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Chronic ethanol consumption impairs receptor-mediated endocytosis of MAA-modified albumin by liver endothelial cells☆

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Abstract

Alcoholic liver disease has been associated with abnormalities in receptor-mediated endocytosis (RME) which results in abnormal degradation of metabolically altered proteins. Model systems using formaldehyde-modified albumin (f-Alb) have shown an impairment in RME following chronic alcohol consumption utilizing both *in situ* perfused rat livers and isolated rat liver endothelial cells (LECs). The discovery that alcohol metabolite derived aldehydes can modify proteins prompted a study to determine if malondialdehyde–acetaldehyde-modified albumin (MAA-Alb) would be degraded similar to that reported for f-Alb, and whether ethanol-fed rats would demonstrate an impaired RME with respect to this ligand which occurs as a consequence of chronic ethanol consumption.

MAA-Alb was degraded slightly more than f-Alb in both *in situ* perfused livers and at the single cell level. This degradation was completely inhibited with 100× unlabeled f-Alb, which suggests the use of a similar receptor. Following alcohol consumption there was a 50–60% decrease in MAA-Alb degradation in whole livers and isolated LECs. Utilizing isolated LECs it was determined that impairment in internalization was the most likely mechanism for the decrease in the amount of MAA-Alb that was degraded. These data show that chronic alcohol consumption by rats does in fact impair RME of alcohol metabolite-derived adducted proteins, and this impairment is due to a defect in the post-internalization step rather than the binding or degradation of the modified protein.

Keywords: Receptor-mediated endocytosis; Aldehyde-modified proteins; Alcohol; Liver endothelial cells; Scavenger receptors; Protein degradation

1. Introduction

RME is a process where ligands are bound to a receptor, internalized and shuttled to lysosomal compartments for degradation, and then returned or expelled from the surface of the cell [1,2]. Alteration of this process has been linked to a number of diseases, including atherosclerosis, diabetes mellitus, and more recently alcoholic liver disease [3–6]. RME by LECs is an important process in the elimination of chemically modified proteins and altered macromolecules including the following: maleylated albumin, acetylated low density lipoprotein, malondialdehyde low density lipoprotein, formaldehyde treated albumin, and acetaldehyde-modified albumin [7].

Previous studies have shown that chronic ethanol administration alters the ability of LECs to perform RME efficiently. This includes the impaired degradation of both acetaldehyde-modified proteins (AA-Alb) and f-Alb [8,9]. Tuma *et al.* [5] have shown that MDA and AA bind to proteins to form a hybrid molecule termed the MAA adduct. Antibodies to this molecule have been detected

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Abbreviations: f-Alb, formaldehyde-modified albumin; MAA-Alb, malondialdehyde–acetaldehyde-modified albumin; MDA, malondialdehyde; AA, acetaldehyde; RME, receptor-mediated endocytosis; ^{125}I, iodine-125; ^{125}I-f-Alb, iodine-125-formaldehyde-modified albumin; ^{125}I-MAA-Alb, iodine-125-MAA-modified albumin; LECs, liver endothelial cells.

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in the serum of alcohol-fed rats and human alcoholics [10,11]. Taking into account that MAA-modification of proteins at lysine residues is analogous to protein modification by other compounds, we hypothesize that MAA-modification of proteins interferes with RME. Therefore, it was the purpose of this study to determine whether MAA-modified proteins are bound and degraded by *in situ* perfused rat livers and to utilize isolated LECs to determine which step in the RME process may be altered by chronic ethanol consumption.

2. Materials and methods

2.1. Rats

Male Wistar rats purchased from Charles River Laboratories were maintained on a Purina rat chow diet, until they reached a weight of 140–150 g, and were divided into three groups. The first group was maintained *ad libitum* on the standard chow diet for 5–7 weeks to serve as a control group ensuring all results were due to ethanol, and not another dietary factor. The second and third groups were housed individually and acclimated to the Lieber–DeCarli liquid control diet from Dyets, Inc. for 3 days [12]. The rats were paired by weight, one rat was given the ethanol-containing diet *ad libitum*, and the other rat was fed an isocaloric amount of the control liquid diet as determined by the pair-fed rat from the day before. Pair feeding was continued for 5–7 weeks. Finally, the ethanol-containing diet consisted of 18% of the total calories as protein, 35% as fat, and 36% as ethanol. In the control diet, ethanol was replaced isocalorically with carbohydrates. All animals were allowed free access to their food and/or water up to 1 hr before sacrifice.

2.2. Chemicals and proteins

Bovine serum albumin (Alb) was purchased from CalBiochem. AA was obtained from Aldrich Chemical Co. MDA was obtained as the sodium salt (MDA ∼ Na) by treatment of tetramethoxypropane (Aldrich Chemical Co.) with NaOH, according to the method of Kikugawa and Ido [13]. Trichloroacetic acid (TCA), phosphotungstic acid (PTA), formaldehyde, and Percoll were purchased from Sigma Chemical Co. Sodium ^{125}I was purchased from Amersham Corporation.

2.3. Preparation and labeling of ligands

Alb was labeled with ^{125}I by the chloramine-T method (^{125}I-Alb), with resulting specific activities between 2800 and 3200 cpm/ng [14]. Briefly, proteins were first labeled with ^{125}I, and modified with formaldehyde or MAA. Formaldehyde treatment of Alb was done using a modification of the methods of Mego *et al.* [15] and Horiuchi *et al.* [16]. As previously described MAA-Alb was prepared by reacting 1.0 mM AA and 1.0 mM MDA with 2 mg of BSA in 0.1 M phosphate buffer, pH 7.2 at 37° for 3 days, followed by dialysis against three changes of 0.1 M sodium phosphate buffer for 24 hr at 4°. The percentage of lysines modified by the different aldehydes was determined by the method of Habeeb [21]. Protein concentrations were determined by the method of Lowry *et al.* [18], and ^{125}I-radioactivity was assessed using a Packard Cobra II gamma-spectrometer.

