Chronic alcohol exposure affects pancreatic acinar mitochondrial thiamin pyrophosphate uptake: Studies with mouse 266-6 cell line and primary cells.

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Running title: Alcohol exposure and mitochondrial TPP uptake

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Abstract

Thiamin is essential for normal metabolic activity of all mammalian cells including those of the pancreas. Cells obtain thiamin from their surroundings and enzymatically convert it into thiamin pyrophosphate (TPP) in the cytoplasm; TPP is then taken up by mitochondria via a specific carrier the mitochondrial TPP transporter (MTPPT; product of the \textit{SLC25A19} gene). Chronic alcohol exposure negatively impacts the health of pancreatic acinar cells (PAC), but its effect on physiological/molecular parameters of MTPPT is not known. We addressed this issue using mouse pancreatic acinar tumor cell line 266-6 and primary PAC of wild-type and transgenic mice carrying the \textit{SLC25A19} promoter that were fed alcohol chronically. Chronic alcohol exposure of 266-6 cells (but not to its non-oxidative metabolites ethyl palmitate and ethyl oleate) led to a significant inhibition in MTPP uptake, which was associated with a decreased expression of MTPPT protein, mRNA, and activity of the \textit{SLC25A19} promoter. Similarly chronic alcohol feeding of mice led to a significant inhibition in expression of MTPPT protein, mRNA, heterogeneous nuclear RNA (hnRNA), as well as in activity of \textit{SLC25A19} promoter in PAC. While chronic alcohol exposure did not affect DNA methylation of the \textit{Slc25a19} promoter, a significant decrease in histone H3 euchromatin markers and an increase in H3 heterochromatin marker were observed. These findings show, for the first time, that chronic alcohol exposure negatively impacts pancreatic MTPPT and that this effect is exerted, at least in part, at the level of \textit{Slc25a19} transcription and appears to involve epigenetic mechanism(s).

Key words: Mitochondria, thiamin pyrophosphate, chronic alcohol exposure, pancreatic acinar cells, uptake.
Introduction

Thiamin in the form of thiamin pyrophosphate (TPP) is essential for normal cell function and metabolism. TPP accounts for about 85-90% of total cellular thiamin and plays an important role in numerous metabolic processes (it acts as a cofactor for the cytosolic transketolase, and for the mitochondrial α-ketoglutarate dehydrogenase, pyruvate dehydrogenase and branched-chain α-ketoacid dehydrogenase) (2). These enzymes are involved in ATP production, oxidative energy metabolism, and reduction of cellular oxidative stress. Thus, deficient/low intracellular levels of thiamin leads to impairment in oxidative energy metabolism (acute energy failure) and predisposes cells to oxidative stress (9); it also negatively impacts the structure and function of the mitochondria (3), an organelle that maintains most (~ 90%) of the cellular TPP content.

The health of the pancreas, an organ with essential exocrine and endocrine functions, is affected by a variety of factors and disease conditions. This organ maintains a high level of thiamin, and utilizes it in exocrine and endocrine functions (39, 40). Thus, a cellular deficiency/low level of this micro-nutrient is expected to exert negative effects on overall pancreatic physiology and health (39, 40). Like all other mammalian cells, PAC cannot synthesize thiamin and must uptake the vitamin from the circulation across the cell membrane. This uptake process is carrier-mediated and involves the high-affinity thiamin transporter-1 and -2 (THTR-1 and THTR-2; proteins encoded by the SLC19A2 and SLC19A3 genes) (46). In the cell, free thiamin is converted to TPP via an enzymatic process that occurs exclusively in the cytoplasm (11, 15). The majority (~ 90%) of the generated TPP is then taken up by the mitochondria [which is unable to synthesize TPP; (1)] for utilization in different metabolic reactions (4). Mitochondrial uptake of TPP involves a specific, carrier-mediated process that involves the MTPPT; product of the SLC25A19 gene (23, 31).
Chronic alcohol use is associated with an increased risk for development of pancreatic injury (36). The mechanism involved in mediating this effect is not fully understood but appears to be multi-factorial. Current belief suggests that chronic exposure to alcohol changes the resting state of the pancreas and lowers its defense mechanisms (10, 33, 37), thus predisposing the organ to the effect(s) of stress conditions/injurious agents or other cell biological events leading to injury (i.e., chronic alcohol exposure “sensitizes” or “primes” the pancreas to subsequent injury/insult) (10, 33, 37). Examples of the mechanisms through which chronic alcohol exposure is believed to exert it’s negatively effects on cell physiology include ATP depletion, oxidative stress, and alterations in gene expression (10, 21, 24). Not only alcohol, but its non-oxidative metabolites like ethyl palmitate and ethyl oleate (fatty acid ethyl esters), may also contribute to the adverse effects on pancreatic acinar cell physiology as has been shown previously (10, 17, 22, 26). Since cellular thiamin deficiency/sub-optimal levels also impact cellular oxidative energy metabolism (ATP production), cell oxidative state, and mitochondrial function, a negative effect of chronic alcohol exposure on PAC thiamin homeostasis could contribute to the adverse effects of alcohol on pancreatic physiology and health. We have previously shown that chronic alcohol exposure/feeding leads to a significant inhibition in thiamin uptake by PAC (44, 48). In the current study, we used as models mouse pancreatic acinar tumor cell line 266-6 that was chronically exposed to alcohol, and wild-type and transgenic mice carrying the SLC25A19 promoter fed alcohol chronically and examined the effect of chronic alcohol exposure on physiological/molecular parameters of MTPPT. Our findings showed, for the first time, that chronic exposure to ethanol (but not to its metabolites ethyl palmitate and ethyl oleate) to negatively impact MTPPT, and that the effect appear to be exerted, at least in part, at the level of
transcription of the *SLC25A19* gene and may involve epigenetic (histone modification) mechanisms.

