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## D<sub>3</sub> Dopamine Receptor Regulation of ETB Receptors in Renal Proximal Tubule Cells From WKY and SHR

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### Abstract

**BACKGROUND**—The dopaminergic and endothelin systems, by regulating sodium transport in the renal proximal tubule (RPT), participate in the control of blood pressure. The D<sub>3</sub> and ETB receptors are expressed in RPTs, and D<sub>3</sub> receptor function in RPTs is impaired in spontaneously hypertensive rats (SHRs). Therefore, we tested the hypothesis that D<sub>3</sub> receptors can regulate ETB receptors, and that D<sub>3</sub> receptor regulation of ETB receptors in RPTs is impaired in SHRs.

**METHODS**—ETB receptor expression in RPT cells was measured by immunoblotting and reverse transcriptase–PCR and ETB receptor function by measuring Na<sup>+</sup>–K<sup>+</sup> ATPase activity. D<sub>3</sub>/ETB receptor interaction was studied by co-immunoprecipitation.

**RESULTS**—In Wistar-Kyoto (WKY) RPT cells, the D<sub>3</sub> receptor agonist, PD128907, increased ETB receptor protein expression, effects that were blocked by removal of calcium in the culture medium. The stimulatory effect of D<sub>3</sub> on ETB receptor mRNA and protein expression was also blocked by nifedipine. In contrast, in SHR RPT cells, PD128907 decreased ETB receptor expression. Basal D<sub>3</sub>/ETB receptor co-immunoprecipitation was three times greater in WKY than in SHRs. The absolute amount of D<sub>3</sub>/ETB receptor co-immunoprecipitation induced by a D<sub>3</sub> receptor agonist was also greater in WKY than in SHRs. Stimulation of ETB receptors decreased Na<sup>+</sup>–K<sup>+</sup> ATPase activity in WKY but not in SHR cells. Pretreatment with PD128907 augmented the inhibitory effect of BQ3020 on Na<sup>+</sup>–K<sup>+</sup> ATPase activity in WKY but not in SHR cells.

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**CONCLUSIONS**—D<sub>3</sub> receptors regulate ETB receptors by physical receptor interaction and govern receptor expression and function. D<sub>3</sub> receptor regulation of ETB receptors is aberrant in RPT cells from SHR.

Endothelin, originally characterized in vascular endothelial cells, is now known to be secreted by renal tubules where it can regulate sodium transport in an autocrine and/or paracrine manner.<sup>1-4</sup> Three endothelin isoforms (ET-1, ET-2, and ET-3) interact with two receptors, ETA and ETB. ETA receptors may contribute to the pathogenesis of hypertension by stimulating vasopressor centers in the brain, and increasing the secretion of aldosterone and the release of catecholamines, renal sodium transport, growth factors, reactive oxygen species, and vascular smooth muscle contractility.<sup>2-5</sup> However, endothelins can also relax vascular smooth muscles by releasing endothelium-derived vasodilators.<sup>3</sup> In addition, ETB receptors can lower blood pressure by decreasing ET-1 levels, and promoting renal loss of sodium and water.<sup>1,4</sup> Indeed, in uninephrectomized rats given deoxycorticosterone acetate and a high-salt diet, spontaneously hypertensive rats (SHRs) and humans with essential hypertension, ETB receptor activation may be a counter regulatory mechanism to the increase in blood pressure.<sup>1,4,6,7</sup> Naturally occurring or induced deletion of the ETB receptor gene and chronic pharmacological blockade of ETB receptors in rats result in salt-sensitive hypertension.<sup>1</sup>

The actions of dopamine on cardiovascular centers in the brain, renal hemodynamics, intestinal and renal epithelial transport, production of reactive oxygen species, and secretion of hormonal/humoral agents, such as aldosterone, catecholamines, endothelin, prolactin, pro-opiomelanocortin, renin, and vasopressin, place dopamine in a central homeostatic position for regulating extracellular fluid volume and blood pressure.<sup>8,9</sup> There are two D<sub>1</sub>-like receptors, D<sub>1</sub> and D<sub>5</sub>, and three D<sub>2</sub>-like receptors, D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub>, expressed in mammalian kidneys. Renal dopamine receptors,<sup>8,9</sup> like ETB receptors,<sup>7,10-14</sup> inhibit sodium transport. Specifically, dopamine, via D<sub>1</sub> and D<sub>3</sub> receptors, decreases renal sodium transport in various segments of the nephron, including the renal proximal tubule (RPT).<sup>8,9</sup> Disruption of either the D<sub>1</sub> or D<sub>3</sub> dopamine receptor gene in mice produces hypertension.<sup>9,15</sup> The hypertension in the D<sub>3</sub> receptor null (D<sub>3</sub><sup>-/-</sup>) mouse is associated with a decreased ability to excrete a sodium load.<sup>15</sup> In a previous study, we found that the natriuretic consequence of D<sub>3</sub> receptor stimulation is attenuated by an ETB receptor antagonist in Wistar-Kyoto (WKY) rats.<sup>16</sup> Because D<sub>3</sub> receptors in the kidney are predominantly expressed in RPTs where ETB receptors are also expressed,<sup>17-24</sup> we hypothesize that the ETB receptor is regulated by the D<sub>3</sub> receptor. Renal D<sub>3</sub> receptor function is impaired in SHRs;<sup>16,18,19</sup> therefore, we also hypothesize that D<sub>3</sub> receptor regulation of the ETB receptor may also be aberrant in SHRs. In order to test the above hypotheses, we studied D<sub>3</sub> and ETB receptor interaction in immortalized RPT cells. These RPT cells behave similarly to nonimmortalized RPT cells, at least with regard to dopamine receptors and responses to G protein stimulation.<sup>25-27</sup>

## METHODS

### Cell culture

Immortalized RPT cells (passages 25–40) from the S<sub>1</sub> segment of nephrons from 4- to 8-week-old WKY and SHRs were cultured at 37 °C in 95% air/5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium/F-12 culture media, as previously described.<sup>18</sup> The cells (80% confluent) were lysed in an ice-cold lysis buffer (phosphate-buffered saline with 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin), sonicated, kept on ice for 1 h, and centrifuged at 16,000 g for 30 min. The

supernatants were stored at  $-70^{\circ}\text{C}$  until use for immunoblotting and/or immunoprecipitation.

