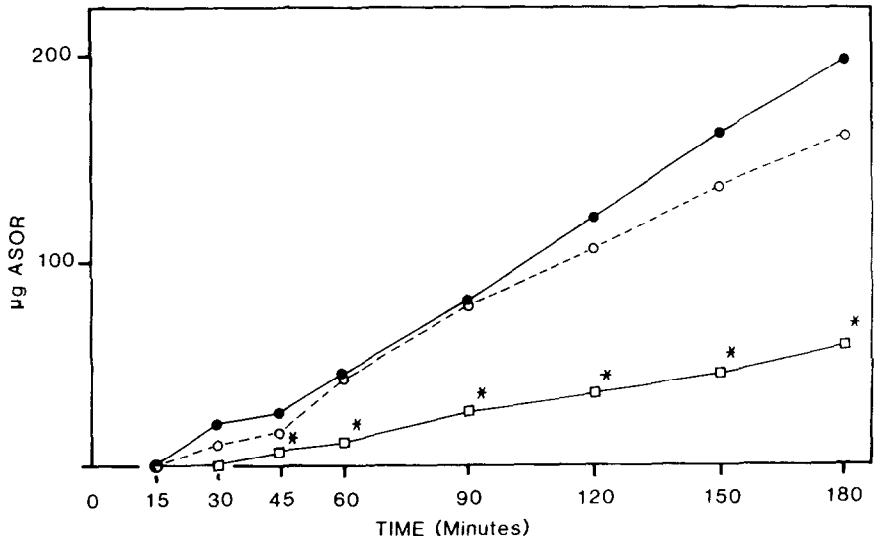

Fig. 3. Degradation of ^{125}I-ASOR by isolated perfused livers from chow-fed (○), pair-fed (●), and ethanol-fed (□) rats. Experimental conditions were identical to those listed for Fig. 2. Aliquots of perfusate were removed at the specified times, and acid-soluble radioactivity (representing degradation products) was determined in the perfusate as described in Materials and Methods. Results are expressed as means of ASOR degraded ± SE for 6–10 determinations in each group. Values significantly different from controls are indicated: (*) $P < 0.01$.

the endosomal fractions after 45 min of perfusion, a shift of radioactivity from the endosomal to the lysosome-containing fraction had become apparent for the control livers. This increase in radioactivity in the lysosomal fraction represents the transfer of ^{125}I-ASOR into the degradative compartment from vesicular fractions. Although this shift was also noted in the rats fed ethanol, the percent of radioactivity shifted was significantly less ($P < 0.01$) than that observed in control animals (Fig. 5). By 90 min, 30% of the radioactivity in control livers was in the lysosomal fraction, whereas only 10% of the ligand was found in this fraction in the ethanol-treated livers. Twenty-five to thirty-five percent of total

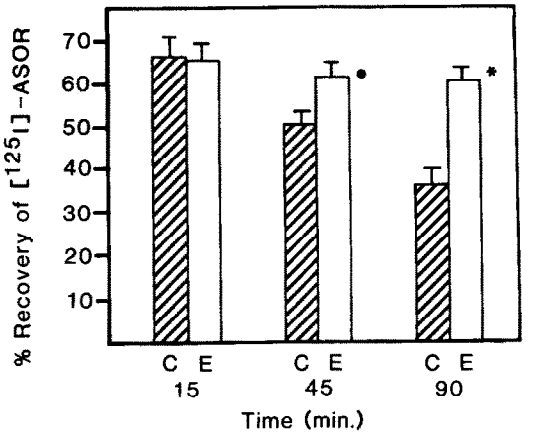

Fig. 4. ^{125}-ASOR content in endosome-containing fractions as a function of time in control (C, hatched bars) and ethanol-fed (E, open bars) rats. Homogenates of perfused livers were subjected to subcellular fractionation. Aliquots of each fraction were analyzed for radioactivity, and the total radioactivity within each compartment was calculated. Data for amounts of ^{125}I-ASOR in the endosome-containing fraction are presented, and values represent the percentage of the sum of radioactivity present in the four subcellular fractions analyzed. Results are expressed as means ± SE for 9–15 determinations. Values significantly different from pair-fed controls are indicated: (●) $P < 0.01$; and (*) $P < 0.001$.

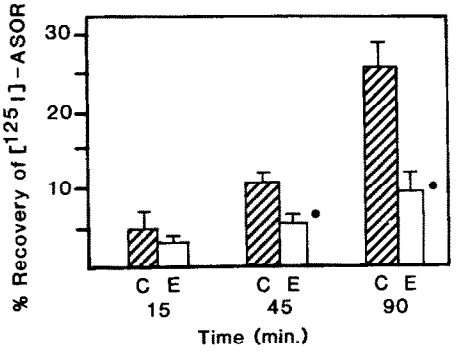

Fig. 5. ^{125}I-ASOR content in lysosome-containing fractions as a function of time in control (C, hatched bars) and ethanol-fed (E, open bars) rat livers. Experimental conditions were exactly as described for Fig. 4. ^{125}I-ASOR in the lysosome-containing fractions represents the percentage of the sum of the radioactivity in the four subcellular fractions analyzed. Results are expressed as means ± SE for 9–15 determinations. Values significantly different from pair-fed controls are indicated: (●) $P < 0.01$.

