Transcriptional modulation of SLC26A3 (DRA) by sphingosine-1-phosphate

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Anbazhagan AN, Priyamvada S, Alakkam A, Kumar A, Borthakur A, Saxena S, Gill RK, Alrefai WA, Dudeja PK. Transcriptional modulation of SLC26A3 (DRA) by sphingosine-1-phosphate. Am J Physiol Gastrointest Liver Physiol 310: G1028–G1035, 2016. First published April 14, 2016; doi:10.1152/ajpgi.00308.2015.—SLC26A3 or Downregulated in adenoma (DRA) is an important chloride/HCO3− exchanger (NHE3) and Cl−/HCO3− exchanger SLC26A3 or DRA (Downregulated in adenoma) (20, 25). Several lines of evidence suggest that dysregulation of DRA function and expression underlies the pathophysiology of inflammatory or infectious diarrhea. Studies have shown that mutations in DRA gene cause congenital chloride diarrhea, a rare genetic disorder characterized by high volume watery diarrhea and a massive loss of chloride (3, 24).

Diarrhea occurs due to the dysregulation of either absorptive or secretory mechanisms of electrolyte transport in the intestine resulting in increased fluid and electrolyte load in the gut (2, 12). One of the predominant mechanisms of electrolyte absorption in the intestine involves electroneutral NaCl absorption in the ileum and colon. This primarily occurs via the coupled operation of apical Na+/H+ exchanger (NHE3) and Cl−/HCO3− exchanger SLC26A3 or DRA (Downregulated in adenoma) (20, 25). The important role of DRA was further evident from studies in DRA knockout mice, which exhibit diarrhea phenotype due to loss of luminal membrane Cl−/base exchange activity (37, 46). Because DRA plays a critical role in the maintenance of total body fluid and electrolyte homeostasis, upregulation of its function and expression in the intestine is now being considered as a novel therapeutic target for diarrheal diseases. In this regard, previous studies from our laboratory have shown upregulation of DRA function and/or expression by probiotics like Lactobacillus acidophilus, Bifidobacterium species, and bioactive lysophosphatidic acid (21, 35, 39–41). Whether DRA function or expression can be directly affected by S1P, an important bioactive lipid metabolite shown to be beneficial in colitis, has not been investigated.

S1P can function both extra- and intracellularly via signaling through cognate S1P receptor or as a second messenger (30). There are five S1P specific G protein-coupled receptors, S1PR1–S5 (6, 13). All the receptor subtypes (S1PR1–S5) have been shown to be expressed in mouse colon (9) and in human colonic cells (44). However, to date, the direct effects of S1P on intestinal ion transport processes in general and DRA in particular have not been studied. The present studies were undertaken to investigate the effects of S1P, including the involvement of S1P receptors, on the apical Cl−/HCO3− exchanger DRA and to elucidate the underlying mechanisms.

Bioactive phospholipids have lately emerged as an important source of signaling molecules, and the role of these phospholipids and their receptors is being emphasized as a target for drug design leading to a new arena of lipodomic-based therapeutics (6, 27). In this regard, sphingosine-1-phosphate (S1P), a sphingolipid metabolite, has been shown to mediate diverse cellular responses such as cell growth, proliferation, actin cytoskeletal organization, and maintenance of tight junction assembly (18, 19, 26). S1P is either produced intracellularly from sphingomyelin (precursor molecule) by activation of enzyme sphingosine kinase (42) or is obtained from diet (29).

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Our results demonstrate that S1P stimulates DRA expression and function in Caco-2 cells via S1P receptors and PI3K/Akt-dependent pathways. Also, S1P induced DRA promoter activity was via enhanced binding of Ying-Yang1 (YY1) transcription factor to S1P-responsive elements in DRA promoter. These findings indicate that S1P or its receptor agonists may have potential anti diarrheal effects and may be of benefit as a therapeutic agent in the treatment of diarrhea associated with inflammatory or infectious disorders of the gut.