### Immunoblotting

Specific polyclonal  $\text{D}_3$  and ETB receptors were used.<sup>17–19,22</sup> Rat RPT cells were treated with vehicle ( $\text{dH}_2\text{O}$ ), a  $\text{D}_3$  receptor agonist (PD128907; Sigma, St Louis, MO),<sup>28</sup> or a  $\text{D}_3$  receptor antagonist (U99194A; Sigma)<sup>29</sup> at the indicated concentrations and durations of incubation.  $\text{D}_3$  and ETB receptor antibodies were specific because no specific bands were observed when the antibodies were preadsorbed with their respective immunizing peptides (1:10 wt/wt) for 12 h at  $4^{\circ}\text{C}$ .<sup>19,30</sup>

The densities of the bands were semiquantified by densitometry using Quantiscan (Ferguson, MO). When appropriate, the densities of the receptor bands were corrected by the densities of the  $\alpha$ -actin bands. All immunoblot bands in one group (receptor of interest or actin) were given a value of 100%. The density of each sample was calculated as a fraction of 100%. The ordinates indicate the ratio of the density of the protein of interest as a fraction of 100% and the density of actin as a fraction of 100%.

### Immunoprecipitation

RPT cells were incubated with vehicle or PD128907 ( $10^{-7}$  mol/l) for 24 h, as described earlier and immunoprecipitation was performed as reported, except that anti- $\text{D}_3$  and anti-ETB receptor antibodies were used.<sup>17–19,22</sup> In order to determine the specificity of the bands, normal rabbit IgG (negative control) and  $\text{D}_3$  receptor antibody (positive control) were used as the immunoprecipitants instead of the ETB receptor antibody. The densities of the bands were semiquantified by densitometry.<sup>17–19,25–27,31</sup>

### Determination of the second messenger(s) involved in the regulation of $\text{D}_3$ receptor on ETB receptor expression in WKY cells

To determine the second messenger(s) involved into the  $\text{D}_3$  receptor-mediated regulation of ETB receptor expression in WKY cells, several inhibitors or agonists were used: PKC inhibitor (19–31,  $10^{-6}$  mol/l), PKA inhibitor (14–22,  $10^{-6}$  mol/l), PKC activator (phorbol 12-myristate 13-acetate,  $10^{-7}$  mol/l), PKA activator (Sp-cAMP-S,  $10^{-7}$  mol/l), calcium channel blocker (nicardipine,  $10^{-6}$  mol/l), and calcium channel agonist (BAY-K8644,  $10^{-6}$  mol/l). PKC inhibitor 19–31, phorbol 12-myristate 13-acetate, Sp-cAMP-S, nicardipine, and BAY-K8644 were purchased from Sigma, PKA inhibitor 14–22 was purchased from Calbiochem (Darmstadt, Germany). The specificities of PKC inhibitor 19–31 and PKA inhibitor 14–22 were reported in our previous studies and those of others.<sup>32,33</sup>

### $\text{Na}^+ - \text{K}^+$ ATPase activity assay

$\text{Na}^+ - \text{K}^+$  ATPase activity was measured as the rate of inorganic phosphate released in the presence or absence of ouabain.<sup>34–36</sup> To prepare membranes for  $\text{Na}^+ - \text{K}^+$  ATPase activity assay, RPT cells cultured in  $21\text{-cm}^2$  plastic culture dishes were washed twice with 5 ml chilled phosphate-free buffer, and centrifuged at  $3,000 g$  for 10 min. The cells were then placed on ice and lysed in 2 ml lysis buffer (1 mmol/l  $\text{NaHCO}_3$ , 2 mmol/l  $\text{CaCl}_2$ , and 5 mmol/l  $\text{MgCl}_2$ ). The cell lysates were centrifuged at  $3,000 g$  for 2 min to remove intact cells, debris, and nuclei. The resulting supernatant was suspended in an equal volume of 1 mol/l NaI, and the mixture was centrifuged at  $48,000 g$  for 25 min. The pellet (membrane fraction) was washed two times and suspended in 10 mmol/l Tris and 1 mmol/l EDTA (pH 7.4). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to 1 mg/ml. The membranes were stored at  $-70^{\circ}\text{C}$  until use.

To measure Na<sup>+</sup>-K<sup>+</sup> ATPase activity, 100- $\mu$ l aliquots of membrane fraction were added to an 800  $\mu$ l reaction mixture (75 mmol/l NaCl, 5 mmol/l KCl, 5 mmol/l MgCl<sub>2</sub>, 6 mmol/l sodium azide, 1 mmol/l Na<sub>4</sub>EGTA, 37.5 mmol/l imidazole, 75 mmol/l Tris-HCl, and 30 mmol/l histidine; pH 7.4) with or without 1 mmol/l ouabain (final volume = 1 ml) and preincubated for 5 min in a water bath at 37 °C. Reactions were initiated by adding Tris-ATP (4 mmol/l) and terminated after 15 min of incubation at 37 °C by adding 50  $\mu$ l of 50% trichloroacetate. For determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures containing ouabain. To quantify the amount of phosphate produced, 1 ml of coloring reagent (10% ammonium molybdate in 10 N sulfuric acid) was mixed thoroughly with the assay mix and centrifuged at 3,000 *g* for 10 min. The formation of phosphomolybdate was determined spectrophotometrically at 740 nm, against a standard curve prepared from K<sub>2</sub>HPO<sub>4</sub>. Na<sup>+</sup>-K<sup>+</sup> ATPase activity was estimated as the difference between total and ouabain-insensitive ATPase activity and expressed as nmol phosphate released per mg protein per min.

