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Differential Effects of Monensin on Asialoglycoprotein Receptor Function after Short-Term Ethanol Administration

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ABSTRACT. Chronic ethanol consumption is associated with multiple impairments in receptor-mediated endocytosis (RME) by the hepatic asialoglycoprotein receptor (ASGP-R). Previous work on this receptor has shown that its activity can be perturbed by the carboxylic ionophore monensin. This agent has been shown to preferentially affect receptor–ligand dissociation and receptor redistribution of one subset (State 2) of ASGP-R, while receptor function in a second subset (State 1 receptors) is unaffected. In the present study, we examined the effect of monensin on ASGP-R activity and intracellular receptor–ligand dissociation after 7–10 days of ethanol feeding, a time when we have shown altered ASGP-R function in ethanol-fed animals. Hepatocytes from male Wistar rats (fed an ethanol-containing or control diet) were utilized. Ethanol administration decreased total ligand binding by 35–40% ($P < 0.01$) without a change in receptor protein content. After monensin treatment, surface receptors on cells from control animals were inactivated and redistributed to the cell interior. In cells from ethanol-fed animals, a similar pattern of monensin-induced inactivation was shown, but no redistribution occurred. Intracellular receptor–ligand dissociation was impaired in both cell types, although the monensin-induced effect on dissociation was significantly less dramatic (two-fold) in the hepatocytes from ethanol-fed animals as compared with controls. Thus, although receptors on both cell types were susceptible to monensin, cells from the ethanol-fed animals were less vulnerable to the added effects of this agent. Since monensin affects functioning of State 2, but not State 1 receptors, a very early effect of ethanol may be a preferential impairment in the State 2 receptor population. BIOCHEM PHARMACOL **55**;10:1603–1609, 1998.

KEY WORDS. asialoglycoprotein receptor; liver; endocytosis; alcohol; monensin

Results from our laboratory have shown that ethanol administration impairs the hepatocellular processes of protein secretion, membrane biogenesis, and especially RME‖ [1–4]. 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 [5, 6]. Within this pathway, the vesicular contents are sorted in acidic endosomal compartments. Once separated, ligands and receptors can be routed to the lysosomes for degradation or recycled back to the cell surface [7, 8].

We have used the hepatic ASGP-R as a model for RME to study the effects of ethanol on liver cell function and have shown that ethanol administration results in impaired RME of ASOR, a ligand for the ASGP-R, at multiple steps along the pathway [1–4, 9–12]. The detrimental effects of ethanol on RME differ in some respects after longer (greater than 5 weeks) periods of ethanol feeding compared with relatively short-term (1–2 weeks) feeding periods. For example, after the shorter feeding period, the receptor appears to be "inactivated" in that it retains the ability to bind an anti-receptor antibody, but does not bind ligand [4]. The ASGP-R pool in the hepatocyte 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%), and has been shown to constitutively recycle (even in the absence of ligand) and to participate in a reversible inactivation/reactivation cycle during the recycling pathway [8]. The second set of receptors, State 1 receptors, do not appear to undergo the inactivation/reactivation process or to be constitutively recycled. Monensin, a carboxylic cationic ionophore that abolishes the acidic interior by dissipating the pH gradient of the endosomes and results in impaired receptor–ligand uncoupling [8, 13–16], affects functioning of State 2 but not State 1 receptors. We have shown previously that both State 1 and State 2 receptor popula-

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‖ *Abbreviations:* RME, receptor-mediated endocytosis; ASGP-R, asialoglycoprotein receptor; and ASOR, asialoorosomucoid.

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tions are affected after long-term ethanol feeding of 10–14 weeks and that the addition of monensin appears to affect cells from both ethanol-fed and control animals in an equal manner after this chronic period of feeding [17]. In the present study, we compared the effects of monensin on ASGP-R function in hepatocytes from ethanol-fed and control rats after a shorter 1-week feeding period during which the receptor inactivation has been shown to occur.

