Age- and tissue-specific induction of NHE3 by glucocorticoids in the rat small intestine

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Kiela, Pawel R., Yigit S. Guner, Hua Xu, James F. Collins, and Fayez K. Ghishan. Age- and tissue-specific induction of NHE3 by glucocorticoids in the rat small intestine. Am J Physiol Cell Physiol 278: C629–C637, 2000.—Of the two known apical isoforms of the Na+/H+ exchanger (NHE) family, only the NHE3 gene is regulated by glucocorticoids. The aim of these studies was to investigate the mechanisms underlying the effects of methylprednisolone (MP) on expression of NHE3 in the proximal and distal small intestine of suckling and adult rats. Immunoblot showed that the glucocorticoid responsiveness in the proximal small intestine was greatest in suckling animals (NHE3/β-actin: 0.43 ± 0.09 control vs. 1.57 ± 0.15 MP; P < 0.001), and responsiveness decreased with age with no effect in adults (0.56 ± 0.14 vs. 0.64 ± 0.17). Distal small intestine was responsive only in adult rats (0.49 ± 0.13 vs. 1.65 ± 0.09; P < 0.001). This pattern was confirmed at the mRNA level and by 22Na uptake. Western blot and [3H]dexamethasone mesylate binding showed that the responsiveness of NHE3 to glucocorticoids is directly related to the expression of glucocorticoid receptor (GR) in the small intestine. These studies suggest that loss and gain of glucocorticoid responsiveness in the proximal and distal small intestine, respectively, are related to age- and segment-dependent expression of GR.

sodium/hydrogen exchange; methylprednisolone; rat development; intestinal epithelium

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),
Rats were killed by CO2 anesthesia followed by cervical injection. The animals were injected four times, once per 12 h. The final injection was performed 2 h before the animals were killed. The rats were killed by CO2 anesthesia followed by cervical dislocation at the desired time (3rd day after the first injection, when suckling rats were exactly 14 days old, weaning rats were 21 days old, adolescent rats were 42 days old, and adult rats were more than 115 days old). The small intestine was excised and flushed with ice-cold PBS, and the mucosa from the proximal and distal one-third was gently scraped.

Chemicals and reagents. Total RNA was isolated from the scraped mucosa using TRIzol reagent and subsequently enriched for poly(A) RNA by oligo(dt) cellulose chromatography (both from GIBCO BRL, Grand Island, NY). RNA mass standard was from GIBCO. 31Na (100–200 C/g (37.0–74.0 TBq/g)) for uptake studies and [32P]dCTP (3,000 Ci/mmol) for Northern blot analyses were purchased from DuPont NEN (Boston, MA). Radioactive probes for Northern blot analyses were generated by random prime labeling, using the DECA Prime II kit from Ambion (Austin, TX). Nitrocellulose membranes (Nitroplus) were from Micron Separations (Westboro, MA). Protein gel electrophoresis reagents, including Kaloedoscope prestained standards were from Bio-Rad (Hercules, CA). X-ray film (X-Omat blue XB-1; Kodak), and horseradish peroxidase (HRP)-linked secondary antibodies were from Amersham. Monoclonal anti-β-actin antibody (clone AC-74) was obtained from Sigma Chemical (St. Louis, MO). The Super Signal chemiluminescent system from Pierce (Rockford, IL) was used for immunoblot processing. Protein was quantitated by a Bradford protein assay reagent and BSA as a standard. Tetramethylammonium hydroxide was made by titrating solutions of tetramethylammonium gluconate. All other chemicals and reagents were purchased from Fisher Biotechnology (Pittsburgh, PA), Sigma, or Bio-Rad.

Immunoblot analysis of rat small intestinal BBM proteins with NHE3-specific antiserum. Intestinal BBM proteins were purified by a MgCl2 precipitation method as previously described (11, 12). Protein (20 μg) was placed in a twofold excess of Laemml solubilization buffer with 2 mM β-mercaptoethanol, boiled for 5 min, and placed on ice. Twenty micrograms of protein were fractionated by 4–12% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were processed with 1:4,000 dilutions of rabbit NHE3-specific polyclonal antibody as previously described (12, 13). This antibody has been raised against a fusion protein encompassing 699–831 amino acids from the COOH-terminal portion of the protein generated by baculovirus-mediated expression in SF9 cells (11). HRP-conjugated anti-rabbit secondary antibody was used at 1:20,000 dilution, except for samples from distal small intestine of suckling rats, where 1:2,000 dilution of secondary antibody was used to enhance sensitivity of detection. Super Signal was used for chemiluminescent detection. Membranes were stripped and subsequently incubated with β-actin antiserum at 1:5,000 dilution. NHE3 band intensities were analyzed by densitometry (ImageMaster GS-700; Bio-Rad) and were normalized for β-actin band intensities on the same blot. All densitometric analyses were performed utilizing Quantity One software (Bio-Rad).

