**Salmonella** infection inhibits intestinal biotin transport: cellular and molecular mechanisms

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**Biotin is an indispensable micronutrient for normal human health, because it is essential for normal cellular metabolism and function. The vitamin acts as a cofactor for five carboxylases that are critical for the metabolism of fatty acids, glucose, and amino acids (reviewed in Refs. 30, 43, 49). Recent studies have also reported a role for biotin in the regulation of gene expression, where expression of more than 2,000 human genes appears to be affected by biotin status (40, 42, 43, 53, 54). Additionally, biotin appears to play a role in the regulation of immune function (1, 4, 14, 17, 24–26, 33, 41, 43) and in the maintenance of normal intestinal homeostasis (16). Deficiency and suboptimal levels of biotin lead to a variety of clinical abnormalities, including dermat abnormalities and neurological disorders (6, 43, 49). Furthermore, at least in animals, biotin deficiency during pregnancy can lead to embryonic growth retardation, congenital malformation, and death (31).

Since mammals, including humans, cannot synthesize biotin, they must obtain the vitamin from exogenous sources via intestinal absorption. The intestinal tract is exposed to two sources of biotin: dietary biotin, and biotin generated by the gut microflora. Absorption of biotin in both the small and large intestines occurs via a carrier-mediated process that involves the sodium-dependent multivitamin transporter (SMVT). A variety of conditions appear to affect the intestinal absorption process of biotin (43a), but nothing is known about the effect of enteric pathogens, like *Salmonella*, on the process. *Salmonella enterica* is a gram-negative, facultative intracellular bacterium that is capable of infecting a number of different hosts, including humans and mice, and causes significant morbidity and mortality worldwide (8, 19, 20, 34, 37, 56). Salmonellosis is one of the most common food- and water-borne diseases, causing ∼20% of all such infections in humans. Of the 2,500 *S. enterica* serovars that cause disease in humans, *S. typhimurium* and *S. enteritidis* are the most common isolates. *S. typhimurium* infection in humans leads to acute gastroenteritis that is associated with inflammatory diarrhea (8, 20, 34, 56). Severe and prolonged cases of *S. typhimurium* infection, as well as dual infection with other pathogens, may also compromise the nutritional status of the infected hosts (12).

During intestinal infection with *S. typhimurium*, the bacterium exploits M cells and enterocytes/colonocytes (and possibly dendritic cells) to traverse the subepithelial compartment (29, 46), where it interacts with, and activates, immune cells (macrophages, neutrophils, dendritic cells), leading to production of proinflammatory cytokines (36). Of the secreted cytokines, TNF-α and IFN-γ play important roles in initiating the inflammatory response (8, 18, 20, 34, 46). These cytokines, however, also have the potential of affecting intestinal absorptive and secretory functions (20, 56). In this study, our goal was to examine the effect of *S. typhimurium* infection on intestinal biotin uptake and to shed light on the mechanism(s) involved. By using a combination of in vitro and in vivo models, we found that *S. typhimurium* infection significantly inhibits intestinal biotin uptake. Moreover, we present evidence that the inhibition is indirect and likely dependent on the upregulation of proinflammatory cytokines that act via the NF-κB-mediated signaling pathway, leading to suppression of transcription of *SLC5A6*, the gene encoding for the SMVT transporter.
MATERIALS AND METHODS

Materials

3H-biotin (specific activity 60 Ci/mmol; radiochemical purity > 97%) was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other cell culture reagents and other chemicals used in this study were of molecular biology grade and were obtained from commercial sources.

Methods

Cell culture. Confluent monolayers of the human-derived colonic epithelial NCM460 cells, conditionally immortalized murine colonic epithelial cells [young adult mouse colonic (YAMC)] cells, and the human-derived intestinal epithelial Caco-2 cells were used in these investigations (American Type Culture Collection, Manassas, VA). NCM460 cells were maintained in F-12 medium (Ham), whereas Caco-2 and YAMC cells were maintained in MEM and DMEM (Gibco) medium, respectively, supplemented with fetal bovine serum and streptomycin (10 mg/mL), under standard condition. Confluent cell monolayers (at 5–6 days postconfluence) were used to examine the effect of cytokines on biotin uptake.

