Sulfate and chloride transport in Caco-2 cells: differential regulation by thyroxine and the possible role of DRA gene

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Alrefai, W. A., S. Tyagi, F. Mansour, S. Saksena, I. Syed, K. Ramaswamy, and P. K. Dudeja. Sulfate and chloride transport in Caco-2 cells: differential regulation by thyroxine and the possible role of DRA gene. Am J Physiol Gastrointest Liver Physiol 280: G603–G613, 2001.—The current studies were undertaken to establish an in vitro cellular model to study the transport of SO₄²⁻ and Cl⁻ and hormonal regulation and to define the possible function of the downregulated in adenoma (DRA) gene. Utilizing a postconfluent Caco-2 cell line, we studied the OH⁻ gradient-driven [³⁵S]SO₄²⁻ and [³⁸Cl]Cl⁻ uptake. Our findings consistent with the presence of an apical carrier-mediated [³⁵S]SO₄²⁻ /OH⁻ exchange process in Caco-2 cells include: 1) demonstration of saturation kinetics [Michaelis-Menten constant (Kₘ) of 0.2 ± 0.08 mM for SO₄²⁻ and maximum velocity of 1.1 ± 0.2 pmol-mg protein⁻¹·2 min⁻¹]; 2) sensitivity to inhibition by DIDS (Kᵢ = 0.9 ± 0.3 μM); and 3) competitive inhibition by oxalate and Cl⁻ but not by nitrate and short chain fatty acids, with a higher Kᵢ (5.95 ± 1 mM) for Cl⁻ compared with oxalate (Kᵢ = 0.2 ± 0.03 mM). Our results also suggested that the SO₄²⁻ /OH⁻ and Cl⁻ /OH⁻ exchange processes in Caco-2 cells are distinct based on the following: 1) the SO₄²⁻ /OH⁻ exchange was highly sensitive to inhibition by DIDS compared with Cl⁻ /OH⁻ exchange activity (Kᵢ for DIDS of 0.3 ± 0.1 mM); 2) Cl⁻ competitively inhibited the SO₄²⁻ /OH⁻ exchange activity with a high Kᵢ compared with the Kₘ for SO₄²⁻ ; 3) DIDS competitively inhibited the Cl⁻ /OH⁻ exchange process, whereas it inhibited the SO₄²⁻ /OH⁻ exchange activity in a mixed-type manner; and 4) utilizing the RNase protection assay, our results showed that 24-h incubation with 100 nM of thyroxine significantly decreased the relative abundance of DRA mRNA along with the SO₄²⁻ /OH⁻ exchange activity but without any change in Cl⁻ /OH⁻ exchange process. In summary, these studies demonstrated the feasibility of utilizing Caco-2 cell line as a model to study the apical SO₄²⁻ /OH⁻ and Cl⁻ /OH⁻ exchange processes in the human intestine and indicated that the two transporters are distinct and that DRA may be predominantly a SO₄²⁻ transporter with a capacity to transport Cl⁻ as well.

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are localized to basolateral membranes of the epithelial cells in all the regions of the human intestine (2). Recent studies (39) from our laboratory have also demonstrated and characterized a sulfate/hydroxylation exchange process in the proximal colonic apical membrane vesicles and shown that this exchanger has very low affinity for chloride compared with sulfate. The results of these studies strongly suggested that the described sulfate/hydroxylation exchange activity was distinct from the previously described Cl⁻/HCO₃⁻ (OH⁻) exchange process. In light of the aforementioned, it is not yet clear whether DRA protein product is in fact the apical anion exchanger responsible for the Cl⁻/HCO₃⁻ exchange across the apical membranes of the human colonic epithelium or whether it is mainly a sulfate and oxalate transporter that could also transport chloride.

These intriguing findings warrant the development of a suitable in vitro model to study the sulfate and chloride uptake, their substrate specificity, and their regulation with various hormones and to examine the role of DRA in intestinal electrolyte transport. The colonic adenocarcinoma Caco-2 cell line has been shown to be a good model to study the mechanisms of electrolyte transport in the intestine (40). Additionally, thyroxine has previously been shown to alter the level of expression and the function of a number of electrolyte transporters in various tissues and cell lines (6, 8, 10). Therefore, our current studies were undertaken to examine whether Caco-2 cell line could serve as a suitable model to study the apical chloride and sulfate uptake mechanisms and their substrate specificity. Also, we examined the possible role of thyroxine in regulation of the expression of human DRA mRNA along with chloride and sulfate uptake activities in Caco-2 cells.

Our current studies demonstrated that Caco-2 cells could serve as an experimental model to study the apical intestinal SO₄²⁻/OH⁻ and Cl⁻/OH⁻ exchange activities. Our data demonstrated that the two transporters are distinct based on the following: 1) in contrast to the Cl⁻/OH⁻ exchange process, the SO₄²⁻/OH⁻ exchange activity was highly sensitive to inhibition by DIDS (the AE inhibitor); 2) DIDS competitively inhibited the Cl⁻/OH⁻ exchange, whereas it inhibited the SO₄²⁻/OH⁻ exchange in a mixed-type manner; 3) chloride inhibited the SO₄²⁻/OH⁻ exchange competitively with a high inhibition constant (Kᵢ) for Cl⁻ compared with the Michaelis-Menten constant (Kₘ) for SO₄²⁻, indicating lower affinity of chloride for this sulfate transporter; and 4) thyroxine significantly reduced the level of expression of human DRA mRNA along with SO₄²⁻/OH⁻ exchange activity, whereas the Cl⁻/OH⁻ exchange activity was not altered by thyroxine. These data indicate that DRA may be primarily responsible for sulfate transport although capable of transporting chloride and that it appears to be distinct from the previously described Cl⁻/HCO₃⁻ (OH⁻) exchanger in the intestinal luminal membrane.

