D$_1$ dopamine receptor regulation of NHE3 during development in spontaneously hypertensive rats

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Received 24 February 2000; accepted in final form 20 December 2000

D$_1$ dopamine receptor regulation of NHE3 during development in spontaneously hypertensive rats. Am J Physiol Regulatory Integrative Comp Physiol 280: R1650–R1656, 2001.—To determine if the defective interactions among D$_1$-like receptors, G proteins, and Na$^+$/H$^+$ exchanger (NHE3) are consequences of hypertension, we studied these interactions in rats, before (2–3 wk) and after (12 wk) the establishment of hypertension. To eliminate the confounding influence of second messenger action on D$_1$ receptor-NHE3 interaction, studies were performed in renal brush-border membranes (BBM) devoid of cytoplasmic second messengers. NHE3 activity increased with age in Wistar-Kyoto (WKY) rats (3 wk = 1.48 ± 0.39, n = 13; 12 wk = 2.83 ± 0.15, n = 16, P < 0.05) but not in spontaneously hypertensive rats (SHRs; 3 wk = 2.52 ± 0.37, n = 11; 12 wk = 2.81 ± 0.20, n = 16). D$_1$ receptor protein tended to decrease, whereas NHE3 protein tended to increase with age in both WKY and SHRs. However, the inhibitory effect of a D$_1$-like agonist, SKF-81297, on NHE3 activity increased with age in WKY rats (3 wk = −40.7 ± 5.3%, n = 10, 12 wk = −58.7 ± 4.6%, n = 12, P < 0.05) but not in SHRs (3 wk = −27.6 ± 5.9%, n = 11, 12 wk = −25.1 ± 3.2%, n = 11). The decreased inhibitory effect of another D$_1$-like agonist, fenoldopam, on NHE3 activity in SHRs was not caused by increased activity and binding of G$_{o/1}$ to NHE3 as has been reported in young WKY rats. G$_{o/1}$ mediates, in part, the inhibitory effect of D$_1$-like agonists on NHE3 activity. In WKY rats, fenoldopam increased G$_{o/1}$ and Na$^+$/H$^+$ binding to the same extent in 2-wk-old (1.5-fold, n = 4) and adult (1.5-fold, n = 4) rats. In contrast, in SHRs, fenoldopam decreased the amount of G$_{o/1}$ bound to NHE3 in 2-wk-old SHRs and had no effect in 4-wk-old and adult SHRs. These studies indicate that the decreased inhibitory effect of D$_1$-like agonists on NHE3 activity in SHRs (compared with WKY rats) precedes the development of hypertension. This may be caused, in part, by a decreased interaction between G$_{o/1}$ and NHE3 in BBM secondary to impaired D$_1$-like receptor function.

DOPAMINE, PRODUCED BY RENAL proximal tubules, is an important paracrine/autocrine inhibitor of renal sodium transport. Under conditions of moderate sodium loading, endogenous renal dopamine accounts for >50% of sodium excreted (28). The natriuresis is caused by inhibition of ion and water transport in proximal and distal tubules (28, 43, 50). In the renal proximal tubule, sodium transported from tubular fluid across the luminal membrane is mediated by cotransporters (e.g., sodium-phosphate cotransporter) and exchangers (e.g., sodium/hydrogen exchanger (NHE) isoform 3). In renal proximal tubules, dopamine inhibits NHE and sodium-phosphate cotransporter activities at the luminal or brush-border membrane (BBM) and Na$^+$/HCO$_3^-$ cotransporter and Na$^+$/K$^+$-ATPase at the basolateral membrane (6, 7, 11–13, 18, 19, 34). The inhibitory effect of dopamine on sodium transport in renal proximal tubules is exerted via D$_1$-like receptors and augmented via D$_2$-like receptors (6, 27, 46).

