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Renal D_3 dopamine receptor stimulation induces natriuresis by endothelin B receptor interactions

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Dopaminergic and endothelin systems participate in the control blood pressure by regulating sodium transport in the renal proximal tubule. Disruption of either the endothelin B receptor (ETB) or D_3 dopamine receptor gene in mice produces hypertension. To examine whether these two receptors interact we studied the Wistar–Kyoto (WKY) and spontaneously hypertensive (SHR) rats by selectively infusing reagents into the right kidney of anesthetized rats. The D_3 receptor agonist (PD128907) caused natriuresis in WKY rats which was partially blocked by the ETB receptor antagonist. In contrast, PD128907 blunted sodium excretion in the SHRs. We found using laser confocal microscopy that the ETB receptor was mainly located in the cell membrane in control WKY cells. Treatment with the D_3 receptor antagonist caused its internalization into intracellular compartments that contained the D_3 receptors. Combined use of D_3 and ETB antagonists failed to internalize ETB receptors in cells from WKY rats. In contrast in SHR cells, ETB receptors were found mainly in internal compartments under basal condition and thus were likely prevented from interacting with the agonist-stimulated, membrane-bound D_3 receptors. Our studies suggest that D_3 receptors physically interact with proximal tubule ETB receptors and that the blunted natriuretic effect of dopamine in SHRs may be explained, in part, by abnormal D_3/ETB receptor interactions.

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The kidney is endowed with two local hormonal systems that play major roles in the regulation of sodium transport across the renal proximal tubule (RPT), namely the dopaminergic and endothelin systems.[1–7] Endothelin regulates epithelial sodium transport through two receptor subtypes (ETA (endothelin A) and endothelin B (ETB)).[5–7] Although it is still not known if the ETB receptor is the subtype responsible for the actions of endothelin at the RPT, there is evidence that endothelin-1 inhibits Na^+–K^+ ATPase activity in this nephron segment.[8] It is generally accepted, however, that dopamine, which is synthesized in and secreted from RPT cells, decreases RPT sodium transport by inhibiting Na^+/H^+ exchanger isoform-3 (NHE3), sodium bicarbonate cotransporter (NBCe1), Na^+–Pi cotransporter type-2 (NaPi2), and Na^+–K^+ ATPase activities, through D_1 and D_3 receptors in this nephron segment.[1–4] Although these two hormonal systems are involved in renal sodium excretion, little is known regarding the physiological interactions of renal dopamine and endothelin in the control of sodium excretion *in vivo*.

Dopamine receptors are classified into D_1- and D_2-like subtypes based on their structure and pharmacology. There are two D_1-like receptors (D_1 and D_5) and three D_2-like receptors (D_2, D_3, and D_4), which are expressed in mammalian kidneys.[1–4] Approximately, 50% of basal sodium excretion is mediated by the paracrine action of renal dopamine exerted on D_1 receptor.[1–4] In the rat kidney, the major D_2-like receptor located in RPTs is the D_3 receptor.[9,10] Disruption of the D_3 receptor gene in mice produces hypertension, which is associated with a decreased ability to excrete a sodium load.[11] Stimulation of the D_3 receptor, synergistically with D_1 receptor, induces natriuresis and diuresis in Wistar–Kyoto (WKY) rats.[12,13] However, the mechanisms of D_3 receptor-mediated natriuresis are incompletely understood.

Endothelin B receptors are expressed in RPT cells.[14] In uninephrectomized spontaneously hypertensive rats (SHRs) treated with deoxycorticosterone acetate and fed a high-salt

diet, and in humans with essential hypertension, ETB receptor activation may function to counter-regulate the increase in blood pressure.[15–17] Naturally occurring or induced deletion of the ETB receptor gene and chronic pharmacological blockade of the ETB receptor in rats result in salt-sensitive hypertension.[17,18] We hypothesize that a D_3/ETB receptor interaction promotes sodium excretion in WKY rats, but not in SHRs. The current study employed a direct renal stimulation of D_3 receptor with PD128907, a selective D_3 receptor agonist, to evaluate its effects on renal sodium excretion in WKY rats and SHRs. We suggest that D_3 receptors regulate RPT ETB receptors by physical receptor interaction and that the impaired natriuretic effect of the D_3 receptor agonist in SHRs may be explained, in part, by an aberrant D_3/ETB receptor interaction.

RESULTS

D_3 and ETB receptor localization in kidney

D_3 receptor staining was found mostly in the subapical areas of the S1, S2, and S3 (with the strongest signal) segments of the proximal tubules in WKY rats and SHRs. The ETB receptor was expressed in the proximal tubules, especially at the brush border membranes of the S3 segment, similar to those noted with the D_3 receptor. No staining was seen when the antibodies were preadsorbed with the immunizing peptide (Figure 1a–d). D_3 and ETB receptor colocalization (laser confocal microscopy) was evident in all proximal tubules of the kidney in WKY rats (Figure 1e).

Stimulation of renal D_3 receptors increases sodium excretion in WKY rats, which was impaired in SHRs

To determine the effect of D_3 receptors on sodium excretion, varying dosages of PD128907 (0.5, 1.0, 5.0, and 10.0 μg/kg/min, with each dose administered for 40 min; $n = 8$), were infused into the right renal artery in WKY rats maintained on low-sodium (0.025% NaCl), normal sodium (1% NaCl), or high-sodium (6% NaCl) diet for 21 days. The intrarenal arterial infusion of the vehicle into the right kidney had no effect on blood pressure, urine flow (V), fractional sodium excretion (FE_{Na}), absolute sodium excretion ($U_{Na}V$), potassium excretion (U_KV), or glomerular filtration rate (GFR) (Table 1). In WKY rats on low-salt diet, PD128907 increased V, $U_{Na}V$, and U_KV, and tended to increase FE_{Na} without affecting GFR (Table 2). In WKY rats on normal or high-salt diet, PD128907 increased V, U_KV, $U_{Na}V$, and FE_{Na} (Tables 3 and 4). The absolute increase in $U_{Na}V$ with PD128907 was greater in rats on high salt than on a normal or low-salt diet, but the percentage increases were the same.

We next studied the effect of the intrarenal infusion of PD128907 (0.5, 1.0, 5.0, and 10.0 μg/kg/min) in SHRs on high-salt diet. PD128907 had no effect on blood pressure, GFR, V, U_KV, $U_{Na}V$, or FE_{Na} in SHRs (Table 5; Figure 2a). We also studied the effect of PD128907 (1.0 μg/kg/min) on renal function in SHRs on normal salt diet and found that PD128907 also had no effect blood pressure, GFR, V, U_KV, $U_{Na}V$, or FE_{Na} at the concentration of PD128907 with 1.0 μg/kg/min (Table 6; Figure 2b).