**Materials and Methods**

**Materials:**

[^3H]-TPP (specific activity 1.8 Ci/mmol; radiochemical purity > 97%) was obtained from Moravek Biochemicals Inc (Brea, CA). Nylon filters (0.45-μm pore size) were from Millipore (Fisher Scientific). Unlabelled TPP and other chemicals, including molecular biology reagents, were from commercial vendors and were of analytical grade. Oligonucleotide primers were synthesized by Sigma Genosys (Sigma, Woodland, TX). MTPPT goat polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and pyruvate dehydrogenase E1 alpha subunit (PDH) monoclonal antibody was purchased from Abcam (Cambridge, MA).

**Methods:**

**Chronic exposure of 266-6 cells to alcohol:**

The mouse-derived pancreatic acinar tumour cell line 266-6 was obtained from American Type Tissue Collection (ATCC; Rockville, MD). The cells (between passages 4 and 20) were cultured in DMEM growth medium (supplemented with 10% FBS and an antibiotic cocktail). The cells were exposed to alcohol (50 mM), or to its non-oxidative metabolites ethyl oleate (50 μM) and ethyl palmitate (50 μM) chronically for 96 h as described before (38, 44). The concentration of alcohol and its metabolites used in this study were similar to those found in the blood of chronic alcoholics (16, 19).

**Generation of transgenic mice and luciferase analysis:**

The *SLC25A19* promoter (1,080 bp; (35)) was fused to the firefly luciferase reporter gene to generate a 3,090 bp DNA fragment. This DNA construct was used to create transgenic
founders by the method of pronuclear DNA injection utilizing the expertise of the transgenic mouse facility at the University of California at Irvine (UCI-TMF) as described by us recently (34, 41). Genotyping of mice was performed by PCR using specific primers for the SLC25A19 promoter and luciferase gene GL2 reverse primer, which would yield a single specific PCR product of 448 bp.

For measuring the firefly luciferase activity, the pancreas was removed and used for the isolation of acinar cells as described before (43, 44, 46). This was then followed by homogenization of the cells in ice-cold passive lysis buffer (Promega), and determination of firefly luciferase activity was carried out using a Luciferase Assay system (Promega). Luciferase activity was normalized relative to total protein concentration in each sample.

**Chronic alcohol feeding of mice and isolation of primary pancreatic acinar cells:**

Wild type and transgenic mice carrying the human SLC25A19 promoter fused to the firefly luciferase reporter gene were fed Lieber-DeCarli ethanol-liquid diet (Dyets, Bethlehem, PA) (ethanol was introduced gradually; ingested amount of calories contributed by ethanol were increased by 5% every day until we achieved 25% (30)] for 4 wks as described by us recently (42, 44). The Institutional Animal Care Use Committee (IACUC) of the Long Beach VA Medical Centre approved the use of these animals. Control transgenic mice littermates that were sex-matched and had similar basal firefly luciferase mRNA expression were pair-fed the same liquid diet in which maltose-dextrin replaced ethanol isocalorically. The mice were euthanized after 4 wks and the pancreas was removed. Wild-type mice were used for RNA and protein estimation studies, while SLC25A19 transgenic mice generated in this study were used for analysis of promoter activity by luciferase assay. Mouse primary PAC were isolated using Worthington collagenase type IV (Lakewood, NJ) digestion method as described previously
The viability of the isolated acinar cells were tested by Trypan blue exclusion method and was >90%.

