Gβ regulation of Na/H exchanger-3 activity in rat renal proximal tubules during development

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The decreased natriuretic action of dopamine in the young has been attributed to decreased generation of cAMP by the activated renal D1-like receptor. However, sodium/hydrogen exchanger (NHE) 3 activity in renal brush-border membrane vesicles (BBMV) can be modulated independent of cytoplasmic second messengers. We therefore studied D1-like receptor regulation of NHE activity in BBMV in 2-, 4-, and 12-wk-old (adult) rats. Basal NHE activity was least in 2-wk-old compared with 4- and 12-wk-old rats. D1-like agonist (SKF-81297) inhibition of NHE activity was also least in 2-wk-old (−1 ± 9%, n = 3) compared with 4 (−15 ± 5%, n = 6) and 12 (−65 ± 4%, n = 6)-wk-old rats. The decreased response to the D1-like agonist in BBMV was not caused by decreased D1 receptors or NHE3 expression in the young. Gαs, which inhibits NHE3 activity by itself, coimmunoprecipitated with NHE3 to the same extent in 2-wk-old and adult rats. Gαs function was also not impaired in the young because guanosine 5'-O-(3-thiophosphorylate) decreased NHE activity to a similar extent in 4-wk-old and adult rats. Gαi3 protein expression in BBMV also did not change with age. In contrast, Gβ expression and the amount of Gβ that coimmunoprecipitated with NHE3 in BBMV was greatest in 2-wk-old rats and decreased with age. Gβ common antibodies did not affect D1-like agonist inhibition of NHE activity in adult rats (8%) but markedly increased it (48%) in 4-wk-old rats. We conclude that the decreased inhibitory effect of D1-like receptors on NHE activity in BBMV in young rats is caused, in part, by the increased expression and activity of the G protein subunit Gβγ. The direct regulation of NHE activity by G protein subunits may be an important step in the maturation of renal tubular ion transport.

D1 dopamine receptor; sodium-hydrogen exchanger 3; Gβγ subunit; renal proximal tubules; ontogeny

The decreased natriuretic effect of dopamine and dopamine agonists in the young of many species, including humans, is well documented (24, 26, 32, 34, 35). The decreased natriuretic effect of dopamine is caused by impaired inhibition of sodium reabsorption via D1-like receptors and activation of sodium reabsorption via α-adrenergic and D2-like receptors (13, 15, 26, 32, 35, 36). The impaired action of D1-like receptors in the young is caused by both decreased renal vasodilatory and tubular effects (13). The decreased renal tubular effect of dopamine may be related, in part, to decreased renal D1-like receptor density. For example, in the rat, renal D1-like receptor density is decreased in the first 3 wk of life (37). However, after 3 wk of age, D1-like receptor density in proximal convoluted tubules of rats no longer differs from adult values, yet the inhibitory effect of dopamine on renal tubular sodium transport is still decreased compared with adult rats (13, 26, 37).

The decreased renal tubular action of D1-like receptors in the young results from impaired inhibitory effects on sodium/hydrogen exchanger (NHE) and Na+-K+-ATPase activities in renal proximal tubule and medullary thick ascending limb of Henle (18, 22, 26). NHE3 can be regulated by phosphorylation/dephosphorylation processes and membrane recycling in intact cells (21, 28, 29, 38, 41, 42). The decreased ability of D1-like receptors to inhibit NHE3 and Na+-K+-ATPase in the young is caused, in part, by decreased production of second messengers (14, 18, 19, 27). Any ontogenic differences in NHE regulatory proteins and protein kinase A are unlikely to be involved because CAMP-mediated inhibition of NHE activity is similar in young and adult rats (22). However, G protein subunits can regulate NHE activity in renal brush-border membranes independent of cAMP (1, 7, 11, 23). In an experimental setup devoid of cytoplasmic second messengers, Gα inhibits while Gβγ dimers stimulate NHE activity in renal brush-border membrane vesicles (BBMV; see Ref. 1). Gβγ dimers may directly regulate effector proteins and ion channels and appear to antagonize the inhibitory action of Gα in NHE activity in BBMV (1, 16).

