Pergamon

Biochemical Pharmacology, Vol. 49, No. 8, pp. 1117–1123, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00011-9

EFFECT OF HYPEROSMOLARITY ON BOTH RECEPTOR-MEDIATED AND FLUID-PHASE ENDOCYTOSIS IN ETHANOL-FED ANIMALS

CAROL A. CASEY,* ROBERT L. WIEGERT and DEAN J. TUMA

Liver Study Unit, VA Medical Center and University of Nebraska Medical Center, Omaha, NE 68105, U.S.A.

(*Received* 27 *December* 1993; *accepted* 26 *October* 1994)

Abstract—We have shown previously that chronic ethanol administration impairs hepatic receptor-mediated endocytosis (RME) of asialoorosomucoid (ASOR), epidermal growth factor and insulin, whereas early uptake by fluid-phase endocytosis (FPE) of a fluorescent dye, Lucifer Yellow (LY), is not altered. Results of these studies suggested that ethanol-induced injury was primarily affecting endocytosis in coated pit areas of the plasma membrane while internalization in noncoated membrane areas was unaffected. In the present study, we investigated the effects of blocking clathrin-coated pit mediated endocytosis by hyperosmolarity on FPE of LY and on RME of ASOR. We also examined the effects of hyperosmolarity on the binding and internalization of insulin, a ligand endocytosed by both RME and FPE. Uptake of LY by noncoated regions of the membrane was not altered in control animals, whereas in hepatocytes from ethanol-fed animals uptake of LY was decreased by 35–40% in the presence of 0.12 M sucrose ($P < 0.05$). These hyperosmolar conditions almost completely inhibited (> 85%) the endocytosis of ^{125}I-ASOR by RME in both ethanol and control cells. Results with insulin showed slight effects (20–30% impairment) on uptake of the ligand in the presence of sucrose. These results are consistent with previous reports that in normal cells the coated pit pathway is impaired by hyperosmolarity, whereas endocytosis in noncoated regions is unaltered. It appears, however, that both FPE and RME in hepatocytes from ethanol-fed animals are susceptible to perturbation by hyperosmolarity. These results indicate that the noncoated pit pathway may be sensitive to stressful conditions such as hyperosmolarity after ethanol treatment.

Key words: receptor-mediated endocytosis; hepatocytes; alcohol; fluid-phase endocytosis; liver; hyperosmolarity

We have shown previously disordered protein trafficking in the livers of rats after acute and chronic ethanol exposure, and recently have focused our interest on internalization events for various molecules into cells by the process of RME† and FPE [1–6]. Multiple steps in the RME pathway for ASOR and EGF appear to be impaired significantly (40–70%) after ethanol administration, including binding, internalization and degradation of the ligands [3–6]. Initial internalization of LY, a molecule internalized by FPE, however, is unaltered [6, 7]. Insulin, a molecule internalized by both RME and FPE, showed intermediate effects in that the binding and internalization of this molecule were altered only slightly (20–30%) after ethanol administration [5]. These previous results suggested that ethanol selectively impaired endocytosis by coated pits, either by altering assimilation of receptors into coated pits on the plasma membrane or by altering invagination of coated pits to form endosomes.

In the present study, we examined the effect of hyperosmolarity on FPE and RME. Hyperosmolar conditions have been employed by other investigators to selectively block the coated pit pathway of RME [8–10]. Hyperosmolarity affects multiple steps in the pathway of RME including formation of coated pits and recycling of clathrin [10, 11], but does not appear to affect internalization of LY, a molecule that is internalized by nonspecific pinocytosis of the plasma membrane [8, 10]. To define more clearly the ethanol-induced alterations in trafficking of ligands, we have studied the effect of hyperosmolarity on endocytosis of ASOR, insulin, and LY in hepatocytes from ethanol-fed rats.