MATERIALS AND METHODS

Materials

Human orosomucoid (α_1 acid glycoprotein), Sephadex G-25, collagenase (Type IV), EDTA, BSA (fraction V), Percoll, neuraminidase (type X), HEPES, 1,3,4,6-tetrachloro-3,6-diphenylglycouril, and monensin were obtained from Sigma. Male Wistar rats (150–175 g) were obtained from the Charles River Laboratories. Na ^{125}I (10–20 mCi/mg) was obtained from Amersham. Eagle's Basal Medium (Grand Island Biological) was supplemented with 2.4 g/L of HEPES (pH 7.4) and 0.22 g/L of sodium bicarbonate. Eagle's/BSA is Eagle's Medium supplemented with 0.1% BSA. Monensin was dissolved in ethanol at concentrations of 0.1 M and diluted with Eagle's Medium before use. All other chemicals were reagent grade.

Nutritionally adequate diets were formulated according to Lieber and DeCarli [18] and purchased from Dyets, Inc. (Bethlehem, PA). 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. ASOR was prepared and iodinated as previously described [9].

Ethanol Treatment of Rats

Animals (180–200 g) were housed in individual cages and acclimated to the Lieber–DeCarli control diet for 3 days prior to the commencement of ethanol feeding. The animals 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. Rats were fed for periods of 7–10 days. The animals were meal-fed during the 24-hr period prior to isolation of the hepatocytes in order to minimize variations in feeding patterns between ethanol-fed rats and their pair-fed controls. This program was approved by the Animal Studies Subcommittee of the Omaha VA Medical Center. Animals were handled in accordance with all applicable local and federal regulations concerning laboratory animals and housed in an American Association for Accreditation of Laboratory Animal Care-Approved Animal Research Facility at the Omaha VA Medical Center.

Hepatocyte Preparation

Hepatocytes were prepared by standard collagenase perfusion as described previously [4, 9] and were purified further using continuous Percoll gradients (40%, v/v). Viability was routinely greater than 80%. After isolation, hepatocytes were preincubated for 30–45 min at 37° to equilibrate the number of cell surface receptors before determining binding and RME [4, 9].

ASOR Binding Assays

Specific surface binding of ^{125}I-labeled ASOR to hepatocytes was determined at 0–4° as previously described [4]. Treatment of the cells with or without monensin was as follows: 10-mL aliquots of hepatocytes obtained after preincubation (1–2 $\times$ 10^6 cells/mL) either were treated with 25 μM of monensin for 60 min at 37° or were untreated. At the end of this incubation period, cells were washed and re-purified by Percoll gradient centrifugation as described above. Washed cells (1 mL at 2 $\times$ 10^6 cells/mL) were used for binding assays. Total (surface and intracellular) binding with ^{125}I-labeled ASOR was determined by the inclusion of digitonin in the incubation medium at a concentration of 0.055% as previously described [10]. Digitonin concentrations are expressed as a weight/volume percentage, and digitonin was made as a stock solution (25%) in absolute ethanol. Characterization and use of digitonin to permeabilize cells to determine total cellular ASOR receptor content have been described by Weigel *et al.* [19]. Nonspecific binding in the presence of 100-fold excess of unlabeled ASOR was routinely less than 5% of specific binding for both surface and total ASOR binding.

Antibody Binding Assays

Hepatocytes (1 $\times$ 10^6 cells), treated with or without 25 μM of monensin as described above, were incubated in the presence (1:100 dilution) of either anti-rat ASGP receptor antibody (a polyclonal rabbit serum prepared after immunization with purified rat ASGP-R) or control nonimmune sera for 1 hr at 0–4° as described previously [4, 10]. Then the cells were washed free of unbound antibody and incubated in the presence of ^{125}I-labeled Protein A (6 μg/mL, 3 $\times$ 10^5 cpm/μg) at 0–4° for 60 min. Total cellular binding (intracellular as well as surface receptors) was determined by the inclusion of digitonin in the initial cell suspension as described above for ASOR binding. Nonspecific binding was routinely less than 10% of specific binding for these assays.