2.4. In situ liver perfusion

After 5–7 weeks of liquid diet, rats were anesthetized with Nembutal and perfused as previously described [9,17]. Briefly, the liver was equilibrated for 15 min with oxygenated Krebs–Ringer bicarbonate buffer containing 1% Alb, followed by a 2 mg/200 mL solution of ^{125}I-modified Alb, MAA-Alb, or f-Alb that was re-circulated for 3 hr through the liver. At various time points, 0.5 mL aliquots were removed and 2.0 mL of 20% TCA and 2% PTA was added to precipitate intact proteins. Degradation was determined by measuring the amount of acid soluble radioactivity left in the supernatant.

2.5. Isolation of liver endothelial cells and Kupffer cells (KCs)

LECs and KCs were prepared by perfusion and differential centrifugation methods as described previously [19]. Briefly, livers were flushed with saline containing HEPES to remove red cells, and perfused *in situ* with 14 mg/300 mL of Liberase from CalBiochem for 15 min. The liver was removed, placed in 200 mL of Dulbecco's phosphate buffered saline (D-PBS) in a sterile 250 mL flask, and placed on a stir plate with a sterile stir bar for 30 min at 37°. The suspended cells were filtered through eight ply-type VII gauze sponges. Hepatocytes and non-parenchymal cells were separated by differential centrifugation at 100 *g* for 5 min and 350 *g* for 10 min at 4°. Cell pellets were pooled, re-suspended in D-PBS, and centrifuged through a 25%/50% Percoll gradient at 900 *g* for 20 min at 4°. The LECs and KCs were collected at the interface, washed with D-PBS, and re-suspended in E-STIM^{TM} media (Cellgro). KCs were isolated by selective adherence to 24-well plates for 20 min at 37°, washed and placed in 10% M199/F12 media. Cells were counted on a hemocytometer and the viability was determined by trypan blue exclusion, and found to be greater than 85% viable. Cell purity by flow cytometry (data not shown) was found to be greater then 90% for LECs and KCs using the following antibodies; mouse anti-RECA-1 for endothelial cells (Serotec), mouse anti-ED2 for Kupffer cells (Serotec), mouse anti-desmin for stellate cells (Sigma), rabbit anti-rat ASGP-receptor for hepatocytes (a generous gift from Dr. Carol Casey, VA Medical Center, Omaha, NE) and

Dil-Ac-LDL for endothelial cells (Biomedica Technologies Inc.).

2.6. Receptor binding assays

Receptor number was determined by the specific binding of ^{125}I-MAA-Alb to LECs at 0°. Typically, cell suspensions of 1×10^6 cells/mL in HBSS containing 1% Alb (HBSS-Alb) were incubated for 30 min at 37° to increase and equilibrate the number of cell surface receptors. Cells were kept in suspension by use of a metabolic shaker operating at 100 rpm. Cell aliquots (typically 1 mL) were added to 4 mL of low pH (pH 3.0) cold HBSS-Alb and mixed gently on ice to remove any endogenously surface-bound ligand. Samples were washed in low pH media by centrifugation at 50 *g* for 3 min, the supernatant fluid aspirated, and the cell pellet gently re-suspended in 10 mL of ice-cold wash medium and re-centrifuged. The washed cells were then re-suspended in 2.0 mL of cold HBSS-Alb (pH 7.4) and incubated at 0° for 60 min with ^{125}I-MAA-Alb as described for f-Alb by Nilsson and Berg [22]. Following incubation, samples were transferred to tubes containing 2.5 mL of ice-cold HBSS-Alb, pelleted at 350 *g* for 5 min, and washed twice with 2.5 mL of ice-cold incubation medium. These cell suspensions contained only surface-bound ^{125}I-MAA-Alb.

To assess specific binding of the ligand, the cell suspensions were divided into two 5 mL aliquots (pH 7.4); buffer was added to one aliquot while low pH HBSS-Alb was added to the other. After gentle mixing on ice for 15 min, suspensions were centrifuged, and aliquots (0.5 mL) of the supernatant fluids were removed to determine free ^{125}I-MAA-Alb. Supernatant fluids were aspirated, and the pellets were re-suspended with 5 mL of HBSS-Alb. Aliquots (0.5 mL) of the pellet suspension were then removed to determine cell-associated radioactivity. Specific binding was defined as the amount of radioactivity displaced by low pH treatment of the cells. Low pH treatment did not affect cell viability or the percentage of single cells. In some cases, specific binding was assessed by including in the binding medium an excess (100-fold) of non-radioactive MAA-Alb. Results of specific binding were similar for both low pH treated cells and cells incubated with excess unlabeled ligand.

2.7. Internalization and degradation of ^{125}I-MAA-Alb by isolated LECs

Degradation of labeled MAA-Alb was followed by measuring the amount of acid soluble radioactivity after precipitating intact protein with 20% TCA/2% PTA [23]. Briefly, LEC suspensions (1×10^6 cells/mL) were incubated at 37° for 30 min in a metabolic shaker to increase and equilibrate the number of cell surface receptors. ^{125}I-MAA-Alb (100 nM, 1500 cpm/ng of protein) was added, aliquots of the cell suspension (1 mL) were removed at various time intervals, added to ice-cold centrifuge tubes, and immediately centrifuged to pellet the cells. A 0.25 mL aliquot of the supernatant was removed and added to 2.0 mL of 20% TCA/2% PTA for determination of acid soluble radioactivity (degradation). After at least 20 min on ice, the mixtures were centrifuged for 5 min at 350 *g*, and radioactivity in the supernatant fluid was determined. At 37° degradation of ^{125}I-MAA-Alb was negligible in the absence of cells.

