Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 20 (2009) 291 – 297
Journal of Nutritional Biochemistry

Role of signaling pathways in the regulation of folate transport in ethanol-fed rats

Abid Hamid, Jyotdeep Kaur*

Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India

Received 5 November 2007; received in revised form 6 February 2008; accepted 6 March 2008

Abstract

Folate is an essential cofactor for normal cellular proliferation and tissue regeneration. Alcohol-associated folate deficiency is common, primarily due to intestinal malabsorption, the mechanism of which needs attention. The aim of the present study was to evaluate the regulatory events of folate transport in experimental alcohol ingestion. For this, male Wistar rats were fed 1 g/kg body weight/day ethanol (20% solution) orally for 3 months and folate transport was studied in isolated intestinal epithelial cells across the crypt–villus axis. The role of different signaling pathways in folate transport regulation was evaluated independently to that of reduced folate carrier (RFC) expression. The results showed that differentiated cells of villus possess high folate uptake activity as compared to mid villus and crypt base cells. During chronic ethanol ingestion, decrease in transport was observed all along the crypt–villus axis but was more pronounced at proliferating crypt base stem cells. Studying the effect of modulators of signaling pathways revealed the folate transport system to be under the regulation of cAMP-dependent protein kinase A (PKA), the activity of which was observed to decrease upon alcohol ingestion. In addition, protein kinase C might have a role in folate transport regulation during alcoholic conditions. The deregulation in the folate transport system was associated with a decrease in RFC expression, which may result in lower transport efficiency observed at absorptive surface in alcohol-fed rats. The study highlights the role that perturbed regulatory pathways and RFC expression play in the decreased folate transport at brush border surface during alcohol ingestion.
© 2009 Elsevier Inc. All rights reserved.

Keywords: Crypt–villus axis; Reduced folate carrier; Alcoholism; Signaling; Protein kinase

1. Introduction

The fast turnover rate of the epithelial cells is one of the characteristic features of the small intestinal tissue, and a constant balance of different cofactors is required for the maintenance of a large number of mature cells [1]. Folate is one such cofactor that not only is required to sustain high rate of DNA synthesis in this highly proliferating tissue but also plays a critical role in maintaining normal metabolic, energy, differentiation and growth status of all mammalian cells [2]. The exogenous folate required by mammals involves the initial step of intestinal uptake across the brush border membrane via reduced folate carrier (RFC) as a major folate transporter. Importantly, the consensus sequences for protein kinase C (PKC) and protein kinase A (PKA) have been shown to exist in cloned RFC [3]. Some recent studies have been directed towards understanding the folate transport regulation by these kinases in different cell lines and suggest an important link between cellular transport system of folate and protein kinases [4]. However, the role of these pathways in folate transport in isolated intestinal epithelial cells under physiological or pathophysiological conditions has not been studied yet.

Deficiency of folate is highly prevalent throughout the world and affects 10% of the U.S. population [5]. Alcohol ingestion has been the major contributor to folate deficiency worldwide [6]. Folate deficiency during alcoholism can develop because of dietary inadequacy, intestinal malabsorption, altered hepatobiliary metabolism, enhanced colonic metabolism and increased renal excretion [7–10]. However, it is evident from the literature that most of the ethanolic effects on folate metabolism are reflected in its effect on

* Corresponding author. Tel.: +91 172 2747585 5181; fax: +91 172 2744401x2745078.
E-mail address: jyotdeep2001@yahoo.co.in (J. Kaur).

0955-2863/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.03.004

intestinal absorption [11]. In addition, the recent overwhelming evidence suggests the existence of intricate mechanisms for controlling patterns of RFC expression and function in response to diverse tissue environments [12]. Hence, we sought to evaluate the regulatory events mediating folate transport across crypt–villus axis epithelium under physiological conditions and their roles in folate malabsorption during chronic alcoholism. Such mechanistic insights could lead to strategies for deducing the folate transport regulation in diverse cellular microenvironments and will be important for designing the therapeutic targets involving regulatory derangements in folate transport systems in primary absorptive epithelia.

