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RESEARCH PAPER

Decreased activity of folate transporters in lipid rafts resulted in reduced hepatic folate uptake in chronic alcoholism in rats

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Abstract Folic acid is an essential nutrient that is required for one-carbon biosynthetic processes and for methylation of biomolecules. Deficiency of this micronutrient leads to disturbances in normal physiology of cell. Chronic alcoholism is well known to be associated with folate deficiency, which is due in part to folate malabsorption. The present study deals with the regulatory mechanisms of folate uptake in liver during chronic alcoholism. Male Wistar rats were fed 1 g/kg body weight/day ethanol (20 % solution) orally for 3 months, and the molecular mechanisms of folate uptake were studied in liver. The characterization of the folate transport system in liver basolateral membrane (BLM) suggested it to be a carrier mediated and acidic pH dependent, with the major involvement of proton coupled folate transporter and folate binding protein in the uptake. The folate transporters were found to be associated with lipid raft microdomain of liver BLM. Moreover, ethanol ingestion decreased the folate transport by altering the *V*max of folate transport process and downregulated the expression of folate transporters in lipid rafts. The decreased transporter levels were associated with reduced protein and mRNA levels of these transporters in liver. The deranged folate uptake together with reduced folate transporter levels in lipid rafts resulted in reduced folate levels in liver and thereby to its reduced levels in serum of ethanol-fed rats. The chronic ethanol ingestion led to decreased folate uptake in liver, which was associated with the decreased number of transporter molecules in the lipid rafts that can be ascribed to the reduced synthesis of these transporters.

Keywords Folate uptake · Liver · Lipid rafts · RFC · PCFT · FBP · Alcohol

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Introduction

Membrane transport of folates and antifolates into cells has been an area of considerable interest because of the important roles that folates play in the biosynthetic processes and antifolates in cancer chemotherapy. Of the different folate derivatives, the physiologically transportable form, 5-methyltetrahydropteroylglutamate (5-CH_3-tetrahydrofolate), is the major one present in the plasma (Wani et al. 2008), urine (Sabharanjak and Mayor 2004), and bile (Birn 2006; Steinberg et al. 1982). Due to exogenous requirement of folate in mammals, there exists a well-developed epithelial folate transport system for the intestinal absorption of folate and in the renal tubular reabsorption (Hamid et al. 2007b; Qiu et al. 2006; Subramanian et al. 2008). The highly sophisticated systems have been developed for the transport of folate. The most well-characterized folate transporters, proton coupled folate transporter (PCFT), and the reduced folate carrier (RFC) mediate folate uptake at pH optima 5.5 and 7.4, respectively, of reduced folates and antifolates and are ubiquitously expressed (Hamid et al. 2007a; Qiu et al. 2006),

hence play a central role in tissue folate homeostasis. In addition to PCFT and RFC, liver and kidney also express high affinity folate transporter folate binding protein (FBP) (Solanky et al. 2010; Wani et al. 2008). After the absorption of folate in intestine, it enters the portal circulation and is taken up by various tissues including liver. Liver is the main storage organ for folates (Corrocher et al. 1985; Ward and Nixon 1990; Hamid et al. 2009b). 5-CH_3-tetrahydrofolate taken up by the hepatocytes is largely secreted in bile and subsequently translocated to the small intestine, where it is reabsorbed (Steinberg 1984; Steinberg et al. 1979). Thus, the enterohepatic cycle of folate is a major factor in the regulation of folate homeostasis and any disturbances in the cycle might lead to folate deficiency. The activities of folate transporters at the liver basolateral membrane play an important role in regulating folate homeostasis. In contrast to the understanding of the molecular mechanisms of folate uptake by absorptive tissues and its excretion by kidneys, not much is currently known about the mechanism and regulation of folate uptake by liver and the interference by ethanol feeding. Moreover, there is not any documentation regarding the presence and involvement of PCFT in folate transport in liver.

Previous studies from our laboratory have shown the association of ethanol feeding with the folate malabsorption in the absorptive tissues such as intestine, colon and kidney and have suggested decreased activity of carrier-mediated folate uptake in these tissues (Hamid and Kaur 2005, 2006; Hamid et al. 2007a; DiBona and Sawin 1982; Wani and Kaur 2011). The reduced uptake in chronic alcoholism was observed to be due to altered kinetic characteristics and downregulation in the expression of folate transporters PCFT and RFC (Hamid and Kaur 2005, 2007a, b; Hamid et al. 2009a; Wani and Kaur 2011; Hamid et al. 2007b). In the current study, we measured the expression of the folate transporters, their distribution in lipid rafts concomitantly with the folate transport kinetics across the liver basolateral membrane in a rat model of chronic alcoholism. This study bears significance in view of the fact that these transporters act as important determinants for the chemotherapeutic potential of various antifolates. The results of the study indicated the existence of a specialized, acidic pH-dependent, carrier-mediated mechanism for folate uptake in liver BLM, with the involvement of PCFT and FBP. The folate transporters were found to be associated with lipid raft microdomains of liver basolateral membrane. There was a decrease in the levels of folate transporters in lipid raft in ethanol-fed rats. The decreased folate transporter levels were accompanied by a decreased protein and mRNA levels in the liver. The deranged folate uptake resulted in reduced folate levels in liver of ethanol-fed rats compared to controls.

Materials and methods

Chemicals

Radiolabelled 5-[^{14}C]-methyltetrahydrofolate, potassium salt with specific activity 24.0 Ci/mmol, was purchased from Amersham Pharmacia Biotech (Hong Kong). Color brust^{TM} electrophoresis marker (M.W.8,000–220,000) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Total RNA Extraction Kit was purchased from Taurus Scientific, Cincinnati, USA. Moloney Murine Leukemia Virus reverse transcriptase (RevertAid^{TM} M-MuLV RT) kit was purchased from the MBI Fermentas, Life Sciences, USA. RNA later (RNA stabilization solution) were obtained from Ambion, Inc. Austin, USA. Enhanced Chemiluminescence detection kit was purchased from biological industries ltd. Kibbutz beit Haemek, Israel. Metal-enhanced DAB substrate kit was purchased from Thermo Fisher Scientific Inc, Rockford, USA. Cryoprotected *L. casei* bacterial strain (MTCC 1423) was purchased from IMTECH, Chandigarh, India.