METHODS

Materials. S1P was purchased from Enzo Life Sciences (Ann Arbor, MI). A sphingosine-1-phosphate receptor-2 agonist and antagonist, FTY-720 and JTE-013, respectively, were obtained from Cayman Chemicals (Ann Arbor, MI). The pharmacological inhibitor LY294002 was purchased from Biomol (Plymouth Meeting, PA), and triciribine was procured from Calbiochem (San Diego, CA). 4',4’-Diisothiocyanostilbene-2,2’ disulfonic acid (DIDS) was obtained from Sigma Aldrich (St. Louis, MO). \(^{125}\)I (NaI) radionuclide was procured from Perkin Elmer (Boston, MA). DRA antibody was raised at the Research Resource Center of the University of Illinois at Chicago (32). Goat anti-rabbit antibody conjugated to horseradish peroxidase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A luciferase assay system was procured from Promega (Madison, WI) and a \(\beta\)-galactosidase assay kit was obtained from Clontech (Mountain View, CA).

S1P preparation. S1P was first dissolved in methanol (0.5 mg/ml) and aliquoted in glass vials. The solvent was evaporated with a stream of nitrogen in a swiveling motion to deposit a thin film on the inside of the glass vial and was stored at \(-80^\circ C\) until further use. At the time of treatment, 400 \(\mu\)l of 0.4% BSA in PBS was added to the stored vial, which was placed in water bath at 37°C for 30 min with repeated vortexing. Dissolved stock solution was then sonicated and used for treatment.

Cell culture. Caco-2 cells [American Type Culture Collection (ATCC), Manassas, VA] were grown routinely in Eagle’s minimum essential medium from ATCC in 5% CO\(_2\)-95% air environment at 37°C in T150 plastic flasks. Growth medium was supplemented with 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 2 mg/l gentamicin, and 20% fetal bovine serum. Cells between passages 25 and 45 were used for the present study. Caco-2 cells were plated on 24-well plates (Costar, Corning, NY) at a density of 2 \times 10^4 cells/well. Fully differentiated Caco-2 monolayers (10–14 days postplating) were treated for the present study. Caco-2 cells were plated on 24-well plates (Costar, Corning, NY) at a density of 2 \times 10^4 cells/well. Fully differentiated Caco-2 monolayers (10–14 days postplating) were treated with 5 \(\mu\)M S1P or vehicle for different time points ranging from 8 to 24 h in serum-free cell culture medium to assess \(\mathrm{Cl}^-/\mathrm{HCO}_3^-\) exchange activity, DRA mRNA, and protein expression. For the promoter studies, cells were plated on a 24-well plate at a density of 5 \times 10^5 cells/well and were transiently transfected by electroporation utilizing an Amaxa Nucleofector system, while still in suspension. For inhibitor studies, transfected cells were pretreated with JTE-013 or LY294002 or triciribine for 1 h and then coculated with S1P for another 24 h.

\(\mathrm{Cl}^-/\mathrm{HCO}_3^-\) exchange activity. \(\mathrm{Cl}^-/\mathrm{HCO}_3^-\) exchange activity was determined by measuring DIDS-sensitive \(^{125}\)I uptake in base-loaded cells as previously described by us (21). After the uptake, the cells were solubilized by incubation with 0.5 N NaOH for 4 h and protein concentration was measured by Bradford method (5). Radioactivity was measured by Packard Tri-Carb 1600 TR liquid scintillation analyzer (Packard Instruments; Perkin Elmer). The \(\mathrm{Cl}^-/\mathrm{HCO}_3^-\) exchange activity was calculated as DIDS-sensitive \(^{125}\)I uptake, and the specific activity was expressed as nanomoles per milligram protein per min.

Quantitative real-time RT-PCR. Total RNA was extracted from control and S1P-treated Caco-2 cells by using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Real-time RT-PCR was used to quantify the relative abundance of DRA or PAT1 mRNA in control and S1P-treated Caco-2 cells after one-step reverse transcription and amplification using Brilliant SYBR Green qRT-PCR Master Mix Kit (Agilent Technologies, Santa Clara, CA). Gene-specific primers used are listed in Table 1.

