**Lactobacillus acidophilus** stimulates the expression of SLC26A3 via a transcriptional mechanism

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Submitted 10 November 2009; accepted in final form 23 December 2009

**Raheja G, Singh V, Ma K, Boumendjel R, Borthakur A, Gill RK, Saksena S, Alrefai WA, Ramaswamy K, Dudeja PK. Lactobacillus acidophilus stimulates the expression of SLC26A3 via a transcriptional mechanism. Am J Physiol Gastrointest Liver Physiol 298: G395–G401, 2010. First published December 31, 2009; doi:10.1152/ajpgi.00465.2009.—Clinical efficacy of probiotics in treating various forms of diarrhea has been clearly established. However, mechanisms underlying anti diarrheal effects of probiotics are not completely defined. Diarrhea is caused either by decreased absorption or increased secretion of electrolytes and solutes in the intestine. In this regard, the electroneutral absorption of two major electrolytes, Na\(^{+}\) and Cl\(^{-}\), occurs mainly through the coupled operation of Na\(^+/\)H\(^{+}\) exchangers and Cl\(^{-}/\)OH\(^{-}\) exchangers. Previous studies from our laboratory have shown that Lactobacillus acidophilus (LA) acutely stimulated Cl\(^{-}/\)OH\(^{-}\) exchange activity via an increase in the surface levels of the apical anion exchanger SLC26A3 (DRA). However, whether probiotics influence SLC26A3 expression and promoter activity has not been examined. The present studies were, therefore, undertaken to investigate the long-term effects of LA on SLC26A3 expression and promoter activity. Treatment of Caco-2 cells with LA for 6–24 h resulted in a significant increase in Cl\(^{-}/\)OH\(^{-}\) exchange activity. DRA mRNA levels were also significantly elevated in response to LA treatment starting as early as 8 h. Additionally, the promoter activity of DRA was increased by more than twofold following 8 h LA treatment of Caco-2 cells. Similar to the in vitro studies, in vivo studies using mice gavaged with LA also showed significantly increased DRA mRNA (~4-fold) and protein expression in the colonic regions as assessed by Western blot analysis and immunofluorescence. In conclusion, increase in DRA promoter activity and expression may contribute to the upregulation of intestinal electrolyte absorption and might underlie the potential anti diarrheal effects of LA.

PROBIOTICS HAVE BEEN USED in clinical trials for the prevention and treatment of various forms of diarrhea such as acute infectious diarrhea, antibiotic-associated diarrhea, and diarrheapredominant irritable bowel syndrome (9). Probiotics are reported to have trophic effects on gut mucosa, e.g., enhancement of intestinal epithelial barrier function, increase in cell survival, and growth and stimulation of mucin synthesis and secretion (22). A number of mechanisms have been proposed to account for these beneficial effects of probiotics, e.g., reduction in luminal pH, inhibition of bacterial adherence, and secretion of some antibacterial compounds, bacteriocins, to remove pathogenic bacteria. Studies have also suggested that probiotics produce trophic factors such as spermine and spermidine (6) and short-chain fatty acids such as butyrate that upregulate the expression of advantageous genes (2, 27). Most of these studies have emphasized the beneficial effects of probiotics; however, their mechanism of action at the molecular level, with respect to their anti diarrheal effects, is not fully understood.

Diarrhea is considered to be a multifactorial event presented owing to either increased secretion of fluid and electrolytes, decreased absorption, or both. Electroneutral absorption of two major electrolytes, Na\(^{+}\) and Cl\(^{-}\), occurs via the coupled operation of sodium hydrogen and anion exchangers localized to the apical membrane of the intestinal epithelial cells. NHE2 and NHE3 are the two major sodium hydrogen exchangers on the apical surface whereas DRA (downregulated in adenoma) and putative anion transporter-1 (PAT-1) are the chloride hydrroxy exchangers. Among these, DRA appears to be the major intestinal anion exchanger since alterations in the function and expression of DRA have been implicated in diarrheal disorders; e.g., mutations in DRA are associated with a rare disorder congenital chloridirrhia (14), presented by voluminous watery diarrhea with an extremely high chloride content. This pathology has been attributed to the lack of chloride absorption from the colon, a major site of chloride uptake in the body (11).