**MATERIAL AND METHODS**

**Cell culture**

Caco-2 cells were obtained from ATCC and cultured at 37°C in an atmosphere of 5% CO₂. Cells were maintained as previously described (33) in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 µg/ml gentamicin, 10 mM HEPES, 1% essential and nonessential amino acids, and 20% fetal bovine serum. For the uptake experiments, cells from passages between 20–25 were plated in 24-well plates at a density of 2 x 10⁵ cells/ml. Confluent monolayers were then used for the transport experiments at day 10 postplating. To study the effect of 100 nM of thyroxine, we rendered cells quiescent by serum removal for 48 h before study. Control cells were treated with equivalent amounts of 100 nM NaOH (vehicle) to thyroxine-treated cells.

**³⁵SO₄²⁻ and ³⁶Cl⁻ uptake**

Sulfate and chloride uptake experiments were performed essentially as described by Olsnes et al. (25) with some modifications. Caco-2 cells were incubated with DMEM base media containing 20 mM HEPES/KOH, pH 8.5, for 1 h at room temperature. All the subsequent steps were performed at room temperature. The media were removed, and the cells were rapidly washed with 1 ml tracer-free uptake mannitol buffer containing 260 mM mannitol, 20 mM MES/Tris, pH 7. The cells were then incubated with the uptake buffer for the indicated time. For SO₄²⁻ uptake studies, the uptake buffer was the mannitol buffer including 50 µM (0.5 µCi/ml of ³⁵SO₄²⁻) sulfuric acid (DuPont). For chloride uptake, mannitol buffer contained 2.7 mM (1.3 µCi/ml of ³⁶Cl⁻) hydrochloric acid. The uptake was terminated by two rapid washes with 1 ml of ice-cold PBS. Finally, the cells were solubilized with 0.5 N NaOH for 4 h. The protein concentration was measured by the method of Bradford (4), and the radioactivity was counted by Packard liquid scintillation analyzer, Tri-CARB 1600-TR (Packard Instrument, Downers Grove, IL). Because the 2-min time point was in the linear range of the uptake for both chloride and sulfate, the uptake was measured at 2 min and was expressed as picomoles per milligram of protein per 2 min and nanomoles per milligram per protein per 2 min for sulfate and chloride, respectively. The uptake values were analyzed for simple Km utilizing a nonlinear regression data analysis from a computerized model (GraphPad, PRISM, San Diego, CA). Lineweaver-Burk analysis (1/v vs. 1/[s]) was used to determine the kinetics parameters [i.e., the apparent Kᵢ and maximum velocity (Vₘₐₓ)] utilizing linear regression data analysis from the same program (GraphPad, PRISM).

**Designing of PCR primers and PCR technique**

The PCR primer sequences for human DRA were designed from the human sequences that have been retrieved from the gene-bank CD-ROM utilizing GeneWorks software and as previously described (37). The primer sequences are 5' primer: ACCATGATTTGACACTCGGTGATGCTGG; 3' primer: ATACACCTGGCGATGATGCTACGC (length of amplified region 910 residues; nt 184–1094 of the human DRA). The PCR was essentially performed according to the manufacturer’s instructions utilizing 2 µg of human colonic cDNA pool obtained from Invitrogen (Carlsbad, CA) as a template, gene-specific human DRA PCR primers, and the proofreading Elongase enzyme mix (GIBCO BRL, Gaithersburg, MD). The reaction was performed in a total volume of 50 µl of PCR mixture containing 60 mM Tris-SO₄⁻ (pH 9.1 at 25°C), 18 mM

Elongase enzyme mix (GIBCO BRL, Gaithersburg, MD). The reaction was performed in a total volume of 50 µl of PCR mixture containing 60 mM Tris-SO₄⁻ (pH 9.1 at 25°C), 18 mM
NH₄SO₄, 1.8 mM MgSO₄, 200 μM each dNTPs, 400 nM of each primers, and 2 μl of the Elongase enzyme mix. The PCR was carried out using a Microcycler programmable heating/cooling dry block (Perkin Elmer, Norwalk, CT) for 40 cycles of amplification (94°C, 30 s; 52°C, 30 s; 68°C, 3 min) followed by 10 min at 68°C. PCR products were separated by electrophoresis on 1% agarose gel containing ethidium bromide (0.5 μg/ml). Bands of expected sizes were visualized under ultraviolet light utilizing Eagle eye II Still Video System (Stratagene, La Jolla, CA). The 910-bp PCR products were excised from the agarose, purified utilizing Sephaglas BandPrep Kit (Amersham Pharmacia Biotech, Piscataway, NJ), and subjected to A-tailing reaction by heating at 70°C for 30 min in a final volume of 10 μl containing 100 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 50 mM KCl, 200 μM dATP, and 5 units of Taq DNA polymerase (GIBCO BRL). Two microliters of the reaction were ligated into a pGEM-T Easy vector (Promega, Madison, WI). The orientation and the sequence of the insert were confirmed by sequencing utilizing the Sequenase Kit (USB, Madison, WI). The constructed DRA vector, p5’DRA, was then utilized for making cRNA probe for the RNase protection assay.

