Dexamethasone Modulates Rat Renal Brush Border Membrane Phosphate Transporter mRNA and Protein Abundance and Glycosphingolipid Composition


With the technical assistance of Shelly A. Scott* and Paul Wilson*

*Department of Internal Medicine, The University of Texas Southwestern Medical Center at Dallas and Department of Veterans Affairs Medical Center, Dallas, Texas 75216; †Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan 48109; ‡Department of Biochemistry, Eunice Kennedy Shriver Center for Mental Retardation, Waltham, Massachusetts 02254; and §Institute of Physiology, University of Zurich, Zurich, Switzerland CH - 8057

Abstract

Glucocorticoids are important regulators of renal phosphate transport. This study investigates the role of alterations in renal brush border membrane (BBM) sodium gradient-dependent phosphate transporter (Na-Pi cotransporter) mRNA and protein abundance in the dexamethasone induced inhibition of Na-Pi cotransport in the rat. Dexamethasone administration for 4 d caused a 1.5-fold increase in the Vmax of Na-Pi cotransport (1785 ± 119 vs. 2759 ± 375 pmol/5 s per mg BBM protein in control, P < 0.01), which was paralleled by a 2.5-fold decrease in the abundance of Na-Pi mRNA and Na-Pi protein. There was also a 1.7-fold increase in BBM glucosylceramide content (528 ± 63 vs. 312 ± 41 ng/mg BBM protein in control, P < 0.02). To determine whether the alteration in glucosylceramide content per se played a functional role in the decrease in Na-Pi cotransport, control rats were treated with the glucosylceramide synthase inhibitor, d-threo-1-phenyl-2-decanoyl-amino-3-morpholino-1-propanol (PDMP). The resultant 1.5-fold decrease in BBM glucosylceramide content (199 ± 19 vs. 312 ± 41 ng/mg BBM protein in control, P < 0.02) was associated with a 1.4-fold increase in Na-Pi cotransport activity (1422 ± 73 vs. 1048 ± 85 pmol/5 s per mg BBM protein in control, P < 0.01), and a 1.5-fold increase in BBM Na-Pi protein abundance. Thus, dexamethasone-induced inhibition of Na-Pi cotransport is associated with a decrease in BBM Na-Pi cotransporter abundance, and an increase in glucosylceramide. Since primary alteration in BBM glucosylceramide content per se directly and selectively modulates BBM Na-Pi cotransport activity and Na-Pi protein abundance, we propose that the increase in BBM glucosylceramide content plays an important role in mediating the inhibitory effect of dexamethasone on Na-Pi cotransport activity. (J. Clin. Invest. 1995, 96:207–216.) Key words: glucocorticoids • glucosylceramide • sphingomyelin • membrane fluidity • Na-Pi-2 protein

Introduction

Glucocorticoids are important regulators of renal phosphate transport. Glucocorticoid excess, e.g., Cushing’s disease, and the administration of glucocorticoids to humans or experimental animals decreases tubular reabsorption of phosphate (1–3). In contrast, glucocorticoid deficiency after adenectomy increases tubular reabsorption of phosphate (4). The primary site of action of glucocorticoids to reduce phosphate reabsorption is the proximal tubule (5), an effect that is independent of endogenous parathyroid hormone activity (6). This inhibitory effect of glucocorticoids on renal tubular phosphate transport is expressed at the level of the proximal tubular apical brush border membranes (7, 8). The decrease in sodium gradient-dependent phosphate transport (Na-Pi cotransport) results from a decrease in the Vmax rather than a change in the affinity for phosphate (8). It is not known whether the decrease in the Vmax reflects a decrease in the number of active Na-Pi cotransport proteins or a change in the transport properties of existing Na-Pi cotransport proteins.

Studies in freshly isolated proximal tubular cells or in primary cultured renal cells indicate that glucocorticoids have a direct and selective inhibitory effect on Na-Pi cotransport demonstrating that the effect is independent of concomitant alterations in renal hemodynamics and/or other phosphate transport regulatory hormones (9–10). In cultured chick renal cells, dexamethasone inhibition of Na-Pi cotransport is blocked by inhibitors of transcription (actinomycin D) and translation (cycloheximide) (10). The nature of the inhibitory products produced by dexamethasone is unknown. They could inhibit of Na-Pi cotransport by causing a decrease in the abundance of Na-Pi cotransport proteins. Alternatively, they might alter membrane lipid composition, since alterations in brush border membrane lipid content or lipid dynamics also regulate Na-Pi cotransport (11). Thus, inhibitors of RNA and protein synthesis could block dexamethasone induced inhibition of Na-Pi cotransport through an effect on the synthesis and content of specific membrane lipids.

Recently the cDNA for a Na-Pi cotransport system of rat kidney cortex (NaPi-2) has been identified by expression cloning (12). Using polyclonal antibodies raised against this renal Na-Pi cotransport system, and using the polymerase chain reaction after reverse transcription of mRNA to microdissected nephron segments, we recently demonstrated that NaPi-2–related mRNA and protein is expressed in the brush border membranes of the proximal tubules in rat kidney (13).

The present study investigates (a) whether the inhibitory effect of dexamethasone on Na-Pi cotransport is mediated by changes in Na-Pi cotransport mRNA and protein abundance.

1. Abbreviations used in this paper: BBM, brush border membrane; Na-Pi cotransport, sodium gradient-dependent phosphate transport.

1Dr. Gross died on November 17, 1992.

Address correspondence to Moshe Levi, M.D., Department of Veterans Affairs Medical Center, 4500 South Lancaster Road (111G1), Dallas, Texas 75216. Phone: 214-372-7041; FAX: 214-372-7948.

