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Cellular and molecular aspects of thiamin uptake by human liver cells: studies with cultured HepG2 cells

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Abstract

The liver is an important site for thiamin metabolism, utilization, and storage. Little is known about the mechanism of thiamin uptake by the human liver. In this study, we examined cellular and molecular aspects of the human liver thiamin uptake process using the human-derived liver HepG2 cells as a model system. Our studies showed that the initial rate of thiamin uptake to be: (1) Na^+-independent and occurs with no detectable metabolic alterations in the transported substrate, (2) highly pH-dependent with diminished uptake upon decreasing incubation buffer pH from 8.0 to 5.0, (3) higher following cell acidification compared to unacidified control cells, (4) saturable as a function of concentration with an apparent K_m of 7.7 ± 1.6 μM, (5) inhibited by the thiamin structural analogues oxythiamin and amprolium but not by the unrelated organic cations tetraethylammonium (TEA) and *N*-methylnicotinamide (NMN), and (6) inhibited in a concentration-dependent manner by the membrane transport inhibitor amiloride. Both of the recently cloned human thiamin transporters, i.e., *SLC19A2* and *SLC19A3*, were found to be expressed in liver HepG2 cells with the former being the predominant form. High promoter activity of the predominant form, i.e., *SLC19A2*, was detected in HepG2 cells, and the minimal region of the *SLC19A2* promoter required for its basal activity in these cells was found to be encoded in a sequence between −356 and −36 and has multiple putative cis-regulatory elements. Mutation of a number of these putative cis-elements diminished promoter activity of the *SLC19A2* minimal region. These results show the involvement of a specialized carrier-mediated mechanism for thiamin uptake by human liver HepG2 cells. In addition, *SLC19A2* was found to be the predominant thiamin uptake carrier expressed in these cells and its promoter displays a high level of activity in them.

Keywords: Thiamin uptake; Human liver; HepG2 cell; Transport mechanism; Gene expression; Gene promoter

1. Background

Thiamin (vitamin B1) is a member of the water-soluble vitamin family of micronutrients. Thiamin is essential for normal cellular functions, growth, and development. It plays a critical role in normal carbohydrate metabolism where it participates in the decarboxylation of pyruvic and α-ketoglutamic acids, and in the utilization of pentose in the hexose monophosphate shunt [1]. These reactions take place predominately in the liver. Thiamin deficiency in humans leads to a variety of clinical abnormalities including neurological and cardiovascular disorders [1–3]. Thiamin deficiency represents a significant nutritional problem in both developed and developing countries. In developed countries, thiamin deficiency has been reported in a high percentage of alcoholic and diabetic patients [4–9], in patients with renal and intestinal diseases [10–12], and in the elderly despite an average daily intake of the vitamin that exceeds the recommended requirement [13]. Thiamin deficiency also occurs in thiamin-responsive megaloblastic anemia (TRMA), an autosomal recessive disorder characterized by manifestations that include sensorineural deafness, and diabetes mellitus [14,15]. A genetic defect in the recently cloned thiamin transporter *SLC19A2* is believed to be the cause of TRMA [16–19]. Thus, studies that are aimed at increasing our understanding of how to optimize thiamin body homeostasis are of significant importance.

All human cells, including those of the liver, lack the ability to synthesize thiamin. Thus, they must obtain the

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vitamin from exogenous sources via transport across the cell membrane. The liver is a major site of thiamin metabolism, utilization, and storage [20,21]; thus it plays an important role in regulating normal thiamin body homeostasis. Previous physiological and biochemical studies with rat liver preparations have shown thiamin uptake to be via a specialized, carrier-mediated mechanism [21–25]. This system transports thiamin in exchange for intracellular H^+ in an electroneutral manner, i.e., thiamin$^+$:H^+ exchange [22]. Very little, however, is known about the mechanism of thiamin uptake by the human liver. The aim of this investigation was to study cellular and molecular aspects of thiamin uptake by the human liver using human-derived liver HepG2 cells as a model system. The results show that thiamin uptake by human liver HepG2 cells occurs via a specialized, carrier-mediated mechanism. The study also shows that both of the recently cloned human thiamin transporters, i.e., *SLC19A2* and *SLC19A3* [16–19,26,27], are expressed in liver HepG2 cells with the level of expression of *SLC19A2* being considerably higher than that of *SLC19A3*. In addition, the results show that the recently cloned *SLC19A2* promoter is highly active in HepG2 cells and that the minimal region required for basal promoter activity is encoded in a sequence between −356 and −36. Furthermore, this region appears to have a number of putative cis-regulatory elements which, when mutated, affect promoter activity.