Isolation of mitochondria from 266 cells:

Mitochondria from 266-6 cells were isolated as described by us previously (5) using a well-established and validated procedure (5, 20, 45). Briefly, alcohol-treated and control 266-6 cells were washed with ice cold PBS, scraped off and suspended in ice-cold buffer A (in mM 10 NaCl, 1.5 MgCl2 and 10 Tris, pH 7.5). Cells were then homogenized (using a dounce glass homogenizer), suspended in equal volume of uptake buffer B (in mM 70 sucrose, 1 KH2PO4, 5 sodium succinate, 5 HEPES, 220 mannitol, 0.1 EDTA, pH 7.4), then centrifuged twice at 500 g for 5 min at 4°C. The resulting supernatant was centrifuged again at 13,000 g for 20 min and the pellet was suspended in uptake buffer B, followed by sonication on ice, and centrifuged at 60,000 g for 90 min at 4°C. This step effectively eliminates remaining membrane/intact cells and the isolated mitochondria were used fresh for uptake investigations. Viability of mitochondria isolated by this method has been established in our laboratory (by showing a respiratory control ratio of 5.64 ± 0.75 when succinate was used as substrate), and by others (20).

Mitochondrial TPP uptake studies:

Mitochondria isolated from 266-6 cells was suspended in suspension buffer containing to obtain a protein concentration of ~15–20 µg/µl, and used for uptake studies immediately using a rapid-filtration technique (45). Briefly, 20 µl of mitochondria suspension was added to 80 µl uptake buffer [suspension buffer supplemented with 10 mM succinate (to maintain the function of mitochondria)] containing ³H-TPP (0.38 µM) and incubated at 37°C for 2 min. The uptake reaction was terminated by adding 1 ml of ice-cold stop solution [in mM: 100 KCl, 100 mannitol, and 10 KH2PO4, pH 7.4] to the uptake reaction mixture. The reaction mixture was then
subjected to rapid filtration on nylon membrane filter, washed twice with stop solution, and radioactivity was counted in a liquid scintillation counter.

**Western blot analysis:**

Western blot analysis was performed using whole cell lysate prepared from 266-6 cells exposed to alcohol chronically and alcohol fed wild-type mouse primary PAC and their respective controls as described previously (42-44). Sixty μg of protein were resolved in premade 10% Bis-Tris minigel (Invitrogen) and electroblotted onto immobilon polyvinylidene difluoride membrane (Fisher Scientific). The electro-blotted membranes were blocked with Odyssey blocking solution (LI-COR Bioscience, Lincoln, NE) for 1 h, followed by overnight incubation with primary antibody of MTPPT (1:100 dilution) goat polyclonal antibody along with pyruvate dehydrogenase E1 alpha subunit (PDH) (1:10,000 dilution) monoclonal antibody. The MTPPT and PDH immunoreactive bands were detected by using donkey anti-goat IRDye-800 for MTPPT and goat anti-mouse IRDye 680 secondary antibodies (1:30,000 dilution). Signals were detected with the Odyssey infrared imaging system (LI-COR Bioscience) and quantified with LI-COR software and normalized to PDH as an internal control.

**Quantitative PCR analysis:**

Total RNA (2 μg) isolated from primary mouse PAC and 266-6 cells were digested with DNase I (Invitrogen). The DNA free RNA was then converted to cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The mice *Slc25a19* and ARPO gene, were amplified using gene-specific primers for mRNA/hnRNA (Table 1) for quantitative PCR (qPCR) analysis using conditions described previously (43, 44). The ARPO was used as endogenous control and the data was quantified using relative relationship method (32).

**Transfection and reporter gene Assay:**
The *SLC25A19* full-length promoter-luciferase reporter construct in pGL-3 Basic vector used in this study was generated previously (35). 266-6 cells in 12-well plates at less than 80% confluency were co-transfected with 2 μg of *SLC25A19* full-length promoter-luciferase reporter construct and 100 ng of pRL-TK (transfection control plasmid *Renilla luciferase*-thymidine kinase) (Promega, Madison, WI) using lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. After 24 h of transfection these cells were exposed to alcohol (50 mM for 96 h) as described above and luciferase activity was measured using the Dual Luciferase Assay system (Promega) as per manufacturer's instructions.