BBM vesicle isolation and transport measurements. BBM vesicles (BBMV) were prepared from mucosal scrapings from the proximal and distal small intestines of groups of 12 suckling (14-day-old) rats or groups of 3–4 adult rats. BBMV were purified by the MgCl2 precipitation technique as previously described (11, 12). The final BBMV pellets were suspended in either preincubation buffer for no pH gradient condition [intracellular pH (pHi) and extracellular pH (pHo) both 7.5] or preincubation buffer with an outwardly directed pH gradient (pHi 5.2, pHo 7.5) and placed at 25°C for 1 h. Protein was quantitated by a modified Bradford protein assay. The purity and enrichment of membrane preparations were assessed by the measurement of alkaline phosphatase activity as previously described (32). Uptake of 22Na+ was measured by a rapid filtration technique as previously described (28). All incubations were carried out at 25°C. Briefly, transport was initiated by adding 20 μl of the final membrane suspension to 80 μl incubation solution, with the addition of 31.25 μCi 22Na+/10 ml. Some studies were performed in the presence of 50 μM (3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate (HOE-694), a specific NHE inhibitor that allows selective inhibition of NHE2 [inhibition constant (Ki) = 5 μM in PS120 cells], without affecting NHE3 (Ki = 650 μM in PS120 cells) (12). HOE-694 was prepared for use by dissolving solid HOE-694 in DMSO to make a 100 mM stock solution and was used within 90 min. The reactions were stopped after 10 s by the addition of 2 ml of ice-cold stop solution. The vesicles were immediately collected on a cellulose nitrate filter and washed with 5 ml of ice-cold stop solution. The amount of radioactive substrate remaining on the filter was determined in a Beckman liquid scintillation counter, with ReadySafe (Beckman, Fullerton, CA) as the liquid scintillant. Radioactivity remaining in the filters after pipetting of incubation medium into the radioactive substrate in the absence of vesicles was used as the background level and was considered in all calculations. Uptake values were determined by subtracting the uptake levels with no pH gradient condition (pHi and pHo 7.5) from those with an outwardly directed pH gradient (pHi 5.2, pHo 7.5). All values are expressed as nanomoles of Na+ uptake per milligram of vesicular protein. Multiple repetitions were performed on three membrane vesicle preparations from different groups of animals at each age.

Northern blot analysis of NHE3 mRNA. Poly(A)+ RNA was isolated from the proximal and distal small intestinal mucosa of at least three rats per group, utilizing TRIzol reagent and oligo(dt) cellulose (GIBCO). Northern blots were carried out as previously described, using 5 μg poly(A)+ RNA/lane (10, 12). A cDNA probe spanning nucleotides 33–2117 of rat NHE3 (36) was used for hybridization. High-stringency washes were performed at 65°C with 0.1× sodium chloride-sodium citrate-0.1% SDS, and blots were placed onto a phosphorimaging K-screen and scanned by Molecular Image FX (Bio-Rad). Northern blots were subsequently reprobed with 18S-specific probe [18S encodes rat cyclophilin (15)]. Ribosomal 18S RNA, as it appeared under ultraviolet light on the membranes, was used to control for loading and transfer efficiency and to normalize for intensity of the NHE3 signal. All densitometric analyses were performed utilizing Quantity One software (Bio-Rad).

