Biotin uptake by human intestinal and liver epithelial cells: role of the SMVT system

Krishnaswamy Balamurugan, Alvaro Ortiz, and Hamid M. Said
Veterans Affairs Medical Center, Long Beach 90822; and University of California, Irvine, California 92697
Submitted 6 February 2003; accepted in final form 14 March 2003

Balamurugan, Krishnaswamy, Alvaro Ortiz, and Hamid M. Said. Biotin uptake by human intestinal and liver epithelial cells: role of the SMVT system. Am J Physiol Gastrointest Liver Physiol 285: G73–G77, 2003. First published March 19, 2003; 10.1152/ajpgi.00059.2003.—It has been well established that human intestinal and liver epithelial cells transport biotin via an Na⁺-dependent carrier-mediated mechanism. The sodium-dependent multivitamin transport (SMVT), a biotin transporter, is expressed in both cell types. However, the relative contribution of SMVT toward total carrier-mediated uptake of physiological (nanomolar) concentrations of biotin by these cells is not clear. Addressing this issue is important, especially in light of the recent identification of a second human high-affinity biotin uptake mechanism that operates at the nanomolar range. Hence, we employed a physiological approach of characterizing biotin uptake by human-derived intestinal Caco-2 and HepG2 cells at the nanomolar concentration range. We also employed a molecular biology approach of selectively silencing the endogenous SMVT of these cells with specific small interfering RNAs (siRNAs), then examining carrier-mediated biotin uptake. The results showed that in both Caco-2 and HepG2 cells, the initial rate of biotin uptake as a function of concentration over the range of 0.1 to 50 nM to be linear. Furthermore, we found that the addition of 100 nM unlabeled biotin, desthiobiotin, or pantothenic acid to the incubation medium had no effect on the uptake of 2.6 nM [³H]biotin. Pretreatment of Caco-2 and HepG2 cells with SMVT specific siRNAs substantially reduced SMVT mRNA and protein levels. In addition, carrier-mediated [³H]biotin (2.6 nM) uptake by Caco-2 and HepG2 cells was severely (P < 0.01) inhibited by the siRNAs pretreatment. These results demonstrate that the recently described human high-affinity biotin uptake system is not functional in intestinal and liver epithelial cells. In addition, the results provide strong evidence that SMVT is the major (if not the only) biotin uptake system that operates in these cells.

sodium-dependent multivitamin transport; biotin uptake; small interfering rna; Caco-2 cells; HepG2 cells

THE WATER-SOLUBLE VITAMIN biotin is essential for normal cellular functions, growth, and development (1, 22). Biotin acts as a coenzyme for five carboxylases that are involved in a variety of metabolic reactions including fatty acid biosynthesis, gluconeogenesis, and catabolism of several branched chain amino acids and odd-carbon-chain fatty acids. Deficiency of biotin leads to a variety of clinical abnormalities including neurological disorders, growth retardation, and skin abnormalities (1, 22). The incidence of biotin deficiency and suboptimal levels have been reported with increasing frequency in recent years and occurs in a variety of conditions such as inborn errors in biotin metabolism and transport, following long-term use of parenteral nutrition, chronic use of certain anticonvulsant medications, and during pregnancy (8, 11, 12, 22, 23, 25).

Humans and other mammals have lost their ability to synthesize biotin, and therefore, must obtain the vitamin from exogenous sources via intestinal absorption. Thus the intestine plays an important role in maintaining and regulating normal biotin body homeostasis. The liver also plays an important role in normal biotin nutrition and physiology, because it represents the major organ for biotin use and metabolism. With the use of a variety of intestinal and liver preparations, previous studies from our laboratory and others (10, 15–20) have characterized the mechanism of biotin uptake by enterocytes and hepatocytes. In both cell types, biotin uptake was shown to occur via an Na⁺-dependent carrier-mediated mechanism that has an apparent Km in the micromolar range (10, 15–20). Subsequent investigations (15–18) have shown that this system is also used by the unrelated water-soluble vitamin pantothenic acid and the metabolically important substrate lipoate; thus it was referred to as the sodium-dependent multivitamin transport system (SMVT). The molecular identity of SMVT has been delineated following its cloning from a number of human and animal tissues and functional identification of its cloned cDNAs in a number of heterologous systems (2, 14, 24).