**Isolation of RNA**

Total RNA was extracted from Caco-2 cells by the method of Chomczynski and Sacchi (7) using RNAzol solution supplied by the manufacturer (Tel-Test, Friendswood, TX) and essentially using the manufacturer’s protocol.

**Generation of cRNA probes and RNase protection assay**

p5’ DRA vector was linearized by digestion with Ava II and transcribed with T7 RNA polymerase in the presence of [³²P]CTP utilizing the riboprobe Gemini transcription system (Promega, Madison, WI). [³²P]cRNA riboprobe for human DRA contained 579 bp, and the protected fragment corresponded to 527 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) vector was constructed, and cRNA riboprobe for GAPDH was generated as described previously by us (11). [³²P]cRNA for GAPDH contained 300 bp, and predicted protected fragment corresponded to 210 bp.

For the RNase protection assay, total RNA (20–30 μg) was coprecipitated with 10⁵ counts/min of high specific activity ³²P-labeled DRA riboprobe and 10⁵ counts/min of low specific activity ³²P-labeled GAPDH riboprobe. Samples were then resuspended in a hybridization buffer containing 75% formamide, 400 mM NaCl, 1 mM EDTA, and 40 mM PIPES, pH 6.4, and were hybridized at 45°C for 12–18 h. Samples were then diluted in 10 vol of 300 mM NaCl, 5 mM EDTA, and 10 mM Tris pH 7.5, and 1,400 units of T1 ribonuclease were added to each sample. After a 45-min incubation at 37°C, samples were added to a stop solution containing 4 M LiCl and 5 μg tRNA and precipitated with 2 vol of ethanol. Precipitates were resuspended in a small volume of dye solution (xylene cyanole and bromophenol blue in 90% formamide and 10 mM EDTA, pH 7.5). The double-stranded ³²P-cRNA fragments that were protected from the RNase digestion were heated at 95°C for 5 min and analyzed by electrophoresis on a denaturing polyacrylamide gel containing 8 M urea. The gels were dried and exposed to storage phosphor screen overnight and then analyzed utilizing a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The relative abundance of DRA was calculated by comparing the number of counts of radioactivity of DRA band in response to thyroxine treatment or vehicle alone after normalization to the number of counts in the GAPDH band. Because GAPDH mRNA is expressed in very high abundance compared with DRA, ~20-fold lower specific activity of ³²P-labeled GAPDH compared with DRA probes were synthesized by including more cold CTP in the transcription reaction. This was specifically done to keep the density of GAPDH band in a readable range on final scan while enabling the detection of DRA band. The procedure of labeling was exactly the same every time, and once the probes were synthesized, the batch of probes was simultaneously utilized for hybridization with equal amounts of RNA.

**Statistical analysis**

Data are means ± SE of at least 3–6 independent determinations (performed in separate wells) repeated on at least two to three occasions. When error bars are not visible in the figures, they are smaller than the symbol. Statistical differences were analyzed by Student’s t-test, and a P value of <0.05 was considered statistically significant.

**RESULTS**

**The OH⁻ gradient-stimulated ³⁵SO₄²⁻ uptake in Caco-2 cells**

Time course of OH⁻ gradient-stimulated ³⁵SO₄²⁻ uptake. Previous studies have shown the protein product of DRA gene to be a sulfate and oxalate transporter. Additionally, Caco-2 cells have previously been utilized to characterize electrolyte transport in the human intestine. Therefore, to establish an in vitro cellular model to study the role of DRA in anion transport in the human intestine and its regulation by the hormones, we first examined the OH⁻ gradient-dependent apical ³⁵SO₄²⁻ uptake in monolayers of 5–7 days post-confluent well-differentiated Caco-2 cells. As shown in Fig. 1, OH⁻ gradient-driven ³⁵SO₄²⁻ uptake in Caco-2 cells was linear as a function of time for up to 7 min and was significantly inhibited by 0.3 mM DIDS (the AE inhibitor). Therefore, a 2-min incubation time was used in all subsequent experiments.

Kinetics of SO₄²⁻/OH⁻ exchange process in Caco-2 cells. To further characterize the apical SO₄²⁻/OH⁻ exchange process in Caco-2 cells, we examined the kinetic parameters of the exchanger by measuring sulfate uptake in the presence of increasing concentrations of extracellular SO₄²⁻. Figure 2A shows that the apical OH⁻ gradient-stimulated ³⁵SO₄²⁻ uptake in Caco-2 cells exhibited saturation kinetics in the presence of increasing concentrations of sulfate. Lineweaver-Burk plot (Fig. 2B) demonstrated a straight line with an apparent Kₘ of 0.2 ± 0.08 mM for sulfate and a Vₘₐₓ of 1.1 ± 0.2 nmol·mg protein⁻¹·min⁻¹. These data indicate a carrier-mediated process for the OH⁻ gradient-driven apical sulfate uptake in Caco-2 cells.

³⁵SO₄²⁻ uptake inhibition by DIDS. As shown above, the SO₄²⁻/OH⁻ exchange process was highly sensitive to inhibition by DIDS. To further examine the characteristics of the exchanger, we studied the effect of DIDS on kinetic parameters of the apical ³⁵SO₄²⁻ uptake in Caco-2 cells. Lineweaver-Burk plot, as shown in Fig. 3, demonstrated that ³⁵SO₄²⁻ uptake was inhibited by 50 μM DIDS in a mixed-type manner, with an increase in the apparent Kₘ for sulfate from 0.22 to 0.73 mM and
a decrease in the $V_{\text{max}}$ from 1.57 to 0.23 nmol·mg protein$^{-1}$·min$^{-1}$. These experiments were repeated using different concentrations of DIDS (0.015, 0.025, and 0.05 mM; not shown) and demonstrated the same pattern of inhibition with a $K_i$ of 0.9 ± 0.3 μM.

**Effect of anions on $^{35}$SO$_4^{2-}$ uptake.** Recent studies (22, 23) have suggested that the DRA protein product is a Cl$^-$/HCO$_3$ (OH$^-$) exchanger. Previously, DRA was shown to be a sulfate and oxalate transporter. To examine the anion specificity of the apical SO$_4^{2-}$/OH$^-$ exchanger in Caco-2 cells, we investigated the effect of various anions on the sulfate uptake in Caco-2 cells. As shown in Fig. 4, 5 mM cis-concentration of butyrate, formate, lactate, succinate, and nitrate failed to significantly inhibit $^{35}$SO$_4^{2-}$ (50 μM) uptake in Caco-2 cells. On the other hand, 5 mM cis-concentration of oxalate and chloride inhibited $^{35}$SO$_4^{2-}$ (50 μM) uptake by ~90 and ~30%, respectively. These findings suggest that oxalate and chloride but not butyrate, formate, lactate, succinate, or nitrate could serve as alternative substrates for the SO$_4^{2-}$/OH$^-$ antiporter in Caco-2 cells. These results also indicate that the exchanger has a markedly higher affinity for oxalate compared with chloride.

The mechanism(s) of $^{35}$SO$_4^{2-}$ uptake inhibition by oxalate and chloride. Because oxalate and chloride significantly inhibited the apical OH$^-$ gradient-stimulated $^{35}$SO$_4^{2-}$ uptake in Caco-2 cells, we further investigated the mechanisms by which oxalate and chloride inhibited the $^{35}$SO$_4^{2-}$ uptake. Figure 5 shows the effect of 0.5 mM concentration of oxalate on the kinetics of $^{35}$SO$_4^{2-}$ uptake, which demonstrates a competitive inhibition. The experiment was repeated using different concentrations of oxalate (0.3, 0.5, and 1 mM; not shown) and showed the same type of inhibition with a $K_i$ of 0.2 ± 0.03 mM for oxalate. On the other hand, as shown in Fig. 6, $^{35}$SO$_4^{2-}$ uptake was also competitively inhibited by 10 mM concentration of chloride. Repeating the experiment with different concentrations of chloride (5, 10, and 25 mM; not shown) revealed the same pattern of inhibition with a $K_i$ of 5.9 ± 1 mM for chloride. These findings suggest that although the
SO₄²⁻/OH⁻ exchanger could use oxalate and chloride as alternative substrates in addition to sulfate, it appears that the antiporter has a markedly higher affinity for both sulfate and oxalate compared with chloride.

OH⁻ gradient-stimulated ³⁶Cl⁻ uptake in Caco-2 cells

Time course of OH⁻ gradient-stimulated ³⁶Cl⁻ uptake in Caco-2 cells

We have previously characterized Cl⁻/HCO₃⁻ (OH⁻) exchange process in the human colonic proximal and distal apical plasma membrane vesicles. To investigate whether Caco-2 cells could also serve as a model to study the chloride uptake, we examined the effect of outwardly directed OH⁻ gradient on the time course of ³⁶Cl⁻ uptake in Caco-2 cells. As shown in Fig. 7, OH⁻ gradient-dependent ³⁶Cl⁻ uptake in Caco-2 cells was linear as a function of time up to 7 min and it was inhibited by DIDS. It should be noted that in contrast to SO₄²⁻/OH⁻ exchange activity, OH⁻ gradient-stimulated ³⁶Cl⁻ uptake was much less sensitive to inhibition by DIDS. These data suggest that in Caco-2 cells the two processes may be occurring via two distinct transporters.

The OH⁻ gradient-stimulated ³⁶Cl⁻ uptake inhibition by DIDS. Because the SO₄²⁻/OH⁻ exchange process was shown to be inhibited by DIDS in a mixed-type manner and Cl⁻/OH⁻ exchange appeared to be more resistant to inhibition by DIDS, we examined the mechanism of Cl⁻/OH⁻ exchange inhibition by DIDS in Caco-2 cells.