To determine the specificity of the D_3 receptor agonist (PD128907) on sodium excretion, a D_3 receptor antagonist (GR103691, which has been reported to be active *in vivo*) was used. GR103691, infused at 1.0 μg/kg/min, did not affect V, $U_{Na}V$, FE_{Na}, or GFR in WKY rats. However, GR103691 blocked the PD128907 (1.0 μg/kg/min)-mediated increase in V, $U_{Na}V$, and FE_{Na} (Figure 3a).

To determine if there was any systemic effect of the drugs selectively infused into the right renal artery, sodium excretion from the left kidney was also measured. We found that the renal function, including sodium excretion, in the left unperfused kidney was not altered by any of the drug treatments (Figure 3b).

Blockade of ETB receptors attenuates D_3 receptor-mediated sodium excretion in WKY rats

We next studied the *in vivo* interaction between D_3 and ETB receptors in WKY rats. The ETB receptor antagonist, BQ788 (5.0 μg/kg/min) did not significantly affect $U_{Na}V$, but partially blocked the natriuretic effect of PD128907, suggesting that a renal D_3/ETB receptor interaction played a role in the D_3 receptor-mediated natriuresis in WKY rats (Figure 3a).

Stimulation of D_3 receptors increases the internalization of ETB receptors and intracellular D_3/ETB colocalization in WKY rat RPT cells, but not in SHR cells

To determine a mechanism for the observed difference in the natriuretic response upon D_3 receptor activation in WKY and SHRs, the distribution of both D_3 and ETB receptors and their colocalization were evaluated by confocal microscopy. In the basal state in WKY rat cells, the D_3 receptor was found in both the plasma membrane and cytoplasm, whereas the ETB receptor was confined mainly in the plasma membrane. Treatment with the D_3 receptor agonist PD128907 promoted the internalization of both D_3 and ETB receptors and increased its intracellular colocalization with D_3 receptors (Table 7). Treatment with the D_3 receptor antagonist GR103691, ETB receptor antagonist BQ788, or with PD128907 in combination with either antagonist, failed to drive the ETB receptors intracellularly in WKY rat cells (Figure 4a).

In SHR RPT cells, the D_3 receptor was found in both the plasma membrane and cytoplasm under basal condition. However, the ETB receptor was expressed exclusively in the cytoplasm, in contrast to its exclusive expression in the plasma membrane in WKY rat RPT cells. D_3 receptor stimulation, as with the other treatments, did not alter the cytoplasmic location of both D_3 and ETB receptors and failed to promote the colocalization of the receptors (Table 7). There was some colocalization between these proteins just below the cell membrane in the basal state and was not altered by D_3 receptor stimulation, alone (Table 7) or with ETB inhibition, presumably arising from the more scattered distribution of the D_3 receptor within the cytoplasm (Figure 4b).

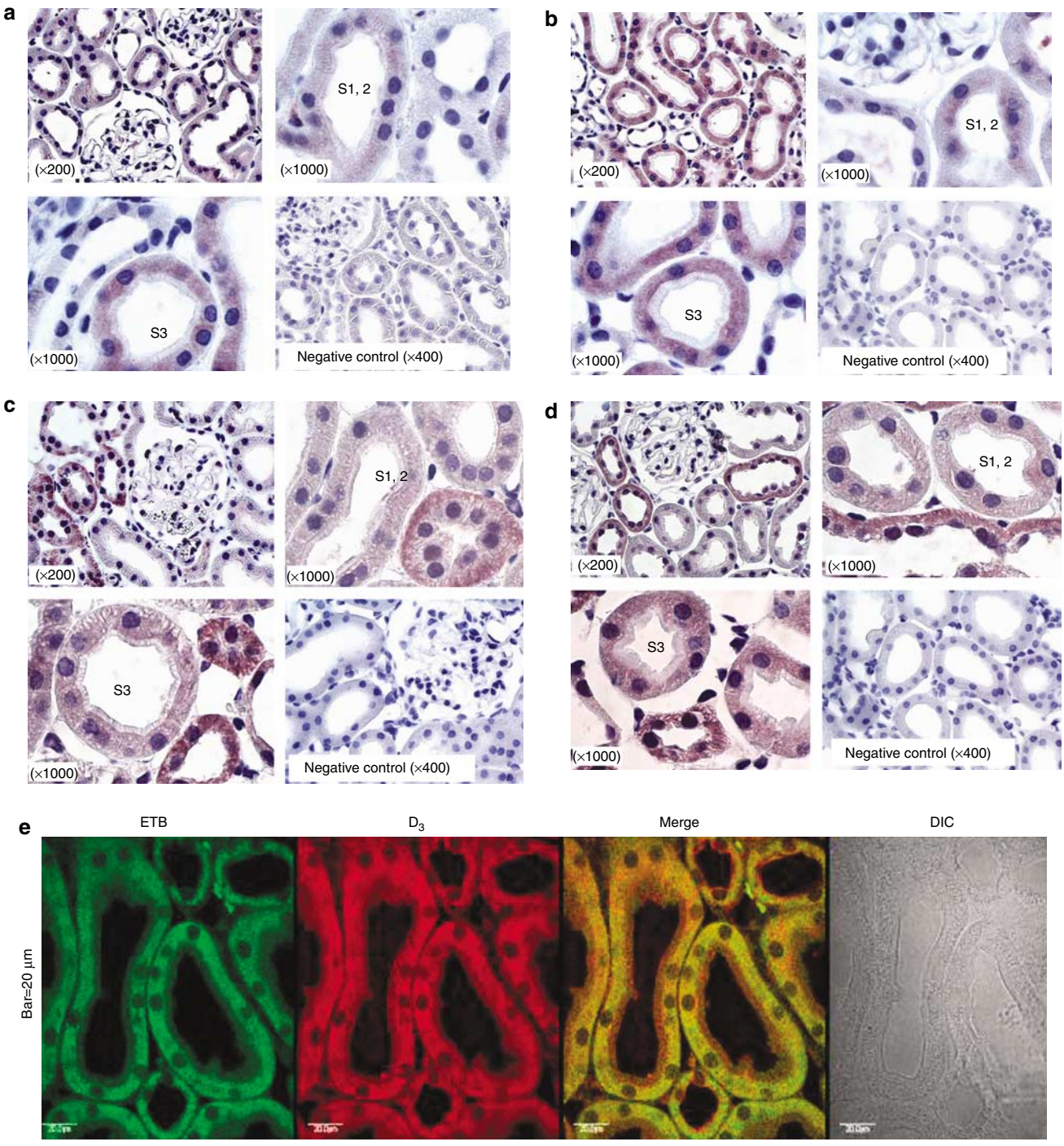

Figure 1 | D_3 and ETB receptor staining in the rat kidneys. (**a**, **b**) D_3 receptor is located in RPTs from WKY rats (**a**) and SHRs (**b**). No staining is seen when the D_3 receptor antibody was preadsorbed with the immunizing peptide. (**c**, **d**) ETB receptor is located in RPTs from WKY rats (**c**) and SHRs (**d**). No staining is seen when the ETB receptor antibody was preadsorbed with the immunizing peptide. These studies were performed at least three times. (**e**) D_3 and ETB receptor colocalization in kidneys from WKY rats. Colocalization appears as yellow after merging the images of fluorescein isothiocyanate-tagged ETB receptor (green) and Alexa 568-tagged D_3 receptor (red). No staining is seen without the antibodies (DIC). DIC, differential interference contrast.