### Reverse transcriptase-PCR of ETB receptors

A total of 2–3  $\mu$ g of total RNA extracted from WKY cells was used to synthesize cDNA which served as template for the amplification of ETB and  $\beta$ -actin (as housekeeping gene).<sup>37</sup> For  $\beta$ -actin, the forward primer was 5'-GTGGGTATGGGTCAGAAGGA-3' and the reverse primer was 5'-AGCGCGTAACCCCTCATAGAT-3' (GenBank accession no. NM031144). The amplification was performed with the following conditions: denaturation at 94 °C for 30 s, annealing for 30 s at 54 °C, and extension for 45 s at 72 °C for 35 cycles. For the ETB receptor, the forward primer was 5'-TTACAAGACAGCCAAAGACT-3' and the reverse primer was 5'-CACGATGAGGACAATGAGAT-3' (GenBank accession no. X57764). The amplification was performed with the following conditions: denaturation at 94 °C for 30 s, annealing for 30 s at 69 °C, and extension for 45 s at 72 °C for 35 cycles. The ETB receptor mRNA expression was normalized for  $\beta$ -actin mRNA.

### Statistical analysis

The data are expressed as mean  $\pm$  s.e.m. Comparison within groups was made by repeated measures analysis of variance with Duncan's test (or Student's *t*-test when only two groups were compared); comparison among groups was made by factorial analysis of variance with Duncan's test. A value of *P* < 0.05 was considered significant.

## RESULTS

### D<sub>3</sub> receptors increase ETB receptor expression in WKY RPT cells, but decrease it in SHR cells

A D<sub>3</sub> receptor agonist, PD128907, increased ETB receptor protein expression in a concentration- and time-dependent manner in WKY RPT cells. The stimulatory effect was evident at 10<sup>-7</sup> mol/l (**Figure 1**), noted as early as 8 h, and maintained for at least 24 h (data not shown). In contrast, in SHR RPT cells, PD128907 (10<sup>-7</sup> mol/l/24 h) decreased ETB receptor expression (WKY: control = 1.0  $\pm$  0.1, PD128907 (10<sup>-7</sup> mol/l) = 1.5  $\pm$  0.1; SHR: control = 1.0  $\pm$  0.1, PD128907 (10<sup>-7</sup> mol/l) = 0.7  $\pm$  0.2 density units (DU); *n* = 9/group) (**Figure 2**), that was also concentration dependent (**Figure 1**).

The specificity of PD128907 as a D<sub>3</sub> receptor agonist was determined using the D<sub>3</sub> receptor antagonist, U99194A, in WKY RPT cells. Consistent with the results shown in **Figure 1**, PD128907 (10<sup>-7</sup> mol/l/24 h) increased ETB receptor expression (control = 0.9  $\pm$  0.09, PD128907 = 1.4  $\pm$  0.08 DU; *n* = 6, *P* < 0.05). U99194A (10<sup>-7</sup> mol/l), by itself, had no effect on ETB receptor expression (0.98  $\pm$  0.06 DU), but reversed the stimulatory effect of PD128907 on ETB receptor expression (0.9  $\pm$  0.08 DU) (**Figure 3**) in WKY RPT cells. The

inhibitory effect of D<sub>3</sub> receptor on ETB receptor expression in SHR cells was also blocked by D<sub>3</sub> receptor antagonist, U99194A (**Figure 3**).

A calcium channel blocker (nicardipine, 10<sup>-6</sup> mol/l), by itself, had no effect on ETB receptor expression, but blocked the stimulatory effect of PD128907 on ETB receptor expression in WKY cells (**Figure 4**). To determine, further, the importance of extracellular calcium entry in the D<sub>3</sub> receptor-mediated upregulation of ETB receptor expression, we studied the effect of PD128907 on RPT cells grown in culture medium with or without calcium. The ability of PD128907 to upregulate the expression of ETB receptor protein was lost when the WKY RPT cells were maintained in calcium-free medium, indicating the requirement for calcium entry in this action (calcium-free medium: control = 0.87 ± 0.09, PD128907 = 0.9 ± 0.02 DU; regular medium with calcium: control = 0.9 ± 0.02, PD128907 = 1.4 ± 0.05 DU; *n* = 5, *P* < 0.05) (**Figure 5**).

We also studied the effect of other cell signaling reagents: PKC inhibitor (19–31, 10<sup>-6</sup> mol/l); PKA inhibitor (14–22, 10<sup>-6</sup> mol/l); PKC activator (phorbol 12-myristate 13-acetate, 10<sup>-7</sup> mol/l), PKA activator (Sp-cAMP-S, 10<sup>-7</sup> mol/l); and calcium channel agonist (BAY-K8644, 10<sup>-6</sup> mol/l). None of those reagents could block the stimulatory effect of D<sub>3</sub> receptor on ETB receptor expression (data not shown).

