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Metabolism Clinical and Experimental 54 (2005) 1056–1064

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Chronic ethanol feeding controls the activities of various sialidases by regulating their relative synthetic rates in the rat liver

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Received 12 January 2005; accepted 7 March 2005

Abstract

We have determined the concentration effects of feeding for 8 weeks 10.8%, 21.6%, and 36% dietary ethanol calories on the activities and relative synthetic rates (RSRs) of various subcellular sialidases of rat liver. The hepatic RSRs of each species of sialidase was determined based on the ratio of 1-hour incorporation of [^{35}S]-methionine into immunoprecipitable sialidase as percent of the incorporation into total protein in each subcellular fraction. Ganglioside sialidase activities in the hepatic subcellular fractions were also determined. Ethanol feeding at 36% dietary calories caused an increase in the ganglioside sialidase activity of the plasma membrane sialidase (PMS) by 232% ($P < .01$) and that of cytosolic sialidase (CS) by 184% ($P < .05$), but decreased the lysosomal membrane sialidase (LMS) by 54% ($P < .01$) when compared with the control animals. The specificity of each antisialidase antibody was verified by immunoblots. The RSR of PMS was increased by 40% ($P < .01$), 67% ($P < .01$), and 220% ($P < .01$) in the 10.8%, 21.6%, and 36% ethanol groups, respectively. Similarly, the RSR of CS was increased by 17% ($P < .01$), 19% ($P < .01$), and 63% ($P < .01$), respectively, in these ethanol groups. In contrast, the RSR of LMS was inhibited by 36% ($P < .01$), 34% ($P < .01$), and 69% ($P < .01$), respectively, in these ethanol groups. Intralysosomal sialidase failed to hydrolyze gangliosides. Thus, PMS and CS, but not LMS or intralysosomal sialidase, may play important roles in ethanol-modulated desialylation of gangliosides and consequent liver injury and behavioral alterations.

1. Introduction

Sialidases (neuraminidases EC 3.2.1.18) are exoglycosidases that catalyze the removal of terminal sialic acid residues, α-ketosidically linked to monosaccharide or oligosaccharide chains of glycoconjugates. Sialic acid is a prominent constituent of many glycoproteins, which are well known to play important roles in such diverse cellular phenomena as molecular transport, cell adhesion, cell-cell recognition and contact inhibition, antigen masking, proliferation, differentiation, and membrane function [1]. Four types of mammalian sialidases differing in their subcellular location, catalytic activity, chromatographic, and immunologic properties, namely, intralysosomal membrane (LS) [2], cytosolic (CS) [3], and 2 membrane-associated sialidases I (plasma membrane [PMS]) and II [4] (lysosomal membrane [LMS]), have been identified. In recent years, various studies have further characterized this functionally important enzyme and studied its specificity toward a number of potential ganglioside substrates as well as its sensitivity to a variety of natural and artificial substrates. Whereas the LS hydrolyzes sialyllactose at pH 4 to 5, but not submaxillary mucin (bovine) or gangliosides [2], LMSs are active toward submaxillary mucin (bovine) and gangliosides [2]. On the other hand, CS hydrolyzes a wider range of sialo-oligosaccharides, sialoglycoproteins, and gangliosides [3], and even after purification, CS can still hydrolyze intact serum glycoproteins and gangliosides [2]. The CS does not hydrolyze bovine submaxillary mucin [2], and GM1 and GM2 gangliosides [2,3]. However, the rat liver PMS exhibits specificity not only toward GM3 as the substrate, but also toward GM2 and GM1 which have been shown to resist a number of other mammalian sialidases [5]

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0026-0495/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2005.03.009

including the one from rat liver cytosol [2]. Removal of sialic acid moieties is a part of the multitude of events involved in the deglycosylation reactions catalyzed by sialidases and has been confirmed as the initial step of degradation of glycoproteins, glycolipids, and gangliosides. Such deglycosylations have been observed in chronic alcohol abusers and patients with alcohol-related liver diseases [6,7], and thus, the general modulatory role of alcohol in deglycosylations has been of interest to various laboratories [8]. Studies involving ethanol treatment have shown a reduction of the presence of sialoconjugates in various mammalian systems [9,10].

The decrease in sialoconjugates could be due to either impaired sialylation or increased desialylation. With regard to impaired sialylation, studies from our laboratory conclusively showed that the increased levels of carbohydrate-deficient glycoconjugates (either in carbohydrate-deficient transferrin or apolipoprotein J) found in alcoholics [11] and ethanol-treated rat model are due to the decreased Gal-β1,4GlcNAc α-2,6-sialyltransferase [12,13].