DISCUSSION

The natriuretic effect of dopamine is best manifested under conditions of moderate sodium intake in normotensive humans,[19,20] dogs,[21] WKY rats,[22,23] and Dahl salt-resistant rats.[24] Disruption of the D_3 receptor gene in mice impairs their ability to excrete a sodium load.[11] Previous studies have found that 7-OH-DPAT, a D_3 receptor agonist, induces a natriuresis in normotensive Dahl salt-sensitive rats on normal sodium diet but not in hypertensive Dahl salt-sensitive rats on high-sodium diet.[25,26] The diminished

Table 1 | Effect of vehicle on renal function in the infused right kidney of WKY rats

	Systolic (mm Hg)	MAP (mm Hg)	Diastolic (mm Hg)	*V* (μl/min)	FE_{Na} (%)	$U_{Na}V$ (nEq/min)	U_KV (nEq/min)	GFR (ml/g kidney per min)
CTRL	135.17 ± 6.94	109.33 ± 2.36	93.33 ± 5.42	3.80 ± 0.54	0.18 ± 0.02	325 ± 54	557 ± 89	0.99 ± 0.06
w/P	135.50 ± 6.25	108.50 ± 3.77	88.33 ± 4.15	3.87 ± 0.46	0.20 ± 0.03	312 ± 55	622 ± 90	0.98 ± 0.06
w/P	131.50 ± 3.97	105.50 ± 2.90	86.00 ± 3.20	3.60 ± 0.33	0.23 ± 0.05	299 ± 53	583 ± 94	0.98 ± 0.05
w/P	131.50 ± 4.72	106.67 ± 3.83	88.83 ± 3.91	4.08 ± 0.64	0.22 ± 0.03	332 ± 69	681 ± 122	1.08 ± 0.07
w/P	132.00 ± 3.30	109.00 ± 2.90	91.00 ± 3.40	4.71 ± 0.69	0.29 ± 0.06	404 ± 73	874 ± 100	1.04 ± 0.07
w/P	124.17 ± 1.85	103.00 ± 2.02	86.00 ± 2.31	4.65 ± 0.67	0.27 ± 0.05	433 ± 107	835 ± 160	0.98 ± 0.04
Recovery	119.83 ± 1.19	100.00 ± 2.31	84.50 ± 2.32	5.07 ± 0.60	0.31 ± 0.06	436 ± 102	856 ± 143	0.98 ± 0.04

FE_{Na}, fractional Na excretion; GFR, glomerular filtration rate; MAP, mean arterial pressure; period CTRL is baseline (vehicle infusion), periods w/P are the placebo (vehicle) infusion periods, and period recovery is the recovery period (vehicle infusion); U_KV, urine K excretion; $U_{Na}V$, urine Na excretion; *V*, urine flow; WKY rat, Wistar–Kyoto rat (body wt 361 ± 13 g, *n*=6).

Table 2 | Effect of PD 128907 (D_3 agonist) on renal function in the infused right kidney of WKY rats on low-salt diet

	Systolic (mm Hg)	MAP (mm Hg)	Diastolic (mm Hg)	*V* (μl/min)	FE_{Na} (%)	$U_{Na}V$ (nEq/min)	U_KV (nEq/min)	GFR (ml/g kidney/min)
CTRL	132.25 ± 1.37	107.37 ± 2.34	94.87 ± 3.00	3.42 ± 0.31	0.04 ± 0.003	58 ± 3.0	549 ± 66	0.95 ± 0.05
0.5 PD	134.12 ± 1.18	106.87 ± 3.09	93.12 ± 4.21	4.47 ± 0.35	0.04 ± 0.004	67 ± 8.0	909 ± 127	1.19 ± 0.08
1.0 PD	131.25 ± 2.16	105.12 ± 2.59	91.75 ± 3.51	5.46 ± 0.76	0.05 ± 0.014	96 ± 35.1	1103 ± 255	1.18 ± 0.11
5.0 PD	131.25 ± 1.92	103.50 ± 1.51	89.50 ± 1.72	6.44 ± 0.92*	0.09 ± 0.041	159 ± 75.4*	1200 ± 237*	1.13 ± 0.08
10.0 PD	127.37 ± 4.14	101.12 ± 2.71	87.87 ± 2.64	6.46 ± 1.58*	0.12 ± 0.057	225 ± 135.1*	995 ± 207*	1.00 ± 0.10
Recovery	125.16 ± 5.76	98.67 ± 3.59	86.00 ± 3.24	7.02 ± 1.09*	0.19 ± 0.115	324 ± 171.2*	1198 ± 278*	1.09 ± 0.07

ANOVA, analysis of variance; FE_{Na}, fractional Na excretion; GFR, glomerular filtration rate; MAP, mean arterial pressure; period CTRL is the baseline (vehicle infusion); periods 0.5 PD, 1.0 PD, 5.0 PD, and 10.0 PD are with infusion of PD 128907 at dosages of 0.5, 1.0, 5.0, and 10.0 μg/kg/min; period recovery is the recovery period (vehicle infusion); U_KV, urine K excretion; $U_{Na}V$, urine Na excretion; *V*, urine flow; WKY rat, Wistar–Kyoto rat (body wt 433 ± 24 g, *n*=8).
*$P<0.05$ vs CTRL, repeated measures ANOVA (ANVR), Duncan's test.