2. Materials and methods

2.1. Animals

Young adult male albino rats (Wistar strain) weighing 100–150 g were obtained from our institute's Central Animal House. The animals were housed in clean wire mesh cages with controlled temperature (23±1°C) and humidity (45–55%) and were subjected to a 12-h dark/12-h light cycle throughout the study. The rats were randomized into two groups of six animals each. The rats in Group I were given a single dose of 1 g ethanol (20% solution)/kg body weight/day and those in Group II received an isocaloric amount of sucrose (36% solution) orally by intragastic intubation daily for 3 months. The dose was administrated daily between 9 and 10 a.m. to avoid perturbance in circadian rhythm. The rats were fed commercially available pellet diet (Ashirwad Industries, Ropar, India) and water *ad libitum*. The body weights of rats were recorded twice weekly. Animals from both groups were sacrificed under anesthesia using sodium pentothal, and blood was drawn for folate estimations.

The protocol of the study was approved by the Institutional Animal Ethical Committee and the Institutional Biosafety Committee.

2.2. Chemicals

Radiolabeled [3′, 5′, 7, 9-^{3}H]-folic acid potassium salt with a specific activity of 24.0 Ci/mmol was purchased from Amersham Pharmacia Biotech, Hong Kong. [γ-^{32}P]-Adenosine-5′-triphosphate triethylammonium salt with a specific activity of 5000 Ci/mmol was procured from Bhabha Atomic Research Centre, Mumbai, India.

2.3. Measurement of folate levels

Serum and RBC folate levels were measured by employing the microbiological assay using *Lactobacillus casei* as described [13].

2.4. Isolation of intestinal epithelial cells

The intestinal epithelial cells were isolated as described [14]. Starting from the ligament of Treitz, the upper two thirds of the small intestine was removed, flushed thoroughly with 0.9% saline. One end of the intestine was tied with thread and filled with rinsing buffer containing 1 mM DTT in normal saline, and the other end was tied as well. The rinsing buffer that filled the intestine was then replaced with a solution consisting of 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4 and 8 mM Na_2HPO_4 and kept at 37°C for 15 min in a beaker containing PBS. We discarded the solution in the intestine, and we filled the intestine with another solution containing 1.5 mM EDTA and 0.5 mM DTT in PBS, which was kept at 37°C for 30 min to isolate total small intestinal epithelial cells or for different time intervals to isolate cells of different origins along the crypt–villus axis. Fraction numbers 1–3 were collected at 4-, 2- and 2-min intervals whereas fraction numbers 4–6 were collected at 3-, 4- and 5-min intervals, respectively. Similarly, fraction numbers 7–9 were collected at 7-, 10- and 15-min intervals, respectively. The three consecutive fractions were pooled and represented villus tip, mid villus and crypt base cells, respectively, from fraction numbers 1–9. The cells were then centrifuged at 3000 rpm for 15 min. Then, 5 ml of the cold PBS was added to the pellet contents and centrifuged at 3000 rpm for 10 min. Two more PBS washings were given to the pellet constituting intestinal epithelial cells.

2.5. Preparation of brush border membrane vesicles (BBMVs)

BBMVs were prepared from the isolated total intestinal cells or cells from different origins at 4°C by the method of Kessler et al. [15] with some modifications. The final pellet containing isolated epithelial cells was homogenized in 2 mM Tris–50 mM mannitol buffer. We added 10 mM $MgCl_2$ to the major portion of the homogenate in 2 mM Tris–50 mM mannitol buffer, followed by intermittent gentle shaking for 10 min. The contents were centrifuged at 3000×*g* for 15 min, and the supernatant was run at 27,000×*g* for 30 min. The pellet thus obtained was mixed with a small amount of loading buffer containing 280 mM mannitol and 20 mM HEPES–Tris, pH 7.4, and hand homogenized followed by centrifugation at 27,000×*g* for 30 min. The final pellet obtained, representing BBMV, was suspended in loading buffer to achieve a final protein concentration of approximately 5 mg/ml. Purity of the membrane preparations was checked by measuring the specific activities of alkaline phosphatase and Na^+, K^+-ATPase in brush border membranes and in the original homogenate [7]. The vesicle preparations from both groups showed enrichment of 10- to 12-fold with respect to alkaline phosphatase and showed negligible amount of Na^+, K^+-ATPase activity. The vesicles used in the study were intact and stable as they showed the properties of a typical brush border membrane as revealed by a transient overshoot of the intravesicular glucose concentration over its equilibrium uptake in the presence of sodium gradient (data not shown).