internalized ligand was not associated with either the isolated lysosome- or endosome-containing fractions. The remaining radioactivity was distributed between nuclei and cytosol in both control and ethanol-fed animals. The amount of radioactivity in these latter fractions was similar for both groups of animals and was probably due to cross-contamination of the nuclei and supernatant fractions with lysosomes and microsomes, since only 70% of the specific marker enzymes, β-hexosamidase and glucose-6-phosphatase, was recovered in their respective fractions. Overall, these results indicate that ethanol treatment interferes with the transfer of ligand from the endosomes to the lysosomes.

DISCUSSION

The results of this study confirm and extend our previous work, showing impaired hepatic RME after chronic ethanol administration [11–13]. In these previous studies, we utilized preparations of isolated hepatocytes to investigate ethanol-induced defects in RME of ASOR. These earlier experiments showed decreased binding, internalization and degradation of ^{125}I-ASOR in hepatocytes isolated from animals after as early as 1 week of ethanol feeding, and these defects persisted during a 7-week feeding period. However, the acute presence of ethanol in the incubation medium did not affect any aspect of RME studies [11, 12]. Since the uptake and degradation of ASOR by the liver have been shown to be hepatocyte-specific [28], the hepatocyte model is particularly useful for comparative studies involving RME of ASGPs. However, since hepatocytes are obtained by enzyme (collagenase) digestion, we wanted to confirm our findings in a system that approaches normal physiology and one in which the potential problems associated with the hepatocyte model, such as collagenase digestion and cell disruption, are eliminated. In the present studies, we used isolated perfused livers, an *in situ* model which has also been used by others to examine RME [29, 30]. The perfused liver has been shown to be a useful model to study the ASGP receptor and the subcellular events it mediates [29, 30]. Isolated perfused livers also offer numerous advantages over an *in vivo* model. In perfused livers, the ligands can be introduced into the liver circulation without dilution by total blood volume and without flow to other organs and the temperature of the perfusion medium (and thus the liver) can be varied over a wide range. Thus, effects on various parameters can be assessed easily, and existing subcellular fractionation procedures for intact liver can still be used with this model. We used a simple perfusion system but one that was fully adequate to maintain liver viability for up to 4 hr at 37°. Data obtained from the present study, employing the perfused liver system, complement the previously reported findings from our laboratory on the effects of ethanol on RME of ASOR in isolated cells and extend these studies by examining post-receptor binding events of RME.

Our experiments are the first to show that ethanol treatment impairs multiple aspects of RME of ASOR in the perfused liver. We report here that binding of ^{125}I-ASOR to cell-surface receptors was decreased by 45–50% after ethanol treatment in isolated livers, a finding which confirms our earlier work using isolated hepatocytes [11]. The decrease in binding, observed in this study, likely reflects a reduction in cell surface receptor number, as previously shown to be the case for isolated hepatocytes [11, 12]. In addition, the clearance of intact ligand from the perfusion medium and the subsequent degradation of intracellular ligand were also decreased markedly

in the perfusion model. These results further demonstrate the dramatic effect of ethanol treatment on the process of RME of ASGPs.

Another important and intriguing finding of this study was that ethanol treatment impaired the movement of internalized ligand from endosomes to lysosomes. In this regard, we showed that in control livers, ligand was confined initially to the endosomal-containing fraction (15–45 min) and after longer times of perfusion, the intact ligand moved to the lysosomal-containing fraction. In the ethanol-treated animals, however, the amount of intact ligand which appeared in the lysosomal fraction was decreased when compared to controls. These results complement previous results from our laboratory which showed that intracellular receptors in hepatocytes from ethanol-fed animals released bound ligand more slowly than did receptors in cells from control animals [13]. Since receptor and ligand are dissociated in endosomal compartments, probably via a decrease in intravascular pH of endosomes [1], ethanol treatment may influence these prelysosomal compartments by affecting acidification. If endosomal acidification is altered after ethanol treatment, the impaired delivery from endosomes to lysosomes could be one result of this impairment. Alternatively, interaction of acetaldehyde, the reactive metabolite of ethanol oxidation, with microtubular protein could also result in impaired movement of ASOR from endosomes to lysosomes, as has been suggested by some of our previous studies involving the effects of ethanol on other protein trafficking events in the liver [31].

In summary, these results, along with our previous work, show that chronic ethanol administration impairs multiple aspects of protein trafficking in hepatocytes. In the present study, we specifically showed impairments in binding, internalization and degradation of ASGPs in perfused livers isolated from ethanol-fed rats. In addition, we showed impairments in ligand trafficking in the liver, specifically in the movement of ligand from endosomes to lysosomes. These changes are providing a basis for further investigation on the effects of ethanol on hepatic protein trafficking, especially those involved with the important process of RME.

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