In genetically hypertensive rats, however, the natriuretic effect of exogenous and endogenous renal dopamine is attenuated markedly (8, 16, 28, 40). This is caused by a decreased inhibitory effect of dopamine and D$_1$-like agonists on NHE3, Na$^+$/HCO$_3^-$ cotransporter, and Na$^+$/K$^+$-ATPase activities (11, 18, 24, 25, 28, 34). The decreased inhibitory effect of D$_1$-like receptors on NHE3 activity in renal proximal tubules and the subsequent failure of D$_1$-like agonists to induce a natriuresis cosegregate with hypertension in spontaneously hypertensive rat (SHR) and normotensive Wistar-Kyoto (WKY) rat crossbreeds (1). The decreased inhibitory effect of D$_1$-like receptors on NHE3 activity in BBM of SHR is caused, in part, by decreased D$_1$-like receptor generation of cAMP (1, 15, 18, 33). However, adenyl cyclase enzyme responsiveness, D$_1$-like receptor density (determined by radioligand binding), and expression of the two D$_1$-like receptors, D$_1$ and D$_5$, (determined by immunoblotting) in renal proximal tubules are not different between WKY and SHRs (1, 15, 24, 33, 53). NHE3 activity in BBM can also be inhibited to a similar extent in WKY and SHRs if the D$_1$-like receptor is bypassed (24, 53). Stimulation
of G proteins by guanosine 5′-O-(3-thiotriphosphate) (GTPγS) inhibits NHE3 activity and increases binding of Gα to NHE3 to a similar extent in BBMVs of WKY rats and SHRs (53). However, D1-like agonist-mediated increases of Gα and NHE3 binding in BBMVs are attenuated in SHRs compared with WKY rats. These studies and the absence of a difference in the coding region of D1 (and D5) receptors in SHRs support the notion that a defective coupling of the D1-like receptors, specifically the D1 receptor, to Gα may be responsible, in part, for the defective inhibitory action of dopamine and D1-like agonists on NHE3 activity in renal BBMVs (1, 18, 24, 53). However, Gβγ can act to oppose Gα action (2). In young WKY rats, the decreased D1 receptor-mediated inhibition of NHE3 activity in BBM is caused, in part, by increased expression and binding of Gβγ dimers to NHE3 (36). It is also possible that the defective interaction among D1-like receptors, G proteins, second messengers, and effectors could be a consequence of the hypertension.

For example, protein kinase A (PKA) inhibits NHE3 activity in BBM to the same extent in WKY rats and SHRs before the establishment of hypertension (24). In adult SHRs, the inhibitory effect of PKA on NHE3 in BBM is attenuated compared with the effect in WKY rats (24). Therefore, we studied these interactions in rats before (2–4 wk) and after (12 wk) the establishment of hypertension. To eliminate the confounding influence of second-messenger action on D1 receptor-NHE interaction, studies were performed in renal BBM devoid of cytoplasmic second messengers (1, 2, 36).

METHODS

Preparation of BBM vesicles. Male WKY and SHRs 2–4 and 10–12 wk of age were used; 12-wk-old rats were considered to be adults. Two- to three-wk-old rats were allowed to nurse ad libitum until the study. All rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). Arterial pressures were measured from the femoral artery before removal of the kidneys. The rats were then killed by an intravenous injection of 100 mg/kg body wt of pentobarbital sodium. Renal BBM vesicles (BBMV) were prepared by MnCl₂ precipitation and differential centrifugation as described previously (1, 2, 12, 13, 24, 36, 53). The purity, assessed by measurement of the BBM enzymes alkaline phosphatase and γ-glutamyltranspeptidase (7- to 8-fold) and the basolateral membrane marker Na⁺-K⁺-ATPase, is not affected by age (24). The functional NHE isofrom in renal BBMVs has been shown to be caused mainly by NHE3 (2, 3, 30, 52). The studies in WKY rats and SHRs were performed concurrently with those reported for WKY rats (36).

Measurement of NHE activity. To eliminate the confounding influence of second messenger action on D1 receptor-NHE interaction, studies were performed in renal BBMVs devoid of cytoplasmic second messengers (2, 36). Therefore, phosphorylation/dephosphorylation, intermediary actions of NHE regulatory factors (NHERFs), and membrane recycling processes should not be involved in any D1-like action observed in these BBMVs (22, 54, 55).