The D1-like receptor family consists of the D1 and D5 receptors in mammalian kidneys (25). Both receptors are expressed in rodent kidneys; however, our studies suggest that D1 receptor function may predominate over that of the D5 receptor in renal proximal tubules (25, 33). Both the D1 receptor and NHE3 are highly expressed in apical membranes of renal proximal tubules (2, 5, 6, 8, 9, 31, 39). Therefore, we studied the...
ontogeny of D1 receptor/G protein subunit regulation of NHE3 in apical membranes in the rat.

MATERIALS AND METHODS

Preparation of BBMV. Male Wistar-Kyoto rats ranging in age from 2 to 12 wk were used; 12-wk-old rats were considered to be adults. Two-week-old rats were allowed to nurse ad libitum until the study. Four- to 12-wk-old rats were allowed ad libitum access to regular rat chow and water. All rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). After measurement of arterial pressures from the femoral artery, the kidneys were harvested, and the cortices were separated from the medullas. The rats were then killed by an intravenous injection of 100 mg/kg body wt of pentobarbital sodium. Renal BBMVs were prepared by MnCl2 precipitation and differential centrifugation as previously described (1, 11, 12, 17, 22). The enrichment of the brush-border membrane enzymes alkaline phosphatase and γ-glutamyl transpeptidase (7- to 8-fold) is not affected by age (22). Moreover, Na+-K+-ATPase, a basolateral membrane marker enzyme, was decreased such that it was barely detectable in brush-border membranes in any age group (22). Renal brush-border membrane express NHE3 but do not express the other NHE isoforms found in the kidney (e.g., NHE1, NHE2, and NHE4; see Refs. 5, 6, 8, 9).

Measurement of NHE activity. Previous reports have indicated that the inhibitory effect of dopamine and D1-like agonists on NHE activity in renal proximal tubules is less in immature than in mature animals (19, 22, 26). In those studies whole tubules were used, or BBMVs were obtained after drug treatment of intact cells. In our experimental setup, BBMVs were obtained before drug treatment. Therefore, phosphorylation/dephosphorylation and membrane recycling processes were not involved in any D1-like action observed in these BBMVs (21, 28, 29, 38, 41).

NHE activity was determined by measuring the 100 μM 5-(N-methyl-N-isobutyl)amiloride (MIA) sensitive uptake of 22Na+ at room temperature by the Millipore rapid filtration technique using 0.65-μm nitrocellulose filters, as previously described (11, 12, 17, 22). NHE activity in rat BBMV is due to NHE3 (39). Guanosine 5'-O-(3-thiotriphosphate) (GTP-γS) and anti-Gi3 antibody (1:100), which require access to the interior of the vesicle interior, were added during vesicle formation (1, 11, 12, 22). The BBMVs were preincubated with the D1-like agonist SKF-81297 for 30 min. Because amiloride-sensitive 22Na+ uptake at 3 s is due mainly to NHE3 activity, comparisons were made at this time period (11, 12, 17, 22). 22Na+ uptake at 1–2 h was assumed to represent equilibrium values and also served as an index of vesicle size (11, 12, 17, 22).

Immunoprecipitation studies. BBMVs were incubated with vehicle or fenoldopam (5 × 10−6 M, a D1-like agonist) for 30 min. The membranes were lysed with ice-cold lysis buffer (PBS with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 1 h and were centrifuged at 14,000 rpm for 30 min. The lysates (supernatant) were then incubated with IgG-purified anti-D1 antibody, affinity-purified anti-NHE-3 antibody, and anti-Gi3 or anti-Gi3 antisera on ice for 1 h and protein A-agarose at 4°C for 2–12 h. The immunoprecipitates were pelleted and washed with lysis buffer (4 times), boiled for 10 min, and subjected to immunoblotting.

