Differential regulation of renal sodium-phosphate transporter by glucocorticoids during rat ontogeny

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The purpose of the present study was to compare and contrast the effects of chronic administration of glucocorticoids on the renal Na-Pi transporter at the level of gene transcription. Northern blot analysis indicated that glucocorticoids do not change mRNA levels in neonatal rabbits (31), whereas a 2.5-fold decrease in mRNA abundance was shown in adult rats after injection with dexamethasone (24). Given the difference in species and the different ages of these animals, these results are difficult to correlate with one another.

Total corticosterone levels in rat serum are lowest during the first 6 days of life, then they surge and peak at around days 21–24 (19, 26). Paradoxically, this pattern is identical to the ontogenic expression of immunoreactive NaPi-2 protein, with lowest levels in suckling rats, highest levels in weanling rats, and decreasing levels with age (33). This, therefore, raises the possibility that the responsiveness of the NaPi-2 to glucocorticoid hormones is correlated during development, with NaPi-2 expression being least sensitive to glucocorticoids during the suckling and weaning periods.

The type II renal Na-Pi transporter is a protein that is specific for the brush-border membranes (BBMs) of the kidney, and it is responsible for the Na-dependent, unidirectional, transepithelial transport of Pi across the proximal tubules (9, 23, 35). It is regulated by a number of metabolic and hormonal stimuli, which include diet, growth factors, and a variety of peptide and steroid hormones. Some of the factors that upregulate transepithelial renal Pi transport are low-phosphate diet (8, 14, 28), thyroid hormone (32), and a variety of peptide hormones, which modulate a large number of behavioral, immunologic, and inflammatory responses. Most of the known physiological effects of glucocorticoids involve regulation of gene transcription and are mediated by an intracellular glucocorticoid receptor (GR), which belongs to a superfamily of steroid/thyroid hormone receptors (4). Nongenomic actions of glucocorticoids have also been described. These may involve binding of the hormone to a membrane-associated steroid receptor, which initiates a second-messenger cascade (38), or modulating membrane fluidity, which results in a change in the activity of certain membrane proteins (24).

During chronic administration of synthetic glucocorticoids and in certain disease states such as Cushing's disease, the urinary excretion of Pi is increased and serum levels of Pi are significantly reduced (21, 22). The effects of glucocorticoids on the type II renal Na-Pi transporter have been investigated previously. Na-dependent Pi uptake studies performed on renal, proximal tubular BBM vesicles (BBMV) isolated from glucocorticoid- and vehicle-injected rats exemplified a decreased maximal reaction velocity without any change in the Michaelis-Menten constant (24). Other studies showed that, under similar glucocorticoid administration parameters, uptake of Pi, and immunoreactive protein levels were significantly decreased in adult rats (24, 36), neonatal rabbits (31), and opossum kidney (OK) cells (20, 37). This decrease in NaPi protein expression was abolished in adrenalectomized rats, such that NaPi-2 protein abundance was shown to increase on immunoblots compared with sham-operated animals (25).

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corticoids on renal NaPi-2 during ontogeny. Furthermore, we sought to contribute new insights into the mechanism by which glucocorticoids alter NaPi-2 expression.

MATERIALS AND METHODS

Experimental animals. Sprague-Dawley male rats (Harlan, Madison, WI) in the following age groups were used: suckling (2 wk or 14 days old), weanling (3 wk or 21 days old), adolescent (6 wk or 42 days old), and adult (96–115 days old) (33). Animals were kept in overhanging cages and fed a normal diet. Rats were treated with methylprednisolone sodium succinate (MP; Upjohn, Kalamazoo, MI) at 30 µg/g body wt (5) or equal volumes of vehicle (PBS). The rats were subcutaneously injected four times once per 12 h. The final injection was performed 2 h before the animals were killed. Rats were killed at the desired day (3rd day after the 1st injection, when suckling rats were exactly 14 days old, weanling rats were 21 days old, adolescent rats were 42 days old, and adult rats were ≤115 days old) by CO2 anesthesia followed by cervical dislocation, and kidneys were harvested immediately. Similarly, adolescent rats were injected subcutaneously with mifepristone (RU-486; Sigma Chemical, St. Louis, MO) at 25 µg/g body wt. This compound was suspended in PBS and sonicated thoroughly before each injection (1, 18). The injections were performed four times, with each one being given before the administration of MP.