Apart from the impaired sialylation, increased desialylation caused by increased sialidase activity could also account for the appearance of sialic acid–deficient glycoconjugates in alcoholic condition [8,14,15]. To understand the possible mechanism underlying the interactions between alcohol and the different sialidases, we have determined the effects of chronic ethanol feeding on the activities and relative synthetic rates (RSRs) of CS, LMS, and PMS in rat liver.

2. Materials and methods

2.1. Chemicals

The protease inhibitors (leupeptine, aprotinine, benzamidine, Tris-HCl), sodium borohydride, bovine serum albumin, glycerol, sucrose, DEAE Sephadex A-25, phenylmethylsulfonyl fluoride, magnesium chloride, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, and potassium phosphate were obtained from Sigma Chemicals, St Louis, Mo. Standardized Pansorbin cells were obtained from Calbiochem, La Jolla, Calif. Monosialoganglioside (G_{M3}) was obtained from Matreya Inc, State College, Pa. Precoated high-performance thin-layer chromatography (HPTLC) plates (Silica Gel 60) were procured from Supelco, Bellefonte, Pa. Cellulose ester Spectra/Por dialysis membrane (MWCO 500) was obtained from Spectrum Laboratories Inc, Rancho Dominguez, Calif. [^{3}H]-sodium borohydride (400 mCi/mmol) and [^{35}S]-methionine (1175 Ci/mmol) were purchased from the American Radiolabeled Chemicals Inc, St Louis, Mo. The Rabbit IgG and Cruz Marker Molecular Weight standards were obtained from Santa Cruz Biotechnology Inc, Santa Cruz, Calif. The 3 rat antisialidases (anti–plasma membrane, anti–lysosomal membrane, and anticytosolic) antibodies with the defined specificities were a generous gift of Prof Taeko Miyagi, Miyagi Prefectural Cancer Center, Miyagi, Japan [4,16]. The cytosolic antibody did not cross-react with the other 2 membrane sialidases. The plasma membrane and lysosomal membrane antibodies also did not cross-react with the cytosolic enzyme. However, there was some cross-reactivity between these 2 membrane sialidase antibodies [4,16]. We have further confirmed these characteristics of these antisialidase antibodies in our laboratory using the Western blot technique. Lieber/Decarli liquid diets were procured from Dyets Inc, Bethlehem, Pa. All other chemicals used were of highest purity commercially available.

2.2. Preparation of ^{3}H-labeled GM3

Monosialoganglioside (G_{M3}) was labeled in the glycosidically bound *N*-acetylneuraminic acid moiety by mild periodate oxidation followed by boro[^{3}H]hydride reduction under conditions maximizing ^{3}H incorporation as described in detail [17]. In brief, 500 μg of G_{M3} was dissolved in 500 μL of 0.1 mol/L sodium acetate buffer in saline (pH 5.5), on ice with continuous stirring. After the addition of 100 μL of 10 mmol/L sodium periodate in water, the vial was recapped and stirred for an additional 10 minutes on ice. To stop the oxidation, 20 μL of glycerol was added to the reaction vial, and the stirring continued for an additional 10 minutes. The total reaction mixture was dialyzed overnight against 3 changes each of 2 L of phosphate-buffered saline (PBS, pH 7.4). The dialyzed mixture was transferred back into a reaction vial, and 40 μL of 0.1 mol/L [^{3}H]-sodium borohydride dissolved in 0.01 mol/L NaOH was added and allowed to stir for 30 minutes. Thereafter, 40 μL of unlabeled 0.1 mol/L $NaBH_4$ in 0.01 mol/L NaOH was added, and the stirring continued for another 30 minutes. The total mixture was again dialyzed, first against 2 changes each of 2 L of 0.1 mol/L acetate buffer (pH 5.5) and then against 2 changes each of 2 L of PBS (pH 7.4). The mixture was further purified by passage through DEAE cellulose spin column (BioRad Laboratories, Hercules, Calif) to collect the ^{3}H-labeled G_{M3} as described [18].

2.3. Animals

Male Wistar rats (Charles River Laboratories, Wilmington, Mass) initially weighing 123 to 132 g were used throughout the study. The rats were housed in plastic cages and had access to food and water ad libitum in a thermostatically controlled room (28°C) under 12-hour dark/light cycle. All protocols dealing with the animals were approved by the institutional animal care and use committee of both The George Washington University and Veterans Affairs Medical Center and followed the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.4. Chronic ethanol feeding

Rats were fed a nutritionally adequate ethanol-containing liquid diet for 8 weeks, essentially formulated according to the modified method of Lieber and DeCarli [19]. Accord-

ingly, 40% of the total energy of the alcohol diet was from fat, 20% from protein, and 4% from carbohydrate. Ethanol administration was maintained at 10.8%, 21.6%, or 36% of total dietary calories in the various groups for 8 weeks. The corresponding isocaloric control diet had equicaloric amounts of dextrin-maltose in place of ethanol. To confirm continuing growth of the rats, the body weights of the animals were measured every week.