Received for publication 5 July 1994 and accepted in revised form 16 March 1995.
and/or a change in brush border membrane lipid composition and lipid dynamics, and (b) whether the alteration in brush border membrane lipid composition per se can directly modulate Na-Pi cotransport activity.

Methods

Experimental animals

The experiments were performed in male Sprague Dawley rats weighing 150–200 grams (Harlan Industries, Indianapolis, IN). Before study, the animals were stabilized on the control diet (0.6% Pi) for 5 d in metabolic balance cages. The rats were then treated with dexamethasone, 100 μg/100 gram body weight, or vehicle (deionized water), administered subcutaneously once a day for four consecutive days. On the fourth day of treatment, urine collection was performed. On the fifth day, the animals were anesthetized with sodium pentobarbital, blood was obtained from the inferior vena cava, and the kidneys rapidly removed and placed in an ice-cold buffer. Blood and urine samples were analyzed for inorganic phosphate by the method of Fiske and Subbarow (14), and for creatinine by a creatinine autoanalyzer (Creatinine II Analyzer, Beckman, Fullerton, CA). Total urinary phosphate excretion and tubular reabsorption were calculated by standard clearance formulae.

In experiments designed to determine the role of alteration in glycosphingolipid content per se in the regulation of Na-Pi cotransport (see Results), control animals were treated with DL-threo-1-phenyl-2-decanoylamino-3-morpholinol-1-propanol (PDMP), an inhibitor of glycosylceramide synthase (15–16). Since previous studies have demonstrated that the circulating plasma levels of PDMP are prolonged in the presence of the cytochrome P450 inhibitor, piperonyl butoxide (16), the butoxide solution was administered before injection of PDMP. The piperonyl butoxide was prepared by dissolving in corn oil at a concentration of 150 mg/ml oil. On the first day of treatment, the butoxide solution was administered intraperitoneally 4 h before PDMP treatment; on days 2 to 4 of treatment, it was administered just before the PDMP injection. PDMP was prepared as an emulsion with the detergent Myrj 52 in normal saline, buffered with sodium acetate. The final concentrations were 0.5 mM PDMP, 6 mg/ml Myrj 52, 12 mg/ml, and sodium acetate 8 mg/ml. The PDMP was given intraperitoneally as a dose of 100 mg/kg body weight.

Brush border membrane isolation

The kidneys were placed in an ice-cold homogenizing buffer consisting of 300 mM mannitol, 5 mM EDTA, 0.1 mM PMSF, 16 mM HEPES, pH 7.50 with Tris. Thin slices were cut from the superficial portion of the kidney and homogenized with a Polytron homogenizer. Brush border membrane (BBM) vesicles were isolated from the resulting homogenate by differential centrifugation and Mg2+ precipitation, as previously described (17). To minimize the potential day-to-day variations in BBM isolation procedure on the resultant measurements, each day BBM from control and dexamethasone-treated rats were isolated simultaneously. For each BBM sample the kidneys from three rats from each experimental group were pooled (n = 1). A total of 36 rats were studied in each group, resulting in n = 12 BBM samples for each experimental group. The final BBM pellet was resuspended in a 300 mM mannitol, 16 mM Heps, 10 mM Tris, pH 7.50 buffer and was aliquoted for simultaneous measurements of (a) enzyme activity, (b) transport activity, (c) protein electrophoresis and Western blotting, (d) lipid composition, and (e) lipid fluidity.

Brush border membrane enzyme activity measurements

The purity of each BBM preparation was determined by measurement of membrane-bound specific enzyme activity, including leucine aminopeptidase (apical marker) and Na,K-ATPase (basolateral marker) in cortical homogenate and in BBM fractions as previously described (18). Enzyme activities were expressed as μmol/h per mg cortical homog- enate or BBM protein. Enrichment (specific activity in BBM/specific activity in homogenate) and recovery (total activity in BBM/total activity in homogenate) were determined using the above enzymes as markers of apical and basolateral membranes. Protein was determined by the method of Lowry et al (19) using crystalline BSA as standard.

Brush border membrane transport measurements

Transport measurements were performed in freshly isolated BBM vesicles by radiotracer uptake followed by rapid millipore filtration. To measure Na+-gradient-dependent 32Pi uptake (Na-Pi cotransport), 10 μl of BBM preloaded in an intravesicular buffer of 300 mM mannitol, 16 mM Heps, 10 mM Tris, pH 7.50 was vortex mixed at 25°C with 40 μl of an extravesicular uptake buffer of 150 mM NaCl, 100 μM K142H2PO4, 16 mM Heps, 10 mM Tris, pH 7.50. Uptake after 5 s (representing initial linear rate) was terminated by an ice-cold stop solution which consisted of 15 mM NaCl, 10 mM Na2 arsenate, 16 mM Heps, 10 mM Tris, pH 7.50. All uptake measurements were performed in triplicate, and uptake was calculated on the basis of specific activity determined in each experiment and expressed as pmol 32Pi/5 s per mg BBM protein.

To determine if the differences in Na-Pi cotransport were specific for Pi, Na+-gradient-dependent glucose and proline uptake were also measured. BBM vesicles were preloaded with the 300 mM mannitol-HEPES-Tris (pH 7.50) intravesicular buffer, and the uptake solution consisted of 150 mM NaCl and either 100 mM D-[1H3]glucose, or 100 μM L-[1H3]proline and HEPES-Tris, pH 7.50. Uptake was terminated by the stop solution, which consisted of 150 mM NaCl, 0.25 M phosphor- din, and Heps-Tris added to pH 7.50.