In additional studies, we measured ETB receptor mRNA expression by reverse transcriptase-PCR and found that the activation of D<sub>3</sub> receptor with PD128907 (10<sup>-7</sup> mol/l) for 24 h increased ETB receptor mRNA expression in WKY cells. However, in the presence of the calcium channel blocker, nicardipine (10<sup>-6</sup> mol/l/24 h), the upregulation of D<sub>3</sub> receptor on ETB receptor mRNA expression was lost (control = 0.82 ± 0.03, PD128907 = 1.0 ± 0.03 DU; nicardipine = 0.79 ± 0.04, nicardipine + PD128907 = 0.83 ± 0.02, *n* = 5, *P* < 0.05).

### D<sub>3</sub> receptor co-immunoprecipitates with the ETB receptor in rat RPT cells

We next determined whether there is a physical interaction between the D<sub>3</sub> and the ETB receptor. ETB receptors were first immunoprecipitated with anti-ETB receptor antibodies and then probed with anti-D<sub>3</sub> receptor antibodies. As shown in **Figure 6**, the density of the 45 kd band, representing the co-immunoprecipitated D<sub>3</sub> receptors with the ETB receptors, was increased by a 24-h treatment with PD128907 (10<sup>-7</sup> mol/l) in WKY and SHR RPT cells (WKY: control = 20 ± 5, PD128907 = 33 ± 4 DU; SHRs: control = 3.4 ± 0.4, PD128907 = 6.0 ± 0.8 DU; *n* = 8, *P* < 0.05). The absolute increase in the amount of D<sub>3</sub>/ETB co-immunoprecipitation with PD128907 (10<sup>-7</sup> mol/l/24 h) treatment was greater in RPT cells from WKY than from SHRs (WKY: 14 ± 6 DU vs. SHR: 2.6 ± 0.8 DU, *n* = 8, *P* < 0.05), although the percent increases were similar. However, basal co-immunoprecipitation was greater in RPT cells from WKY than those from SHRs.

### Pretreatment with PD128907 increases the inhibitory effect of ETB receptor on Na<sup>+</sup>-K<sup>+</sup> ATPase activity in WKY cells but not in SHR cells

To investigate the physiological significance of the D<sub>3</sub>/ETB receptor interaction, the effects of D<sub>3</sub> or/and ETB receptor stimulation on Na<sup>+</sup>-K<sup>+</sup> ATPase activity were determined in WKY and SHR cells. Stimulation of ETB receptors by BQ3020 (10<sup>-8</sup> mol/l/15 min; Sigma) decreased Na<sup>+</sup>-K<sup>+</sup> ATPase activities in WKY cells, which could be blocked by the ETB receptor antagonist, BQ788 (10<sup>-8</sup> mol/l/15 min; Sigma) (control = 0.39 ± 0.02, BQ3020 = 0.26 ± 0.03; BQ788 = 0.40 ± 0.06, BQ3020 + BQ788 = 0.37 ± 0.04 nmol/mg protein/min; *n* = 5, *P* < 0.05). In contrast, the inhibitory effect of ETB receptor on Na<sup>+</sup>-K<sup>+</sup> ATPase activity was lost in SHR cells (**Figure 7**). Pretreatment with PD128907 (10<sup>-7</sup> mol/l) for 24 h augmented the inhibitory effect of BQ3020 (10<sup>-8</sup> mol/l/15 min) on Na<sup>+</sup>-K<sup>+</sup> ATPase activity

in WKY but not in SHR cells. The basal level of  $\text{Na}^+\text{-K}^+$  ATPase activity was higher in SHR than in WKY cells (**Figure 7**).

## DISCUSSION

ETB 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*.<sup>20-22,38,39</sup> An inhibitory effect has been found in collecting ducts and medullary thick ascending limbs of Henle.<sup>3,4,12,13</sup> However, no effect, inhibitory, and stimulatory effects have been reported in the proximal tubule.<sup>10,13,14,39</sup> Short-term stimulation of ETB receptors in opossum kidney cells, a proximal tubular cell line, activates the sodium hydrogen exchanger, NHE3.<sup>39</sup> In contrast, a 6-h exposure of these opossum kidney cells to endothelin-1 inhibits NHE3 expression and activity via ETB receptors.<sup>14</sup> It is of interest that the ability of the  $\text{D}_3$  receptor agonist to increase ETB expression also occurs within the same time frame, 8 h, as the ability of ETB to inhibit NHE3 expression and activity. In this study, we showed that activation of ETB receptors in the short term (15 min) could inhibit  $\text{Na}^+\text{-K}^+$  ATPase activity in RPT cells from WKY rats.

The natriuretic effect of dopamine is exerted mainly via  $\text{D}_1$ -like receptors.<sup>8,9</sup> The effect of  $\text{D}_2$ -like receptors, independent of  $\text{D}_1$ -like receptors, on sodium excretion has ranged from antinatriuresis, no effect, to natriuresis.<sup>8,9</sup> It is possible that these discrepant effects are related to the use of drugs that have poor selectivity to the  $\text{D}_2$ -like receptor subtypes.<sup>28,29,40,41</sup> 7-OH-DPAT and PD128907, which are  $\text{D}_3$  receptor agonists with preferential selectivity for  $\text{D}_3$  over  $\text{D}_2$  and  $\text{D}_4$  receptors,<sup>28,29</sup> decrease sodium transport. The acute intravenous administration of 7-OH-DPAT in Dahl salt-resistant rats increases sodium and water excretion without affecting blood pressure.<sup>42</sup> The selective intrarenal infusion of PD128907 in WKY rats also induces a natriuresis that is attenuated by a  $\text{D}_3$  receptor antagonist, GR103691.<sup>16</sup> That  $\text{D}_3$  receptors can mediate natriuresis is supported by the decreased ability of  $\text{D}_3$  receptor deficient mice to excrete an acute saline load.<sup>15</sup>

