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Sodium butyrate enhances intestinal riboflavin uptake via induction of expression of riboflavin transporter-3 (RFVT3)

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Abstract

Background: Uptake of riboflavin (RF) by intestinal epithelial cells occurs via a specific carrier-mediated process that involves the apically localized RF transporter-3 (RFVT3). Previous studies have shown that sodium butyrate (NaB) affects intestinal uptake of other substrates and expression of their membrane transporters, but its effect on intestinal uptake of RF and expression of RFVT3 has not been examined.

Aims: To investigate the effect of NaB on intestinal RF uptake process and expression of the RFVT3.

Methods: Two experimental models were used in this study: Human-derived intestinal epithelial Caco-2 cells and *ex vivo* mouse colonoids. ^{3}H-RF uptake assay, western blot, RT-qPCR and chromatin immunoprecipitation (ChIP) assay were performed.

Results: Treating Caco-2 cells with NaB led to a significant increase in carrier-mediated RF uptake. This increase was associated with a significant induction in the level of expression of the hRFVT3 protein, mRNA and heterogenous nuclear RNA (hnRNA) Similarly, treating mouse colonoids with NaB led to a marked increase in the level of expression of the mRFVT3 protein, mRNA and hnRNA. NaB did not affect hRFVT3 mRNA stability, rather it caused significant epigenetic changes (histone modifications) in the *SLC52A3* gene where an increase in H3Ac and a reduction in H3K27me3 levels were observed in the NaB treated Caco-2 cells compared to untreated controls.

Conclusion: These findings demonstrate that NaB up-regulates intestinal RF uptake and that the effect appears to be mediated, at least in part, at the level of transcription of the *SLC52A3* gene and may involve epigenetic mechanism(s).

Keywords

Riboflavin; RFVT3; intestine; sodium butyrate; colonoids

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Introduction

Riboflavin (RF), a member of the water-soluble family of vitamins, is essential for normal human health. In its coenzyme forms [flavin mononucleotide (FMN) and flavin dinucleotide (FAD)], RF plays key metabolic roles in biological oxidation-reduction reactions [1]; it also plays a role in protein folding [2] and has both anti-oxidant and anti-inflammatory properties [3, 4]. A role for RF (and for the microbiota-generated metabolites used in its biosynthesis) in normal immune function has also been demonstrated [5–7]. Deficiency of RF occurs in conditions like inflammatory bowel diseases (IBD) [8], chronic alcoholism [9], and diabetes mellitus [10]; it also occurs in patients with infantile Brown-Vialetto-Van Laere Syndrome (BVVLS), a rare neurological disorder linked to genetic mutations in RF transporters, where supplementation with pharmacological doses of RF brings about a significant improvement in the clinical symptoms of the affected subjects [11–14].

Mammals, including humans, obtain RF from exogenous sources through intestinal absorption as they lack of ability to synthesize the vitamin endogenously. The intestine encounters RF from two sources: a dietary source, which is absorbed in the small intestine, and a bacterial (gut microbiota) source, which is absorbed in the large intestine [15–18]. Studies have shown that the small and large intestinal RF uptake processes are similar and both occur via a specific carrier-mediated mechanism [17, 19–21]. While all the three recently cloned RF transporters (RFVT-1, −2 and −3, products of the *SLC52A1, SLC52A2*, and *SLC52A3* genes, respectively) were found to be expressed in the gut, expression of RFVT3 has been shown to be the highest [22–26]. At the cellular level, expression of the RFVT3 was also found to be exclusively restricted at the apical membrane domain of the absorptive cells [26]. An essential role for RFVT3 in intestinal RF uptake has been demonstrated in studies using *in vitro* knockdown (siRNA) and *in vivo* intestinal-specific knockout approaches [26, 27]. Finally, several genetic mutations in the RFVT3 transporter have been identified in humans and shown to lead to clinical manifestations [12, 28].

Butyrate is one of the important short chain fatty acids (SCFAs) produced in the large intestinal lumen by bacterial fermentation of dietary carbohydrates, specifically resistant starch and dietary fibers [29, 30]. The normal colonic lumen contains approximately 100–150 mM total SCFAs, which consists of acetate, propionate and butyrate in the ratio of 6:2:2, respectively [30–33]. Of these three SCFAs, butyrate is the preferred fuel for the colonic epithelial cells and is important for the maintenance of their health [34]. Recent studies have shown that the microbiota generated butyrate exerts a significant effect on the intestinal absorptive function [35–39], but its effect on intestinal absorption of RF and on expression of RFVT3 has not been examined. Here we tested the effect of NaB on intestinal RF uptake using *in vitro* and *ex vivo* models. The results showed that NaB stimulates intestinal RF uptake and that the effect is in part mediated at the level of transcription of the *SLC52A3* gene and may involve epigenetic mechanism(s).