Brush border membrane protein electrophoresis and Western blots

BBM were denatured for 2 min at 95°C in 2% SDS, 10% glycerol, 1 mM EDTA, 95 mM Tris/HCl, pH 6.8 (final concentrations) and 40 μg BBM protein per lane were electrophoresed on 10% polyacrylamide gels according to Laemmli (20), as previously described (21). After electrophoretic transfer onto nitrocellulose membranes (BA83; Schleicher & Schuell, Keene, NH) Western blots were performed using antiserum against Na-Pi-2 and 5'-nucleotidase at dilutions of 1:4000 and 1:5000, respectively. Production of polyclonal antibodies against COOH- and NH2-terminal synthetic peptide of a recently cloned Na-Pi cotransport system of rat kidney cortex (Na-Pi-2) (12) has been recently described (13). Polyclonal antibodies against renal ecto-5'-nucleotidase were obtained against the purified enzyme as described and characterized (22). Primary antibody binding was visualized using goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio Rad Laboratories, Richmond, CA) and quantified by densitometry. Prestained molecular weight marker proteins (Bio Rad Laboratories) were run in parallel.

Brush border membrane lipid composition measurements

Lipids from brush border membrane were extracted by the method of Bligh and Dyer (23), as we have previously described (17–18).

To determine free cholesterol content. An aliquot of the lipid extract was injected into a 530 μm 50% phenylnethil silicone column in a Hewlett-Packard model 5890 gas chromatograph with a flame ionization detector, run isothermally at 280°C, with coprostanol serving as an internal standard. Area ratios were completed with a Hewlett-Packard 3392A integrator, and cholesterol was expressed as nmol per mg brush border membrane protein (11).

To determine individual phospholipid polar head group species. An aliquot of the lipid extract was applied to thin layer chromatography plates (Silica Gel 60, E. Merck, Darmstadt, FRG) and individual phospholipids including sphingomyelin, phosphatidylcholine, phosphatidyl- ethanolamine, phosphatidylserine, and phosphatidylinositol were separated by a two-dimension solvent system (24), as we have previously described (17–18). Phospholipid content in total and individual phospholipids was determined by measuring phosphorus content by the method of Ames and Dubin (25).

To determine the glycopephalolipid composition. An aliquot of the lipid extract was evaporated to dryness and subjected to alkaline metha- nalysis. Briefly, the dried lipids were dissolved in 2 ml of chloroform.
The reaction was started by adding 1 ml of 0.21 N sodium hydroxide in methanol, continued for 1 h at 37°C and terminated by adding 0.8 ml of 0.2 M acetic acid. The resultant mixture was centrifuged for 5 min at 800 grams. The upper layer was removed. The lower layer was washed with 1 ml of methanol plus 0.8 ml of water. After the same centrifugation, the lower layer was transferred into another glass tube and dried under a stream of nitrogen gas. The lipids were then chromatographed on high performance thin layer chromatography plates (HPTLC, E. Merck 5641): glucosylerceramide was separated with a solvent system consisting of chloroform:methanol:water (65:25:4) on plates which were pretreated with 2.5% borax in methanol:water (1:1). The lipid bands were visualized by impregnating the plates with a modified charring reagent (100 grams of CuSO4 .5H2O in conc. H3PO4/water/methanol (100:750:400) (16). The charred TLC plates were scanned with a video densitometer. The glucosylerceramide bands were quantitated by comparing the density of each spot with the density of the corresponding standard curve.

To determine the glycosphingolipid molecular species. The lipid extracts were dissolved in chloroform:methanol, 2:1, vol/vol, and partitioned into lower and upper phase GSL according to Folch et al. (26). The lower phase GSL were put over a Unisil column and eluted with acetone:methanol, 9:1, vol/vol. After alkaline hydrolysis the samples were dried and perbenzoylated in 10% benzoyl chloride in pyridine for 16 h at 37°C. The perbenzoylated glycosylerceramide bands were then dissolved in dioxane-tetrachloride, and injected onto a silica gel column (Zipax, E.L. Dupont, Wilmington, DE, 2.1 mm x 50 cm) and eluted with a 13-min linear gradient of 1–20% dioxane in hexane with a flow rate of 2 ml/min (27). Absorption was read at 230 nm. The individual GSL peaks were integrated by a Dynaxam system on a Macintosh SE computer.

Brush border membrane lipid fluidity measurements

BBM fluidity was determined by the steady-state and time-resolved fluorescence measurements of (a) 1,6-diphenyl-1,3,5,7-tetrazine (DPH), and (b) 6-dodecanoyl-2-diethylaminonaphthalene (Laurdan) (Molecular Probes, Eugene, OR). The steady-state emission spectra of Laurdan was measured in a spectrofluorometer (SLM 4800C, Urbana, IL). Excitation wavelength was 340 nm, and emission was measured at 440 nm and 490 nm. In phospholipid vesicles and in BBM the emission maximum for Laurdan is 440 nm in the gel phase and 490 nm in the liquid-crystalline phase (28–29). The emission spectra of Laurdan is quantitated by the generalized polarization (GP) equation:

\[ GP_{Laurdan} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \]

where \( I_{440} \) and \( I_{490} \) are the emission intensities at 440 and 490 nm, respectively (28–29).

The steady-state anisotropy of DPH (\( r_{copp} \)) was measured in the same spectrofluorometer equipped with excitation and emission polarizers. Excitation wavelength was 360 nm and emission was viewed through a KV 399 nm filter. \( r_{copp} \) is determined by:

\[ r_{copp} = \frac{I_2 - I_1}{I_2 + 2I_1} \]

where \( I_2 \) and \( I_1 \) represent the intensities of the parallel and perpendicular components of the emission respectively (17).

