Ibuprofen-Induced Changes in Sulfate Renal Transport

KAZUKO SAGAWA, LISA JO BENINCOSA, HEINI MURER and MARILYN E. MORRIS
Department of Pharmaceutics, State University of New York at Buffalo, Amherst, New York (K.S., L.J.B., M.E.M.) and Institute of Physiology, University of Zürich, Zürich, Switzerland (H.M.)

Accepted for publication July 13, 1998 This paper is available online at http://www.jpet.org

ABSTRACT
Nonsteroidal anti-inflammatory drugs (NSAIDs) increase sulfate renal clearance and decrease the fractional reabsorption of sulfate by the kidneys. The mechanism of this alteration of inorganic sulfate homeostasis is unknown. The objectives of this study were 1) to investigate if sulfate renal transport is altered in isolated membrane vesicles after pretreatment of animals in vivo with ibuprofen (IBU), and 2) to determine the cellular mechanism of changes in sulfate renal transport. Female Lewis rats received IBU at a i.v. dose of 27 mg/kg followed by an infusion of 33 μg/min for 4 hr. Sulfate transport was studied using brush border (BBM) and basolateral membrane (BLM) vesicles isolated from rat kidney cortex. The V_{max} for the sodium-dependent sulfate cotransport (NaSi-1) in BBM was significantly lower in the IBU group compared with the control group (0.79 ± 0.23 vs. 1.25 ± 0.17 nmol/mg protein/10 sec, respectively; P < .05) with no change in K_{m}. There were no significant differences between the study groups in sulfate anion exchange kinetics in BLM vesicles. NaSi-1 transporter mRNA level in kidney cortex and protein level in BBM were significantly lower in animals pretreated with IBU compared with that in control animals. There was no change in membrane fluidity of BBM and BLM isolated from IBU-treated animals as measured by the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene. These results indicate that IBU treatment alters sodium-dependent sulfate cotransport by a downregulation of mRNA and protein of NaSi-1 transporter in BBM.

Inorganic sulfate is eliminated from the body predominantly in unchanged form by urinary excretion (Walser et al., 1953). Capacity-limited renal reabsorption is of primary importance in sulfate homeostasis. Sulfate reabsorption occurs predominantly from the renal proximal tubule. Inorganic sulfate is transported across the BBM predominantly via sodium-dependent sulfate cotransport, which is distinct from sodium-dependent phosphate, glucose or amino acid cotransport (Lücke et al., 1979; Turner, 1984). Inorganic sulfate exits the proximal tubule cell across the BLM via sulfate anion exchange transport for which bicarbonate is the most effective counterion (Pritchard and Renfro, 1983).

Renal sulfate reabsorption is regulated by various pharmacological agents and physiological conditions. An increased renal sulfate reabsorption is found in infants, young children and pregnant women, resulting in increased serum sulfate concentrations (Cole et al., 1982; Morris and Levy, 1983). Renal reabsorption of sulfate increases under conditions of sulfate deficiency (Benincosa et al., 1995) while sulfaturia occurs after ingestion of a high sulfate diet (Sagawa et al., 1998a). A decreased V_{max} for sodium sulfate cotransport occurs in chick kidney BBM after dexamethasone treatment (Renfro et al., 1989), and in BLM isolated from vitamin D-deficient rats (Fernandes et al., 1997), while an increased V_{max} occurs in methionine-deficient rats after ingestion of a low methionine diet (Benincosa et al., 1995). The altered sodium/sulfate cotransport in methionine-deficient and vitamin D-deficient rats is associated with changes in both BBM sodium sulfate cotransport (NaSi-1) protein and mRNA content.