Materials and Methods

Materials:

Human-derived intestinal epithelial Caco-2 were obtained from American Type Culture Collection (ATCC, Manassas, VA). ^{3}H-RF (specific activity 12.5 Ci/mmole; radiochemical purity > 97%) was purchased from Moravek Biochemicals (Brea, CA). All other cell culture and molecular biology reagents were of analytical grade and were obtained from commercial vendors. The primers used to perform RT-qPCR analysis were purchased from Sigma Genosys (Woodlands, TX).

Cell culture and RF uptake:

Caco-2 cells were grown in EMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin at 37°C in 5% CO_2-95% air environment. Confluent Caco-2 cells grown in 12 wells plates were serum starved in EMEM containing 0.5% FBS for 24 hrs and treated with different concentrations of NaB (1, 2, 5, and 10 mM), or with sodium acetate (1 mM) and sodium propionate (1mM) for 24 hrs and used to determine the initial-rate (3 min) of RF uptake. Briefly, Caco-2 cells were incubated in KR buffer (133 mM NaCl, 4.93 mM KCl, 1.23 mM MgSO4, 0.85 mM $CaCl_2$, 5 mM glucose, 5 mM glutamine, 10 mM HEPES, and 10 mM MES, pH 7.4) at 37°C in the presence of ^{3}H-RF (25 nM). After incubation, cells were subjected for radioactivity measurement in a liquid scintillation counter (Beckman Coulter) as described before [20, 40].

Mouse colonoids preparation:

For *ex vivo* studies, we used 8–10-weeks old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) to generate colonoids as described before [41–43]. Mice were euthanized, the colonic tissue was removed and prepared colonic crypts as described before [41–43]. The generated colonoids were treated with 1mM NaB for 24 hrs and used for mRFVT3 protein, mRNA and hnRNA expression studies. These animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Veteran Administration Medical Center (VAMC), Long Beach, CA.

RNA extraction and RT-qPCR:

To determine the RFVT3 mRNA and hnRNA expression levels, total RNA from Caco-2 cells and mouse colonoids treated with NaB and untreated controls was extracted as described [26, 40]. Total RNA (2 μg) was reverse transcribed to cDNA using i-Script reverse transcriptase (RT) kit (Bio-Rad, CA). RT-qPCR was performed using gene specific primers for determining the mRNA (hRFVT3 forward 5'-CCTTTCCGAAGTGCCCATC-3'and reverse 5'- AGAAGGTGGTGAGGTAGTAGG-3'; mRFVT3 forward 5'-GGATCAGTGGAAGCCAGTG-3' and reverse 5'GACCTGTTAGGCAGGAAGATG-3'; hβ-actin forward 5'-AGCCAGACCGTCTCCTTGTA-3' and reverse 5'-TAGAGAGGGCCCACCACAC-3'; mβ-actin forward 5'ATCCTCTTCCTCCCTGGA-3' and reverse 5'-TTCATGGATGCCACAGGA-3') and hnRNA (hRFVT3 forward 5'-TCTCAGCACTTGGCTTTATC −3'and reverse 5'- CTCCCATGCGTATGTATGTA −3'; mRFVT3 forward 5'-GCCTAGGAAAACCTTGCCCAGT-3' and reverse 5'-

CAGAGGGACTGGTGGAGGACA-3’; hβ-actin forward 5'TTCCTGGGTGAGTGGAG-3’ and reverse 5'- GGACTCCATGCCTGAGAG −3'; mβ-actin forward 5’-AGATGACCCAGGTCAGTATC-3’ and reverse 5’-GAGCAGAAACTGCAAAGAT-3’) expression levels in a CFX96 real-time icycler (Bio-Rad). After initial denaturation at 95°C for 5 min, the amplification program was repeated 45 times (95°C with a 30-s hold, 55°C with 15-s hold followed by 72°C with 30-s hold for extension and fluorescence measurement). The mRNA and hnRNA expression levels of RFVT3 were normalized relative to β-actin and the expression level was calculated following relative relationship method as described before [44].

hRFVT3 mRNA stability assay:

Caco-2 cells were pretreated with 5 μg/ml actinomycin D (AcD; a potent transcription inhibitor) for 1 hr to stop the transcription and incubated with 1 mM NaB for specified time points (0, 4, 8, 12 and 24 hrs). Total RNA (2 μg) was extracted and hRFVT3 mRNA expression levels were determined by RT-qPCR using hRFVT3 specific primers and relative relationship method as mentioned above.