2.6. Transport of [^{3}H]-folic acid in BBMV across the crypt–villus axis

Uptake studies were performed at 37°C using the incubation buffer consisting of 100 mM NaCl, 80 mM mannitol, 10 mM HEPES and 10 mM 3-[*N*-morpholino] ethanesulfonic acid (MES), pH 5.5. We added 10 μl of vesicles (50 μg protein) prepared from different cell types to an incubation buffer containing 0.5 μM [^{3}H]-folic acid. Initial rate of transport was determined by stopping the reaction after 30 s unless otherwise mentioned by adding ice-cold stop solution containing 280 mM mannitol and 20 mM HEPES–Tris, pH 7.4, followed by rapid vacuum filtration [16]. Nonspecific binding to the filters was determined by residual filter counts after filtration of the incubation buffer and labeled substrate without vesicles. Also, in the preliminary experiments, vesicular transport of [^{3}H]-folic acid in the presence of excess of unlabeled folic acid was measured and amounted to a negligible value, suggesting the specificity of the transport process. The radioactivity that remained on the filters was determined by liquid scintillation counting (Beckman Coulter LS 6500).

2.7. Characterization of transport in cells

2.7.1. Time course and kinetics of folate transport in epithelial cells

The reaction was initiated by adding 10 μl of epithelial cells (100 μg protein) to 0.5 μM of [^{3}H]-folic acid in incubation buffer (pH 5.5). The reaction was stopped at different time intervals, namely, 10, 20, 30, 60 and 120 s. For determining the K_m and V_{max} values, the [^{3}H]-folic acid concentration was varied from 0.125 to 1.00 μM in the reaction medium and reaction was allowed for 30 s after cell addition.

2.7.2. Intracellular regulation of folic acid uptake in epithelial cells

During total cell isolation as described above, the concentration of EDTA and DTT used was 0.75 and 0.25 mM, respectively. A small aliquot from each isolated cell preparation was checked for viability by trypan blue exclusion. The viable cells (50 μg protein) were then incubated in the incubation buffer consisting of 100 mM NaCl, 80 mM mannitol, 10 mM HEPES and 10 mM MES, pH 5.5, containing activators or inhibitors of PKC-, PKA- and cAMP-mediated pathways, for 30 min as per an earlier study [1]. Transport reaction was initiated with 0.5 μM [^{3}H]-folic acid and stopped with the stop solution of 280 mM mannitol and 20 mM HEPES–Tris, pH 7.4, after 30 s. The protein kinase inhibitor (1.33 μg/50 μl reaction mixture), atropine (3 mM), dibutyryl cAMP (DbcAMP) (1 mM), chelerythrine chloride (7 μM) and phorbol-12-myristate-13-acetate (PMA) (10–30 μM) were the various modulators used in the study.

2.7.3. Estimation of cAMP-dependent PKA activity

During assay, the phosphotransferase activity of the viable total intestinal epithelial cells was monitored using histone IIA as a substrate by employing the method of Roskoski [17] with modifications. We added 5 μl of the substrate histone (1 mg/ml) to a microcentrifuge tube containing 10 μl of the assay dilution buffer containing 250 mM 3-[*N*-morpholino]propanesulfonic acid, 50 mM $MgCl_2$ and 1.25 mg/ml BSA. Then, 5 μl of cAMP (50 μM) was added, followed by 5 μl of diluted [γ-^{32}P]-ATP (100 μM), and volume was made to 30 μl with the distilled water. The 10 μl of cell suspension (50 μg protein) was added to the whole reaction mixture and incubated at 37°C for 5 min with constant agitation. Two controls for each sample, either without the external addition of cAMP or histone substrate, were run simultaneously. Aliquots of 25 μl were transferred and spotted on phosphocellulose strips (Whatman P81, 1×2 cm). The strips were washed with 10 ml of 75 mM phosphoric acid and swirled gently for 5 min. Phosphoric acid was decanted, and phosphocellulose strips were washed twice more in phosphoric acid with gentle agitation. The strips were dried, and radioactivity was measured as counts per minute in a scintillation cocktail.