To determine the internalization of ^{125}I-MAA-Alb, pelleted and equilibrated LECs were incubated with ^{125}I-MAA-Alb. At various times, LECs were re-suspended and washed twice with 1 mL of ice-cold HBSS-Alb, and three times with HBSS-Alb at pH 3.0 to remove surface bound ligand. After the low pH treatment, the cell suspension was centrifuged and the pellet re-suspended in HBSS-Alb. An aliquot of this suspension (0.25 mL) was added to 2 mL of 20% TCA/2% PTA for determination of internalized ligand. Acid precipitates were washed 20% TCA/2% PTA and radioactivity in the precipitated pellet determined. This value represented the amount of intracellular intact ligand. Treatment with low pH HBSS-Alb media and subsequent centrifugation had no effect on cell viability as assessed by exclusion by trypan blue.

2.8. Statistical analysis

Statistics were performed using ANOVA analysis comparing the different experimental and control groups. Statistical significance was achieved if P values were less than 0.05. All statistical analysis was performed using SigmaStat (Jandel, Scientific, 1994).

3. Results

3.1. In-situ degradation of ^{125}I-MAA-Alb

Previous studies on the degradation of f-Alb in livers of chow-fed rats showed that 400–500 μg of ^{125}I-f-Alb was degraded over a 3 hr period of time [9,17]. When these same studies were performed in ethanol rats, there was a 40–60% decrease in f-Alb degradation. Knowing that ethanol consumption would impair f-Alb degradation, experiments were performed to determine the effects of ethanol on the degradation of the newly characterized protein–MAA adduct.

As shown in Fig. 1, perfusion of chow-fed or pair-fed rat livers with the MAA adducted Alb (that forms in the presence of 1 mM AA with 1 mM MDA), resulted in the degradation of approximately 450 μg of MAA-Alb over a 3 hr time period. This degradation was shown to be decreased by 50–70% in the livers of rats chronically consuming ethanol in their diet. Additionally, as a positive control a little more than 400 μg of f-Alb, which has been used previously by us [17] and others [24–27] as a standard

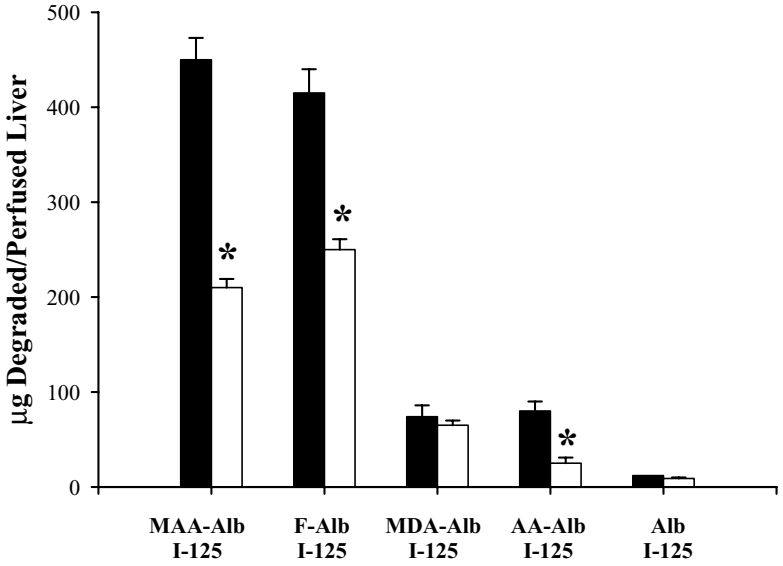


Fig. 1. Degradation of ^{125}I-MAA-Alb by *in situ* perfused livers of chow-fed (filled bars) or ethanol-fed (empty bars) rats perfused with 10 μg/mL of ^{125}I labeled: MAA-Alb, f-Alb, MDA-Alb, AA-Alb, and Alb. These ligands were re-circulated via perfusion for 3 hr, aliquots taken, and TCA/PTA precipitated to determine the amount of acid soluble protein (degradation). Experiments were performed using six animals in each group, and significance reported at $^{*}P < 0.05$. Animals perfused with ^{125}I-MAA-Alb showed significantly higher degradation than those perfused with ^{125}I-MDA-Alb, ^{125}I-AA-Alb, or ^{125}I-Alb, but not ^{125}I-f-Alb. Additionally, chronic ethanol consumption significantly decreased the amount of degradation of every ligand except for ^{125}I-Alb.

ligand of the scavenger receptor, was degraded in the livers of chow-fed rats, with a 40–50% decrease in this degradation observed in the livers of rats chronically consuming ethanol. Aldehyde control experiments with AA-Alb and MDA-Alb used iodinated Alb that had been exposed to 1 mmol/L of AA or MDA for 3 days at 37° and pH 7.4. As shown in Fig. 1, AA-Alb and MDA-Alb were taken up and degraded by the perfused liver (75 μg), at levels that were much lower than those observed with the adduct composed of each of these two aldehydes, MAA-Alb (400–500 μg). Interestingly, the exposure of Alb to either of these two aldehydes resulted in products that where <1% of the total lysine residues available (Table 1). These data certainly may explain the decreased ability of AA-Alb and MDA-Alb to be degraded as compared to MAA-Alb where it was demonstrated that 35.6% of the lysines were modified.

Table 1
Modification of lysine residues on Alb with different aldehydes

Type of adduct	Concentration	Incubation time	Percent modification
f-Alb	20%	1 hr	46.7
AA-Alb	1 mM	3 days	<1
MDA-Alb	1 mM	3 days	<1
MAA-Alb	1 mM AA + 1 mM MDA	3 days	35.6

Note: All adducts were prepared in sealed Eppendorf tubes, wherein 2 mg of ^{125}I-BSA was reacted with each concentration of aldehyde in either sodium phosphate buffer (pH 7.4) or 0.2 mol/L carbonate buffer (pH 10.0) at 37° for their specific incubation time. f-Alb was produced using 20% formaldehyde. Each product was dialyzed for 16 hr against 0.02 mol/L of sodium phosphate buffer containing 0.15 mol/L of NaCl at 4° with several changes of the buffer. The percentage of modification of lysine residues by the different aldehydes was determined by the method of Habeeb [21]. $P < 0.05$.

However, the extent of lysine modification must not be the only factor as f-Alb (46.7%) is modified much more than MAA-Alb, and is degraded at a slightly but significantly lower level.

To determine whether this degradation was specific for each ligand, the reaction was performed in the presence of a 100× concentration of unlabeled f-Alb or MAA-Alb. Incubation with f-Alb (100×) completely inhibited the degradation of either MAA-Alb or f-Alb indicating the receptor(s) responsible for binding and degrading these ligands may be similar (data not shown). Unfortunately, the addition of 100× MAA-Alb resulted in hepatotoxic effects characterized by dissociation of liver tissue, liver leakage, and rupture. Therefore, the liver did not remain intact and the inhibition of ^{125}I-MAA-Alb degradation by unlabeled MAA-Alb was not able to be performed. This has led to other investigations into the effects of MAA-Alb on the liver.

To determine the kinetics of this degradation, rats were fed for 6 weeks on pair, ethanol, and chow diets. Figure 2 shows the degradation of MAA-Alb over a 3 hr period of *in situ* perfusion. Chow-fed and pair-fed animals degraded approximately 500 μg of MAA-Alb, with the ethanol animals degrading only about 200 μg of MAA-Alb. The kinetics of this 60% decrease in MAA-Alb degradation indicated that the degradation of MAA-Alb is impaired in ethanol-fed animals similar to that observed when f-Alb was utilized as the ligand in the perfusate [9].

3.2. Purification and characterization of LECs and KCs

Previous studies have shown that LECs or KCs are the cells most likely involved in the degradation of these

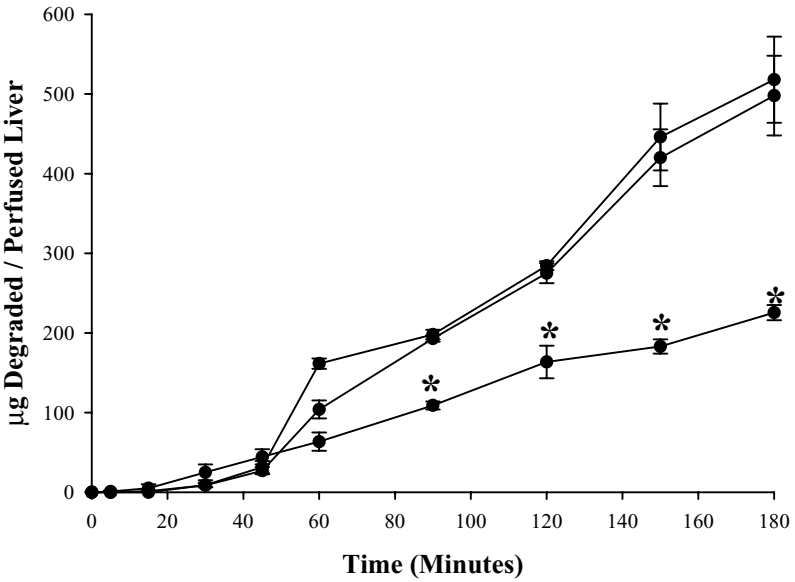


Fig. 2. Effects of ethanol feeding on *in situ* degradation of ^{125}I-MAA-Alb by livers from chow-fed (■), pair-fed (●), and ethanol-fed (▲) rats. Livers were perfused with 10 μg/mL of ^{125}I-MAA-Alb, aliquots were taken at indicated times over a 3 hr period and intact protein precipitated with TCA/PTA. Acid soluble radioactivity (protein degradation) in the supernatant was determined. Results are expressed as means ± SD (N = 6 in each group) and values significantly different from control rats at $^{*}P < 0.01$ are indicated.

ligands. Therefore, steps were taken to isolate these cells as described in Section 2. Using a fluorescent activated cell sorter (FACSCalibur), LECs was found to be >90% pure by staining with anti-RECA and Dil-Ac-LDL. Staining with anti-ED-2 (Kupffer cell marker) showed that <5% of the cells were Kupffer cells, with <2% of the cells staining with anti-desmin (stellate cell marker; data not shown). Similarly, KCs were found to be >90% pure by staining with anti-ED-2 (Kupffer cell marker). Staining with anti-RECA and Dil-Ac-LDL showed <5% contamination with LECs, and <5% contamination with stellate cells (anti-desmin). Finally, the absence of hepatocytes was confirmed by using an antibody to the asialoglycoprotein receptor. The purity of the LECs and KCs did not differ among chow-fed, pair-fed or ethanol-fed rats (>90% pure).

3.3. Degradation of ^{125}I-MAA-Alb by isolated LECs and KCs

To determine the role of LECs in degrading ^{125}I-MAA-Alb, isolated cell suspensions from chow-fed, pair-fed, and ethanol-fed animals were incubated with 25 μg of ^{125}I-MAA-Alb, and the amount of degradation determined by counting the acid soluble protein in the supernatant (Fig. 3A and B). As indicated, LECs isolated from chow-fed and pair-fed rat livers degraded 8 μg of ^{125}I-MAA-Alb/10^6 cells (Fig. 3A). This was about 2 μg more than observed for f-Alb [9]. However, the ability of LECs isolated from ethanol-fed animals to degrade the protein was decreased by 50% resulting in only 3.8 μg degraded over the same period of time.