Western blot analysis:

Caco-2 cells and mouse colonoids treated with NaB and controls were lysed in 1X protease cocktail inhibitor mixture (Roche, Indianapolis, IN) containing RIPA buffer (Sigma) and the total protein was prepared as described [45, 46]. Sixty micrograms of total protein was loaded on 4–12% premade mini gels (Invitrogen) and protein was transferred to polyvinylidene difluoride (PVDF) membranes. After transfer the membranes were blocked in Odyssey blocking buffer (LI-COR) and then probed either with hRFVT3 (1:200 dilution) (custom antibody generated by Alpha diagnostic, San Antonio, TX) or mRFVT3 (1:500 dilution) (Biosis Antibodies, Woburn, MA) and β-actin (Santa Cruz Biotechnology) polyclonal antibodies. The specificity of both the antibodies were established before using variety of cell lysates [27, 45, 46]. The membranes were washed then probed with corresponding secondary antibodies (LICOR Biosciences) in 1:30,000 dilutions for 1 hr at room temperature and the specific bands were quantified using Odyssey application software supplied in an Odyssey Infrared imaging system (LI-COR Biosciences).

Chromatin immunoprecipitation (ChIP) assay and qPCR:

NaB-treated and untreated Caco-2 cells were subjected for ChIP analysis as described before [40, 47]. The cross-linked chromatin was incubated overnight with 1–2 μg of the specific antibody [H3, H3Ac, H3K27me3 and IgG (Millipore, Billerica, MA)] and the immunoprecipitated samples were subjected to DNA purification and analyzed by qPCR using *SLC52A3* promoter specific primers (forward 5'GGGTTCGCTCAGTGAAGGTA-3' and reverse 5'-AGCAGGAGTGTGTCCGTTGTG-3') to amplify the region −199 to +8 relative to transcriptional start site (relative to TSS as +1) using the PCR conditions as described before [40].

Statistical analyses:

Carrier-mediated RF uptake data presented in this study are means ± SE of multiple independent experimental determinations. RF uptake by the carrier-mediated process was determined by subtracting uptake of ^{3}H-RF in the presence of 1 mM unlabeled RF from total uptake (i.e., from uptake in the absence of unlabeled RF). Statistical significance was determined using the student's *t*-test/ Mann-Whitney Ranksum test with statistical significance set at $P < 0.05$. Western blot, RT-qPCR and ChIP-qPCR analyses are expressed as means ± SE of at least from three to six independent experiments.

Results

Effect of NaB on RF uptake by Caco-2 cells:

To determine the effect of NaB treatment on intestinal RF uptake, we examined the initial-rate of carrier-mediated ^{3}H-RF (25nM) uptake in confluent monolayers of Caco-2 cells treated with 1mM NaB for 24 hrs. The results showed that such a treatment leads to a significant ($P < 0.01$) induction in RF uptake. When higher concentrations of NaB were used (2, 5, and 10 mM), a slight-dose dependent increase in RF uptake was observed above what was seen with 1mM NaB (Fig. 1). We used 1 mM NaB as our working concentration in all subsequent studies. In another investigation, we examined and compared the effect of the other SCFAs that exist in significant amount in the colonic lumen (i. e., acetate and propionate) on ^{3}H-RF uptake by Caco2 cells and found that NaB to be the most potent inducer of the vitamin uptake (Fig. 2).

Effect of NaB on the level of expression of RFVT3 protein, mRNA and hnRNA in Caco-2 cells (in vitro) and mouse colonoids (ex vivo):

To investigate the basis of the observed induction in carrier-mediated RF uptake after NaB treatment, expression levels of RFVT3 protein was examined. After 24 hrs of NaB treatment, Caco-2 cells were harvested and total cell homogenate was subjected to western blot analysis to determine the level of hRFVT3 protein expression. The results showed that NaB treatment leads to a significant ($P < 0.01$) induction in hRFVT3 protein expression compared to untreated cells (Fig. 3A). Similar finding was observed when *ex vivo* mouse colonoids were exposed to NaB (1mM NaB; 24 hrs) in that a significant ($P < 0.05$) induction in mRFVT3 protein expression was observed in colonoids treated with NaB compared to untreated controls.

To further investigate the mechanism responsible for the observed NaB-dependent induction of RF uptake, we also examined the effect of NaB on hRFVT3 and mRFVT3 mRNA expression levels in Caco-2 cells and mouse colonoids, respectively by RT-qPCR. The results showed a significant ($P < 0.01$) induction in the levels of expression of hRFVT3 and mRFVT3 mRNA in both Caco-2 cells and mouse colonoids after NaB treatment compared to their respective untreated controls (Fig. 4A & B).

Changes in mRNA level could occur via changes in RNA stability and/or changes in transcription rate of the relevant gene (*SLC52A3* in this case). To investigate if changes in RNA stability caused the observed increase in intestinal hRFVT3 mRNA expression levels

during NaB treatment, we performed RNA stability assay as described by us and others before [35, 48]. Briefly, Caco-2 cells were pre-incubated with 5μg/ml actinomycin D (AcD), a potent RNA synthesis inhibitor, and then treated with 1mM NaB for different time points (0, 4, 8, 12, and 24 hrs). The results showed that hRFVT3 mRNA expression level in cells treated with AcD alone or with AcD plus NaB to be similar (Fig. 5). This suggests that NaB does not affect hRFVT3 mRNA stability rather the effect is transcriptionally mediated. To investigate the rate of transcription of *SLC52A3* gene after NaB treatment, the expression level of RFVT3 (*SLC52A3*) heterogenous RNA (hnRNA) (the first product of gene transcription and whose level of expression reflects the rate of transcription of a given gene) was determined by RT-qPCR. The results showed a significant ($P < 0.01$ for Caco-2 cells, and $P < 0.05$ for mouse colonoids) induction of both the hRFVT3 and mRFVT3 hnRNA expression in NaB-treated groups compared to their respective untreated controls (Fig. 6A & B).

Involvement of epigenetic mechanisms in the regulation of SLC52A3 transcriptional activity by NaB:

As described above, NaB stimulated intestinal RF uptake by inducing the expression of RFVT3 and that this effect is mediated, at least in part, via transcriptional mechanism(s) involving the *SLC52A3* gene. Since NaB is known to exert epigenetics effects (via acting as histone deacetylase inhibitor and its suppression of histone de-acetylation lead to accumulation of multi-acetylated forms of histone, which in turn alters the compactness of chromatin therefore affecting DNA folding and gene expression [49, 50]), we examined possible involvement of epigenetic mechanisms (e.g., histone modifications) in the effect of NaB on expression of the RFVT3. We also examined possible involvement of DNA methylation (specifically trimethylation of histone 3 lysine 27; H3K27me3) since this histone modification also influence gene expression [40, 51, 52]. For this, we performed a ChIP-qPCR analysis and focused on the *SLC52A3* promoter region (−199 to +8) since it is essential for driving basal activity of the promoter and is involved the regulation of the RF uptake process in intestinal epithelial cells under other conditions [40, 53]. The results showed that treating Caco-2 cells with NaB (1 mM; 24 hrs) to be associated with a significant ($P < 0.01$) increase in the histone acetylation marker (H3Ac) at *SLC52A3* promoter region compared to untreated control cells (Fig. 7A). We also observed a significant ($P < 0.05$) decrease in the level of repressor marker H3K27me3 in *SLC52A3* promoter region in cells treated with NaB compared to untreated cells (Fig.7B). Together, these findings suggest that the up-regulation of hRFVT3 expression in the presence of NaB may involve induction in histone hyper-acetylation and selective histone methylation at the *SLC52A3* gene promoter region.

Discussion

As mentioned earlier, humans and other mammals obtain RF from dietary and microbiota sources via absorption in the small and large intestine, respectively. This occurs via a specific carrier-mediated process that involves the RFVT3, a transporter that is exclusively localized at the apical membrane domain of the polarized intestinal epithelial cells [20, 22, 26]. It has also been known for some time that NaB causes induction in intestinal uptake of

certain substrates and in the level of expression of the uptake systems involved in these processes [35–39]. Little is known about the effect of NaB on intestinal/colonic uptake of water-soluble vitamins including RF. Thus, our aim in this investigation was to address this issue using the *in vitro* human-derived intestinal epithelial Caco-2 cells and *ex vivo* mouse colonoids.

Our results showed that treatment of Caco-2 cells with NaB to lead to a significant induction in RF uptake and this up-regulation was associated with the marked increase of hRFVT3 protein and mRNA expression. Similarly, treating mouse colonoids with NaB was found to lead to an induction in mRFVT3 protein and mRNA expression. As it is known, the increase in the level of expression of a given mRNA could be due to cause by changes in level of its stability, and/or could be an indication for the involvement of transcriptional mechanism(s). To test the first possibility, we performed mRNA stability assay and found no change in hRFVT3 mRNA stability in cells treated with NaB compared to untreated controls. To test the second possibility, we determine the level of expression of the *SLC52A3* hnRNA in Caco-2 cells and mouse colonoids treated with 1mM NaB. The results indeed showed a significant increase in the level of expression of the RFVT3 hnRNA in cells treated with NaB compared to untreated controls. This clearly suggest the involvement of transcriptional mechanism in mediating the effect of NaB on intestinal RF uptake and on expression of the uptake system involved.

Since epigenetic mechanisms play an important role in the regulation of gene expression, and that NaB is known to induce epigenetic alterations in the genes that it affects [35, 40, 47, 50–52], we also searched for possible epigenetic alterations in the *SLC52A3* promoter after NaB treatment. We focused on histone modifications since it plays important role in regulating transcriptional activities. Our findings showed that a significant increase in the activity of H3Ac in cells treated with NaB compared to untreated controls. In contrast the activity of the heterochromatin (repressor) H3K27me3 was found to be significantly reduced in NaB treated cells. Together, these findings suggest that possible involvement of epigenetic mechanism(s) in the *SLC52A3* promoter activity by NaB.

In summary, our investigation shows that the NaB stimulates intestinal RF uptake process and that this occurs via induction in the level of expression of the RFVT3 in the intestine. In addition, these results suggest that this effect occurring at the level of transcription of the *SLC52A3* gene and may involve epigenetic mechanism(s).

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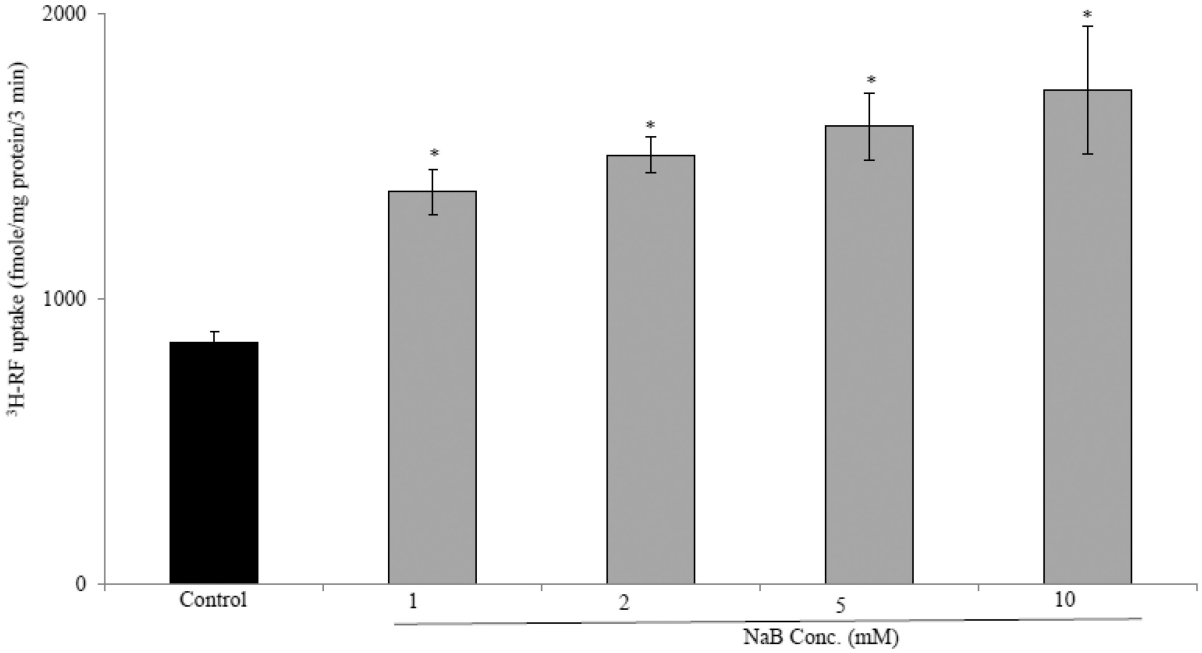

Figure 1. Effect of NaB on RF uptake by Caco-2 cells.

Caco-2 cells were treated with increased concentration (1, 2, 5 and 10 mM) of NaB for 24 hrs and RF uptake was assayed. Data are means ± SE of multiple determinations from three (n=3) independent experiments * $P < 0.01$.