2. Methods

^{3}H-Thiamin (sp. act. 15–25 Ci/mmol; radiochemical purity >97%) was obtained from American Radiolabeled Chemicals Inc. (ARC), St. Louis, MO. Routine biochemicals and cell culture reagents were purchased from Sigma Chemical Company (St. Louis, MO), and Life Technologies Incorporated (Rockville, MD). Fetal bovine serum (FBS) was obtained from Omega Scientific (Tarzana, CA). Liver HepG2 cells were obtained from American Type Culture Collection (Rockville, MD). The radiochemical purity of stock ^{3}H-thiamin and the degree of metabolism of the transported thiamin into HepG2 cells were checked by means of thin-layer chromatography using cellulose gel pre-coated plates and a solvent system of isopropanol/0.5 M acetate buffer, pH 4.5/water (65:15:20, v/v) [28].

Liver HepG2 cells were grown and sub-cultured as described by us previously [29,30]. Cells were used between passages 29 and 39. Uptake studies were performed on confluent monolayers (3–5 days post-confluence) incubated at 37 °C in Krebs–Ringer buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 $MgSO_4$, 0.85 $CaCl_2$, 5 glucose, 5 glutamine, 10 HEPES and 10 MES, pH 7.4; unless otherwise specified). In certain experiments, cells were pretreated with the compound under study for a specific period of time prior to the addition of ^{3}H-thiamin and the start of uptake experiments. Unless otherwise specified, uptake was examined over a period of 3 min, i.e., initial rate. Uptake reaction was terminated by the addition of ice-cold buffer followed by rinsing and digestion of cells with NaOH, neutralization with HCl, and counting the radioactive level. Parallel wells were used to determine protein concentrations using a Bio-Rad kit (Richmond, VA).

In the study on the effect of cell acidification on thiamin uptake by HepG2 cells, confluent monolayers of these cells were incubated for 30 min in Krebs–Ringer buffer (K–R buffer) in which NaCl was replaced with NH_4Cl [28]. This was followed by removal of the buffer, washing the monolayers with KCl containing K–R buffer (KCl replaced NaCl), and incubating the cells with ^{3}H-thiamin in the same KCl containing K–R buffer for 3 min. Results were compared to ^{3}H-thiamin uptake by cells pre-incubated for 30 min with a KCl containing K–R buffer (instead of NH_4Cl containing K–R buffer), with the rest of cells being handled similarly.

2.1. Expression of SLC19A2 and SLC19A3 in liver HepG2 cells: semiquantitative RT-PCR

Semiquantitative PCR with specific primers for *SLC19A2* and *SLC19A3* was performed on first-strand cDNA generated from RNA (5 μg) isolated from mature confluent monolayers of HepG2 cells. The sequence of the primers was as follows, for *SLC19A2* they were: F 5′-GCTGCTGCAGTGTATATCATG-3′; R 5′-CACCAAA-TACTAGGGCATAG-3′. For *SLC19A3* they were: F 5′-ACCTGACCAGTGCAGAG-3′; R 5′-GGTAATGAT-GAAACTGATACATAC-3′. The RT reaction was primed with either an oligo-dT primer, a random hexamer, or a gene-specific primer. Each of these RT reactions were used in separate PCRs with gene-specific primers and identical results were obtained. A SuperScript™ First-Strand Synthesis for RT-PCR kit (Life Technologies) was used as described by the manufacturer. PCR conditions were: denaturation at 95 °C for 5 min; 30 cycles of 95 °C denatured for 30 s, 56 °C annealing for 30 s, 72 °C extension for 1 min, and a final 7 min extension at 72 °C. The PCR product was separated through a 2% agarose gel with images captured using an Eagle Eye™ II system (Stratagene, La Jolla, CA). The amplified RT-PCR products were normalized to the amplified β-actin RT-PCR product using the densitometry program on the Eagle Eye™ II System.