The  $\text{D}_3$  receptor may also interact with other G protein-coupled receptors to increase sodium excretion. We have reported synergistic effects between renal  $\text{D}_1$  and  $\text{D}_3$  receptors,<sup>43</sup> and antagonistic effects between  $\text{D}_3$  and  $\text{AT}_1$  receptors on their expressions<sup>18,31</sup> and blood pressure regulation.<sup>15</sup> Our previous study also showed that  $\text{D}_3$  and ETB receptors colocalize in RPTs of WKY rats similar to the colocalization observed in immortalized RPT cells in this study. The natriuretic effect of the  $\text{D}_3$  receptor agonist, PD128907 in WKY rats, is attenuated by an ETB receptor antagonist (BQ788).<sup>16</sup> It is possible that the ability of the  $\text{D}_3$  receptor to affect the cellular trafficking of the ETB receptor participates in the acute interaction between  $\text{D}_3$  and ETB receptors to affect sodium transport acutely.

It is likely that  $\text{D}_3$  receptors can regulate ETB receptor expression in the long term. To determine the mechanisms by which the  $\text{D}_3$  receptor regulates ETB receptor, we studied the influence of a  $\text{D}_3$  receptor agonist (PD128907) on ETB receptor expression in RPT cells. We found that stimulation of the  $\text{D}_3$  receptor increases ETB receptor protein in WKY RPT cells. The  $\text{D}_3$  receptor regulation of ETB receptor expression cannot be ascribed to increased endothelin synthesis, because stimulation of  $\text{D}_3$  receptors with PD128907 for 24 or 48 h has no effect on endothelin production in WKY RPT cells (data not shown). However, pretreatment of WKY cells with a  $\text{D}_3$  receptor agonist for 24 h augments the ETB receptor-mediated inhibitory effect on  $\text{Na}^+\text{-K}^+$  ATPase activity in WKY cells. Therefore, it is possible that the long-term natriuretic effect of  $\text{D}_3$  receptors may also be, in part, mediated by ETB receptors.

The D<sub>3</sub> receptor has been shown to increase calcium flux in isolated nerve terminals and human embryonic kidney 293 cells stably expressing human D<sub>3</sub> receptors and Gα(qo5).<sup>44,45</sup> It has also been reported that activation of the L-type calcium channel stimulates protein synthesis in peripheral blood mononuclear cells.<sup>46</sup> We find in the current study that the stimulatory effect of D<sub>3</sub> receptor on ETB receptor expression is blocked by a calcium channel blocker and the removal of calcium in the cell culture medium. Therefore, the D<sub>3</sub> receptor stimulates ETB receptor expression by increasing intracellular calcium concentrations by activation of calcium channels. Furthermore, the regulation may occur at the transcriptional/post-transcriptional level. The detailed mechanisms remain to be elucidated in the future.

The interaction between D<sub>3</sub> and ETB receptors is different between WKY and SHRs. D<sub>3</sub> receptor stimulation does not elicit a natriuresis in SHRs,<sup>16</sup> and pretreatment of SHR cells with a D<sub>3</sub> receptor agonist for 24 h does not augment the ETB receptor-mediated inhibitory effect on Na<sup>+</sup>-K<sup>+</sup> ATPase that is observed in WKY cells. In contrast to the stimulatory effect of a D<sub>3</sub> receptor agonist on ETB expression in WKY rats, the opposite occurs in SHRs. The aberrant interaction between D<sub>3</sub> and ETB receptor might be involved into the pathogenesis of hypertension.

We also find that D<sub>3</sub> and ETB receptors can physically interact, as determined by their co-immunoprecipitation. D<sub>3</sub> receptor stimulation results in an increase in the interaction co-immunoprecipitation of D<sub>3</sub> and ETB receptors in WKY RPT cells. The increase in D<sub>3</sub>/ETB receptor co-immunoprecipitation in RPT cells following D<sub>3</sub> receptor agonist stimulation could have been caused by the increase in the expression of either the ETB or D<sub>3</sub> receptors. We have reported that D<sub>3</sub> receptor stimulation increases its own receptor expression in RPT cells from WKY rats.<sup>31</sup> It is also possible that the D<sub>3</sub> receptor agonist could have increased the physical interaction between the D<sub>3</sub> and ETB receptors. However, further studies are needed to determine whether the increased interaction between these two receptors in WKY rats is a direct or an indirect mechanism, possibly by the alteration of an adapter gene or adapter proteins.

In the basal state, there is less D<sub>3</sub>/ETB receptor co-immunoprecipitation in SHR RPT cells compared to WKY RPT cells. This could be due to the decreased D<sub>3</sub> receptor expression in SHR cells relative to WKY cells, but could not be caused by differences in ETB receptor expression, because the expression of renal ETB receptor is the same in WKY and SHRs. Moreover, the percent increase in D<sub>3</sub>/ETB co-immunoprecipitation with the D<sub>3</sub> receptor agonist is similar in the two rat strains. The mechanism for the decreased basal D<sub>3</sub>/ETB co-immunoprecipitation in SHR cells remains to be determined.

In summary, we have demonstrated that D<sub>3</sub> receptors regulate the expression and function of ETB receptors in immortalized rat RPT cells. Furthermore, D<sub>3</sub> and ETB receptors co-immunoprecipitate in RPT cells and D<sub>3</sub> receptor agonist stimulation enhances the interaction between these two G protein-coupled receptors. In RPT cells from SHRs, this interaction between D<sub>3</sub> and ETB receptors is aberrant, which may participate in the increased renal sodium transport in spontaneous hypertension.