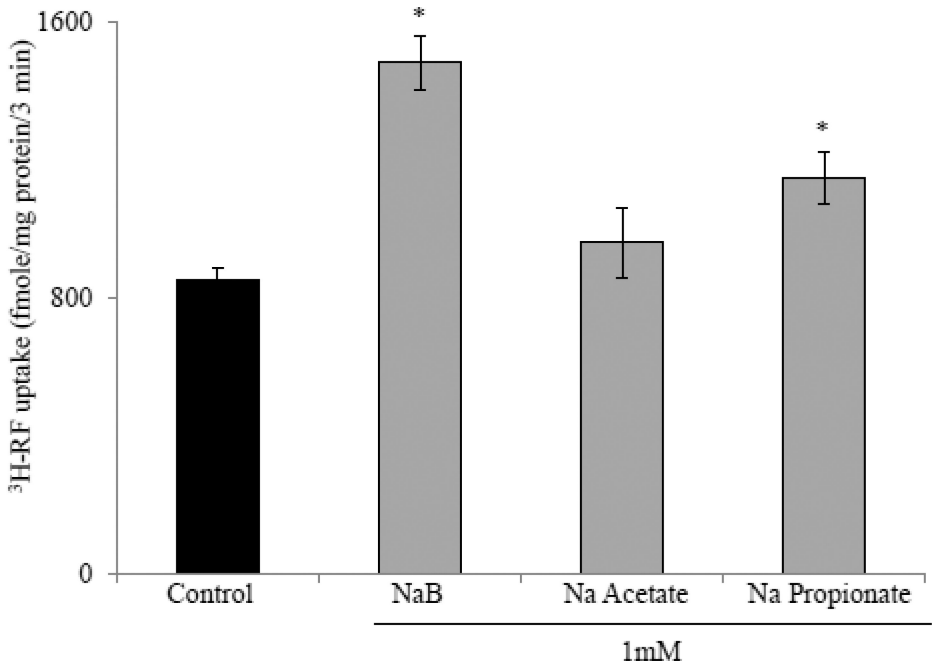

Figure 2. Effect of different short chain fatty acids (SCFAs) on intestinal RF uptake.

Confluent Caco-2 monolayers were treated with different SCFAs (1mM) for 24 hrs followed by determination of RF uptake. Data are means ± SE of multiple determinations from three (n=3) independent experiments * $P < 0.01$.

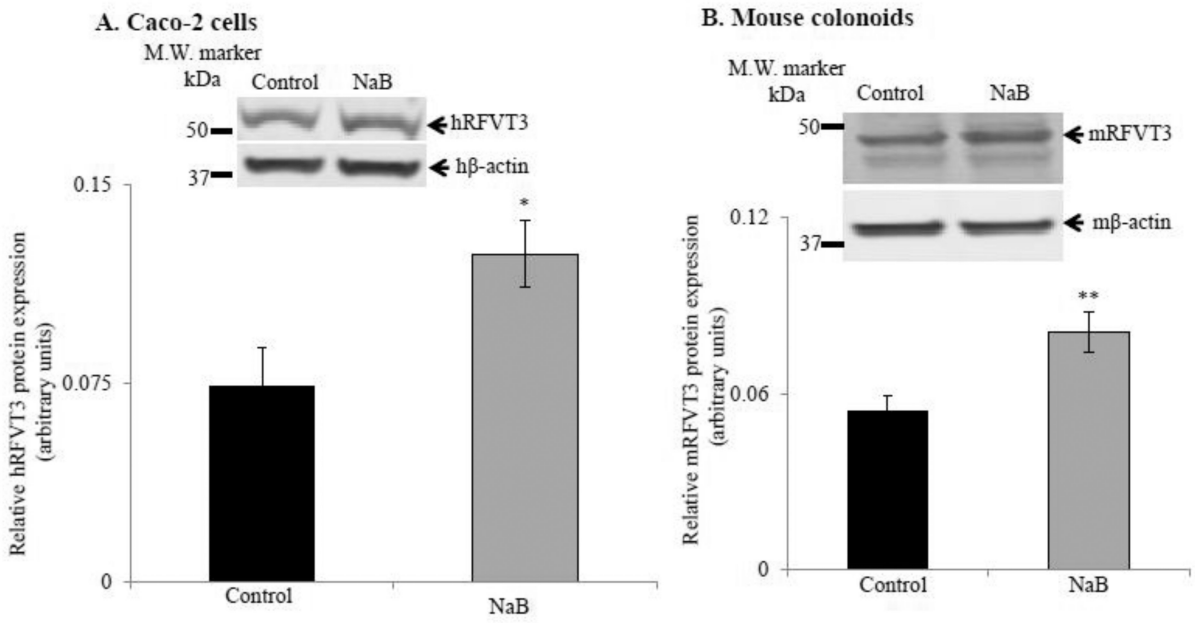

Figure 3. Effect of NaB on level of expression of the RFVT3 protein in Caco-2 cells and in mouse colonoids.

Western blot analysis was performed to determine the expression of hRFVT3 (A) and mRFVT3 (B) protein levels in Caco-2 cells (n=6) and mouse colonoids (n=5), respectively, after NaB treatment as described in “Materials & Methods”. The images are representative of multiple independent experiments with similar results. Data are means ± SE of at least from five independent experiments **P < 0.05, * P < 0.01.

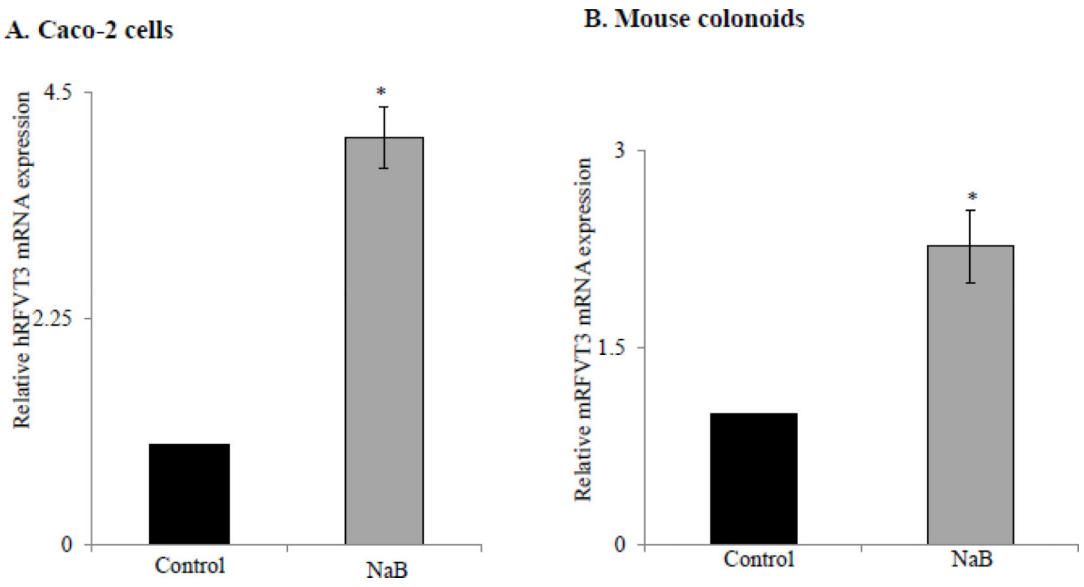

Figure 4. Effect of NaB on level of expression of RFVT3 mRNA in Caco-2 cells and in mouse colonoids.

RT-qPCR was performed to determine the hRFVT3 (A) and mRFVT3 (B) mRNA expression in NaB treated and untreated Caco-2 cells (n=3) and colonoids (n=4) using gene specific primers as described in “Materials & Methods”. Data are means ± SE of at least from three independent experiments * $P < 0.01$.

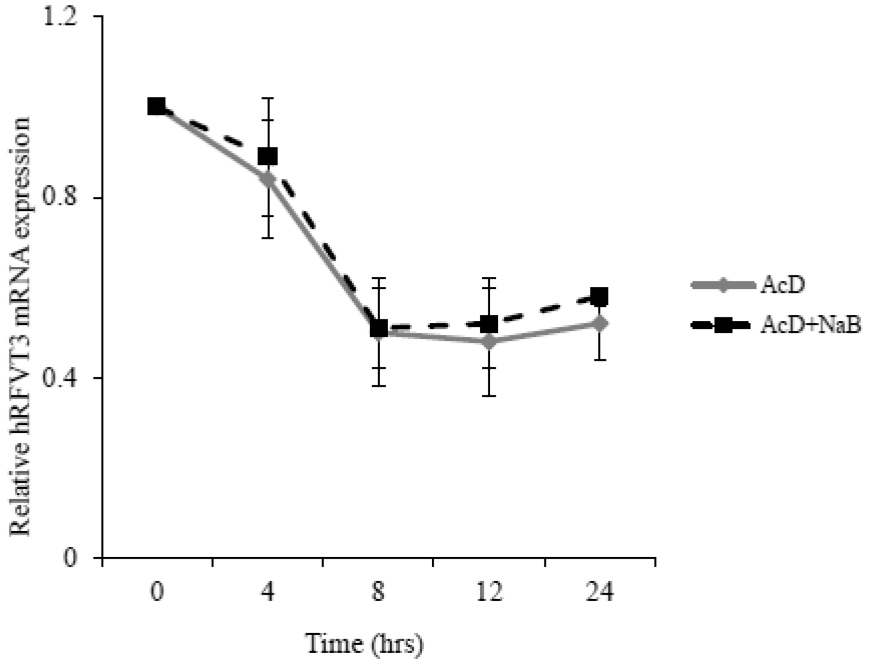

Figure 5. Effect of NaB on hRFVT3 mRNA stability in Caco-2 cells.

Caco-2 cells were pre-incubated with 5μg/ml actinomycin D (AcD) for 1hr, then treated with NaB (1mM) for 24 hrs. hRFVT3 mRNA expression levels were quantified by RT-qPCR. Data are means ± SE of six independent experiments.