MATERIALS AND METHODS

Materials

Human orosomucoid (α_1 acid glycoprotein), CM-cellulose, collagenase (Type IV), EDTA, BSA (fraction V), phosphotungstic acid, Percoll, neuraminidase (type X), HEPES, 1,3,4,6-tetrachloro-3,6-diphenylglycouril, Lucifer Yellow CH (LY, lithium salt), mouse EGF (receptor grade), porcine insulin (24.1 U/mg) and chloramine T 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 of iodine), ^{125}I-insulin (A14), and ^{125}I-EGF were from Amersham. Eagle's Medium (Grand

*Corresponding author: Carol A. Casey, Ph.D., Liver Study Unit, VAMC (151), 4101 Woolworth Ave., Omaha, NE 68105. Tel. (402) 346-8800, Ext. 3547; FAX (402) 449-0604.

† Abbreviations: RME, receptor-mediated endocytosis; ASOR, asialoorosomucoid; EGF, epidermal growth factor; LY, Lucifer Yellow; FPE, fluid-phase endocytosis; and KRB, Krebs–Ringer buffer.

Island Biological, Catalog No. 420-1400) was supplemented with 2.4 g/L HEPES (pH 7.4) and 0.22 g/L sodium bicarbonate. Eagle's/BSA is Eagle's Medium supplemented with 0.1% BSA. LY was dissolved in Eagle's/BSA at stock concentrations of 1 mg/mL and filtered (0.2 μm) before use. All other chemicals were reagent grade.

Nutritionally adequate diets were formulated according to Lieber and DeCarli [12] and purchased from Bio-Serv, 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. Human orosomucoid was desialylated and iodinated by the method of Oka and Weigel [13] as previously described [3].

Methods

Ethanol treatment of rats. Animals were maintained initially on a Purina rat chow diet until they reached 150–160 g and then were divided into two groups. The rats were housed in individual cages and acclimated to the Lieber–DeCarli control diet for 3 days. Then the rats were 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 5–7 weeks. Chow-fed rats were not included in this study as an additional control group since we have shown previously that changes in RME and FPE after 5–7 weeks of feeding are a result of ethanol consumption and not due to other dietary factors [3, 4, 7]. The animals were meal-fed during the 24 hr prior to isolation of the hepatocytes in order to minimize variations in feeding patterns between the ethanol-fed rats and their pair-fed controls. This program was approved by the Animal Studies Subcommittee of the Omaha DVA Medical Center. Animals were handled in accordance with all applicable local and federal regulations concerning laboratory animals. Animals were housed in an American Association for Accreditation of Lab Animal Care-approved Animal Research Facility at the Omaha VA Medical Center.

Hepatocyte preparation. Hepatocytes were prepared by the collagenase perfusion method of Seglen [14], as described previously [3]. Cells were further purified using continuous Percoll gradients. Viability was routinely greater than 85% after centrifugation with Percoll; this treatment did not affect endocytosis as tested in this study. After isolation, hepatocytes were preincubated for 30 min at 37° to equilibrate the number of cell surface receptors before determining binding, RME and FPE. For studies using ASOR and LY, cells were washed and transferred to Eagle's/0.1% BSA for the analyses. For studies using insulin, cells were washed after preincubation and resuspended in KRB/3% BSA. This procedure of incubating cells in buffer containing 3% BSA has been shown to be appropriate for studies of insulin RME, and very little insulin-degrading activity is present in the medium [15]. For all studies, a cell density of 2–3 × 10^6 cells/mL was used, in a metabolic shaker at 37°.

Internalization of ASOR, insulin and LY. The fluorescent dye LY was used as a marker for FPE, and either ^{125}I-ASOR or ^{125}I-insulin was used in the same samples for measurement of RME. At various times, the cell suspensions (1.2 mL at 2–3 million cells/mL) were removed and the cells were washed twice with 4 mL of ice-cold Eagle's/BSA. Surface-bound and cell-associated radioactivity was determined as described here. For insulin, cells were incubated in 0.2 M acetic acid containing 0.5 M NaCl for 10 min at 0°. At this pH (2.5), surface-bound ligand dissociates from cells and internalized ligand remains cell-associated [16]. In studies using ASOR, surface-bound ligand was removed by the addition of 20 mM EDTA to the Eagle's/BSA medium [17]. Radioactivity representing internalized ligand was equal to the total radioactivity in the cell pellets after acid or EDTA stripping. The amount of radioactivity released by acid or EDTA treatment was equal to surface-bound ligand. Results of binding experiments were corrected for nonspecific binding (amount of radioactivity attached to the cell pellet at time zero in the presence of acid or EDTA). For measurements of FPE, the washed cells were solubilized in 0.5 mL of 0.05% Triton X-100 containing 1 mg/mL BSA. Cell-associated LY was quantitated using a Perkin–Elmer fluorescence spectrometer model LS 5B, with excitation at 430 nm and emission at 540 nm as described by Oka *et al.* [8]. Simultaneous endocytosis of ^{125}I-ASOR and ^{125}I-insulin was assessed by measuring cell-associated radioactivity in an LKB gamma spectrometer.