Table 3 | Effect of PD 128907 (D_3 agonist) on renal function in the infused right kidney of WKY rats on normal salt diet

	Systolic (mm Hg)	MAP (mm Hg)	Diastolic (mm Hg)	*V* (μl/min)	FE_{Na} (%)	$U_{Na}V$ (nEq/min)	U_KV (nEq/min)	GFR (ml/g kidney/min)
CTRL	132.25 ± 1.31	110.00 ± 2.12	99.25 ± 3.12	4.45 ± 0.27	0.06 ± 0.005	115 ± 1.9	716 ± 60	1.23 ± 0.16
0.5 PD	134.25 ± 1.65	104.00 ± 2.54	89.25 ± 2.56	5.85 ± 0.94	0.08 ± 0.011	163 ± 12.9	1085 ± 153	1.22 ± 0.09
1.0 PD	127.75 ± 4.82	100.25 ± 5.76	88.00 ± 7.56	7.12 ± 0.64*	0.09 ± 0.015*	199 ± 39.1	1384 ± 86*	1.34 ± 0.12
5.0 PD	130.50 ± 4.92	102.50 ± 6.38	88.50 ± 7.69	8.25 ± 0.75*	0.11 ± 0.008*	252 ± 27.1*	1756 ± 114*	1.31 ± 0.11
10.0 PD	127.50 ± 6.29	95.75 ± 6.79	79.75 ± 7.12	8.30 ± 0.25*	0.17 ± 0.021*	334 ± 53.1*	1637 ± 06*	1.16 ± 0.07
Recovery	127.75 ± 7.02	96.75 ± 7.43	81.25 ± 7.64	7.94 ± 0.43*	0.25 ± 0.009*	392 ± 59.9*	1538 ± 213*	0.92 ± 0.08

ANOVA, analysis of variance; FE_{Na}, fractional Na excretion; GFR, glomerular filtration rate; MAP, mean arterial pressure; period CTRL is the baseline (vehicle infusion); periods 0.5 PD, 1.0 PD, 5.0 PD, and 10.0 PD are with infusion of PD 128907 at dosages of 0.5, 1.0, 5.0, and 10.0 μg/kg/min; period recovery is the recovery period (vehicle infusion); U_KV, urine K excretion; $U_{Na}V$, urine Na excretion; *V*, urine flow; WKY rat, Wistar–Kyoto rat (body wt 435 ± 23 g, *n*=4).
*$P<0.05$ vs CTRL, repeated measures ANOVA (ANVR), Duncan's test.

Table 4 | Effect of PD 128907 (D_3 agonist) on renal function in the infused right kidney of WKY rats on high-salt diet

	Systolic (mm Hg)	MAP (mm Hg)	Diastolic (mm Hg)	*V* (μl/min)	FE_{Na} (%)	$U_{Na}V$ (nEq/min)	U_KV (nEq/min)	GFR (ml/g kidney/min)
CTRL	139.25 ± 3.41	110.00 ± 3.42	96.71 ± 4.95	5.25 ± 0.93	0.35 ± 0.08	924 ± 199	763 ± 211	1.14 ± 0.07
0.5 PD	136.85 ± 1.81	109.71 ± 3.10	97.85 ± 4.57	7.23 ± 1.40	0.48 ± 0.16	1241 ± 330	1122 ± 253*	1.19 ± 0.13
1.0 PD	136.71 ± 1.68	107.28 ± 2.03	92.71 ± 2.79	8.74 ± 1.52*	0.66 ± 0.20	1768 ± 551	1339 ± 206*	1.09 ± 0.14
5.0 PD	135.28 ± 1.81	108.86 ± 3.39	95.00 ± 4.81	9.94 ± 2.02*	0.71 ± 0.22	2092 ± 702*	1503 ± 227*	1.16 ± 0.11
10.0 PD	134.43 ± 1.25	107.71 ± 2.68	94.28 ± 3.67	9.81 ± 1.25*	0.96 ± 0.20*	2266 ± 516*	1387 ± 190*	0.99 ± 0.11
Recovery	131.14 ± 2.31	104.71 ± 2.25	90.43 ± 2.17	10.38 ± 1.16*	1.20 ± 0.21*	2605 ± 466*	1344 ± 142*	0.94 ± 0.05

ANOVA, analysis of variance; FE_{Na}, fractional Na excretion; GFR, glomerular filtration rate; MAP, mean arterial pressure; period CTRL1 is the baseline (vehicle infusion); periods 0.5 PD, 1.0 PD, 5.0 PD, and 10.0 PD are with infusion of PD 128907 at dosages of 0.5, 1.0, 5.0, and 10.0 μg/kg/min; period recovery is the recovery period (vehicle infusion); U_KV, urine K excretion; $U_{Na}V$, urine Na excretion; *V*, urine flow; WKY rat, Wistar–Kyoto rat (body wt 438 ± 20 g, *n*=7).
Data are expressed as mean ± s.e.
*$P<0.05$ vs CTRL repeated measures ANOVA (ANVR), Duncan's test.

natriuretic response to D_3 receptor stimulation in the hypertensive Dahl salt-sensitive rat is associated with decreased ^{3}[H]-7-OH-DPAT binding to renal membranes.[25,26] However, the same group did not find a strain-dependent effect of 7-OH-DPAT in WKY rats and SHRs.[27] The interpretation of this study, however, is confounded by the systemic administration of 7-OH-DPAT; activation of D_3 receptors and non-D_3 dopamine receptors outside the kidney makes interpretation difficult. For example, activation of D_3 receptors could decrease renin production in tissues other than the kidney, decrease aldosterone secretion, or inhibit sympathetic activity resulting in a decrease in blood pressure.[1–4] To

Table 5 | Effect of PD 128907 (D_3 agonist) on renal function in the infused right kidney of SHR on high-salt diet

	Systolic (mm Hg)	MAP (mm Hg)	Diastolic (mm Hg)	*V* (μl/min)	FE_{Na} (%)	$U_{Na}V$ (nEq/min)	U_KV (nEq/min)	GFR (ml/g kidney/min)
CTRL	213.00 ± 4.51	182.00 ± 5.19	166.33 ± 6.17	3.48 ± 0.75	0.13 ± 0.008	220 ± 23.6	292 ± 123	1.04 ± 0.03
0.5 PD	211.00 ± 5.13	180.33 ± 6.01	165.33 ± 6.96	3.29 ± 0.65	0.08 ± 0.010	171 ± 31.1	409 ± 158	1.28 ± 0.02
1.0 PD	204.00 ± 2.52	171.33 ± 0.33	154.67 ± 0.88	3.31 ± 0.67	0.10 ± 0.025	178 ± 51.6	479 ± 54	1.09 ± 0.01
5.0 PD	204.00 ± 5.29	172.00 ± 6.56	155.67 ± 7.17	3.38 ± 0.58	0.08 ± 0.027	161 ± 17.1	643 ± 193*	1.35 ± 0.25
10.0 PD	202.33 ± 1.45	171.33 ± 3.18	155.33 ± 4.81	3.51 ± 0.43	0.14 ± 0.034	240 ± 39.4	675 ± 69*	1.09 ± 0.19
Recovery	201.66 ± 0.67	170.33 ± 2.33	154.67 ± 3.53	3.64 ± 0.44	0.16 ± 0.033	242 ± 51.9	670 ± 207*	0.97 ± 0.05

ANOVA, analysis of variance; FE_{Na}, fractional Na excretion; GFR, glomerular filtration rate; MAP, mean arterial pressure; period CTRL is the baseline (vehicle infusion); periods 0.5 PD, 1.0 PD, 5.0 PD, and 10.0 PD are with infusion of PD 128907 at dosages of 0.5, 1.0, 5.0, and 10.0 μg/kg/min; period recovery is the recovery period (vehicle infusion); SHR, spontaneously hypertensive rat (body wt 373 ± 13 g, *n*=3); U_KV, urine K excretion; $U_{Na}V$, urine Na excretion; *V*, urine flow.
*$P<0.05$ vs CTRL, repeated measures ANOVA (ANVR), Duncan's test.