Assays for ^{125}I-labeled ASOR Internalization

Internalization of ^{125}I-labeled ASOR was followed by the accumulation of acid-insoluble radioactivity in the cell pellet as previously described [9]. Briefly, cells that had been preincubated were placed at 37° in 25-mL Erlenmeyer flasks at a concentration of 2–3 $\times$ 10^6 cells/mL with ^{125}I-labeled ASOR at concentrations of 10 μg/mL. At the indicated times of incubation, aliquots were removed and washed with Eagle's/BSA/EDTA to remove surface-bound ligand, and the radioactivity in the cell pellet was determined.

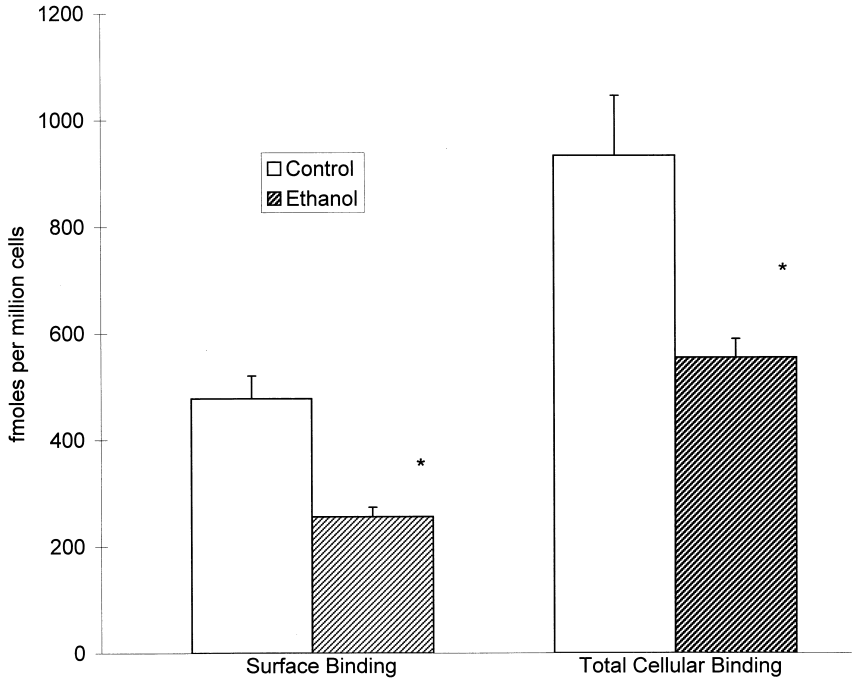

FIG. 1. Determination of surface and total cellular ASGP-R activity after 1–2 weeks of ethanol administration. Isolated hepatocytes were obtained by collagenase perfusion from animals pair-fed an ethanol-containing or control liquid diet for 7–10 days. The binding of ^{125}I-labeled ASOR to surface or total cellular receptors was performed at 4° as described under Materials and Methods. Results are expressed as femtomoles bound per million cells from control and ethanol-fed animals and are means ± SEM for 6–8 determinations. ⋆Values were significantly different from those of control animals, $P < 0.01$.

Assays for Intracellular Receptor–Ligand Dissociation

The internalized ligand in the cellular pellets obtained as described above for the internalization assays was either attached to receptor (bound ligand) or dissociated from its receptor (free ligand). Measurement of bound and free ligand was determined by measuring the ability of digitonin to release intracellular radioactive ASOR in the cells as described by Oka and Weigel [20]. Briefly, cell aliquots (previously treated with 10 mM of EDTA to remove surface-bound ligand) were incubated in the presence of Eagle's Medium containing 0.055% digitonin for 10 min on ice. Then samples were centrifuged at 2000 *g* for 2 min to separate permeabilized cells (containing ligand bound to the receptor) from incubation medium (containing free, unbound ligand released during digitonin treatment). Radioactivity in the respective fractions was then determined. To confirm that the radioactivity remaining in the permeabilized cells was indeed receptor-bound ligand, we resuspended some of the digitonin permeabilized cells in Eagle's Medium containing 20 mM of EDTA to dissociate receptor–ligand complexes. This EDTA treatment released greater than 90% of the radioactivity into the isolation medium, confirming that the radioactive ligand was receptor-bound.