Determination of the endocytic rate constant (k_e) for ASOR. Radioactivity representing internalized ligand (In) is equal to the total radioactivity in the cell pellets after EDTA stripping. The amount of radioactivity released by EDTA is equal to surface-bound ligand (Sur). The endocytosis rate constant was calculated by the method of Wiley and Cunningham [18]. The constant (k_e) is the slope of the In/Sur ratio plotted as a function of time. Results of binding experiments were corrected for nonspecific binding as described above.

Osmolarity. Sucrose was dissolved to a concentration of 0.48 M in deionized water. Eagle's stock was made as a 2-fold concentrated solution. One volume of the concentrated medium and an equal volume of 0.48 M sucrose were combined to give a stock solution of 0.24 M osmolite in Eagle's/BSA. This medium was then added to a cell suspension (in Eagle's) to give a desired final concentration of 0.12 M sucrose. We chose this concentration to examine because RME was inhibited almost completely (85–95%), whereas FPE was unaffected (data not shown). At higher sucrose concentrations, the initial uptake of LY was impaired in the control cells (data not shown).

General. Protein was determined by the method of Lowry *et al.* [19] using BSA as a standard. Centrifugations of cell suspensions were performed at 50 × *g* for 2 min. ^{125}I-Radioactivity was determined using an LKB gamma spectrometer. Cell number was determined using a hemocytometer. Results are expressed as femtomoles ASOR or insulin bound or internalized per million cells. Statistical analyses were carried out using Student's *t*-test.

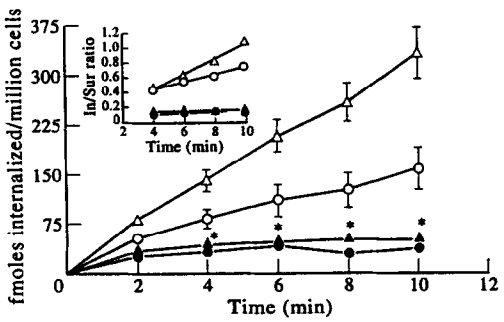

Fig. 1. Effect of hyperosmolarity on internalization of ASOR in cells from ethanol-fed (○,●) and control (△,▲) animals. Cells from the respective animal types were resuspended in Eagle's/BSA medium either with (▲,●) or without (△,○) the addition of 0.12 M sucrose at 37° with 5 μg/mL [^{125}I]ASOR. At the indicated times, samples were removed, and the amount of ligand internalized was determined as described in Materials and Methods. Values are expressed as means ± SEM for 6–10 determinations. Internalization in ethanol-fed animals was significantly different from controls in the absence of sucrose ($P < 0.05$). In addition, the presence of 0.12 M sucrose impaired internalization in both cell types by > 80% ($P < 0.01$, indicated by *). Inset: Effect of hyperosmolarity on In/Sur ratios as a function of incubation time for ASOR in control and ethanol-fed rats. Cell surface-bound ASOR (Sur) values were determined by incubating the cells with 20 mM EDTA to strip cell-associated surface ligand. Internalized ligand (In) values were obtained at each time as indicated in the main portion of the figure.