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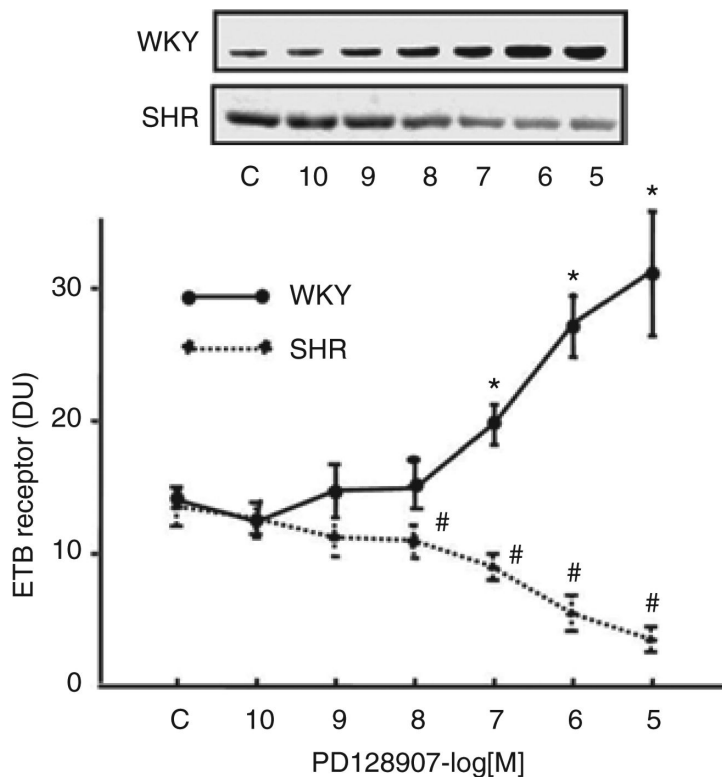
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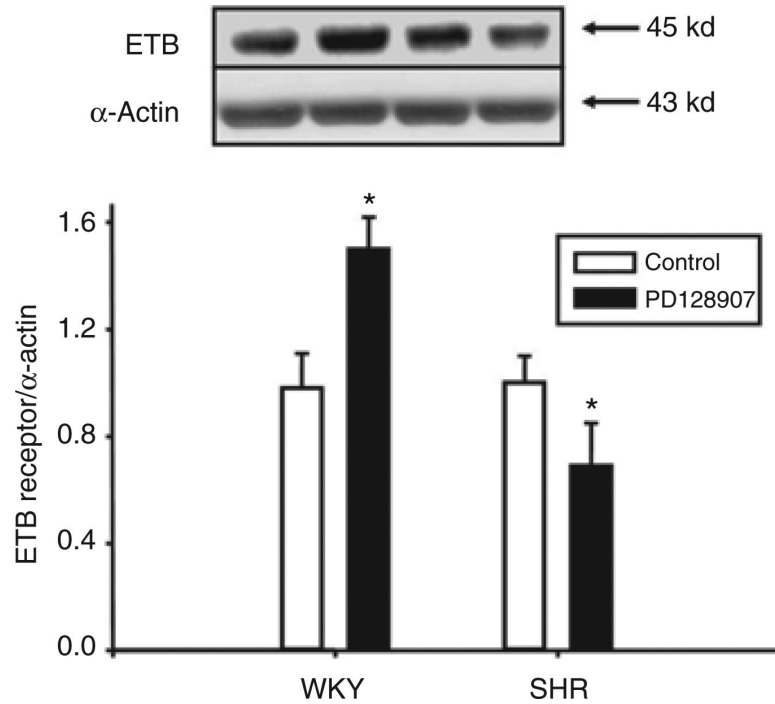


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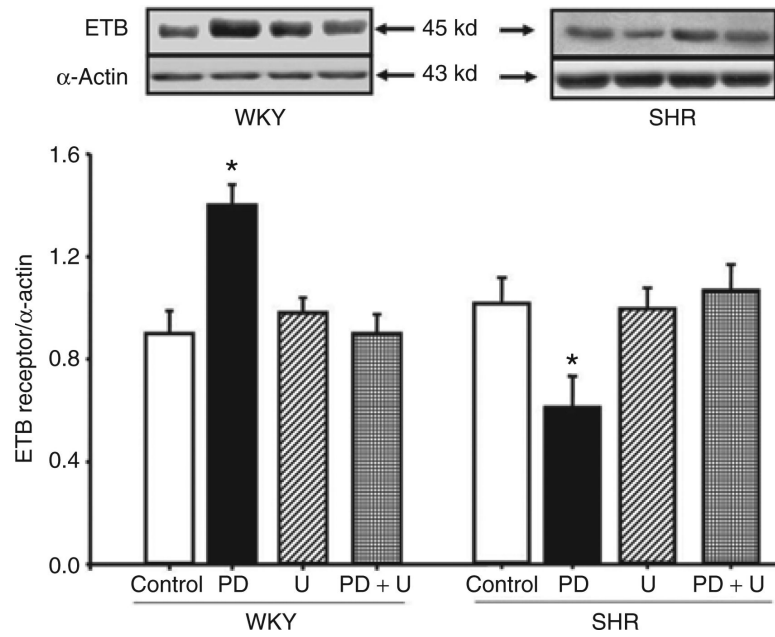
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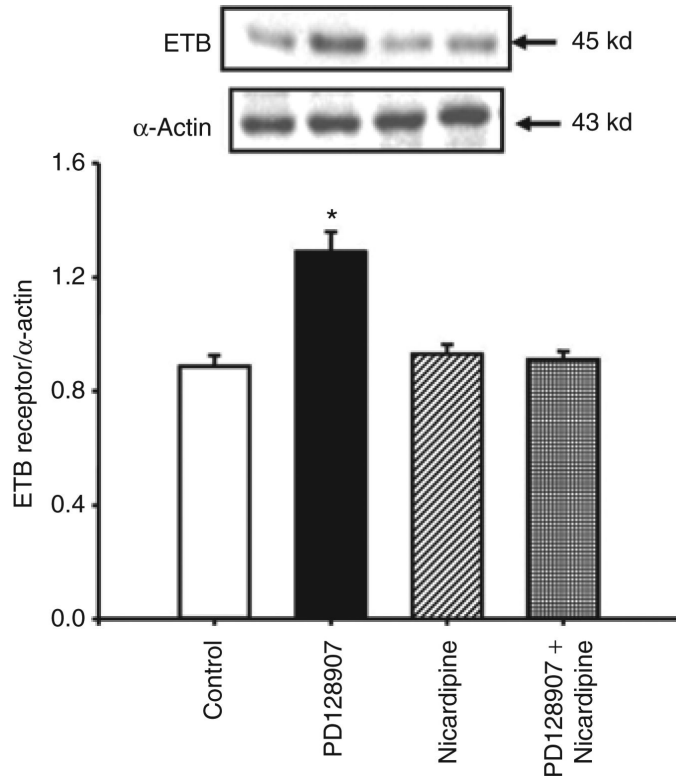
**Figure 1.** Concentration response of ETB receptor protein expression to the D<sub>3</sub> receptor agonist, PD128907 in renal proximal tubule cells from SHR and WKY rats. Immunoreactive ETB receptor band was quantified after 24-h incubation with the indicated concentrations of PD128907. Results are expressed as density units (DU) as a fraction of 100% and the seven samples from each rat strain set at 100% (WKY: *n* = 5, \**P* < 0.05 vs. control (C); SHR: *n* = 10, #*P* < 0.05 vs. C, analysis of variance, Duncan's test). SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto.