Fig. 3. Effect of DIDS on the kinetics of SO₄²⁻/OH⁻ exchange activity in Caco-2 cells. Postconfluent Caco-2 cells were preincubated for 1 h at room temperature in HEPES/KOH medium adjusted to pH 8.5. OH⁻ gradient-stimulated SO₄²⁻ uptake in Caco-2 cells, in the presence (○) or the absence (●) of 0.05 mM DIDS, was determined as described in Fig. 2 legend. Lineweaver-Burk plot for data is shown. Results are means ± SE of 4 uptake determinations performed on 2 separate occasions.

Fig. 5. Effect of oxalate on the kinetics of SO₄²⁻/OH⁻ exchange process in Caco-2 cells. OH⁻ gradient-stimulated SO₄²⁻ uptake in Caco-2 cells in the presence (○) or the absence (●) of 0.5 mM concentration of oxalate was determined as described in Fig. 2 legend. Lineweaver-Burk plot for data is shown. Results are means ± SE of 8 uptake determinations performed on 3 separate occasions.

Fig. 4. Effects of anions on OH⁻ gradient-driven ³⁵SO₄²⁻ uptake in Caco-2 cells. Caco-2 cells preincubated with the HEPES medium, pH 8.5, were incubated with ³⁵SO₄²⁻ uptake buffer, containing 50 μM of ³⁵SO₄²⁻, in the presence of 5 mM concentration of different anions. Results are a percentage of uptake in the presence of each anion compared with control (100%) and are means ± SE of 6 uptake determinations (*P < 0.05) from 3 separate occasions.

Fig. 6. Effect of Cl⁻ on ³⁵SO₄²⁻ in Caco-2 cells. pH gradient-driven SO₄²⁻ uptake in postconfluent Caco-2 cells was measured in the presence (○) or the absence (●) of 10 mM concentration of Cl⁻ as described in Fig. 2 legend. Lineweaver-Burk plot for data is shown. Results are means ± SE of 4 uptake determinations performed on 2 separate occasions.
significant changes occurred in the V_{max}.

These results further support the notion of the presence of distinct SO_4^{2-}/OH^- and Cl^-/OH^- transporters in Caco-2 cells. To further analyze the effect of thyroxine on the SO_4^{2-}/OH^- exchange process in Caco-2 cells, we examined the effect of thyroxine treatment on the kinetic parameters of 35SO_4^{2-} uptake. As shown in Fig. 10, the V_{max} of 35SO_4^{2-} uptake was significantly decreased (3.4 ± 0.28 compared with 5.1 ± 0.42 nmol·mg protein^{-1}·min^{-1}) in response to thyroxine treatment compared with vehicle alone. The differences in V_{max} values of vehicle-treated cells alone compared with our basal values in Caco-2 cells appear to be due to a different batch of Caco-2 cells utilized here. Also, thyroxine treatment resulted in a decrease in the K_{m} for SO_4^{2-} (0.09 ± 0.04 compared with 0.18 ± 0.05 mM in vehicle alone).

The effect of thyroxine on the relative abundance of human DRA mRNA

To investigate the possible mechanism of reduced SO_4^{2-}/OH^- exchange activity in Caco-2 cells, we examined the effect of thyroxine on the level of expression of DRA mRNA, utilizing an RNase protection assay. The [32P]cRNA probes for human DRA and GAPDH (internal standard) were hybridized to total RNA extracted from control and thyroxine-treated Caco-2 cells. Subsequently, RNase-digested bands with the predicted sizes were observed in quantitative manner for human DRA and GAPDH mRNA. Figure 11A shows a representative RNase protection assay blot for human DRA and GAPDH in Caco-2 cells treated with 100 nM thyroxine or vehicle alone for 12, 20, and 24 h. The data demonstrate protected fragments for human DRA and

Thyroxine has previously been shown to alter the level of expression and the function of various electrolyte transporters in a number of tissue and cell lines (6, 8, 10). To investigate the role of thyroxine in the possible regulation of chloride and sulfate uptake in Caco-2 cells, we incubated Caco-2 cells with 100 nM of thyroxine for 24 h and studied its effect on both SO_4^{2-}/OH^- and Cl^-/OH^- exchange processes. As shown in Fig. 9A, the OH^- gradient-driven 36Cl^- uptake was significantly reduced in Caco-2 cells incubated with thyroxine by 45.3 ± 8.6% compared with control (vehicle alone). In contrast, 36Cl^- uptake showed no significant changes after thyroxine treatment (Fig. 9B).
GAPDH with the appropriate expected sizes. The human DRA mRNA, as shown in Fig. 11A, was reduced in a time-dependent manner in response to thyroxine treatment compared with vehicle alone with a maximal reduction at 24-h time point. Analysis of the quantification for human DRA mRNA in Caco-2 cells after 24-h incubation with thyroxine or vehicle alone is depicted in Fig. 11B (n = 4). The relative abundance of human DRA mRNA was calculated by taking the ratio of their representative densities to that of GAPDH. As shown in the Fig. 11B, incubation of Caco-2 cells for 24 h with 100 nM thyroxine reduced the relative abundance of DRA mRNA by 57.5 ± 0.81% compared with control. These data along with the reduction in the V_max of \( \text{SO}_4^{2-} \)/OH\(^{-} \) with no changes in the \( \text{Cl}^{-}/\text{OH}^{-} \) uptake in these cells suggest that DRA may be involved directly in the apical \( \text{SO}_4^{2-}/\text{OH}^{-} \) but not in \( \text{Cl}^{-}/\text{OH}^{-} \) exchange in Caco-2 cells.