General

Protein was determined by the method of Bradford [21] using BSA as a standard. Centrifugation of cell suspensions was performed at 50 *g* for 3 min. ^{125}I-Radioactivity was determined using an LKB gamma spectrometer. Cell number was determined using a hemocytometer. Results are expressed as femtomoles of ASOR bound or internalized per million cells. Statistical analyses were carried out using Student's *t*-test.

RESULTS

Determination of Surface and Total Binding of ASOR to Hepatocytes

Hepatocytes, isolated from pair-fed and ethanol-fed rats, were initially characterized by determining the ability of ASOR to bind to the cell surface and to intracellular receptors. The distribution of surface and total cellular binding of ^{125}I-labeled ASOR in the cell preparations is shown in Fig. 1. Similar to results from previous studies, ethanol administration significantly impaired both surface and total ASOR binding by 40–50% (Fig. 1). When the data were expressed as a ratio of surface binding to total binding to get a measure of receptor distribution in the two cell types, no differences were observed between cells from ethanol-fed and control animals (ratios of 0.46 and 0.51, respectively). Thus, the receptor activity as determined by ligand binding was equally deceased on the surface and inside the cell after ethanol treatment, and approximately 50% of the receptor binding capacity was on the surface and 50% was intracellular in both cell types (Fig. 1).

Determination of Surface and Total Binding of anti-ASGP-R Antibody to Hepatocytes

We also examined antibody binding to obtain a measure of receptor protein content in the two cell types (Fig. 2). Ethanol feeding caused a significant 30% decrease in surface antibody binding as compared with controls. Total (intracellular and surface) binding of the antibody was affected only minimally (less than 10%) by ethanol feeding.

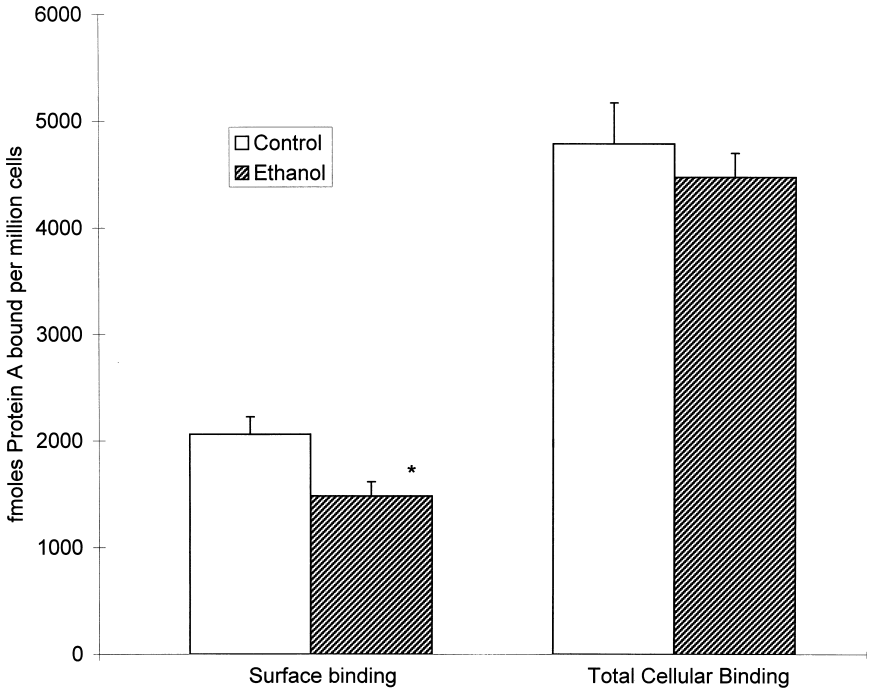

FIG. 2. Determination of surface and total cellular anti-ASGP-R antibody binding after 7–10 days of ethanol administration. Isolated hepatocytes were obtained by collagenase perfusion from animals pair-fed ethanol or control diet for 7–10 days. ASGP-R content was determined by the ability of anti-ASGP-R antibody followed by ^{125}I-labeled Protein A to bind to surface or total cellular receptors, as described under Materials and Methods. Results are expressed as femtomoles of ^{125}I-labeled Protein A bound per million cells for control and ethanol-fed animals and are means ± SEM for 6–8 determinations. ⋆Values were significantly different from those of control animals, $P < 0.01$.