Immunoblotting studies. The proteins were separated by electrophoresis (7.5% SDS-polyacrylamide gel) and then were electrophoretically transferred to nitrocellulose membranes. The transblots were probed with the indicated antibodies and were detected by peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system (Amersham Life, Arlington Heights, IL). The densities of the appropriate bands were determined using Quantscan (Biosoft, Ferguson, MO).

Materials. Rabbit polyclonal anti-NHE3 and anti-D1 receptor antibodies were produced against a synthetic oligopeptide from the amino acid sequence of rat NHE3 (amino acids 633–646) or rat D1 receptor (amino acids 299–307; Research Genetics, Huntsville, AL; see Refs. 2 and 31). The antibodies were specific to their respective proteins, as determined by Western blotting with preimmune sera or preadsorbed antibody and immunoprecipitation similar to previous reports (2, 31).

Other materials included GTP-γS (Calbiochem, La Jolla, CA); MIA (Research Biochemicals, Natick, MA); SKF-81297 (Smith Kline Beecham, King of Prussia, PA); G protein subunit antibodies (NEN Life Science Products, Boston, MA); and other reagents (all from Sigma Chemical, St. Louis, MO).

Statistical analysis. Data are expressed as means ± SE. Differences within groups were analyzed by ANOVA for repeated measures, followed by Scheffé’s test; paired t-test was used when only two groups were compared. Differences among groups were analyzed by one-way ANOVA, followed by Scheffé’s test; t-test was used when only two groups were compared.

RESULTS

Animal data. The body weights, combined left and right kidney weights, and mean arterial pressures are listed in Table 1. As expected, body and kidney weights and blood pressures increased with age.

Table 1. NHE activity and Gαi expression in developing kidneys

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>2</th>
<th>n</th>
<th>4</th>
<th>n</th>
<th>12 (Adult)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>33.0 ± 6.2</td>
<td>8</td>
<td>82.3 ± 4.7*</td>
<td>6</td>
<td>352 ± 31*</td>
<td>6</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.19 ± 0.03</td>
<td>8</td>
<td>0.46 ± 0.03*</td>
<td>6</td>
<td>1.37 ± 0.11*</td>
<td>6</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>42 ± 2</td>
<td>8</td>
<td>76 ± 2*</td>
<td>6</td>
<td>118 ± 7*</td>
<td>6</td>
</tr>
<tr>
<td>Basal NHE activity, nmol·min⁻¹·mg protein⁻¹</td>
<td>1.32 ± 0.24</td>
<td>3</td>
<td>3.38 ± 0.41*</td>
<td>6</td>
<td>2.82 ± 0.38*</td>
<td>6</td>
</tr>
<tr>
<td>SKF-81297 (5 × 10⁻⁴ M)-inhibited NHE activity, % of control values</td>
<td>−1 ± 9</td>
<td>3</td>
<td>−15 ± 5*‡</td>
<td>6</td>
<td>−65 ± 4*‡</td>
<td>6</td>
</tr>
<tr>
<td>GTP-γS (3 × 10⁻⁴ M)-inhibited NHE activity, % of control values</td>
<td>ND</td>
<td>−56 ± 2†</td>
<td>3</td>
<td>−65 ± 6†</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Gαi protein in brush-border membranes, density units/10 μg protein</td>
<td>35 ± 2</td>
<td>3</td>
<td>36 ± 2</td>
<td>3</td>
<td>29 ± 3</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. MAP, mean arterial pressure; NHE, Na/H exchanger; GTP-γS, guanosine 5'-O-(3-thiotriphosphate); ND, not done. *P < 0.05 vs. 2 wk. †P < 0.05 vs. 4 wk. ‡P < 0.05 vs. vehicle.
NHE activity. Using BBMVs obtained before any drug treatment, we found that basal NHE activity in BBMV increased from 2 to 4 wk of age but not from 4 to 12 wk of age. The inhibition of NHE activity by the D1 agonist SKF-81297 was least in the youngest rats and increased with age (Table 1). These results are in agreement with our previous studies using another D1-like agonist, fenoldopam (22).