Animals

Young adult male albino rats (Wistar strain) weighing 100–150 g were obtained from Institute's Central Animal House. The rats were housed in clean wired mesh cages with controlled temperature (23 ± 1 °C) and humidity (45–55 %) having a 12-h dark–light cycle throughout the study. The rats were randomized into two groups of 12 animals each, such that the mean body weights and the range of body weights for each group of animals were similar. The rats in group I were given 1 g ethanol (20 % solution)/kg body weight/day and those in group II received isocaloric amount of sucrose (36 % solution) orally by Ryle's tube daily for 3 months. The rats were fed a commercially available pellet diet (Ashirwad Industries, India) containing 2 mg folic acid per kg diet and water ad libitum. The body weights of rats were recorded twice a week. Overnight fasted rats were sacrificed under anesthesia using sodium pentothal.

The protocol of the study was approved by "Institute Animal Ethics Committee" (40/IAEC/149) Postgraduate Institute of Medical Education and Research, Chandigarh, India, and "Institutional Biosafety Committee" (IBC-2008/173) Postgraduate Institute of Medical Education and Research, Chandigarh, India.

Estimation of folate by microbiological assay

The folate estimations were determined by microtitre plate assay using *Lactobacillus casei* as described earlier (Hamid et al. 2007b; DiBona and Sawin 1982; Wani et al. 2011).

Isolation of liver basolateral membrane vesicles (BLMV)

Liver basolateral membrane vesicles were isolated by the method of Inoue (Inoue et al. 1982), with some modifications. Liver was perfused in situ with 30 mL of ice-cold buffer-1 (0.25 M sucrose containing 10 mM Hepes–KOH buffer, pH 7.4 and 0.2 mM $CaCl_2$). The liver was then excised, minced, and homogenized with 20 strokes in 120 mL of same buffer in a loose-fitting dounce homogenizer at 4 °C. After filtration through cheese cloth, the homogenate was diluted to 320 mL with buffer-2 (0.25 M sucrose containing 10 mM Hepes–KOH buffer, pH 7.4) and EGTA was added to give a final concentration of 1 mM. After centrifugation for 10 min at 1,500*g*, the supernatant fraction was collected and centrifuged again for 10 min at 7,600*g*. The supernatant fraction was centrifuged at 23,000*g* for 30 min. The resulting pellet was resuspended in 4 mL of ice-cold buffer-2 containing 1 mM EGTA. The 6-mL suspension was layered on a discontinuous density gradient consisting of 24 mL of 23.5 % (w/v) sucrose per 4 % ficoll and 10 mL of 20 % (w/v) sucrose per 1 mM EGTA in 10 mM Hepes–Tris buffer (pH 7.4). The gradient was centrifuged for 2 h at 100,000*g*. The white band at the 20–23.5 % interphase was collected and washed with 4 volumes of ice-cold Buffer-2 for 40 min at 65,000*g*. The pellet was resuspended in 5 mL of loading buffer and washed again as described. The final pellet obtained was suspended in loading buffer (280 mM mannitol, 20 mM Hepes–Tris, pH 7.4) to achieve a final protein concentration of approximately 5 mg/mL.

Measurement of the activity of basolateral membrane (BLM) marker enzyme Na^+ K^+ ATPase in isolated purified BLMV and in initial homogenates was performed to determine the relative purity of the final BLMV preparations. The vesicle preparations from both the groups showed enrichment of 12–15-fold with respect to Na^+, K^+-ATPase activity with minimal or no contamination from other membrane markers.

Transport of 5-[^{14}C]-methyltetrahydrofolate

Uptake studies were performed at 37 °C using the incubation buffer of 100 mM NaCl, 80 mM mannitol, 10 mM HEPES, 10 mM 2-morpholinoethanesulfonic acid (MES), pH 5.5 and 0.5 μM of 5-[^{14}C]-methyltetrahydrofolate, unless otherwise mentioned. Ten microliters of isolated liver basolateral membrane vesicles (50 μg protein) from the control and the ethanol-fed rats for different specific assays was added to incubation buffer containing 5-[^{14}C]-methyltetrahydrofolate of known concentration. Reaction was stopped by adding ice-cold stop solution containing 280 mM mannitol, 20 mM Hepes–Tris, pH 7.4, followed by rapid vacuum filtration. Non-specific binding to the filters was determined by residual filter counts after filtration of the incubation buffer and labeled substrate without vesicles, as described earlier (Wani and Kaur 2011; Dev et al. 2010; Hamid et al. 2007b). The radioactivity retained by the filters was determined by liquid scintillation counting (Beckman Coulter LS 6500). For the determination of kinetic constants K_m and V_{max}, transport of 5-[^{14}C]-methyltetrahydrofolate (referred to as folate subsequently) was measured by varying the concentration of 5-[^{14}C]-methyltetrahydrofolate from 0.125 to 8.0 μM in the incubation buffer of pH 5.5.

Binding of 5-[^{14}C]-methyltetrahydrofolate

Ten microliters of BLMV (50 μg protein) was incubated at 4 °C in the 90 μL of binding buffer consisting of 100 mM NaCl, 80 mM mannitol, 10 mM HEPES, and 10 mM MES, at pH 5.5 containing 5-[^{14}C]-methyltetrahydrofolate (0.5 μM). After a specified time, the reaction was stopped by the ice-cold stop solution and bound folate was separated from unbound one by vacuum filtration (Hamid and Kaur 2005, 2007b). The radioactivity remained on the filters was determined by liquid scintillation counting (Beckman Coulter LS 6500).

Isolation of detergent-soluble and detergent-insoluble fractions from rat liver basolateral membrane vesicles

Detergent-soluble (DS) and insoluble (DI) fractions of liver BLM were prepared essentially as described (Federiconi et al. 2011). Briefly, 3 mg of liver BLMVs were centrifuged for 30 min at 100,000*g* at 4 °C and suspended in MES buffer containing 50 mM MES (pH 6.5), 60 mM NaCl, 3 mM EGTA, 5 mM $MgCl_2$, 1 % Triton X-100, and 1× complete protease inhibitor cocktail. Membrane vesicles were then incubated with MES buffer on a rotary shaker for 30 min at 4 °C. At the end of the incubation, BLMVs were centrifuged at 100,000*g* at 4 °C for 30 min, and supernatant was designated as DS fraction. The pellet was resuspended in buffer containing 15 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 1 % Triton X-100, 0.1 % SDS, and 1× complete protease inhibitor cocktail and was designated as DI fraction. Both DS and DI fractions were analyzed by Western blotting using anti-PCFT, anti-RFC, and anti FBP antibodies.