RESULTS

Effect of hyperosmolarity on the internalization of ^{125}I-ASOR

We first examined the effect of 0.12 M sucrose on the steady-state continuous rate of internalization of ^{125}I-ASOR over a 10-min time course at 37° in hepatocytes from ethanol- and pair-fed control animals (Fig. 1). These conditions have been shown previously to disrupt the coated pit pathway in rat hepatocytes [8–10]. In both control and ethanol-fed animals, internalization was linear over the 10-min time course. Cells from ethanol-fed animals internalized 40–50% less ligand over the time course than did corresponding controls, results that agree with previous data from our laboratory [3, 20]. Endocytosis was inhibited almost completely in the presence of the hyperosmolar medium at a 0.12 M concentration for both cell types (Fig. 1). No further inhibition of ASOR uptake was observed by suspensions in up to 0.4 M sucrose (data not shown).

Effect of hyperosmolarity on the k_e for ASOR

To evaluate quantitatively the effect of hyperosmolarity on the internalization of ASOR into the cells during steady-state endocytosis, we examined its effect on the endocytic rate constant (k_e). This constant is derived from the slope of the line of the In (internalized ligand)/Sur (surface-bound ligand) values plotted versus time of incubation. To use the In/Sur ratio as a basis for calculating the endocytic rate constant, surface-bound ligand should be constant during the time of measurement and no degradation of ligand should occur. These criteria were met for the experiments described here (data not shown). We have shown the data from Fig. 1 represented as k_e functions in the inset of Fig. 1 and the data tabulated and numerically presented in Table 1. ASOR was internalized in a linear fashion in the absence of hyperosmolarity over the 10 min of incubation (Fig. 1, inset). The calculated k_e for control cells was twice that for ethanol cells (Table 1), results that show significant impairment in internalization of bound ASOR in the ethanol-fed animals and that agree with previous results from our laboratory [6]. When the effect of hyperosmolarity was examined, the ability of cells to internalize bound ligand was nearly abolished (Fig. 1 inset), and the values for k_e were inhibited by > 90% in both cell types (Table 1). These data indicate that in the presence of hyperosmolar conditions surface-bound ligand on both control and ethanol cells was unable to be internalized into the cell, probably as a result of impaired internalization into coated pits and/or impaired functioning of coated pits.

Effect of hyperosmolarity on the internalization of LY

Molecules may also enter the cells through noncoated areas of the plasma membrane by FPE. To address the combined effects of ethanol administration and hyperosmolarity on the noncoated pit pathway, we utilized uptake of a fluorescent dye, LY, which is internalized by FPE. Results from our previous studies showed that the initial rates of LY uptake were not altered significantly by chronic ethanol administration [6, 7], and these results are confirmed in our present study (Fig. 2). When we examined the effect of hyperosmolarity on the uptake of LY, we found that the addition of 0.12 M sucrose to the medium did not alter the initial uptake of the dye in control cells, results that are in agreement with other researchers [8, 10]. In cells from ethanol-fed animals, however, hyperosmolarity significantly impaired the initial uptake of LY by 30–40% (Fig. 2) during the time course of incubation. These results indicated that the addition of sucrose to the medium was not tolerated as well in cells from ethanol-fed animals as in controls, and that the noncoated areas of membrane internalization were thus affected after ethanol feeding.

Effect of hyperosmolarity on uptake of insulin

We also examined internalization of insulin over a 10-min time course. Insulin has been shown to enter the cells by both a receptor-mediated mechanism (which may be located in both coated and noncoated regions of the plasma membrane) as well as by nonreceptor-mediated or fluid-phase endocytosis [21–23]. The studies reported here were performed at two concentrations of insulin (0.05 and 10 nM). The lower concentration is in the range of reported values for peripheral circulation [24], and the higher concentration is similar to concentrations used by other investigators to show that endocytosis of insulin is mediated by both coated and noncoated

Table 1. Effect of hyperosmolarity on surface binding and k_e values for ASOR after ethanol administration

Cell type	Condition	Surface ASOR binding* (fmol/10^6 cells)	K_e†
Control	+ None	310 ± 17	0.117
	+ 0.12 M sucrose	325 ± 22	0.007
Ethanol	+ None	202 ± 19‡	0.050‡
	+ 0.12 M sucrose	198 ± 18‡	0.004

* Means ± SEM, N = 6–10.