NSAIDs IBU, indomethacin and tiaprofenic acid, when administered at doses that inhibit renal prostaglandin synthesis, decrease the in vivo renal reabsorption of sulfate in rats (Morris and Benincosa, 1992). However, the renal sulfate transport in animals pretreated with NSAIDs has not been studied; it is not known whether there are changes in the BBM sodium/sulfate cotransport or the BLM anion exchange of sulfate. Moreover, the mechanism of the altered renal reabsorption of sulfate after NSAID treatment is unknown. The objectives of this present investigation were to

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; BBM, brush border membrane; BLM, basolateral membrane; IBU, ibuprofen; mRNA, messenger RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; DPH, 1,6-diphenyl-1,3,5-hexatriene; PGE_2, prostaglandin E_2; cAMP, cyclic AMP.
determine the effect of IBU pretreatment in rats on 1) renal sulfate transport in BBM and BLM, 2) membrane motional order (fluidity) of BBM and BLM, and 3) sodium/sulfate cotransporter (NaSi-1) mRNA and protein expression in BBM.

Materials and Methods

Infusion study design. Female Lewis rats (Charles River, Wilmington, MA) had right jugular cannulae implanted 2 days prior to the study day. Animals received either IBU (IBU group) or the vehicle only (control group). Ibuprofen (Sigma Chemical, St Louis, MO) was dissolved in a small volume of 2 M NaOH, diluted to the final volume with normal saline and the pH of the solution was adjusted to 7.4. The dosage regimen for IBU was designed to achieve steady-state concentrations of 70 μg/ml (0.34 mM). This concentration is similar to the peak concentration seen clinically after administration of a single 800 mg oral dose of IBU in humans (Lockwood et al., 1983; Lee et al., 1984). Preliminary studies demonstrated that this IBU dosage regimen produced >80% inhibition of the renal synthesis of prostaglandins as measured by the urinary excretion of PGE_2 (Morris and Benincosa, 1992). The bolus dose and infusion rate of IBU were calculated using kinetic parameters obtained from published data (Shah and Jung, 1987). The dosage of IBU was 27 mg/kg by i.v. bolus injection followed by 33 μg/min infusion through jugular vein cannula for 4 hr at which time the animals were sacrificed and kidney cortex samples were obtained.

Renal vesicle preparations. BBM and BLM vesicles were prepared from kidney cortex right after the infusion was stopped by previously described procedures (Benincosa and Morris, 1993a). The tissue from the animals in the same study group was combined for the membrane vesicle preparations. Briefly, the freshly isolated rat kidney cortex was homogenized in homogenizing buffer (250 mM sucrose, 10 mM triethanolamine-HCl, pH 7.6). BBM and BLM vesicles were separated using a Percoll (Sigma Chemical) density gradient centrifugation. The BBM fraction was further purified by MgCl_2 precipitation. The final BBM and BLM preparations were resuspended in 300 mM mannitol, 10 mM HEPES, pH 7.4, and in 100 mM mannitol, 50 mM HEPES, pH 7.4, respectively. Alkaline phosphatase activity was measured as a marker enzyme for BBM vesicles using a commercially available kit (Sigma Diagnostic, kit #245, St. Louis, MO). Na^+-K^+ ATPase activity was measured as a marker enzyme for BLM vesicles using the method of Johansen and Skou (1970).

Sulfate transport studies. The sulfate transport was examined by measuring the uptake into membrane vesicles using a rapid filtration method (Goldinger et al., 1984). Vesicles were diluted to 6 mg/ml with preincubation buffer (300 mM mannitol, 10 mM HEPES for BBM; 100 mM mannitol, 50 mM HEPES, 50 mM KCl, for BLM; pH 7.5) for 1 hr prior to the uptake study. Experiments were begun by diluting vesicles 1:10 (to yield a final protein concentration of 0.6 mg/ml) with the uptake medium (100 mM mannitol, 10 mM HEPES, 100 mM NaCl or KCl for BBM; 200 mM mannitol, 50 mM HEPES, with or without 20 mM potassium thiosulfate for BLM; pH 7.5) containing [35S]Na_2SO_4 (DuPont/NEN, Boston, MA) and various concentrations of K_2SO_4. The filters (pore size 0.45 μm, Millipore, Bedford, MA) were presoaked overnight in stop solution (300 mM mannitol and 10 mM HEPES for BBM; 200 mM mannitol, 50 mM HEPES for BLM; pH 7.5) containing 100 mM K_2SO_4 to minimize any binding of sulfate to the membrane. Binding to the filter was determined by filtering uptake medium without vesicles. All uptake studies were performed at room temperature.