The fluorescence lifetimes of DPH and Laurdan were measured with use of a photon counting multifrequency phase and modulation spectrofluorometer (ISS K2, Urbana, IL) using the 351 nm excitation line of an argon ion laser (30). The dynamic polarization of DPH was also measured with use of a multifrequency differential phase and modulation spectrofluorometer using the 351 nm excitation line of an argon ion laser. The time-resolved fluorescence data were analyzed with Globals Unlimited software (Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, IL) (31).

RNA isolation

Thin slices were cut at 4°C from the superficial cortex and homogenized with Polytron in a 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol denaturation solution. Sequentially, 0.1 vol 2 M sodium acetate, pH 4.0, 1 vol water-saturated phenol, and 0.2 vol chloroform:isoamyl alcohol mixture (49:1) were added to the homogenate. Total RNA was isolated as previously described (32), and resuspended in water treated with diethyl pyrocarbonate (DEPC). Absorbance at 260 and 280 nm were obtained to quantify and assess the purity of the RNA fraction.

**Formaldehyde agarose gel electrophoresis and Northern blot analysis**

After denaturation of RNA samples in formaldehyde, 20 μg total RNA per lane was size fractionated using 0.66 M formaldehyde, 1% agarose (final concentration) gels (BioRad Laboratory). RNA size standards (GIBCO BRL, Gaithersburg, MD) were run in parallel. After electrophoresis the gel was placed onto a vacuum-blotting device (BioRad Laboratories) and vacuum of 60 cm H2O was applied for 4 h using 20× SSC (0.3 M NaCl, 0.3 M Na2 citrate, pH 7.0) as blotting buffer, which resulted in complete transfer of RNA. The RNA was blotted onto GeneScreen Plus nylon membranes (NEN/DuPont, Boston, MA) and the RNA was immobilized by irradiation with ultraviolet (U.V.) light (UV crosslinker; BioRad Laboratories). Prehybridization (4 h at 42°C) and hybridization (18 h at 42°C) of the RNA blots were performed with a buffer (250 μl/cm2) consisting of 5 × SSPE (0.75 M NaCl, 50 mM NaH2PO4, 5 mM EDTA, pH 7.40), 5% Denhardt's solution, [0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA (fraction V)], 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 50% deionized formamide as previously described (33). cDNA probes of NaP2-12 (12), β-Antin (34) and GAPDH (35), all full length, were labeled by random priming (Pharmacia Biotech Inc., Piscataway, NJ) using [γ-32P]dCTP (NEN-DuPont, Boston, MA). After hybridization, the blots were washed twice for 15 min each time in 2× SSPE, 0.1% SDS at room temperature, twice for 15 min each time in 0.1× SSPE, 0.1% SDS at 37°C, and twice for 15 min each time in 0.1× SSPE, 0.1% SDS at 50°C. Autoradiography was performed at ~70°C with NEN-DuPont Reflection film using a DuPont intensifying screen (NEN-DuPont). Membranes were stripped (0.1× SSCE, 0.1% SDS at 95°C for 5 min) before another hybridization was performed. mRNA levels for NaP2-1 were quantitated by densitometry and normalized to the density of the corresponding GAPDH and β-actin mRNA.

**Data analysis**

The data are expressed as the mean±SE. The statistical significance of the results between samples obtained from control and experimental rats was determined by the unpaired student's t test or one way analysis of variance with Student-Newman Keul analysis for multiple comparisons. Significance was accepted at the P < 0.05 level.

**Results**

The effect of dexamethasone on urinary phosphate excretion.

Dexamethasone administration caused a significant increase in urinary excretion of Pi and a decrease in the tubular reabsorption of Pi. These occurred despite a decrease in serum Pi concentration (Table I).

The effect of dexamethasone on BBM enzyme activity.

Dexamethasone had no effect on the activity of the apical membrane enzyme marker leucine aminopeptidase. Enrichment and recovery of leucine aminopeptidase was similar in BBM isolated from control and dexamethasone-treated rats. As previously described (36), dexamethasone caused an increase in the activity of the basolateral membrane enzyme marker Na,K-ATPase. Enrichment and recovery of Na,K-ATPase was, however, minimal and similar in BBM isolated from control and dexamethasone-treated rats (Table II). BBM isolated from control and dexamethasone-treated rats were, therefore, highly and similarly purified.
Table I. The Effect of Dexamethasone on Serum Phosphate and Urinary Phosphate Excretion

<table>
<thead>
<tr>
<th></th>
<th>S&lt;sub&gt;p&lt;/sub&gt; mg/dl</th>
<th>U&lt;sub&gt;p&lt;/sub&gt; x V mg/24 h</th>
<th>TRPi %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.9±0.4</td>
<td>22.4±3.6</td>
<td>86.5±1.8</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>6.6±0.4*</td>
<td>54.3±13.0*</td>
<td>44.8±5.3*</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SE for 6 rats in each group. * P < 0.05, † P < 0.001 by unpaired t-test. S<sub>p</sub>, serum phosphate concentration; U<sub>p</sub> x V, urinary phosphate excretion; TRPi, tubular reabsorption of phosphate defined as 1 - C<sub>p</sub>/C<sub>G</sub>; C<sub>p</sub>, clearance of phosphate; C<sub>G</sub>, clearance of creatinine.