These antibody binding data indicate that there was an inactivation of the receptor in the ethanol-fed animals, since total antibody binding was not different between the two cell types, while ligand binding was decreased significantly. In addition, the receptor distribution was altered in ethanol-fed animals, as shown by an accumulation of intracellular receptor protein (the surface/total binding ratio was 0.33 for ethanol-fed as compared with 0.44 for the control animals). These data confirm previous results from our laboratory which showed that after this early 1-week feeding period there is an inactivation and redistribution of the receptor in ethanol-fed animals [4]. In contrast, when animals are fed ethanol for longer periods of time (greater than 5 weeks), both antibody and ligand binding are decreased [4, 10].

Effects of Monensin on Surface and Total Cellular Ligand and Antibody Binding

To examine more closely the effect of ethanol administration on altered function of one or the other of the two subpopulations of the ASGP-R, we examined the effects of monensin on binding properties of the ASGP-R after these early times of feeding. Previous work from our laboratory had led us to speculate that the State 2 receptors may be preferentially impaired by ethanol treatment [10], since many of the parameters of ethanol-induced defects were characteristic of alteration of State 2 rather than State 1 receptors. Monensin is an agent that has been shown to decrease ligand binding and impair receptor–ligand dissociation preferentially in State 2 receptors in normal rat hepatocytes [13, 20].

When we examined the effect of preincubation with 25 μM of monensin on ligand and antibody binding, we found decreased surface ligand binding by an average of 57 and 51% in cells from control and ethanol-fed animals, respectively (Table 1). Likewise, total cellular ligand binding was decreased significantly, although to a lesser extent, in both cell types (47% decreases in both cell types) (Table 1). The data on ligand binding showed a slight redistribution in receptor ligand binding ability in control animals from surface to intracellular compartments after monensin treatment, most likely caused by

TABLE 1. Effects of monensin treatment on surface and total ASGP-R activity and content in control and ethanol-fed animals

Animal type		^{125}I-ASOR bound*		^{125}I-Protein A bound*	
		− Monensin	+ Monensin	− Monensin	+ Monensin
Control	Surface	478 ± 63	203 ± 35†	2063 ± 211	1330 ± 130†
	Total	934 ± 133	498 ± 100†	4789 ± 513	4265 ± 484
Ethanol-fed	Surface	256 ± 35	132 ± 16†	1484 ± 109	1314 ± 229
	Total	555 ± 63	298 ± 24†	4476 ± 462	3925 ± 583

Binding experiments were performed with cell preparations that had been preincubated at 37° for 45 min and subsequently incubated either with or without the addition of 25 μM of monensin for 60 min, as described under Materials and Methods.

*Data are presented as fmoles ASOR or Protein A bound per million cells are the means ± SEM for 6–10 experiments.

†Significantly different from untreated samples, $P < 0.01$.

