Chronic alcohol exposure inhibits biotin uptake by pancreatic acinar cells: possible involvement of epigenetic mechanisms

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Srinivasan P, Kapadia R, Biswas A, Said HM. Chronic alcohol exposure inhibits biotin uptake by pancreatic acinar cells: possible involvement of epigenetic mechanisms. Am J Physiol Gastrointest Liver Physiol 307: G941–G949, 2014. First published September 11, 2014; doi:10.1152/ajpgi.00278.2014.—Chronic exposure to alcohol affects different physiological aspects of pancreatic acinar cells (PAC), but its effect on the uptake process of biotin is not known. We addressed this issue using mouse-derived pancreatic acinar 266-6 cells chronically exposed to alcohol and wild-type and transgenic mice (carrying the human SLC5A6 5′-promoter) fed alcohol chronically. First we established that biotin uptake by PAC is Na+ dependent and carrier mediated and involves sodium-dependent multivitamin transporter (SMVT). Chronic exposure of 266-6 cells to alcohol led to a significant inhibition in biotin uptake, expression of SMVT protein, and mRNA as well as in the activity of the SLC5A6 promoter. Similarly, chronic alcohol feeding of wild-type and transgenic mice carrying the SLC5A6 promoter led to a significant inhibition in biotin uptake by PAC, as well as in the expression of SMVT protein and mRNA and the activity of the SLC5A6 promoters expressed in the transgenic mice. We also found that chronic alcohol feeding of mice is associated with a significant increase in the methylation status of CpG islands predicted to be in the mouse Slc5a6 promoters and a decrease in the level of expression of transcription factor KLF-4, which plays an important role in regulating SLC5A6 promoter activity. These results demonstrate, for the first time, that chronic alcohol exposure negatively impacts biotin uptake in PAC and that this effect is exerted (at least in part) at the level of transcription of the SLC5A6 gene and may involve epigenetic/molecular mechanisms.

Biotin; pancreatic acinar cells; alcohol; transport

THE WATER-SOLUBLE VITAMIN biotin, a member of the B family of vitamins, is essential for normal cellular functions, growth, and development. Biotin acts as a cofactor for five carboxylases that are critical for fatty acid, glucose, and amino acid metabolism (reviewed in Ref. 40). Recent studies have also shown a role for biotin in the regulation of gene expression (where expression of more than 2,000 human genes appears to be affected by biotin status; 39, 49), and in immune function (1, 21, 22, 38). Thus it is not surprising that deficiency/suboptimal levels of this essential micronutrient leads to a variety of clinical abnormalities (e.g., neurological disorders, growth retardation, dermatological abnormalities) (28, 40, 48). Such deficiency/suboptimal levels occur in conditions of chronic alcoholism (4, 12), long-term parenteral nutrition (13, 29), and inborn errors of biotin metabolism (28, 48) and in subjects on long-term therapy with certain anticonvulsant drugs (17, 18).

The pancreas is a complex organ with important exocrine and endocrine functions. A variety of disease conditions and factors affect the health of this vital organ leading to significant morbidity and mortality. Cells of this organ, like all other mammalian cells, cannot synthesize biotin endogenously and thus must obtain the vitamin from their surroundings via transport across the cell membrane. Nothing is known about how pancreatic acinar cells (PAC) take up biotin. Previous studies have identified the sodium-dependent multivitamin transporter (SMVT) as a major and highly efficient system for biotin transport across mammalian cell membranes (2, 14, 34, 35). These studies have delineated the molecular identity of the SMVT system from a number of species including human, rat, and mouse (34, 35) and have cloned and characterized the 5′ regulatory region (promoters) of its gene (SLC5A6) (7, 11, 37). Also, different aspects of transcriptional and posttranscriptional regulation of the SMVT system and its cell biology have been delineated in recent years (15, 37, 41).