Lactic acid bacteria, particularly lactobacilli, are one of the predominant commensal bacteria in the gut microflora and are most commonly used probiotics for the prevention and treatment of diarrheal disorders (35). Lactobacillus acidophilus (LA) has been shown to prevent enteroinvasive *Escherichia coli*-mediated disruption of intestinal epithelial barrier function in Caco-2 cells (26). We have previously demonstrated stimulation of Cl\(^{-}/\)OH\(^{-}\) exchange activity via enhanced DRA function in response to short-term (3 h) LA treatment of Caco-2 cells (3). In the clinical trials, probiotics are generally given for a number of days for the treatment of diarrhea; however, nothing is known about the long-term effects of probiotics on the function and expression of intestinal chloride transporters. We hypothesized that, in addition to the short-term benefits (3), clinical efficacy of probiotics may also involve upregulation of genes involved in electrolyte absorption in the intestine. Therefore, the studies were designed to examine the long-term effects of LA on SLC26A3 and SLC26A6 expression in both the in vitro and in vivo models. Our data show that LA enhances the DRA expression in the intestine via a transcriptional mechanism.

MATERIALS AND METHODS

**Materials.** Caco-2 cells were procured from ATCC. Radionucleotide \(^{38}\)Cl was obtained from PerkinElmer. RNAeasy kits for RNA
extraction were obtained from Qiagen, and real-time quantitative RT-PCR (qRT-PCR) kits were from Stratagene. 4,4′-Disothiocyanate-stibine-2,2′-disulfonic acid (DIDS) and 2-(N-morpholino)ethanesulfonic acid (MES) were procured from Sigma-Aldrich (St. Louis, MO). Common reagents for SDS-PAGE such as ammonium persulfate, acrylamide, and bis-acrylamide were from Fisher Scientific (Pittsburgh, PA).

Cell culture. Caco-2 cells were grown in T-75 cm² culture flasks at 37°C in a 5% CO₂-95% O₂ incubator. The medium in which the cells were grown consisted of minimum essential medium, 20% FBS (fetal bovine serum), 20 mM HEPES, 100 IU/ml penicillin, and 100 mg/ml streptomycin; 2 × 10⁴ cells were plated per well in a 24-well culture plate and were used between passages 25 and 45. Fully differentiated confluent monolayers were used for the experiments (10–12 days postplating).

Bacterial culture. LA was procured from ATCC (4357). Bacteria were grown overnight in MRS broth at 37°C in a 5% CO₂-95% O₂ incubator. The next day, bacteria were spun down by centrifuging at 3,000 rpm for 10 min. For in vitro studies, culture supernatant was separated from spun-down bacteria, filtered through a 0.22-μm filter, and mixed with cell culture media for further use; for in vivo studies, 3 × 10⁹ colony-forming units (CFUs) of bacteria were gavaged per animal for 14 to 24 h.

Cl⁻/OH⁻ exchange activity. Cl⁻/OH⁻ exchange activity was assessed as ³⁶Cl⁻ uptake carried out in well-differentiated polarized Caco-2 cells as described previously (13). After treatment of Caco-2 cells with LA culture supernatant diluted 50 times in culture media (LA-CM) for different time intervals, cells were incubated in base medium containing 20 mM HEPES pH 8.5 at room temperature. After 30 min, base medium was removed and the cells were rapidly washed with 1 ml tracer-free mannitol uptake buffer containing 260 mM mannitol and 20 mM Tris-MES, pH 7.0. This was followed by incubation with or without 600 μM DIDS in uptake buffer for 5 min since this time period is within the linear range of Cl⁻ uptake in cells. The radioactive ³⁶Cl⁻ (2.9 mM) in hydrochloric acid (specific activity 17.12 mCi/mg) was added to the uptake buffer at a concentration of 1.4 μCi/ml. Uptake was stopped by removing the radioactivity-containing buffer and washing the cells rapidly two times with ice cold phosphate-buffered saline (PBS), pH 7.2. Furthermore, the cells were solubilized by 0.5 N NaOH for 4 h and protein concentration was measured by the Bradford method (4). Radioactivity was measured by a Packard Tri-Carb 1600 TR liquid scintillation analyzer (Packard Instruments; PerkinElmer). The Cl⁻/OH⁻ activity was then calculated as DIDS-sensitive ³⁶Cl⁻ uptake, and the specific activity is expressed as nanomoles per milligram protein per 5 min.