2.5. Metabolic labeling in vivo

Metabolic labeling in all the studies was performed as previously described by us with a slight modification [20]. Briefly, at the termination of the ethanol-treatment experiments, all rats were anesthetized by intraperitoneal injection of 50 mg/kg body weight of pentobarbital. Then, each rat was injected with 0.2 mL of saline solution containing 0.2 mCi of [^{35}S]-methionine through the portal vein. After 60 minutes, the liver was perfused with cold 0.25 mol/L sucrose solution containing protease inhibitors (2 μg/mL) to wash out the free labeled methionine and retain the newly synthesized labeled proteins intact. During this procedure, the animal was killed by exsanguination. The liver was carefully dissected out and immediately processed individually for the isolation of lysosomal, plasma membrane, and cytosolic fractions.

2.6. Preparation of the lysosomal, plasma membrane, and cytosolic fractions

The liver subcellular fractions were prepared as described previously [21,22]. All procedures were performed at 0°C to 4°C. Briefly, each liver was placed in cold homogenizing medium (0.25 mol/L sucrose, 10 mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid and 1 mmol/L magnesium chloride) supplemented with protease inhibitors (2 μg/mL) and phenylmethylsulfonyl fluoride (10 μg/mL) cocktail. A portion of the liver (5 g) was minced with scissors into small pieces of not more than 30 μL each and homogenized using a Glas-Col Variable Speed homogenizer (Glas-Col, Terre Haute, Ind). The homogenate was diluted to 1:5 (wt/vol) with homogenizing medium. After centrifugation (500*g* for 10 minutes), the pellet was resuspended in the same volume of homogenizing medium, homogenized, and recentrifuged at 500*g* for 10 minutes. The nuclear pellet was discarded. The postnuclear supernatant was combined with the initial supernatant, mixed, and centrifuged at 6000*g* for 5 minutes. The pellet was resuspended in 20 mL of the homogenizing medium, rehomogenized, and centrifuged at 6000*g* for 5 minutes. The resulting pellet was collected as the lysosomal fraction and stored at −80°C until use. The postlysosomal supernatant fractions were combined and centrifuged (16 000*g* for 5 minutes). The supernatant and the upper part of the pellet were combined and centrifuged again at 16 000*g* for 20 minutes. The fluffy layers were rehomogenized in 5 mL of homogenizing medium, combined with the supernatant, and recentrifuged at 16 000*g* for 20 minutes. The pellet was collected as the plasma membrane fraction and stored at −80°C until use. The 16 000*g* supernatant fraction was subjected to centrifugation at 105 000*g* for 90 minutes to yield the supernatant collected as the cytosolic fraction.

The purity of each isolated subfraction was verified by measuring the activities of the marker enzymes such as acid phosphatase for the lysosomal fraction and alkaline phosphatase for the plasma membrane following the methods of Sommer [23]. Both purity and recovery of each subcellular fraction were found to be consistent with our previous reports [12,24] and those of other investigators [21].

2.7. Assay of sialidase activity

The sialidase activities of the rat liver fractions (lysosomal membrane, plasma membrane, and cytosolic fractions) were determined using the ^{3}H-labeled monosialoganglioside (G_{M3}) as a substrate. The standard assay mixture contained ^{3}H-labeled G_{M3} such that each incubation contained 8.4 × 10^3 disintegrations per minute (dpm) and 60 nmol of G_{M3}; specific activity 1400 dpm/nmol, 6.7 μL of 0.75 mol/L sodium acetate buffer pH 4.8, 2.4 μL of 0.1 mol/L sodium deoxycholate, 2 μL of bovine serum albumin (5 mg/mL), and the enzyme source (50-100 μg protein) in a final volume of 100 μL. The control samples contained identical aliquots of the sample that had been previously heated for 20 minutes in boiling water. After incubation for 2 hours at 37°C, the reaction mixture was cooled on ice and lyophilized. The samples were dissolved in a total of 20 μL of chloroform/methanol (2:1) solution and subjected to HPTLC using chloroform/methanol/water (58:34:8) with 20 mg/dL of $CaCl_2 \cdot 2H_20$ as the solvent system [25]. For quantitation, the HPTLC adsorbent in each lane was cut into 1-cm strips and scrapped into a scintillation vial (Packard Bioscience, Meriden, Conn) containing Ultima Gold high flash-point liquid scintillation cocktail (PerkinElmer Life and Analytical Sciences, Boston, Mass). The fraction corresponding to the liberated [^{3}H] sialic acid was quantified by counting in a Beckman Multipurpose Liquid Scintillation Counter LS 6500 (Beckman Inc, Palo Alto, Calif). In all cases, 1 U of sialidase activity is defined as 1 nmol of sialic acid released per hour per milligram of protein.