† k_e Values represent the slope of the lines from data analyzed by least squares analysis of means ± SEM of In/Sur ratios versus incubation time, as shown in Fig. 1 inset.

‡ Significantly different from corresponding controls, $P < 0.05$.

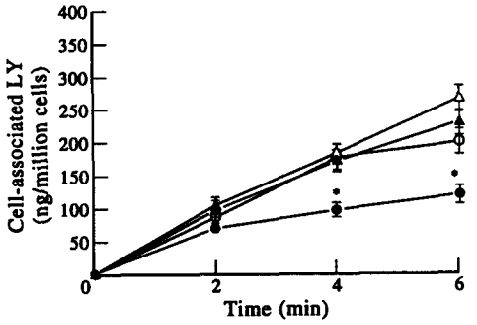

Fig. 2. Uptake of LY by isolated hepatocytes from control (△,▲) and ethanol-fed (○,●) animals suspended in hyperosmolar medium. LY (0.1 mg/mL) was added to cells, at 37°, suspended in control medium (△,○) or medium containing 0.12 M sucrose (▲,●). Values are expressed as means ± SEM for 6–10 determinations. In control cells, no impairments in LY uptake in the presence of sucrose were observed. In ethanol cells, values significantly different from samples in isosmotic medium are indicated by an asterisk (*), $P < 0.05$.

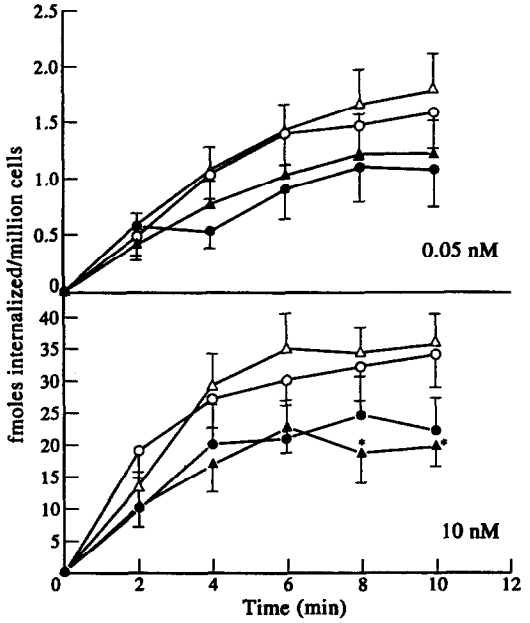

Fig. 3. Internalization of insulin at two concentrations, 0.05 and 10 nM, by control (△,▲) and ethanol (○,●) cells suspended in hyperosmolar medium. Cells from the respective animal types were resuspended in KRB/3% BSA either with (▲,●) or without (△,○) the addition of 0.12 M sucrose at 37° with 0.3 ng/mL ^{125}I-insulin (0.05 nM, top panel) or 60 ng/mL ^{125}I-insulin (10 nM, bottom panel). At the indicated times, samples were removed, and the amount of ligand internalized was determined as described in Materials and Methods. Values are expressed as means ± SEM or 6–10 determinations. Internalization that was significantly different in the presence of sucrose is indicated by an asterisk (*), $P < 0.05$.

pit endocytosis [10]. No differences were noted in the ability of the ligand to enter the cells between control and ethanol-fed animals at either concentration (Fig. 3). The addition of 0.12 M sucrose to the medium decreased internalization slightly at both concentrations, by 20–40%, although these results were significantly different only for control animals at the longer times of incubation when examining internalization at 10 nM (Fig. 3, bottom panel). Thus, internalization of insulin is intermediately affected by hyperosmolarity, a result that would be expected for a molecule internalized by both RME and FPE.