Western blotting. Control and S1P-treated Caco-2 cells were washed with ice-cold 1× PBS to remove residual media. The cells were scraped in 1× PBS and cell lysates were prepared as described previously (32). Equal amounts (75 \(\mu\)g/sample) of cell lysates were solubilized in SDS-gel loading buffer, loaded on a 7.5% SDS-polyacrylamide gel, and transblotted to nitrocellulose membrane. The membrane was blocked for 1 h at room temperature with 5% nonfat dry milk in 1× PBS followed by overnight incubation with affinity-purified anti-DRA (1:100 dilution) or GAPDH antibody (Sigma; 1:3,000 dilution) in 1× PBS and 2.5% nonfat dry milk at 4°C. The membrane was washed five times with the wash buffer (1× PBS and 0.1% Tween-20) for 5 min and probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:2,000 dilution) for 1 h followed by detection with ECL (enhanced chemiluminescence, from Bio-Rad, Hercules, CA). Expression levels of phosphorylated and total Akt were detected by using specific antibodies from Cell Signaling (Danvers, MA) at recommended dilutions in 1× TBST (Tris-buffered saline with 0.1% Tween 20) and 1% BSA buffer overnight at 4°C. The membranes were washed five times with the wash buffer (1× TBST) for 5 min and probed with HRP-conjugated goat anti-rabbit antibody (1:2,000 dilution) for 1 h.

Measurement of DRA promoter activity. Caco-2 cells were transiently transfected with full-length DRA promoter and different deletion constructs cloned upstream of the luciferase reporter gene by electroporation utilizing an Amaxa Nucleofector system as previously described by us (36). p-Cytomegalovirus (CMV)-\(\beta\)-galactosidase mammalian expression vector (BD Biosciences, Clontech, Palo Alto, CA), was also transfected that served as an internal control for transfection efficiency. After 24 h, cells were treated with S1P or S1PR2 agonist/antagonist or PI3K/Akt inhibitors for different time points ranging from 8 to 24 h. Control cells were treated with vehicle (0.4% BSA-1× PBS). Cells were then washed with 1× PBS and lysed with passive lysis buffer (Promega, Madison, WI). The activities of both firefly luciferase and \(\beta\)-galactosidase were measured by luminometer according to the manufacturer’s instructions with kits from Promega and Clontech, respectively.

Nuclear extract and EMSA. Caco-2 cells were treated with 5 \(\mu\)M S1P or vehicle and nuclear extracts were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagents from Pierce (Rockford, IL) according to the manufacturer’s instructions. YY1 double-stranded oligo used as a probe for the gel shift assay was designed and obtained from IDT (Integrated DNA Technologies, Coralville, IA) (5’-AACACAACACTATACCCTGCTATCTTTGG-3’). The sequence of the potential YY1-A binding sites utilized for electrophoretic mobility shift assay (EMSA) lies between –790 to –392 bp. The consensus binding site for YY1-A is 5’-CGCTCCGCGGCGATCCTTGCGGCG-3’ and the mutant oligonucleotide used in the study is 5’-CGCTCAGCGATTATCTTGGCGGCG-3’. Gel shift assay

**Table 1. Human primers used for real-time PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>SLC26A3 or DRA</td>
<td>(F) 5’-TTCAAGGTTGAGGAGCTTATTCC-3’</td>
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<tr>
<td></td>
<td>(R) 5’-GTTGTCTGCTGGTTGCTTCTG-3’</td>
</tr>
<tr>
<td>SLC26A6 or PAT-1</td>
<td>(F) 5’-AGATGCGGCTATCCTTCTTCTT-3’</td>
</tr>
<tr>
<td></td>
<td>(R) 5’-ATCCACGACAGCCTTCTGCTT-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>(F) 5’-GAATGCGGCTATCCTTCTTCTT-3’</td>
</tr>
<tr>
<td></td>
<td>(R) 5’-GAATGCGGCTATCCTTCTTCTT-3’</td>
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was performed by a nonradioactive digoxigenin labeling method utilizing a commercially available kit from Roche Diagnostics (Mannheim, Germany). The double-stranded oligonucleotide was end labeled with digoxigenin-11, and binding reactions were performed as previously described (14, 36); 6% nondenaturing gels were used to separate the DNA-protein complexes by electrophoresis. Electotransferred complexes on the membrane were detected by use of anti-digoxigenin antibody.

Statistical analysis. Results are expressed as means ± SE of three to five independent experiments. Statistical analysis was performed by Student’s t-test or one-way analysis of variance followed by Tukey’s test. A P value of 0.05 or less was considered statistically significant.