2.8. Specificity of each antisialidase serum: Western blot characterization of the respective product

To confirm the specificity of each antisialidase serum provided by Professor Miyagi, each specific liver subcellular fraction was subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis followed by electroblotting to polyvinylidene difluoride membrane and finally analyzed using the corresponding sialidase antiserum by the Western blot technique. In brief, protein samples (14 μg) determined according to the Bradford method [26] were solubilized in 2× Laemmli buffer in the presence of 2-mercaptoethanol and separated on a 4% to 20% Tris-HCl Criterion Precast gel

(BioRad Laboratories). The separated proteins were transferred by electroblotting to an Immun-Blot polyvinylidene difluoride membrane (BioRad Laboratories). Nonspecific binding sites were blocked overnight at 4°C with Tris-buffered saline containing 0.05% Tween 20 (TTBS) and 5% dried skimmed milk. The respective antisialidase serum (1:5000) was diluted in TTBS–5% dried skimmed milk and incubated overnight at 4°C. After washing with TTBS, the membrane was incubated for 1 hour at room temperature with the secondary antibody of rabbit anti-IgG peroxidase-conjugated polyclonal antibody (1:1000 in TTBS–5% dried skimmed milk). After washing the membrane with TTBS and then Tris-buffered saline, the respective sialidase protein bands were visualized by the enhanced chemiluminescence detection technique using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer Life and Analytical Sciences). A Cruz standard marker (Santa Cruz Biotechnology Inc) was used for molecular weight identification of the immunoreactive proteins.

2.9. Measurements of the incorporation rates of [^{35}S]-methionine into newly synthesized sialidases in various subcellular fractions of the livers

Labeled hepatic lysosomal membrane, plasma membrane, and cytosolic fractions prepared as described above were subjected to Pansorbin (Calbiochem) immunoprecipitation essentially as adapted by us [27]. In all the assays, rat anti-LMS, anti-PMS, and anti-CS antibodies courtesy of Prof Taeko Miyagi were used [4,16]. An aliquot (1 mL) of each liver fraction was mixed with an equal volume of radioimmunoprecipitation (RIPA) buffer (150 mmol/L NaCl, 50 mmol/L Tris [pH 8.0], 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) supplemented with protease inhibitors cocktail and incubated on ice for 30 minutes. This fraction was then mixed with the pellet obtained from 100 μL of prewashed Pansorbin cells in cold PBS, pH 7.4, and the resulting suspension was incubated for 2 hours at 4°C with intermittent mixing. After incubation, the suspension was then subjected to centrifugation for 5 minutes at 3000*g*. The supernatant fractions were incubated overnight at 4°C with the various antisialidase antibodies, then for 1 hour on ice with the pellet from 100 μL of Pansorbin cells, and precipitated as described above. The Pansorbin pellet was washed 4 times with 1 mL RIPA buffer followed by centrifugation at 10 000*g* for 1 minute. The supernatants were aspirated, and the pellet was resuspended in 1 mL of super wash buffer (100 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 7.4], 2 mmol/L EDTA, 2% Triton X-100, and 0.5% SDS), transferred into a scintillation vial (Packard Bioscience) containing Ultima Gold high flash-point liquid scintillation cocktail (PerkinElmer Life and Analytical