The effect of dexamethasone on BBM transport activity. Dexamethasone caused a significant decrease in Na-Pi cotransport activity. The decrease in Na-Pi cotransport was selective as dexamethasone did not cause changes in Na-glucose or Na-proline cotransport activities (Fig. 1). The decrease in Na-Pi cotransport in dexamethasone-treated rats was not caused by a faster dissipation of the Na" gradient via the enhanced activity of the Na/H antiporter, as, in the presence of 3 mM amiloride, Na-Pi cotransport was still significantly decreased (95±4.22 in dexamethasone vs. 138±123 pmol 32Pi/5 s per mg BBM protein in control, P < 0.01).

The effect of dexamethasone on BBM Na-Pi cotransport kinetics. Kinetics of Na-Pi cotransport in the presence of 150 mM NaCl and as a function of extravesicular Pi ranging from 25 to 800 μM Pi indicated that dexamethasone caused a decrease in V<sub>max</sub> of Na-Pi cotransport, whereas, dexamethasone did not change the affinity for Pi (Fig. 2). Similarly, kinetics of Na-Pi cotransport in the presence of 800 μM Pi and as a function of extravesicular NaCl ranging from 0 to 150 mM NaCl indicated that dexamethasone caused a decrease in the V<sub>max</sub> of Na-Pi cotransport, whereas, dexamethasone did not change the affinity for Na, or the stoichiometry for Na-Pi cotransport (data not shown).

Table II. The Effect of Dexamethasone on Brush Border Membrane Enzyme Activity, Enrichment, and Recovery

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Control</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine Aminopeptidase</td>
<td>2.10±0.07</td>
<td>1.95±0.13</td>
</tr>
<tr>
<td>BBM</td>
<td>22.44±0.88</td>
<td>20.24±0.57</td>
</tr>
<tr>
<td>ENR</td>
<td>10.7±0.4</td>
<td>10.7±0.9</td>
</tr>
<tr>
<td>REC</td>
<td>34.8±1.9</td>
<td>40.6±2.8</td>
</tr>
<tr>
<td>Na,K-ATPase</td>
<td>25.8±0.9</td>
<td>34.2±1.1*</td>
</tr>
<tr>
<td>BBM</td>
<td>29.8±1.2</td>
<td>41.1±1.4*</td>
</tr>
<tr>
<td>ENR</td>
<td>1.2±0.1</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>REC</td>
<td>3.8±0.2</td>
<td>4.6±0.2</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SE for 12 BBM preparations in each group. * P < 0.001 by unpaired t test. The enzyme activities are expressed as μmol/h per mg cortical homogenate (CH) or brush border membrane (BBM) protein. ENR (enrichment) and REC (recovery) were determined as indicated in Methods.

Figure 1. Effect of dexamethasone on BBM sodium gradient-dependent phosphate (Na-Pi), glucose (Na-Glu), and proline (Na-Pro) transport activity. The data are expressed as the mean±SE for 12 BBM in each group.

The effect of dexamethasone on renal cortical Na-Pi mRNA abundance. To determine if the V<sub>max</sub> of Na-Pi cotransport was associated with a decrease in Na-Pi mRNA, RNA isolated from control and dexamethasone treated rats were analyzed by Northern blots. As illustrated in Fig. 3, NaPi-2 mRNA shown by a 2.6-kb transcript is significantly decreased in dexamethasone treated rats. Ethidium bromide staining of the gel and rehybridization of the Northern blot with GAPDH or β-actin cDNA probes indicate that the decrease in NaPi-2 mRNA abundance is not due to differences in RNA loading on the gels. The abundance of NaPi-2 mRNA relative to GAPDH or β-actin mRNA, determined by densitometric analysis, indicates that NaPi-2 mRNA is decreased by 2.5-fold in dexamethasone treated rats.

The effect of dexamethasone on BBM Na-Pi cotransporter abundance. To determine if the dexamethasone induced decrease in the V<sub>max</sub> of Na-Pi cotransport was associated with a decrease in Na-Pi cotransporter abundance, BBM isolated from control and dexamethasone treated rats were analyzed by Western blots using the anti (NaPi-2)-antiserum. As illustrated in Fig. 4, a strong reaction with a 80–90-kD protein bands was observed in BBM from control and dexamethasone rats. Densitometric analysis of the reaction with these protein bands showed that in BBM isolated from dexamethasone treated rats there was a 2.5-fold decrease in the staining intensity when compared to BBM isolated from control rats. The staining pattern is specific since these protein bands were not visualized in the presence of preimmune serum and were completely protected by antigenic peptide (13). As a control the same BBM were analyzed for the presence of ecto-5' -nucleotidase by Western blots. Densitometric analysis of the staining intensity showed no difference in BBM isolated from dexamethasone treated vs control rats.

The effect of dexamethasone on BBM lipid composition. Dexamethasone caused significant changes in BBM lipid composition. Dexamethasone increased glucosylceramide mass content (528±63 in dexamethasone vs. 312±41 ng/mg BBM protein in control, P < 0.02). Dexamethasone also caused significant alterations in neutral glycosphingolipid species, in that there are increases in the relative amounts of hydroxylated and/or phytosphingosine containing glucosylceramide species (Table III). Dexamethasone caused significant alterations in phospholipid species as well, including an increase in sphingomye-
lin, a decrease in phosphatidylcholine, resulting in an increase in the sphingomyelin to phosphatidylcholine mole ratio (Table IV). Dexamethasone had no effect on phosphatidylethanol-amine, phosphatidylserine, or phosphatidylinositol mole content (Table IV). In addition, dexamethasone also did not significantly alter cholesterol (360.7±8.4 in dexamethasone vs. 356.8±22.1 nmol/mg BBM protein in control, P = NS), total phospholipid (485.5±10.3 in dexamethasone vs 450.7±13.6 nmol/mg BBM protein in control, P = NS) mole content, or the cholesterol to phospholipid mole ratio (0.75±0.02 in dexamethasone vs. 0.79±0.04 with control, P = NS).