Chronic alcohol use/exposure is associated with increased risk of pancreatic injury (16, 19, 30). The mechanism involved in mediating this effect is not fully understood and appears to be multifactorial. It is believed that chronic exposure to alcohol alters the resting state of the pancreas and lowers its defense mechanisms (27, 31, 33). This in turn predisposes the organ to the effect of stress conditions/injurious agents or to other cell biological events causing injury (i.e., alcohol “sensitizes” or “primes” the pancreas to subsequent injury) (16, 19, 30, 31, 33). Chronic alcohol exposure was recently shown to negatively impact biotin transport events in intestinal and renal epithelial cells (45, 46), effects that may contribute to the observed low biotin levels found in chronic alcoholics (4, 12). The effect that chronic alcohol exposure has on the physiology of biotin uptake by PAC is not known. Negative effects of chronic alcohol exposure on pancreatic physiology of biotin (a micronutrient essential for normal cellular functions) may weaken pancreatic health and contribute to reduction in its defense mechanisms. Thus the aim of this study was to examine the effect of chronic alcohol exposure on biotin uptake by PAC. We used an in vitro model of alcohol exposure using the mouse-derived pancreatic acinar 266-6 cells and an in vivo model of alcohol exposure utilizing wild-type and transgenic mice carrying the human SLC5A6 promoters that were fed alcohol chronically. Since nothing is known about the mechanism of biotin uptake by PAC, we first delineated the mechanism involved. Our results show, for the first time, that biotin uptake by PAC is via a Na+−dependent, carrier-mediated process and that chronic alcohol exposure and feeding significantly inhibit this process. The latter appears to be exerted, at least in part, at the level of transcription of the SLC5A6 gene and may involve epigenetic/molecular mechanisms.
MATERIALS AND METHODS

Materials

\[^{[1]}H\]Biotin (specific activity 20 Ci/mmol; radiochemical purity > 98%) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Nitrocellulose filters (0.45-µm pore size) were from Millipore (Fisher Scientific). Unlabeled biotin and other chemicals including molecular biology reagents were from commercial vendors and were of analytical grade. Oligonucleotide primers used in this study were synthesized by Sigma Genosys (Sigma, Woodland, TX).

METHODS

Cell culture and uptake studies. Mouse-derived pancreatic acinar 266-6 cells (266-6) are an acinar pancreatic cell line derived from the pancreas of the young adult mouse. The tumor was induced with an elastase/ISV-40 T antigen fusion gene and expresses detectable levels of a number of digestive enzymes. mRNAs were obtained from American Type Tissue Collection (ATCC; Rockville, MD). Cells between passages 2 and 20 were cultured in DMEM growth medium containing 10% FBS and an antibiotic cocktail. Biotin uptake was measured at 37°C in Krebs-Ringer (K-R) buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 MgSO4, 0.85 CaCl2, 5 glucose, 5 glutamine, 10 HEPES, and 10 MES; pH 7.2) as described before (44, 46). The viability of acinar cells was tested by the Trypan blue exclusion method and was of analytical grade. Oligonucleotide primers used in this study were synthesized by Sigma Genosys (Sigma, Woodland, TX).

Isolation of mouse primary pancreatic acinar cells and biotin uptake. Mouse primary PAC were isolated from the pancreas of adult mice by the Worthington collagenase type IV (Lakewood, NJ) digestion method as described previously (44–46). Isolated cells were suspended in K-R buffer, and uptake was performed within 1 h of isolation. The viability of acinar cells was tested by the Trypan blue exclusion method and was >90%. Cells were suspended in K-R buffer and either \[^{[1]}H\]labeled or unlabeled biotin was added to initiate uptake. The uptake reaction was terminated by adding 1 ml ice-cold K-R buffer, and the cell suspension was placed on nitrocellulose filters under negative pressure by use of a vacuum manifold (Hoefer Scientific Instruments, Holliston, MA), washed with 5 ml of ice-cold K-R buffer, and uptake was measured with a scintillation counter (Beckman Coulter LS6500, Brea, CA). Digested samples were taken for determination of protein concentration (Bio-Rad Dc protein assay kit).

Chronic exposure of 266-6 cells to alcohol and biotin uptake. Mouse-derived pancreatic acinar 266-6 cells were chronically (96 h) exposed to alcohol [50 mM; a concentration that mimics the level of alcohol found in blood of chronic alcoholics (10)] in DMEM growth medium containing 10% FBS as described before (32, 44). After 96 h of exposure biotin uptake was measured at 37°C in K-R buffer and then measured for radioactive content by using a scintillation counter as described above.