2.7.4. Western blot analysis for RFC expression in BBMV

For expression studies, brush border membrane proteins (100–150 μg) isolated from intestinal epithelial cells were resolved on 10% SDS-PAGE and transferred to nitrocellulose membrane for 4–5 h at 4°C, and the transfer was carried out at 25 V or 300 mA. Equal amounts of BBMV protein from the two groups of rats were loaded for Western blot analysis using the procedure described by Towbin et al. [18] employing polyclonal primary antibodies of rabbit anti-rat RFC (1:500 dilutions), kindly provided by Dr. Hamid M. Said (Professor, Physiology and Biophysics, School of Medicine, University of California, Irvine, CA, USA), and were raised against specific region of rat RFC synthetic peptide corresponding to amino acids 495–512 of the rat RFC. The polyclonal antibodies against leucine aminopeptidase (LAP; an intestinal brush border peptidase), which are rabbit anti-rat LAP (1:500), were used and served as an internal control. Secondary antibodies used were HRP-labeled goat anti-rabbit IgG (1:2000 dilution). The quantification of blots was carried out by using Scion image software.

2.7.5. Statistical analysis

The data were computed as mean±S.D. Group means were compared by using the Student's *t* test, and analysis of variance was used wherever necessary. The acceptable level of significance was $P<.05$ for each analysis.

3. Results

3.1. Growth and folate levels of rats

There was no significant change in the relative body weights of rats during the course of ethanol administration as reported earlier by us [16]. At the time of sacrifice, the mean body weights of rats in control and ethanol-fed groups were 201±8 and 196±9 g, respectively. The mean serum folate levels were 49.64±5.29 and 33.71±4.95 μg/L ($P<.001$), and the mean

RBC folate values were 950±29.84 and 624±49.73 μg/L (P<.001) in control and ethanol-fed rats, respectively.

3.2. Determination of kinetic constants

Time course of folate uptake at various intervals revealed that the folate uptake in both groups showed maxima up to 30–45 s and attained plateau level throughout thereafter. In addition, K_m and V_{max} values under physiological conditions in rats were determined in the presence of increasing [^{3}H]-folic acid concentration from 0.125 to 1.0 μM at pH 5.5. The observed saturation phenomena with increasing substrate concentration are indicative of Michaelis–Menten kinetics with a K_m value of 0.75±0.001 μM and a V_{max} value of 50.21±2.14 pmol/30 s/mg protein as calculated by means of a Lineweaver–Burk plot (Fig. 1).

3.3. Transport of [^{3}H]-folic acid in BBMV across the crypt–villus axis

The cell fractions (F1–F9) isolated from the small intestine were characterized by an approximately eight-fold decrease in a villus cell marker enzyme, alkaline phosphatase specific activity from F1 (villus tip) to F9 (crypt base) (data not shown). On the basis of the distribution patterns of the villus and the crypt cell markers, the nine cell fractions were grouped as the villus tip (F1–F3), mid villus (F4–F6) and crypt (F7–F9) cells, representing differentiated, differentiating and proliferating enterocytes, respectively. BBMVs were isolated from these pooled cell fractions, and the initial rate of [^{3}H]-folic acid transport was studied (Fig. 2). Folate transport was observed to be significantly higher at the villus tip as compared to the crypt base in both the control and the ethanol-fed group (P<.01 and P<.001, respectively). Ethanol feeding resulted in a significant decrease in folate transport all along the crypt–villus axis, with the decrease being maximum (50%) at the crypt base.

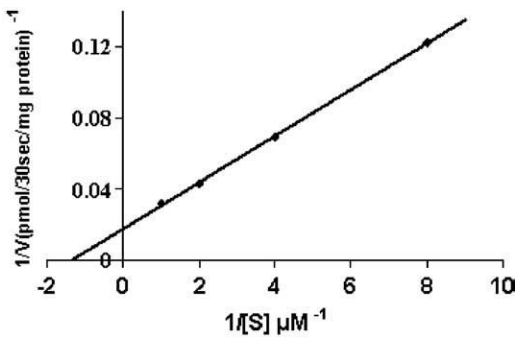

Fig. 1. Lineweaver–Burk plot showing [^{3}H]-folic acid uptake in intestinal epithelial cells as a function of substrate concentration. Uptake was measured by varying [^{3}H]-folic acid concentration from 0.125 to 1.0 μM in the incubation medium (pH 5.5) after incubating cells (100 μg) for 30 s. Each data point is the mean±S.D. of three separate uptake determinations carried out in duplicate.