More recently, a second human high-affinity Na⁺-dependent biotin uptake system, with an apparent Km in the nanomolar range (2.6 nM), has been described in peripheral blood mononuclear cells (PBMCs) (26) and in keratinocytes (6). Although the molecular identity of...
this system has not been established, functional impairment in this transporter is believed to be the cause of the recently identified genetic defect in biotin transport in a child (11). The latter study (11) has also suggested that the defect in biotin transport may not be limited to PBMCs but may also involve the biotin uptake process in the other tissues including the small intestine and liver.

To date, however, little is known about the relative contribution of the human SMVT (hSMVT) toward total carrier-mediated uptake of physiological (nanomolar) concentrations of biotin by intestinal and liver cells and whether the recently described high-affinity biotin uptake system is functional in these cells. To address these issues, we examined biotin uptake at the low-nanomolar range in the human-derived intestinal Caco-2 cells and the liver HepG2 cells. The suitability of these two in vitro cellular model systems for studying the finer details of biotin uptake mechanisms has been previously established in our laboratory (10, 17).

We also used the new approach of small interfering RNA (siRNA) to selectively silence the hSMVT gene (via degradation of its mRNA), an approach that has been well established in recent years (7, 9). The results show that intestinal and liver epithelial cells do not have a functional high-affinity biotin uptake system. Rather, hSMVT appears to be the major (if not the only) biotin uptake system in human intestinal and liver epithelial cells.

MATERIALS AND METHODS

[3H]biotin (specific activity 58.2 Ci/mmol; radiochemical purity 97%) was obtained from DuPont New England Nuclear (Boston, MA). The culture medium and all cell culture ingredients were obtained from Sigma (St. Louis, MO). All other chemicals used in this investigation were of analytical grade and were obtained from commercial sources.

The human-derived intestinal epithelial Caco-2 cells and the liver HepG2 cells (passages 20 and 19, respectively, ATCC, Manassas, VA) used in this study were grown as described previously (10, 20). Uptake studies were performed on confluent cell monolayers 3–5 days following confluence. The initial rate of [3H]biotin uptake (i.e., 3 min; see Refs. 10 and 20) was examined in cells incubated in Krebs-Ringer buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 MgSO4, 0.85 CaCl2, 5 glucose, 5 glutamine, 10 HEPES, and 10 2-(y-morpheline)-ethane sulfonic acid, pH 7.4) at 37°C. Labeled and unlabeled biotin were added to the incubation medium at the onset of the uptake experiment, and the reaction was terminated by the addition of 2 ml ice-cold buffer followed by immediate aspiration. Cells were then rinsed twice with ice-cold buffer, digested with 1 ml of 1 NaOH, neutralized with HCl, and then counted for radioactivity. The protein content of cell digests was measured in parallel wells using a kit (Bio-Rad, Richmond, VA).

Pretreatment of monolayers with siRNAs. Two custom-made hSMVT gene-specific siRNAs (double-stranded RNAs of 21 nucleotides; siRNA-I: 5’-aa gctgggcatgtctacttt dTdT-3’; siRNA-II: 5’-aa tggctgctcggctgc dTdT-3’; GeneBank accession no. AF081571) were chemically synthesized by a commercial vendor (Qiagen-Xeragon, Germantown, MD). Both the sense and antisense strands of these two siRNAs were modified at their 3' ends to increase stability (5). The chosen sequence of the two siRNAs corresponded to the coding regions 51–71 and 195–215, respectively, to the first nucleotide of the start codon (ATG) of the hSMVT gene. Before their use in transfection studies, the siRNAs were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) at 1 min at 90°C followed by 1 h at 37°C. Transient transfection of subconfluent (~70%) Caco-2 and HepG2 cells with 1 µg siRNAs/well was performed using the oligofectamine reagents as per the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Control cells were transfected with scrambled siRNAs (5’-aa cgegccagaageacg dTdT-3’). Cells were maintained until 3–5 days following confluence, then used in the specific experiments. Uptake studies with these cells were performed as described earlier.

Semiquantitative PCR analysis. The PCR was used to determine the level (amount) of endogenous hSMVT in siRNA-pretreated and control cells. Total RNA was isolated from siRNA-pretreated and control Caco-2 and HepG2 cells using TRIzol reagent as per the manufacturer’s instructions (Life Technologies, Rockville, MD). Five micrograms of the total RNA were then reverse transcribed with oligo(dT) and random hexamer primers using Superscript II (Life Technologies). Five micrograms of the total RNA were then reverse transcribed with oligo(dT) and random hexamer primers using Superscript II (Life Technologies) enzyme. After the reverse transcription, three different dilutions were made and used for semiquantitative PCR assays. The hSMVT primers and the PCR conditions used were forward primer: 5’-CGATCTTAATTTACTGTTGGAGT-3’; reverse primer 5’-GGACAGCCACAGATCAAAGC-3’; and 95°C/10 min for 1 cycle and 95°C/30 s, 57°C/30 s, and 72°C/30 s for 22–30 cycles, respectively. For β-actin, the primers and the PCR conditions were F01: 5’-CATCTTGCTGCTGACCT-3’; reverse 5’-TAATGTCAGCACGATTTCC-3’, and the conditions were the same as mentioned above. A negative control without cDNA template was run with every assay. We also
measured the mRNA level of the unrelated human thiamine transporter THTR-1 in control and hSMVT siRNAs cells pretreated to confirm the specificity of the siRNAs used in the study. In all cases, the final PCR products were analyzed on 3% agarose gels and data were normalized relative to the human β-actin using the Eagle Eye II System (Stratagene).

**Western blot analysis.** Western blotting was used to determine the level (amount) of endogenous hSMVT protein in control and siRNA-pretreated cells. Membranous proteins isolated (21) from Caco-2 and HepG2 cells (~200 μg/lane) were resolved on a 10% SDS-PAGE and electroblotted on Hybond enhanced chemiluminescent nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were then blocked with 5% dried milk in phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 and were then incubated overnight at 4°C with specific rabbit polyclonal anti-peptide antibodies raised against the LYHACRGWGRHTVGELLMADRK peptide of the human (and rat) SMVT (alpha Diagnostics, San Antonio, TX). The specificity of these polyclonal antibodies has been demonstrated in our laboratory recently (13). Immunodetection was performed using goat anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase and an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Specific bands were quantitated using the Eagle Eye II System (Stratagene).

**Data presentation and statistical analysis.** Transport data presented in this paper are means ± SE of multiple separate uptake determinations and were expressed in terms of femtomoles per milligram of protein per 3 min. Uptake of biotin by the carrier-mediated process was calculated by subtracting the passive diffusion component (determined from the slope of the uptake line between a high pharmacological concentration of biotin in mM and the point of origin, i.e., multiplication of the slope by individual concentration) from total biotin uptake at each concentration. Statistical analysis was performed using the Student’s t-test, with statistical significance being set at 0.05 (P < 0.05). All transient transfection studies, semiquantitative PCR, and Western blot analysis were performed on at least three separate occasions with comparable results. Data from a representative set of experiments are presented.

**RESULTS AND DISCUSSION**

**Uptake of nanomolar concentrations of biotin by the human-derived intestinal Caco-2 and liver HepG2 cells.** Our aim in these studies was to determine whether the recently described high-affinity biotin uptake mechanism of the human PBMCs (26) is also functional in human intestinal and liver epithelial cells. To do so, we searched for evidence of saturation in the initial rate of biotin uptake (i.e., 3 min; see Refs. 10 and 20) as a function of concentration within the nanomolar range (0.1–50 nM). This range was chosen because it is within the capacity of the high-affinity biotin uptake system, which has a reported apparent K_m of 2.6 nM (26). The results showed carrier-mediated biotin uptake as a function of concentration within the stated range to be linear in both Caco-2 and HepG2 cells (r = 0.99 in both cases; Fig. 1, A and B) and occurred at a rate of 13.3 ± 2.7 and 33.0 ± 1.5 fmol·mg protein^{-1}·min^{-1}, respectively. The lack of saturation in biotin uptake over the concentration range, where the high-affinity carrier system supposedly functions (26), suggests that the latter system does not function in Caco-2 or HepG2 cells.