**DNA methylation analysis:**

The methylation status of mouse *Slc25a19* promoter was analyzed by bisulfite sequencing as described previously (29, 42). DNA was isolated from 266-6 cells exposed to alcohol and primary PAC isolated from mice fed alcohol (25% of total calories) for 4 wks and from their pair-fed controls using wizard Genomic DNA purification kit (Promega). Bisulfite reactions were performed using EpiTect Bisulfite Kit (Qiagen) resulting in conversion of all the unmethylated cytosines to uracil, but not the 5-methylcytosines. The bisulfite-treated DNA was PCR amplified using primers (see Table 1) designed by MethPrimer (28) which span areas of CpG islands in the promoter of *Slc25a19*. Primer sequences were designed to exclude CG dinucleotides. PCR conditions for amplification was: 3 min at 95°C; 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C; and finally extension of 20 min at 72°C. Amplified products were cloned into the pGEM-T easy vector via TA cloning (Promega). A minimum of ten clones (per amplicon of control and alcohol) were sequenced and data represented are from 3 independent experiments from multiple sets of samples. The sequence was analyzed using QUMA methylation analysis tool (25).
Histone modifications:

ChIPs were performed using SimpleChIP™ Enzymatic Chromatin IP Kit (Agarose Beads) (Cell Signaling, Danvers, MA) as per manufacturer’s recommendation. The antibodies for H3 histone modifications (H3, H3K4me3, H3K9Ac, H3K27me3 and IgG) were purchased from Millipore. Briefly, 266-6 cells (control and alcohol exposed) were crosslinked using formaldehyde (final concentration of 1 %) for 10 mins. The cross-linking was stopped by addition of 0.1 volume glycine, washed with PBS containing protease inhibitor cocktail (PIC) and nuclei was isolated from the scraped cells following the manufacturer’s protocol. The nuclei were digested with micrococcal nuclease followed by sonication. An aliquot of the sonicated chromatin preparation was purified using DNA spin columns provided with the kit and checked for chromatin digestion and concentration. Agarose gel electrophoresis of the digested chromatin showed the presence of DNA fragments of approximately 150-900 bp. 5 µg of chromatin was diluted in 500 µl Chip buffer containing PIC and 10 µl of this sample was stored to be used as 2% input in qPCR. The chromatin preparation was immunoprecipitated (IP) overnight at 4°C in Chip buffer containing PIC using specific antibodies for H3, H3K4me3, H3K9Ac, H3K27me3 and IgG (IgG was used as negative control). The IP samples and the 2% input were purified following the manufacturer’s protocol. The 2 µl of purified DNA from alcohol exposed and control 266-6 cells were subjected to qPCR analysis using primers that spans -266 to -137 and -131 to +10 (TSS = +1) region of the mouse Slc25a19 promoter (see Table-1). The data was normalized to percent input and represented percent of enrichment relative to H3 compared to the control.). No amplification was detected in IgG negative control.
Statistical Analysis:

Uptake data with isolated mitochondria from 266-6 cells is mean ± SE of a minimum of three separate experiments and are expressed as percentage relative to simultaneously performed controls. MTPP uptake was determined by subtracting simple diffusion from total uptake. Protein, mRNA, hnRNA, luciferase activity and histone modification determinations were performed from at least three sets of samples prepared at different occasions. The Student's t-test was used for statistical analysis, and $P < 0.05$ was considered statistically significant.
Results

Chronic alcohol exposure of pancreatic acinar tumor cell line 266-6 inhibits MTPP uptake:

Chronic exposure of mouse-derived pancreatic acinar tumor cell line 266-6 to alcohol [ethanol; 50 mM, 96 h; (16)] resulted in a significant inhibition ($P < 0.01$) in carrier-mediated MTPP uptake by freshly isolated mitochondria (Fig. 1A). On the other hand, chronic exposure (96 h) of the cells to the alcohol non-oxidative metabolites ethyl oleate and ethyl palmitate [both at 50 µM; (19)] was without an effect on MTPP uptake (106 ± 7.2 % and 110 ± 9.0 % relative to simultaneously performed controls respectively). Thus, we focused on the effect of chronic exposure of PAC to ethanol on MTPP uptake in all subsequent investigations.

In other studies, we examined the effect of chronic alcohol exposure of 266-cells on level of expression of the mouse MTPPT protein (Western blotting), with the results showing a significant ($P < 0.01$) reduction in the level of the MTPPT protein in compared to control cells (Fig. 1B). The level of expression of mouse endogenous Slc25a19 mRNA in 266-6 cells exposed chronically to alcohol was also analyzed (by qPCR using primers specific for the transporter) and found to be significantly ($P < 0.01$) lower than that of control cells (Fig. 2A) (exposure of the cells to the alcohol metabolites ethyl oleate and ethyl palmitate was again without an effect on level of expression of $Slc25a19$ mRNA; data not shown).