Immunoblot analysis of glucocorticoid receptor protein in small intestinal mucosa. Mucosa scraped from proximal and distal segments of the small intestine was immediately placed in ice-cold 8 M urea, sonicated on ice, and centrifuged at 100,000 g for 1 h. The supernatant was assayed for protein concentration, mixed with Laemmli solubilization buffer with 2 mM β-mercaptoethanol, boiled for 5 min, and placed on ice. Fifty micrograms of protein were fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were processed with rat glucocorticoid receptor (GR)-specific...
antiserum (monoclonal mouse antibody, doneBuGR2; Affinity-BioReagents, Golden, CO) and with a 1:5,000 dilution of HRP-conjugated anti-mouse secondary antibody. Super Signal (Pierce) was used for chemiluminescent detection.

\[ ^{3}H \]dexamethasone labeling of intestinal GR. Enterocytes were isolated from proximal and distal segments of the small intestine of suckling (14-day-old; 3 groups of 3 rats) and three individual adult rats essentially as described by Weiser (40). Intestinal segments were excised from animals, rinsed thoroughly with 154 mM NaCl plus 1 mM dithiothreitol (DTT), filled with citrate buffer (15 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH₂PO₄, 5.6 mM Na₂HPO₄, pH 7.3), and incubated at 37°C for 15 min. The contents were emptied, and segments were filled with ice-cold PBS (with no Ca²⁺ or Mg²⁺) with 0.5 mM DTT and palpated on ice for 5 min. The intestinal segments were then drained into 15-ml conical tubes. Mixed populations of enterocytes obtained this way were washed several times with ice-cold DMEM-high glucose medium (Irvine Scientific; Santa Ana, CA), and the final pellet was resuspended in one volume of DME-high glucose medium containing 50 nM \[^{3}H\]dexamethasone mesylate (19) with or without the addition of 25 µM unlabeled dexamethasone as a competitor. Cells were incubated on ice for 2 h, pelleted by centrifugation (900 g, 15 min), washed with ice-cold DMEM, and the final pellet was sonicated on ice in 1 vol of 8 M urea. Samples were centrifuged at 100,000 g for 40 min, washed several times with ice-cold DME-high glucose, and the final pellet was sonicated on ice in 1 vol of 8 M urea. Samples were centrifuged at 100,000 g for 1 h and mixed 1:1 with Laemmli sample buffer with 2 mM b-mercaptoethanol, boiled for 5 min, and placed on ice. Fifty micrograms of protein were fractionated by 10% SDS-PAGE. The gels were subsequently fixed in a solution containing 10% acetic acid and 30% methanol overnight and impregnated with scintillators (EN³HANCE; NEN) for 1 h. Scintillators were precipitated in gels by 1-h equilibration in 1% glycercol/5% polyethylene glycol 8,000 solution. Gels were dried and exposed to X-ray film at –70°C for 2 wk. To confirm that the band observed on film represents GR, duplicate gels were transferred to nitrocellulose membranes and processed as described above for immunoblot analysis of GR.

Statistical analysis. Collected data were statistically analyzed by Student’s t-test using StatView software (v. 4.53; Abacus Concepts, Berkeley, CA). A statistical difference of \( P < 0.05 \) was considered significant.

**RESULTS**

Western blot analysis of NHE3 in the proximal and distal small intestine. Mucosa from proximal and distal segments of the small intestines of 14-day-old and adult rats was obtained, BBM proteins were purified and separated by SDS-PAGE. Protein was then electroblotted onto nitrocellulose membranes, which were reacted with polyclonal antiserum specific for NHE3 and monoclonal antibody against b-actin. Administration of MP affected immunoreactive NHE3 protein (~85 kDa) in a different fashion in the proximal and distal small intestines of 14-day-old and adult rats (Fig. 1). Responsiveness to MP in the proximal small intestine was the highest in suckling animals (NHE3/b-actin ratio: 0.43 ± 0.09 control vs. 1.57 ± 0.15 MP; \( P < 0.001 \)), with no effect seen in adults (0.56 ± 0.14 vs. 0.64 ± 0.17). In contrast, the pattern observed in the distal small intestine showed no response in suckling rats (0.12 ± 0.02 vs. 0.11 ± 0.03) and showed highest induction in adults (0.49 ± 0.13 vs. 1.65 ± 0.09; \( P < 0.001 \); \( n = 5–7 \) for all data; Fig. 1). It should be noted that the amount of NHE3 immunoreactive protein in the distal small intestine of suckling rats cannot be adequately compared with the other groups, since much higher concentrations of secondary antibody had to be used to visualize NHE3 in the distal segment. The fact that sensitivity of the assay had to be significantly enhanced to detect and quantitate changes in the distal small intestine of suckling rats indicates, however, that...
the amount of the brush-border NHE3 is significantly lower in the distal than in the proximal segment.