RESULTS

S1P stimulates Cl−/HCO3− exchange activity. To determine the long-term effects of S1P on Cl−/HCO3− exchange activity, Caco-2 monolayers were treated with different doses of S1P (1–10 μM) for 24 h. Cl−/HCO3− exchange activity was measured as DIDS-sensitive 125I− uptake after base loading the cells (as described in METHODS). As shown in Fig. 1, S1P significantly increased the DIDS-sensitive iodine uptake in a dose-dependent manner with a moderate increase at 1 μM (~31%) and a significantly higher increase at 5 μM concentration (~72%) compared with control with no further change at 10 μM. Therefore, 5 μM S1P was used in all subsequent experiments. However, no significant increase in Cl−/HCO3− exchange activity was observed at 8 and 16 h by 5 μM S1P (data not shown).

S1P increases DRA mRNA and protein expression. We next investigated the effect of S1P on DRA mRNA expression in Caco-2 cells. As shown in Fig. 2A, DRA mRNA was significantly increased by incubation of Caco-2 cells with 5 μM S1P. A significant increase in DRA mRNA expression was observed as early as 8 h (~1.5-fold) of S1P treatment. S1P-mediated increase in DRA expression was sustained up to 16 h (Fig. 2A). Both DRA and PAT-1 (putative anion transporter-1) are characterized as the apical membrane Cl−/HCO3− exchangers of intestinal epithelial cells that belong to the family of SLC26 anion exchangers (40). However, in contrast to its effects on DRA, S1P did not show any effect on PAT-1 mRNA levels at any given time point (Fig. 2B). These results suggest that the effects of S1P are specific to DRA. Additionally, Western blot analysis also demonstrated a significant increase in DRA protein levels (Fig. 3A). Densitometric analysis of the protein bands showed that S1P treatment increased DRA protein levels by ~50% compared with control (Fig. 3B). The results show that the increase in DRA function in response to S1P was concomitant with an increase in DRA mRNA and protein expression.

S1P increases DRA promoter activity. Because S1P increased DRA mRNA expression, we next investigated whether the increase is mediated via transcriptional regulation of DRA at the promoter level. Caco-2 cells were transiently transfected with full-length DRA promoter (~1183/+114) along with pCMV-βgal as an internal control. The cells were then incubated with 5 μM S1P for different time points and the promoter activity was determined by the firefly luciferase assay and the measurement of β-galactosidase to normalize for the transfection.
S1PR2 receptor. /H11002

region of the DRA promoter (Fig. 5).

show that the S1P response elements are located in the
pared with the respective controls (taken as 100%). These data
S1P-induced stimulatory effects on the promoter activity com-
were observed with the deletion construct
1183/
H11002
H11001
(114) in response to S1P treatment. Similar results
promoter activity was measured by luciferase assay. As shown
fected cells were then treated with S1P (5
- deletion constructs of DRA promoter. Transiently trans-
fected cells were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The blot was immunostained with rabbit anti-DRA antibody. A: representative blot of 3 separate experiments is shown. B: data were quantified by densitometric analysis and expressed as percent of control. Values represent means ± SE of 3 different experiments. *P < 0.05 compared with control.

Identification of S1P-responsive region in DRA promoter. To identify the S1P-responsive region involved in mediating the stimulatory effect of S1P on DRA promoter activity, Caco-2 cells were transiently transfected with progressive 5'-deletion constructs of DRA promoter. Transiently transfected cells were then treated with S1P (5 µM) for 8 h and the promoter activity was measured by luciferase assay. As shown in Fig. 5, a significant increase in the promoter activity (2-fold) was observed for the full-length DRA promoter construct (−1183/+114) in response to S1P treatment. Similar results were observed with the deletion construct −790/+114. However, further deletion to −398/+114 fragment abolished the S1P-induced stimulatory effects on the promoter activity compared with the respective controls (taken as 100%). These data show that the S1P response elements are located in the −790/−398 region of the DRA promoter (Fig. 5).