Isolation of lipid rafts by Optiprep density gradient

Lipid rafts were isolated by floatation on Optiprep density gradient (Haupt et al. 1982). Briefly, liver basolateral membrane vesicles were centrifuged at 100,000*g* for 30 min at 4 °C and then resuspended and incubated for

30 min at 4 °C in TNE buffer containing 25 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 1 % Triton X-100 supplemented with 1× complete protease inhibitor cocktail. The membranes were then adjusted to 40 % final concentration of Optiprep (Sigma Aldrich, USA) and layered at the bottom of density gradient with steps of final concentrations of 35, 30, 25, and 20 % of Optiprep in TNE buffer. TNE buffer was laid on the top of the gradient, which was then centrifuged at 215,000*g* for 4 h at 4 °C. Fractions were collected from the top of the gradient and then analyzed by Western blotting. Proteins in the top four fractions are considered to be raft associated (Stickelmeyer et al. 2000). Protein concentrations in each fraction were assessed by using a Bradford kit.

Western blot analysis

For total protein isolation, 100 mg of each liver tissue sample were homogenized in ice-cold RIPA buffer and liver BLMV (100 mg) were resolved on 10 % SDS-PAGE and transferred to PVDF membrane for 20 min at 15 V. Western blotting was performed using the procedure described by Towbin (Li et al. 2004); using polyclonal primary antibodies as rabbit anti-rat RFC (1:800 dilutions) raised against specific region of rat RFC synthetic peptide corresponding to amino acids 494–512 (Said et al. 2000). The polyclonal antibodies against PCFT (1:1,000 dilutions) were raised against specific region of rat PCFT synthetic peptide corresponding to amino acids 442–459. The primary antibodies against FBP or FR-α were purchased from Santa Cruz Biotechnology (Delaware Avenue Santa Cruz, CA, USA). Secondary antibodies used were goat anti-rabbit IgG-HRP-labeled (1:20,000 dilutions). The bands were visualized by either metal enhanced DAB substrate kit or enhanced Chemiluminescence detection (Morin et al. 2003) kit according to the manufacturer's instructions. The quantification of blots was carried out by using "Scion image".

Reverse transcriptase (RT)-PCR analysis

Total RNA was isolated from the liver by using total RNA extraction kit, and cDNA synthesis was carried out from the purified and intact total RNA according to manufacturer's instructions. Expression of *RFC*, *PCFT, FBP,* and *GAPDH* was evaluated using sequence specific primers corresponding to the sequence in the open reading frame. PCR mixture (20 µL) was prepared in 1× PCR buffer consisting of 0.6 U of Taq polymerase, 2 µM of each primer for *GAPDH*, *PCFT, FBP* and *RFC* along with 200 µM of each dNTP. In optimized PCR, the initial denaturation step was carried out for 2 min at 95 °C. The denaturation, annealing, and elongation steps were carried out respectively for 1 min at 94 °C, 45 s at 64 °C (*PCFT*) or 56 °C (*GAPDH*) or 62.5 °C (*FBP*) and 1 min at 72 °C for 35 cycles. In case of *RFC* denaturation, annealing and elongation steps were carried out respectively for 30 s at 94 °C, 30 s at 52.1 °C, 30 s at 72 °C for 35 cycles. The final extension step was carried out for 10 min at 72 °C. The primers were designed using Primer3 Input (version 0.4.0). The sequences of the primers used were as follows: 5′-CATGCTAAGC GAACTGGTGA-3′(sense) and 5′-TTT CCACAGGACATGGACA-3′ (antisense) for RFC, 5′-AA GCCAGTTATGGGCAACAC-3′ (sense) and 5′-GGATAG GCTGTGGTCAAGGA-3′ (antisense) for *PCFT*, ATGAG TGTTCCCCGAACTTG (sense) and GCATAGAACCTC GCCACTTC (antisense) for *FBP* and 5′-CCTTCATTG ACCTCAACTACAT-3′ (sense) and 5′-CCAAAGTTGTC ATGGATGACC-3′ (antisense) for *GAPDH*. The expected PCR products of size 120, 300, 370, and 400 bp were obtained for *RFC*, *PCFT, FBP,* and *GAPDH,* respectively, when electrophoresed on 1.2 % agarose gel. The densitometric analyses of products were determined by using "Scion image" software.

Statistical analysis

Each uptake assay was performed thrice with 4 independent preparations from each group. Statistical analysis was performed with Graphpad Prism software (Avenida de la Playa La Jolla, USA). The data were computed as mean ± SD. Group means were compared by using Student's *t* test and ANOVA. Kinetic parameters of the saturable folate uptake process (i.e., maximal velocity (*V*max) and the apparent K_m) were calculated by performing non-linear least square (NLLSQ) fitting using Graphpad Prism software (Avenida de la Playa La Jolla, USA). The acceptable level of significance was $p < 0.05$ for each analysis.

Results and discussion

Folate levels

After absorption of folate in the intestine, it is transported to the liver, where methylated folates are rapidly and excreted into bile, whereas non-methylated folates are either methylated and transported into bile or incorporated into a hepatic polyglutamated pool of folate (Wani et al. 2008). Since the present study dealt with the mechanistic insights of folate malabsorption during alcoholism, so it was important to determine the folate levels in liver and serum. The total folate levels were significantly decrease by 21 % ($p < 0.01$) in the liver and (33 %, $p < 0.001$) in serum of ethanol-fed rats as compared to respective

controls (Fig. 1a, b), suggesting the association of chronic ethanol ingestion with reduced folate levels in liver, the main storage organ for folates (Corrocher et al. 1985; Ward and Nixon 1990; Hamid et al. 2009b). This decreased folate levels might led to its decreased secretion in bile and subsequent translocation to small intestine, where it is reabsorbed (Steinberg 1984; Steinberg et al. 1979) thereby might contribute to reduced folate levels in serum and