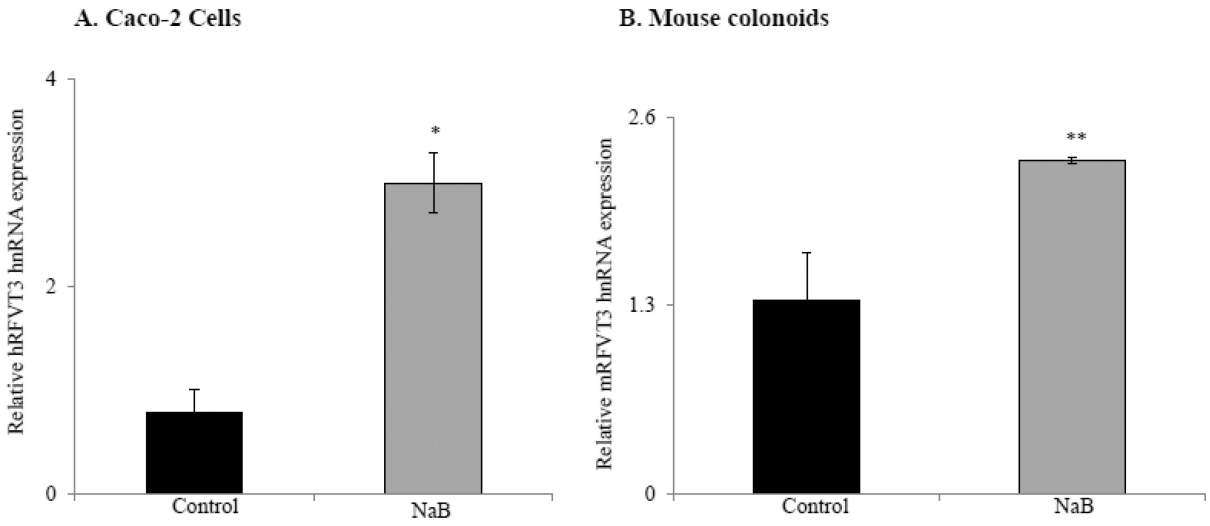

Figure 6. Effect of NaB on level of expression of RFVT3 hnRNA in Caco-2 cells and in mouse colonoids.

RT-qPCR was performed to determine the hRFVT3 (A) and mRFVT3 (B) hnRNA expression levels in NaB treated and untreated Caco-2 cells (n=3) and colonoids (n=4) using gene specific primers as described in “Materials & Methods”. Data are means ± SE of at least from three independent experiments * $P < 0.01$; ** $P < 0.05$.

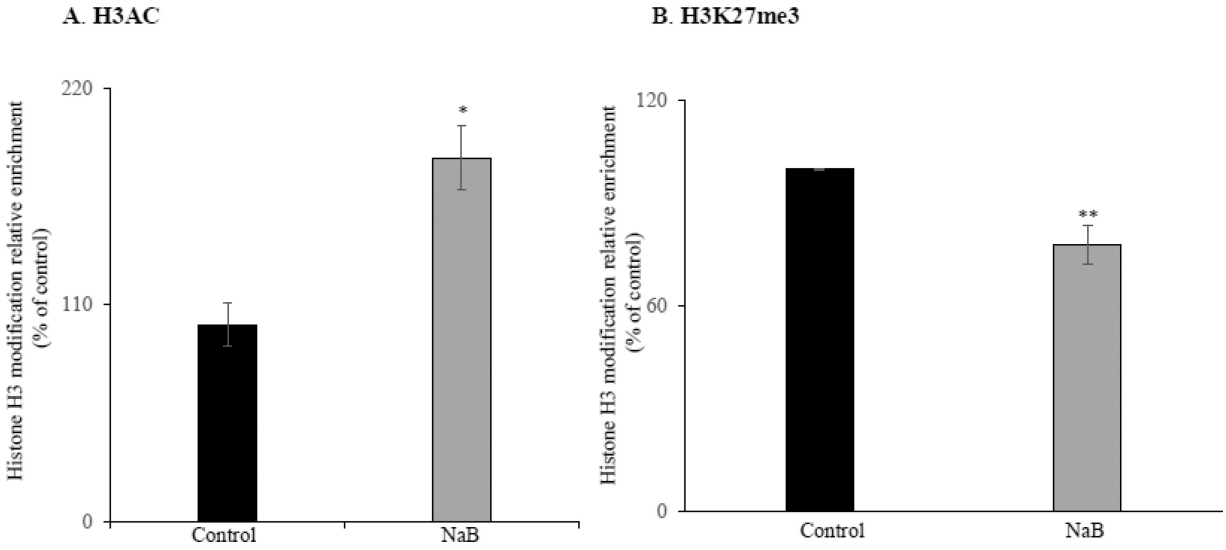

Figure 7. ChIP-qPCR analysis of H3Ac and H3K27me3 in immunoprecipitated DNA fragments on *SLC52A3* promoter in Caco-2 cells treated with NaB.

ChIP-qPCR data showing the ratio of H3Ac (A) or H3K27me3 (B) relative to total H3 levels in Caco-2 cells treated with NaB and untreated control cells. Values are normalized to total input DNA and expressed as means ± SE of three (n=3) independent experiments from different sample preparations. * $P < 0.01$; **$P < 0.05$.