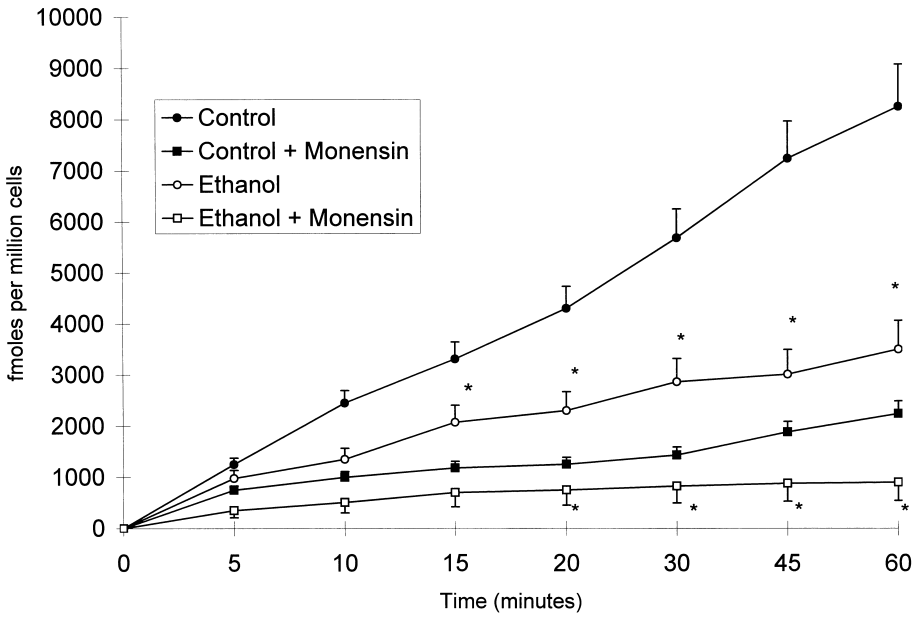

FIG. 3. Kinetics of internalization of ^{125}I-labeled ASOR in the presence and absence of 25 μM of monensin in animals fed an ethanol or control diet for 1–2 weeks. Cells (2–3 × 10^6/mL in Eagle's/BSA) were incubated with 10.0 μg/mL of ^{125}I-labeled ASOR at 37° in the presence or absence of 25 μM of monensin. At the indicated times, aliquots (0.4 mL) were removed and added to buffer containing 10 mM of EDTA to remove surface-bound ligand. Cells were washed twice, and total internalized ASOR in the cell pellets was determined. Results are expressed as femtomoles of ASOR internalized per million cells and are means ± SEM for 6–8 pairs of animals. ⋆Values were significantly different from those of control animals, $P < 0.05$.

impaired receptor recycling. No receptor redistribution was apparent in the cells from ethanol-fed animals. When antibody binding was examined to obtain a measure of receptor content, monensin treatment caused a decrease in surface receptor content by approximately 35% in cells from control animals and had a minimal, albeit significant, 12% decrease in surface antibody binding in cells from ethanol-fed animals (Table 1). Total cellular receptor content was unaltered in both cell types after monensin treatment (Table 1). The antibody binding data indicate a redistribution of surface receptors to the interior in cells from control animals, as shown by changes in surface/total binding ratios after monensin treatment from 0.42 to 0.30. In cells from ethanol-fed animals, the surface/total antibody binding ratios (0.33) were unchanged after monensin treatment. Thus, monensin treatment caused an inactivation of receptors (as defined by decreased total ligand binding without decreased total antibody binding) in both cell types; however, a redistribution of receptor from surface to intracellular sites was observed only in the control animals.

Effects of Monensin on Intracellular Processing of ^{125}I-Labeled ASOR during Continuous Endocytosis

To examine the effects of monensin on intracellular processing of ^{125}I-labeled ASOR and to identify possible impairments in the function of intracellular receptors, post-internalization events of the ASOR–receptor complex were studied. In the first series of experiments, total internalized ligand over a time course of incubation was measured (Fig. 3). Cells from the control animals internalized significantly more ligand at each time point than did cells from the ethanol-fed animals. Monensin, included at a final concentration of 25 μM in each flask, significantly decreased the total uptake of ASOR in cells from both control and ethanol-fed animals (Fig. 3).