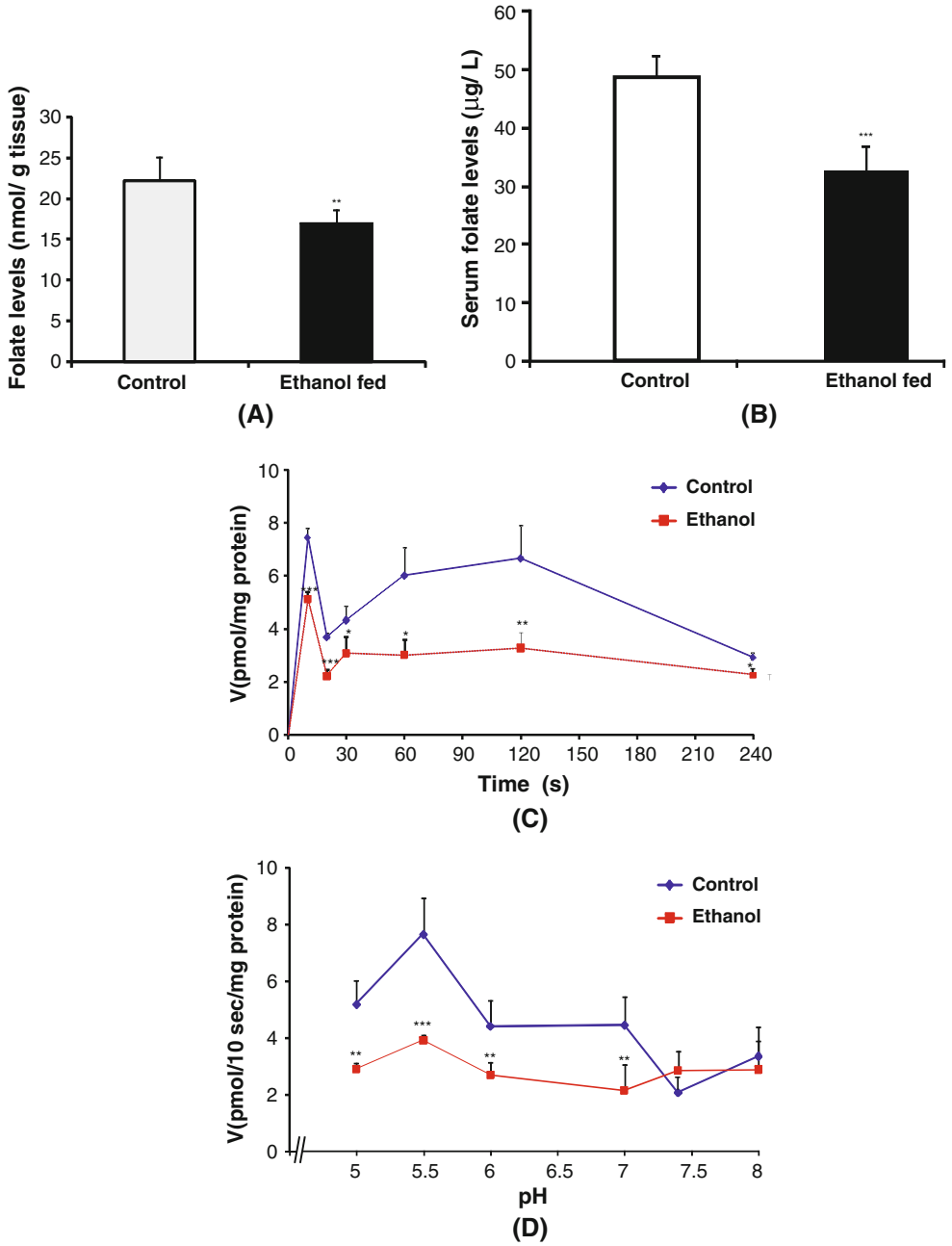

Fig. 1 Folate levels and folate uptake as a function of time and pH. **a** Folate levels in liver of control and ethanol-fed rats. **b** Folate levels in serum of control and ethanol-fed rats estimated by microtitre plate assay using *L. casei*. **c** Time course of folate uptake in the liver basolateral membrane vesicles (BLMV). BLMV were incubated at 37 °C in buffer (100 mM NaCl, 80 mM mannitol, 10 mM HEPES, 10 mM 2-morpholinoethanesulfonic acid (MES) pH 5.5) for different time intervals. 5-[^{14}C]-Methyl-THF (0.5 µM) was added to the incubation medium at the start of uptake. Each point represents the mean ± SD of four determinations. **d** Uptake of 5-[^{14}C]-Methyl-THF in the rat liver BLMV as a function of pH optimum. Uptake was measured by varying the pH of incubation buffer [100 mM NaCl, 80 mM mannitol, 10 mM HEPES, 10 mM 2-morpholinoethanesulfonic acid (MES)] from 5.0 to 8.0, keeping intravesicular pH 7.4 at 0.5 µM substrate concentration for 10 s. Each data point is mean ± SD of 4 separate uptake determinations. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control

supply to other tissues. Thus, the disturbance in enterohepatic cycle of folate by ethanol develops the clinical symptoms of folate deficiency.

Kinetic characterization of folate transport across the liver basolateral membrane (BLM)