To further confirm this suggestion, we examined the effect of unlabeled biotin, the biotin structural analog desthiobiotin, and that of the unrelated compound pantothenic acid (all at 50 and 100 nM) on the initial rate of carrier-mediated [3H]biotin (2.6 nM) uptake. The results showed that none of the tested compounds...
significantly affected carrier-mediated [3H]biotin uptake by Caco-2 and HepG2 cells (Fig. 2). This is unlike the inhibition in the uptake of nanomolar concentration of [3H]biotin by unlabeled biotin reported in PBMCs (26). Our findings provide further support for the above-stated suggestion that the high-affinity biotin uptake system reported in PBMCs is not functional in human intestinal Caco-2 and liver HepG2 cells. Rather biotin uptake appears to be occurring via a carrier-mediated system that does not saturate at the nanomolar concentration range examined. This system could be SMVT that has an apparent $K_m$ in the micromolar range (10, 15–20) (see below).

Effects of selective silencing of the endogenous SMVT gene on carrier-mediated biotin uptake by Caco-2 and HepG2 cells. Our aim in these experiments was to examine the effect of selectively knocking down the endogenous hSMVT of Caco-2 and HepG2 cells on carrier-mediated uptake of nanomolar concentration of biotin. We elected to use the recently established approach of siRNA to silence the hSMVT gene, because this approach has been proven to be highly effective and selective in silencing a targeted gene (7, 9). Two hSMVT-specific siRNAs were used in our studies. First we verified that the siRNAs were able to silence the hSMVT gene in these cells. This was performed by determining the level of hSMVT mRNA by semiquantitative PCR in siRNA pretreated and control cells. The results showed that pretreating Caco-2 and HepG2 cells with siRNAs substantially reduced the level of the endogenous hSMVT mRNA compared with control cells (Fig. 3). mRNA levels of the human β-actin (Fig. 3) and the human thiamin transporter THTR-1 (data not shown), on the other hand, were not affected by the siRNA treatment, i.e., they were similar in siRNA-pretreated and control Caco-2 and HepG2 cells. The latter findings confirm the specificity of the selected siRNAs for SMVT. We also determined (by Western blot analysis) the level of the SMVT protein in siRNA-pretreated and control Caco-2 and HepG2 cells and found the level to be substantially reduced in the siRNA-pretreated cells compared with controls (Fig. 4). In contrast, no changes in the protein level of the unrelated thiamin THTR-1 were found in siRNA-pretreated and control Caco-2 and HepG2 cells (data not shown). These findings clearly demonstrate the effectiveness of our siRNA approach in selectively silencing the SMVT gene in these cells.

With the use of siRNA-pretreated Caco-2 and HepG2 cells, we then examined the initial rate of carrier-mediated uptake of 2.6 nM biotin and compared the results with that of controls. Our results (Fig. 5) showed that biotin uptake was severely (>85%; $P < 0.01$) inhibited in siRNA-pretreated Caco-2 and HepG2 cells compared with controls. These results clearly demonstrate that SMVT is the main (if not the only) carrier system for biotin uptake in these cells. It is worth mentioning here that uptake of pantothenic acid (another substrate for SMVT) was also severely inhibited in hSMVT siRNA-pretreated compared with control cells (data not shown).

In summary, our results demonstrate that the recently reported high-affinity biotin uptake system is not functional in human intestinal and liver epithelial cells. Rather, SMVT appears to be the main (if not the only) carrier system involved in biotin uptake in these cells.
REFERENCES