S1P-mediated stimulation of DRA promoter activity involves S1PR2 receptor. S1P exerts its effects via G protein-coupled receptors S1PR1−3 (7). To identify the receptor subtype involved, DRA promoter activity was measured in response to FTY-720, a S1P receptor agonist, that shows dose dependent affinity toward different S1P receptor subtypes (7). When Caco-2 cells transfected with DRA promoter were treated with FTY-720 in the nanomolar range (known to activate the receptors S1PR1 and S1PR3), no activation of DRA promoter was observed (data not shown). However, as shown in Fig. 6A, treatment of transiently transfected Caco-2 cells with FTY-720 at 5 µM concentration (known to activate S1PR2) for 8 h resulted in significant stimulation of DRA promoter activity (145.7 ± 4.9), similar to S1P. These results indicate that S1PR2 is involved in mediating the effects of S1P on DRA promoter activity. Involvement of S1PR2 was further confirmed utilizing JTE-013, a specific S1PR2 antagonist. As shown in Fig. 6B, S1P alone resulted in a significant increase in DRA promoter activity; JTE-013 treatment alone had no effect on DRA promoter. However, cotreatment of S1P with JTE-013 completely blocked the stimulatory effects of S1P on DRA promoter activity. Overall, these studies indicate that S1P via S1PR2 increases DRA promoter activity in Caco-2 cells.

Stimulatory effects of S1P on DRA promoter activity are PI3K/Akt dependent. Previous studies in HMEC-1 (intestinal epithelial cell line) have shown that S1P mediates its effects via the activation of PI3K/Akt pathway (4). We next sought to examine the role of PI3K/Akt in mediating the stimulatory effects of S1P on DRA promoter activity. Caco-2 cells transiently transfected with DRA promoter were pretreated with PI3K inhibitor (LY294002, 50 µM) or Akt inhibitor (triciribine, 1 µM) for 1 h followed by coincubation with S1P for another 8 h. Our results showed that inhibition of PI3K (Fig. 7A) and Akt (Fig. 7B) completely blocked the stimulatory effects of S1P on DRA promoter, suggesting that S1P stimulates DRA promoter activity via the activation of PI3K/Akt pathway. Because S1P-mediated increase in DRA promoter activity was blocked by the S1PR2 antagonist JTE-013, we next evaluated whether JTE-013 also inhibits S1P-mediated activation of Akt. This was confirmed by examining the phosphorylation levels of Akt (pAkt) in response to S1P treatment of Caco-2 cells in the presence or absence of JTE-013. Time course of S1P treatment revealed that activation of Akt occurred as early as 15 min and persisted till 8 h posttreatment (data not shown). As shown in Fig. 7C, S1P (5 µM) treatment for 15 min significantly increased pAkt levels compared with

Fig. 3. S1P increases DRA protein expression in Caco-2 cells. Lysates prepared from untreated or S1P (5 µM, 24 h)-treated Caco-2 cells were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The blot was immunostained with rabbit anti-DRA antibody. A: representative blot of 3 separate experiments is shown. B: data were quantified by densitometric analysis and expressed as percent of control. Values represent means ± SE of 3 different experiments. *P < 0.05 compared with control.

Fig. 4. S1P stimulates DRA promoter activity. Caco-2 cells were transiently transfected with DRA luciferase promoter construct (−1183 to +114-Luc) (Luc) along with the mammalian expression vector for β-galactosidase (pCMV β-gal). Cells were treated with 5 µM S1P in 1% FBS in cell culture medium for 8, 16, and 24 h. The promoter activity was expressed as a ratio of luciferase to β-galactosidase (relative luciferase activity) in each sample expressed as % of control. All transfections were performed in triplicate and repeated at least thrice with separate batches of cells. Results are expressed as % of control and represent means ± SE of 4 separate experiments. *P < 0.05 compared with control.
G1032 SPHINGOSINE-1-PHOSPHATE ENHANCES DRA FUNCTION AND EXPRESSION

Fig. 5. The region between −790 and −398 of DRA promoter harbors the S1P response elements. Caco-2 cells were transiently transfected with full-length DRA promoter and its progressive 5′ deletion constructs and were treated with S1P (5 µM, 8 h) in 1% FBS in cell culture medium. The promoter activity was expressed as a ratio of luciferase to β-galactosidase (relative luciferase activity). Results are expressed as % of respective control for each promoter construct and represent means ± SE of 3 separate experiments. *P < 0.05 compared with control.

Fig. 6. S1PR2 receptor is involved. Caco-2 cells transfected with DRA luciferase promoter construct were treated with FTY-720 (5 µM) S1PR2 agonist (A) or with S1PR2 antagonist JTE-013 (50 nM) (B) (pretreatment for 1 h followed by coincubation with S1P 5 µM for 8 h). The promoter activity was expressed as a ratio of luciferase to β-galactosidase (relative luciferase activity). Results are expressed as % of respective control for each promoter construct and represent means ± SE of 3 separate experiments. *P < 0.05 compared with control.

The untreated control cells. Pretreatment of Caco-2 cells with JTE-013 alone had no effect on pAkt levels. However, cotreatment of cells with JTE-013 and S1P markedly suppressed S1P-induced phosphorylation of Akt (Fig. 7C). These data indicate that the stimulatory effect of S1P on DRA expression was mainly via S1PR2 and involves the activation of PI3/Akt pathway.

S1P induces YY1 transcription factor that binds to the response element in DRA promoter. In silico analysis (using ALGGEN PROMO) of −790/−398 region of DRA promoter revealed the binding sites for the transcription factor YY1 and GATA (Fig. 8A). Our earlier studies demonstrated the involvement of the transcription factors GATA and YY1 in mediating the effects of butyrate on DRA promoter activity (1). To examine the potential involvement of these transcription factors in mediating the stimulatory effects of S1P on DRA promoter activity, EMSA was performed utilizing YY1 and GATA cis-elements as digoxigenin-end-labeled probes. The binding of GATA to nuclear extracts was not affected in presence of S1P (data not shown). However, as shown in Fig. 8B, the binding of labeled YY1 to nuclear extracts was significantly increased in the presence of S1P (lane 3) compared with control (lane 2). The binding specificity of the complexes was examined by competition experiments in which excess of unlabeled cold, consensus, or mutant oligo of YY1 was used. S1P-induced increase in YY1 binding to the Caco-2 nuclear proteins was competed out in the presence of excess of unlabeled probe (lane 4) or unlabeled consensus YY1 oligo (lane 5) but only partially competed by the mutant YY1 oligo (lane 6). The identity of the binding protein in this complex was confirmed by addition of a specific YY1 antibody. The presence of the anti-YY1 antibody abrogated the formation of the DNA-protein complex (lane 7). This indicates that YY1 antibody potentially binds to a site on the transcription factor that is essential for its DNA binding activity and this in turn blocks the formation of the DNA-protein complex. Overall these results suggest that S1P stimulates DRA promoter activity by increasing the binding of YY1 to the DRA promoter.
DISCUSSION

S1P is a bioactive sphingolipid metabolite produced abundantly in intestinal tissues and known to perform diverse functions (34, 44, 47). For example, increase in transepithelial resistance with a concomitant decrease in paracellular permeability in response to S1P has recently been reported (18). This barrier-protective effect of S1P was attributed to an increase in level and distribution of E-cadherin (18). Recent studies showed that oral treatment of S1P agonist protected experimental colitis by improving tight junction-dependent barrier function (11). However, there are no studies pertaining to its effects on intestinal epithelial absorptive processes, which are fundamental to diarrhea associated with IBD (33).

The SLC26A3 gene product DRA plays critical role in intestinal chloride absorption. In this regard, previous studies from our laboratory and others have shown dysregulation of DRA function and expression in the pathogenesis of diarrhea associated with inflammation (33, 38, 39) or enteric infections (37). The S1P response cis-element is located in the −790/−398 region of DRA promoter. A: schematic diagram of transcription factor YY1 and GATA binding region in S1P-responsive region of DRA promoter (using ALGGEN PROMO). B: YY1 binds to DRA promoter. Nuclear extracts from control (untreated) or S1P-treated (5 μM, 24 h) Caco-2 cells were incubated with DIG-labeled potential YY1 oligo. Lane 1 shows only the free probe. DNA-protein binding in control (lane 2) was significantly increased in response to S1P treatment (lane 3). DNA-protein complexes were competed by excess of unlabeled potential YY1 probe (lane 4) or excess of YY1 consensus sequence (125-fold excess, lane 5), but only partially by mutant consensus YY1 oligo (lane 6). Addition of YY1 antibody blocked the formation of DNA protein complex (lane 7). A representative gel of 3 separate experiments is shown.
out, suggesting that it is less important in mediating bulk absorption. Parallel to the increase in DRA mRNA expression, S1P treatment significantly increased the promoter activity of DRA gene, suggesting the involvement of transcriptional regulation.