Effect of hyperosmolarity on surface binding of insulin

Surface binding of insulin at 37° over 10 min of incubation in the presence and absence of 0.12 M sucrose was also determined. In these studies, we show that no differences in insulin binding to control or ethanol cells occurred (Fig. 4) and that the addition of 0.12 M sucrose to the medium did not change this binding during the first 6 min of incubation at either concentration. Surface receptor binding did drop off slightly between 6 and 10 min of incubation at the higher concentration of insulin in the absence of sucrose, results that indicate receptor down-regulation. This decrease in receptor

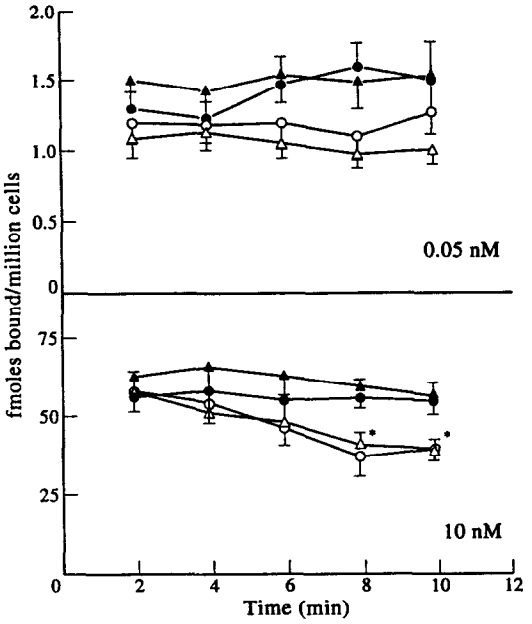

Fig. 4. Effect of hyperosmolarity on insulin binding during continuous endocytosis. Surface binding of insulin was determined in cells from ethanol-fed (○,●) or pair-fed control (△,▲) animals in the presence (▲,●) or absence (△,○) of hyperosmolarity in the medium. Samples were incubated as described in Fig. 3, and ligand bound to the cell surface was displaced in acid conditions as described in Materials and Methods. No differences were noted in binding between control and ethanol-fed animals in either the presence or absence of sucrose at any time point. A pattern of decreased insulin binding in the 10 nM incubations for 8 and 10 min (as compared to the initial 2-min time point) was noted for both control and ethanol cells in the absence of sucrose (*, $P < 0.05$).

number did not occur in the presence of sucrose (Fig. 4, bottom panel). At the lower (0.05 nM) concentration of insulin, no evidence for receptor down-regulation was seen (Fig. 4, top panel) in either the presence or absence of sucrose.

DISCUSSION

In the present series of experiments, we examined the effect of hyperosmolarity on both receptor-mediated and fluid-phase endocytosis in hepatocytes isolated from control and ethanol-fed animals. Our previous research showed that RME was especially altered after ethanol treatment. Early intake of a fluorescent dye, LY, by noncoated membrane areas was not affected after ethanol administration, while long-term net uptake was impaired. This effect on net uptake appeared to be due to enhanced efflux of ingested material after ethanol treatment [7]. All together, our previous data indicated that ethanol administration affected mainly endocytosis by coated pit areas of the plasma membrane, while initial internalization in noncoated membrane areas was unaltered.

In the present study, we have further investigated these findings by examining the effect of hyperosmolarity on endocytosis (both FPE and RME) in ethanol-fed animals. Hyperosmolarity has been shown to impair RME of ligands internalized by coated pits, presumably by interfering with coated pit formation and internalization. We examined whether endocytosis in ethanol cells showed a pattern of internalization different from that in control animals after the addition of an osmolite, sucrose, to the medium. We found that early events of both FPE (using LY as a marker) and RME (using ASOR as a ligand) were altered by hyperosmolarity in ethanol-fed animals, in contrast to control animals where only RME was impaired. Internalization of ASOR was inhibited almost completely in both cell types, consistent with the known predominance of the coated pit pathway by the asialoglycoprotein receptor [25] and findings by other investigators [8–10]. When internalization of insulin, a molecule internalized by both RME and FPE, and LY, a molecule internalized strictly by FPE, was examined, we showed intermediate impairments in both control and ethanol-fed animals. This partial inhibition indicates that cells from ethanol-fed animals may be susceptible to stressful conditions such as hyperosmolarity when examining noncoated pit mechanisms of internalization or ligands internalized by a combination of coated pit and noncoated pit endocytosis.