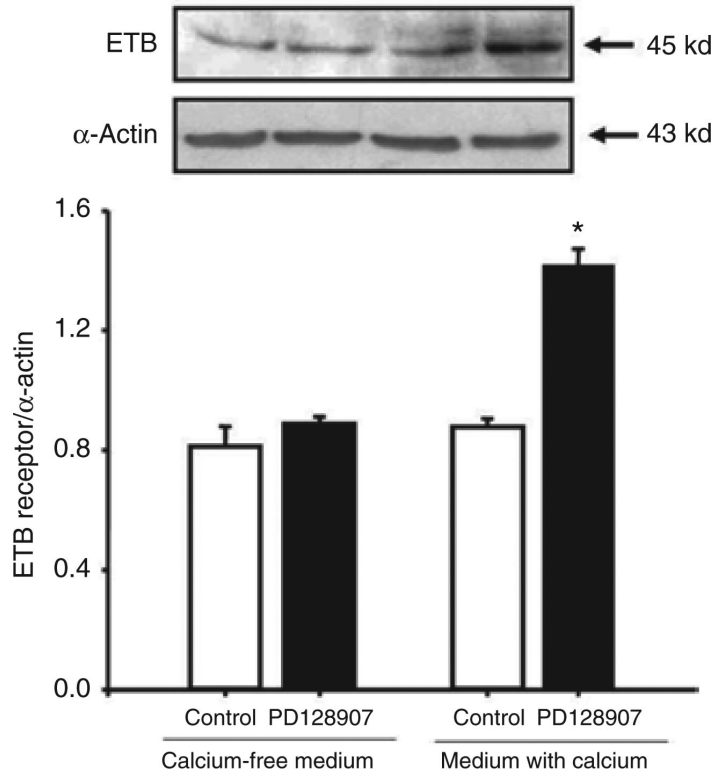
**Figure 2.** Differential effect of a D<sub>3</sub> receptor agonist, PD128907 ( $10^{-7}$  mol/l/24 h), on ETB receptor protein in WKY and SHR renal proximal tubule cells. The cells were incubated at the indicated time and concentration. Results are expressed as the ratio of ETB receptor to  $\alpha$ -actin densities ( $n = 11$ , \* $P < 0.05$  vs. control, analysis of variance, Duncan's test). SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto.



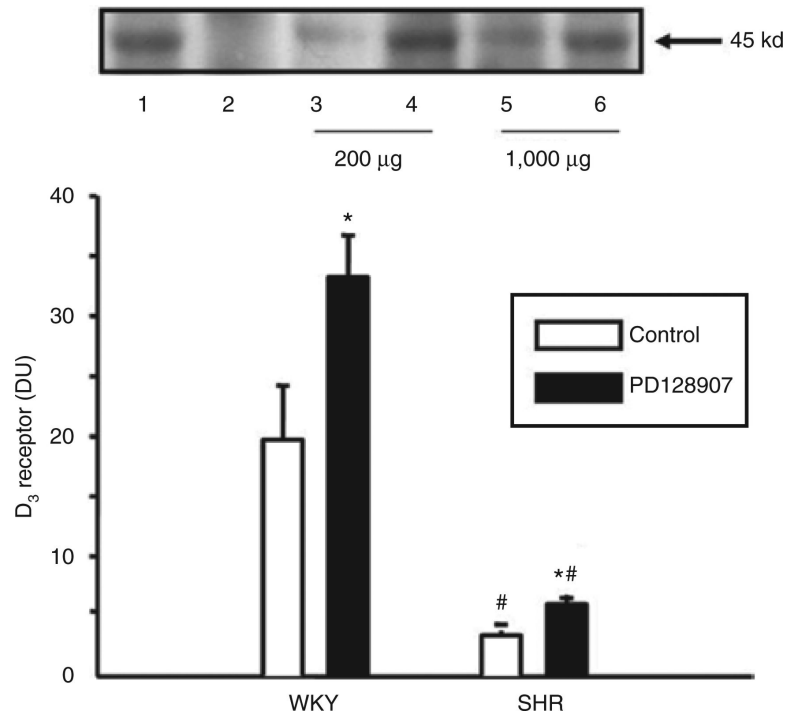
**Figure 3.** Effect of a D<sub>3</sub> receptor agonist (PD128907) and a D<sub>3</sub> receptor antagonist (U99194A) on ETB receptor protein in renal proximal tubule cells from SHR ( $n = 5$ ) and WKY ( $n = 6$ ) rats. The cells were incubated with the indicated reagents (PD128907,  $10^{-7}$  mol/l; U99194A,  $10^{-7}$  mol/l) for 24 h. Results are expressed as the ratio of ETB receptor to  $\alpha$ -actin densities ( $*P < 0.05$  vs. others, analysis of variance, Duncan's test). PD, PD128907; U, U99194A. SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto.