Kidney cortexes were removed and snap-frozen in liquid nitrogen and three adolescent rats were killed from each group. Kidney cortices were removed and snap-frozen in liquid nitrogen. Poly(A)+ RNA was isolated by using a commercially available kit according to the manufacturer’s protocol. With use of 4 µg/lane, the poly(A)+ RNA was fractionated by 10.220.32.247 on September 7, 2017 http://ajpcell.physiology.org/ Downloaded from

Chemicals and reagents. Poly(A)+ RNA was isolated using the FastTrack kit (Invitrogen, La Jolla, CA). RNA mass standard was obtained from Gibco-BRL (Grand Island, NY). Isotope for Northern blot analyses ([32P]dCTP, 3,000 Ci/mm mol; Redivue) was purchased from Amersham (Piscataway, NJ). Radioactive probes for Northern blot analyses were generated by random prime labeling with use of the MegaPrime system (Amersham). Nitrocellulose membranes (Nitroplus) were obtained from Micron Separations (Westboro, MA), protein gel electrophoresis reagents from Bio-Rad (Hercules, CA) and Novex (San Diego, CA), Rainbow Marker and Kaleidoestest Prestained Standards from Amersham and Bio-Rad, respectively, and X-ray film (X-Omat Blue X-ray, Kodak) and horseradish peroxidase-linked secondary antibodies from Amersham. Mouse NaPi-2 antibody [rabbit polyclonal antibody raised against mouse NaPi-2 (33)] at a dilution of 1:2,000 for these experiments. Anti-rabbit IgG was utilized as a secondary antibody [rabbit polyclonal antibody raised against mouse NaPi-2 (33)] at a dilution of 1:3,000. Anti-rabbit IgG was utilized as a secondary antibody for efficiency of RU-486 antagonism at a dilution of 1:5,000 dilution. Anti-rabbit IgG was utilized as a secondary antibody (anti-rabbit IgG) at 1:2,000 dilution for 40 min. Finally, the membranes were washed with PBST-0.1% milk four times for 10 min each and then incubated with the secondary antibody (anti-rabbit IgG) at 1:2,000 dilution for 40 min. The membranes were blotted onto nitrocellulose membranes at 4°C. The nitrocellulose membranes were blocked overnight in PBS with 0.05% Tween 20 (PBST) and 5% nonfat dry milk. The next day the membranes were rinsed with PBST-0.1% milk and incubated for 40 min at room temperature with the primary antibody [rabbit polyclonal antibody raised against mouse NaPi-2 (33)] at a dilution of 1:2,000 dilution. This antibody has been shown previously to cross-react with high specificity with the rat NaPi-2 (33). The membranes were washed with PBST-0.1% milk four times for 10 min each and then incubated with the secondary antibody (anti-rabbit IgG) at 1:2,000 dilution for 40 min. The membranes were blotted onto nitrocellulose membranes at 4°C. The nitrocellulose membranes were blocked overnight in PBS with 0.05% Tween 20 (PBST) and 5% nonfat dry milk. The next day the membranes were rinsed with PBST-0.1% milk and incubated for 40 min at room temperature with the primary antibody [rabbit polyclonal antibody raised against mouse NaPi-2 (33)] at a dilution of 1:2,000 dilution. This antibody has been shown previously to cross-react with high specificity with the rat NaPi-2 (33). Three adolescent rats were killed from each group. Kidney cortices were removed and snap-frozen in liquid nitrogen. Poly(A)+ RNA was isolated by using a commercially available kit according to the manufacturer’s protocol. With use of 4 µg/lane, the poly(A)+ RNA was fractionated by denaturing agarose gel electrophoresis. After the samples were blotted onto nitrocellulose membranes, the blots were hybridized with rat NaPi-2-specific probe (33). High-strin-

Uptake of phosphate and glucose was measured by a rapid filtration technique. P1 transport was initiated by incubating 20 µl of the vesicular suspension with 80 µl of 100 mM NaCl or KCl, 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, and 10 mM KH2PO4. Na-dependent glucose uptake measurements were performed to determine whether the changes in Pi transport were specific to Na-Pi cotransport. For these experiments, 20 µl of the vesicular suspension were mixed with 80 µl of 100 mM NaCl, 100 mM mannitol, 10 mM HEPES-Tris (pH 7.4), 0.1 mM α-d-glucose, and tracer amounts of [3H]glucose. After 10 s (which is within the linear rate phase) (16), the reaction was terminated by addition of 2 ml of stop solution (100 mM NaCl, 10 mM mannitol, 10 mM HEPES-Tris, pH 7.4, and 0.1 mM phlorizin).

In both experiments the vesicles were collected on 0.45-µm nitrocellulose filters and washed several times with 2–3 ml of ice-cold stop solution. The filters were dissolved in Ready-Protein scintillant (Beckman, Fullerton, CA), and radioactivity was measured by a scintillation counter. Radioactivity bound to filters in the absence of vesicles was used as background and was subtracted from the counts. The Na-dependent component of the Pi uptake was obtained by subtracting uptake in the presence of KCl from uptake in the presence of NaCl. Values are means ± SE for each group and represent the results of at least three separate uptake reactions with samples isolated from different groups of animals.

 Protein gel electrophoresis and Western blot analysis. BBMVs were purified as described above (see BBMV isolation and transport measurements) from suckling, weanling, adolescent, and adult rats. The BBMVs isolated from the renal cortex were diluted in at least an equal volume of Laemmli solubilization buffer (2% SDS, 10% glycerol, 1 mM EDTA, and 2 mM β-mercaptoethanol, pH 6.8) and placed on ice for 30 min. After the protein standards and samples were fractionated on a 4–12% gradient Tris-glycine gel, they were electroblotted onto nitrocellulose membranes at 4°C. The nitrocellulose membranes were blocked overnight in PBS with 0.05% Tween 20 (PBST) and 5% nonfat dry milk. On the next day the membranes were rinsed with PBST-0.1% milk and incubated for 40 min at room temperature with the primary antibody [rabbit polyclonal antibody raised against mouse NaPi-2 (33)] at a dilution of 1:2,000 dilution. This antibody has been shown previously to cross-react with high specificity with the rat NaPi-2 (33). The membranes were washed with PBST-0.1% milk four times for 10 min each and then incubated with the secondary antibody (anti-rabbit IgG) at 1:2,000 dilution for 40 min. Finally, the membranes were washed with PBST-0.1% milk four times for 10 min each, reacted with the chemiluminescence reagent for 60 s, and then exposed to film. The membranes were incubated with β-actin antibody in the same manner at 1:5,000 dilution of the primary antibody and 1:2,000 of the secondary antibody (anti-mouse IgG). β-Actin signal was used as an internal standard to depict the changes in immunoreactive NaPi-2 levels. Furthermore, Na/H-exchanger isoform 3 (NHE3)-specific antibody (12, 13) labeling was utilized to control for efficiency of RU-486 antagonism at a dilution of 1:3,000. Anti-rabbit IgG was utilized as a secondary antibody at a dilution of 1:12,000 for these experiments.

mRNA isolation and Northern blot analysis. Six suckling and three adolescent rats were killed from each group. Kidney cortices were removed and snap-frozen in liquid nitrogen. Poly(A)+ RNA was isolated by using a commercially available kit according to the manufacturer’s protocol. With use of 4 µg/lane, the poly(A)+ RNA was fractionated by denaturing agarose gel electrophoresis. After the samples were blotted onto nitrocellulose membranes, the blots were hybridized with rat NaPi-2-specific probe (33). High-strin-
REGULATION OF NaPi-2 BY GLUCOCORTICOIDS DURING ONTOGENY

RESULTS

Western blot analysis of BBM proteins with use of NaPi-2- and β-actin-specific antisera. A predominant band at 73 kDa for NaPi-2 was seen in all age groups, as previously described (8, 11, 33). This predominant band was normalized with respect to β-actin at 47 kDa (Fig. 1A).

The data were analyzed for statistical significance by Student’s t-tests or ANOVA followed by Fisher’s protected least significant difference post hoc test by using the Statview software package (version 4.53, Abacus Concepts, Berkeley, CA).