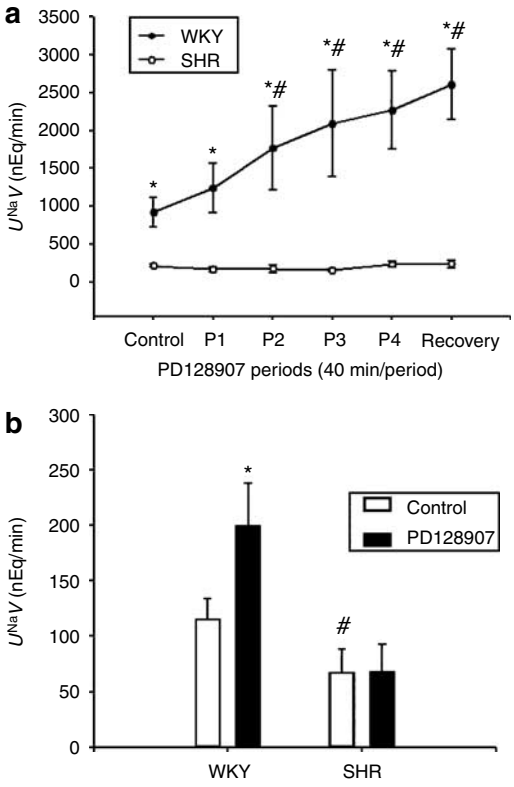

Figure 2 | Effect of the renal infusion of a D_3 receptor agonist on sodium excretion in WKY rats and SHRs. (**a**) Varying dosages of the D_3 receptor agonist, PD128907 (0.5–10.0 μg/kg/min), were infused into the right renal artery of rats on high-salt diet. *$P<0.05$ vs SHR, #$P<0.05$ vs control, $n=6$. There is no error bar when the symbol is bigger than the error bar. (**b**) PD128907 (1.0 μg/kg/min) was infused into the right renal artery of rats on normal salt diet. *$P<0.05$ vs control, #$P<0.05$ vs WKY rat, $n=3$.

overcome these limitations, we studied the renal effects of another selective D_3 receptor agonist, PD128907, which may be more selective for the D_3 receptor than 7-OH-DPAT, infused directly into the right renal artery, through the right suprarenal artery in WKY rats and SHRs on low-, normal, and high-salt diet. Activation of D_3 receptor with PD128907 induces natriuresis in WKY rats on low-, normal, or high-NaCl diet. In contrast, the effect of PD128907 on sodium excretion is impaired in SHRs fed on normal or high-salt diet.

Endothelin B receptors, which are expressed in RPTs, medullary thick ascending limbs of Henle, and collecting ducts of the kidney, have been shown to affect sodium transport *in vivo* and *in vitro*.[14,28,29] An inhibitory effect of the ETB receptor on ion and water transport in the renal medulla has been reported.[6,15,30] However, no effect, and inhibitory and stimulatory effects have been reported in the RPT.[15,30–33] Short-term stimulation of ETB receptors in opossum kidney cells, a proximal tubular cell line, activates the sodium hydrogen exchanger, NHE3.[33] However, a 6-h exposure of these opossum kidney cells to endothelin-1 inhibits NHE3 expression and activity, through ETB receptors.[34]

The mechanisms underlying the D_3 receptor-mediated natriuresis and diuresis are not completely understood. We have suggested that the D_3 receptor may interact with other G-protein-coupled receptors (GPCRs) to increase sodium excretion.[4] There are synergistic effects between renal D_1 and D_3 receptors,[12] and antagonistic effects between D_3 and AT_1 receptors on their expressions and blood pressure regulation.[35] Our current study shows that an ETB receptor antagonist, by itself, does not significantly affect sodium excretion but partially blocks the natriuretic effect of a D_3 receptor agonist in WKY rats, indicating that the natriuretic and diuretic effects of D_3 receptor are, in part, mediated by a mechanism involving ETB receptors. However, the interaction between D_3 and ETB receptors is different between WKY rats and SHRs; the D_3 receptor-mediated natriuresis in SHRs is impaired.

The mechanism(s) of the impaired D_3 receptor-mediated natriuresis in SHRs is not completely understood. Our previous study showed that D_3 receptor expression in the renal cortical membrane and RPT cells is decreased in SHRs relative to those from WKY rats.[12] To study another mechanism that may explain the difference in the natriuretic response to D_3 receptor stimulation between WKY rats and SHRs, the cellular distribution of D_3 and ETB receptors in rodent RPT cells was ascertained by confocal microscopy. Our study shows that both D_3 and ETB receptors are found in the plasma membrane and cytoplasm in WKY rat RPT cells at the basal state, indicating that ETB receptors are accessible for trans-regulation by agonist-activated membrane D_3 receptors. Indeed, agonist stimulation of D_3 receptors promotes the internalization of both D_3 and ETB

Table 6 | Effect of PD 128907 (D_3 agonist) on renal function in the infused right kidney of SHR on normal salt diet

	Systolic (mm Hg)	MAP (mm Hg)	Diastolic (mm Hg)	V (μl/min)	FE_{Na} (%)	$U_{Na}V$ (nEq/min)	U_KV (nEq/min)	GFR (ml/g kidney/min)
CTRL	167 ± 11	143 ± 11	123 ± 12	3.32 ± 1.06	0.052 ± 0.019	67 ± 21	442 ± 123	1.21 ± 0.09
w/PD	158 ± 5	121 ± 5	103 ± 7	3.84 ± 1.23	0.041 ± 0.012	70 ± 23	613 ± 113	1.40 ± 0.07
w/PD	163 ± 8	127 ± 5	106 ± 7	3.78 ± 0.73	0.046 ± 0.014	68 ± 25	683 ± 82	1.32 ± 0.13
w/PD	164 ± 6	130 ± 5	107 ± 6	3.39 ± 0.28	0.043 ± 0.015	67 ± 27	690 ± 126	1.38 ± 0.17
w/PD	165 ± 8	130 ± 7	107 ± 6	3.08 ± 0.53	0.057 ± 0.014	68 ± 13	599 ± 164	1.35 ± 0.18
w/PD	171 ± 4	130 ± 7	106 ± 8	2.40 ± 0.11	0.048 ± 0.017	69 ± 25	583 ± 86	1.25 ± 0.16
Recovery	171 ± 9	137 ± 11	109 ± 9	2.50 ± 0.29	0.040 ± 0.016	54 ± 18	545 ± 162	1.31 ± 0.16

FE_{Na}, fractional Na excretion; GFR, glomerular filtration rate; MAP, mean arterial pressure; NS, not significant; period CTRL is baseline (vehicle infusion); periods w/PD are the PD 128907 (1 μg/kg/min) infusion periods; period recovery is the recovery period (vehicle infusion); SHR, spontaneously hypertensive rat (body wt=297 ± 4 g, n=3; U_KV, urine K excretion; $U_{Na}V$, urine Na excretion; V, urine flow.
Data are expressed as means ± s.e. P=NS vs CTRL.