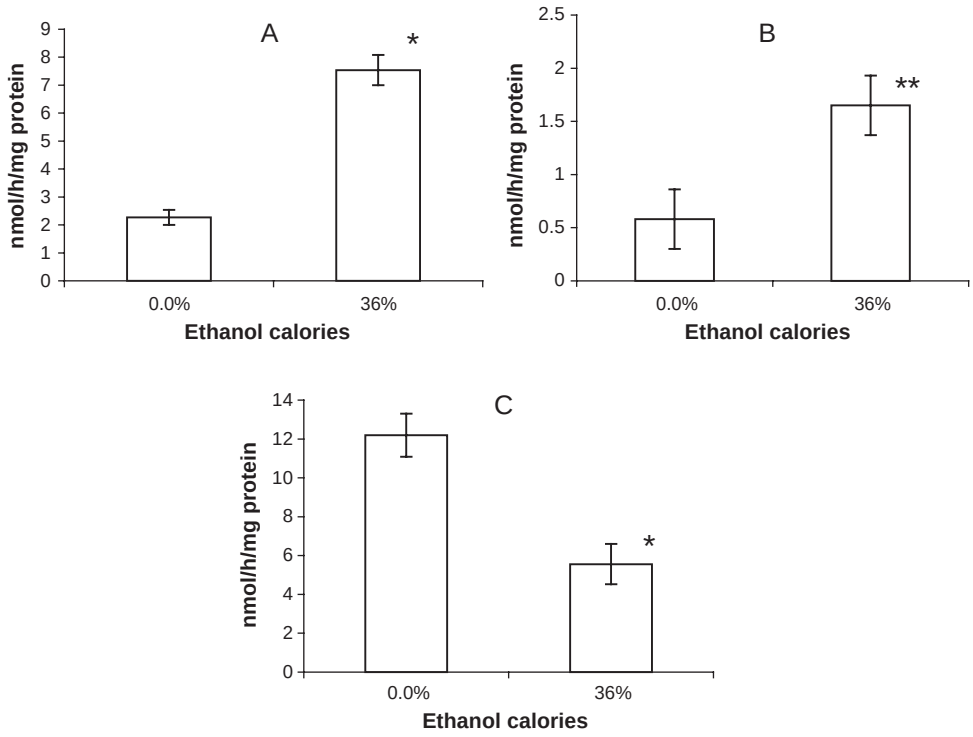

Fig. 1. A-C, Effect of chronic ethanol feeding on the sialidase activity in various liver subcellular compartments. Plasma membrane, cytosol, and lysosomal membrane fractions were isolated from the livers of rats maintained on 36% dietary ethanol calories for 8 weeks, and an aliquot of each fraction was assayed for sialidase activity as described in Materials and methods. Results are expressed as mean ± SE for 4 animals per group. The differences between the ethanol-treated group and control group were analyzed by the 1-way ANOVA test followed by Tukey test. The *P* values of statistical significance in the ethanol groups compared with the corresponding control groups are as follows: (A) plasma membrane fraction **P* < .01; (B) cytosolic fraction ***P* < .05, and (C) lysosomal membrane fraction **P* < .01.

Sciences), and counted in a Beckman Multipurpose Liquid Scintillation Counter LS 6500 (Beckman Inc). The corresponding [^{35}S]-methionine incorporation into the newly synthesized total proteins in an equal volume of each subcellular fraction of the liver from each rat was determined by measuring the radioactivity in the trichloroacetic acid (10% vol/vol) precipitable pellet in each fraction.

2.10. Hepatic RSRs of various sialidases

To normalize the differences in the endogenous pool size of methionine and the amount of tracer administered from one animal to another in all the groups, the incorporation of [^{35}S]-methionine into newly synthesized specific protein is expressed as the incorporation into immunoprecipitable specific sialidase protein as percent of incorporation into total proteins. Thus, the RSRs of each species of sialidase are defined as the [^{35}S]-methionine incorporation into immunoprecipitable sialidase in an aliquot of a specific subcellular fraction as percent of the [^{35}S]-methionine incorporation into trichloroacetic acid precipitable radioactivity in an identical volume of the specific subcellular fraction. This method of expression truly reflects the synthetic rate of a specific protein relative to that of total liver proteins in any given subcellular fraction, and therefore, any observed changes in the RSRs between the groups should be due to the specific alcohol treatment.

2.11. Protein assays

The protein concentrations of the various fractions were determined according to the Bradford method [26] using bovine serum albumin as a standard.