Northern blot analysis. Poly(A)$^+$ RNA was purified from groups of rats at 14 days of age and from adults, fractionated by denaturing agarose gel electrophoresis, and blotted onto nitrocellulose membranes. Blots were probed with NHE3 cDNA-specific probes. Top: NHE3 band, which was quantitated by densitometry and normalized with respect to the ribosomal 18S RNA as visualized on the nitrocellulose membrane (shown below). C, control group injected with saline; MP, experimental group injected with methylprednisolone. Bottom: graphic summary of data. *Differences (Student's t-test) between bars representing NHE3 in proximal small intestine of suckling rats (C vs. MP, P < 0.01; n = 4) and in proximal (C vs. MP, P < 0.05; n = 5) and distal small intestine (C vs. MP, P < 0.05; n = 5) of adults.

MP administered to suckling rats increased the relative abundance of NHE3 mRNA in the proximal (4.76 ± 0.16 control vs. 8.45 ± 0.15 MP; P < 0.01, n = 4) but not in distal small intestine (1.37 ± 0.3 vs. 1.56 ± 0.22, n = 4; Fig. 2). In adult rats, NHE3 mRNA was increased by MP both in the proximal (3.6 ± 1.1 vs. 8.7 ± 2.5; P < 0.05, n = 5) and distal small intestine (2.15 ± 0.04 vs. 4.67 ± 0.4; P < 0.05, n = 5; Fig. 2).

Uptake analysis of NHE3 activity in intestinal BBMV. BBMV were purified from proximal and distal segments of the small intestine of 14-day-old and adult rats. Alkaline phosphatase enrichment was 10- to 12-fold in all groups, which suggested an equivalent enrichment for BBM in all samples. $^{22}$Na$^+$ uptake studies were performed with or without 50 µM HOE-694 to determine the NHE3-mediated component of measured Na$^+$/H$^+$ exchange. Inhibition by HOE-694 was effective, with its effectiveness dependent on the

Fig. 3. Initial rate of $^{22}$Na$^+$ uptake in intestinal BBM vesicles. BBM vesicles were prepared from proximal and distal small intestine of suckling (2 wk) and adult rats. Uptake was performed in presence of 0 µM or 50 µM HOE-694; presented data depict only the NHE3 component of Na$^+$/H$^+$ exchange. Open bars, control (saline-injected) rats; solid bars, MP-injected animals. *Statistical differences (Student's t-test) between bars representing proximal (C vs. MP, P < 0.01; n = 4) and distal small intestine (C vs. MP, P < 0.05; n = 4) of suckling rats, and distal small intestine of adults (C vs. MP, P < 0.01; n = 4).
age and segment of the small intestine. The contribution of NHE2 (measured by the difference between $^{22}$Na$^+$ uptake in the absence and presence of 50 µM HOE-694) and NHE3 to total Na$^+$/H$^+$ exchange activity in BBMV from proximal small intestines of suckling rats was 40% and 60%, respectively. In adult rats, the relative contributions of NHE2 and NHE3 were 15%, and 85%, respectively. These data are in accordance with previously published observations (7). In the distal segment of the rat small intestine, contribution of NHE3 increased with age from 75% in sucklings to 98% in adults. In all cases, the NHE2 component of the measured Na$^+$/H$^+$ exchange was not affected by MP treatment (data not shown). Analysis of NHE3 activity confirmed the changes seen in immunoreactive NHE3 protein, with the one discrepancy being a significant induction of NHE3 activity by MP in the distal segment of the small intestine of suckling rats (Fig. 3).

Western blot analysis of GR in the proximal and distal small intestine. To determine whether the ability of MP to induce expression of NHE3 protein correlates with the abundance of GR, which may be the rate-limiting factor in glucocorticoid hormone action, we analyzed GR protein in proximal and distal small intestinal mucosa obtained from suckling and adult rats. GR protein expression was significantly higher in the proximal than in the distal segment of the small intestine of suckling rats ($P < 0.01; n = 4$), whereas in adult rats levels of immunoreactive GR were much higher in the distal part ($P < 0.01; n = 4$; Fig. 4). In some cases the amount of GR protein in the proximal small intestine of adult rats was below the detection limit. Treatment with MP caused significant downregulation of GR protein in most of the tissues, except for proximal small intestine of adult rats, where the difference did not reach the level of significance (Fig. 4).