Uptake of 5-methyltetrahydrofolate when studied as a function of time revealed transport maxima at 10 s in both the groups (Fig. 1b). However, the uptake was 21–50 % ($p < 0.05$, $p < 0.01$, $p < 0.001$) less in ethanol-fed group studied at different time points from 10 to 240 s. In order to determine the driving force for folate transport across the liver basolateral membrane, pH of incubation buffer was varied from 5 to 8, keeping the intravascular pH constant at 7.4. Figure 1c illustrates the results on the effect of a pH gradient, imposed across the vesicle membrane, on the uptake of 5-methyltetrahydrofolate into rat liver BLMVs. The results demonstrated that the uptake increased when the pH was decreased from 7.0 to 5.5 and suddenly decreased at pH 5.0, with maximum uptake at pH 5.5 in both the groups and demonstrated that folate is transported across the liver BLM via an H^+ gradient-dependent carrier-mediated mechanism. This argument is supported by the fact that oxidation of NADH at plasma membrane (Hamid and Kaur 2009) and/or Na^+–H^+ exchange (Salcedo-Sora et al. 2011) at liver BLM generating a "localized" proton gradient that could energize active transport of 5-methyltetrahydrofolate. Similar folate transport characteristics have been observed in a variety of human and rat tissues and cell lines, which were previously thought to be mediated by RFC, the differences between the pH optimum for RFC-mediated transport and substrate specificities in these tissues versus cell lines of hematopoietic origin were unexplained till the discovery of PCFT (Kumar et al. 1998). However, at different pH, points studied the uptake was 38–48 % ($p < 0.01$, $p < 0.001$) less at pH of 7.0–4.5 and the change was not significant at pH 7.4 and 8.0 in the ethanol-fed rats as compared to the control. To determine whether folate uptake at the liver BLM is a carrier-mediated process, kinetic studies were performed in the presence of increasing concentrations of the substrate from 0.125 to 8.0 μM. The uptake at pH 5.5 showed saturation phenomena with plateau at 1.5 μM of substrate concentration indicative of Michaelis-Menten kinetics (Fig. 2a). At different concentrations of the folate, the uptake was found to be 20–54 % less in the ethanol-fed group. The apparent K_m values were 1.07 ± 0.09 and 1.10 ± 0.12 μM in the control and the chronic ethanol-fed groups, respectively, as calculated by non-linear least square analysis. The observed value of K_m, that is, 1.07 μM is in agreement with the earlier studies (Horne et al. 1992a, b). In addition, the V_{max} values were 25.25 ± 2.3 and 14.49 ± 3.6 pmol/10 s/mg

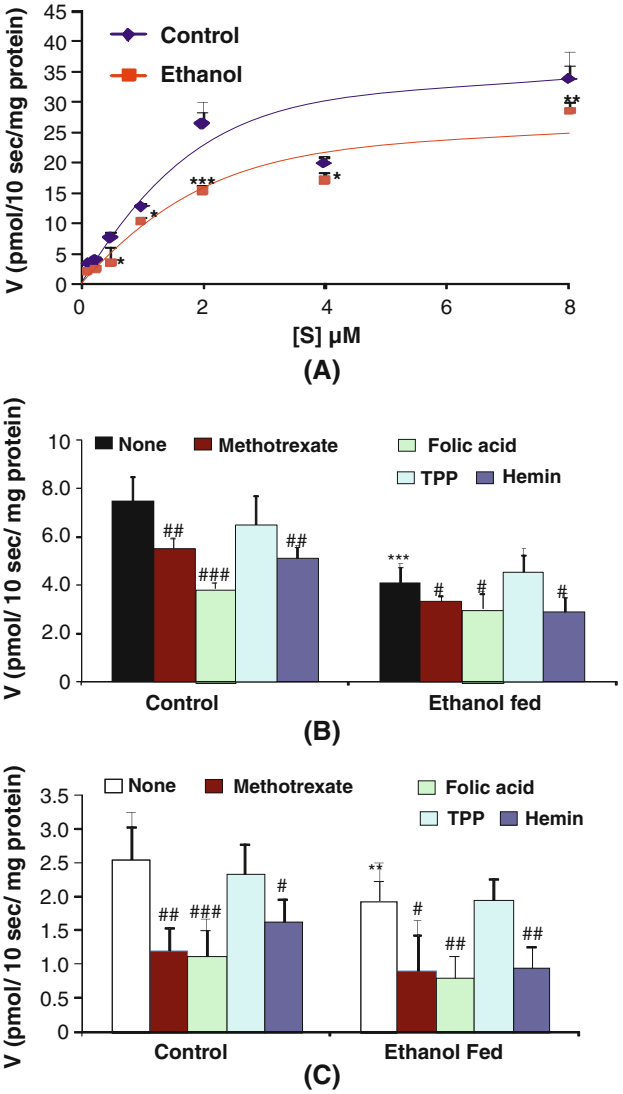

Fig. 2 Uptake of 5-[^{14}C]-Methyl-THF in the liver BLMV as a function of substrate concentration, structural analogs, and binding of 5-[^{14}C]-Methyl-THF to liver BLM. **a** Uptake was measured by varying 5-[^{14}C]-Methyl-THF concentration from 0.125 to 8.0 μM in the incubation medium (100 mM NaCl, 80 mM mannitol, 10 mM HEPES, 10 mM 2-morpholinoethanesulfonic acid (MES), pH 5.5) after incubating liver BLMV for 10 s. **b** Effect of structural analog and inhibitors on the uptake of 5-[^{14}C]-Methyl-THF in the liver BLMV. Uptake of 5-[^{14}C]-Methyl-THF (0.5 μM) was measured with and without analogs (5 μM folic acid and 5 μM methotrexate)/inhibitor (5 mM thymine pyrophosphate (TPP) and 25 μM hemin) in incubation buffer of pH 5.5. Bars are mean ± SD of 4 separate uptake determinations. **c** Binding of 5-[^{14}C]-Methyl-THF to liver BLM and the contribution of transporters to folate binding. Binding of 5-[^{14}C]-Methyl-THF (0.5 μM) was determined by incubating liver BLMV at 4 °C in the binding buffer (100 mM NaCl, 80 mM mannitol, 10 mM HEPES, 10 mM MES, pH 5.5) for 10 s, and the binding of 5-[^{14}C]-Methyl-THF (0.5 μM) to liver BLM was measured in presence and absence of structural analogs (5 μM folic acid and 5 μM methotrexate)/inhibitor (5 mM thymine pyrophosphate (TPP) AND 25 μM hemin) in incubation buffer of pH 5.5. Each data point is mean ± SD of 4 separate binding determinations. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control and $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus none

protein in the control and the ethanol-fed groups, respectively ($p < 0.05$), suggesting decrease in the number of carrier molecules on the liver BLM without any change in affinity of transporter(s) for folate was responsible for decreased activity of folate transporters at membrane surfaces during chronic alcoholism.