Effect of MP on Na-dependent Pi and D-glucose uptake by rat renal BBMVs. BBMVs were prepared from suckling and adolescent animals by the Mg2+-precipitation method, as described in MATERIALS AND METHODS. Alkaline phosphatase activity was 10- to 12-fold enriched in all groups compared with the crude homogenate (data not shown). There was no change in Pi uptake by BBMVs isolated from suckling rats (0.186 ± 0.022 and 0.246 ± 0.061 nmol Pi·mg protein−1·10 s−1 for control and MP, respectively, n = 3); however, there was a significant decrease after MP injection in adolescent rats (0.619 ± 0.012 and 0.323 ± 0.034 nmol Pi·mg protein−1·10 s−1 for control and MP, respectively, n = 5, P < 0.0001; Fig. 2). There was no difference between control and injected groups in terms of glucose uptake in suckling (0.284 ± 0.038 and 0.439 ± 0.089 nmol glucose·mg protein−1·10 s−1 for control and MP, respectively, n = 3, P = 0.1842) or adolescent rats (0.215 ± 0.041 and 0.191 ± 0.052 nmol glucose·mg protein−1·10 s−1 for control and MP, respectively, n = 4; Fig. 2, inset). This finding is in agreement with previously published data (15) and confirms the specificity of MP action on NaPi-2 activity.

Northern blot analysis. To determine the age-specific response to glucocorticoids on the level of NaPi-2 mRNA abundance, suckling and adolescent animals were studied. After hybridization with NaPi-2-specific cDNA probe, a 2.6-kb band (8, 14) was quantitated by densitometry and normalized with respect to 18S ribosomal RNA (Fig. 3A). The hybridization signal obtained for NaPi-2 demonstrates that glucocorticoids do not alter mRNA expression in suckling or adolescent rats. These data are depicted graphically in Fig. 3B.
**Effect of RU-486 on NaPi-2 and NHE3 immunoreactive protein levels.** Adolescent rats were injected with RU-486 and MP to determine whether the GR antagonist would block the glucocorticoid-induced repression of NaPi-2 protein abundance. Immunoreactive NaPi-2 protein levels decreased, as expected, with MP injection (Fig. 4). This effect of MP was not neutralized after MP and the GR antagonist RU-486 were injected in close succession (Fig. 4). The antagonist was not able to reverse the downregulation of NaPi-2 protein levels ($P = 0.0012$, C vs. MP; $P = 0.0002$, C vs. RU-486 + MP ($n = 8$)). Inset: antagonism of MP-induced upregulation of NHE3 in rat kidney cortex by RU-486. $^*P = 0.032$, C vs. MP; $P = 0.011$, MP vs. RU-486 + MP ($n = 4$).

**DISCUSSION**

Proper maintenance of $P_i$ homeostasis is crucial for development and bone formation. Conditions such as hypophosphatemic rickets (9, 10), which develops early in life, are associated with growth retardation and lower skeletal abnormalities. Chronic administration of synthetic glucocorticoids and disease states such as Cushing’s disease (adrenal hyperplasia) increase the risk of development of osteoporosis in part because of a continuous renal leak of $P_i$. The present investigation sought to determine the effects of chronic MP injection on the ontogeny of the renal NaPi-2 and to determine the mechanism by which glucocorticoids affect renal epithelial $P_i$ transport.

**Fig. 3.** Northern blot analysis of NaPi-2 mRNA levels. mRNA was isolated from suckling and adolescent rats. After electrophoresis on 1% denaturing agarose gel, RNA was transferred to nitrocellulose membranes. Blots were probed with NaPi-2 cDNA-specific probes. A: NaPi-2 band at 2.6 kb (top), which was quantitated by densitometry and normalized with respect to 18S ribosomal RNA (bottom), as visualized on nitrocellulose membrane. C, control group injected with PBS. B: quantitation of NaPi-2 mRNA levels and 18S ribosomal RNA band by scanning densitometry. Results are expressed as ratio of NaPi-2 to 18S ribosomal RNA. Values are means ± SE of 4 experiments for suckling (2W) and 8 experiments for adolescent (6W) rats. No differences in NaPi-2 mRNA levels were detected at either age with MP treatment.