We also examined whether monensin treatment differentially affected receptor–ligand dissociation in these two cell types. In cells from control animals, Weigel and coworkers [8, 13, 14, 20] have shown that monensin impairs ligand processing by State 2, but not State 1, receptors. In studies from our laboratory using cells from animals fed ethanol for longer periods (10–14 weeks), monensin did not show preferential effects on either cell type [17]. Since we previously showed that the ASGP-R is inactivated after early times of ethanol administration, we wanted to examine any potential differential effects of monensin after this time period as well. For these studies, we utilized a digitonin technique to differentiate between internalized ligand that is free (i.e. dissociated) and that which is receptor-bound during steady-state conditions of endocytosis [17, 19, 20]. Cells were incubated as described above at 37° in the presence of saturating amounts of ^{125}I-labeled ASOR; amounts of bound and free ligand were then determined in washed cell pellets after various times of incubation. The results from these studies are presented in Fig. 4. Data are expressed as a ratio of bound to free ligand present in the cell at any one time in order to normalize the values for internalization between the cell samples, since absolute amounts of internalized ligand varied between control and ethanol-fed animals (Fig. 3). When we examined results over a 60-min time course of incubation, we found that the presence of monensin significantly increased the bound to free ligand ratios in cells from both control and ethanol-fed animals over the entire time course (Fig. 4), indicating the impairment in intracellular dissociation of ligand from its receptor induced by this ionophore. In addition, these data indicated that the monensin effect was not as severe in cells from ethanol-fed animals as in cells from control animals,

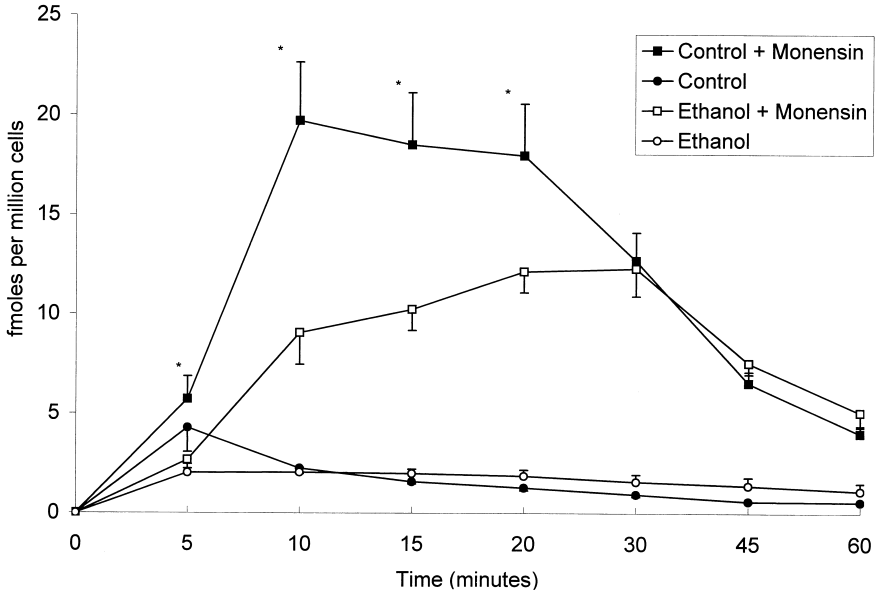

FIG. 4. Kinetics of ligand dissociation and the effects of monensin on this process in rat hepatocytes isolated from pair-fed control and ethanol-fed animals. Cells were incubated as described in Fig. 3. The washed cell aliquots were incubated with digitonin (0.06%) to permeabilize the cells. Intact ligand in the permeabilized pellet represents "bound" ligand, while the intact ligand in the supernatant is "free." Data are expressed as bound/free ratios of intact ligand at each time point for the respective cell types and incubation conditions and are means ± SEM for 6–8 pairs of animals. ⋆Values were significantly different from those of control animals, $P < 0.05$.

especially during the first 20 min of monensin exposure, since the bound/free ligand ratio did not increase to as large an extent in cells from the ethanol-fed animals as compared to controls at these times (Fig. 4).