2.2. Promoter analysis: cell transfection and firefly luciferase assay

Four micrograms of *SLC19A2* promoter deletion constructs generated in our laboratory in a previous study ([31]; see Table 1) were transfected separately into human liver HepG2 cells using Lipofectamine reagent (Life Technologies, Gaithersburg, MD) as described by us previously

[31,32]. To normalize for transfection efficiency, the cells were co-transfected with 100 ng of pRL-TK (Promega) plasmid along with the promoter constructs. Total cell lysate was prepared from cells 24 h post-transfection and firefly luciferase activity was assayed using the Dual luciferase kit (Promega) and a Turner Design 20/20 Luminometer (Sunnyvale, CA). The activity was normalized to the Renilla luciferase activity from pRL-TK in the same extract.

2.3. Mutational analysis

Mutations were introduced into the *SLC19A2* minimal promoter-luciferase constructs using a Stratagene site-directed mutagenesis kit and the manufacturers protocols. The sequences were verified by the Laragen Sequencing Facility (Los Angeles, CA). The mutated constructs were then transiently transfected into HepG2 cells using the identical method described earlier.

2.4. Statistical analysis

All transport data described in this paper are the result of multiple separate uptake determinations and are expressed as mean ± S.E. in mol/mg protein/unit time. Kinetic parameters of the saturable component of thiamin uptake (i.e., V_{max} and apparent K_m) were determined using a computerized model of the Michaelis–Menten equation as described by Wilkinson [33]. Data on promoter activity are expressed as mean ± S.E. of at least three independent experiments and are given as fold expression over pGL3-Basic expression set arbitrarily at one. Data were analyzed by regression analysis and the Student's *t*-test.

3. Results

3.1. General characteristics of thiamin uptake by human liver HepG2 cells

Uptake of thiamin (15 nM) by HepG2 cells as a function of time was linear for 3 min of incubation and occurred at a rate of 9.2 fmol/mg protein/min (data not shown). Uptake during this period occurred without metabolic alterations as 97% of the transported radioactivity was found, by thin-layer chromatography (see Methods), to be in the form of intact thiamin.

The role of Na^+ in the incubation medium in thiamin uptake by HepG2 cells was also examined by testing the effect of its isosmotic replacement with another monovalent cation (Li^+) or with mannitol on the vitamin uptake. The results showed that such replacement had no significant effect on the initial rate of thiamin (15 nM) uptake (61.4 ± 1.9, 69.5 ± 0.8, and 69.6 ± 1.8 fmol/mg protein/3 min in the presence of Na^+ (control), Li^+, and mannitol, respectively). The effect of pretreating HepG2 (for 30 min) cells with 0.5 or 1 mM of the Na–K–ATPase inhibitor ouabain on uptake of 15 nM thiamin was also examined. The result showed no effect of such treatment on thiamin uptake (76.5 ± 3.6, 74.7 ± 0.5 and 77.5 ± 0.5 fmol/mg protein/3 min, in the absence and presence of 0.5 and 1 mM ouabain, respectively).

In another study, we investigated the effect of changing incubation buffer pH and intracellular pH on thiamin uptake by HepG2 cells. Decreasing incubation buffer pH from 8.0 to 5.0 was found to be associated with a progressive decrease in the initial rate of thiamin (15 nM) uptake (Fig. 1). Lowering the intracellular pH of HepG2 cells (achieved as described in Methods) led to a significant ($P<0.01$) increase in thiamin (15 nM) uptake compared to control (80.7 ± 1.3 and 281.8 ± 3.4 fmol/mg protein/3 min for control and acidified cells, respectively). We also examined the effect of the membrane transport inhibitor amiloride on thiamin (15 nM) uptake by HepG2 cells. The results showed amiloride to cause significant ($P<0.01$ for all) inhibition in thiamin uptake (72.6 ± 1.1, 61.7 ± 0.7, and 53.0 ± 0.9 fmol/mg protein/3 min for control and in the presence of 0.1 and 0.5 mM amiloride, respectively).

We also examined the effect of incubation temperature on the initial rate of thiamin (15 nM) uptake. The results showed a significant ($P<0.01$) decrease in the vitamin uptake upon lowering the incubation temperature from 37 to 4 °C (72.8 ± 4.9 and 14.6 ± 0.8 fmol/mg protein/3 min, respectively).