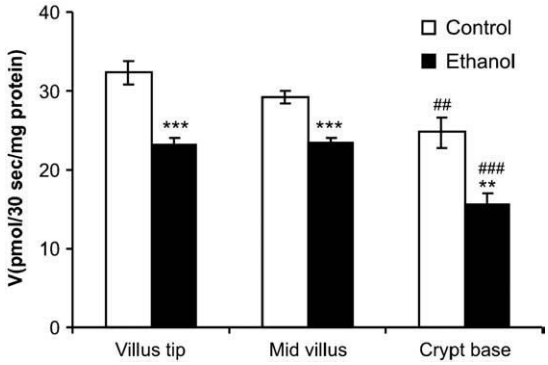

Fig. 2. [^{3}H]-Folic acid uptake in BBMVs isolated from villus tip, mid villus and crypt base cells of the intestine. Each data point is the mean±S.D. of three separate uptake determinations carried out in duplicate. **P<.01, ***P<.001 versus Control and $^{##}P$<.01, $^{###}P$<.001 versus Villus tip.

3.4. Intracellular regulation of folic acid transport in epithelial cells

Using trypan blue, we found that the isolated intestinal cells used for transport study were 70–80% viable in both groups of rats. Importantly, none of the modulators affected the viability of cells when cells were checked 1 h after incubation. A 10-μl aliquot of cells containing an equal amount of protein (100 μg) was taken from both groups of animals for measurement of folate transport. Since there was no alteration in histology of the two groups of animals with regard to villus height and architecture [19], it was assumed that the number of cells per milligram of protein is equal in the two groups (data not shown). The cells were pretreated with various activators and inhibitors for 30 min before measurement of transport activity (Fig. 3). The results revealed that when the cells were incubated in the presence of protein kinase inhibitor specific to cAMP-dependent kinases, there was a 66% and 51% decrease (P<.001) of initial rate of folic acid transport in control and ethanol-fed rats, respectively. Interestingly, when the cells were incubated in a compound known to increase the intracellular cAMP level, for example, DbcAMP, the initial rate of [^{3}H]-folic acid (0.5 μM) uptake was found to increase by 20% and

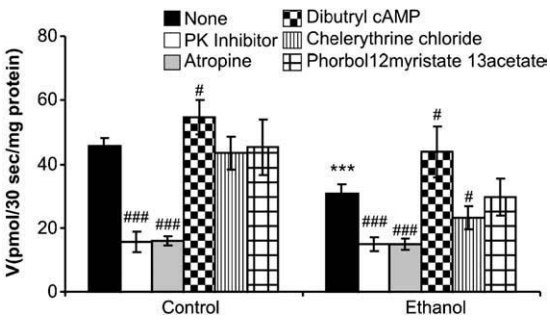

Fig. 3. Effect of positive and negative modulators of PKA and PKC on [^{3}H]-folic acid uptake in viable isolated intestinal epithelial cells. Each data point is the mean±S.D. of 12 independent observations. ***P<.001 versus Control, $^{#}P$<.001, $^{###}P$<.001 versus None.

43% ($P<.01$), respectively, in the control and ethanol-fed rats. Atropine, which is an adenylyl cyclase inhibitor and lowers intracellular cAMP levels, resulted in reduction of folic acid uptake of 65% and 51% ($P<.001$), respectively, in pretreated epithelial cells from control and ethanol-fed rats. The observed changes (increase or decrease) in folate transport activity are attributed to the effects of these modulators on cAMP levels.

For the investigation of the role of PKC-mediated pathway in the regulation of folic acid uptake, the epithelial cells were pretreated for 30 min with PKC activator (PMA) or the specific inhibitor chelerythrine chloride. None of these pretreatments significantly affected the folic acid uptake in the control group; however, under similar conditions, chelerythrine chloride could inhibit the transport significantly by 24% ($P<.05$) in the ethanol-fed group. Interestingly, PMA could not impart any effect in the ethanol-fed group even when its concentration was increased from 10 to 30 μM.

3.5. cAMP-dependent PKA activity in epithelial cells

To correlate the observed increase in folate transport activity in response to exogenous cAMP levels with PKA activity, we incubated cells in the presence or absence of cAMP or histone (substrate) and we monitored PKA activity using [γ-^{32}P]-ATP in intestinal epithelial cells of control and ethanol-fed rats (Fig. 4). Total PKA activity was decreased by 21% ($P<.01$) upon ethanol feeding to rats. In control rats, there was 35% and 49% ($P<.001$) of total PKA activity in the absence of cAMP or histone, respectively. In ethanol-fed rats, the levels of PKA activity under these conditions were 43% and 55% ($P<.001$), respectively.