DISCUSSION

In our current studies, we have established an in vitro cellular model to study sulfate and chloride transport and have defined the possible role of DRA in the anion transport in the human intestine. Our results demonstrated that the Caco-2 cell line could serve as a suitable model to study sulfate and chloride transport. The data of our studies demonstrated and characterized the presence of an apical \( \text{SO}_4^{2-}/\text{OH}^{-} \) exchange process in Caco-2 cells. The findings consistent with a carrier-mediated activity for the \( \text{OH}^{-} \) gradient-stimulated \( \text{SO}_4^{2-} \) uptake are: 1) \( \text{SO}_4^{2-} \) uptake exhibited saturation kinetics; 2) \( \text{SO}_4^{2-} \) uptake was significantly inhibited by DIDS (the AE inhibitor); and 3) oxalate and chloride (but not butyrate, formate, lactate, succinate, and nitrate), competitively inhibited the \( \text{SO}_4^{2-}/\text{OH}^{-} \) exchange process, indicating the presence of an anion antiporter that could use oxalate and chloride as alternative substrates in addition to sulfate. Furthermore, the results of the present studies indicated that the apical \( \text{SO}_4^{2-}/\text{OH}^{-} \) and \( \text{Cl}^{-}/\text{OH}^{-} \) exchange in Caco-2 cells are distinct processes based on the following: 1) \( \text{SO}_4^{2-}/\text{OH}^{-} \) exchange activity was more sensitive to inhibition by DIDS compared with \( \text{Cl}^{-}/\text{OH}^{-} \) exchange process; 2) \( \text{SO}_4^{2-}/\text{OH}^{-} \) exchange process was inhibited by DIDS in a mixed-type manner, whereas \( \text{Cl}^{-}/\text{OH}^{-} \) exchange was competitively inhibited by DIDS; 3) incubation with 100 nM thyroxine inhibited the \( \text{OH}^{-} \) gradient-stimulated \( \text{SO}_4^{2-} \) but not \( \text{Cl}^{-} \) uptake in Caco-2 cells; and 4) in parallel to reduced \( \text{SO}_4^{2-} \) uptake, thyroxine treatment also reduced the relative abundance of DRA mRNA.

Previous studies have shown that postconfluent differentiated Caco-2 cells possess many of the functional and structural characteristics of the native enterocyte including similar transport mechanisms and regulatory pathways (27). Therefore, Caco-2 cells have been previously used as a model to characterize the electrolyte transport in the small intestine and colon (40).
Silberg et al. (37) have recently shown that the DRA gene is expressed in postconfluent but not preconfluent Caco-2 cells. Consistent with the previous studies of Silberg et al., our results showed that 5–7 days postconfluent but not preconfluent Caco-2 cells expressed the DRA gene (data not shown) and possess apical SO$_4^{2-}$/OH$^-$ and Cl$^-$/OH$^-$ exchange activities. Therefore, these cells have been utilized here as an in vitro cellular model to study the regulation of the DRA expression by thyroxine and delineate the interactions between sulfate, chloride, and oxalate transport in the human intestine.

The apical SO$_4^{2-}$/OH$^-$ exchange in the polarized monolayers of Caco-2 cells appeared to be a carrier-mediated process. The SO$_4^{2-}$/OH$^-$ exchanger in Caco-2 cells exhibited kinetic characteristics similar to SO$_4^{2-}$/OH$^-$ or SO$_4^{2-}$/HCO$_3^-$ exchangers in other systems. For instance, $K_m$ of 0.2 mM for SO$_4^{2-}$ uptake in Caco-2 cells is comparable to SO$_4^{2-}$/OH$^-$ exchanger in rabbit ileal BBM (0.475 mM; Ref. 34), SO$_4^{2-}$/HCO$_3^-$ exchanger in rabbit ileal BLM (0.122 mM; Ref. 20), rat liver lysosomal sulfate transporter (0.213 mM; Ref. 8), and human proximal colon BBM (0.8 mM; Ref. 39). Furthermore, the SO$_4^{2-}$/OH$^-$ exchanger in Caco-2 cells appears to be highly sensitive to DIDS. Our results showed that the effect of DIDS on $^{35}$SO$_4^{2-}$ uptake occurs in a linear mixed type of inhibition with a $K_i$ of 0.9 ± 0.3 μM for DIDS. This $K_i$ value is also comparable with the $K_i$ of DIDS inhibition of SO$_4^{2-}$/HCO$_3^-$ exchanger in the rabbit ileum BLM (6 μM; Ref. 20). Although the mechanism of inhibition by DIDS of the SO$_4^{2-}$/OH$^-$ exchange process needs more detailed characterization, this kind of inhibition suggests that DIDS and SO$_4^{2-}$ have different binding sites on the exchanger and that DIDS, upon binding to its site, may alter the affinity of the transporter for substrates probably via inducing conformational changes in the antiporter.