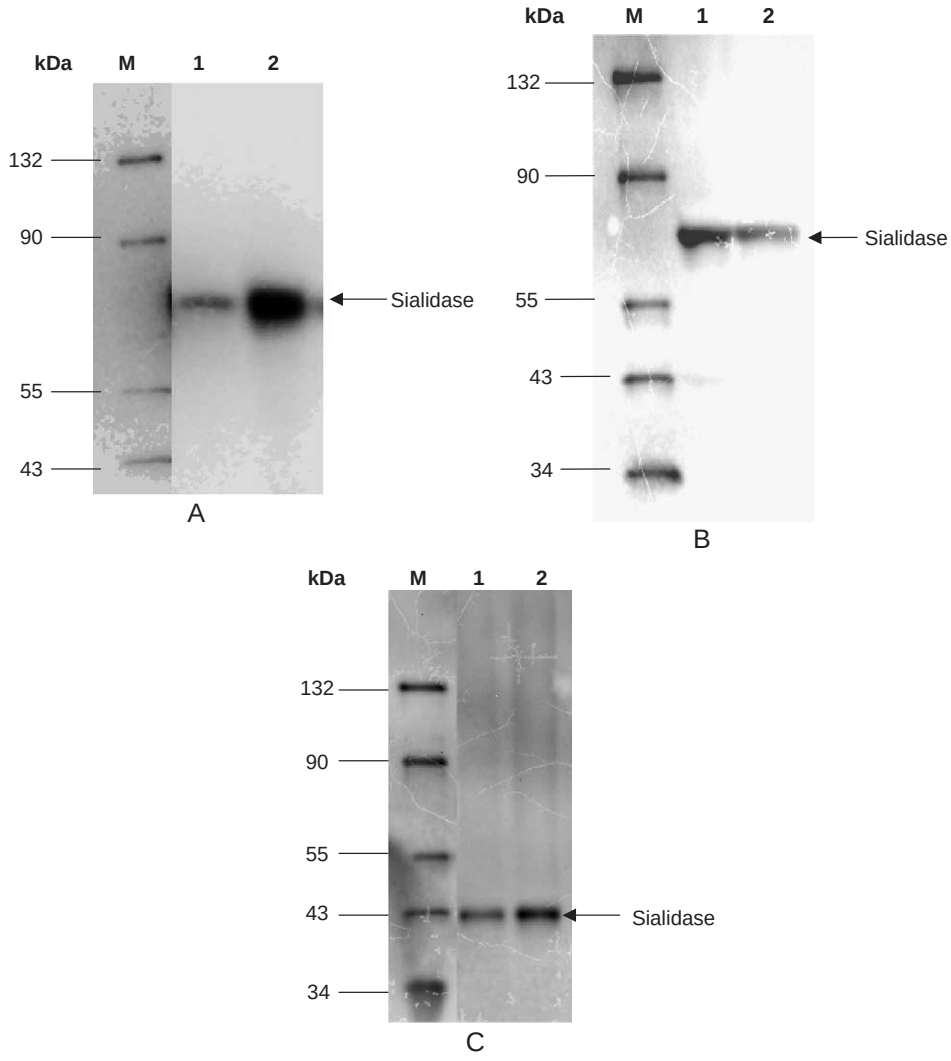

Fig. 2. A-C, Characterization of the specificities anti-PMA (A), anti-LMA (B), and anti-CS (C) sera by Western blot technique. The various liver subcellular fractions (plasma membrane, lysosomal membrane, and cytosol fractions) were prepared from rats maintained on 0% (CN) or 36% dietary ethanol calories (ETOH) for 8 weeks. An aliquot of each fraction was subjected to Western blot analysis with the corresponding antisialidase antibody as described in Materials and methods. Lane M, molecular weight standard; lane 1, extract from CN liver fraction; lane 2, extract from ETOH liver fraction.

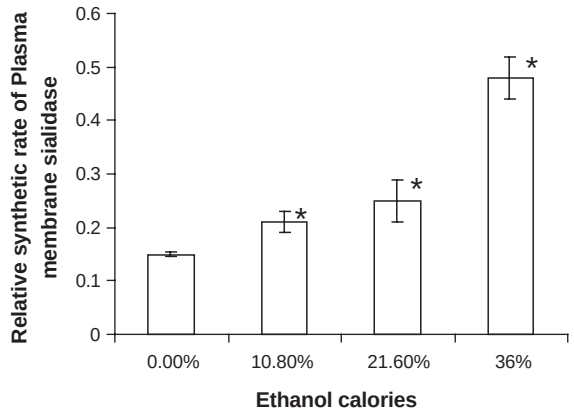

Fig. 3. Effect of chronic ethanol feeding on the RSRs of PMSs. Each plasma membrane fraction from each group was analyzed for RSRs of its sialidase as described in Materials and methods. The results are expressed as mean ± SE for 6 animals per group except in 36% dietary ethanol calories group where the number was 4. The differences between the ethanol-treated groups and control group were analyzed by the 1-way ANOVA test followed by Tukey test. *The value is significantly different from the corresponding value in the control group at $P < .01$.

2.12. Statistical analysis

The data are expressed as mean ± SE. All groups within each data set were compared by 1-way analysis of variance (ANOVA) test followed by the Tukey test.

3. Results

3.1. Effect of chronic ethanol administration on body weight

The growth profiles of all the rats in the study followed the same pattern as reported in our earlier studies [12,28]. In the present analysis, the total body and liver weights of the animals at the end of the study were 385 ± 6 and 12 ± 0.2 g, respectively, in the control group, 395 ± 8 and 12 ± 0.3 g in 10.8% ethanol group, 387 ± 6 and 11 ± 0.2 g in 21.6% ethanol group, and 392 ± 12 and 13 ± 0.4 g in 36% ethanol group. Consequently, there was no statistical significance in the final liver/body weight ratio profiles of the animals among the various experimental groups.

3.2. Effect of ethanol on sialidase activity in the rat plasma membrane, lysosomal membrane, and cytosolic fractions

Using the sensitive radiometric assay with the physiological ganglioside, G_{M3}, as the substrate, our present results show that chronic ethanol administration (36% of total dietary calories) significantly increased the activity of PMS (Fig. 1A) by 232% ($P < .01$) and that of CS by 184% ($P < .05$) as shown in Fig. 1B. In contrast, the activity of the lysosomal sialidase was decreased by 54% ($P < .01$) when compared with the control animals (Fig. 1C). The intralysosomal fraction prepared according to Miyagi and Tsuiki [2] showed no activity toward G_{M3} as a substrate as compared with the intact lysosomal sialidase that hydrolyzed the ganglioside at the rate of 12.19 ± 1.11 nmol h^{-1} mg^{-1} protein.

3.3. Specificity of the antisialidase serum

Miyagi et al [4] have confirmed the specificities of the various polyclonal sialidase antibodies using both the protein A–bacterial adsorbent 1and Protein A–Sepharose 4B column technique. Using the Western blot analysis in our present study, each of the antigen-antibody complex isolated from the respective subcellular fraction showed a consistent single polypeptide band that corresponded well to the previously reported apparent molecular mass of the various rat sialidases as shown in Fig. 2A-C [4,16]. Thus, whereas PMS (Fig. 2A) and LMS (Fig. 2B) showed the presence of a single 70-kDa band, CS (Fig. 2C) showed the presence of a single 43-kDa protein as described [4,16].

3.4. Effect of ethanol on the hepatic RSR of rat PMS, LMS, and CS

To determine whether the observed changes in sialidase activities in the hepatic compartments were due to corresponding alterations in the RSRs of these enzymes, we determined the incorporation of labeled methionine into the newly synthesized sialidases from various subcellular fractions by a highly sensitive immunochemical method. In all our studies, we used the 3 rat antisialidases (anti-PMS, anti-LMS, and anti-CS) antibodies provided by Prof Taeko Miyagi, Miyagi Cancer Center, Japan [4,16]. Using the Western blot technique, we also confirmed that although there was no cross-reaction between CS antibody and the 2 membrane sialidases (LMS and PMS) antibodies, there was some cross-reactivity between the 2 membrane sialidase antibodies [4,16].

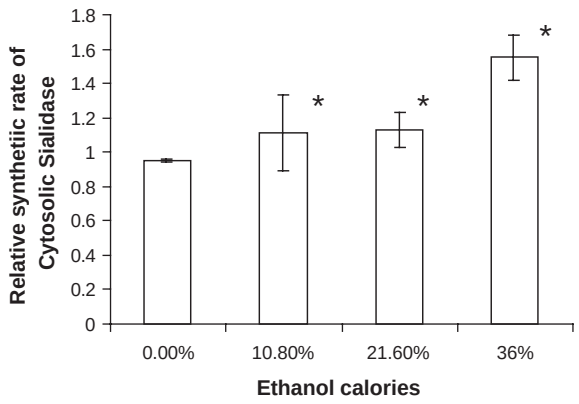

Fig. 4. Effect of chronic ethanol feeding on the RSRs of CS. Each cytosolic fraction from each group was analyzed for RSRs of its sialidase as described in Materials and methods. The results are expressed as mean ± SE for 6 animals per group except in 36% dietary ethanol calories group where the number was 4. The differences between the ethanol-treated groups and control group were analyzed by the 1-way ANOVA test followed by Tukey test. *The values in ethanol group were significantly different from the corresponding values in the control group at $P < .01$.

Because the Western blot analysis of each species of sialidase was done on the specifically isolated intracellular organelle, there should be little contribution attributable to any species other than that characteristic of each isolated fraction. Therefore, the immunoprecipitation data reported in the present study should be valid for each particular species of sialidase. For example, although PMS and LMS antibodies do cross-react, the plasma membrane fraction had very little contamination from lysosomal fraction and vice versa. Thus, the interpretations of our findings are valid.

In the plasma membrane and cytosolic fractions, chronic ethanol administration exhibited a dose-dependent increase in the RSRs of the various sialidases as we increased the ethanol concentration from 10.8% to 36% of total dietary calories. Fig. 3 shows that the RSRs of PMSs was increased by 40% ($P < .01$) on administration of 10.8% ethanol calories, by 67% ($P < .01$) with 21.6% ethanol calories, and by 220% ($P < .01$) with 36% of ethanol calories for 8 weeks when compared with animals on the control diet. Similarly, there was a dose-dependent increase in the RSRs of CS by 17% ($P < .01$), 19% ($P < .01$), and 63% ($P < .01$) in animals that received 10.8%, 21.6%, and 36% of ethanol calories in the diet, respectively (Fig. 4). However, we observed a decreased hepatic RSRs of LMS by 36% ($P < .01$) in 10.8% ethanol calories group, by 34% ($P < .01$) in 21.6% ethanol calories group, and by a marked 69% ($P < .01$) when the animals were maintained on 36% of ethanol calories in diet for 8 weeks as compared with the control group (Fig. 5). Unfortunately, the RSRs of LS could not be measured in the present study because an appropriate antibody for this species of sialidase is not yet available.