Bacterial Culture and Infection

Salmonella enterica serovar Typhimurium strain IR715 [a nalidixic-acid resistant derivative of strain ATCC 14028 (21)] and an isogenic noninvasive, nonpathogenic mutant [SPN487; invA spb (37)] were used. For the in vivo studies, C57BL/6 mice were treated with streptomycin (20 mg/mouse) to induce cecal and colonic inflammation (5) and then challenged with S. typhimurium wild-type or with the invA spb mutant (100 μL of 10^8 bacteria/mL). Mice were killed 72 h later, and their intestinal tissues were collected and analyzed.

For the in vitro studies, overnight bacterial cultures were grown in Luria-Bertani broth, washed with PBS, and diluted to a multiplicity of infection = 10 in the respective cell culture medium without fetal bovine serum and antibiotics. Following a 90-min infection, the intestinal epithelial monolayers were treated with gentamycin (50 μg/mL) to remove adherent bacteria (15), allowed to recover for 6 h, and used for biotin uptake investigations.

Biotin Uptake

For mouse studies, we used the in vivo intestinal (jejunal) closed-loop technique (16), as well as the isolated colonic sheet approach (48). In the former studies, we introduced 83 nM 3H-biotin into the loop, followed 5 min later (after animal was killed) by removal of loop tissue, processing, and counting of the 3H content (using liquid scintillation counter). In the colonic sheet investigations, equal pieces (1 cm) of colonic sheets were incubated (5 min) in vitro in Krebs-Ringer (KR; pH 7.4) buffer in the presence of 64 nM 3H-biotin, washed, and then processed for 3H content.

For the studies with cultured cells, confluent monolayers of NCM460, YAMC, and Caco-2 cells treated with heat-stable toxins were used, as described previously (15). In brief, cells were incubated (5 min; initial rate) in KR buffer (pH 7.4) at 37°C in the presence of 5 nM 3H-biotin. The cells were then washed twice with ice-cold KR buffer, lysed with 1 N NaOH (followed by neutralization with 10 N HCl), and counted for radioactivity in a liquid scintillation counter.

Real-Time PCR Analysis

Total RNA was isolated from infected cells or mice tissues in TRizol (Life Technologies), as described in the manufacturer’s protocol, and then treated with DNase. The RNA pool was reverse transcribed to cDNA using i-script kit (Bio-Rad) and then used for quantitative real-time PCR using specific primers [mSlc5a6 F: 5′-GGATCTGTGGGACTGTGA-3′; R: 5′-CACATCTGGTCAGATGAC-3′; mβ-actin, F: 5′-ATCCCTTTCCTCTTCTGGA-3′, R: 5′-TTCATGGATGCG-ACAGGA-3′; hSlc5a6 F: 5′-GGATCTGTGGGACTGTGA-3′, R: 5′-TAGCCCAATGGACGAGA-3′; hβ-actin F: 5′-CATCCCTGCGTCACCTAC-3′, R: 5′-AAATCTCAGGAAATGTTCTCC-3′].

Statistical Analysis

All uptake data presented in the study are means ± SE of multiple separate determinations. Uptake was expressed as femtomoles per milligram protein per 5 min. Biotin uptake by the carrier-mediated process was determined by subtracting uptake of a physiological concentration of 3H-biotin in the presence of a high pharmacological concentration of unlabeled biotin (1 mM) from uptake in its absence. All other determinations are presented as means ± SE of at least three independent experiments; promoter activity (in arbitrary units) is expressed as fold over pGL3-Basic. Statistical significance was de-
RESULTS

S. typhimurium Infection Inhibits Intestinal Biotin Uptake by Downmodulating the Expression of the Vitamin Transporter SMVT