3.2. Evidence for involvement of a specialized, carrier-mediated mechanism for thiamin uptake by HepG2 cells

Two sets of studies were performed to address this issue. In the first study, we examined the initial rate of thiamin

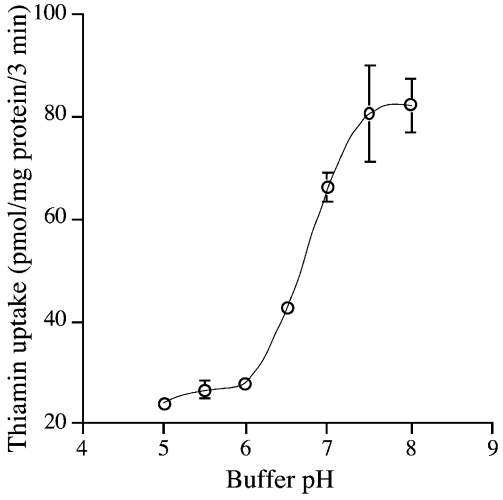

Fig. 1. Effect of incubation buffer pH on thiamin uptake by liver HepG2 cells. Monolayers were incubated in Krebs–Ringer buffer of varying pH at 37 °C. ^{3}H-Thiamin (1 μM) was added to the incubation medium at the onset of incubation. Initial rate of uptake (3 min) was then determined. Data are mean ± S.E. of four to six separate uptake determinations.

uptake (3 min) as a function of substrate concentration in the bathing medium (0.015–20 μM). The results showed thiamin uptake to include a saturable component (Fig. 2). Uptake by this component was determined by subtracting uptake by simple diffusion from total thiamin uptake by HepG2 cells at each substrate concentration. Uptake by simple diffusion was calculated from the slope of the line between uptake at high substrate concentration (1 mM) and the point of origin. The apparent K_m and V_{max} of the saturable component were then determined as described in Methods and found to be 7.7 ± 1.6 μM and 5.8 ± 0.6 pmol/mg protein/3 min, respectively.

In the second study, we tested the effect of a 25 μM concentration of the thiamin structural analogs oxythiamin and amprolium, and a 100 μM concentration of the unrelated organic cations tetraethylammonium (TEA), and *N*-methylnicotinamide (NMN) on the initial rate of ^{3}H-thiamin (15 nM) uptake by confluent HepG2 cells. The results showed that while thiamin structural analogs oxythiamin and amprolium cause a significant ($P<0.01$) inhibition in ^{3}H-thiamin uptake (64.6 ± 2.1, 49.3 ± 1.0, and 42.6 ± 0.8 fmol/mg protein/3 min for control and in the presence of oxythiamin and amprolium, respectively), no inhibition in thiamin uptake was observed in the presence of the unrelated organic cations (64.6 ± 2.1, 69.2 ± 1.5, and 69.4 ± 2.3 fmol/mg protein/3 min, for control and in the presence of TEA and NMN, respectively).

3.3. Expression of SLC19A2 and SLC19A3 in HepG2 cells

In this study, we determined the relative expression of the recently cloned human thiamin transporters *SLC19A2* and *SLC19A3* in HepG2 cells. We used semiquantitative PCR with specific primers for *SLC19A2* and *SLC19A3* as described in Methods. The results showed that although both of the human thiamin transporters are expressed in HepG2 cells, the level of expression of *SLC19A2* is approximately 2.5-fold higher than that of *SLC19A3* (Fig. 3).

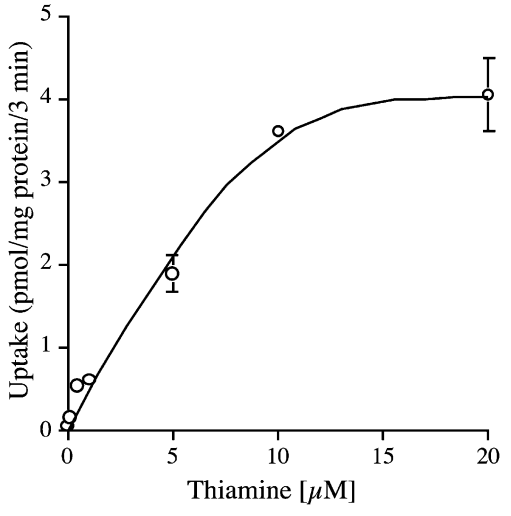

Fig. 2. Initial rate of thiamin uptake by liver HepG2 cells as a function of concentration. Monolayers were incubated for 3 min (initial rate) in Krebs–Ringer buffer pH 7.4 at 37 °C in the presence of different concentrations of thiamin. Uptake by the saturable component was determined as described in Results. Data are mean ± S.E. of four to seven separate uptake determinations.

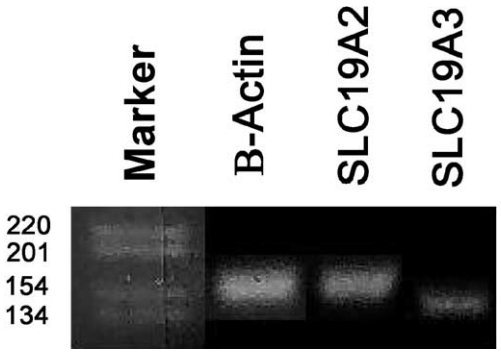

Fig. 3. Expression of *SLC19A2* and *SLC19A3* in liver HepG2 cells. Reverse transcription reaction was performed using 5 μg of total RNA isolated from HepG2 cells using oligo-dT primer and an Invitrogen Superscript kit. Subsequent PCRs were performed with equal amounts of first-strand cDNA product using gene-specific primers in separate reactions since the products were all similar in size. The final PCR product was run on a 2% agarose gel with a GibcoBRL 1 kb marker.

3.4. Analysis of SLC19A2 promoter in HepG2 cells

SLC19A2 appears to be the predominant thiamin transporter in HepG2 cells. Therefore, we examined the activity of its promoter in these liver cells. We used the recently cloned 5′ regulatory region of the *SLC19A2* gene (2210 bp) fused to the firefly luciferase reporter gene [31]. The results showed that the activity of the of *SLC19A2* promoter in DNA transfected HepG2 cells to be approximately 300-fold higher compared to cells transfected with vector alone (Fig. 4). We then extended the study to determine the minimal region of the *SLC19A2* promoter that is required for its basal activity in HepG2 cells. This was performed using deletion constructs of the *SLC19A2* promoter fused to the firefly luciferase reporter gene (Table 1) that were previously

Table 1
Relative position and size of the *SLC19A2* promoter and promoter deletion constructs

Construct name	Position	Size
PGL3-P	−2250 to −36	2214
PGL3-PΔ1	−2179 to −36	2143
PGL3-PΔ2	−1645 to −36	1609
PGL3-PΔ3	−450 to −36	414
PGL3-PΔ4	−429 to −36	393
PGL3-PΔ5	−356 to −36	320
PGL3-PΔ6	−317 to −36	281
PGL3-PΔ7	−275 to −36	239
PGL3-PΔ8	−139 to −36	103
PGL3-PΔ9	−108 to −36	72

A of the translation initiation codon is considered as 1.

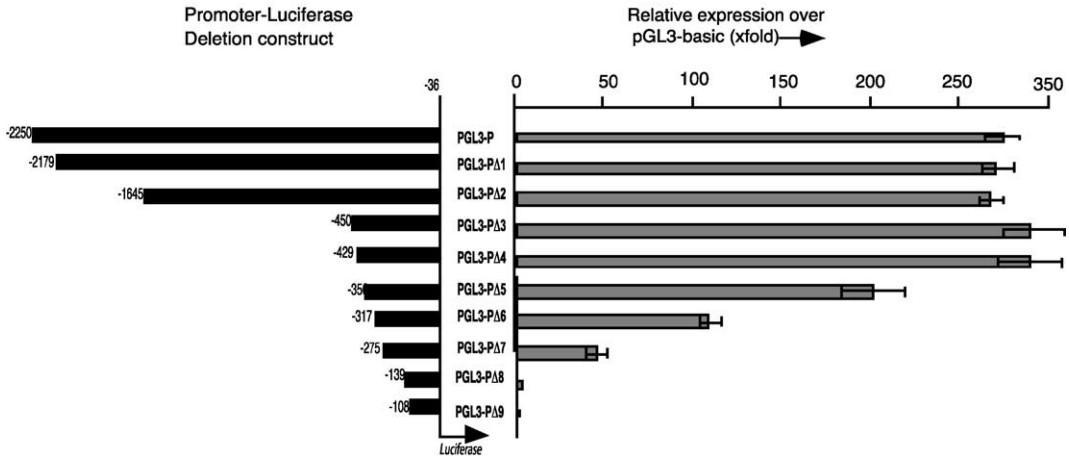

Fig. 4. Activity of *SLC19A2* promoter in HepG2 cells: identification of the minimal region required for basal activity. The size and position of different promoter-luciferase constructs are shown on the left. The results of a luciferase assay for each construct following transient transfection into liver HepG2 cells are shown on the right. Firefly luciferase activity was normalized relative to the activity of simultaneously expressed Renilla luciferase. The results are expressed relative to the pGL3-basic vector which was set at 1, and represent the average of three independent experiments.