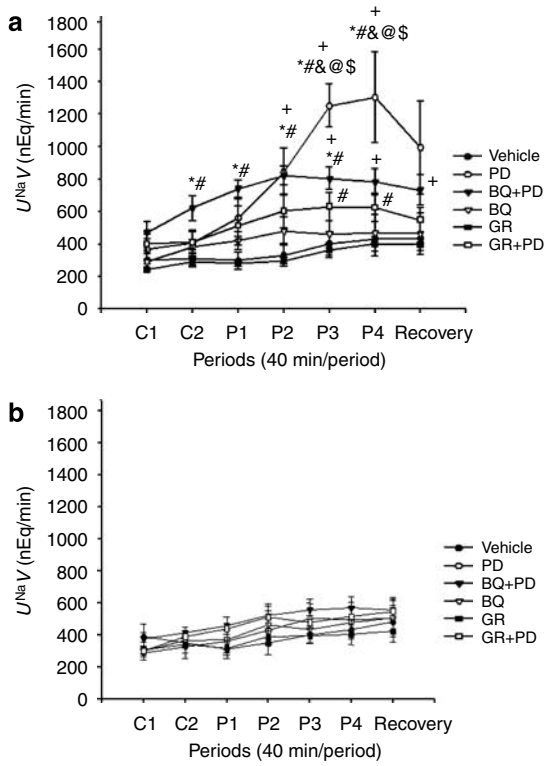

Figure 3 | Effects of D_3 or/and ETB receptors on sodium excretion in WKY rats. Effects of vehicle (n = 6), D_3 receptor agonist PD128907 (1.0 μg/kg/min; n = 6), D_3 receptor antagonist GR103691 (1.0 μg/kg/min; n = 4), ETB receptor antagonist BQ788 (5.0 μg/kg/min; n = 4), or in combination (PD128907 + GR103691, PD128907 + BQ788, n = 6/group) on sodium excretion ($U_{Na}V$) from the right kidney (**a**) and left kidney (**b**) of anesthetized WKY rats on high-salt diet. During control period 1 (C1), only the vehicle was infused. During control period 2 (C2), the vehicle was infused into both the vehicle and PD128907 groups; GR103961 was infused into the GR103961 group and the PD128907 + GR103961 group; and BQ788 was infused into the BQ788 group and the PD128907 + BQ788 group. During periods 3–6 (P1, P2, P3, and P4, respectively), PD128907 was infused into the PD128907 group, PD128907 and GR103961 were co-infused into the PD128907 + GR103691 group, and PD128907 and BQ788 were co-infused in the PD128907 + BQ788 group. During period 7, only the vehicle was infused into all groups; this was considered the recovery period. Data are expressed as mean ± s.e. $^*P<0.05$ vs vehicle, $^{\#}P<0.05$ vs GR, $^{\&}P<0.05$ vs GR + PD, $^{@}P<0.05$ vs BQ, $^{\$}P<0.05$ vs BQ + PD (factorial ANOVA), $^{+}P<0.05$ vs control (repeated measures ANOVA), Duncan's test.

Table 7 | Effect of a D_3 receptor agonist on the colocalization of D_3 and ETB receptors in cell membrane, and cytoplasm in WKY and SHR RPT cells

	WKY		SHR	
Structure	Basal	PD128907	Basal	PD128907
Membrane (%)	37.76 ± 3.00*	10.03 ± 3.13	6.04 ± 1.65	7.02 ± 1.99
Cytoplasm (%)	12.87 ± 2.41	40.95 ± 2.46*	20.01 ± 0.02	21.25 ± 0.64

ANOVA, analysis of variance; ETB, endothelin B; SHR, spontaneously hypertensive rat; RPT, renal proximal tubule; WKY rat, Wistar–Kyoto rat.
Twenty to forty regions of interest (cell membrane and cytoplasm) per cell (n=3/rat strain) were randomly selected and the extent of colocalization was determined as the degree (in %, Metamorph 6.1) of overlap between the fluorescent signals of D_3 and ETB receptors. Data are expressed as mean ± s.e.m.
$^*P<0.05$ vs others (same subcellular compartment), one-way factorial ANOVA Scheffe's test, n=3 experiments.

receptors, as well as their intracellular colocalization. However, in the basal state while the D_3 receptor is found in both the plasma membrane and cytoplasm, in SHR RPT cells, the ETB receptor is found exclusively in the cytoplasm making the receptor inaccessible to extracellular ligand stimulation. The intracellular distribution of ETB receptors may also explain the previously reported inability of the ETB receptor agonist, BQ3020, to increase ETB protein expression in SHR RPT cells. The D_3 receptor signal transduction pathway in SHR RPT cells is intact; treatment with the D_3 receptor agonist 7-OH-DPAT decreases the activity of NHE3, one of the downstream effectors of activated D_3 receptors, in SHR cells in a concentration-dependent manner, albeit to a lesser extent compared to those observed in WKY rats.[36] Thus, it is conceivable that the aberrant distribution of the ETB receptors in SHR cells hinders the access of other GPCRs, for example, D_3 receptors, and extracellular agonists to trans-regulate and activate other GPCRs, ETB receptor in this instance.

As observed in other GPCRs, agonist stimulation results in signal transduction and internalization of the agonist-occupied GPCR.[37,38] This results initially in desensitization but is required for resensitization, allowing the GPCR to respond to subsequent agonist stimulation. The decreased amount of D_3 receptors in plasma membrane in SHR cells (relative to WKY rat cells) and the failure of the few D_3 receptors at the plasma membrane of SHR cells to be