Although different mechanisms can mediate a change in mRNA level of a given gene, a predominant mechanism is via changes in the rate of transcription of that gene (i. e., changes in activity of gene promoter). Thus, we also examined the effect of chronic exposure of pancreatic acinar 266-6 cells to alcohol on activity of the human $SLC25A19$ promoter transfected into these cells. The results showed a significant ($P < 0.01$) reduction in activity of the $SLC25A19$ promoter in cells chronically exposed to alcohol compared to control cells (Fig. 2B). These findings
suggest that the effect of chronic exposure to alcohol on PAC MTPP uptake is exerted, at least in part, at the level of transcription of the *SLC25A19* gene.

**Chronic alcohol feeding of mice negatively affects pancreatic acinar MTPPT:**

To confirm our *in vitro* findings on the effect of chronic alcohol exposure of 266-6 PAC on physiological and molecular parameters of the MTPPT in an *in vivo* setting of alcohol exposure, we examined the effect of chronic alcohol feeding of wild-type and transgenic mice carrying the *SLC25A19* promoter on the level of expression of protein, mRNA, hnRNA and *SLC25A19* promoter activity (luciferase activity). Wild-type mice were fed the Lieber - DeCarli ethanol-liquid diet (25% of total ingested calories were provided by ethanol) for 4 wks; control mice were pair-fed the same liquid diet but without alcohol (ethanol was iso-calorically substituted with maltose-dextrin) as described previously (42, 44, 47). All molecular parameters were determined using freshly isolated PAC. The effect of chronic alcohol feeding of mice on the expression of the mouse MTPPT protein in PAC was analyzed by Western blotting. The results showed a significantly (*P* < 0.001) lower level of expression of MTPPT protein in PAC of mice chronically fed alcohol compared to their pair-fed controls (Fig. 3).

In another study, we examined (by means of qPCR) the effect of chronic alcohol feeding of mice on the level of expression of mouse endogenous *Slc25a19* mRNA in PAC. The results showed a significant (*P* < 0.01) reduction in the expression of the *Slc25a19* mRNA in PAC of mice chronically fed alcohol compared to pair-fed controls (Fig. 4A).

The level of hnRNA of a given gene reflects the rate of transcription of that gene (i. e., it reflects the activity of the promoter. To examine if chronic alcohol feeding of mice affects the transcription rate of the *Slc25a19* gene in PAC, we performed qPCR to determine the relative expression of mouse *Slc25a19* hnRNA in PAC of alcohol-fed mice and their pair-fed controls.
The results showed a significantly ($P < 0.01$) lower level of expression of the $Slc25a19$ hnRNA in alcohol-fed mice compared to pair-fed controls (Fig. 4B). To further confirm this finding, we generated transgenic mice carrying the $SLC25A19$ human promoter fused to firefly luciferase reporter gene and examined the effect of chronic alcohol feeding of these transgenic mice on the activity of the $SLC25A19$ promoter expressed in PAC; results were compared to those of transgenic mice that were pair-fed (littermates) the same diet but without alcohol. The results showed a significantly ($P < 0.01$) lower $SLC25A19$ promoter activity in PAC of transgenic mice chronically fed alcohol compared to activity in the pair-fed transgenic controls (Fig. 4C).