In contrast, KCs demonstrated the degradation of 0.0175 μg of ^{125}I-MAA-Alb/10^6 cells, with no effect of ethanol on this degradation (Fig. 3B). This degradation was approximately 50-fold less than the amount degraded by a similar number of LECs, and strongly suggests that the major cells involved in the degradation of this ligand are the LECs. Thus, the rest of the studies reported in this manuscript will focus only on LECs.

3.4. Binding of ^{125}I-MAA-Alb to isolated liver endothelial cells

To compare the binding affinities for ^{125}I-MAA-Alb between LECs from chow-fed, pair-fed, and ethanol-fed animals, ^{125}I-labeled MAA-Alb was added to cell suspensions as indicated in Section 2. The ability of ^{125}I-MAA-Alb to bind LECs from chow-fed and pair-fed rats was similar (300 fmol/10^6 cells). As seen in Fig. 4, there was a very small but statistical difference in the binding of MAA-Alb to each of the treatment groups, indicating that the abundance of receptor(s) and the affinity for MAA-Alb were slightly different in the presence of ethanol in the diet. However, this difference is not enough to result in a 50–70% decrease in degradation.

3.5. Ethanol feeding affects internalization and degradation of ^{125}I-MAA-Alb by isolated LECs

As shown in Fig. 5, ethanol feeding resulted in a 20–40% decrease in ^{125}I-MAA-Alb internalization, which was significantly decreased between 40 and 180 min of incubation. To further determine the role of RME in the observed ethanol-induced defect, LECs were allowed to bind the ligand at 4° and washed three times with HBSS-Alb. The cells were warmed to 37°, aliquots of the cells harvested,

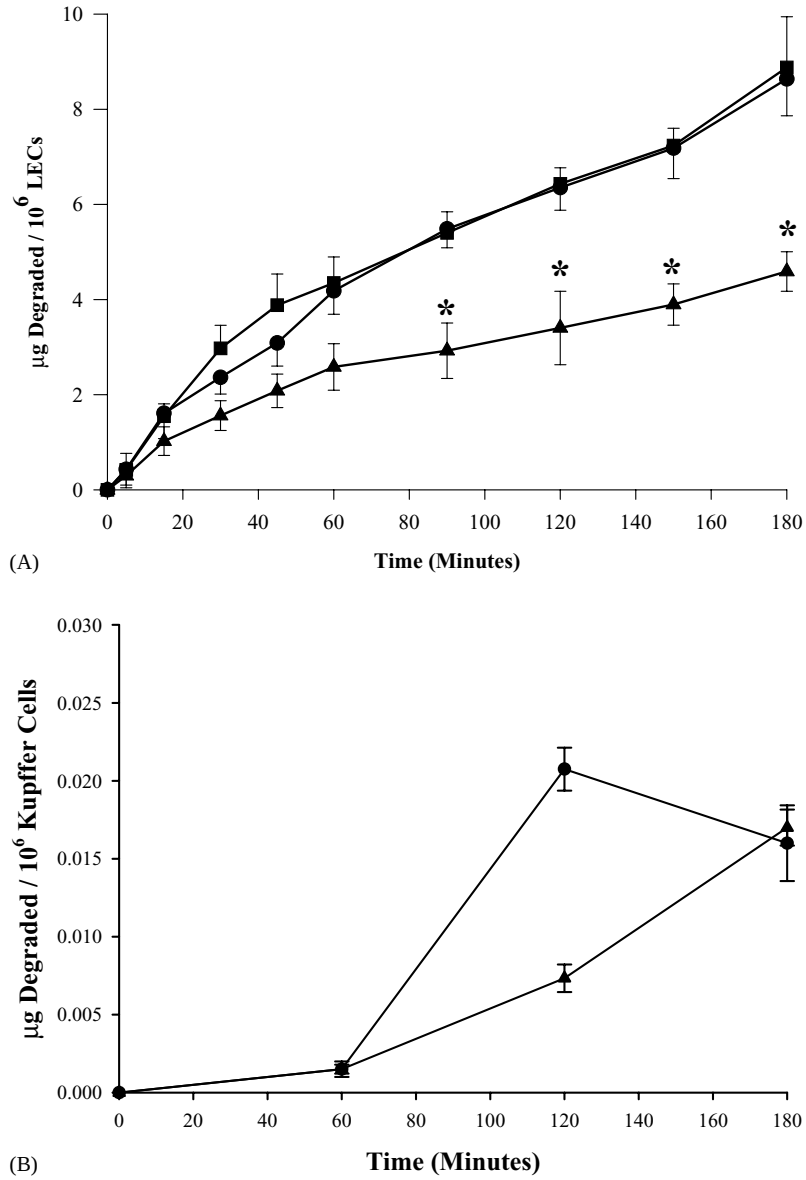


Fig. 3. (A, B) Kinetics of ^{125}I-MAA-Alb degradation by isolated LECs (A) and KCs (B) from chow-fed (■), pair-fed (●), and ethanol-fed (▲) rats. LEC and KC suspensions (1×10^6 cells/mL) were incubated at 37° with ^{125}I-MAA-Alb (25 μg/mL of protein) for the indicated amount of time, aliquots of the cell suspensions (1 mL) were removed, and acid-soluble radioactivity was determined as described in Section 2. Results are expressed as means ± SD (N = 6 in each group), and values significantly different from controls ($^*P < 0.01$) rats are indicated.

and the ^{125}I-MAA-Alb that was internalized and subsequently degraded was determined. By performing these studies, it was possible to trace ^{125}I-MAA-Alb as it traverses into the cell in a single round of binding, internalization, and degradation. As indicated in Fig. 6A, internalization of surface bound ligand was decreased by 50% in LECs from ethanol-fed animals when compared to cells from the control groups. Additionally, the decrease in surface bound internalization contributed to the decrease in the percent degraded of the total surface bound ligand (Fig. 6B), indicating that the decrease in MAA-Alb degradation resulted from a defect in the internalization of surface bound MAA-Alb. In fact, when the degradation of MAA-Alb was normalized to the amount of surface bound MAA-Alb (Fig. 7), there was no significant statistical difference in the rate of MAA-Alb degradation between the groups supporting the hypothesis that reduced degradation of adducted proteins by LECs from alcohol-fed animals results from a defect in internalization of modified proteins and not from a reduced rate of intracellular protein degradation.