**Figure 4.** Effect of a D<sub>3</sub> receptor agonist (PD128907) and a calcium channel blocker (nicardipine) on ETB receptor protein in Wistar-Kyoto renal proximal tubule cells. The cells were incubated with the indicated reagents (PD128907,  $10^{-7}$  mol/l; nicardipine,  $10^{-6}$  mol/l) for 24 h. Results are expressed as the ratio of ETB receptor to  $\alpha$ -actin densities ( $n = 5$ /group, \* $P < 0.05$  vs. others, analysis of variance, Duncan's test).



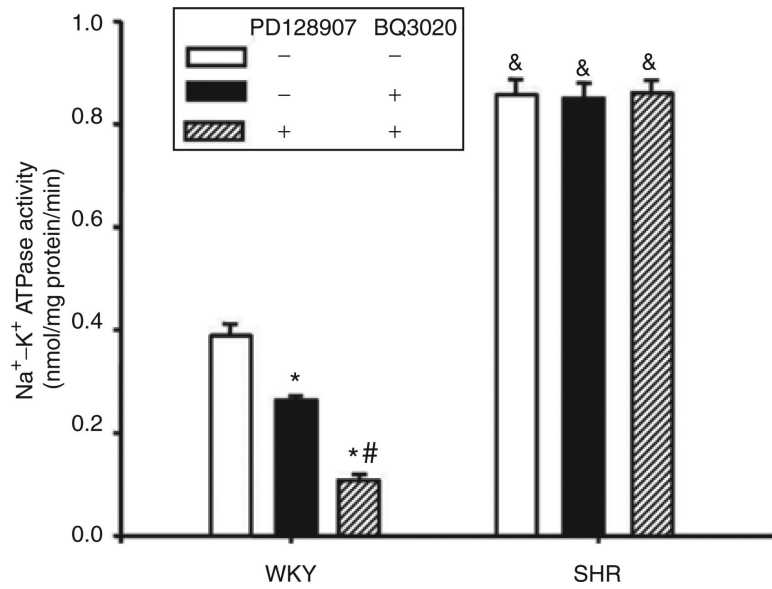
**Figure 5.** Effect of a D<sub>3</sub> receptor agonist, PD128907, on ETB receptor expression in Wistar-Kyoto cells grown in medium with or without calcium. The cells were incubated with PD128907 (10<sup>-7</sup> mol/l) for 24 h. Results are expressed as the ratio of ETB receptor to α-actin densities (n = 5, \*P < 0.05 vs. others, analysis of variance, Duncan's test).



**Figure 6.**

Effect of a D<sub>3</sub> receptor agonist, PD128907, on the co-immunoprecipitation of D<sub>3</sub> and ETB receptors in WKY and SHR RPT cells. The cells were incubated with PD128907 ( $10^{-7}$  mol/l) for 24 h. Thereafter, the samples were immunoprecipitated with an anti-ETB receptor antibody and immunoblotted with an anti-D<sub>3</sub> antibody (\* $P < 0.05$  vs. control, # $P < 0.05$  vs. WKY  $n = 8$ /group, analysis of variance, Duncan's test). One immunoblot (45 kd) is depicted in the upper panel: (lane 1, positive control; lane 2, negative control; lane 3, vehicle-treated RPT cells from WKY rats; lane 4, PD128907-treated RPT cells from WKY rats; lane 5, vehicle-treated RPT cells from SHRs; lane 6, PD128907-treated RPT cells from SHRs). For a positive control, anti-D<sub>3</sub> antibodies (1 µg/ml) were used as the immunoprecipitant; for a negative control, normal rabbit IgG (1 µg/ml) was used as the immunoprecipitant, instead of the anti-ETB antibodies, and immunoblotted with anti-D<sub>3</sub> antibodies as above. The amount of D<sub>3</sub> receptor antibody needed to immunoprecipitate ETB receptors in SHR RPT cells (1,000 µg, lanes 5 and 6) is five times that used in WKY RPT cells (200 µg, lanes 3 and 4). RPT, renal proximal tubule; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto.





**Figure 7.**

Effect of pretreatment with a D<sub>3</sub> receptor agonist, PD128907, on the inhibitory effect of ETB receptor on Na<sup>+</sup>-K<sup>+</sup> ATPase activity in WKY and SHR RPT cells. The cells were pretreated with PD128907 (10<sup>-7</sup> mol/l) or vehicle (dH<sub>2</sub>O) for 24 h. After washing for 15 min, the cells were treated with the ETB receptor agonist, BQ3020 (10<sup>-8</sup> mol/l), for 15 min. Results are expressed as nmol phosphate released per mg protein per min (\**P* < 0.05 vs. control, #*P* < 0.05 vs. BQ3020, &*P* < 0.05 vs. WKY, *n* = 6/group, analysis of variance, Duncan's test). RPT, renal proximal tubule; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto.