Conditional knockout of the Slc5a6 gene in mouse intestine impairs biotin absorption

Abhishek Ghosal, Nils Lambrecht, Sandeep B. Subramanya, Rubina Kapadia, and Hamid M. Said

Departments of Medicine and Physiology/Biophysics, University of California, School of Medicine, Irvine, and Medical Research Service, Veterans Affairs Medical Center, Long Beach, California

Submitted 26 September 2012; accepted in final form 19 October 2012

Ghosal A, Lambrecht N, Subramanya SB, Kapadia R, Said HM. Conditional knockout of the Slc5a6 gene in mouse intestine impairs biotin absorption. Am J Physiol Gastrointest Liver Physiol 304: G64–G71, 2013. First published October 25, 2012; doi:10.1152/ajpgi.00379.2012.—The Slc5a6 gene expresses a plasma membrane protein involved in the transport of the water-soluble vitamin biotin; the transporter is commonly referred to as the sodium-dependent multivitamin transporter (SMVT) because it also transports pantothenic acid and lipoic acid. The relative contribution of the SMVT system toward carrier-mediated biotin uptake in the native intestine in vivo has not been established. We used a Cre/lox technology to generate an intestine-specific (conditional) SMVT knockout (KO) mouse model to address this issue. The KO mice exhibited absence of expression of SMVT in the intestine compared with sex-matched litters as well as the expected normal SMVT expression in other tissues. About two-thirds of the KO mice died prematurely between the age of 6 and 10 wk. Growth retardation, decreased bone density, decreased bone length, and decreased biotin status were observed in the KO mice. Microscopic analysis showed histological abnormalities in the small bowel (shortened villi, dysplasia) and cecum (chronic active inflammation, dysplasia) of the KO mice. In vivo (and in vitro) transport studies showed complete inhibition in carrier-mediated biotin uptake in the intestine of the KO mice compared with their control littersmates. These studies provide the first in vivo confirmation in native intestine that SMVT is solely responsible for intestinal biotin uptake. These studies also provide evidence for a casual association between SMVT function and normal intestinal health.

sodium-dependent multivitamin transporter; intestinal uptake; gene knockout

BIOTIN, a water-soluble vitamin, is required for normal cellular functions, growth, and development. The vitamin (a carboxyl carrier) acts as a cofactor for five carboxylases that catalyze critical steps in essential pathways in intermediary metabolism, e.g., gluconeogenesis, fatty acid synthesis, fatty acid entry into mitochondria for β-oxidation, and catabolism of odd-carbon fatty acids and branched chains amino acids (18, 21, 37). Additional roles for biotin in regulating normal immune function, cell proliferation, and gene expression have also been reported in recent years (reviewed in Refs. 21, 30). Thus, it is not surprising that deficiency of this essential micronutrient leads to negative health consequences, e.g., growth retardation, neurological disorders, dermatological abnormalities (reviewed in Ref. 42). Biotin deficiency and suboptimal levels occur in patients with inborn errors of biotin metabolism (6, 39), patients with inflammatory bowel diseases (10, 40), those on long-term therapy with anticonvulsant drugs (reviewed in Refs. 4, 14, 15), where inhibition in intestinal biotin absorption believed to be a contributing factor (33, 27), patients on long-term parenteral nutrition (11, 19), and in substantial numbers of alcoholics (5, 9), where impairment in intestinal biotin absorption process believed to be a contributing factor (38).

Humans and other mammals cannot synthesize biotin and, thus, must obtain the vitamin from exogenous sources via intestinal absorption. The human (mammalian) intestine is exposed to two sources of biotin: a dietary source, and a bacterial source, i.e., normal microflora of the large intestine (1, 45). Studies from our laboratory and others have used a variety of small and large intestinal preparations to show the involvement of a Na+-dependent, carrier-mediated process in biotin uptake (reviewed in Ref. 35). Subsequent studies showed that this transport process is also utilized by two other functionally unrelated nutrients, namely pantothenic acid, a water-soluble vitamin that is essential for the synthesis of coenzyme A and acyl carrier proteins, and lipoate, a potent intracellular and extracellular antioxidant (35, 26, 34). For this reason, the transport system that mediates this process is referred to as the sodium-dependent multivitamin transporter (SMVT). Other studies have shown that changes in dietary levels of biotin exert an adaptive regulatory effect on intestinal uptake of the vitamin (32, 28) although the same does not appear to apply in the case of changes in dietary levels of pantothenic acid on August 18, 2017 http://ajpgi.physiology.org/ Downloaded from http://ajpgi.physiology.org/ by 10.220.33.6 on August 18, 2017 http://ajpgi.physiology.org/ Downloaded from http://ajpgi.physiology.org/ by 10.220.33.6 on August 18, 2017
transport is particularly timely. Thus, in this study, we generated an intestinal specific (conditional) SMVT KO mouse model and used it to study biotin (and pantothenic acid) uptake in vivo. The results presented here provide evidence that the SMVT system is exclusively responsible for biotin uptake system in the native intestine in vivo. Also, an unexpected pathological intestinal phenotype was observed in the SMVT KO mice that support a role for functional SMVT in maintaining normal intestinal health.

MATERIALS AND METHODS

Materials. 

In vivo and in vitro intestinal uptake studies. In the in vivo intestinal uptake studies, we used a previously described jejunal loop approach (29). The loop (1 cm) was filled with 100 μL of Krebs-Ringer buffer containing 3H-biotin (or pantothenic acid, ascorbic acid), and uptake was measured 5 min later (uptake was linear during this period, data not shown). Substrate uptake was expressed in fmol/mg tissue wet weight/5 min.

In the in vitro uptake studies, isolated intestinal cells were prepared from the jejunum of adult mice (about 6–8 wk) as described before (29). Briefly, mice were euthanized, their jejunum was removed and washed, and the enterocytes were isolated by incubation (2 × 15 min) at 37°C with shaking in Hank’s balanced salt solution supplemented with 0.5 mM dithiothreitol and 1.5 mM EDTA. The cells were collected by centrifugation (1,000 g for 5 min), and pellet was resuspended in KR buffer (pH 7.4). Cell viability was estimated by the Trypan blue exclusion method and found to be greater than 84% for both wild-type and KO mice. 3H-Biotin uptake was measured by the rapid filtration method as described by us before (29). Protein concentrations were measured using a Bio-Rad (Hercules, CA) protein determination kit. Uptake data were expressed in fmol/mg protein/5 min.

All in vivo and in vitro uptake experiments with KO mice were run simultaneously with sex-matched wild-type littermate mice.

Quantitative real-time PCR. Total RNA was isolated from different mouse tissues using Trizol (Invitrogen, Carlsbad, CA) following

Fig. 1. Generation of the sodium-dependent multivitamin transporter (SMVT) conditional knockout (KO) mice and genotyping. A: schematic representation of the homologous recombination to introduce the loxP sites at the upstream and downstream regions of exons 4 and 6 of the Slc5a6 gene, respectively. A 5' loxP recombination site was inserted into intron 3 of the Slc5a6 gene together with a Neo cassette, and a second 3' loxP recombination site was inserted into intron 6 of the gene. Exons 4, 5, and 6 of the Slc5a6 gene were then conditionally removed when animals were crossed with transgenic mice expressing the Cre-recombinase only in intestinal epithelial cells under the villin promoter. B: PCR analysis of mouse ear DNA samples using 2 primers, showing presence of loxP (i) (+/+) and Cre transgenes (ii). Conditional deletion of Slc5a6, exon 4 to exon 6, was confirmed in enterocytes (iii).
manufacturer protocol. RNA sample was treated with DNaseI, and cDNA was prepared using i-Script kit (Bio-Rad). Quantitative real-time PCR was performed using gene-specific primers (for SMVT, forward 5’-GGATCTGTGGGACTGTGA-3’ and the reverse 5’-CATCCTGATGGACAGA-3’; for β-actin, the forward 5’-ATCTCTTCTCCCTTGGA-3’ and the reverse 5’-TTCATGATGCGCAGA-3’) in CFX96 real-time i-cycler (Bio-Rad), and relative expression was quantified by normalizing Ct values with corresponding β-actin.

**Western blot analysis.** For Western blot analysis, cells were minced and homogenized in RIPA buffer (Sigma) containing complete protease inhibitor cocktail (Roche, Nutley, NJ). The soluble fraction was isolated by centrifugation at 8,000 g for 5 min, and equal amounts of protein were resolved on a 10% mini gel (Invitrogen). The protein was transferred to PVDF membrane and probed simultaneously with affinity-purified mouse SMVT antibodies (raised in rabbit) and mouse monoclonal β-actin antibody (raised in mouse). The blot was then incubated with anti-rabbit IR 800 dye and anti-mouse IR 680 dye (LI-COR) secondary antibodies (1:25,000) for 1 h at room temperature. Relative expression was quantified by comparing the fluorescent intensities in an Odyssey Infrared imaging system (LI-COR) using Odyssey application software (version 3.0) with respect to corresponding β-actin.

**Estimation of biotin status.** Biotin status was estimated as described previously (7, 16, 19) by measuring the total level of biotinylated proteins in the liver of SMVT KO mice and their sex-matched littermate controls using Western blot analysis. In these studies, we first incubated the membrane with anti-mouse anti-β-actin antibodies, followed by labeling of the biotinylated proteins with avidin IR 800 dye, and the anti-β-actin primary antibodies with anti-mouse IR 680 dye (LI-COR).

**Phenotype assessment.** We assessed differences in body weight, bone density and length, and gross body structure of the KO mice and their sex-matched littermates. For X-ray imaging, animals were anesthetized and placed in a Kodak Image station. Bone density and bone length in X-ray images were quantitatively analyzed using Scion Image 4.0.3 (Scion, Frederick, MD).

**Histopathology analysis.** Tissue sections from the gastrointestinal tract, brain, heart, lungs, liver, pancreas, spleen, and kidneys from sex-matched wild-type littermate controls and KO mice were removed immediately after euthanasia, sectioned, fixed in 10% formalin overnight, and paraffin embedded. Hematoxylin and eosin-stained slides were prepared using standard histological techniques (Long Beach Veterans Medical Center Clinical Histology Laboratory). Gross and microscopic evaluations and reporting were performed by a board-certified anatomic pathologist.

**Statistical analysis.** All data presented are means ± SE of at least three independent experiments. Uptake of 3H-biotin by the carrier-mediated process was determined by subtracting uptake in the presence of unlabeled biotin from that in its absence; the same was done in determining carrier-mediated uptake of pantothenic acid and ascorbic acid. In all studies, simultaneously performed controls (same-sex littermates) were used. Significance was set at P < 0.05 and was calculated using the Student’s t-test.

**RESULTS**

**Generation and establishment of the SMVT conditional KO mouse colony.** As mentioned earlier, the mouse has a similar intestinal biotin uptake process to that of human (and to all mammals examined so far, e.g., rat, rabbit), and intestine of these species express SMVT (reviewed in Ref. 35). Furthermore, SMVT of these species share a high degree of sequence homology (reviewed in Ref. 35). Thus the mouse is an excellent model to use to gain a better understanding of the overall
physiological importance of SMVT and its role in intestinal biotin absorption process in native intestine in vivo.

A heterozygous mouse (Slc5a6+/loxP) was generated (inGenious Targeting Laboratory) and was further inbred in our facility to generate homozygous mice (Slc5a6+/loxP; Fig. 1A). The latter was then used to generate an intestinal-specific SMVT KO mouse line by breeding with a mouse expressing intestinal specific Cre-recombinase under the control of the villin promoter. The presence of Cre-recombinase and loxP sites (+/+ or +/loxP) in the offsprings was established by genomic PCR (Fig. 1B, i and ii). Veriﬁcation of the intestinal speciﬁc deletion of the Slc5a6 gene was conﬁrmed by RT-PCR using intestinal mRNA (Fig. 1B, iii).

Observed phenotype of the KO mice. About two-thirds of the KO mice died prematurely between 6 and 10 wk of age with the cause of death being due to acute peritonitis. The phenotype of the SMVT conditional KO mice showed significant differences compared with their age- and sex-matched wild-type littermate controls (Fig. 2). They displayed significant growth retardation, decreased bone density and length, lethargic behavior, and hunched back posture compared with their litters (Fig. 2, A and B). Mean body weight of the KO mice at 4–6 wk was signiﬁcantly ($P < 0.01$) lower than that of their sex-matched litters (Fig. 2C). Also, X-Ray image analysis of four different sets of KO mice and their sex-matched wild-type litters showed a significant decrease in bone density of the femoral and humoral head, as well as a signiﬁcantly shorter pelvic, tibial, and femoral bones ($P < 0.01$ for all) (Fig. 2D and E). Autopsy of ﬁve euthanized KO mice, compared with their sex-matched wild-type littermates, showed distended and thinned ileal and cecal walls. On the other hand, no gross abnormalities were observed with the brain, heart, lungs, liver, pancreas, spleen, and kidneys.
Microscopic analysis of all major organs of the SMVT conditional KO mice did not show any histological abnormalities except for sections of the small bowel and cecum. Small bowel villi of all five KO mice were significantly (P<0.01) shorter than that of sex-matched wild-type littermates (Fig. 3, A and B). Also, KO mice showed focal areas of low-grade dysplasia (Fig. 3C). Sections of the cecum of all KO mice showed significant (P<0.01) chronic active inflammation with focal cryptitis/crypt abscesses and a significant (P<0.01) increase in the number of neutrophils in the mucosa (Fig. 3, D and E). We also observed low-grade adenomatous changes and extensive submucosal edema (Fig. 3F). Similar features (but less extensive) were seen in the colon.