Assessment of GR function by $[\text{H}]$dexamethasone mesylate binding. Ligand binding experiments were performed with enterocytes isolated from proximal and distal small intestine of untreated suckling and adult rats, using the covalent labeling technique with $[\text{H}]$dexamethasone mesylate, and were visualized by fluorography. One to three bands were usually observed with a predominant band of ~40 kDa. These bands represent products of proteolytic degradation of GR, because all of them reacted with monoclonal anti-GR antibody (BuGR2). BuGR2 antibody reacts with a single epitope within the DNA binding domain of the GR (21); therefore more bands containing the antigenic epitope, but not steroid-binding domain, were seen on immunoblots than on fluorography. Although all bands showed the same pattern in terms of differences between ages and tissues, only the ~40-kDa band, which corresponds to a 42-kDa chymotryptic fragment of GR containing both the hormone-binding and DNA-binding domains (26) is presented (Fig. 5). The results were identical to those observed for the immunoreactive GR protein. The highest intensities of affinity-labeled GR were observed in enterocytes isolated from the proximal small intestine of suckling rats and the distal segment of adult rats, and lowest intensities were obtained from distal small intestine of suckling rats and proximal small intestine of adult rats (Fig. 5). In all cases, labeling with $[\text{H}]$dexamethasone mesylate could be partially competed out by addition of an excess of unlabeled dexamethasone.
Glucocorticoid hormones have long been suggested to play a role in enhancing intestinal capacity to absorb Na$^+$ (4, 39). Of the two known apical NHE isoforms, NHE2 and NHE3, only expression of NHE3 can be induced by glucocorticoids (12, 43). This suggests that induction of electroneutral transport of Na$^+$ in the small intestine is largely mediated via an increase in expression and activity of NHE3. Cho et al. (8) demonstrated that stimulatory effects of dexamethasone on NHE3 expression in adult rats are tissue dependent, being apparent in the ileum and proximal colon but not in jejunum, distal colon, or kidney. Our earlier observations, however, showed that MP can induce NHE3 expression in the jejunum of suckling rats (12). The present studies were therefore designed to investigate the effects of glucocorticoids on NHE3 expression and activity in both proximal and distal segments of the rat small intestine during development. Initially, we analyzed the effects of MP on NHE3 protein expression by Western blot analysis utilizing BBM isolated from 2-wk-old, 3-wk-old, 6-wk-old, and adult rats (data from 3- and 6-wk-old rats not presented). We showed that NHE3 could be significantly induced in the proximal small intestine of 2-wk-old rats, with the response to MP gradually decreasing with age until no induction was noticed in adults. The distal segment of the small intestine showed the opposite trend, with a lack of stimulating effects of MP in suckling rats and a significant induction of NHE3 protein in adults. Therefore, in further experiments we narrowed the focus of this study to 2-wk-old and adult rats.

Analysis of NHE3 mRNA by Northern blot revealed a similar pattern to protein expression, with one exception being a significant increase in NHE3 mRNA in the proximal small intestine of adult rats. This observation seems to be in contrast with observations published by Cho et al. (8), which concluded that jejunal NHE3 mRNA does not change in response to dexamethasone treatment in adult rats. However, Northern blots shown in the cited investigation seem to show at least a twofold increase in both NHE3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; constitutive control) signals in the jejunum of dexamethasone-treated rats. GAPDH does not seem to be a suitable constitutive marker for normalization of Northern blot data in this particular experimental design. Therefore, Cho et al. (8) could have consequently underestimated the NHE3 mRNA induction by glucocorticoids in the jejunum of adult rats. Additionally, the data obtained in our studies are based on five repetitions as opposed to three repetitions analyzed by Cho et al. (8). An increase in mRNA without parallel changes in protein abundance and activity in response to glucocorticoid treatment has been reported previously in studies on sucrase-isonaltase (SI) gene expression. In 10- and 16-day-old rats injected with dexamethasone, the increase in steady state of SI mRNA levels after dexamethasone administration was proportionally higher than the increase in sucrase activity (33). It was suggested that synthesis of active protein is significantly slower than the accumulation of SI mRNA. Whether this is also the case with NHE3 in the jejunum of adult rats or whether there are other factors involved, like posttranscriptional regulation of NHE3 induced by glucocorticoids, remains to be determined.