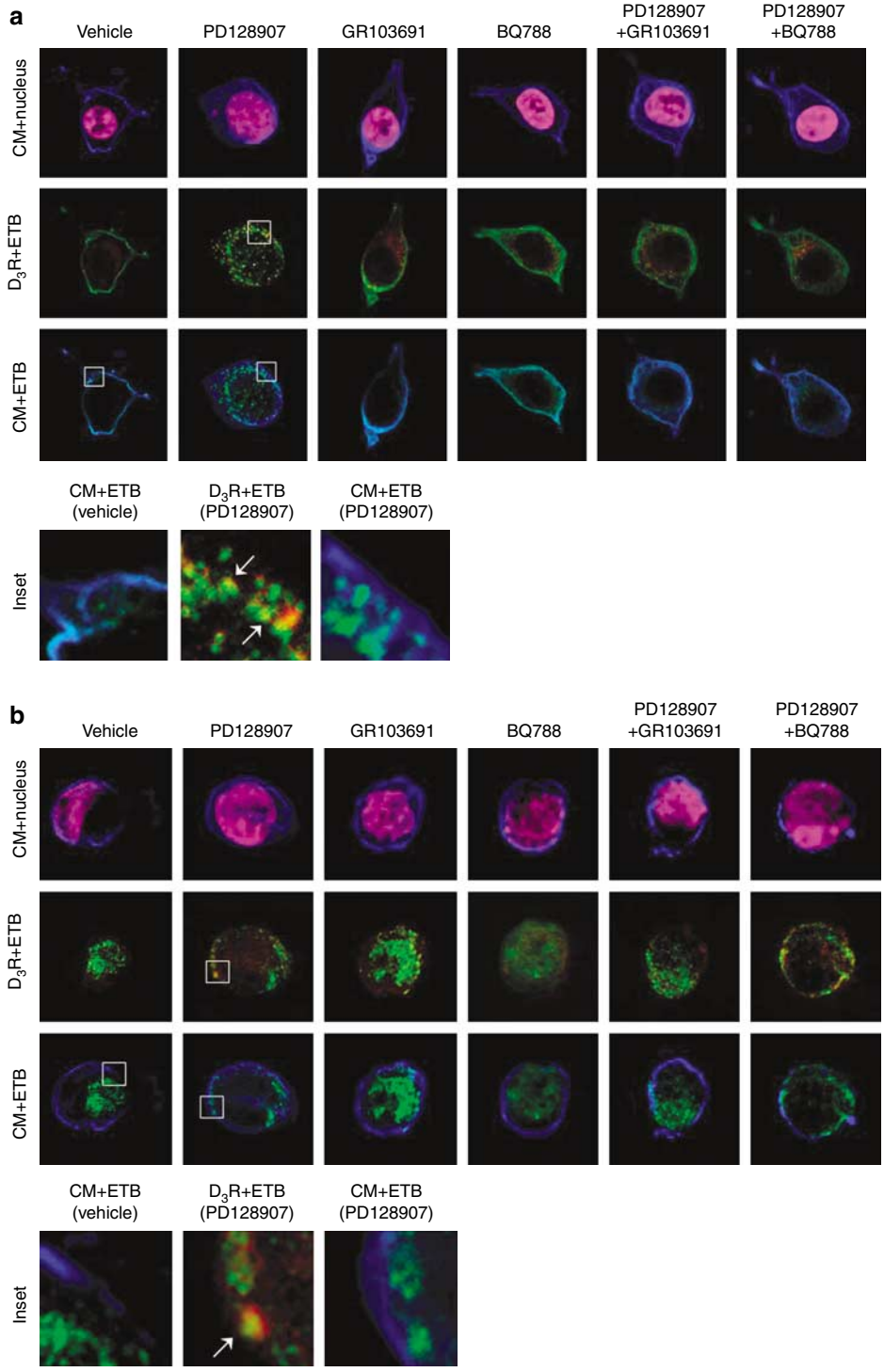

Figure 4 | Effect of D_3 receptor stimulation on ETB receptor cellular localization in immortalized RPT cells from WKY rats (a) and SHRs (b). The cells were serum-starved for 1 h and treated with the D_3 receptor agonist PD128907, D_3 receptor (D_3R) antagonist GR103691, ETB receptor antagonist BQ788, PBS (vehicle), or a combination of D_3 receptor agonist with either antagonist for 30 min. Images obtained by laser scanning confocal microscopy of D_3 receptor (pseudocolored red), ETB receptor (green), cell membrane (CM, blue), and nucleus (magenta) were overlain using Olympus Fluoview FV300 version 3C Acquisition Software. Inset images represent magnified versions of selected regions of the cell. Colocalization between D_3 and ETB receptors is shown in yellow (inset, white arrows), whereas that of ETB receptor and the cell membrane is in cyan (inset), $n = 3$ independent experiments.

internalized following receptor occupation prevent their resensitization, resulting in decreased agonist responsiveness of the D_3 receptor to current and subsequent stimulation in SHRs. It is possible that differences in total basal renal expression of D_3 and ETB receptors in WKY rats and SHRs could also participate in the differential effect of D_3 receptor

stimulation in these two rat strains. We have reported that RPT cell D_3 receptor expression is decreased in SHRs, relative to WKY rats.[35] Therefore, a decreased renal D_3 expression and D_3 and ETB interaction in SHRs may explain the decreased natriuretic effect of D_3 agonists in this rat strain. Although the proximal tubule is responsible for most of sodium reabsorption along the nephron, both D_3 and ETB receptors are present in more distal nephron segments, for example, medullary collecting duct.[14,39–42] Therefore, the D_3-receptor-mediated increase in sodium excretion involving the ETB receptor may involve nephron sites other than the proximal tubule.

In summary, we have demonstrated that the renal activation of the D_3 receptor induces natriuresis and diuresis in WKY rats on low-, normal, or high-NaCl diet. An ETB receptor antagonist, by itself, has no effect but partially blocks the natriuretic effect of a D_3 receptor agonist in WKY rats. In contrast, the D_3 receptor agonist does not affect sodium excretion in SHRs. In WKY rat RPT cells, the ETB receptors are located mainly at plasma membranes and thus are available for interaction with agonist-activated, membrane-located D_3 receptors. However, under basal conditions, the ETB receptors in SHR RPT cells are found mainly in the cytoplasm and are inaccessible to agonist-stimulated, membrane-located D_3 receptors. The aberrant ETB receptor distribution in the SHR RPT cells may explain, in part, the impaired ability to excrete a sodium load in spontaneous hypertension.

MATERIALS AND METHODS

Immunohistochemistry

The rat kidneys were cleared of blood with oxygenated saline and kept in Histochoice (Amresco, Kaysville, UT, USA) for 1–2 days at 4°C. The samples were embedded in paraffin and 4-μm sections mounted on slides. Immunostaining was performed, as reported,[43,44] using rabbit anti-rat ETB receptor antibody (Alomone Labs, Jerusalem, Israel) or rabbit anti-rat D_3 receptor antibody at 4°C overnight. The specificity of the antibodies has been proved in our previous experiments.[12,43] Biotinylated anti-rabbit-immunoglobulin-G and diaminobenzidine detection system (Vectastain ABC Kit; Vector Labs, Peterborough, UK) were used for color development.[44] The samples were counterstained with hematoxylin. Controls included antibody preadsorbed with its immunizing peptide.