Real-time PCR. RNA was extracted from LA-treated and untreated Caco-2 cells and mice samples by use of Qiagen RNeasy kits. RNA was reverse transcribed and amplified with Brilliant SYBR Green qRT-PCR Master Mix kit (Stratagene). Human DRA (accession no. BC025671) was amplified with gene-specific primers (sense primer 1250 bp, 5′-TTCAGTGCCAGTCTATTC-3′; antisense primer 1416 bp, 5′-CCGTTGCTCTGTGCCG-3′). Human histone was amplified as an internal control utilizing gene-specific primers (sense primer, 5′-ACGCAGCTTCTGTTACAGG-3′; antisense primer, 5′-CTTGGGCTGAAATAGCCAGA-3′). Mouse DRA (accession no. BC139273) was amplified with gene-specific primers (sense primer 454 bp, 5′-TCTGACGGAAGTGTGCTAAC-3′; antisense primer 607 bp, 5′-CCAGGAGCAACTGAATGAT-3′). Human histone was amplified as an internal control by utilizing gene-specific primers (sense primer, 5′-GAGATCTCGAGCTCAGATAGAA-3′; antisense primer, 5′-CAAAAGGGCAACAGGAAAG-3′). Human PAT-1 was amplified with the gene-specific primers (sense primer, 5′-CTGCTACTGCTGACCTCT-3′; antisense primer, 5′-CTTGGGTGGAATGTAAGCT-3′), and mouse PAT-1 was amplified with gene-specific primers (sense primer, 5′-GAAATGGAGCTGCA-GAGGA-3′; antisense primer, 5′-GCTGGACGAGAAGAATGGG-3′). Relative levels of DRA and PAT-1 mRNA were expressed as percent of control normalized to histone.

Transfections. Caco-2 cells were transfected with DRA promoter (p-1183/+114) fragment cloned upstream of the luciferase reporter gene in pGL2-Basic and β-galactosidase expression vector by electroporation using Amaxa Nucleofactor System as described previously (2). Activities of firefly luciferase and β-galactosidase were measured according to the manufacturer’s instructions (Promega). DRA promoter activity was expressed in terms of relative luciferase activity normalized to β-galactosidase activity.

In vivo studies. In vivo studies performed in C57Bl/6j mice were approved by the Animal Care Committee of University of Illinois at Chicago and Jesse Brown Veterans Affairs Medical Center. Mice were gavaged with LA (3 × 10⁹ CFUs) or sterile PBS as vehicle for 14 to 24 h. Intestines were resected and mucosa was scraped for RNA and protein extraction. A part (~2 cm) of the different regions of intestine (ileum and colon) was immediately snap frozen in optimal cutting temperature embedding medium (Tissue-Tek OCT compound; Sakura) for immunofluorescence studies. RNA was extracted and real-time qRT-PCR was performed as described above.

Western blotting. Tissue lysates were prepared from the scraped mucosa of ileum and colon by use of cell lysis buffer (Cell Signaling). Lysates were run on a 8% gel and then transferred onto nitrocellulose membrane. Immunoblotting was carried out with anti-DRA affinity purified antibody as previously described (3).

Immunofluorescence. Snap-frozen tissues were cut into 5-μm sections on the slides by use of a cryostat and were preserved at −80°C until further use. For immunostaining, these sections were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and permeabilized with 0.5% Nonidet P-40 in PBS for 5 min, followed by blocking in 5% normal goat serum (NGS) for 120 min at room temperature. Rat anti-DRA antibody (1:50 dilutions of cell culture supernatant (CCS), LA-CM, LA culture supernatant in culture media) was added and incubated overnight at 4°C, followed by peroxidase-conjugated goat anti-rat antibody (1:2,000 dilution of goat anti-rat HRP-conjugated antibody; Animal Systems). DRA expression in mouse ileum and colon was assessed using 4,4′-Diaminobenzidine (DAB) as substrate. For negative controls, the primary antibody was omitted. In some experiments, β-galactosidase was used as an internal control for DRA localization. Mucosal expression of firefly luciferase and DRA was assessed as described above.