The effect of dexamethasone on BBM fluidity. Dexametha-
sone increased the steady-state fluorescence anisotropy of DPH (Fig. 5 left). Dexamethasone also significantly increased the fluorescence lifetime of DPH. DPH lifetime data in biological membranes is best fitted assuming a Lorentzian distribution rather than a single or multiple discrete lifetime components (36). There was a significant increase in the center (9.94 in control vs. 10.50 ns in dexamethasone, P < 0.01), and a significant decrease in the width (1.60 in control vs. 0.93 ns in dexamethasone, P < 0.01) of the lifetime distribution of DPH in BBM isolated from dexamethasone-treated rats (Fig. 6 A). Dexamethasone also significantly decreased the rotational diffusion of DPH as measured by the dynamic polarization of DPH. Dexamethasone caused an increase in the limiting anisotropy, and caused decreases in the fast and slow rotational diffusion rate of DPH within the BBM lipid bilayer (Table V).

Dexamethasone also increased the generalized polarization of the phase sensitive fluorescence probe Laurdan (Fig. 5 right). Dexamethasone caused a significant increase in the center (4.33 in control vs. 4.58 ns in dexamethasone, P < 0.05) and a significant decrease in the width (0.43 in control vs. 0.15 ns in dexamethasone, P < 0.01) of the lifetime distribution of Laur-
dan (Fig. 6 B). The steady-state and lifetime measurements with DPH (rotational probe) and Laurdan (phase-sensitive probe),
therefore, indicate that dexamethasone causes a decrease in BBM lipid fluidity.

The effect of PDMP on BBM glucosylceramide content and Na-Pi cotransport activity. The administration of the glucosylceramide synthase inhibitor PDMP to control rats significantly decreased glucosylceramide content (199±19 mg/mg BBM protein in control, P < 0.02). The effect of PDMP on glucosylceramide content was quite specific as PDMP caused no changes in BBM cholesterol, total phospholipid, sphingomyelin, or phosphatidylcholine content (Table VI). The effect of PDMP to lower BBM glucosylceramide content was associated with a significant increase in BBM Na-Pi cotransport activity (Fig. 7). The effect of PDMP was specific and selective for Na-Pi, as PDMP did not significantly alter Na-glucose or Na-proline cotransport activities (Fig. 7). The effect of PDMP to increase BBM Na-Pi cotransport activity was associated with a 1.5-fold increase in BBM NaPi-2 protein abundance (Fig. 4). Interestingly, the increase in NaPi-2 protein abundance was not associated with an increase in NaPi-2 mRNA abundance (Fig. 3). The effect of PDMP to lower BBM glucosylceramide content was also associated with an increase in BBM lipid fluidity, as reflected by a significant decrease in the steady-state fluorescence anisotropy of DPH (Fig. 8).

Discussion

This study demonstrates that dexamethasone selectively inhibits BBM Na-Pi cotransport activity, an effect mediated by a decrease in the Vmax. There was no change in the affinities of the transporter for Na or Pi. These results agree with previous studies also showing that the inhibitory effect of dexamethasone on NaPi cotransport is independent of endogenous parathyroid hormone activity and of dietary Pi content (6, 8).

The decrease in Vmax of renal cortical BBM NaPi cotransport in dexamethasone treated rats correlated with parallel decreases in renal cortical NaPi-2 mRNA content and renal cortical BBM NaPi-2 protein abundance. The decreases in transcription and translation of Na-Pi cotransporter plays an important role in the dexamethasone induced inhibition of Na-Pi cotransport. Since in a previous study actinomycin D and cycloheximide blocked the inhibition of Na-Pi cotransport, it is possible that dexamethasone may activate a regulatory protein which then inhibits Na-Pi specific transcription and translation.

The decrease in the Vmax of Na-Pi cotransport also correlated with increases in BBM glucosylceramide and sphingomyelin content. Dexamethasone was shown to increase the sphingomyelin content of 3T3-L1 and HeLa cells by increasing the rate of sphingomyelin synthesis from phosphatidylcholine (38, 39). The present study is the first to determine the effect of dexamethasone on renal BBM sphingomyelin composition. We found that dexamethasone increased sphingomyelin content by 9.3%. This was paralleled by an 8.0% decrease in phosphatidylcholine content. These results are compatible with observations on many cell lines, including the kidney, that sphingomyelin is

### Table III. The Effect of Dexamethasone on Neutral Glycosphingolipid Molecular Species as Determined by HPLC

<table>
<thead>
<tr>
<th>Glycosphingolipid Description</th>
<th>Control</th>
<th>Dexamethasone</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mole %</td>
<td>mole %</td>
<td></td>
</tr>
<tr>
<td>Nonhydroxy fatty acid — C18 sphingosine containing glucosylceramide</td>
<td>33.7±2.9</td>
<td>12.2±0.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Nonhydroxy fatty acid — C18 phytosphingosine containing glucosylceramide</td>
<td>8.3±1.3</td>
<td>15.9±1.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Hydroxy fatty acid — glucosylceramide and galactosylceramide</td>
<td>30.4±3.7</td>
<td>45.2±1.9</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Nonhydroxy fatty acid — C18 sphingosine containing galactosylceramide</td>
<td>4.8±0.6</td>
<td>10.3±0.5</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Nonhydroxy fatty acid — C18 phytosphingosine containing globotetraglycosylceramide</td>
<td>9.3±0.5</td>
<td>6.7±1.2</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Hydroxy fatty acid — C18 sphingosine containing globotetraglycosylceramide</td>
<td>6.0±0.4</td>
<td>3.0±0.5</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Hydroxy fatty acid — C18 phytosphingosine containing globotetraglycosylceramide</td>
<td>6.3±0.4</td>
<td>6.2±0.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SE for 6 BBM in each group.