4. Discussion

LECs have been shown to bind and degrade formaldehyde and acetaldehyde-modified proteins [8,9,17], and chronic ethanol consumption has been shown to result

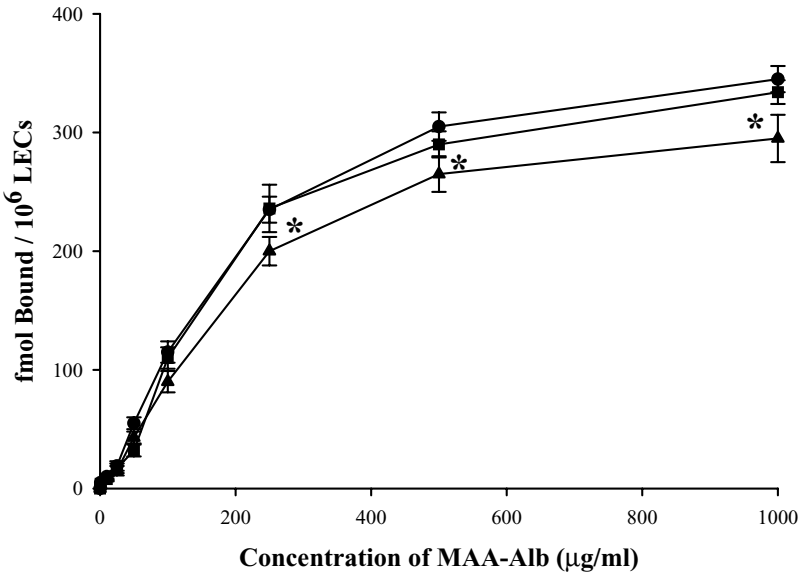


Fig. 4. Binding of ^{125}I-MAA-Alb to LECs. Cell suspensions (1×10^6/1.0 mL in HBSS) were incubated at 37° for 30 min to increase and stabilize the receptor number, and then chilled on ice. One milliliter of the cold cell suspension was added to tubes containing different concentrations of ^{125}I-MAA-Alb (from 1 to 1000 μg/mL). Total volume was 2.0 mL. The samples were incubated at 0–4° for 90 min with constant gentle rotation, diluted with cold HBSS, centrifuged, 0.5 mL of the supernatant fluid was removed to determine free ^{125}I-MAA-Alb, and cell pellets were washed once with excess HBSS. Specific binding was determined as described for binding assays in Section 2 and reported as saturation of the binding of ^{125}I-MAA-Alb LECs isolated from chow-fed (■), pair-fed (●), and ethanol-fed (▲) rats. Specific binding was found to be 76–88% at all concentrations tested. Results are shown as the means ± SD for 8 determinations, and significant differences between ethanol-fed and chow- or pair-fed controls reported at $^{*}P < 0.05$.

in a significant decrease (50–60%) in the ability of LECs to degrade these ligands. The f-Alb ligand has been used extensively to characterize LECs as; it is almost exclusively taken up by only this cell type [3,25,26]. Additionally, the degradation of f-Alb has been shown to be impaired when LECs were isolated from the livers of rats chronically consuming ethanol, suggesting that the removal of this ligand by RME was affected at the post-internalization step in the process [8,9]. However, while formaldehyde-modified proteins are readily degraded by

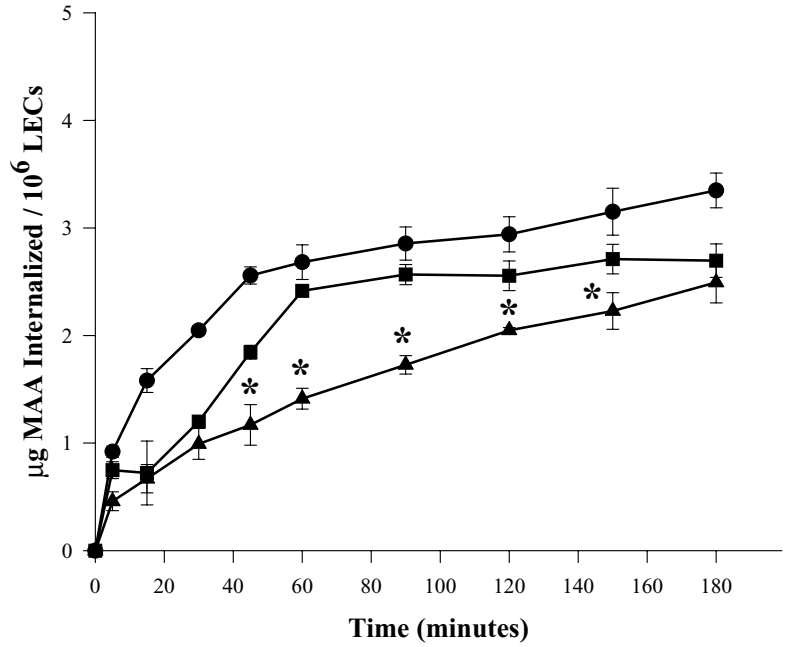
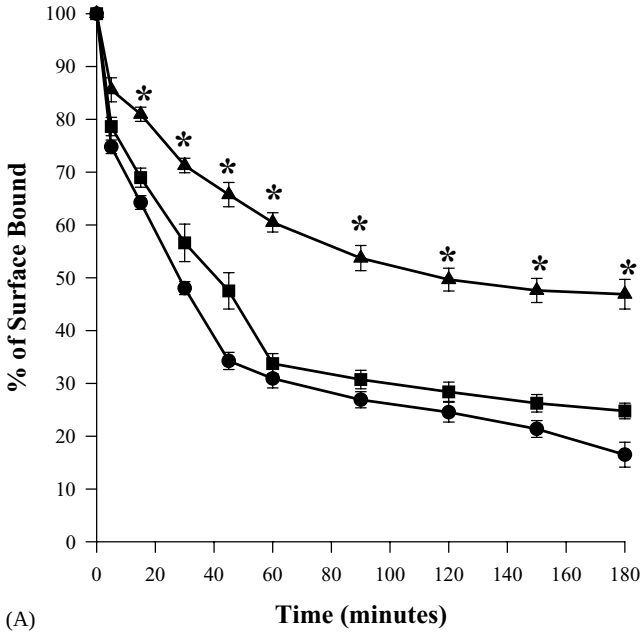