SIP can act both intracellularly as a second messenger as well as extracellularly via interaction with S1P receptors designated as S1PR1–5. The S1P receptor subtypes exhibit differential coupling to various heterotrimeric G proteins that in turn regulate numerous downstream signaling pathways (6, 43). Interestingly, S1P-mediated effects on DRA promoter activity in Caco-2 cells were found to be receptor dependent since the stimulatory effects of SIP on DRA promoter activity were completely blocked in presence of the potent S1PR2 antagonist JTE-013. Similar to SIP, DRA promoter activity was stimulated by pharmacological activation of S1PR2 by FTY720 (5 μM). FTY-720 serves as an S1PR2 agonist at higher (micromolar) concentrations (45) and is reported to exert protective effects in amelioration of oxazolone colitis in Balb/c mice (9) and in treatment of colon cancer in ulcerative colitis patients (28).

S1PR2 in particular has been shown to couple through G\textsubscript{12/13} (6). Signaling through G\textsubscript{12/13} has been shown to be associated with activation of 1) GTPase-Ras/Erk pathway, 2) PI3K/Akt pathway, 3) PI3K/Rac pathway, and 4) protein kinase C and phospholipase C (6). S1P-induced activation of the PI3/Akt pathway through G\textsubscript{12/13} has also been shown to prevent apoptosis and promote survival (4, 17) in intestine. Our results clearly showed that the increase in DRA promoter activity by SIP was abolished in the presence of LY294002 (PI3 kinase inhibitor) and triciribine (Akt inhibitor), indicating involvement of PI3/Akt signaling pathway in mediating SIP effects on DRA expression. PI3K/Akt signaling is considered as a prosurvival mediator and is also reported to be involved in alterations in the expression of various genes like cyclin D1 in intestinal epithelial cells (41).

Progressive 5'-deletions of the DRA promoter from −790/+114 to −398/+114 region completely abrogated the stimulatory effects of SIP. These data indicated that potential cis-elements present in −790 to −398 region of DRA promoter might have contributed to SIP-mediated stimulation of DRA promoter activity. Sequence analysis of this region of DRA promoter revealed potential binding sites for YY1 and GATA transcription factors. The involvement of YY1 in S1P-induced stimulation of DRA promoter was further confirmed by EMSA, demonstrating increased binding of YY1 to its cis-elements in DRA promoter in response to SIP. YY1 is a multifunctional zinc finger transcription factor associated with embryogenesis, cellular proliferation, and differentiation (16). In the intestine, YY1 has been shown as an essential transcription factor involved in regulation of intestinal stem cell homeostasis and renewal (31). Depletion of YY1 also results in increased crypt length due to imbalance in the ratio of crypt to villus cell populations. With respect to its role in regulating gene expression, YY1 can either activate or repress transcription and can also serve as initiator element (16). For example, YY1 has been shown to be involved in suppression of IFN-γ promoter in T cells (48). On the other hand, our earlier studies have shown the involvement of YY1 and GATA transcription factors in mediating butyrate-induced increase in DRA promoter activity in LS174T colonic cells (1).

In summary, our findings provide novel data on the upregulation of DRA function and expression by SIP via transcriptional mechanisms. S1P-induced increase in DRA promoter activity occurred via S1PR2 mediated by YY1 through a PI3/Akt-dependent mechanism, subsequently increasing DRA function and expression. These data highlight the potential proabsorptive and hence antidiarrheal role of S1P. Future studies are needed to investigate whether SIP can counteract decreased DRA expression observed in models of gut inflammation or diarrheal disorders.

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

AUTHOR CONTRIBUTIONS
A.N.A. and P.K.D. conception and design of research; A.N.A., S.P., A.A., and A.K. performed experiments; A.N.A. analyzed data; A.N.A., A.B., and P.K.D. interpreted results of experiments; A.N.A. prepared figures; A.N.A., P.K.D. edited and revised manuscript; P.K.D. approved final version of manuscript.

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