NHE activity was determined by measuring the 100 μM 5-(N-methyl-N-isobutyl)-amiloride-sensitive uptake of ⁴²Na⁺ at room temperature by the Millipore rapid filtration technique using 0.65-μm nitrocellulose filters as previously described (1, 2, 12, 13, 24, 36, 53). The BBMV were preincubated with the D1-like agonist SKF-81297 for 30 min. Because amiloride-sensitive ⁴²Na⁺ uptake at 3 s is due mainly to NHE activity, comparisons were made at this time period (13). ⁴²Na⁺ uptake at 1–2 h was assumed to represent equilibrium values and also served as an index of vesicle size (13, 24).

Immunoprecipitation and immunoblotting studies. BBMVs were incubated with vehicle or a D1-like agonist (fenoldopam, 5 × 10⁻⁶ M) 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 centrifuged at 14,000 rpm for 30 min. The lysates (supernatant) were then incubated with affinity-purified anti-NHE-3 antibody, anti-Gα, anti-Gα13, or anti-G protein common subunit antisera for 1 h and protein A-agarose for 2–12 h at 4°C. The immunoprecipitates were pelleted and washed with lysis buffer (4 times), boiled for 10 min, and subjected to immunoblotting. The proteins were separated by electrophoresis (7.5% SDS-polyacrylamide gel) and then electrophoretically transferred to nitrocellulose membranes. The blots were probed with the indicated antibodies, detected by the peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system (Amersham Life, Arlington Heights, IL), and quantified using Quantiscan (Biosoft, Ferguson, MO; see Ref. 2). For immunoblotting, 50–100 μg of protein were loaded on a polyacrylamide gel. The amount of protein transferred to the nitrocellulose membrane was verified by Ponceau-S stain.

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 3). The antibodies were specific to their respective proteins as determined by Western blotting with preimmune sera or preadsorbed antibodies (NEN Life Science Products, Boston, MA); all other reagents were from Sigma (St. Louis, MO).

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

RESULTS

Blood pressures. Systolic blood pressure was slightly greater in SHR than in WKY rats at 3–4 wk of age (109 ± 3 vs. 97 ± 3 mmHg, respectively, n = 4/group, P < 0.05, t-test) and markedly greater in SHR (n = 22) than in WKY rats (n = 16) at 12 wk of age (204 ± 6 vs. 122 ± 3 mmHg, respectively, P < 0.05, t-test). D₁ receptors tended to decrease from 2 to 4 wk of age in both WKY rats and SHRs, reaching significance in the latter rat strain (P < 0.05, Fig. 1A). NHE3 increased from 2 to 4 wk of age in WKY rats and tended to increase from 4 wk to adult age in SHRs, but statistical
significance was not reached ($P < 0.05$ ANVR, Scheffe’s test; Fig. 1B). There were no significant differences between WKY rats and SHR at any age.

**NHE3 activity.** NHE3 activity in BBMV increased with age in WKY rats but not so in SHRs (Fig. 2A). NHE activity was greater in SHRs than in WKY rats at 3 wk but not at 12 wk of age. The ability of SKF-81297, a $D_1$-like agonist, to inhibit NHE3 activity in BBMV was less at 3 wk than at 12 wk of age, whereas baseline NHE3 activity was greater in 12-wk-old WKY rats (Fig. 2B). No difference in the effect of SKF-81297 was noted between the 3- and 12-wk-old SHR. Regardless of age, SKF-81297 inhibited NHE3 activity to a greater extent in WKY rats than in SHRs. The decreased ability of SKF-81297 to inhibit NHE3 activity in BBMV in the SHR compared with WKY rats at any age could not be explained by any strain differences in $D_1$ receptor or NHE3 protein expression.