To evaluate the time course of sulfate uptake, the vesicles were incubated with 100 μM K_2SO_4 containing 5 nCi/μl [35S]Na_2SO_4, and the amounts of sulfate present in the vesicles were measured at various time points (10, 30, 60, 180 sec and 1 hr). Preliminary studies demonstrated that uptake at 10 sec represents the linear uptake process and that equilibrium conditions were obtained at 60 min in both BBM and BLM vesicle preparations. Therefore, 10 sec uptake values were used for the further concentration-dependent sulfate uptake studies to evaluate the Michaelis-Menten parameters. Sodium-dependent sulfate uptake was evaluated by measuring the sulfate uptake into BBM vesicles; the vesicles were incubated with various concentrations of K_2SO_4 (0.1–8 mM, containing 5 nCi/μl [35S]Na_2SO_4) in the presence or absence of 100 mM NaCl. The difference between these two values represents the sodium-dependent cotransport process. To examine sulfate uptake by anion exchange transport, the BLM vesicles were incubated with various concentrations of K_2SO_4 (0.05–1.2 mM, containing 5 nCi/μl [35S]Na_2SO_4). Diffusional uptake of sulfate was determined by measuring sulfate uptake into BBM vesicles with a competitive inhibitor for this transport, potassium thiosulfate (20 mM). The mannitol concentration was reduced when thiosulfate was present to maintain the same osmolarity. Filters were dissolved in 1 ml of 2-methoxyethanol (Amersham Co, Arlington Heights, IL) prior to counting by liquid scintillation spectroscopy (Model 1900 CA Tri Carb Liquid Scintillation Counter, Packard Instrument, Downers Grove, IL). Vesicle protein concentrations were determined by the Coomassie blue binding method (Bradford, 1976).
using a positive pressure transfer apparatus (Posiblot Transfer Apparatus, Stratagene). The RT-PCR products were loaded on the gel in duplicate. Hybridization probe was 300 bp NaSi-1 cDNA at positions 492–792, and was prepared by PCR. The random primer labeling reaction was prepared using a random primer labeling kit (Prime-It, Stratagene, La Jolla, CA). Matrices were prehybridized for a minimum of 4 hr and hybridized overnight in hybridizing solution (5× SSC, 1% SDS, 5× Denhardt’s 50% formamide, 100 μg/ml sheared salmon sperm DNA) at 42°C. Matrices were washed 5 times in 2× standard saline citrate (SSC), 0.1% SDS at room temperature, then 2 times in 0.1× SSC, 0.1% SDS at room temperature followed by 0.1× SSC, 0.1% SDS at 65°C until the radioactivity was decreased to the background levels. Hybridization signals were visualized and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The RT-PCR results were expressed as a ratio between amplified native NaSi-1 mRNA signal and amplified deletion standard cRNA signal, added as an external standard, normalized by the amount of total RNA.

**Sandwich-type ELISA procedure.** NaSi-1 polyclonal and monoclonal antibodies were raised against rabbits and mice, respectively as previously described (Sagawa et al., 1997). The assay plates (polystyrene flatbottom microtiter plates, Maxisorp, Nunc, Denmark) were coated with the NaSi-1 monoclonal antibody (10 μg/ml), then incubated with 5% Blotto/PBS overnight at 4°C to block nonspecific absorption. Wells were washed and incubated with samples or sample buffer (2.5% Triton-X in 1× PBS) only (negative control) at 4°C overnight. The wells were incubated with NaSi-1 antiserum or preimmune serum (1:600 diluted in 0.3% BSA/PBS), then incubated with horseradish peroxidase (HRP) conjugated mouse anti-rabbit IgG. After washing, freshly prepared substrate solution (0.5 mg/ml o-phenylenediamine dihydrochloride, 0.045% H2O2) was added. The reaction was stopped with 2 M sulfuric acid and OD at 490 nm was measured using a Microkinetics Reader (Bio-Tek instruments, Winooski, VT). The amounts of NaSi-1 in the tissue were calculated using a constructed standard curve using serial dilution of the NaSi-1 standard protein (5.58–164 fmol) (Sagawa et al., 1998b).