**Fig. 4.** Effects of RU-486 (RU) and MP on NaPi-2 and Na/H exchanger isoform 3 (NHE3)-immunoreactive protein levels. Effects of RU-486 on glucocorticoid-injected rats were analyzed by Western blot analysis. A dual injection with RU-486 and MP (RU + MP) was administered to adolescent rats. A: effect on NaPi-2 (73 kDa) protein levels. B: effect on NHE3 protein (85 kDa) levels. $\beta$-Actin was used as an internal standard to control for loading and transfer efficiency. C, control (placebo injected).
The levels of immunoreactive NaPi-2 protein levels in the control groups showed the anticipated maturational decrease that has been documented by our group (i.e., lowest in suckling rats, highest in weanling animals, and declining with age) (33). As shown in Figs. 1 and 2, there was a 60% reduction in protein abundance after MP administration in adult and adolescent rats. This marked decrease closely resembles those that have been documented by other groups (24, 25). However, no change in NaPi-2 protein levels after glucocorticoid treatment of suckling and weanling animals is a novel observation (Fig. 1). Because suckling and weanling rats and adolescent and adult rats showed similar results in regard to MP responsiveness, we decided to pursue the remainder of the investigation with only the suckling (2 wk) and adolescent (6 wk) age groups. NaPi-2 activity, as estimated by initial rate 32P uptake studies with renal BBMVs, paralleled the changes observed in immunoreactive protein. There was a 48% decrease in P1 transport induced by MP in adolescent animals. These data match earlier studies performed in adult rats, where P1 transport was shown to decrease significantly after glucocorticoid treatment (24). Consistent with NaPi-2 protein levels, P1 uptake was unaffected by MP treatment in suckling rats. To control for the specificity of MP effect on P1 transport in adolescent rats, we performed Na-dependent glucose uptake experiments. As predicted and in agreement with previously published experiments (18), there was no effect of MP on glucose uptake in BBMVs isolated from adolescent rat kidneys. In addition, when the control groups are compared, there is about a threefold increase in P1 uptake in adolescent animals as opposed to sucklings, which is in agreement with our previously published results (33).

As determined by Northern blotting, the NaPi-2 mRNA levels were not altered in response to MP in suckling and adolescent rats (Fig. 3). In suckling rats, this is in agreement with the levels of immunoreactive protein and Na-dependent P1 uptake, neither of which was affected by MP. There is, however, an inconsistency between increased NaPi-2 protein and its activity and unaltered mRNA levels observed in adolescent rats. Because the data conflict with previously published results (24), which showed an ~2.5-fold decrease in relative levels of NaPi-2 mRNA in response to dexamethasone, we increased the number of repetitions of Northern analyses to eight. It is difficult to explain the discrepancy between the previously published results and the presented data. In both experimental designs, rats of approximately the same age were utilized. One could speculate that the potency of dexamethasone used by Levi et al. (24) is much greater (≥5-fold higher) than that of MP used in our studies. However, in our experimental design, we used MP at 30 μg/g body wt, which should correct for and exceed the respective dose and potency of dexamethasone. On the basis of the time effectiveness of dexamethasone (long acting) and MP (intermediate acting), the administration schedule [once a day for dexamethasone (24) and every 12 h for MP (our studies)] is also unlikely to be the cause of the described differences. In the studies by Levi et al., the authors used deionized water for control injections, whereas dexamethasone as a water-insoluble compound must have been injected as ethanol solution. On the basis of published information, it is difficult to assess the amount and concentration of ethanol administered in this study. In our present studies we used MP sodium succinate, which is very soluble in aqueous solutions and was dissolved in PBS, which was also used for control injections. It is, however, difficult to speculate whether this discrepancy could account for the observed differences in NaPi-2 mRNA levels in adolescent rats. In support of the hypothesis that the rat NaPi-2 may be mainly regulated at the posttranscriptional level are ontogenic changes in NaPi-2 expression. It has been documented that the developmental increase in protein expression and P1 transport does not involve changes in NaPi mRNA (33). An example of another transport protein that is affected by glucocorticoids in a similar fashion is the pancreatic cell glucose transporter GLUT-2. Dexamethasone had no effect on the mRNA levels but decreased protein abundance by 65% (17). In addition, when dexamethasone was administered to neonatal (3- to 5-day-old) rabbits, the activity of the Na-P1 transporter and protein decreased, without any change in mRNA levels (31), which further indicates a transcription-independent pathway for down-regulation of Na-P1 transporter expression.