**G protein subunits.** We have reported that $G_\alpha\alpha$ protein expression in BBM slightly decreased with age in WKY rats (36); there was also a trend for an age-related decrease in SHRs, but significance was not achieved. We have also reported that another $D_1$-like agonist, fenoldopam (5 $\mu$M), increased the amount of $G_\alpha\alpha$ bound to NHE3 to a similar extent in 2- and 12-wk-old WKY rats (36). In the SHRs, fenoldopam had no such effect and actually decreased the amount of $G_\alpha\alpha$ bound to NHE3 (compared with basal) at 2 wk of age (Fig. 3). These studies suggest that the failure of fenoldopam in the SHR to increase the amount of $G_\alpha\alpha$ bound to NHE3 with age may be the cause of absence of the ontogenic increase in the inhibitory action of fenoldopam on NHE3 activity.

$G_\alpha\gamma_3$ protein expression did not change with age in WKY rats (36) but decreased with age in SHRs (Fig. 4). In SHRs, there were no differences in the amount of $G_\alpha\gamma_3$ bound to NHE3 with age under basal conditions (Fig. 4). In SHRs (Fig. 4), as in WKY rats (data not shown), fenoldopam (5 $\mu$M) decreased the amount of $G_\alpha\gamma_3$ bound to NHE3 at 2 wk of age. Therefore, in SHRs, as in WKY rats (36), $G_\alpha\gamma_3$ protein does not seem to change with age.

**Fig. 2.** NHE3 activity assessed as amiloride-sensitive $^{22}\text{Na}^+$ uptake in renal BBM vesicles in 3- and 12-wk-old WKY rats and SHRs. A: basal NHE3 activity with age. NHE activity increased with age in WKY rats but not in SHRs. *$P < 0.05$ vs. other groups, ANOVA, Duncan’s test. B: effect of $5 \times 10^{-6}$ M SKF-81297 ($D_1$ agonist) on NHE3 activity. SKF-81297 inhibition of NHE3 activity increased with age in WKY rats but not in SHRs. **$P < 0.05$ vs. other groups. 

**#**$P < 0.05$ vs. SHR, ANOVA, Duncan’s test.
to influence basal or fenoldopam-mediated effects on NHE3 activity at any age.

We have reported that in WKY rats, $G_b$ protein expression in BBMs decreased with age (36). In addition, fenoldopam increased the amount of $G_b$ bound to NHE3 in BBMs of 2- and 4-wk-old but not in 12-wk-old WKY rats (36). In the current studies, in SHRs as in WKY rats, $G_b$ expression decreased with age. However, in contrast to WKY rats, the amount of $G_b$ bound to NHE3 did not change with age, either under basal conditions or after fenoldopam (5 $\mu$M) stimulation (Fig. 5). Therefore, in SHRs, unlike that observed in 2- and 4-wk-old WKY rats (36), $G_b$ protein does not seem to influence basal or fenoldopam-mediated effects on NHE3 activity at any age.