### Table IV. The Effect of Dexamethasone on Brush Border Membrane Phospholipid Composition

<table>
<thead>
<tr>
<th>Phospholipid Description</th>
<th>Control</th>
<th>Dexamethasone</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mole %</td>
<td>mole %</td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>40.2±0.9</td>
<td>44.0±1.1</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>28.8±0.9</td>
<td>26.5±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Sphingomyelin/Phosphatidylcholine</td>
<td>1.43±0.06</td>
<td>1.70±0.08</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>20.7±1.7</td>
<td>18.8±2.2</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td>9.8±0.7</td>
<td>10.7±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphatidinositol</td>
<td>3.0±0.2</td>
<td>2.6±0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SE for 12 BBM in each group. * P < 0.05, † P < 0.01 by unpaired t test.
Fraction (40, mide effects potential BBM the golipid content rat creased effects plus myelin net result is opposite change from neogenesis in tion of Lipids increase atered by sylceramide content.

Dexamethasone 1.87±0.23* 2.71±0.25 Control data was data diffusion rotational rotational

Table V. The Effect of Dexamethasone on the Rotational Diffusion Rate of Lipids as Revealed by the Dynamic Polarization of DPH

<table>
<thead>
<tr>
<th></th>
<th>D1</th>
<th>D2</th>
<th>r_a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.71±0.25</td>
<td>15.51±1.64</td>
<td>0.183±0.001</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.87±0.23 *</td>
<td>9.52±0.331</td>
<td>0.194±0.0022</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SEM for 6 BBM in each group. * P < 0.05, \* P < 0.01 by unpaired t test. The dynamic polarization data was analyzed by the Globals Unlimited software. D1, the fast rotational diffusion rate of DPH; D2, the slow rotational diffusion rate of DPH; r_a, the limiting anisotropy; DPH, diphenylhexatriene.

Figure 6. Effect of dexamethasone on the fluorescence lifetime distribution of (A) DPH and (B) Laurdan. Dexamethasone causes an increase in the center and a decrease in the width of the fluorescence lifetime distributions of DPH, and Laurdan.

Our study indicates that the dexamethasone induced increase

Table VI. The Effect of PDMP on Brush Border Membrane Lipid Composition

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PDMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (nmol/mg BBM protein)</td>
<td>422.8±5.7</td>
<td>419.7±6.3</td>
</tr>
<tr>
<td>Total Phospholipid (nmol/mg BBM protein)</td>
<td>464.9±10.2</td>
<td>456.1±4.0</td>
</tr>
<tr>
<td>Sphingomyelin (mole %)</td>
<td>41.6±0.6</td>
<td>40.5±1.3</td>
</tr>
<tr>
<td>Phosphatidylcholine (mole %)</td>
<td>20.5±0.4</td>
<td>19.8±0.5</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±SE for 6 BBM in each group.

Dexamethasone Modulates Renal Phosphate Transport

synthesized from phosphatidylycholine by the addition of ceramide (40, 41). Also, we and others have found that an alteration in BBM sphingomyelin content is associated with a similar but opposite change in phosphatidylycholine content (17, 18). The net result is no overall change in the sum total of BBM sphingomyelin plus phosphatidylycholine content.

Dahiya and Brasitus had reported that dexamethasone increased rat renal cortical and medullary total neutral glycosphingolipid content (42). In our study, we specifically measured the effect of dexamethasone on superficial cortical BBM glucosylceramide content since we were interested in correlating the effects of dexamethasone on BBM Na-Pi cotransport with the potential effects of dexamethasone on BBM glycosphingolipid composition. Our study does not determine whether the effect of dexamethasone to increase glucosylceramide content is mediated by increased rate of synthesis or decreased rate of degradation of glucosylceramide (15). Dexamethasone enhances gluconeogenesis in rat kidney cortex (8). Increased gluconeogenesis may increase UDP-glucose, providing increased substrate for glucosylceramide synthesis (15), even in the absence of changes in the activities of glucosylceramide synthase or glucosylceramide β-glucosidase. These later enzymes regulate glucosylceramide synthesis and hydrolysis, respectively (15). Dahiya and Brasitus had reported that dexamethasone increased the concentration of neutral glycosphingolipids and the short chain hydroxy fatty acids of glucosylceramide. While these data are not directly comparable to those presented here in that a different strain of rats (Sherman vs. Sprague Dawley) was used and total kidney rather than cortical BBM was analyzed, the data are, nevertheless, consistent in that there appears to be an increase in the hydroxylated neutral glycosphingolipid species following dexamethasone (42). Interestingly, in addition to the effect of increased levels of sphingomyelin, an increase in the relative amounts of the more polar hydroxylated glycosphingolipid species would also be expected to cause a decrease in membrane fluidity (43).

Our study indicates that the dexamethasone induced increase...
in BBM glucosylceramide content is correlated with a selective decrease in Na-Pi cotransport. On the other hand, in rats treated with the glucosylceramide synthase inhibitor PDMP, the decrease in BBM glucosylceramide content is associated with a selective increase in BBM Na-Pi cotransport activity. Thus, our studies establish an inverse correlation between BBM glucosylceramide content and BBM Na-Pi-2 protein abundance. Our results therefore suggest that the dexamethasone induced alteration in BBM glucosylceramide content and/or other related glycosphingolipids may mediate the inhibitory effect of dexamethasone on Na-Pi cotransport activity and BBM NaPi-2 protein abundance.