**Epigenetic changes in mouse $Slc25a19$ promoter in PAC due to chronic alcohol exposure:**

The above-described studies showed that *in vitro* and *in vivo* exposure of PAC to alcohol led to an inhibition in TPP uptake and this inhibition is, at least in part, mediated at the level of transcription of the $Slc25a19$ gene. Such an effect could be mediated via changes in expression of a nuclear factor(s) that is needed for activity of the $Slc25a19$ promoters or through epigenetic mechanisms (e.g., DNA methylation, histone modifications or both). Example of the former is the nuclear factor NF-Y, which plays an important role in regulating basal activity of the $SLC25A19$ promoter (35). Thus, we examined the effect of chronic alcohol exposure of mouse pancreatic acinar tumor cell line 266-6 on the expression of the transcription factor NF-Y. The results, however, showed no significant changes in the expression of this factor in 266-6 cells chronically exposed to alcohol compared to control cells (data not shown). We therefore moved to examine the possible involvement of epigenetic mechanisms in the effect of chronic alcohol exposure of PAC on $Slc25a19$ transcription. We focused on possible changes in DNA methylation at CpG islands and specific modifications in the N-terminal tails of histones since they represent important mechanisms through which a particular condition (including chronic
alcohol exposure) affects transcriptional activity of a particular gene (6-8, 12-14). To investigate if chronic alcohol feeding/exposure affects the methylation status of the Slc25a19 promoter of mouse PAC, we first identified and cloned the mouse Slc25a19 promoter. The predicted promoter region (-1076 to +154 relative to TSS) was identified using the EPD database (18), then amplified by PCR using forward and reverse adapter primer consisting of NheI and HindIII restriction sites (Tabel-1), and cloned in to the pGL3-Basic-luciferase vector to give pGL3-Slc25a19. The resulting clone was sequenced using GL2 and Rv3 primers (pGL3-Basic sequencing primers) and the nucleotide sequence has been deposited in GenBank (Accession No. KT225481). The pGL3-Slc25a19 was transfected into 266-cells and luciferase activity found to be significantly \( (P < 0.01; 200 \text{ folds}) \) higher than that of the pGL-3 Basic empty vector (Fig. 5A). This confirms the authenticity of the Slc25a19 cloned promoter. We then used Methprimer (28) to screen for CpG islands in the promoter region using the default parameters. Our analysis identified two closely placed CpG islands at -335 to -178 and -143 to -38 relative to TSS. These CpG islands in the Slc25a19 promoter were PCR amplified using the bisulfite converted DNA from control and alcohol fed/exposed mice PAC or 266-6 cells, cloned in pGEM-T Easy Vector and subjected to sequencing as described previously (42). The results showed no significant alterations in DNA methylation at the CpG islands of the Slc25a19 promoter as a result of chronic alcohol feeding/exposure. These findings suggest that mechanism(s) other than DNA methylation at the CpG islands is involved in mediating the inhibitory effect of chronic alcohol feeding/exposure on activity of the Slc25a19 promoter.

Role of histone modifications in mediating the inhibitory effect of chronic alcohol exposure on Slc25a19 transcriptional activity was examined in mouse pancreatic acinar tumor cell line 266-6 chronically (96 h) exposed to alcohol. Results of the Chip-qPCR analysis of
promoter regions -266 to -137 and -131 to +10 (TSS = +1) showed a significant ($P < 0.05$ for the -266 to -137 region, and $P < 0.01$ for the -131 to +10 region) decrease in the H3K4me3 and H3K9Ac euchromatin markers, whereas the heterochromatin marker H3K27me3 increased significantly ($P < 0.01$ for both the regions) (Fig. 5 B and C). These results indicate that chronic alcohol exposure favors heterochromatin pattern at the Slc25a19 promoter, which may contribute to the reduction in promoter activity and in level of expression of the MTTP transporter mRNA.

**Discussion**

This study examined the effects of chronic exposure of mouse pancreatic acinar tumor cell line 266-6 as well as native pancreatic acinar cells from mice fed alcohol chronically on physiological/molecular parameters of MTPPT. The MTPPT is responsible for delivering TPP (the major form of thiamin in cells) from the cytoplasm to the mitochondria (an organelle that contains and utilizes around 90% of total cellular TPP) as there is no synthesis of TPP in this organelle. Our approach employed both an *in vitro* model of chronic exposure to alcohol (mouse pancreatic acinar tumor cell line 266-6) and an *in vivo* model of exposure (mice fed the Lieber-DeCarli ethanol-liquid diet for 4 weeks with pair-feeding of controls).

The results showed that chronic exposure of 266-6 cells to alcohol (but not to its non-oxidative metabolites ethyl palmitate and ethyl oleate) led a significant inhibition in carrier-mediated TPP uptake by isolated mitochondria. Chronic alcohol exposure of 266-6 cells was also associated with a significant reduction in the level of expression of the MTPPT protein and mRNA (again, ethyl palmitate and ethyl oleate were without an effect on Slc25a19 mRNA). The finding that activity of the SLC25A19 promoter transfected into PAC was also significantly reduced by chronic alcohol exposure indicates that the suppression in MTPP uptake and
expression of the MTPPT mRNA is, at least in part, mediated at the level of transcription of the Slc25a19 gene.

In the in vivo model of alcohol exposure, chronic alcohol feeding of mice was associated with a significant reduction in the level of expression of PAC MTPPT protein, mRNA, and hnRNA. The latter finding again suggests that the effect of chronic alcohol exposure in vivo is, at least in part, mediated at the level of transcription of the Slc25a19 gene. This was confirmed in studies utilizing transgenic mice carrying the human SLC25A19 promoter we generated for this purpose. In the latter studies a marked reduction in the activity of the luciferase reporter gene in PAC of mouse fed alcohol chronically was observed compared to pair-fed controls. These results confirm and complement our in vitro findings and suggest that chronic alcohol exposure/feeding negatively impacts the MTPP uptake process and that the effect is most likely mediated at the level of transcription.