The apical SO$_4^{2-}$/OH$^-$ exchange process in Caco-2 cells appeared to be specific for SO$_4^{2-}$, Cl$^-$, and oxalate but not for the other anions such as nitrate and short chain fatty acids. Our kinetic studies demonstrated that both Cl$^-$ and oxalate inhibited the OH$^-$ gradient-stimulated $^{35}$SO$_4^{2-}$ uptake in Caco-2 cells in a competitive manner. The $K_i$ values for Cl$^-$ and oxalate suggest that although the exchanger can transport Cl$^-$ and oxalate in addition to SO$_4^{2-}$ ($K_m$ = 0.2 mM) and oxalate ($K_i$ = 0.2 mM) compared with Cl$^-$ ($K_i$ = 5.9 mM). In agreement with the previous studies of Silberg et al. (37), which demonstrated that DRA gene product is a sulfate and oxalate transporter, our results indicate that DRA may be responsible for the SO$_4^{2-}$/OH$^-$ exchange process in Caco-2 cells. On the other hand, previous studies in the human proximal colon and rabbit ileum (18, 21) have shown that nitrate and bromide could substitute for chloride in the Cl$^-$/HCO$_3^-$ (OH$^-$) exchange processes. Because the SO$_4^{2-}$/OH$^-$ exchanger in the current study exhibited lower affinity for chloride and nitrate did not alter the OH$^-$ gradient-stimulated $^{35}$SO$_4^{2-}$ uptake, the data of the current study suggest that SO$_4^{2-}$/OH$^-$ and Cl$^-$/HCO$_3^-$ (OH$^-$) exchange activities in the human intestine may be distinct processes. We have previously characterized a Cl$^-$/HCO$_3^-$ (OH$^-$) exchange process in the human small intestine and proximal colon (21, 29). These studies demonstrated that Cl$^-$ uptake into the human colonic apical membrane vesicles was stimulated in the presence of a pH gradient. The OH$^-$ gradient-stimulated Cl$^-$ uptake into these vesicles was further stimulated in the pres-
ence of a HCO$_3^-$ gradient. The data of these studies clearly demonstrated that in the human small intestine and colon, Cl$^-$/HCO$_3^-$ and Cl$^-$/OH$^-$ exchange activities were mediated via the same transporter. In the present study, we intended to examine whether Caco-2 cells could also serve as a suitable model to study chloride transport. Our data demonstrated the presence of an outwardly OH$^-$ gradient-stimulated $^{36}$Cl$^-$ uptake that was linear as a function of time and could be inhibited by DIDS. Because Caco-2 cells were derived from human colon adenocarcinoma and when confluent demonstrate the characteristics of differentiated enterocytes (27), it is most likely that the OH$^-$ gradient-driven $^{36}$Cl$^-$ uptake into these cells represents the activity of the same transporter that is also responsible for Cl$^-$/HCO$_3^-$ exchange process. In this regard, previous studies by Rajendran and Binder (28) have shown the presence of two distinct transporters, Cl$^-$/OH$^-$ and Cl$^-$/HCO$_3^-$ exchangers in rat distal colonic apical membrane vesicles. In these studies, the pH gradient-dependent $^{36}$Cl$^-$ uptake was not (unlike our previous studies with human colonic apical membrane vesicles) affected by imposing a HCO$_3^-$ gradient. Additionally, in these studies with rat colonic apical membrane vesicles, bumetanide was shown to preferentially inhibit the Cl$^-$/OH$^-$ but not the Cl$^-$/HCO$_3^-$ exchange process (28). In contrast, in our current studies, bumetanide inhibited both $^{38}$Cl$^-$ and $^{35}$SO$_4^{2-}$ uptake to the same extent (~30–40%, data not shown) ruling out the possibility that, similar to rat distal colon, Caco-2 cells may also possess two different Cl$^-$/HCO$_3^-$ and Cl$^-$/OH$^-$ exchange processes with one that could take $^{35}$SO$_4^{2-}$ as a substrate.

Similar to our previous findings in the human proximal colonic apical membrane and to other intestinal AEs such as of rabbit and human ileum (13, 18, 21, 29), the Cl$^-$/OH$^-$ exchange process in Caco-2 cells appeared to be relatively less sensitive to inhibition by DIDS. It has previously been suggested that the possible explanation for the poor inhibition may be a result of competition with the substrate (5). In agreement with that, our findings showed that Cl$^-$/OH$^-$ exchange process in Caco-2 is competitively inhibited by DIDS with a $K_i$ of 0.3 mM. The $K_i$ value for Cl$^-$ uptake inhibition by DIDS is also comparable with other systems such as Cl$^-$/HCO$_3^-$ exchange in the rabbit ileal basolateral membrane, with a $K_i$ of 0.28 mM (19). The fact that SO$_4^{2-}$/OH$^-$ and Cl$^-$/OH$^-$ exchange activities in Caco-2 cells have different sensitivities and mechanisms of inhibition by DIDS with different $K_i$ values further supports the notion that they are mediated via distinct transporters. Recent studies (22, 23) have shown that DRA is capable of transporting Cl$^-$ and suggested that DRA is the intestinal apical Cl$^-$/HCO$_3^-$ exchanger. The data in the current study showed that SO$_4^{2-}$/OH$^-$ uptake is competitively inhibited by Cl$^-$ with a $K_i$ of 5.9 mM indicating that SO$_4^{2-}$/OH$^-$ exchanger is able to transport Cl$^-$ but with a low affinity compared with SO$_4^{2-}$.