Fig. 1. Cl⁻/OH⁻ exchange activity (DIDS-sensitive ³⁶Cl⁻ uptake) in Caco-2 cells in response to treatment for 24 h with different dilutions (A) and for indicated time periods with 1:50 dilutions of Lactobacillus acidophilus (LA) culture supernatant (B). LA-CM, LA culture supernatant in culture media. Values are means ± SE of 3 independent experiments performed in triplicate. Differences between groups, control vs. LA treated, are statistically significant at ***p < 0.001, **p < 0.01.
temperature. Sections were then incubated with primary antibodies anti-DRA (1:100) and anti-villin (1:100) in 1% NGS for 120 min. After being washed with 1% NGS, sections were incubated with secondary antibodies, Alexa Fluor 568-conjugated goat anti-rabbit IgG and Alexa Fluor 488-conjugated goat anti-mouse IgG, for 60 min and then mounted with slow-fade DAPI by using coverslips. Microscopy was performed with a Zeiss LSM 510 laser scanning confocal microscope equipped with a ×63 water immersion objective.

Statistical analysis. Data are presented as means ± SE of three to five independent experiments. Difference between control vs. treated was analyzed by one-way analysis of variance with Tukey’s test. Differences were considered significant at \( P < 0.05 \).

RESULTS

Long-term (6–24 h) LA exposure increases \( \text{Cl}^-/\text{OH}^- \) exchange activity in Caco-2 cells. Previous studies from our laboratory have shown that short-term exposure to LA for 3 h copy was performed with a Zeiss LSM 510 laser scanning confocal microscope equipped with a ×63 water immersion objective.

Statistical analysis. Data are presented as means ± SE of 4 different experiments. Differences between groups, control vs. LA treated, are statistically significant at *\( P < 0.01 \).
increased the Cl⁻/OH⁻ exchange activity in Caco-2 cells. To examine the long-term effects of LA, Caco-2 monolayers were treated with LA-CM (different dilutions) for 6–24 h and DIDS sensitive ³⁶Cl⁻ uptake was measured after base loading the cells. As shown in Fig. 1A, Cl⁻/OH⁻ exchange activity was significantly increased at all the dilutions of LA-CM used. In addition, Cl⁻/OH⁻ exchange activity was significantly enhanced at all time points in response to LA-CM treatment ranging from 6 to 24 h (Fig. 1B).

**LA-mediated stimulation of Cl⁻/OH⁻ exchange activity occurs via increased levels of DRA but not PAT-1.** Two members of the SLC26 gene family, DRA (SLC26A3) and PAT-1 (SLC26A6) have been implicated as candidate genes for apical Cl⁻/OH⁻ (HCO₃⁻) exchange activity in the mammalian intestine (11). Therefore, to examine the effect of LA on DRA and PAT-1 mRNA levels, Caco-2 monolayers were treated with LA-CM (1:50 dilution) for 8 and 24 h and mRNA levels of DRA and PAT-1 were determined by real-time quantitative PCR. As shown in Fig. 2A, mRNA levels of DRA were found to be significantly increased as early as at 8 h of LA-CM treatment and remained elevated until 24 h. PAT-1 mRNA levels, however, did not change significantly following LA-CM 24 h treatment as shown in Fig. 2B. Furthermore, levels of DRA protein were also elevated by about ~1.5 fold in Caco-2 cells after 24 h of LA treatment as revealed by the densitometry analysis of the Western blot (Fig. 3).

**LA increases DRA promoter activity.** We next investigated whether the increase in the DRA mRNA expression was through a transcriptional mechanism involving increased promoter activity. Caco-2 cells were transfected with DRA promoter reporter plasmid, 24 h posttransfection, cells were treated with LA-CM (1:50 dilution) for different time intervals and DRA promoter activity was assessed. DRA promoter activity was markedly increased by more than twofold in response to LA-CM following 8 h of treatment without any change at the later time points studied (Fig. 4). These results demonstrate that long-term LA treatment increases DRA expression through a transcriptional mechanism in human intestinal epithelial cells.

**In vivo studies.** Our in vitro studies in Caco-2 cells clearly demonstrated that LA increased Cl⁻/OH⁻ (HCO₃⁻) exchange activity by increasing DRA expression. However, in vitro studies do not give a comprehensive idea of these absorptive processes in native intestine. Therefore, we next investigated the effects of LA on DRA expression in an in vivo mouse model.