Promoter activity of a gene under a given condition could be regulated by various molecular mechanisms that include an increase or a decrease in the level of expression of a nuclear factor that is needed for promoter activity of the particular gene, and/or epigenetic mechanisms like histone modifications and DNA methylation. We investigated both of these possibilities for their role in mediating the inhibitory effect of chronic alcohol exposure on pancreatic acinar MTPP uptake. Our results, however, showed that in PAC the level of expression of the transcription factor that plays a major role in regulating basal transcriptional activity of SLC25A19, i.e., NF-Y (35), is not affected by chronic alcohol exposure.

As to possible involvement of epigenetic mechanisms (histone modifications and/or DNA methylation) in mediating the inhibitory effect of chronic alcohol exposure on Slc25a19 transcription, these mechanisms are known to be involved in mediating the effect of chronic
alcohol exposure on the expression of a variety of genes (12, 13, 42). With regards to histone modifications, Histone H3 protein is a major target of specific modification via addition or removal of four classes of chemical groups: methyl, acetyl, phosphate, and ubiquitin (27). We focused mainly on three specific histone modifications, trimethylation of histone 3 at the lysine 4 (H3K4me3), acetylation of histone 3 at the lysine 9 (H3K9Ac) as euchromatin markers, and trimethylation of histone 3 at the lysine 27 (H3K27me3) as a heterochromatin marker. The results showed that chronic alcohol exposure leads to a significant decrease in euchromatin formation (by decreasing H3K4me3 and H3K9Ac histone modifications), and an increase in the formation of heterochromatin (by decreasing H3K27me3). Increase in heterochromatin structure maintains the DNA in condensed form and thereby prevent the accessibility of transcriptional factors or chromatin associated proteins which leads to transcriptional repression of the associated genes (27). Our in vitro and in vivo results, showed no changes in the methylation status of the CpG islands in the SLC25A19 promoter in the alcohol exposed PAC compared to controls suggesting no role for DNA methylation.

Previous in vivo and in vitro studies from our laboratory have shown that chronic alcohol exposure/feeding negatively affects uptake of free thiamin by PAC across cell membrane and the effect is mediated at the level of transcription of the SLC19A2 and SLC19A3 genes that encodes the plasma membrane thiamin transporter-1 & -2, i. e., THTR-1 and THTR-2 (44, 48). When combined with the findings of the present study showing that chronic alcohol exposure also affect MTPPT, it becomes clear that the physiologic/molecular parameters of vitamin B1 transport into and within PAC are negatively impacted by chronic exposure to alcohol. Such adverse effects may impact energy metabolism and health of PAC leading to changes in their resting state and lowering of their defense mechanisms.
In summary, results of these investigations show, for the first time, that chronic alcohol exposure negatively impacts the physiological and molecular parameters of pancreatic MTPPT and that the effect appears to be mediated, at least in part, at the level of transcription of the SLC25A19 gene and may involve epigenetic mechanisms.
References


Figure legends

Fig. 1. Effect of chronic alcohol exposure of mouse pancreatic acinar tumor cell line 266-6 on mitochondrial [\(^3\)H]-TPP uptake (A), and level of expression of MTPPT protein (B). 266-6 cells were exposed chronically to alcohol (50 mM, 96 h) and carrier-mediated [\(^3\)H]-TPP uptake was determined in freshly isolated mitochondria. Level of protein expression was determined by Western blotting. Data are mean ± SE of at least 3 independent experiments. *P < 0.01.

Fig. 2. Effect of chronic alcohol exposure of mouse pancreatic acinar tumor cell line 266-6 on Slc25a19 mRNA (A) and on activity of the SLC25A19 promoter (B). RNA was isolated from 266-6 cells exposed chronically to alcohol. Mouse endogenous Slc25a19 mRNA was quantitated using qPCR. Full-length SLC25A19 promoter in pGL3-Basic were transfected into 266-6 cells were exposed to alcohol followed by determination of luciferase activity and data were normalized relative to Renilla luciferase activity. Data are mean ± SE of at least 3 independent experiments. *P < 0.01.

Fig. 3. Effect of chronic alcohol feeding of mice on expression of MTPPT protein in PAC. Level of protein expression was determined by Western blotting using total cell lysate from primary PAC isolated from mice fed alcohol (25 % of total calories) for 4 wks and from their pair-fed transgenic mice controls. mRNA was quantitated using qPCR. Data are mean ± SE of at least 3 independent experiments from different sets of mice. *P < 0.01, **P < 0.05.