Functional analysis of NHE3 activity was performed by initial rate$^{22}$Na$^+$-uptake studies. Previous investigations in 2-, 3-, and 6-wk-old rats (28) determined that 10-s uptake represented the initial rate in all groups and that there were no differences between age groups in the following experimental parameters: enrichment of membrane preparations for BBM markers and impoverishment of membrane preparations for basolateral membrane, mitochondrial membrane, and endoplasmic reticulum membrane markers. These observations suggested that the phenomenon observed in the present studies utilizing this BBMV purification and uptake method is based solely on the activity of the apical membrane NHEs. To assess the contribution of NHE3, NHE2 was selectively inhibited with 50 µM HOE-694 (K$i$ = 5 µM in PS120 cells) (14). The NHE3 contribution could be estimated by comparing the uptake in the absence of HOE-694 (which represents the activity of both NHE2 and NHE3) with uptake rates in the presence of 50 µM HOE-694 [which represents the activity of only NHE3 ($K_i = 650$ µM)] (14). The calculated relative contributions of NHE2 and NHE3 in...
proximal small intestine of suckling and adult rats are in close agreement with previously published data (12), with NHE3 contributing to Na\(^+\)/H\(^+\) exchange from \(~60\%\) in suckling rats to 85% in adult rats. By comparison, the contribution of NHE3 in the distal segment of the small intestine was much higher, ranging from 75% to 98% in suckling and adult rats, respectively. This indicates that, in both ages studied, NHE3 represents a more critical component of Na\(^+\)/H\(^+\) exchange in the distal than in the proximal small intestine.

MP treatment caused an increase in NHE3 activity that paralleled the increases observed in NHE3 protein abundance, with one exception being significantly higher activity of NHE3 in distal small intestine of suckling rats, in which no increase of immunoreactive NHE3 protein or its mRNA was documented. Such an increase of activity of a membrane carrier in response to glucocorticoid hormones could be attributed to changes in membrane fluidity induced by glucocorticoids. Dudeja et al. (18) demonstrated an increase in maximum velocity of Na\(^+\)/H\(^+\) exchange paralleled by an increase in the lipid fluidity of dexamethasone-treated membrane vesicles isolated from the rat distal colon. Later studies performed on transfected fibroblasts (5), however, showed that the membrane fluidizer, benzyl alcohol, causes a 50% decrease in NHE3 specific activity. It is, therefore, unlikely that altered membrane fluidity leads to the increase of NHE3 activity observed in the distal small intestine of suckling rats. One could also expect that such a physical interaction should not be tissue or age dependent, yet we did not observe changes in NHE3 activity in response to MP in proximal small intestine of adult rats. The mechanism underlying the increase in Na\(^+\)/H\(^+\) exchange in the distal small intestine of suckling rats in response to MP remains, therefore, to be determined.

The interaction with a functional glucocorticoid receptor is the rate-limiting step in genomic actions of glucocorticoid hormones (2). To search for possible mechanisms underlying the described age- and tissue-dependent effects of MP on NHE3 expression, we analyzed the abundance of GR in the small intestinal epithelium in both segments and ages studied. GR in the rat intestinal epithelium is extremely unstable and undergoes rapid degradation, even in the presence of multiple protease inhibitors. Repeated attempts to use nondenaturing methods of sample preparation, using a wide spectrum and differing concentrations of protease inhibitors including nonreversible trypsin and chymotripsin inhibitors (N\(_\alpha\)-p-tosyl-L-lysine chloromethyl ketone and N-tosyl-L-phenylalane chloromethyl ketone, respectively) or including BSA as a competitive substrate, failed to yield full-size (\(~95\) kDa) bands of rat GR on immunoblots. Therefore, Western blots were performed on tissues homogenized in 8 M urea as a chaotrope agent, which significantly reduced degradation of GR. These studies demonstrated that the epithelium of small intestinal segments in which MP does not induce NHE3 expression, namely distal small intestine in suckling rats and proximal small intestine of adult rats, contains significantly less GR protein. In other words, changes in tissue- and age-dependent stimulation of NHE3 expression in response to glucocorticoid treatment are paralleled by changes in the abundance of glucocorticoid receptor protein. We also observed significant downregulation of the GR protein abundance by MP treatment as could be expected from previously published investigations (35).