generated in our laboratory [31]. The results showed the minimal region to be encoded by a sequence between −356 and −36 (using the A of the ATG initiator methionine as position +1) (Fig. 4). Using computer analysis (MAT Inspector and Ali Baba), we identified several putative cis-regulatory elements in this minimal promoter region which may be involved in the regulation of *SLC19A2* expression in human liver HepG2 cells. This includes putative binding sites for gut-enriched Krupple-like factor (GKLF), nuclear factor 1 (NF1), activator protein 1 (AP1) and stimulating factor 1 (SP1). To determine if any of these putative cis-elements is important for activity of the minimal promoter, we mutated these sites individually, then measured promoter activity of the mutated constructs as described earlier. We also examined the effect of introducing a non-specific mutation outside these putative cis-elements for comparison. The results showed that mutating the AP1 site at −311, the SP1 site at −347, or introducing the non-specific mutation at −278 has no effect on core promoter activity. However, mutating the NF1 site at −291 or the GKLF/SP1 at −234 led to a significant decrease ($P<0.01$ for all) in promoter activity (Fig. 5). The GKLF site and SP1 site at −234 overlap and the mutation could effect either site.

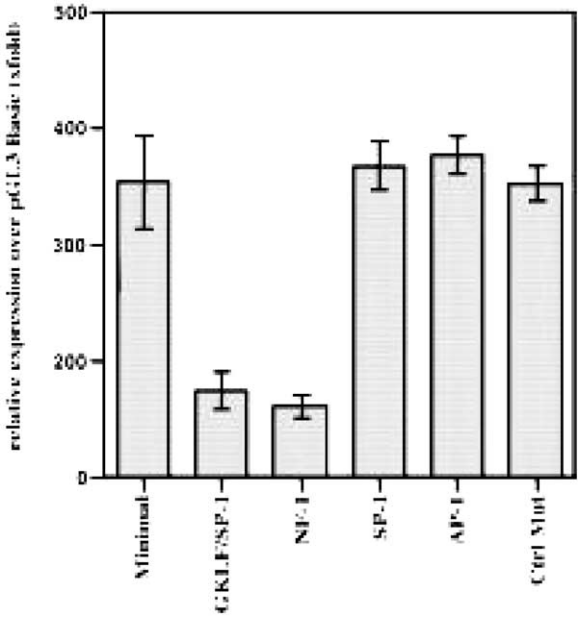

Fig. 5. Effect of mutating putative cis-elements in the *SLC19A2* minimal promoter region on promoter activity in HepG2 cells. Legend is as in Fig. 4. Mutations were introduced as described in Methods.

4. Discussion

The liver is a major site of thiamin metabolism, utilization, and storage. Little, however, is known about the mechanism of thiamin uptake by human liver cells. The present study was aimed at addressing this issue using the human-derived liver HepG2 cells as a model system. These cells were chosen because they have proven to be a good model in similar physiological investigations and have been used to characterize transport of other nutrients and substrates [29,30,34]. Uptake of thiamin by HepG2 cells was Na^+-independent as replacing Na^+ in the incubation medium with Li^+ or mannitol did not affect thiamin uptake and that ouabain (the inhibitor of Na^+-K^+-ATPase) failed to affect the uptake process. Uptake, however, was found to be highly pH dependent. Decreasing the incubation buffer pH from 8.0 to 5.0 was associated with a progressive decrease in thiamin uptake. On the other hand, cell acidification was associated with a marked increase in thiamin uptake. These findings are in line with the concept that thiamin is being transported via a thiamin$^+$:H^+ exchange mechanism as

suggested for the vitamin uptake by isolated basolateral membrane vesicles of rat liver [22]. According to this mechanism, increasing the magnitude of the outwardly directed H^+ gradient leads to an increase in thiamin uptake, while decreasing the magnitude of the gradient leads to a decrease in the vitamin uptake as observed in the present study. It is interesting to also report here that amiloride, the inhibitor of the Na^+:H^+ exchanger also inhibits thiamin uptake by HepG2 cells. A similar effect for amiloride on thiamin uptake has been observed in other cell types including intestinal and renal epithelial cells [28,35–38]. These findings raise the possibility that long-term use of this diuretic may lead to interference with normal thiamin body homeostasis. Such a situation may exist, for example, in the case of alcoholic patients with liver cirrhosis who are on long-term use of diuretics to treat their fluid overload. Many of these alcoholic patients are also thiamin-deficient but it is unclear if the use of diuretics contributes to this abnormality. Detailed investigation is required to address this issue.