DISCUSSION

An increase in NHE3 protein abundance and activity in BBM with age has been reported in rabbits and sheep (5, 20). We also found an increase in NHE3 protein abundance and activity in BBM between 3 and 12 wk of age in WKY rats (24, 36); the current data suggest that a similar ontogenic increase in NHE3 protein abundance occurs in SHRs. NHE3 activity in proximal tubules has been reported to be greater in SHRs than in WKY rats studied before 8 wk of age; thereafter, most studies could not find a difference in basal NHE activity between these two rat strains (10, 18, 23, 24, 31, 39, 42). Because a difference was noted between WKY rats and SHR in S1 but not in S2 segments, differences among reports could have been caused by different proportions of S1 and S2 segments in any given preparation (10). In spite of these experimental limitations, a decreased ability of D1-like agonists to inhibit NHE3 activity in SHR compared with WKY rats has been found consistently (1, 18, 24). The decreased ability of D1-like agonists to inhibit NHE3 activity in SHRs compared with WKY rats could not be accounted for by differences in D1-like receptor density (24). However, two D1-like receptors, $D_1$ and $D_5$, are expressed in renal proximal tubules (4, 44). We now report a decreased D1-like agonist inhibition of NHE3 activity in BBM in SHRs compared with WKY rats. It was present at different ages and cannot be accounted for by differences in D1 receptor or NHE3 expression. Except for a trend for lower NHE3 protein abundance in SHRs than in WKY rats at 4 wk of age, NHE3 expressions in renal BBM were similar in WKY rats and SHRs, in agreement with some studies using proximal tubule homogenates (23, 31, 53).
We have previously noted a decreased ability of D1-like agonists to inhibit NHE3 activity in young WKY rats (24, 36). This was not due to nonregulatable NHE3 activity because GTPγS and PKA decreased NHE3 activity to a similar extent in young and adult WKY rats (24, 36). Because forskolin also stimulated cAMP production in renal proximal tubules to a similar extent in young and adult WKY rats, we suggested that the decreased stimulatory effect on adenylyl cyclase activity and decreased inhibitory effect on NHE3 activity in BBM by D1-like agonists in young rats were not caused by age-related differences in effector proteins (15, 33). Recently, the inhibitory effect of PKA on NHE3 activity has been found to be aided by proteins called NHERF, two of which have been identified (22). The inhibition of phosphorylated NHE3 by ligands other than dopamine, e.g., parathyroid hormone, has also been shown to be associated with its translocation from the plasma membrane to intracellular organelles (54). Abnormalities of any of these pathways may be responsible for the decreased inhibitory effect of D1-like receptors on NHE3 activity in renal proximal tubules of SHRs. Indeed, PKA inhibited NHE3 activity in renal proximal tubules to a lesser extent in SHRs than in WKY rats (24). To avoid a confounding effect of cytoplasmic signal transducers, we performed our studies in BBMV devoid of cytoplasmic components.

G protein subunits can directly regulate NHE3 activity in renal BBMV (2). Therefore, we wondered whether differences in the coupling of D1-like receptors to G protein subunits may also explain the decreased D1-like inhibitory effect on NHE3 activity in the SHR. Such a differential coupling might also explain the differences in D1-like stimulation of adenylyl cyclase in renal proximal tubules in WKY rats and SHRs (15, 18, 33). Gαi can directly inhibit NHE3 activity while Gβγ can directly increase NHE3 activity (2). We have suggested that increased expression and activity of Gβγ subunits contributed to the attenuated D1-like receptor inhibition of NHE3 activity in BBM of young normotensive WKY rats (36). In SHRs, as in WKY rats, Gβ1 common expression in BBM decreased with age (36). In young, but not adult WKY rats, D1-like agonist stimulation increased Gβ1 common binding to NHE3 (36). However, in SHRs, regardless of age, D1-like agonist stimulation did not affect the binding of Gβ1 common to NHE3. Thus the attenuated effect of D1-like agonists on NHE3 activity in BBM was not due to alterations in Gβγ action.

Gαi,3 decreased with age in BBM in SHR but not in WKY; however, fenoldopam did not affect the amount of Gαi,3 bound to NHE3 in adult rats and decreased it in young rats in both WKY rats and SHRs. Therefore, changes in Gαi,3 expression and binding to NHE3 do not explain the differences in D1-like action with age in WKY rats or the differences between WKY rats and SHRs. Furthermore, Gαi,3 did not participate in the D1-like receptor inhibition of NHE3 activity in BBMV (2). Antibodies to Gαi,3 also did not affect D1-like stimulated binding of GTPγS in renal proximal tubules in either WKY rats or SHRs (26).