**Evaluation of membrane motional order (fluidity).** The motional order of BBM and BLM obtained from IBU-treated or control rat kidney cortex was determined by examining the fluorescence polarization of DPH, as previously described (Sarkar et al., 1993; Balasubramanian et al., 1997). To incorporate the probe, 2 μl of 2 mM DPH in tetrahydrofuran was added to the membrane vesicles, and incubated at 37°C for 1 hr. Fluorescence polarization measurements were done on a SLM Aminco (SLM Aminco, Urbana, IL) 8000 spectrofluorometer with film polarizers (FPI 110). Samples were excited at 355 nm and the emission was monitored at 430 nm with 4-nm excitation and emission slits. The lipid order parameter (S) was calculated from the steady-state polarization value by the equation: 

\[ S^2 = \frac{(4r^3)}{3} - 0.11/r_n \]

where \( r_n \) is the maximal fluorescence anisotropy value in the absence of any rotational motion (taken as 0.40) and \( r \) is the steady-state anisotropy (Pottel et al., 1983).

**Statistical analysis.** Results were expressed as the mean ± S.D., unless stated otherwise. The differences in the kinetic parameters, mRNA, and protein levels between the study groups were analyzed using unpaired t tests.

**Results**

**Transport studies.** Each vesicle preparation was characterized by comparing the enrichment of marker enzymes in the purified preparations with that found in the initial homogenate. The BBM exhibited enrichment ratios of alkaline phosphatase of 15 ± 2.1 (mean ± S.D., n = 8) while the enrichment ratios for Na+-K+ATPase in BLM were 9.2 ± 3.6, which are similar to values reported by others (Kinsella et al., 1979; Knox and Haramati, 1985). The time course for sulfate uptake was examined in BBM and BLM vesicles (data not shown). Sulfate uptake exhibited a characteristic overshoot during the first minute of incubation in both BBM and BLM vesicles indicating a transient intravesicular accumulation of sulfate. There were no differences in the 60 min (equilibrium) uptake rates in either membrane preparation suggesting that there were no changes in sulfate binding or vesicle volume. Sulfate uptake by sodium-dependent sulfate transport in BBM was decreased at the 10 sec time point in the IBU group when compared to that obtained in control group. There was no change in sulfate anion exchange transport in BLM between groups.

The concentration-dependent sulfate uptake into BBM was determined by incubating the vesicles with or without sodium in the uptake medium. The difference between the two represents the sulfate uptake via sodium-dependent sulfate cotransport. Sulfate uptake into BBM vesicles increased linearly in the absence of sodium. The data were fit using the Michaelis-Menten equation to obtain estimates of \( K_m \) and \( V_{max} \). A representative fit of the sodium-dependent uptake process using nonlinear regression analysis is shown in figure 1. The \( V_{max} \) value in the IBU group was significantly lower compared with the control group (P < .05). The \( K_m \) for sulfate BBM transport was not significantly different between groups. The concentration-dependent sulfate uptake into BLM was determined with or without the inhibitor, thiosulfate. The difference between the two represents the sulfate uptake via sulfite anion exchange transport. There were no significant differences in \( K_m \) and \( V_{max} \) for sulfite anion exchange transport (table 1).

**NaSi-1 mRNA abundance.** Because the transport studies showed a difference in \( V_{max} \) for sodium-dependent sulfate transport, the level of the mRNA for this transport gene (NaSi-1) was measured in tissue, and compared between the study groups. The southern hybridization blot is shown in figure 2A. The RT-PCR amplification efficiency was normalized by taking the hybridization signal ratio between native RNA (700 bp) and deletion standard cRNA (600 bp). The native NaSi-1 mRNA were expressed as the signal ratio to native and standard, normalized with the amount of total RNA loaded on the gel. The average of 4 lanes from the same study group was used for statistical analysis (fig. 2).