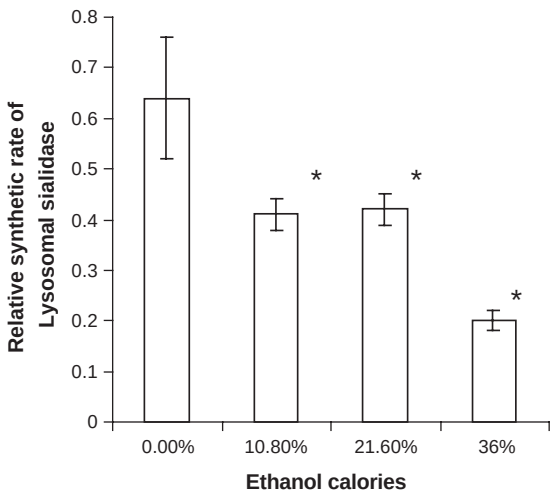

Fig. 5. Effect of chronic ethanol feeding on the RSRs of LMS. Each lysosomal membrane fraction from each group was analyzed for RSRs of its sialidase as described in Materials and methods. The results are expressed as mean ± SE for 6 animals per group except in 36% dietary ethanol calories group where the number was 4. The differences between the ethanol-treated groups and control group were analyzed by the 1-way ANOVA test followed by Tukey test. *The values in ethanol group were significantly different from the corresponding values in the control group at $P < .01$.

4. Discussion

In the present report, we have conclusively demonstrated that chronic ethanol-mediated increased activities of liver PMS and CS (Fig. 1A and B) are due to their corresponding increased hepatic RSRs (Figs. 3 and 4). This clearly supports our earlier observations that chronic ethanol administration increases the activities of sialidases in the liver [12] and all the compartments of the blood including serum, leukocytes, and erythrocytes [28]. It is suggested that the increase in serum sialidase activity can be used as a viable biomarker of alcoholism and alcohol-related pathology [15].

Miyagi and Tsuiki [2] have shown that, in contrast to LMS that hydrolyzes gangliosides, the LS acts only on other oligosaccharide substrates and synthetic substrates such as 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid. We have confirmed this in the present study. Therefore, it is plausible to postulate that the LS has no physiological role in the desialylation of gangliosides based on our present studies. Because we have used the ganglioside, G_{M3}, as the substrate for all the liver subcellular sialidases in the present study, the observed lysosomal sialidase activity must be exclusively due to LMS. Therefore, it is reasonable to conclude that chronic ethanol-induced decreased lysosomal sialidase activity (Fig. 1C) must be due to a concomitant decreased RSR of LMS (Fig. 5). Similarly, the marked increase in PMS and CS in the ethanol-treated group compared with the control group must be due to the corresponding changes in their RSRs. This is again supported by parallel changes in the intensities of the immunoblots of these sialidases in the ethanol group compared with the control group.

The profound increases in the activities of PMS (232%) and CS (184%) on one hand, and the marked decrease of the lysosomal sialidase (54%) caused by chronic ethanol exposure on the other hand, imply the possible significant roles of the former 2 forms of sialidases but not those of LMS in ethanol-modulated desialylation of various sialoconjugates. Evidence exists supporting the significance of plasma membrane–bound sialidase in the catabolism of sialoconjugates [29]. Furthermore, a positive correlation between the activity of PMS and the turnover rate of radiolabeled sialic acid compounds on the cell surface was observed with Rous sarcoma virus–transformed chick embryonic fibroblasts [30].

It is known that most sialic acids are associated with plasma membrane in eukaryotic cells [31]. Thus, it is most likely that the increased presence of asialoconjugates [14,15] and free sialic acid observed after alcohol exposure [32] is promoted by the increased sialidase activity in the plasma membrane, which, in turn, is due to its increased relative biosynthetic rate. The functional significance of alcohol-induced up-regulation of these sialidases may have clinical implications in the appearance of carbohydrate-deficient transferrin and other asialoconjugates commonly observed in human alcoholics.

The physiological and clinical relevance of our present study is exemplified by the fact that alcohol profoundly alters the ganglioside sialidase in the liver. Presumably, ethanol is distorting the orientation of sialoconjugates in the lipid bilayer of gangliosides resulting in greater susceptibility of their sialic acid residues to degradation by endogenous sialidases. This is consistent with the observation that brain synaptosomal free sialic acid is markedly increased in chronic alcohol-fed rats [33]. This finding is strongly supported by the fact that exogenous ganglioside administration has a protective effect against alcohol intoxication and other adverse effects of ethanol [34-40]. Certainly, further studies on these pertinent questions are required to understand the actions of alcohol.

Acknowledgment

This research was supported by a grant from the National Institute on Alcohol Abuse and Alcoholism (no. AA08149).

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