Further, in order to determine the specificity of the folate transporters in the liver BLM (Fig. 2b), inhibition of 5-methyltetrahydrofolate transport by the structural analogs and inhibitors was examined. The structural analogs methotrexate and folic acid decreased the uptake by 25 % ($p < 0.01$) and 48 % ($p < 0.001$) in the control and 18 % ($p < 0.05$) and 24 % ($p < 0.05$) in the ethanol-fed rats, respectively. Investigating the contribution of the PCFT and RFC in the transport of 5-methyltetrahydrofolate across the liver BLM, the transport was measured in presence of inhibitors, thiamine pyrophosphate (inhibitor of RFC) and hemin (inhibitor of PCFT). The inhibitor hemin decreased the uptake by 31 % ($p < 0.01$) in the control and 27 % ($p < 0.05$) in the ethanol-fed rats while the inhibitor thiamine pyrophosphate did not change the uptake significantly, suggesting the major involvement of PCFT while minimal involvement of RFC in the folate uptake across liver BLM. This again supported the above results that showed maximum uptake at acidic pH, characteristics of folate uptake by the PCFT, as observed in case of intestinal BBM (Hamid et al. 2007b; Dev et al. 2010) and colon apical membrane (Wani and Kaur 2011; Dudeja et al. 1997).

Binding of folate to the liver basolateral membrane

We measured the binding of folate in the membrane preparations. Upon determining the binding component of the total folate transport in both the groups (Fig. 2c), it was found that the binding contributes to 33 and 38 % of total transport in the control and the ethanol-fed group, respectively, and there was a significant decrease (24 %) in binding of 5-methyltetrahydrofolate to the liver BLM in the ethanol-fed group as compared to the control group ($p < 0.01$), thereby contributing to the observed folate malabsorption across the liver BLM. The structural analogs (Fig. 2c), methotrexate and folic acid, decreased the binding by 52 % ($p < 0.01$) and 56 % ($p < 0.001$) in the control and 52 % ($p < 0.05$) and 58 % ($p < 0.01$) in the ethanol-fed rats, respectively, and the inhibitor hemin decreased the binding by 36 % ($p < 0.05$) in the control and 50 % ($p < 0.01$) in the ethanol-fed rats. However, there was not any significant change in the binding of folate in the presence of thiamine pyrophosphate in both the groups.

These results demonstrated that folate binding to the liver basolateral membrane was reduced in chronic alcoholism and there was major involvement of the PCFT mediated binding of folate to the liver BLM. The high affinity of folate transporter FBP also contributes to binding and transport across liver BLM as its role has already been established (Abdel Nour et al. 2007; Gates et al. 1996; Mendelsohn et al. 1996).

Distribution of folate transporters in lipid microdomains in the liver

Lipid rafts (LRs) are the specialized microdomains of the plasma membrane that are essential for the normal functioning of various membrane transporters (Anderson and Jacobson 2002). Proteins might enter LR at the Golgi level and their shuttling between the Golgi and cell membranes allows cell to exert regulatory control over the surface expression of their proteins. To find out whether that the folate transporters (PCFT and RFC) are associated with lipid microdomains of the liver BLM of rat, partitioning of the PCFT, FBP, and RFC between detergent-soluble (DS) and detergent-insoluble (DI) fractions of rat liver BLM was performed, which revealed the presence of these transporters in the DI fractions of the liver BLM (Fig. 3a).

We examined the distribution of the PCFT and the RFC of rat liver basolateral membrane on Optiprep density gradient. We have validated this technique earlier by measuring the specific activity of alkaline phosphatase (well-known marker for lipid rafts) in all the fractions collected from gradient using colon apical membranes (Wani and Kaur 2011). The pattern of specific activity of alkaline phosphatase in all these fractions revealed a gradient with considerable activity in the top floating fractions (1–4), indicating these fractions contained lipid rafts. Besides our studies, one of the study carried out in rat liver hepatocytes revealed similar graduation of proteins in sucrose density gradient with the lipid rafts floating and concentrating in low sucrose density and raft clustering in ethanol-treated hepatocytes without any change in distribution of lipid raft protein flotillin (Nourissat et al. 2008). So, the fractions isolated from the gradient using the liver BLM were subjected to western blotting for the PCFT and the RFC expression (Fig. 3b–e). The distribution of the PCFT and the RFC of rat liver basolateral membrane on Optiprep density gradient revealed the presence of the PCFT and the RFC protein in the top 5 fractions (20–30 %) with negligible or no expression thereafter in the Optiprep density gradient (Fig. 3b–e). However, our experiments using antibodies against FBP in these fractions were not successful, which might be explained by the fact that phosphatidyl Inositol–Specific phospholipase C (PI–PLC) gets translocated to the raft fraction during ethanol treatment in rat hepatocytes there by might cleaves GPI anchor of FBP protein leading to their release in extracellular