Chronic alcohol feeding of mice. Wild-type and transgenic mice carrying the full-length human SLC5A6 promoter fused to the firefly luciferase reporter gene, previously generated and characterized by this laboratory (37), were used (approval for their use was obtained from the Institutional Animal Care Use Committee of the Long Beach VA Medical Center). Mice were fed Lieber-DeCarli ethanol-liquid diet (Dyets, Bethlehem, PA) [ethanol provided 25% of total ingested calories and was introduced gradually; calories were increased by 5% every day until we achieved 25% (25)] for 4 wk as described by us recently (44, 46). Control littermates (sex-matched that had similar basal firefly luciferase mRNA expression) were pair-fed with the same liquid diet but without ethanol (maltose-dextrin replaced ethanol isocalorically). The mice were euthanized after 4 wk and the pancreas was removed and primary PAC were isolated from mice by a collagenase type-V (Sigma, St. Louis, MO) digestion method as described above. Freshly isolated PAC were used for uptake analysis as described above, with a portion stored at −80°C for protein, mRNA, and firefly luciferase analysis.

Western blot analysis. Whole cell lysate prepared from primary mouse PAC and 266-6 cells chronically fed/exposed to alcohol and their respective controls were used for Western blot analysis as described previously (44, 47). The cells were suspended in 200 µl of RIPA buffer (Sigma) according to manufacturer’s protocol and supplemented with protease inhibitor cocktail (Roche). Sixty micrograms of pancreatic acinar cell protein were resolved in premaid 10% Bis-Tris minigel (Invitrogen) and electroblotted onto immobilon poly-vinylidene difluoride membrane (Fisher Scientific). The electroblotted membranes were blocked with Odyssey blocking solution (LI-COR Bioscience, Lincoln, NE) followed by overnight incubation with primary antibody of SMVT/KLF-4 (1:200 dilution) rabbit polyclonal antibody along with β-actin (1:3,000 dilution) monoclonal antibody. The SMVT (68.6 kDa)/KLF-4 (53 kDa) and β-actin (42 kDa) immunoreactive bands were detected by using goat anti-rabbit IRDye-800 for SMVT and goat anti-mouse IRDye 680 secondary antibodies (1:3,000 dilution). Signals were detected with the Odyssey infrared imaging system (LI-COR Bioscience) and quantified with LI-COR software and normalized to β-actin as an internal control.

Real-time PCR analysis. Total RNA (2 µg) isolated from primary acinar cells and 266-6 cells were treated with DNase I (Invitrogen) and converted to cDNA using iScript CDNA synthesis kit (Bio-Rad, Hercules, CA). The coding region of mice SLC5A6 gene and ARPO, were PCR amplified using gene-specific primers (Table 1) for quantitative PCR study. Quantitative PCR conditions were same as described previously (44, 47). The data were normalized to ARPO and then quantified by a relative relationship method (26).

Table 1. Primers used for amplification coding region of the respective genes by quantitative PCR and bisulfite PCR primers for amplifying mice SLC5A6 Cpg islands