Biotinylation, confocal microscopy, and quantification of D_3 receptor, and ETB receptor colocalization in rat RPTs and RPT cells

The kidneys were perfused with saline and flash-frozen in Optimal Cutting Temperature Compound.[44] The frozen kidney sections were mounted on slides, fixed with cold methanol for 10 min, permeabilized (0.1% Triton X-100 in phosphate-buffered saline (PBS)) for 30 min at room temperature, and blocked by 5% normal goat serum in PBS.

The WKY rat and SHR RPT cells are immortalized cell lines[45] and have characteristics similar to freshly obtained RPT brush border membranes and RPTs, at least with regard to D_1 receptors and their responses to G-protein stimulation.[46,47] The WKY rat and SHR RPT cells (<23 passages) were grown on coverslips to 40–50% confluence and treated for 30 min with D_3 receptor agonist (PD128907, 10^{-6} M), D_3 receptor antagonist (GR103691, 10^{-6} M), ETB receptor antagonist (BQ788, 10^{-6} M), vehicle (PBS), or a combination of the D_3 receptor agonist with either antagonist after serum starvation for 1 h. The cells were washed three times with ice-cold PBS and the cell membrane was labeled with the cell-impermeant EZ-link sulfo-NHS-SS-Biotin (1 mg/ml) for 30 min on ice. The cells were washed with ice-cold PBS, supplemented with 10 mM glycine, pH 7.0 to stop the reaction and remove the excess biotin, and then fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 in PBS. The RPTs or RPT cells were double immunostained for D_3 receptor and ETB receptor using monoclonal anti-D_3 receptor antibody (1:200; Zymed, South San Francisco, CA, USA) and the affinity-purified rabbit polyclonal anti-ETB receptor antibody (1:200), and then with goat anti-mouse secondary antibody conjugated with Alexa 633 and goat anti-rabbit secondary antibody conjugated with Alexa 568 (1:200 for each; Molecular Probes, Carlsbad, CA, USA). After incubating the cells with FITC-conjugated avidin for 10 min, the cells were washed with PBS and distilled water before mounting on glass slides using Vectashield mounting medium with 4,6-diamidino-2-phenylindole. Nail polish was used as sealant. Images were obtained for D_3 receptor (pseudocolored red), ETB receptor (pseudocolored green), cell membrane (pseudocolored blue), and the nucleus (pseudocolored magenta) through laser confocal microscopy. Corresponding images were overlain using the Olympus Fluoview FV300 version 3C Acquisition Software.[35,44] The experiments were repeated three times. Quantitative analysis of the extent of colocalization between D_3 and ETB receptors in both cells lines was performed (Metamorph 6.1; Molecular Devices). After subtraction of background fluorescence and thresholding, 30–40 regions of interest (cell membrane and cytoplasm) were selected and the degree (expressed as %) of overlapping signals for each region of interest was obtained using the 'colocalization' application of the software.

In vivo studies

Male WKY rats and SHRs (Taconic Farms, Germantown, NY, USA), ranging in age from 9 to 16 weeks and fed low- (0.06%), normal (0.4%), or high-(6%) sodium diet for 21 days[48] prior to the performance of the experiments, were anesthetized with pentobarbital (50 mg/kg body wt, intraperitoneally), placed on a heated table to maintain rectal temperature between 36 and 37°C, and tracheotomized (PE-240). Anesthesia was maintained by the infusion of pentobarbital at 0.8 mg/100 g body wt per h.[12] Catheters (PE-50) were placed into the external jugular and femoral veins, and femoral artery. Systemic arterial pressure was monitored electronically (Cardiomax II; Columbus Instruments, Columbus, OH, USA). Laparotomy was performed, and both the right and left ureters were catheterized (PE-10). The right renal artery was exposed, and the right suprarenal artery, which originates from the right renal artery, was catheterized (PE-10 heat stretched to 180 μm) and the vehicle (saline) or drugs was infused at a rate of 40 μl/h.[12] The duration of the surgical procedures was about 60 min. Fluid losses during surgery were replaced with 5% albumin at 1% body weight over 30 min. GFR was determined by the clearance of [^{14}C]-inulin (NEN, Boston, MA, USA) in normal saline infused at 5 ml/100 g body wt for 30 min, followed by a rate of 0.8 ml/100 g body wt per h until the end of the experiment, as previously reported.[12] After an equilibration period of 120 min, urine was collected every 40 min for clearance measurements.

In vivo studies, groups

Control group. In the control group, normal saline (vehicle) was infused into the right suprarenal artery.

Dose–response (PD128907) groups. After a baseline period, the WKY rats (on low-, normal, or high-salt diet) and SHRs (normal or high-salt diet) were infused, through the right renal artery, with PD128907 at a dose of 0.5, 1.0, 5.0, and 10.0 μg/kg/min.[13,35,48,49] Thereafter, the infusate was changed to the vehicle (recovery period); each period lasted 40 min.

Single-dose infusion groups. The WKY rats were divided into six groups: (1) vehicle; (2) D_3 receptor agonist (PD128907); (3) D_3 receptor antagonist (GR103691);[50] (4) ETB receptor antagonist (BQ788);[51] (5) combined D_3 receptor agonist and antagonist (PD128907 + GR103691); and (6) combined D_3 receptor agonist and ETB receptor antagonist (PD128907 + BQ788).

The vehicle group was treated as described for the control group. For the D_3 receptor agonist group, two baseline periods were obtained. Thereafter, PD128907 was infused (1.0 μg/kg/min) for four time periods, followed by one recovery period in which the drug infusion was stopped but the vehicle infusion was continued for another 40 min.

To determine the effect of blockade of D_3 or ETB receptors on basal renal function, GR103691 (1.0 μg/kg/min) or BQ788 (5.0 μg/kg/min) was infused during the second baseline period and continued for four periods followed by recovery. To determine the effect of a D_3 receptor blocker on D_3 receptor agonist effect, GR103691 was infused during the second baseline period, and then co-infused with PD128907 for four time periods, followed by recovery. To determine the effect of an ETB receptor blocker on D_3 receptor agonist effect, BQ788 was infused during the second baseline period, and then co-infused with PD128907 for four periods, followed by recovery. During the recovery period, all drug infusions were stopped and only the vehicle was infused; each period lasted 40 min.

Blood samples were obtained before starting the infusion of [^{14}C]-inulin, before the first collection period, and at the end of the experiment. Radioactivity, and sodium and potassium concentrations in the blood and urine samples were analyzed. The rats were killed by an overdose of pentobarbital (100 mg/kg body wt).

Statistical analysis

The data are expressed as mean ± s.e.m. Comparison within groups was made by repeated measures analysis of variance (ANOVA) with Duncan's or Holm–Sidak test (or paired *t*-test when only two groups were compared); comparison among groups was made by one-way factorial ANOVA with Duncan's, Holm–Sidak, or Scheffe's test. A value of $P < 0.05$ was considered significant.

DISCLOSURE

All the authors declared no competing interests.

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