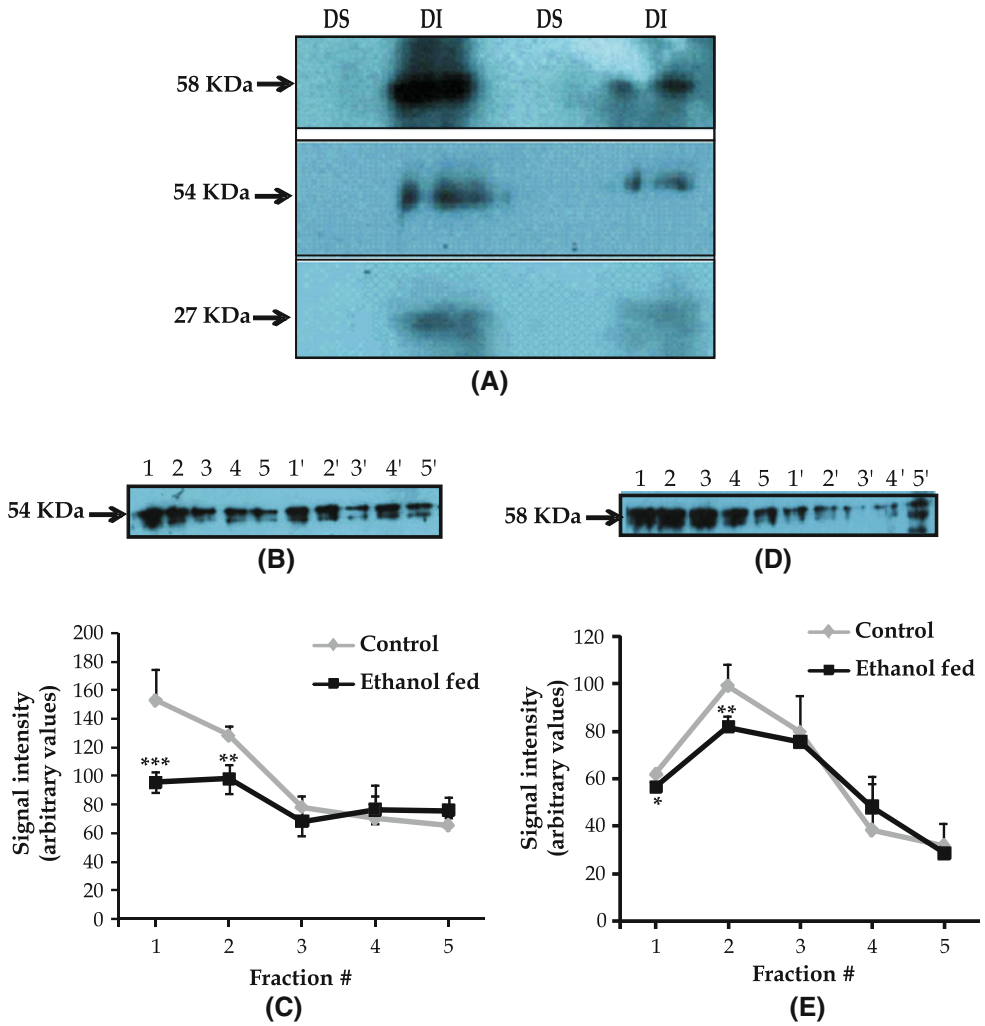

Fig. 3 Association of folate transporters (PCFT, FBP, and RFC) proteins with lipid rafts in liver BLM. **a** Association of PCFT (54 kDa), FBP (27 kDa), and RFC (58 kDa) with DI fractions of liver BLM. Liver BLMVs were centrifuged for 30 min at 100,000*g* at 4 °C and suspended in MES buffer containing 50 mM MES (pH 6.5), 60 mM NaCl, 3 mM EGTA, 5 mM $MgCl_2$, 1 % Triton X-100, and 1× complete protease inhibitor cocktail. Membrane vesicles were then incubated with MES buffer on a rotary shaker for 30 min at 4 °C. At the end of the incubation, BLMVs were centrifuged at 100,000*g* at 4 °C for 30 min, and supernatant was designated as DS fraction. The pellet was resuspended in buffer containing 15 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 1 % Triton X-100, 0.1 % SDS, and 1× complete protease inhibitor cocktail and was designated as DI fraction. Proteins (50 μg) from DI and DS fractions were separated by electrophoresis on 10 % polyacrylamide gel and then analyzed by Western blotting for PCFT, FBP, and RFC expression. **b**, **d** The liver BLM was subjected to floatation on Optiprep density gradients, and fractions were collected from top of the gradients (fractions 1–4 represent detergent-resistant membrane). Fractions were separated by electrophoresis and analyzed by Western blotting using **a** anti-PCFT (54 kDa) and **b** RFC (58 kDa) antibodies. **c**, **e** Blots were scanned, and the intensity of bands was determined by densitometric analysis. Data are mean ± SD of 4 separate experiments. The represented blots shown for PCFT and RFC expression as, *lane* 1–5: Control; *lane* 1′–5′: ethanol fed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control

medium (Nourissat et al. 2008). The data thus obtained provide strong evidence that the majority of the PCFT and the RFC pool were associated with the DI lipid raft microdomains. Moreover, ethanol ingestion significantly decreased the lipid rafts associated transporters on liver BLM as compared to the control (Fig. 3b–e). The extent of decrease was more for the PCFT 25–40 % ($p < 0.01$, $p < 0.001$) and 10–20 % for the RFC ($p < 0.05$, $p < 0.01$), respectively. Our findings, demonstrated for the first time that the presence of PCFT and RFC in LR of the liver BLM of rats in agreement to our earlier studies in the colon BLM (Wani and Kaur 2011), suggest the alteration of the lipid composition of liver basolateral membrane by chronic alcoholism (Puddey et al. 1995; Gutierrez-Ruiz et al. 1995; Kaur 2002), which might result in disruption of LR in chronic alcoholism.