Fig. 5. Kinetics of ^{125}I-MAA-Alb internalization by isolated LECs from chow-fed (■), pair-fed (●), and ethanol-fed (▲) rats. Incubation conditions are identical to those listed in Fig. 3. After the cell suspension was centrifuged, the medium was decanted, and the cells incubated in the presence of ice-cold HBSS (pH 3.0) to remove surface-bound ligand. Acid-precipitable radioactivity remaining in the centrifuged cell pellet was then determined as described in Section 2. The amount of internalized ligand was expressed as the mean ± SD for 6–8 determinations. Values were considered significantly different at $^{*}P < 0.01$.



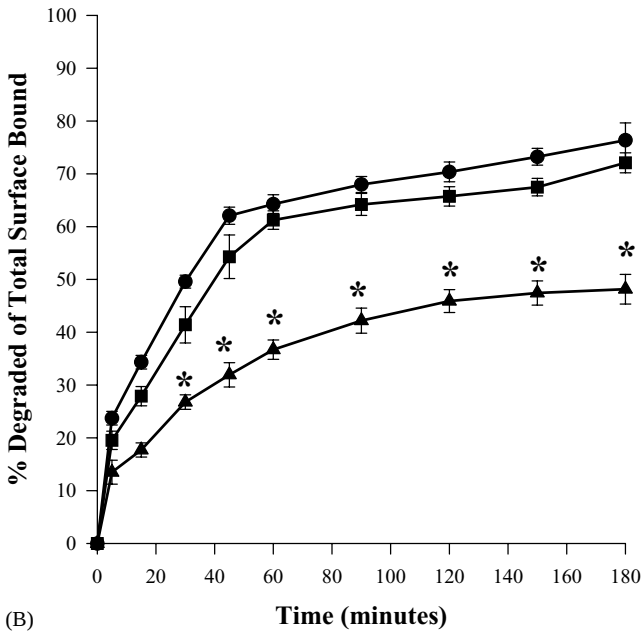


Fig. 6. (A, B) Internalization and degradation of cell surface-bound ^{125}I-MAA-Alb by isolated LECs from chow-fed (■), pair-fed (●), and ethanol-fed (▲) rats. LEC suspensions (1×10^6 cells/mL in HBSS medium) were allowed to bind to ^{125}I-MAA-Alb at 4° as described in Section 2. The degradation rate of cell surface-bound ligand was determined at various time points following treatment with HBSS medium at pH 3.0 (A). Additionally, the rate of degradation of the labeled ligand was determined as described in Section 2 (B). Each point is the mean ± SD of 8 experiments. Both the amount of cell surface-bound ligand (A) and rates of degradation (B) are expressed as percent of the initial amount of surface-bound ligand. Values significantly different from controls are indicated at $^{*}P < 0.05$.

LECs, their physiological relevance in disease processes has been questioned with respect to the extent the protein is modified, and the source of the formaldehyde. In contrast, acetaldehyde adducts that could form following chronic ethanol consumption are physiologically relevant, but do

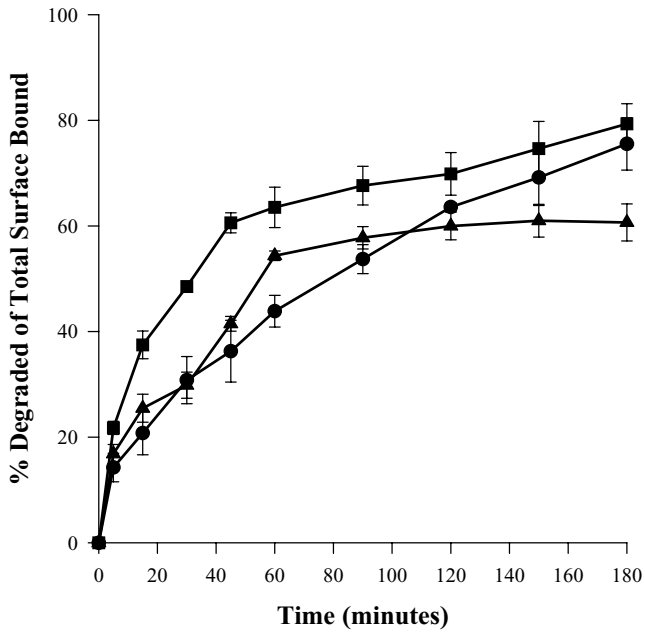


Fig. 7. Degradation of cell-associated ^{125}I-MAA-Alb by LECs isolated from chow-fed (■), pair-fed (●), and ethanol-fed (▲) rats. Experimental conditions were identical to those listed for Fig. 6. Rates of degradation of the internalized ligand were assessed as described in Section 2. Briefly, the rate of degradation is expressed as percent of the maximal amount of internalized ligand for each time point. Each point is the mean ± SD of 8 experiments, and no significant differences ($P < 0.05$) were observed between ethanol and control cells at any of the time points tested.

not result in high levels of degradation (approximately 100 μg or less) depending upon the level of modification [8]. Thus, the significance of impaired RME in LECs has been, to this point, lessened by the lack of a physiological relevant ligand (adduct).