DISCUSSION

Previous results from our laboratory have shown that long-term ethanol administration alters RME of ASGPs by impairing binding, ligand internalization, and receptor–ligand dissociation in hepatocytes [2, 4, 9, 10, 17]. These differences were apparent after as early as 1 week of ethanol administration and continued through long-term (up to 14 weeks) feeding. We have also shown that despite the marked impairments in uptake of ligand, the return of intact ligand to the medium (a process called diacytosis) is not impaired after long-term ethanol feeding [10]. These results were interesting to us, especially in light of studies from Weigel's group [8, 13–16], which have shown that the ASGP-R population is composed of two distinct populations (State 1 and State 2) of receptors that are differentially regulated, both thermodynamically and kinetically. These populations exist on the surface and inside the hepatocyte in a relative 50–50 proportion [8]. State 2 receptors appear to be responsible for bulk endocytic activity (>80%) by the ASGP-R and are sensitive to a variety of agents (including the carboxylic cationic ionophore monensin). This subset undergoes a transient, reversible inactivation/reactivation process that occurs during constitutive receptor recycling [15, 16]. State 1 receptors, on the other hand, are not perturbed by these agents and do not appear to undergo the inactivation–reactivation process. The State 1 pathway appears to be responsible for diacytosis. In the present study, we tested the hypothesis that ethanol administration preferentially affects the constitutively recycling State 2 pathway rather than the minor State 1 pathway. To accomplish this, we examined the effects of monensin on the functioning of ASGP-Rs in cells from ethanol-fed and control rats. The findings presented here indicate that after short-term ethanol administration of 7–10 days, the receptors on cells from both control and ethanol-fed animals were susceptible to the effects of monensin as shown by impaired ligand binding and processing. Because not all of the receptors are affected by monensin, the receptor population on both cell types appears to be a mixture of State 1 and State 2 receptors. Despite the fact that both cell types were affected by monensin, we did find some differential effects in the degree of monensin-impaired RME between the cell types. In ethanol-fed animals, there was not an apparent receptor redistribution after monensin addition, as was seen for controls. In addition, the cells from ethanol-fed animals were less susceptible to alterations in receptor/ligand uncoupling than were controls. Because receptor redistribution and receptor/ligand uncoupling of State 2 receptors are a target for monensin effects, a very early effect of ethanol on ASGP-R function in hepatocytes may be a preferential impairment in the function of State 2 receptor populations.

In collaboration with Weigel's group, we recently analyzed another phenomenon of State 2 receptor function, that of the transient inactivation/reactivation cycle, which has been shown to occur in permeabilized hepatocytes [22]. These studies were performed after 7–10 days of ethanol feeding using the same cell populations as were used for the present study. We examined the dynamic fatty acylation/deacylation cycle of State 2 ASGP-Rs, which is thought to be responsible for the transient inactivation/reactivation of these receptors to determine whether that may be the molecular basis for the inactivation of ASGP-Rs in the ethanol-fed rats. Results from those studies indicated that State 2 receptors were not affected preferentially in ethanol-fed animals for the reversible fatty acylation process

after 1 week of feeding [22]. In the present study, we see similar findings after monensin treatment, in that the cells from ethanol-fed animals appear to contain both State 1 and State 2 receptors and that both cell types are affected by the addition of this ionophore. However, the results of these latter studies using monensin indicate that there may be some preferential impairment in State 2 receptor populations after the short-term ethanol administration.

In conclusion, after both short-term (7–10 days) and long-term (5 weeks or greater) ethanol feeding, binding of ASOR to the ASGP-R is impaired. After the longer feeding periods, receptor content is also decreased as shown by decreased antibody binding, while after the earlier periods the receptors appear to be inactivated and accumulated intracellularly. The functional hepatocytic receptor population in ethanol-fed animals, although decreased, appears to be composed of both State 1 and State 2 receptors after both of these feeding periods. Although State 2 ASGP-Rs do not appear to be preferentially affected after long-term ethanol administration for 10–14 weeks [17], data from the present study indicate that there are some differences in susceptibility to monensin after the early times of feeding. In addition to these differential effects of monensin, recent data from our laboratory also showed that differential effects occurred during internalization in the presence of colchicine, a microtubule-depolymerizing agent that affects State 2 but not State 1 receptor function (unpublished data). Since receptor inactivation occurs after this early time period and the inactivation/reactivation cycle is a phenomenon of State 2 but not State 1 receptors, it would appear that the early effects of ethanol administration on ASGP-R RME may show some preferential impairment in the function of State 2 receptors or, alternatively, alter the proportion of State 1 and State 2 receptors.

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