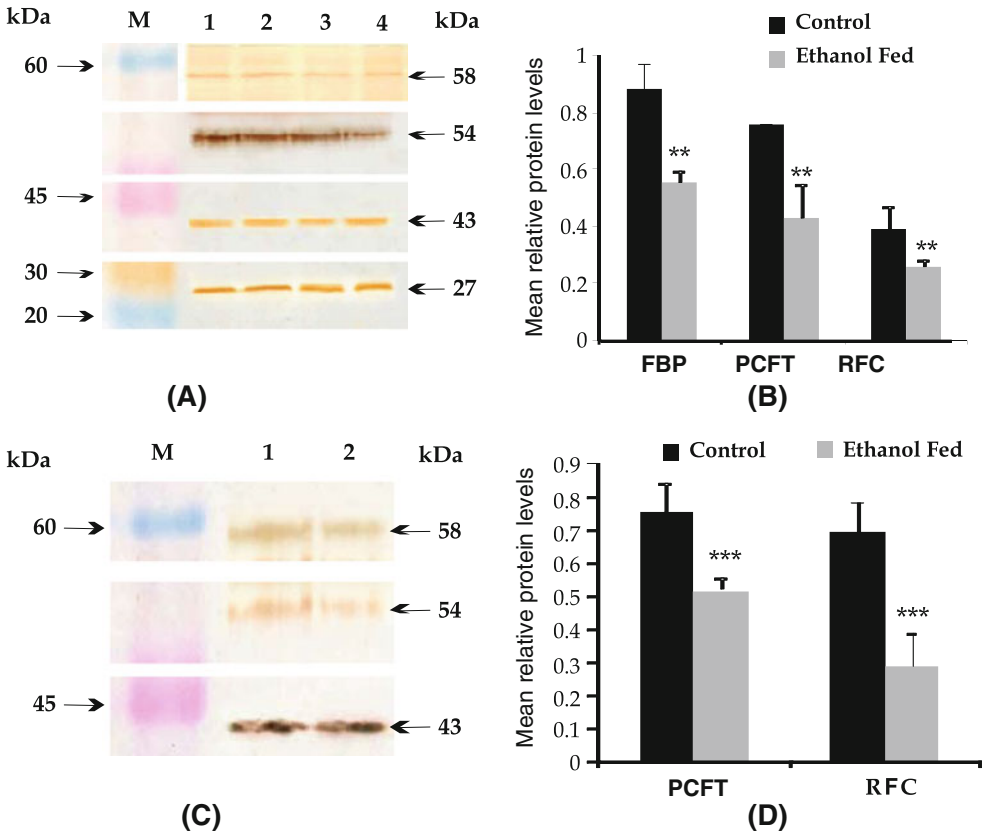

Fig. 4 Western blot analysis of folate transporters in liver. Western blots analysis of liver tissue lysate (**a**) using anti-RFC (58 kDa), anti-PCFT (54 kDa), and anti FBP (27 kDa) antibodies with β-actin (43 kDa) as an internal control (**b**) *Graph* represents summary data of densitometric analysis, *lane* M- marker, *lane* 1, 2: control; 3, 4: ethanol fed. Western blot analysis of the liver BLM (**c**) using anti-RFC (58 kDa), anti-PCFT (54 kDa) antibodies. **d** *Graph* represents summary data of densitometric analysis. Data are expressed are mean ± SD of four separate experiments. *Lanes*: 1, control; 2, ethanol fed. $^{**}p < 0.01$; $^{***}p < 0.001$ versus control

Expression of the PCFT, FBP, and RFC protein in the liver

The finding that the ethanol ingestion resulted in significant reduced levels of the PCFT and the RFC proteins in lipid rafts at the liver BLM led us to study whether this decrease in association of protein levels in lipid rafts was due to the decreased protein synthesis. The effect of chronic alcoholism on the level of expression of the PCFT, FBP, and RFC protein in the liver tissue and in basolateral membrane vesicles isolated from liver was analyzed by western blotting. The results showed a significant decrease in the level of expression of the PCFT ($p < 0.001$), FBP ($p < 0.01$), and the RFC ($p < 0.001$) proteins in the liver tissues of the ethanol-fed rats (Fig. 4a, b). The extent of decrease was 2.3-fold (57 %) for RFC, 1.7-fold (36 %) for FBP, and 1.6-fold (31 %) for the PCFT in ethanol-fed rats as compared to the respective controls. Parallel to the observed decrease in protein expression in tissue a significant decrease in the level of expression of the PCFT ($p < 0.001$) and the RFC ($p < 0.001$) proteins was found in the liver basolateral membranes of the ethanol-fed rats (Fig. 4c, d). In this context, the observed downregulation in the *PCFT*, *FBP*, and the *RFC* protein levels in lipid rafts corresponds with the reduced protein levels.

Expression of mRNA corresponding to the *RFC*, *PCFT*, and *FBP* in the liver

We studied the mRNA expression of all these transporters in order to have mechanistic insights of decreased levels of folate transporters in the liver during chronic alcoholism. RT-PCR analysis was performed using sequence specific primers in the coding regions of *PCFT*, *RFC*, and *FBP* genes in order to obtain the fragments of expected sizes, that is, 300, 120, and 370 bp, respectively, for the RFC, *PCFT*, and *FBP* during the amplification reaction using *GAPDH* (400 bp) as an internal control. The mRNA expression levels of *PCFT*, *RFC*, and *FBP* were significantly lower in the ethanol-fed group (Fig. 5a, b). The expression levels of *PCFT*, *RFC*, and *FBP* is decreased by 1.9-fold (47 %), 1.5-fold (30 %), and 2.3-fold (56 %), respectively, ($p < 0.001$) in the ethanol-fed group (Fig. 5a, b). Thus, the observed downregulation in the *PCFT*, *FBP*, and the *RFC* protein levels corresponds with the reduced mRNA expression, suggesting some transcriptional or posttranscriptional regulatory mechanism

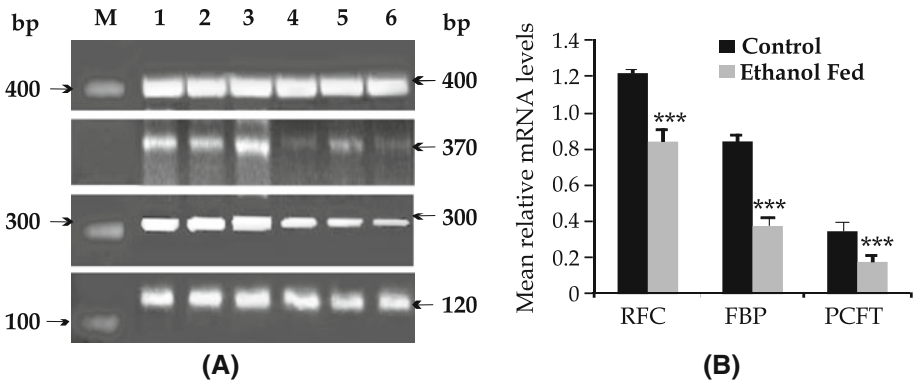

Fig. 5 RT-PCR analysis of *RFC* (120 bp), *FBP* (370 bp), and *PCFT* (300 bp) with *GAPDH* (400 bp) as an internal control in liver. **a** Resolved on 1.2 % agarose gel electrophoresis and **b** densitometric analysis representing relative change in PCFT, FBP, and RFC mRNA expression. Data shown are representative of 5 separate sets of experiments. *Lane* M: ladder; *Lanes* 1–3: control; 4–6: ethanol fed. ***$p < 0.001$ versus control

responsible for reduced folate transporter levels in liver of ethanol-fed rats.

Conclusion

In conclusion, the folate uptake across liver basolateral membrane was carrier mediated and occurs maximally at acidic pH range. There was a minimal contribution of the RFC in the transport of folate across the liver BLM. The majority of folate transport might occur via FBP (already established) and PCFT, and the results show that chronic ethanol ingestion leads to decreased liver folate uptake. The folate transporters were associated with the lipid raft microdomains at the liver basolateral membrane. Ethanol feeding resulted in reduced association of folate transporter proteins with lipid rafts that can be ascribed to their decreased synthesis. However, further studies are needed to delineate the exact molecular events, which could explain the role of the transcriptional, translational, and posttranslational and/or the trafficking events that regulate the number of transporter molecules in the liver BLM during chronic alcoholism in rats.

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