NHE3 and D1 receptor expression. The expression of NHE3 was least in the young and tended to increase with maturation, in agreement with reports of others (3, 4, 20). D1 receptor expression did not change with age (Fig. 1 and Table 1).

G protein subunit analyses. G proteins have been shown to mediate the second messenger-independent regulation of NHE activity in renal brush-border membranes (1, 7, 11, 23). Therefore, we next studied the ontogeny of G protein subunit expression. We found that there was a decrease in Gsα expression in brush-border membranes with maturation (Fig. 2). The amount of Gsα that coimmunoprecipitated with NHE3 under basal conditions was similar among the groups. We did not try to resolve the two species of Gsα because only one species could be resolved with the immunoprecipitation studies. After treatment with the D1-like agonist fenoldopam (5 × 10⁻⁶ M), the amount of Gsα that coimmunoprecipitated with NHE3 increased to a similar extent in 2-wk-old (60 ± 25%) and 12-wk-old rats (68 ± 24%; Fig. 2). In contrast, the D1-like agonist did not affect the amount of Gsα that coimmunoprecipitated with NHE3 in 4-wk-old rats. However, Gsα function was not impaired in renal BBMVs of these 4-wk-old rats because GTPγS, the nonhydrolyzable analog of GTP, inhibited NHE activity to the same extent in 4-wk-old and 12-wk-old rats (Table 1). Giα expression in brush-border membranes under basal conditions also did not change with age (Table 1).

We have reported that, in BBMVs, Gβγ subunits can antagonize the inhibitory effect of Gsα on NHE activity (1). In the current studies, we found that Gβ expression in brush-border membranes was highest in 2-wk-old rats and decreased with age (Fig. 3). The amount of Gβ that coimmunoprecipitated with NHE3 under basal conditions also did not change with age (Table 1).
The decreased inhibitory effect of D1-like agonists on NHE activity in the young is not caused by diminished D1 receptor expression. We found that D1 receptor expression in brush-border membranes does not change during maturation. D1-like receptor density in renal proximal tubules has been previously reported to increase from 1 to 3 wk of age, at which age adult values were achieved (14, 26, 27, 37). However, D1-receptor subtype expression in brush-border membranes was not studied (14, 26, 27, 37), and these studies did not distinguish between the two D1-like receptors (D1 and D3) expressed in mammalian nephrons (25). The major D1-like receptor in renal proximal tubules, the D1 receptor, is expressed to a greater extent at luminal than at basolateral membranes (31, 33). Thus increased expression/activity of NHE3 and decreased D1 receptor expression in brush-border membranes of young animals cannot explain the decreased inhibitory action of D1-like agonists on NHE activity in kidneys of immature animals.

cAMP and protein kinase A have been shown to be important mediators in the inhibition of NHE activity in renal brush-border membranes (12, 19, 21, 22, 28, 29, 38, 42). D1 receptors have been reported to inhibit NHE activity via Protein kinase A (12). The limited ability of D1-like receptors to increase adenylyl cyclase activity in the young is not due to decreased expression of D1 receptors in brush-border membranes (14, 18, 19, 22, 27). Decreased production of second messengers after receptor occupation may explain, in part, the decreased D1-like receptor inhibition of NHE activity in the young. However, this mechanism does explain our observations because NHE activity was measured in the absence of cytoplasmic second messengers. Redistribution of NHE3 protein away from plasma membranes because of high perfusion pressure is unlikely because blood pressure is less in younger than in older animals (41). A defective response of NHE3 to cAMP/protein kinase A is also unlikely since cAMP inhibited NHE activity in BBMVs to a similar extent in young and adult rats (22).