Gαi expression tended to decrease with age in both WKY rats and SHRs, but no differences were noted between the two strains (25, 36, 38, 47). In renal BBMs of WKY rats, there were age-related differences in the amount of Gαi bound to NHE3 after D1-like receptor stimulation (36). In contrast, in young (current studies) and adult SHRs, the amount of Gαi bound to NHE3 was not increased by D1-like receptor stimulation (53). GTPγS binding to renal proximal tubular basolateral membranes after D1-like agonist stimulation was also less in SHRs than in WKY rats at any age (26). The influence of other Ga subunits was not studied. Gα expression in the kidney was decreased in adult but not in young SHR (26, 38). Although alterations in Gα expression may play a role in the uncoupling of the D1-like receptor in adult hypertensive animals, this may not be the case in young SHR (26, 28). In the presence of calcyon, the D1 receptor becomes linked to phospholipase C-β (14, 17, 29); phospholipase C-β and protein kinase C activation by D1 receptors may be involved in the D1-like receptor inhibition of Na+–K+-ATPase activity in the kidney (29). Protein kinase C inhibited NHE3 activity in cells heterologously expressing NHE3 other than the kidney (42). However, D1-like receptor-mediated inhibition of luminal NHE activity has been reported to be independent of phospholipase C (14, 48). Moreover, neither D1 nor D5, the two D1-like receptors expressed in renal proximal tubules, is directly linked to Gαi (17, 32, 37). The D1 receptor is also not linked to the other family of Ga proteins, Gα12 and Gα13 (37).

The cause of the impaired D1-like receptor function in renal proximal tubules in genetic hypertension remains to be determined (28). Although abnormalities of G protein subunits have been implicated in genetic hypertension, including the SHR, a primary abnormality in G proteins is unlikely in the SHR (21, 49). GTPγS inhibited NHE activity in BBM to a similar extent in WKY rats and SHRs (53). GTP and 5′-guanylyl imidodiphosphate stimulated cAMP production in isolated proximal tubules to a similar extent in WKY rats and SHRs (15, 33). Moreover, reconstitution of D1 receptors from SHRs with exogenous G proteins failed to induce formation of a high-affinity binding site (47). We have not found differences in the sequence or expression in renal proximal tubules of the D1 and D5 receptors between WKY rats and SHRs (unpublished observations). We have suggested, however, that an abnormal posttranslational modification, specifically increased serine phosphorylation of the D1-like receptor, may be responsible for its uncoupling from the G protein-effector complex in genetic hypertension (45).

Modification of receptors by oxygen radicals produced in excess in hypertension has been raised as a possible mechanism for the decreased function of D1-like receptors in the kidney (51). While this is possible, one would have to propose a restricted oxygen radical formation. The uncoupling of the D1-like receptor in rodent genetic hypertension has been described mainly in proximal tubules and medullary thick ascending limbs of Henle (1, 7, 8, 11, 15, 18, 24–26, 28, 33, 40, 53).
The uncoupling was also not found in the striatum of the brain where D1 receptors are expressed in greater abundance than in renal proximal tubules (28). A general increase in oxygen radical formation also fails to explain the organ and nephron segment receptor-specific D1-like defect in hypertension (28). Parathyroid hormone receptor (15, 26, 33, 34, 41), CCK-A receptor (unpublished studies), and β-adrenergic receptor functions (38) were not impaired in young SHRs when increased reactive oxygen species is already evident (9).

We conclude that the decreased inhibitory effect of D1-like agonists on NHE3 activity in SHRs (compared with WKY rats) precedes the development of hypertension. In WKY rats, the enhanced NHE3 inhibition by D1-like receptors in older rats is caused by an age-dependent decrease in Gβγ action and maintenance of the level of Gαo action. In SHRs, the decreased NHE3 inhibition by D1 receptors is not caused by Gβγ action but rather by a decreased interaction between Gαo and NHE3. Proteins like NHERF1 and NHERF2 that regulate NHE3 activity are unlikely to be involved in our experiments utilizing BBMV because the regulation of NHE3 by these proteins involves cAMP (22).

**Perspectives**

The cause(s) of essential hypertension remains elusive, probably because it is a heterogeneous disease in which both genetics and environment contribute to elevate blood pressure. The blood pressure difference between a hypertensive strain of rat and a normotensive strain. (38) were not impaired in young SHRs when the brain where D1 receptors are expressed in greater abundance than in renal proximal tubules (28). A general increase in oxygen radical formation also fails to explain the organ and nephron segment receptor-specific D1-like defect in hypertension (28).

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