To test whether the amounts of detected GR correlate with ligand-binding capacity, \([^{3}H]dexamethasone\) mesylate binding studies were performed with isolated enterocytes. We chose this approach to study the efficacy of ligand binding by GR, because dexamethasone mesylate creates a covalent bond with GR (38). Such a complex can then be analyzed under denaturing conditions that help prevent GR degradation. However, most likely because of the time lag necessary for enterocyte preparation and incubation with \([^{3}H]dexamethasone\) mesylate, we could not prevent GR from being partially degraded, and we observed a fairly strong band of \(~40–45\) kDa. This band most likely corresponds to a 42-kDa chymotryptic fragment of GR containing both the hormone-binding and DNA-binding domains (26). The binding of \([^{3}H]dexamethasone\) mesylate could be partially competed out by addition of excess unlabeled dexamethasone in the incubation medium, and Western blotting of duplicate gels confirmed the 40- to 45-kDa band to be a proteolytic fragment of the GR (data not shown). It must be pointed out that a strict interpretation of the competition data is impossible, since the interaction of GR with dexamethasone mesylate is irreversible, and this may explain why competition was incomplete. These two controls suggest that this assay gives a good indication of the ligand-binding capacity of GR.

There is an alternative method for studying GR ligand binding, which is noncovalent \([^{3}H]dexamethasone\) binding followed by scintillation counting (24, 30). This, however, seems to be of little value, since it does not account for GR degradation or nonspecific binding to the mineralocorticoid receptor (MR). Dexamethasone has been shown to bind the MR with a higher affinity than that for the GR \((K_d = 0.5 \text{ nM for MR vs. } 2.5–5.0 \text{ nM for GR (16)})\). MR mRNA is expressed throughout the small intestine and undergoes ontogenic changes (20), which could interfere with the specificity of the noncovalent \([^{3}H]dexamethasone\) binding assay. In pilot studies, we demonstrated that, in adult rats, stimulation with MP is much more effective when MP was administered together with canrenone acid (CA), a water-soluble metabolite of MR antagonist spironolactone. The NHE3 activity in the distal small intestine increased \(~2.5-\)fold over control values when rats were treated with MP alone, whereas MP and CA combined resulted in a 6-fold increase in NHE3-mediated Na\(^+\) uptake (unpublished observations). This suggests that GR and MR compete for glucocorticoid hormones in the small intestinal epithelium. However, we chose not to use CA in the current investigation, since it could obscure the physiological responses to MP treatment. These results suggested that the effects of MP observed in our present studies were indeed medi-
ated by GR and not by MR. All of these facts, when considered together, suggest that covalent labeling of GR with \(^{3}H\) dexamethasone mesylate followed by fluorography is the best alternative to assay for GR ligand binding. The results of this assay showed that ligand binding capacity of GR in the proximal and distal small intestine parallels the levels of immunoreactive GR. These data support the hypothesis that the differential responsiveness of the proximal and distal small intestine is most likely due to differential expression of GR.

Loss of glucocorticoid responsiveness in the proximal small intestine at a relatively early stage of development is believed to be a common feature of both brush-border and lysosomal enzymes of the small intestine, such as sucrase, maltase, acid \(\beta\)-galactosidase, and lactase (23). The documented differences in GR protein expression and ligand binding capacity in the proximal small intestine of suckling and adult rats very likely represent the mechanism of the reported loss of glucocorticoid responsiveness in the proximal small intestine. The described age-related increase in glucocorticoid responsiveness in the distal small intestine is a novel observation and adds to the complexity of glucocorticoid regulation of intestinal gene expression.

In summary, membrane transporter NHE3 now joins the group of intestinal proteins, whose expression in the proximal small intestine is regulated by glucocorticoid hormones only at earlier stages of postnatal development. These data suggest a common mechanism of age-related desensitization of the proximal small intestine to glucocorticoids into adulthood. Glucocorticoid responsiveness in the distal small intestine follows the opposite pattern, with no effects in suckling animals and a significant response in adults. Tissue- and age-specific expression of GR in the small intestinal epithelium is postulated to be the mechanism of the reported changes of the glucocorticoid responsiveness of NHE3 in the rat small intestine.

The proximal part of the small intestine of sucklings is more prone to mucosal injuries resulting, e.g., from viral infections. Maintaining the responsiveness to glucocorticoid hormones in this segment may be helpful in counteracting these injuries and aid the regenerative processes. On the other hand, it is also likely that the role of glucocorticoids in postnatal increases in enzyme and membrane transporter gene expression is more critical in the proximal than in the distal segment of the small intestine. Induction of active transport of Na\(^{+}\) by glucocorticoids in adults is of more physiological importance in the distal small intestine, since the proximal part is believed to function as a “leaky” epithelium where fine tuning of water and electrolytes would not be essential (7). Therefore, in situations requiring Na\(^{+}\) retention (such as volume depletion), activation of active Na\(^{+}\) absorption would occur predominantly in the distal, not proximal, small intestine.

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