Expression of SMVT in intestine and other tissues of the KO mice. Expression of SMVT at the mRNA and protein levels was examined using real-time quantitative PCR and Western blot analysis, respectively. Only residual expression of the SMVT mRNA and protein was found in the intestinal mucosal scraping in the KO mice compared with the sex-matched littermates (Fig. 4, A and B). This residual SMVT expression in the intestinal mucosal scraping is most probably contributed by contaminating submucosal tissue. On the other hand, no difference was found in the level of expression of the SMVT mRNA and protein in the liver and kidneys of the conditional KO mice compared with their littermate controls (Fig. 4, A and B).

Effects of loss of SMVT on biotin level and on intestinal uptake of biotin and pantothenic acid. We determined the level of biotin in the liver of KO mice and their sex-matched littermates (liver level of biotin is a good indicator of overall biotin status) (16, 36, 7) using an established procedure (see MATERIALS AND METHODS). The results showed a significantly (P<0.01) lower biotin level in the liver of the KO mice compared with their controls (ratio of biotinylated proteins/β-actin of 254.1 ± 23.6 and 120.1 ± 15.1, respectively).

In another study, we examined biotin uptake, both in vivo and in vitro in the jejunum of KO mice and compared the findings to uptake in the jejunum of their sex-matched littermate controls. Our in vivo loop studies showed a near complete disappearance (P<0.01) of the carrier-mediated uptake for biotin (83 nM) in the jejunum of the KO mice (Fig. 5A). In the in vitro studies, we used isolated jejunal epithelial cells and observed a significant inhibition (P<0.01) in carrier-mediated biotin (64 nM) uptake in the KO mice compared with their control littermates (Fig. 5B). The residual biotin uptake seen in the isolated and suspended enterocytes of the KO mice is most probably due to uptake of the vitamin across the exposed basolateral membrane domain, which is known to have a non-SMVT uptake system (31). Similarly, uptake of panto-
Ascorbic acid (100 nM), which shares the same uptake system with biotin in the intestine, was significantly ($P < 0.01$) inhibited in jejunal loops of KO mice compared with their control littermates (Fig. 5C). In contrast to the inhibition observed in intestinal biotin and pantothenic acid uptake in the SMVT KO mice, no inhibition in intestinal uptake of the unrelated ascorbic acid (77 μM) was observed (Fig. 5D) (intestinal uptake of ascorbate occurs via different and distinct transport systems; reviewed in Ref. 35). The latter clearly shows specificity of the SMVT KO model.

We also examined biotin uptake (64 nM) in the kidney of the SMVT KO mice and compared the findings to biotin uptake by the kidney of their sex-matched littermates using an established brush-border membrane vesicle (BBMV) techniques (22). Our results showed similar biotin uptake by renal BBMV of the two mice groups (Fig. 5E).

**DISCUSSION**

The aim of this investigation was to gain a better understanding of the role of the SMVT system in intestinal absorption of the water-soluble vitamin biotin in the native intestine in vivo. Biotin is indispensable for life, and, because mammals cannot synthesize the vitamin endogenously, they must obtain it from exogenous sources via intestinal absorption. The vitamin plays critical roles in normal cellular metabolism, immune function, and cell proliferation (reviewed in Refs. 21 and 30). Our approach involved the generation of an intestinal-specific (conditional) SMVT KO in mice using the Cre/lox technology. We verified suitability of the model by demonstrating a near absence of expression of the SMVT protein and mRNA in the intestine of the KO mice compared with the intestine of their sex-matched littermates, whereas expression of SMVT in other tissues was similar between the KO mice and their wild-type littermates.

Overall biotin status, reflected by level of the vitamin in the liver, was found to be significantly lower in the KO mice compared with their sex-matched littermates. About two-thirds of the KO mice died prematurely between the age of 6 and 10 wk. Also, a characteristic phenotype was observed in the KO mice compared with the wild-type littermates. All KO animals exhibited growth retardation (a much smaller body weight), decreased bone density and length, as well as lethargy, and hunched back posture, also termed kangaroo posture, that have been reported in biotin-deficient rats (20) and the biotinidase KO mouse (25). Upon autopsy, we also observed significant distention and thinning of the wall of the small bowel and cecum, whereas all other organs appeared normal. Furthermore, significant histological changes were observed in both the small bowel and cecum. In the small bowel, the villi were significantly shorter in the KO mice compared with their sex-matched littermates, and focal areas of low-grade dysplasia were observed. In the cecum of the KO mice, chronic active inflammation with focal cryptitis/ crypt abscesses, as well as low-grade adenomatous changes and extensive submucosal edema, were observed although the mechanisms that mediate these inflammatory responses in the SMVT KO mice are under
ROLE OF SMVT IN INTESTINAL BIOTIN UPTAKE IN VIVO

investigation. We speculate that these pathological findings are related to the roles played by biotin in maintaining normal innate and adaptive immune functions (reviewed in Refs. 21 and 30). An example of the latter is the essentiality of biotin for normal activity of intestinal NK cells (24). These cells play an important role in promoting antipathogen responses and in maintaining intestinal epithelial homeostasis (43). It is worth mentioning here that activity of these cells and level of biotin are both decreased in patients with Crohn’s disease (2, 13, 23, 24, 43). Further studies are needed to delineate the mechanism(s) involved in the inflammatory responses observed in the intestine of the SMVT KO mice.

Our studies demonstrated that knocking out SMVT in the mouse intestine caused complete loss of carrier-mediated intestinal uptake in vivo. Uptake of biotin by isolated enterocytes in suspension was also severely blunted in the KO mice compared with uptake by isolated enterocytes from their littermate controls. We speculate that the residual uptake of biotin observed in the isolated enterocytes is attributable to basolateral uptake by a system normally responsible for biotin exit from the enterocytes; a non-SMVT biotin uptake system is known to exist at the basolateral membrane domain of these cells (31), and enterocytes in the preparation are exposed to 3H-biotin. Uptake of pantothenic acid, which also shares the intestinal absorption process with biotin, was similarly abolished in the KO mice. In contrast, uptake of the unrelated water-soluble vitamin C (ascorbate) was similar in the KO and control groups. These findings clearly indicate that the SMVT system is the only system that is involved in the uptake of biotin (and pantothenic acid) in the intestine and that the observed inhibition in uptake of these two vitamins in the SMVT KO mice is specific and does not affect uptake of other nutrients. Our observation that the carrier-mediated renal biotin uptake is similar in the KO mice and their sex-matched wild-type littersmates demonstrates that the disruption in the SMVT function is, as intended, being limited to the gut.

With the establishment of SMVT as the biotin and pantothenic acid transport in the native intestine, it is worth mentioning that the system appears to respond to fluctuation in dietary biotin (but not pantothenic acid) levels. This conclusion is based on previous observations showing that dietary biotin levels adaptively regulate intestinal biotin uptake (via transcriptionally mediated mechanism) (32, 28), whereas changes in dietary levels of pantothenic acid have no effect its intestinal uptake (37).

In summary, our study represents the first in vivo confirmation in native tissue for an exclusive role for SMVT in intestinal biotin uptake. Also, knocking out the intestinal SMVT system leads to the development of a characteristic intestinal phenotype that may be relevant to intestinal health and normal homeostasis.

GRANTS

This work was supported by grants from the Department of Veterans Affairs and the National Institutes of Health (DK58057 and DK56061).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.G., N.W.G.L., S.B.S., and R.K. performed experiments; A.G., N.W.G.L., R.K., and H.M.S. analyzed data; A.G., N.W.G.L., and R.K. drafted manuscript; N.W.G.L., S.B.S., and H.M.S. edited and revised manuscript; H.M.S. conception and design of research; H.M.S. approved final version of manuscript.

REFERENCES