3.6. Expression of the RFC at the brush border membrane surface

The effect of chronic alcoholism on the level of expression of the RFC protein at the brush border membrane surface was studied by Western blot analysis to identify the RFC using polyclonal antibodies raised against a specific

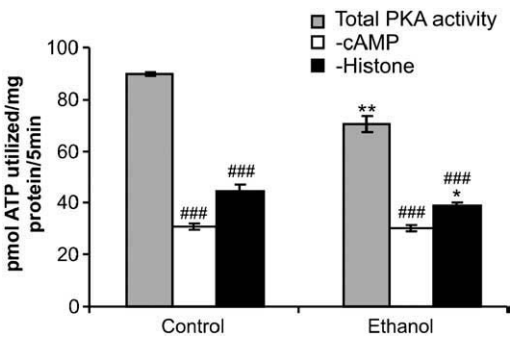

Fig. 4. PKA activities in isolated intestinal epithelial cells in the presence of either exogenous cAMP or histone IIA or both. Each data point is the mean±S.D. of 12 independent observations. *$P<.05$, **$P<.01$ versus Control and $^{###}P<.001$ versus Total PKA activity.

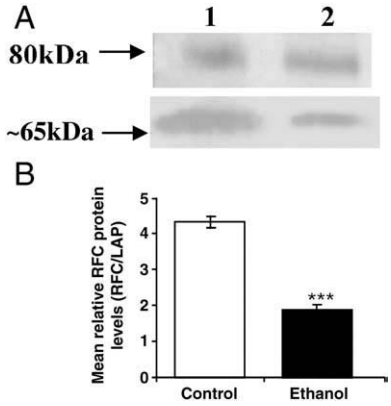

Fig. 5. (A) Western blot analysis of intestinal BBMV using anti-RFC (65 kDa) and anti LAP (80 kDa) antibodies. (B) Densitometric analysis representing relative change in RFC protein levels. Data shown are the mean of five separate sets of experiments. Lane 1, Control; lane 2, Ethanol. ***$P<.001$ versus Control.

region of rat RFC, and reactivity was found at ~65 kDa. Also, there was no cross-reaction of RFC antibodies against any protein in the vesicular preparations used. Antisera against the LAP showed reactivity at 80 kDa, which served as an internal control. LAP is a membrane-bound amino-peptidase present in intestinal brush border membrane, whose activity has been found to be unaffected by chronic ethanol feeding [20,21]. The expression of the RFC protein was observed to be three-fold higher in BBMV from the control group when compared with the chronic, ethanol-fed group (Fig. 5).

4. Discussion

In the present study, the higher transport rate of folate at villus tip cells was observed as compared to mid villus or crypt base cells, which suggested that with maturation of the intestinal stem cells, the specialized folate transport system is predominantly active in villus tip cells. Under such conditions, ethanol treatment for 3 months reduced the folate transport capability all across the crypt–villus axis, which resulted in an appreciable decrease in serum and RBC folate levels. For the validation of the present cell model under physiological conditions, we observed kinetic constants in the intestinal epithelial cells comparable to that of the purified brush border membrane.

Using viable epithelial cells, the intracellular regulation of folic acid transport was focused on PKA- and PKC-mediated pathways because consensus sequences for these kinases have been found in cloned folate transporters (i.e., RFC) in different mammalian species [4]. In the presence of inhibitor of cAMP-dependent protein kinase, a significant decrease in folic acid transport implied the involvement of protein kinases for efficient folate transport process to be operative. Since the best defined target of cAMP is PKA, which, in turn, mediates most of the physiological effects of cAMP in eukaryotes, in order to deduce the role of cAMP in the folate