The thyroid hormone thyroxine has been shown to have a widespread effect on membrane transport of amino acids (12), glucose (35), and ions (6, 8, 10) in various tissues and cell lines. For instance, Cano et al. (6) demonstrated a stimulation of renal Na$^+$/H$^+$ exchanger by transcriptional activation in OK cell line by thyroxine. Furthermore, Chou et al. (8) have reported that the lysosomal sulfate transport in the rat liver is decreased by thyroxine treatment. Additionally, thyroxine has been previously shown to play an important role in the developmental changes of a number of intestinal digestive enzymes and transporters (14). In this regard, Chow et al. (10) have previously shown that thyroxine treatment to suckling rats was followed by a 60% decrease in the expression of AE2 in the small intestine. The novel finding in the current study is that, in Caco-2 cells, 24-h treatment with thyroxine significantly decreased the OH$^-$-gradient-driven sulfate but not chloride uptake. This differential regulation indicates a specific effect of thyroxine on the sulfate transporter and negates its generalized effect via possible changes in membrane composition and fluidity as has been observed under some circumstances (26, 32).

In the current studies, both the $V_{max}$ and the $K_m$ for $^{35}$SO$_4^{2-}$ uptake in Caco-2 cells were significantly decreased in response to thyroxine treatment compared with vehicle alone. Although most chronic biological effects of thyroxine are believed to be mediated via alterations at the level of gene transcription (30), studies have shown that limited exposure to thyroxine may also affect membrane transport via changes in the cytoplasmic calcium concentration as shown in the case of glucose uptake in chick embryo myocytes (36) or by activating other systems such as kinases because phosphorylation was observed in the case of GLUT-1 and GLUT-4 (31). Therefore, the changes in the $K_m$ for SO$_4^{2-}$ in response to thyroxine could be speculated to occur by a possible involvement of protein kinases and/or other pathways in the regulation of sulfate uptake in Caco-2 cells. Further studies will be required to investigate the detailed mechanisms of the regulation of SO$_4^{2-}$/OH$^-$ exchange process by thyroxine. Consistent with the decrease in the $V_{max}$ of sulfate uptake, our current results demonstrated that thyroxine treatment in Caco-2 cells also significantly decreased the relative abundance of DRA mRNA. Because it has previously been demonstrated that the DRA gene product is a sulfate and oxalate transporter that exhibits a high homology to other sulfate but not chloride bicarbonate transporters, it is most likely that in Caco-2 cells, DRA is responsible for the pH-driven bicarbonate uptake that is regulated by thyroxine. Additionally, our results suggest that, in Caco-2 cells, the OH$^-$ gradient-driven chloride uptake occurs via a transporter that is distinct from the DRA. In this regard, Melvin et al. (22) have shown that mouse DRA directly mediated the Cl$^-$/HCO$_3^-$ exchange process in 293 cells transfected with mouse DRA. These elegant studies, however, have some limitations: for example, 1) the studies ignored the fact that DRA was initially shown to be a sulfate and oxalate transporter and did not investigate the effect of SO$_4^{2-}$ on the Cl$^-$/HCO$_3^-$ exchange.
process; 2) also, the studies did not take into consideration the previous data that showed that SO$_4^{2-}$ does not affect Cl$^{-}$ transport in the human colonic apical membranes; 3) the study did not provide evidence for the targeting of the exogenous mouse DRA protein to the membrane of the transfected cells; and 4) these studies did not rule out the possibility of DRA functioning as part of a protein complex that is responsible for the described Cl$^-$/HCO$_3^-$ exchange process.

On the other hand, the findings of the current studies suggest that chloride transport in Caco-2 cells is mediated by two distinct transporters: 1) a SO$_4^{2-}$/OH$^-$ exchanger, which could be DRA, with ability to transport Cl$^-$ as well but with low affinity; and 2) a distinct Cl$^-$/OH$^-$ exchanger. Previously, Kere et al. (17) have proposed different models regarding DRA and apical epithelial chloride transport. They suggested that DRA might be contributing to the apical Cl$^-$/HCO$_3^-$ exchange activity by itself or that DRA may be part of a multiprotein complex with Cl$^-$/HCO$_3^-$ exchange function, or that the Cl$^-$/HCO$_3^-$ exchange may require two or more transporters having functions that are tightly coupled by a common substrate. Along with our previous findings in the human proximal colonic apical membrane that demonstrated that sulfate did not alter the Cl$^-$/HCO$_3^-$ (OH$^-$) exchange process, our results appear to support the last proposed model of Kere et al. (17). In this model, a mutation in only one of the proteins could block the functions of both transporters and cause the observed defect in the Cl$^-$/HCO$_3^-$ (OH$^-$) exchange in CLD patients.

In conclusion, our current studies raise questions about the direct role of DRA in intestinal chloride transport. Our results indicate for the first time that in Caco-2 cells the apical Cl$^-$/OH$^-$ and SO$_4^{2-}$/OH$^-$ exchange processes are distinct and that DRA may be directly responsible for the SO$_4^{2-}$/OH$^-$ exchange activity.

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