The insensitivity of NaPi-2 to glucocorticoids in suckling and weanling rats could represent a physiological adaptation to keep serum P1 levels at a maximum during early development, since it is crucial for proper formation and mineralization of bone. In light of the suppressive effects of glucocorticoids in adult rats, lack of responsiveness to these hormones around weaning may provide the necessary conditions for the developmental increase in NaPi-2 protein levels and P1 transport activity observed in rats (33; present data). We, therefore, hypothesize that it is not glucocorticoids per se that are responsible for developmental changes in NaPi-2 expression but, rather, an unidentified mechanism responsible for sensitization/desensitization of NaPi-2 to glucocorticoids. In an earlier study the response of neonatal rabbits to dexamethasone was investigated, and it was concluded that glucocorticoids did play a role in the maturational decrease in proximal tubule P1 transport (2). Given the difference in species, however, it is difficult to correlate these findings with those of the present investigation.

No change in NaPi-2 mRNA levels in response to MP in adolescent rats suggests that decreases observed in immunoreactive protein and P1 uptake represent nongenomic actions of glucocorticoids. Because transcriptional activation or repression by glucocorticoid hormones is mediated through the GR, we studied the influence of a specific inhibitor of GR, RU-486. As expected, the effect of MP was not inhibited by the RU-486 (Figs. 4 and 5), proving our hypothesis that rat renal Na-P1 transporter is not regulated at the level of gene transcription by glucocorticoids. The effectiveness of RU-486 inhibition of MP-induced transcription was
confirmed by analyzing NHE3 protein, a transporter known to be regulated at a transcriptional level by glucocorticoids (6, 7, 12, 25). It was observed that RU-486 did indeed inhibit the induction of NHE3 (Figs. 4 and 5). These results further implicate a nongenomic action of MP on NaPi-2 and support the observed lack of change in NaPi-2 mRNA in response to MP.

In vitro studies on the effects of dexamethasone on Na-Pi cotransport in OK cells (37) showed that Pi uptake was significantly higher in cells treated with RU-486 and dexamethasone than in cells treated with dexamethasone only. This suggests that GR is the key element for glucocorticoid action in OK cells. This study was solely trying to assess the short-term (acute) regulation by glucocorticoids on the OK cell Na-Pi transporter, whereas the present investigation was based on long-term in vivo effects of MP administration.

One can only speculate on the exact mechanism of the nongenomic downregulation of NaPi-2. Glucocorticoids are known to alter cell membrane fluidity (24), which can affect the function of certain membrane transporters. However, evidence exists that changes of membrane fluidity do not specifically affect Pi transport (2). More attention is given to membrane-initiated actions of glucocorticoids mediated by a specific membrane-associated GR (38). In this model, glucocorticoids exert their effect via a second-messenger pathway. Recently, it was documented that dexamethasone-treated adult rats exhibit an increase in intracellular immunohistochemical staining of NaPi-2 (25). It is possible that glucocorticoids are altering the amount of apical membrane-associated NaPi-2 via a hormone-initiated cell-signaling cascade, leading to the internalization of the transporter. Internalization of the transporter may be followed by its degradation. Dexamethasone-induced degradation of GLUT-2 was demonstrated by pulse-chase experiments (17). This is particularly of interest, because dexamethasone decreases GLUT-2 protein levels without affecting mRNA levels (17). Furthermore, parathyroid hormone-induced degradation of NaPi-2 has been demonstrated as well (29), which shows that there is already a cascade of hormone-mediated events that can lead to the degradation of the type II renal Na-Pi transporter.

In view of our present data, we conclude that the responsiveness of rat NaPi-2 to glucocorticoids is age dependent, with suckling and weanling rats being least sensitive to MP-induced downregulation. We hypothesize that lack of responsiveness of this transporter to glucocorticoids around weaning plays a permissive role in the maturational increase in NaPi protein abundance and activity. The obtained results suggest also that the observed changes in NaPi-2 expression and activity in adolescent rats represent nongenomic actions of glucocorticoids and do not involve changes in NaPi-2 steady-state mRNA levels.

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