transport, activator such as DbcAMP, which increases the intracellular cAMP levels, was employed. The observed 2.5-fold increase in folate transport in ethanol-fed rats under such conditions suggested that cAMP may form an important component of signaling cascade, it being affected in chronic alcoholism. Moreover, atropine, which results in decreased intracellular cAMP levels by inhibiting the activity of adenylyl cyclase, inhibited the folic acid transport and again emphasizes the role of cAMP in folate transport and its perturbance during chronic alcoholism. Thus, these observations suggest that the cAMP-mediated PKA pathway may be involved in the regulation of folic acid uptake in intestinal epithelial cells. Such a statement is plausible because PKA represents a downstream target of cAMP action and can transduce cAMP signal either by phosphorylating the preexisting proteins in the cytoplasm or by activating the transcription factors involved in the gene expression [22,23]. Earlier studies have suggested that intracellular cAMP may negatively affect the folic acid uptake process via PKA-independent mechanism in NCM460 [24], IEC-6 [1] and PaCa-2 cell lines [25]. However, the increased cAMP levels were found to positively correlate with the folic acid uptake in the present study. Such a discrepancy may be related to differences in the experimental setup employed in the previous studies using cultured cell lines unlike viable epithelial cells in our study. In addition, the observed increase in PKA activity in the presence of exogenous cAMP levels in two groups of rats and the fact that there was significant decrease in PKA activity in ethanol-fed rats further substantiated our results. Therefore, cAMP-mediated PKA can be proposed to regulate the intestinal folate transport system; the regulatory role of which gets altered during alcoholism-associated folate malabsorption via an unknown mechanism.

Furthermore, the lack of effect of PKC modulators on folic acid uptake in the control group showed that the PKC pathway has no role in folic acid transport system under physiological conditions. Nevertheless, the significant effect of the PKC inhibitor chelerythrine chloride in ethanol-fed group proposes that PKC may play a role in folic acid transport regulation during alcoholism. However, the absence of any effect of the PKC activator PMA in the ethanol-fed group may suggest that either PMA as such or its pharmacological range concentrations used in the study were not sufficient to revert the observed reduction in folate transport after ethanol feeding. Overall, these findings suggest that protein kinases via cAMP are involved in physiological folic acid transport regulation, and after chronic ethanol ingestion in rats, PKC could also play a vital role via yet unidentified mechanism(s). This can be explained by the presumption that phosphorylation of PKC site has no subsequent effect on the transport activity under physiological conditions or the site gets accessibility only in alcoholism due to change in membrane fluidity or conformation. Although no direct evidence exists regarding the intracellular regulation of folate transporters (e.g., RFC) by kinases, the effect of modulators on transport activity and the presence of consensus sequences for these kinases in cloned RFC makes it a fascinating field to study, especially in pathophysiological conditions of folate malabsorption such as alcoholism. The disturbance in regulation of the folate transport system via kinases reflected its effect as decreased expression of the RFC at intestinal brush border membrane observed in the present study. Since the level of RFC message is a reflection of functional uptake across the intestinal surface, such results suggest that a decrease in the number of transport carriers due to perturbed signaling is the possible reason of the folate malabsorption during alcoholism. These observations substantiated our earlier study in which chronic ethanol feeding resulted in altered kinetic characteristics of folate transport process, reflected by decreased V_{max} in rat intestine [19,26]. The decreased RFC protein molecules in the brush border membrane can occur by greater turnover or degradation, which results in lesser number of transporter molecules available at the brush border membrane surface for the uptake of folic acid during alcoholism. Future studies should focus on whether the cAMP–PKA network is directly involved in phosphorylating the RFC and/or substrate or whether it influences the expression of the RFC at the transcriptional or posttranslational level. In addition, the role of signaling intermediates of these pathways on the expression of RFC will help in determining the target of alcohol interference in the folate transport system.

Taken together, the decreased transport all along the crypt–villus axis associated with altered regulating pathways was reflected as low expression of the RFC in the intestine during alcoholism.

Acknowledgments

Abid Hamid acknowledges the Council of Scientific and Industrial Research (CSIR), New Delhi, India, for fellowship. The authors also thank ICMR, New Delhi, for grant support.

References

[1] Said HM, Ma TY, Ortiz A, Tapia A, Valerio CK. Intracellular regulation of intestinal folate uptake: studies with cultured IEC-6 epithelial cells. Am J Physiol Cell Physiol 1997;272:C729–36.
[2] Matherly LH, Goldman ID. Membrane transport of folates. Vitam Horm 2003;66:403–56.
[3] Said HM, Mohammed ZM. Intestinal absorption of water-soluble vitamins: an update. Curr Opin Gastroenterol 2006;22:140–6.
[4] Said HM. Recent advances in carrier-mediated intestinal absorption of water-soluble vitamins. Annu Rev Physiol 2004;66:419–46.
[5] Novakovic P, Stempak JM, Sohn KJ, Kim YI. Effects of folate deficiency on gene expression in the apoptosis and cancer pathways in colon cancer cells. Carcinogenesis 2006;27:916–24.
[6] Sakuta H, Suzuki T. Alcohol consumption and plasma homocysteine. Alcohol 2005;37:73–7.
[7] Hamid A, Kaur J. Chronic alcoholism alters the transport characteristics of folate in rat renal brush border membrane. Alcohol 2006;38:59–66.