In a report by Tuma *et al.* [5] it was shown that reacting MDA and AA with proteins results in a hybrid aldehyde-modified protein adduct that has been designated MAA. This adduct is interesting as it forms at physiologically relevant levels, results in a very stable and highly modified proteins, and induces immune responses in the absence of adjuvants. The MAA adduct has many interesting characteristics that suggest it may be a good ligand for removal by LECs. Therefore, it was the purpose of these experiments to investigate how MAA-Alb is removed from the liver, and whether chronic alcohol consumption affects this process.

The process of a ligand binding to a specific receptor, internalizing, and then being degraded is commonly known as RME. Step 1 in this process is the specific binding of a modified protein to a receptor located on the surface of the cell. The scavenger receptor (SR) family of receptors is a good candidate for this as they bind a number of chemically modified polyanionic compounds. Additionally, studies by other investigators have indicated this family of receptors are involved in the removal of a number of modified proteins [16,20]. Step 2 in the process is the internalization of the modified protein, and transport

through the cytoplasm in endosomes to the lysosomes. The last step is the degradation of the protein by proteolytic enzymes in the lysosome, followed by transportation of the degraded products out of the cell.

Previous studies by our laboratories have shown that f-Alb and AA-Alb are degraded very efficiently by *in situ* perfused rat livers [5,6,8–12], and that chronic ethanol consumption impairs this process. Also in these studies, it was possible to show that the removal of KCs by gadolinium chloride did not alter the degradation of f-Alb or AA-Alb *in situ*. Additional studies using isolated LECs or KCs, confirmed this observation and strongly suggest that LECs are the principle cell type in the liver capable of degrading these modified aldehydes.

In studies described in this manuscript, isolated LECs from chow-fed, pair-fed, and ethanol-fed rats were shown to bind MAA-Alb through receptor(s) present on the cell surface. Further analysis of the binding of MAA-Alb to LECs, demonstrated a small but significant decrease in the binding of this ligand to LECs from ethanol-fed rats when compared to both control groups. Since the binding was significantly lower, but not enough to account for the 50–60% decrease in degradation that has been reported, it was felt that the internalization and/or degradation of MAA-Alb must also be altered by chronic ethanol consumption. Thus, experiments were performed using a single round of MAA-Alb binding to assess internalization and degradation. Results of these experiments indicated a decrease in the internalization of the surface bound ligand that is consistent with the reported 50–60% decrease in degradation.

When the data were normalized for the amount of MAA-Alb degraded that was actually internalized, results between the three groups became relatively equal. This equal amount of degradation indicated that once the protein was internalized, it was degraded. Thus, these studies demonstrated that ethanol affects RME of MAA-Alb primarily at the post-internalization step rather than during degradation.

In order to assure that the binding and degradation were a result of the MAA modification of the protein; AA-Alb and MDA-Alb were used as negative controls. In these studies, it was shown that proteins modified with these aldehydes at 1 mM concentrations (levels that are used to form the MAA adduct) do not result in significant levels of lysine modification (<1%) and are degraded very poorly (approximately 75 μg) when compared to MAA-modification (35.6%) and degradation (400–500 μg). Thus, these aldehydes individually do not modify proteins very well, and they are not efficiently recognized by the receptors on LECs. More importantly, the combination of these two aldehydes at the same concentration (1 mM), can cause an increase in both the modification of the protein and its subsequent degradation. Since both of these aldehydes have been shown to be present during chronic ethanol consumption, it is reasonable to believe that the MAA adduct may have a physiologically relevant role in aldehydes.

One point that has been difficult to investigate is the specificity of the binding of MAA-modified proteins. Normally, specificity is determined by inhibition with 10× or 100× cold ligand. However, in these studies 100× MAA-Alb caused an alteration in the liver structure, which resulted in the leakage of the perfusate from the liver within a short period of time. This resulted in the loss of ligand in the liver making it impossible to calculate binding and degradation. With respect to isolated LECs, the cell viability decreased in the presence of 100× MAA-Alb. Analysis of supernatants has shown that there was an increase in the level of tumor necrosis factor alpha (TNF-α) into the medium following exposure to MAA-Alb (data not shown), which could explain the cellular damage. Based on this observation, it is enticing to speculate that impaired RME of MAA-Alb could result in the stimulation of LECs to release cytokines/chemokines and other inflammatory products. Studies are currently underway to better assess these observations

In summary, MAA-Alb is a physiologically relevant ligand that forms following chronic ethanol consumption and appears to primarily be bound, internalized, and degraded by LECs. Following chronic ethanol consumption the ability of these cells to degrade the MAA-modified proteins is decreased (50–60%) as a result of a defect in both the binding and internalization processes. Regardless, these alterations in RME may lead to a series of events by which these products would be left in the circulation and could: (1) stimulate LECs to release cytokine/chemokine; and/or, (2) circulate back to lymph nodes and/or the spleen to induce immune responses as has been previously reported. In either case, MAA-modified proteins may induce abnormal responses that could play significant roles in the development and/or progression of alcoholic liver disease.

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