Other receptors do not recycle continuously and may not always reside in clathrin-coated areas of the plasma membrane. An example of one such receptor is the insulin receptor. Moss and Ward [10] have shown evidence that some of the insulin that is taken up by the cells is receptor mediated but not coated pit dependent. Some insulin may also be internalized by FPE [21]. The multitude of pathways for insulin entrance into the cell probably accounts for the fact that no difference between control and ethanol cells on insulin uptake was seen when effects of hyperosmolarity were examined. We previously showed that long-term uptake and saturation binding of the insulin receptor was impaired by 25–30% after ethanol treatment [5], but we did not see differences when we examined steady-state binding during early times of incubation [6]. In the present study when we examined the effects of hyperosmolarity on these early uptake events for insulin, we showed impairments intermediate to those shown for ASOR and LY. We also showed that, if anything, enhanced binding of insulin was seen in the presence of hyperosmolarity. We did find some evidence for down-regulation of the insulin receptor at high (10 nM) concentrations in that the receptor number on the cell surface was lower at 8 and 10 min of incubation than at the earlier times (0–6 min, see Fig. 4). This down-regulation was not observed in the presence of hyperosmolarity, presumably since not all receptors would be recycled. At subsaturation levels of insulin (0.05 nM), no receptor down-regulation was seen. Since the ligand levels were so far below saturation (~1.5 fmol/million cells bound vs a total receptor population of around 200 fmol/million cells), there would presumably be plenty of receptors to bind the ligand over the 10 min of incubation without the need for receptor recycling.

We also examined uptake of LY, a molecule internalized by FPE. Hyperosmolar conditions have been shown not to affect FPE in normal cells [8], since FPE does not proceed via a coated pit pathway. In control cells, the coated pit pathway and FPE can be distinguished from each other by the effect of hyperosmolarity on each pathway. Such is not the case in cells from ethanol-fed animals. In ethanol-fed animals we had reported previously that the initial influx of dye into the cells is unaffected, even though RME is decreased dramatically [6]. Hyperosmolar conditions result in intermediately impaired FPE in the ethanol cells, results that indicate that the noncoated pit pathway may be able to operate normally under ideal conditions, but may be more susceptible to stressful conditions such as changes in osmolarity.

We also tried to investigate two potential mechanisms which may be responsible for the differences we saw between ethanol-fed and control animals. The first would be an increased sensitivity of ethanol cells to sucrose, and the second would be an inability of the ethanol cells to retain loaded dye. We were not able to determine whether cells from ethanol animals displayed a higher sensitivity than control cells to added sucrose, mainly because the ethanol cells were already so impaired compared with controls that we were unable to obtain an accurate concentration–response curve (data not shown). To investigate the second possibility, we performed some experiments to examine whether added sucrose affected the ability of the cells from ethanol-fed animals to retain dye. Our results indicated that in cells loaded with LY for 30–60 min, added sucrose (up to 0.4 M) did not alter patterns of efflux (data not shown). Since we needed to load the cells with dye to measure efflux accurately, we were not able to determine whether hyperosmolarity affected immediate retention of dye by our methods. We cannot discount at this time the possibility that ethanol cells are susceptible to sucrose because of either an increased sensitivity to sucrose or an inability to retain LY.

In conclusion, chronic ethanol administration altered plasma membrane dynamics in isolated hepatocytes. These impairments were especially noticeable when examining ligands internalized by the coated pit, or RME, pathway. Receptor-ligand internalization, trafficking, and receptor recycling were all impaired. The process of noncoated pit endocytosis, or FPE, was less affected by ethanol administration. Indeed, initial rates of LY uptake into cells were not altered during basal conditions of incubation, but the addition of sucrose to the medium impaired FPE in ethanol-fed, but not control, animals. All together, these results indicate that the plasma membranes from ethanol-fed animals may be susceptible to increased stress. This defective surface membrane could interfere with the ability of hepatocytes to respond to the external environment, alter transport processes and modify other liver functions.

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