[8] Homann N, Tillonen J, Salaspuro M. Microbially produced acetaldehyde from ethanol may increase the risk of colon cancer via folate deficiency. Int J Cancer 2000;86:169–73.
[9] Schalinske KL, Nieman KM. Disruption of methyl group metabolism by ethanol. Nutr Rev 2005;63:387–91.
[10] Villanueva J, Chandler CJ, Shimasaki N, Tang AB, Nakamura M, Phinney SD, et al. Effects of ethanol feeding on liver, kidney and jejunal membranes of micropigs. Hepatol 1994;19:1229–40.
[11] Villanueva JA, Devlin AM, Halsted CH. Reduced folate carrier: tissue distribution and effects of chronic ethanol intake in the micropig. Alcohol Clin Exp Res 2001;25:415–20.
[12] Rajgopal A, Sierra EE, Zhao R, Goldman ID. Expression of the reduced folate carrier SLC19A1 in IEC-6 cells results in two distinct transport activities. Am J Physiol Cell Physiol 2001;281:C1579–86.
[13] Tamura T. In: Picciano MF, Stokstad ELR, Gregory JF, editors. Folic acid metabolism in health and disease. New York: Wiley-Liss; 1990. pp. 121–37.
[14] Weiser MM. Intestinal epithelial cell surface membrane glycoprotein synthesis, I. An indicator of cellular differentiation. J Biol Chem 1973; 248:2536–41.
[15] Kessler M, Acuto O, Storelli C, Murer H, Muller M, Samenza G. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport. Biochem Biophys Acta 1978;506:136–54.
[16] Hamid A, Kaur J. Kinetic characteristics of folate binding to rat renal brush border membrane in chronic alcoholism. Mol Cell Biochem 2005;280:219–25.
[17] Roskoski Jr R. Assays of protein kinase. Methods Enzymol 1983;99: 3–6.
[18] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350–4.
[19] Hamid A, Wani NA, Rana S, Vaiphei K, Mahmood A, Kaur J. Down-regulation of reduced folate carrier may result in folate malabsorption across intestinal brush border membrane during experimental alcoholism. FEBS J 2007;274:6317–28.
[20] Kaur J, Virender Jaswal MS, Nagpaul JP, Mahmood A. Chronic ethanol feeding and microvillus membrane glycosylation in normal and protein malnourished rat intestine. Nutrition 1992;8:338–42.
[21] Kaur J, Nagpaul JP, Mahmood A. Expression of brush border enzymes in ethanol fed rat intestine. Indian J Med Res 1994;100:289–94.
[22] Muller U, Brandsch M, Prasad PD, Fei YJ, Ganapathy V, Leibach FH. Inhibition of the H^+/peptide cotransporter in the human intestinal cell line Caco-2 by cyclic AMP. Biochem Biophys Res Commun 1996; 218:461–5.
[23] Velasco G, Iglesias CF, Dominguez P, Barros F, Gascon S, Lazo PS. Protein kinase C from small intestine epithelial cells. Biochem Biophys Res Commun 1986;139:875–82.
[24] Kumar CK, Moyer MP, Dudeja PK, Said HM. A protein tyrosine kinase-regulated pH dependent, carrier mediated uptake system for folate in human normal colonic epithelial cell line NCM460. J Biol Chem 1997;272:6226–31.
[25] Nabokina SM, Ma TY, Said HM. Mechanism and regulation of folate uptake by human pancreatic epithelial MIA PaCa-2 cells. Am J Physiol Cell Physiol 2004;87:C142–8.
[26] Hamid A, Kaur J, Mahmood A. Evaluation of the kinetic properties of the folate transport system in intestinal absorptive epithelium during experimental ethanol ingestion. Mol Cell Biochem 2007;304: 265–71.