Fig. 4. Effect of chronic alcohol feeding of mice on Slc25a19 mRNA (A) and hnRNA (B), and on activity of the human SLC25A19 promoter expressed in transgenic mice (C) in
PAC. RNA was isolated from PAC of alcohol fed mice and their pair-fed controls. Mouse endogenous Slc25a19 mRNA was quantitated using qPCR. Luciferase activity of SLC25A19 promoters was determined and is presented as percentage relative to pair-fed transgenic mice controls. Mouse Slc25a19 hnRNA was quantitated using qPCR. Data are means ± SE of at least 3 independent experiments from multiple sets of mice. *P < 0.01.

Fig. 5. (A) Confirmation of mouse Slc25a19 promoter luciferase activity, and (B) analysis of histone modifications. pGL3-Basic and mouse promoter luciferase construct- pGL3-Slc25a19 were transfected into mouse pancreatic acinar tumor cell line 266-6, luciferase activity was determined, and data were normalized relative to Renilla luciferase activity. 266-6 cells were exposed chronically to alcohol (50 mM, 96 h) and the formaldehyde cross-linked chromatin was immunoprecipitated using antibodies specific to Histone H3, H3K4me3, H3K9Ac and H3K27me3. DNA was purified from the immunoprecipitated complexes, followed qPCR of the purified DNA fragments. Mouse Slc25a19 promoter spanning the region -266 to -137 and -131 to +10 was amplified separately. The data was normalized relative to input DNA and expressed as percentage of enrichment relative to H3. *P < 0.01, **P < 0.05.
Fig. 1.

**A**

Carrier-mediated $[^{3}H]$ TPP uptake (% of simultaneously performed controls)

- Control
- Alcohol

**B**

Relative MTPPT protein expression (% of simultaneously performed controls)

- Control
- Alcohol

[Image of bar charts showing data]
Relative expression of the endogenous Slc25a19 mRNA (% relative to simultaneously performed control) for Control and Alcohol.

Relative Slc25a19 luciferase activity (% relative to simultaneously performed control) for Control and Alcohol.

Fig. 2.
Fig. 3.
Fig. 4.
A

Mouse Slc25a19 luciferase activity

B

Histones modifications Slc25a19 promoter (-266 to -137)

C

Histones modifications Slc25a19 promoter (-131 to +10)

Fig. 5.
**Table-1.** Primers used for qPCR, cloning of mouse *Slc25a19* promoter, bisulfite PCR primers for amplifying mice *Slc25a19* CpG islands and qPCR primers for histone modification studies.

<table>
<thead>
<tr>
<th>Primers used in the study: Forward and Reverse Primers (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primers for real time PCR</strong></td>
</tr>
<tr>
<td>ARPO mRNA: GCTGAACATCTCCCCCCTTCTC; ATATCCTCATCTGATTCCTCC</td>
</tr>
<tr>
<td><em>Slc25a19</em> mRNA: TCCAGATTGAACGCTGTG; GACAGCTCCGATGCCTATGGAC</td>
</tr>
<tr>
<td>ARPO hnRNA: GGCACTCCATGTTGGTCC; TTGACACAGCCCCCAC</td>
</tr>
<tr>
<td><em>Slc25a19</em> hnRNA: CTGCTGTGATCCTCTGTAG; ATCTTTCTGCTTTGTCTTC</td>
</tr>
<tr>
<td><strong>Primers for genotyping SLC25A19 transgenic mice.</strong></td>
</tr>
<tr>
<td>CCCTCGAGGTCAGCTGCTGCTGCTGGTGGTC; TTTATGTTTTTGGGTGCTTCCA</td>
</tr>
<tr>
<td><strong>Primers for cloning mouse Slc25a19 promoter</strong></td>
</tr>
<tr>
<td>CTAGCTAGCCCTGGCTGCTGCTGAAGCTTAC; CCAAGCTTTATACCTGCTGCTGCTGAC</td>
</tr>
<tr>
<td><strong>Primers for Slc25a19 promoter Bisulfite PCR analysis (two pairs)</strong></td>
</tr>
<tr>
<td>TTAATGAGAGGGTTTTTATAGAGGT; ATACCGCAACAGAAAACCCATAGAC</td>
</tr>
<tr>
<td>TTTTTAAGATTATTTGATTTGAAAAATTT; ACCCCCTTTAAAAACTTAAAAAACT</td>
</tr>
<tr>
<td><strong>Primers for histone modification studies of the Slc25a19 promoter</strong></td>
</tr>
<tr>
<td>-266 to -137: GCGGTATCTGGACAGTGACA; TGTAGCCCAACAGCAAAACTT</td>
</tr>
<tr>
<td>-131 to +10: TAAAAGCCCAACTTGCCATC; CTGTAATCTCCTCCGCTTCCA</td>
</tr>
</tbody>
</table>