**LA increases DRA but not PAT-1 mRNA levels.** DRA mRNA levels were assessed by real-time qRT-PCR in samples from the mice gavaged with LA. Jejunum (Fig. 5A) and ileum (Fig. 5B) did not show a significant change in the mRNA expression of DRA compared with the controls; however, colon showed a highly significant increase of about fourfold ($P < 0.01$ vs. control) after 24 h of LA treatment as shown in Fig. 5C. However, PAT-1 mRNA levels remained unchanged in all the intestinal regions following LA treatment (Table 1).

**LA induces DRA protein expression.** Since we observed an increase in DRA mRNA levels in the colon, we next assessed

| PAT-1 levels remain unchanged following Lactobacillus acidophilus exposure in mice |
|-------------------------------|----------------|----------------|----------------|
| LA, h | Jejunum | Ileum | Colon |
| 0 | $1.0 \pm 0.0$ | $1.0 \pm 0.0$ | $1.0 \pm 0.0$ |
| 14 | $1.58 \pm 0.39$ | $0.83 \pm 0.24$ | $1.17 \pm 0.17$ |
| 18 | $1.09 \pm 0.22$ | $1.01 \pm 0.35$ | $1.01 \pm 0.18$ |
| 24 | $1.01 \pm 0.14$ | $0.84 \pm 0.18$ | $0.97 \pm 0.13$ |

*Results represent means ± SE of 5 independent experiments. Values of mRNA levels for putative anion transporter (PAT-1) were normalized against histone mRNA levels and further compared to 0 h treatment considered as $1.0 \pm 0.0$. LA, Lactobacillus acidophilus; PAT-1, putative anion transporter-1.*
DRA protein expression upon LA treatment. Western blot analysis revealed that DRA protein expression was enhanced in the LA-treated colonic tissues at all time points with a maximal increase observed at 24 h. Furthermore, densitometric analysis revealed that there was a significant increase in the DRA protein levels (3-fold) in LA-treated samples compared with the controls (Fig. 6). These results were further corroborated by immunofluorescence imaging. As shown in Fig. 7, significant increase in the expression of DRA protein can be clearly visualized on the apical surface of colonic sections from LA-gavaged mice compared with those from control mice.

DISCUSSION

Recently, an upsurge has been observed regarding the reports on beneficial effects of probiotics. Many probiotic strains are being used in clinical trials for the improvement of intestinal health. For example, LA has been shown to reduce antibiotic-associated diarrhea whereas *L. plantarum* and *Bifidobacterium breve* are helpful in irritable bowel syndrome, inflammatory bowel disease, and diarrhea (23, 24). However, the mechanisms for their action at a molecular and cellular level still remain elusive. In this regard, we have previously demonstrated that the probiotic LA stimulated Cl−/OH− exchange activity in human intestinal epithelial Caco-2 cells within 3 h. This effect was dependent on PI-3 kinase activity and involved increased surface levels of DRA, the major apical Cl−/OH− exchanger (3). This was the first report showing involvement of a signaling pathway in the short-term stimulation of Cl− absorption by probiotics. Under in vivo conditions, intestinal epithelial cells are continuously exposed to probiotic strains and hence their secreted factors are available to these cells for longer periods of time. To the best of our knowledge, currently there are no reports regarding the long-term effects of probiotics on intestinal ion absorptive mechanisms. Therefore, the present studies were designed to examine the long-term effects of LA utilizing both in vitro and in vivo models.

Probiotics *B. infantis*, *E. coli* 1917, and *L. plantarum* improve epithelial barrier function by increasing the expression of tight junction proteins such as zonula occludens (ZO)-1, ZO-2, and occludin (30, 36). In the present study we found that Cl−/OH− exchange activity was also enhanced after 24-h LA treatment, which suggested that some additional mechanisms might be operating, most probably through increased transcrip-
tion of SLC26A3 gene. Interestingly, DRA promoter activity was found to be increased after 8 h of LA treatment and DRA mRNA expression was enhanced between 8–24 h without any change in the PAT-1 mRNA expression. However, DRA promoter activity returned back to normal at 12 and 24 h, implying that an increase in the promoter activity at 8 h was sufficient to maintain enhanced mRNA and protein expression observed until 24 h. This increase in DRA expression by LA is an exciting observation considering the fact that DRA is most likely the major luminal Cl⁻/OH⁻ exchanger in colon, since mutations in this gene give rise to congenital chloride diarrhea (10) and DRA knockout mice also exhibit similar symptoms (29). In contrast, PAT-1 knockout mice have not been shown to produce chloride diarrhea (33). It is also possible that PAT-1 (i.e., SLC26A6) is not regulatable by LA via transcriptional mechanism. Based on our combined results from previous and present studies, it can be inferred that LA can upregulate DRA at the apical surface, most probably through trafficking, and, when the cells are treated with secreted factors of LA on a long-term basis as in the present study (8–24 h), DRA expression is also enhanced, secondary to the increased transcription.

We further validated our in vitro results of Caco-2 cells utilizing an in vivo mouse model. Consistent with the in vitro results, in vivo data also showed that DRA mRNA expression was significantly enhanced in the colon of LA-gavaged mice whereas mRNA levels of DRA were unchanged in jejunum and ileum. This could be due to the relative expression of DRA and its functional role along the length of mouse intestine (2, 18, 20). Colonic regions have been shown to express significantly higher DRA, which plays an important role in electroneutral Cl⁻ absorption in colonic regions compared with the small intestine (21). It could also be due to the fact that Lactobacilli strains predominantly colonize in the colon compared with the small intestine (1, 32) and hence they can affect the gene expression in colon either directly or through their secreted factors (7, 12). Another reason for observed effects in colon could be the rapid transit of probiotics from the small intestine compared with colon where they stay for a longer period of time. Levels of PAT-1, which is predominantly expressed in jejunum and ileum, remained unchanged following LA exposure, which could again be due to the preferential colonization or slow transit of LA in colonic regions compared with small intestine. It is possible that increased mRNA levels might not necessarily translate into increased protein expression because of potential regulation by specific micro-RNAs or changes in mRNA stability (8). However, increased DRA protein expression as assessed by Western blotting and confocal immunofluorescence clearly demonstrated that increased mRNA levels did translate into protein.

The mechanism of action of probiotics is not well characterized at the molecular level. Some studies using microarray analysis reported up- and downregulation of various genes involved in inflammation, cell signaling, cell cycle, and lipid metabolism following probiotics exposure (17, 25, 31). We report for the first time that expression of the chloride transporter gene, DRA, is enhanced in response to LA treatment; however, the identity of secreted bioactive factors is unclear at present. Few recent studies have suggested that probiotics secrete some bioactive trophic factors such as spermine and spermidine that act as growth promoters, resulting in overall increased mucosal mass and increased expression of brush border enzymes such as sucrase and alkaline phosphatase (5, 6). Conjugated linoleic acids produced by Lactobacillus are shown to be effective in Helicobacter pylori-infected gastric epithelial cells (16) and some soluble proteins derived from Lactobacillus rhamnosus GG have been reported to regulate intestinal epithelial cell survival and growth (34). Another report has shown that conditioned media from some of the individual strains of VSL#3 (a probiotics mixture) improved epithelial barrier function both in vitro and in vivo (19). Probiotics are well known to produce short-chain fatty acids such as butyrate from complex carbohydrates by bacterial fermentation, which in turn are reported to stimulate NHE3 (15) and DRA (2) expression. Therefore, the stimulation of DRA expression as observed in our present studies could arise from any known bioactive factors documented above or could be due to some unidentified bioactive molecules secreted by these bacteria. We speculate that stimulation of DRA expression in our in vivo studies could be partly via the production of short-chain fatty acids by probiotics. Since DRA is one of the most critical transporters involved in coupled electroneutral NaCl absorption from intestine, our findings hold significance in contributing to the molecular mechanism for the beneficial effects of probiotics in treatment of diarrheal disorders. Further studies are needed to examine the potential beneficial effects of probiotic strains on sodium absorption as well as to delineate signaling mechanisms used by the bioactive factors of probiotics that elicit these beneficial effects.

GRANTS
These studies were supported by the Department of Veterans Affairs and the National Institute of Diabetes and Digestive and Kidney Diseases Grants DK 54016 (P. K. Dudeja), DK 81858 (P. K. Dudeja), Program Project Grant DK 067887 (P. K. Dudeja and K. Ramaswamy), DK 74459 (R. K. Gill), and CCFA Grant 1942 (S. Saksena).

DISCLOSURES
No conflicts of interest are declared by the author(s).

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AJP-Gastrointest Physiol • VOL 298 • MARCH 2010 • www.ajpgi.org