**Fig. 1.** Concentration-dependent sulfate uptake into BBM vesicles. Data shown are from a representative uptake study. Each point represents the mean ± S.D. from triplicate determinations from one vesicle preparation.
The steady-state mRNA levels in IBU group was significantly lower (decrease of 39.5%) compared with that in control group (P < .01).

**NaSi-1 protein abundance.** For the quantitative ELISA, the NaSi-1 protein standard was prepared from the purified NaSi-1 fusion protein. A linear relationship between the amounts of NaSi-1 standard protein vs. OD 490 was obtained (r^2 = .99). The BBM and BLM samples (25 µg protein per well) were assayed in quadruplicate to compare the NaSi-1 protein in BBM between study groups. IBU-treated animals exhibited a significantly lower NaSi-1 protein level in the BBM compared with the BBM isolated from control animals (P < .05, n = 4). Small amounts of NaSi-1 protein were detected in BLM, possibly due to cross-contamination with BBM, and there was no difference in NaSi-1 protein level in BLM between the groups (fig. 3).

**Membrane motional order (fluidity).** The fluorescence polarization studies with DPH demonstrated that BLM and BBM differ from one another in that the motional order of BBM is less than that of BLM. This is consistent with previous reports that examined the fluidity of these membranes (Benincosa et al., 1995; Balasubramanian et al., 1997). We have previously demonstrated that the addition of benzyl alcohol in vitro results in increased membrane fluidity in BBM and BLM as measured by the fluorescence polarization of DPH (Balasubramanian et al., 1997), while the in vitro addition of cholesterol produces decreased membrane fluidity (manuscript submitted for publication). However, treatment of animals with IBU did not produce any changes in membrane fluidity or the lipid order parameter for either BBM or BLM (fig. 4).

**Discussion**

IBU, indomethacin and tiaprofenic acid are NSAIDs that inhibit the renal reabsorption of inorganic sulfate in vivo (Morris and Benincosa, 1992; Benincosa and Morris, 1993b). The present investigation examined sulfate renal membrane transport after IBU treatment. Pretreatment with IBU de-

### TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>IBU</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBM</td>
<td>V_{max} (nmol/mg protein/10 sec)</td>
<td>1.25 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>K_m (mM)</td>
<td>0.33 ± 0.17</td>
</tr>
<tr>
<td>BLM</td>
<td>V_{max} (nmol/mg protein/10 sec)</td>
<td>0.33 ± 0.17</td>
</tr>
</tbody>
</table>

Values are reported as mean ± S.D. of 3 preparations.

*a* Significantly different, P < .05 (n = 3).

---

**Fig. 2.** A, Southern hybridization signals of RT-PCR products. The top bands (700 bp) are reverse transcribed and amplified NaSi-1 RNA in the tissue. The bottom bands (600 bp) are reverse transcribed and amplified deletion cRNA added as an external standard. The signals are duplicate lanes from one preparation, and two RNA samples were prepared from one tissue pool. B, The comparison of NaSi-1 mRNA level between the study groups. The mRNA levels were compared as RT-PCR products that were expressed as the volume ratio of coamplified NaSi-1 DNA and deletion DNA. Values are presented as mean ± S.D. (n = 4). There was a significant difference between IBU and control group (**, P < .01).
creased the transport capacity for sodium-dependent sulfate cotransport in BBM vesicles compared with that in the control group, with no apparent change in $K_m$. IBU treatment did not alter the sulfate anion exchange transport in BLM vesicles. NaSi-1 mRNA level in kidney cortex and protein level in BBM were significantly lower in animals pretreated with IBU compared with that in control animals. Thus, it appears that the mechanism of decreased sulfate uptake in renal BBM after IBU treatment involves, at least in part, downregulation of NaSi-1 transport.

Other mechanisms which could contribute to the IBU-induced decreased sodium/sulfate cotransport include 1) a direct (competitive) inhibition of sodium/sulfate cotransport by IBU, 2) changes in membrane fluidity and 3) inhibition of prostaglandin synthesis by IBU.

NSAIDs, such as IBU, indomethacin and tiaprofenic acid, when added at high concentrations in vitro to membrane vesicle preparations inhibit sulfate renal transport, exerting a pronounced effect at the BLM (Benincosa and Morris, 1993a). Organic anions such as salicylic acid and probenecid can inhibit sulfate renal transport at high concentrations, although this effect also occurs predominantly on the anion exchange process in BLM (Darling et al., 1994). Previously, we demonstrated that IBU inhibited sulfate uptake into BBM and BLM rat kidney vesicles by 22% and 38%, respectively, when the vesicles were incubated with 1 mM of IBU (Benincosa and Morris, 1993a). The unbound IBU concentration present in vivo in treated animals in this study would be $\sim 0.03$ mM, much lower than concentrations that produce significant inhibition of sulfate transport at either the BBM or BLM. Thus, IBU, at the steady-state plasma concentration achieved in this study, would be expected to exhibit little direct inhibition of sodium-dependent sulfate cotransport in BBM.

Changes in membrane composition and/or fluidity can alter sodium-dependent transport processes in the proximal tubule. We have found that changes in membrane composition and fluidity can produce altered sodium sulfate cotransport (unpublished data). However, no changes in membrane fluidity of BBM or BLM were observed in membranes isolated from IBU-treated animals. The fluorescent probe, DPH, used for membrane fluidity measurement in this study, is incorporated into the hydrophobic core of the lipid bilayer, and therefore, changes at the hydrophilic region of the membrane will not be detected by this probe. The motional order of the membrane surface might be altered by IBU treatment, and that could be responsible for the altered NaSi-1 transport capacity. The effect of IBU on membrane motional order in the hydrophilic region of the membrane is unknown at this time.

Another possible mechanism that could be involved in the IBU-mediated decrease in sodium/sulfate cotransport is inhibition of prostaglandin synthesis in the kidney. It was hypothesized that the mechanism of NSAIDs on sulfate urinary excretion involved the inhibition of prostaglandin synthesis because the effect of NSAIDs on sulfate reabsorption in vivo was abolished with concomitant prostaglandin $E_2$ treatment (Morris and Benincosa, 1992). We found that the NaSi-1 mRNA level in the kidney cortex obtained from animals pretreated with IBU and concomitant prostaglandin $E_2$ ($PGE_2$) was similar to that in animals pretreated with IBU only (data not shown). The role of prostaglandins in the reabsorption of sulfate, if any, is not understood at this time. Prostaglandins are formed mainly in the medullary collecting tubule (Bonvalet et al., 1987), and act directly at the site of synthesis (Levenson et al., 1982). Thus, the previous in vivo results that demonstrated that concomitant PGE$_2$ administration could reverse the effects of IBU on the fractional reabsorption of sulfate by the kidneys may reflect an action of PGE$_2$ at a site other than the kidney cortex or may reflect other physiological or pharmacological effects of prostaglandins. Previously, we investigated the influence of a cyclic AMP (cAMP) analog, dibutyryl-cAMP on IBU induced alterations of renal sulfate clearance in rats since the mechanism of action of prostaglandins in many biological systems involves the activation of adenylate cyclase and an increase in cellular cAMP concentrations (Kuehl, 1974; Schlendorff et al., 1978). We observed that dibutyryl-cAMP did not reverse the major IBU-induced changes in renal sulfate clearance (Benincosa and Morris, 1992), suggesting that the effect of PGE$_2$ is not mediated through cAMP. The role of prostaglandins in the regulation of sodium renal homeostasis remains to be elucidated.

In summary, IBU when present in plasma at therapeutic concentrations produces a decrease in $V_{\text{max}}$ for sodium sulfate cotransport in BBM. The molecular mechanism of this interaction involves a down-regulation of mRNA and a resulting decrease in the abundance of NaSi-1 protein in BBM.

References


Send reprint requests to: Marilyn E. Morris, Ph.D., 527 Hochstetter Hall, Department of Pharmaceutics, State University of New York at Buffalo, Amherst, NY 14260. E-mail: memorris@acsu.buffalo.edu