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward and Reverse Primers (5’-3’)</th>
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<tbody>
<tr>
<td>SLC5A6</td>
<td>GACTCTGCTGGGACCTGCG; CAATCTCTGGCAATGACA</td>
</tr>
<tr>
<td>KLF-4</td>
<td>AGAAGGCTGAGAGTTCTTACAA; TTGAAAGGATTCGTTGCTTC</td>
</tr>
<tr>
<td>ARPO</td>
<td>GCTGAAACTCTTGCCCCCTCTG; ATATGCTCATGATTCTCTG</td>
</tr>
<tr>
<td>Bisulfite PCR Primers (4 pairs)</td>
<td></td>
</tr>
<tr>
<td>SLC5A6 promoter</td>
<td>TTTTTTTTTGTATTGTTTATGTGCTTGGTT; ATATACAAAATTTAAACGCGTTTATAACGTTT</td>
</tr>
<tr>
<td></td>
<td>GGATCTGTGGGACTGTGA</td>
</tr>
<tr>
<td></td>
<td>ACTGACATTCTTATGAAAGTTGAGG</td>
</tr>
<tr>
<td></td>
<td>GATAGATTTATTATTTTTTTATTTTTTTTG</td>
</tr>
<tr>
<td></td>
<td>TGTATTTAGGTTTATGTTGTTTATGTTT</td>
</tr>
<tr>
<td></td>
<td>TTTTTATGTCAGATTTGAAAAGTTG; CATTTAATATTTAAATCTCGCAACCC</td>
</tr>
<tr>
<td></td>
<td>AGTTTGCGAATTTTTTATTatatGTTT</td>
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<tr>
<td></td>
<td>GCTGCGATCTGAAATCTGAGTACCT</td>
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Transfection and reporter gene assay. The SLC5A6 full-length promoter–luciferase reporter constructs used in this study were generated previously (37). Transfection was performed with Lipofectamine 2000 reagent (Invitrogen) according to manufacturer’s instructions. For luciferase assay, 266-6 cells were cotransfected in 12-well plates at least 80% confluency with 2 μg of each test construct and 100 ng of the Renilla transfection control plasmid Renilla luciferase-thymidine kinase (pRL-TK) (Promega, Madison, WI). The day after transfection these cells were exposed to alcohol (50 mM for 96 h) as described above. After 96 h of alcohol exposure, luciferase activity was determined by using the Dual Luciferase Assay system (Promega) per manufacturer’s instructions.

Bisulfite conversion and sequencing. Methylation status of mouse Slc5a6 promoter was assessed by bisulfite sequencing as described previously (24, 43). Primary PAC were isolated from mice fed alcohol (25% of total calories) for 4 wk and from their pair-fed controls. DNA was isolated by using the wizard Genomic DNA purification kit (Promega), and bisulfite reactions were performed with the EpiTect Bisulfite Kit (Qiagen) under conditions that allowed for complete conversion of unmethylated cytosines to uracil, but not 5-methylcytosines. Subsequently, bisulfite-treated DNA was amplified by using primers (see Table 1) designed by MethPrimer (23) to span areas of CpG islands in the promoter of Slc5a6. Primer sequences were designed to exclude CG dinucleotides. The bisulfite-modified DNA was amplified by PCR, under the following conditions: 3 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C; and finally 20 min at 72°C. Amplified products were cloned into the pGEM-T easy vector via TA cloning (Promega). At least 10 clones were sequenced (per amiplocum of control and alcohol) and data represented are from three independent experiments from multiple sets of mice. The sequence was analyzed with the QUAMA (QUantification tool for Methylation Analysis) tool (20).

Statistical analysis. Uptake data with mouse primary PAC are means ± SE of at least three separate experiments from different mice and are expressed as percentage relative to simultaneously performed controls. Uptake with the 266-6 cells is also mean ± SE from at least three separate experiments. Carrier-mediated biotin uptake was determined by subtracting uptake by simple diffusion from total uptake. Protein, RNA, and luciferase activity determinations were performed from at least three sets of samples prepared at different times. The Student’s t-test was used for statistical analysis, and P < 0.05 was considered statistically significant.

RESULTS

Physiological Aspects of Pancreatic Acinar Biotin Uptake Process

We examined the general characteristics of the pancreatic acinar biotin uptake process with regard to the role of extracellular Na⁺ and H⁺ (i.e., pH), the effect of incubation temperature, and the presence of biotin structural analogs as well as pantothenic acid and lipoate, and the saturability of the uptake process as a function of concentration. The role of extracellular Na⁺ in biotin uptake was tested by examining the effect of isosmotic replacement of Na⁺ with choline on the initial rate of [³H]biotin (5 nM) uptake. The results showed a significant (P < 0.01 for both) increase in biotin uptake by 266-6 cells (171.2 ± 7 and 61.9 ± 2 fmol·mg protein⁻¹·min⁻¹) and by freshly isolated primary mouse PAC (83.1 ± 13.4 and 35.1 ± 15 fmol/mg protein) in the presence of Na⁺ compared with its absence, respectively. The effect of pH was examined by testing the initial rate of biotin (5 nM) uptake at pH 7.4 and 5.5 with results showing a significantly higher (P < 0.01 for both) uptake at pH 7.4 than pH 5.5 in both 266-6 cells (as percentage 100 ± 2 and 27.7 ± 12.5) and in mouse primary PAC (as percentage 100 ± 7.2 and 57.7 ± 6.1). The effect of the incubation temperature on initial rate of biotin uptake (5 nM) by 266-6 mouse PAC was also examined and found to be significantly (P < 0.01) higher at 37°C compared with 21 and 4°C (as percentage 100 ± 2.5, 65.8 ± 7.3, and 12.2 ± 3.7, respectively). In other studies, we examined the effect of unlabeled biotin, that of the biotin structural analog desthiobiotin, and that of pantothenic acid, and lipoate (all at 100 μM) on the initial rate (5 min) of carrier-mediated [³H]biotin (5 nM) uptake. The results showed that all compounds tested caused a significant (P < 0.01 for all) inhibition of [³H]biotin uptake (as percent of simultaneously performed control: 100 ± 2.2, 25.2 ± 1.9, 37.5 ± 0.1, 47.3 ± 3.0, and 40.2 ± 7.0, for control and presence of unlabeled biotin, desthiobiotin, pantothenic acid and lipoate, respectively). Finally, we examined the initial rate of biotin uptake by 266 cells as a function of substrate concentration in the incubation medium and obtained evidence for the existence of a saturable component (Fig. 1). Kinetic parameters, the apparent Km and Vmax, of the saturable component were 17.3 ± 6.3 μM and 1,409 ± 172.6 fmol·mg protein⁻¹·min⁻¹, respectively. These findings suggest the involvement of a Na-dependent carrier-mediated process that bears the characteristics of the involvement of the SMVT in biotin uptake by PAC. Indeed, Western blotting and PCR studies showed that the SMVT system is significantly expressed in both primary mouse PAC and in 266-6 cells (Fig. 1B).

![Fig. 1. Uptake of biotin by cultured mouse pancreatic acinar (PAC) and 266-6 cells as a function of substrate concentration (A) the expression of sodium-dependent multivitamin transporter (SMVT) in mouse primary PAC and 266-6 cells by means of qPCR (left) and Western blot (right) (B). The 266-6 cells were incubated for 5 min at 37°C in K-R buffer pH 7.4 in the presence of micromolar (5–100 μM) concentrations of biotin. Uptake by the carrier-mediated system was calculated as described in MATERIALS AND METHODS. Data are means ± SE of at least 3 independent experiments. PCR was performed with mouse gene-specific primers for Slc5a6 and β-actin; cDNA was synthesized from RNA isolated from 266-6 cells and mouse primary PAC. Western blot was performed by using total cells lysate of 266-6 cells and mouse primary PAC.](http://ajpgi.physiology.org/)
Effect of Chronic Alcohol Exposure of Mouse-Derived Pancreatic Acinar 266-6 Cells on Biotin Uptake: In Vitro Studies

We examined the effect of chronic exposure of alcohol [50 mM, 96 h (32, 44, 47)] on the mouse-derived pancreatic acinar 266-6 cells on the initial rate of biotin (5 nM) uptake. The result showed a significant inhibition ($P < 0.01$) in carrier-mediated biotin uptake by cells chronically exposed to alcohol compared with control (no alcohol) cells (Fig. 2A). Next we examined the effect of chronic alcohol exposure on the expression of the mouse SMVT protein and found a significant ($P < 0.05$) reduction in level of expression of the transporter in cells chronically exposed to alcohol compared with control cells (Fig. 2B).

In another study, we examined the effect of chronic alcohol exposure on the expression of $Slc5a6$ mRNA. The results showed a significant ($P < 0.01$) reduction in the level of expression of $Slc5a6$ mRNA in 266-6 cells chronically exposed to alcohol compared with controls (Fig. 2C). Although changes in mRNA level could be mediated via different mechanisms, a common mechanism involves changes in the transcription rate of the particular gene. To test this possibility, we examined the effect of chronic alcohol exposure of pancreatic acinar 266-6 cells transfected with the human $SLC5A6$ promoter. The results showed a significant ($P < 0.01$) inhibition in promoter activity in alcohol-exposed cells compared with control cells (Fig. 2D). The above findings show that chronic in vitro exposure of PAC to alcohol leads to an inhibition in biotin uptake and suggest that the effect is exerted, at least in part, at the level of transcription of the $SLC5A6$ gene.

Effect of Chronic Alcohol Feeding of Mice on Biotin Uptake by Pancreatic Acinar Cells: In Vivo Exposure Studies

To confirm the above-described findings on the effect of in vitro chronic alcohol exposure of PAC on the physiological and molecular parameters of pancreatic acinar biotin uptake process in an in vivo model, we examined the effect of chronic alcohol feeding of mice on biotin uptake by native PAC. In these studies mice were fed Lieber-DeCarli ethanol liquid diet (ethanol provided 25% of total ingested calories) for 4 wk, while control mice were pair-fed the same liquid diet but without alcohol (maltose-dextrin substituted for ethanol isocalorically) as described previously (44, 46). The results showed

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Fig. 2. Effect of chronic alcohol exposure of pancreatic acinar 266-6 cells on carrier-mediated [$^3$H]biotin uptake (A), and level of expression of SMVT protein (B), mRNA (C), and $SLC5A6$ promoter activity (D). Cells were exposed chronically to alcohol (50 mM, 96 h) and carrier-mediated [$^3$H]biotin uptake was determined. Level of protein expression was determined by Western blotting. Levels of mRNA were determined by qPCR. Full-length $SLC5A6$ promoter in pGL3-Basic was transfected into 266-6 cells exposed to alcohol followed by determination of luciferase activity (data were normalized relative to Renilla luciferase activity). Data are means ± SE of at least 3 independent experiments. *$P < 0.01$; **$P < 0.05$. 

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G944 ALCOHOL INHIBITS PANCREATIC BIOTIN UPTAKE

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a significant ($P < 0.01$) inhibition in the initial rate of biotin (5 nM) uptake by freshly isolated PAC from the alcohol-fed mice compared with those from pair-fed controls (Fig. 3A). We also examined, by means of Western blotting, the effect of chronic alcohol feeding on the expression of the mouse SMVT protein in PAC and found a significant ($P < 0.001$) reduction in SMVT protein in cells from the alcohol-fed mice compared with those from pair-fed controls (Fig. 3B).

Additionally, we examined (by means of quantitative PCR) the effect of chronic alcohol feeding of mice on the level of expression of Slc5a6 mRNA. The results showed a significant ($P < 0.05$) reduction of expression of Slc5a6 mRNA in PAC of mice chronically fed alcohol compared with their pair-fed controls (Fig. 3C). To determine whether the reduction in Slc5a6 mRNA level in the alcohol-fed mice is mediated via an effect on the rate of SLC5A6 transcription, we used our previously generated and characterized (37) transgenic mice that carry the human SLC5A6 promoters fused to the luciferase reporter gene. The results showed that chronic alcohol feeding of these transgenic mice led to a significant ($P < 0.05$) reduction in the activity of the SLC5A6 promoter in PAC compared with its activity in pair-fed transgenic control mice (Fig. 3D).

**Fig. 3. Effect of chronic alcohol feeding of mice on carrier-mediated [3H]biotin uptake (A), expression of SMVT proteins (B) and mRNA (C) of PAC, and activity of SLC5A6 promoter in transgenic mice (D). Primary PAC were isolated from mice fed alcohol (25% of total calories) for 4 wk and from their pair-fed transgenic mice controls. Carrier-mediated biotin uptake was determined. Level of protein expression was determined by Western blotting. mRNA levels were determined by qPCR. Luciferase activity of SLC5A6 promoters were determined and are presented as percentage relative to pair-fed transgenic mice controls. Data are means ± SE of at least 3 independent experiments from multiple sets of mice. *$P < 0.01$.**

**Effect of Chronic Alcohol Feeding on DNA Methylation Profile of the Mouse Slc5a6 Promoter, and on Level of Expression of the Transcription Factor KLF-4 in Pancreatic Acinar Cells**

The in vitro and in vivo exposure of PAC to alcohol lead to an inhibition in biotin uptake and this inhibition is, at least in part, mediated at the level of transcription of the SLC5A6 gene. Such an effect could be mediated via epigenetic mechanisms (e.g., DNA methylation) and/or via changes in expression of an important nuclear factor(s) that is necessary for normal activity of the SLC5A6 promoters. Nuclear factor KLF-4, which plays a major role in regulating the basal activity of the SLC5A6 promoter, has multiple cis-binding sites in the minimal (core)
promoter region and is a target of regulation of *SLC5A6* expression by external factors (36, 37). With regards to DNA methylation, this mechanism involves covalent addition of methyl groups to cytosine where hydrogen H5 is replaced with a methyl group. This usually takes place in short stretches of the vertebrate genome (500 bp-2 kb) that are enriched in CpG dinucleotides, known as CpG islands. These CpG islands are frequently in regions within gene promoters and are often involved in transcriptional regulation. In general, hypermethylation of CpG islands leads to gene silencing whereas hypomethylation leads to gene activation (9). Since chronic alcohol is known to alter the methylation status of other genes (3, 5, 6), we investigated whether chronic alcohol feeding of mice affects the methylation status of the *Slc5a6* promoters in PAC. We used the Eukaryotic Promoter Database (http://epd.vital-it.ch/) to predict the location of the mouse *Slc5a6* 5′ regulatory region, followed by MethPrimer (23) to screen for CpG islands as described previously (43). Our analysis suggests that the mouse *Slc5a6* 5′ regulatory region is between −6460 and −3699, whereas our MethPrimer analysis identified two CpG islands, one between −5040 and −5070 and the other between −5060 and −4386, using the translational start as +1 (Fig. 4).

Using results of the MethPrimer analysis we designed primers that did not contain CpG dinucleotide sequences (Table 1) and amplified the CpG regions in the promoter after bisulfite conversion of DNA isolated from PAC of mice chronically fed alcohol and their pair-fed controls. We then sequenced the identified regions as described previously (43). The sequences were analyzed by use of QUMA (20). The results showed significant (*P* < 0.05) alterations in DNA methylation at certain CpG sites in the −5060 to −4386 region of *Slc5a6* promoter as a result of chronic alcohol feeding (Fig. 4).

In other studies, we examined the effect of chronic alcohol feeding of mice on the expression of the transcription factor KLF-4 in PAC. Previous studies from our laboratory using mutational analysis, EMSA (electrophoretic mobility shift assay), and supershift assays, as well as a transfection approach, have established an important role for this transcription factor in driving the activity of the *SLC5A6* promoter (37). Expression of this nuclear factor appears to be inhibited by chronic alcohol exposure in other tissues (8). The results showed a significant (*P* < 0.05) decrease in the expression of KLF-4 at both the protein (Fig. 5A) and mRNA levels (*P* < 0.01) (Fig. 5C) in the alcohol-fed mice compared with their pair-fed controls. We also determined the expression level of KLF-4 protein and mRNA in 266-6 cells chronically exposed to alcohol compared with their controls. The results showed that chronic alcohol exposure of 266-6 cells leads to a significant decrease in the level of expression of KLF-4 at both the protein (*P* < 0.05) (Fig. 5B) and mRNA levels (*P* < 0.01) (Fig. 5D).

**DISCUSSION**

Biotin is essential for normal cellular metabolic reactions and plays important roles in immune function and gene expression (1, 21, 22, 38, 39, 49). Thus disturbances in normal biotin homeostasis lead to negative consequences on human health. The pancreas is an important organ of the digestive system and diseases of this organ lead to significant morbidity and mortality. Chronic alcohol exposure negatively impact many physiological aspects of PAC, but its effect on biotin uptake by these cells is not known, and therefore the aim of the study was to address this issue. Since the mechanism of biotin uptake by PAC is unknown, we addressed this issue first using as models the mouse-derived pancreatic acinar 266-6 cells and mouse primary PAC as model we showed that [³H]biotin uptake by these cells is Na⁺ dependent and is sensitive to the presence of unlabeled biotin, the biotin structural analog des-

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**Fig. 4.** Effect of chronic alcohol feeding of mice on *Slc5a6* promoter CpG island methylation. The methylation status of the mice *Slc5a6* promoter was assessed by bisulfite sequencing. DNA was isolated from primary PAC of alcohol-fed mice and from their pair-fed controls. Bisulfite reactions were performed. The bisulfite-modified DNA was amplified by PCR, using primers listed in Table 1 and cloned into the pGEM-T easy vector via TA cloning (Promega). At least 10 clones were sequenced (per amplicon of control and alcohol) and data represented are from 3 independent experiments from multiple sets of mice. The sequence was analyzed by using the QUMA (QUantification tool for Methylation Analysis) tool. Open and shaded bar indicates level of methylation of CpG islands in the control and alcohol-fed mice PAC. **P* < 0.05.
thiobiotin, as well as that of pantothenic acid and lipoate. The latter findings are hallmark features of the sodium-dependent multivitamin transporter, SMVT, a system that is markedly expressed in mouse (and human) pancreatic cells. The observed saturation in the initial rate of biotin uptake as a function of concentration by PAC further supports the involvement of a carrier-mediated mechanism for the vitamin uptake by these cells.

To study the effect of chronic alcohol exposure on biotin uptake by PAC, we used an in vitro model of chronic alcohol exposure (pancreatic acinar 266-6 cells) and an in vivo model of exposure (wild-type and transgenic mice carrying the SLC5A6 promoters). The results showed that chronic exposure of 266-6 cells to alcohol leads to a significant inhibition in carrier-mediated biotin uptake. The inhibition was associated with a significant reduction in the expression of SMVT at protein and mRNA levels. Although changes in mRNA levels could occur via different mechanisms, one such mechanism is a change in the transcription rate. The latter appears to be the case here as significant reduction in the activity of the SLC5A6 promoter transfected into 266-6 cells was observed upon chronic exposure to alcohol.

Chronic alcohol feeding of mice also led to a significant inhibition in biotin uptake by freshly isolated primary PAC, and this inhibition was again associated with a significant reduction in the expression of mouse SMVT at the protein and mRNA levels. Involvement of transcriptional mechanism in the latter effects was confirmed in studies using transgenic mice carrying the SLC5A6 promoters and chronically fed alcohol and significant reduction in the activity of the SLC5A6 promoter in PAC of transgenic mice fed alcohol chronically was observed compared with the activity in PAC of pair-fed transgenic animals was observed. The results of our complementary in vitro and in vivo approaches suggest that chronic alcohol feeding of mice negatively affects pancreatic acinar biotin uptake process, and at least part of this effect is exerted at the level of transcription of the SLC5A6 gene.

Inhibition of promoter activity of a given gene under certain condition could be mediated, among other things, via epigenetic mechanisms, e.g., DNA methylation, and/or via a de-
increase in the level of expression of a transcription factor(s) that is needed for promoter activity. With regards to DNA methylation, hypermethylation (especially in the promoter region) is in general associated with gene silencing whereas hypomethylation is associated with gene activation (9). Since chronic alcohol exposure is known to alter the methylation status of a number of other genes (3, 5, 6), we investigated whether it changes the methylation status of the Slc5a6 promoters in PAC. To address this issue, we first screened [using MethPrimer (23)] the predicted mouse Slc5a6 promoters for CpG islands and obtained evidence to suggest their existence. We then examined the effect of chronic alcohol feeding of mice on the methylation status of these CpG islands in the Slc5a6 promoter. The results showed significant alterations in the DNA methylation status of the Slc5a6 promoter in the alcohol-fed mice compared with their pair-fed controls; these findings also suggest that these alterations may contribute to the inhibition seen in Slc5a6 transcription upon chronic alcohol exposure. Further studies, however, are needed to confirm the latter suggestion.

We have previously shown that the transcription factor KLF-4 is important for basal activity of the SLC5A6 promoter, has multiple cis-binding sites in its minimal (core) promoter region, and is the target for regulation of SLC5A6 activity by external factors (36, 37). Thus we also examined whether chronic alcohol exposure is known to alter the methylation status of the Slc5a6 gene in mouse intestine and liver epithelial cells compared with their paired-fed controls; these findings also suggest that these alterations may contribute to the inhibition seen in Slc5a6 transcription upon chronic alcohol exposure. Further studies, however, are needed to confirm the latter suggestion.

In summary, our findings demonstrate for the first time that chronic alcohol exposure leads to a significant reduction in pancreatic acinar KLF-4 expression and intracellular zinc in the lung. Alcohol Clin Exp Res 37: 361–371, 2013.