The process of thiamin uptake by HepG2 cells appears to be carrier-mediated as indicated by the saturation in the substrate uptake as a function of concentration and the inhibition in ^{3}H-thiamin uptake by the thiamin structural analogs oxythiamin and amprolium. The fact that none of the unrelated organic cations affected the transport of the cationic thiamin indicates that the uptake process is specific in nature.

Our current investigation has also shed light on the molecular nature of the transport system(s) involved in thiamin uptake by the human liver HepG2 cells. Both of the recently cloned human thiamin transporters *SLC19A2* and *SLC19A3* were found to be expressed in HepG2 cells. However, the expression of *SLC19A2* was more predominant than that of *SLC19A3*, suggesting that the former transporter may play a more prominent role in thiamin uptake in liver cells. Focusing on the predominant *SLC19A2* transporter, we examined the promoter activity of this gene in liver HepG2 cells. This was performed by transfecting HepG2 cells with the previously identified *SLC19A2* promoter fused to the firefly luciferase reporter gene [31] and assaying for luciferase activity. Our results showed a very high level of activity of *SLC19A2* promoter in HepG2 cells. This activity is significantly higher than that previously observed in the human-derived intestinal epithelial Caco-2 cells (300- compared to 150-fold above activity of vector transfected cells; Ref. [31]). It is interesting to also mention here that the level of expression of endogenous RNA for *SLC19A2* in HepG2 and Caco-2 cells correlates well with the activity of the transfected promoter (unpublished observations). These findings show that *SLC19A2* is efficiently expressed in HepG2 cells, and thus, may play an important role in the normal liver thiamin uptake process. In analyzing the activity of the *SLC19A2* promoter in HepG2 cells, we also sought to determine the minimal region of the promoter that is required for its basal activity in this cell type. The results showed the minimal region to be encoded in a sequence between −356 and −36 (relative to the A of the ATG initiation codon). This region was found to have a CAAT box at position −305, is GC-rich, and has several putative cis-elements including NF1, AP1, GKLF, and SP1 that may be involved in the regulation of transcription of the *SLC19A2* gene in liver cells. To test for a possible involvement of these elements in the activity of the minimal promoter in liver cells, we mutated these putative sites individually and examined promoter activity of the mutated constructs in HepG2 cells. We also examined the effect of introducing a non-specific mutation outside these elements on promoter activity of the minimal region. The findings showed that while the non-specific mutation and the mutations introduced at the AP1 or the SP1 site had no effect on activity of the minimal promoter region, mutating the NF1 or GKLF/SP1 site led to a significant decrease in promoter activity. These results suggest that the latter cis-elements may play a role in the functionality of the *SLC19A2* promoter in liver cells. Further studies are required to confirm this suggestion. It is of interest to mention here that a similar promoter region was found to be responsible for basal activity of the *SLC19A2* gene in intestinal epithelial Caco-2 cells [31]. Mutational analysis of the minimal *SLC19A2* promoter in Caco-2 cells showed the GKLF/SP1 and NF1 sites were important for activity, however, the SP1 site was also determined to contribute to activity in these intestinal cells (unpublished observations). This may suggest that the observed differential activity of the *SLC19A2* promoter in Caco-2 and HepG2 cells is partly due to the SP1 cis-regulatory element. Additional studies, however, are needed to confirm this possibility.

In summary, results of this investigation demonstrate the involvement of a specialized carrier-mediated mechanism for thiamin uptake by human liver HepG2 cells. In addition, *SLC19A2* appears to be the predominant thiamin transporter expressed in these cells and its promoter displays a high degree of activity. Furthermore, the minimal region of the *SLC19A2* promoter required for its basal activity in HepG2 cells was found to be encoded by a sequence between −356 and −36 and contains a number of putative cis-elements which may be involved in regulating its activity.

Acknowledgements

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