At the present time it is not known how alterations in BBM lipid composition modulate Na-Pi cotransport activity. Recent studies indicate that alterations in glycosphingolipid content control cellular functions by diverse mechanisms. In several cell types 1,25 dihydroxy vitamin D3, TNF-α, and γ-interferon have been shown to induce hydrolysis of membrane sphingomyelin and generation of ceramide (44–46). Ceramide has been shown to have diverse second messenger functions including (a) ceramide-activated protein kinase and phosphatase, (b) phosphorylation of the epidermal growth factor receptor, (c) activation of Raf, MEK, MAP kinase and phospholipase A2, (d) degradation of IkB, the physiologic inhibitor of the transcription factor NK-kB, with subsequent translocation to the nucleus, and (e) downregulation of c-myc expression (44–47). In addition, glycosphingolipids may also modulate protein delivery from the endoplasmic reticulum–golgi complex to the plasma membrane. In baby hamster kidney cells, inhibition of glucosylceramide synthase with PDMP markedly decreases plasma membrane glucosylceramide content and also impairs plasma membrane delivery of the vesicular stomatitis virus G (VSV-G) protein (48). Therefore, glycosphingolipids can modulate cell function by transcriptional/translational as well as posttranslational mechanisms. Our data indicates that in dexamethasone treated rats the increase in BBM glucosylceramide content is correlated with decreases in NaPi-2 mRNA and NaPi-2 protein abundance. In contrast, in PDMP treated rats the decrease in BBM glucosylceramide content is correlated with an increase in NaPi-2 protein abundance, independent of changes in NaPi-2 mRNA abundance.

Alterations in BBM lipid composition could also alter the lipid mobility and/or the lipid dynamics which could in turn modulate Na-Pi cotransport activity (11). In our study, we found dexamethasone induced increases in BBM glucosylceramide and sphingomyelin content were associated with significant changes in steady-state anisotropy, lifetime distribution, and rotational diffusion rate of the fluorescent probe DPH, as well as significant changes in the emission spectra and lifetime of the phase-sensitive fluorescent probe Laurdan. These changes indicate that dexamethasone causes less "fluid" and/or more "ordered or gel-like" microenvironments in the BBM lipid bilayer (29). In agreement with our previous findings (11) these changes are associated with a selective modulation of NaPi cotransport, while there are no changes in Na-glucose or Na-proline cotransport activities. The effects of increases in membrane glycosphingolipid content and/or dexamethasone administration to decrease membrane fluidity occurs in several (49–51), but not all (52–54) cells of non-renal origin. The effect of PDMP to decrease BBM glucosylceramide content and increase BBM Na-Pi cotransport activity, on the other hand, is associated with an increase in BBM fluidity. Thus, in addition to the inverse correlation between BBM glucosylceramide content and BBM Na-Pi cotransport activity (Fig. 9), there is also...
a direct correlation between BBM fluidity and BBM Na-Pi co-
transport activity. In summary, these studies indicate that dexamethasone in-
duced decrease in the \( V_{\text{max}} \) of BBM Na-Pi co-transport is corre-
lated with decreases in renal cortical NaPi-2 mRNA and BBM NaPi-2 protein abundance, and with an increase in BBM glyco-
sphingolipid content. Since primary alteration in BBM glucos-
syleramide content selectively modulates BBM Na-Pi co-transport activity and NaPi-2 protein abundance, we propose that the dexamethasone induced increase in BBM glucosyleramide content, or a closely related glycosphingolipid, plays an im-
portant role in mediating the inhibitory effect of dexamethasone on Na-Pi co-transport activity.

Acknowledgments

The authors dedicate this manuscript to the beloved memory of the late Sonja K. Gross who has always been and will continue to be a source of inspiration to all of us.

The authors thank Drs. Dave M. Jameson (University of Hawaii, Honolulu, HI) and Enrico Gratton (Laboratory for Fluorescence Dy-
namics, University of Illinois, Urbana-Champaign, IL) for the use of their spectrometers and laboratories for fluorescence lifetime and dy-
namic polarization measurements, Sandra F. Nickerson and Julie Propes for their secretarial assistance, and the Biomedical Medical Service at the Dallas VAMC for the illustrations.

This work was supported by grants from Department of Veterans Affairs Merit Review (M. Levi and J. A. Shayman), National Institutes of Health grants DK-41487 (J. A. Shayman) and NS-15037 (R. H. Murer), Swiss National Science Foundation grants 32.28664.90 (J. Biber), and 32.30785.91 (H. Murer), and in part by the Department of Mental Retardation of the Commonwealth of Massachusetts, Contract No. 100220023C. J. A. Shayman is an Investigative Director of the American Heart Association.

References

1. Roberts, K. E., and R. F. Pitts. 1953. The effect of cortisone and desoxycorticosta-
costerone on the renal tubular reabsorption of phosphate and the excretion of


3. Anderson, J., and J. B. Forster. 1959. Effect of cortisone on urinary phos-
5. Webster, S. K., A. Haramati, and F. G. Knox. 1986. Effect of dexamethasone on
tion of the reabsorption of inorganic phosphate in the proximal tubule in the
Na"+--H"+ exchange and decrease the Na"+-gradient-dependent-phosphate-uptake
79:4932–4936.
glucocorticoid effect on renal transport of phosphate. Am. J. Physiol. 243:C227–
C236.
F81.
metabolic acidosis-induced renal transports of inorganic phosphate, calcium,
and NH,.
12. Noronha-Blob, L., and B. Sacktor. 1986. Inhibition of glucocorticoids of
2169.