Heterotrimeric G proteins may regulate NHE activity independent of cytoplasmic second messengers (1, 7, 11, 23). We have reported that Gs can directly inhibit NHE activity (1). During ontogenesis of normotensive rats, expression of the short form of Gs increased, whereas expression of the long form of Gs remained unchanged in whole kidney membranes (30). In the current report, we also found that Gs did not change with age in renal proximal tubules (data not shown). However, in brush-border membranes, Gs expression decreased with age. The amount of NHE3 bound to Gs did not change with age, but D1-like agonist stimulation increased the amount of Gs that communoprecipitated with NHE3 at 2 wk and 12 wk but not at 4 wk. However, the nonhydrolyzable GTP analog GTPγS inhibited NHE activity, presumably via Gs, to a similar extent in 4-wk-old and adult rats. These data indicate that decreased Gs binding to NHE3 in the young cannot fully account for the decreased D1-like
receptor inhibition of NHE activity in BBMVs of immature rats.

G\(_{i}\alpha\) may mediate the stimulatory effect of G proteins on NHE activity in BBMVs (10). G\(_{i}\alpha,3\) expression in kidneys has been reported to be higher in younger than older rats (30). However, in our studies, the expression of G\(_{i}\alpha,3\), the major G\(_{i}\alpha\) in renal brush-border membranes, did not change with age. Therefore, we presumed that increased G\(_{i}\alpha\) expression could not explain the decreased D\(_1\)-like action on NHE activity in the young. Furthermore, pertussis toxin treatment of renal BBMVs, which inhibited G\(_{i}\alpha\) activity, did not have any effect on D\(_1\)-like receptor action in the young (unpublished observations). Therefore, we turned our attention to the role of the G\(_{b}\gamma\) subunits on D\(_1\)-like action on NHE activity.

G\(_{b}\gamma\) subunits have been reported to directly affect effector proteins such as adenyl cyclase and ion channels (16). We also have preliminary evidence that G\(_{b}\gamma\) subunits can directly affect NHE activity in renal BBMVs in adult rats (1). Thus G\(_{b}\gamma\) subunits can stimulate NHE3 activity and oppose the inhibitory action of G\(_{i}\alpha\) subunits. In the current report, we found that G\(_{b}\gamma\) expression in renal brush-border membranes decreased with age. We also found that D\(_1\)-like receptor stimulation increased the quantity of G\(_{b}\gamma\) dimers that coimmunoprecipitated with NHE3 in 2- and 4-wk-old but not in adult rats. Furthermore, addition of anti-G\(_{b}\gamma\) common antibodies in BBMVs enhanced the inhibitory effect of a D\(_1\)-like agonist on NHE activity in immature but not in adult rats, suggesting increased activity of G\(_{b}\gamma\) dimers in BBMVs of immature rats. These studies indicate that the decreased inhibitory effect of the D\(_1\)-like agonist on NHE activity in immature rats was, in part, due to increased expression and activity of G\(_{b}\gamma\).

In summary, we have confirmed that inhibition of NHE activity in renal BBMV by D\(_1\)-like agonist increases with age. The decreased inhibitory effect of D\(_1\)-like agonists is not due to age-related differences in the expression of NHE3 and D\(_1\) receptors. Although decreased D\(_1\) receptor stimulation of cAMP production in the young may contribute to the decreased inhibition of NHE activity in renal BBMV, increased expression of G\(_{b}\gamma\) dimers and binding to the NHE3 may also play a role. The changing relationships and interplay of G protein subunits may be responsible for the changes seen in the maturation of renal tubular ion transport.

Perspectives

The regulation of NHE3 activity in brush-border membranes of renal proximal tubule cells by D\(_1\)-like receptors occurs at multiple levels. These regulatory pathways involve G protein-second messenger-dependent and G protein-second messenger-independent pathways. The latter pathway may be more important in the immature than in the mature state.

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