To determine the effect of S. typhimurium infection on intestinal biotin uptake, we infected C57BL/6 mice with wild-type S. typhimurium by oral gavage. At 72 h postinfection, we determined $^{3}$H-biotin uptake in the small intestine and in the colon compared with sex- and age-matched control mice gavaged with PBS. Our results showed a significant inhibition of $^{3}$H-biotin uptake, both in the small intestine and in the colon ($P < 0.01$ for both) (Fig. 1, A and B). In contrast, we did not observe any changes in jejunal and colonic biotin uptake when mice were gavaged with a S. typhimurium $\Delta$invA $\Delta$spiB mutant, which is avirulent because it does not invade the intestinal mucosa nor replicate within the host (Fig. 1C and data not shown). As our results indicated that S. typhimurium infection decreases biotin uptake, we next investigated whether this effect was dependent on the inhibition of the vitamin transporter SMVT, which our laboratory recently found to be the only biotin uptake system in the intestinal tract (16). To this end, we examined whether S. typhimurium infection would reduce the expression of the Slc5a6 mRNA and the SMVT protein in the jejunum and in the colon. Our results (Fig. 2) show a significant ($P < 0.01$) decrease in the level of expression of both the Slc5a6 mRNA and the SMVT protein in the jejunum and colon of S. typhimurium-infected mice, compared with uninfected controls.

Because changes in the mRNA of a given gene could be mediated by changes in the transcription rate of that gene, we examined the effect of S. typhimurium infection on the expression levels of the endogenous Slc5a6 hnRNA; the level of hnRNA reflects rate of transcription (13, 47). We found a significant ($P < 0.01$) decrease in the level of Slc5a6 hnRNA in the jejunum of the S. typhimurium-infected mice compared with controls (Fig. 3), suggesting that S. typhimurium infection reduces the transcription rate of the Slc5a6 gene. Similar changes were also found in the colon of S. typhimurium-infected mice (data not shown). Taken together, our results indicate that S. typhimurium infection reduces the transcription rate of the Slc5a6 gene, resulting in reduced production of the SMVT transporter and, consequently, in reduced biotin uptake during infection.

The Proinflammatory Cytokines Tnf-$\alpha$ and Ifn-$\gamma$ Are Induced During S. typhimurium Infection and Inhibit Biotin Uptake In Vitro

Next, we sought out to determine the mechanisms by which S. typhimurium inhibits the expression of SMVT and biotin uptake. To this end, we set up in vitro culture models, and we infected mouse colonic epithelial YAMC cells, human colonic epithelial NCM460 cells, and confluent Caco2 cells, which resemble enterocytes from the small intestine, with wild-type S. typhimurium (multiplicity of infection = 10) for 90 min. We then measured the initial rate of $^{3}$H-biotin (5 nM) uptake. Surprisingly, we detected similar biotin uptake in uninfected cells and in cells infected with S. typhimurium in all of the three cell types used (Fig. 4). These findings suggest that the observed inhibition of jejunal and colonic biotin uptake in mice infected with S. typhimurium is most likely indirect in nature.

It is known that Salmonella infection in humans leads to an increase in serum levels of proinflammatory cytokines, including that of TNF-$\alpha$ and IFN-$\gamma$ (46). Exposure of intestinal epithelial cells to elevated levels of proinflammatory cytokines affects the intestinal transport of a variety of substrates (2, 10, 39, 44). It is, therefore, possible that the inhibitory effect of S. typhimurium infection on mouse intestinal biotin uptake in vivo is mediated by an effect of proinflammatory cytokines on the vitamin uptake process. To test this hypothesis, we first determined whether S. typhimurium infection of mice cause an increase of Tnf-$\alpha$ and Ifn-$\gamma$, two proinflammatory cytokines known to affect intestinal physiology, in both the serum and in the gut mucosa (Fig. 5). We found a significant ($P < 0.01$; ~64 and 27-fold for TNF-$\alpha$ and IFN-$\gamma$, respectively) induction in serum levels of TNF-$\alpha$ and IFN-$\gamma$ in S. typhimurium-infected mice, compared with controls (Fig. 5, A and B). Moreover, we also detected a significant ($P < 0.01$ for both) upregulation of

![Fig. 1. Effect of Salmonella infection on intestinal biotin uptake in vivo. A: carrier-mediated in vivo biotin uptake in jejunal loops in sex-matched littermates. B: carrier-mediated biotin uptake in colonic sheets of infected mice and sex-matched littermate controls. C: carrier-mediated biotin uptake after infection with the nonpathogenic S. typhimurium $\Delta$invA $\Delta$spiB mutant in vivo. Values are means ± SE of 3–6 independent observations from 3–6 different sets of mice. *$P < 0.01$.](http://ajpgi.physiology.org/doi/abs/10.1152/ajpgi.00112.2015)
are means in jejunal (i) and colonic (ii) mucosa. Values are means ± SE of at least 3 separate sets of mice. * P < 0.01.

Fig. 2. Expression of the Slc5a6 gene and of the sodium-dependent multivitamin transporter (SMVT) protein in jejumon and colon of Salmonella-infected mice. A: quantitative PCR using reverse transcribed total RNA from jejunum (i) and colon (ii) of control and S. typhimurium-infected mice. B: Western blot analysis of the SMVT protein expression in jejunal (i) and colonic (ii) mucosa. Values are means ± SE of at least 3 separate sets of mice. * P < 0.01.

Tnf-α and Ifnγ mRNA in the small intestine and in the colon (Fig. 5, C and D). In contrast, no upregulation of these proinflammatory genes was observed when the mice were infected with the nonpathogenic S. typhimurium ΔinvA ΔspiB mutant (data not shown).

We next examined the effect of exposure of human colonic epithelial NCM460 cells to TNF-α and IFN-γ on the biotin uptake mechanisms. In these studies, cells were treated with TNF-α and IFN-γ (20 and 30 ng/ml, respectively) for 24 h, followed by the examination of the initial rate of biotin uptake. Consistent with our prediction, we observed a significant (P < 0.01) inhibition of biotin uptake in cells treated with these cytokines compared with untreated controls (Fig. 6A). As we observed in S. typhimurium-infected mice, inhibition of biotin uptake was associated with a significant (P < 0.01) reduction in the levels of expression of the SMVT protein and SLC5A6 mRNA (Fig. 6, B and C). To further examine the mechanisms of biotin-uptake inhibition, we next investigated the effect TNF-α and IFN-γ on the activity of the SLC5A6 promoter; we focused our study on the SLC5A6 promoter-1, since it is the most active in the intestine (11). We found a significant (P < 0.01) decrease in the activity of the SLC5A6 promoter in cells pretreated with the cytokines, compared with untreated controls (Fig. 6D). These findings indicate that the inhibition of intestinal biotin uptake caused by proinflammatory cytokines is, at least in part, mediated at the level of transcription of the SLC5A6 gene.

Role of the NF-κB Pathway in Mediating the Inhibitory Effect of Proinflammatory Cytokines on SLC5A6 Transcription and on Biotin Uptake

One of the important intracellular signaling mechanisms that mediate the effect of proinflammatory cytokines on intestinal epithelial cells is mediated by the NF-κB signaling pathway (32, 55). Also, a computer analysis (Alibaba 2.1; http://www.gene-regulation.com/pub/programs/alibaba2/index.html) showed that the human and mouse SLC5A6 promoters both have a putative site for NF-κB. To determine whether the inhibitory effect of TNF-α and IFN-γ on the activity of the SLC5A6 promoter and on biotin uptake is dependent on NF-κB signaling, we used the NF-κB inhibitor MG132, which blocks activation of NF-κB by preventing the degradation of the NF-κB inhibitor IκB (22, 32). In these inves-
tigations, we first confirmed that treatment of NCM460 cells with TNF-α and IFN-γ leads to translocation of the NF-κB subunit p65 into the nucleus and to the degradation of IκB in the cytosol (Fig. 7A, i–ii). We then examined the effect of pretreating NCM460 cells for 24 h with TNF-α and IFN-γ in the presence of the NF-κB inhibitor MG132 (1 \( \mu \text{M} \)) on the activity of the SLC5A6 promoter and on biotin uptake (Fig. 7, B and C). Our results showed that NF-κB inhibition significantly \((P < 0.01 \text{ for both})\) attenuates the degree of cytokine-mediated inhibition of SLC5A6 promoter activity.

![Graph A](image1)

**Fig. 4.** Effect of *Salmonella* infection on intestinal biotin uptake in vitro. Mouse colonic YAMC cells, human colonic NCM460 cells, and human Caco-2 enterocytes were serum-starved overnight 3–4 days postconfluence and then infected with wild-type *S. typhimurium* at a multiplicity of infection of 10. Biotin uptake was subsequently measured as described in MATERIALS AND METHODS. **A:** effect of *Salmonella* on carrier-mediated biotin uptake on YAMC cells. **B:** effect of *Salmonella* on carrier-mediated biotin uptake on NCM460 cells. **C:** effect of *Salmonella* on carrier-mediated biotin uptake on Caco-2 cells. Values are means ± SE of at least 3 experiments.

![Graph B](image2)

**Fig. 5.** Level of Tnf-α and Ifn-γ expression in serum and intestinal mucosa of *Salmonella* -infected mice and sex-matched littermate controls. Fold changes in the serum level of Tnf-α (A) and Ifn-γ (B) were measured by ELISA. C: quantitative PCR was performed to determine Tnf-α mRNA expression using total RNA from jejunum (i) and colon (ii). D: quantitative PCR was performed to detect Ifn-γ mRNA expression using cDNA prepared from total RNA from jejunum (i) and colon (ii). Values are means ± SE of at least 3–4 separate sets of mice. *P < 0.01.
and of biotin uptake. These findings suggest that activation of the NF-κB pathway mediates the inhibition of intestinal biotin uptake observed during TNF-α and IFN-γ treatment.

**DISCUSSION**

The aim of this study was to examine the effect of *Salmonella* infection on the uptake of the water-soluble vitamin biotin, and to determine the mechanism(s) involved in the observed effect. Salmonellosis is one of the major public health concerns worldwide, affecting millions of people globally. Prolonged and severe cases of this infection have the potential to negatively impact normal body homeostasis of water-soluble vitamins, including biotin. Thus far, however, no studies have examined the possible effects of *Salmonella* infection on intestinal biotin uptake.

**Fig. 6.** Effect of TNF-α and IFN-γ on biotin uptake, SMVT protein level, and SLC5A6 mRNA expression in NCM460 cells. A: carrier-mediated biotin uptake in colonic NCM460 cells followed by incubation with proinflammatory cytokines (20 ng/ml and 30 ng/ml of TNF-α and IFN-γ, respectively). B: Western blot assay of hSMVT after normalization with β-actin as internal control; inset shows representative gel picture in absence and presence cytokines. C: quantitative real-time PCR to analyze SLC5A6 expression in cytokine-treated NCM460 cells compared with the untreated control. D: effect of cytokines on the SLC5A6-P1 promoter. Relative firefly luciferase activity was normalized by renilla firefly activity and the value expressed as fold over basic. Values are means ± SE of at least 3–4 separate sets of experiments (mice). *P < 0.01.

**Fig. 7.** Role of NF-κB in the inhibitory effect of proinflammatory cytokine on biotin uptake by NCM460 cells; translocation of p65 and degradation of IκB. A: Western blot analysis using nuclear (i) and cytosolic (ii) fractions of cytokine-treated NCM460, showing the relative amounts of NF-κB and IκB, respectively. B: effect of the NF-κB inhibitor, MG132, on the proinflammatory cytokine-induced inhibition on SLC5A6-P1 promoter activity (i) and carrier-mediated biotin uptake (ii). Values are means ± SE of at least 3 separate sets of experiments. *P < 0.05, **P < 0.01.
testinal absorption of water-soluble vitamins, including biotin. Here we investigated this question, using both in vivo and in vitro models of Salmonella infection: the streptomycin-pre-
treated mouse model (27, 35), and cultured intestinal and colonic epithelial cell lines.

Our results showed that infection of mice with wild-type S. typhimurium, but not with a noninvasive, nonpathogenic S. typhimurium mutant, leads to a significant inhibition of biotin uptake, both in the small intestine and in the colon. These findings indicate that absorption of both dietary biotin, which is usually absorbed in the small intestine, as well as the biotin that is generated by large intestinal microbiota (3), is inhibited by Salmonella infection. Notably, the inhibition of intestinal biotin uptake was found to be associated with a significant reduction in the expression of the Slc5a6 gene and of its gene product SMVT (SMVT is the only biotin uptake system that operates in the intestinal tract; Ref. 16). The inhibition in SMVT expression in the intestine of S. typhimurium-infected mice appears to be occurring, at least in part, at the level of transcription of the Slc5a6 gene, as suggested by the significant reduction in the expression level of the Slc5a6 hnrRNA (level of hnrRNA reflects changes in transcription rate of genes). In contrast to the inhibitory effect in intestinal biotin uptake observed in mice infected with S. typhimurium, no such inhibition was seen during infection of mouse and human intestinal or colonic epithelial cells. Collectively, these in vivo and in vitro findings suggest that the effect of S. typhimurium infection on intestinal biotin uptake is indirect in nature. This idea is consistent with the present understanding of how Salmonella infection affects gut mucosal physiology, i.e., mainly via the proinflammatory response that it elicits (36, 45). To further test this hypothesis in the present investigation, we first determined whether activation of the NF-κB pathway by TNF-α and IFN-γ, was induced in our S. typhimurium-infected mice. Consistent with the above-described prediction, we found that the levels of both TNF-α and IFN-γ were markedly induced in the intestinal mucosa and in the serum of S. typhimurium-infected mice. We then examined the effect of exposing intestinal epithelial cells to these cytokines and observed a significant inhibition of biotin uptake, as well as inhibition in the level of expression of the SMVT mRNA and activity of the Slc5a6 promoter. The cytokine-mediated inhibition in intestinal biotin uptake and level of expression of the SMVT protein are in contrast to the stimulatory effect they cause in intestinal uptake of peptides and in level of mRNA expression of the involved transporter, pepT1 (9, 52).

Because the NF-κB pathway is a major intracellular hub that mediates inflammatory responses, and because our bioinformatic analysis predicted that the 5′-regulatory region of the Slc5a6 gene contains putative NF-kB sites, we determined whether activation of the NF-kB pathway by TNF-α and IFN-γ would result in the inhibition of biotin uptake by intestinal epithelial cells. As we predicted, our findings showed that inhibition of the NF-kB pathway with the blocker MG132 significantly reverted the inhibitory effect of TNF-α and IFN-γ on both the Slc5a6 promoter activity and on biotin uptake in the human intestinal NCM460 cell line. Together, our results indicate that the proinflammatory cytokines TNF-α and IFN-γ exert their inhibitory effect on intestinal biotin uptake via, at least in part, an NF-κB-dependent down-modulation of the Slc5a6 gene transcription, resulting in reduced expression of the multivitamin transporter SMVT. Moreover, our findings are likely relevant to other conditions that are associated with intestinal inflammation and with an increase of both TNF-α and IFN-γ, like inflammatory bowel diseases; of note, significantly lower biotin levels have been observed in inflammatory bowel disease patients (7, 18, 23, 46).

In conclusion, our findings show that infection with S. typhimurium leads to a significant inhibition of small intestinal and colonic biotin uptake, and that this inhibition is indirect in nature, as it is mediated by TNF-α and IFN-γ, two proinflammatory cytokines highly induced during S. typhimurium infection. Furthermore, the inhibitory effect of these cytokines appears to be, at least in part, exerted at the level of transcription of the Slc5a6 gene via the NF-κB signaling pathway, resulting in lower levels of the SMVT vitamin transporter. Finally, the inhibition of intestinal biotin uptake by proinflammatory cytokines observed in this study may also provide an explanation for the low biotin levels found in patients with chronic intestinal inflammation (14, 52).

REFERENCES


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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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