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Impaired Stimulatory Effect of ETB Receptor on D_3 Receptor in Immortalized Renal Proximal Tubule Cells of Spontaneously Hypertensive Rats

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Key Words
Essential hypertension · Dopamine receptor · Endothelin receptor

Abstract
Background: Activation of renal D_3 receptor induces natriuresis and diuresis in Wistar-Kyoto (WKY) rats; in the presence of ETB receptor antagonist, the natriuretic effect of D_3 receptor in WKY rats is reduced. We hypothesize that ETB receptor activation may regulate D_3 receptor expression in renal proximal tubule (RPT) cells from WKY rats, which is impaired in RPT cells from spontaneously hypertensive rats (SHRs). ***Methods:*** D_3 receptor expression was determined by immunoblotting; the D_3/ETB receptor linkage was checked by coimmunoprecipitation; Na^+-K^+-ATPase activity was determined as the rate of inorganic phosphate released in the presence or absence of ouabain. ***Results:*** In RPT cells from WKY rats, the ETB receptor agonist BQ3020 increased D_3 receptor protein. In contrast, in RPT cells from SHRs, BQ3020 did not increase D_3 receptor. There was coimmunoprecipitation between D_3 and ETB receptors in RPT cells from WKY and SHRs. Activation of ETB receptor increased D_3/ETB coimmunoprecipitation in RPT cells from WKY rats, but not from SHRs. The basal levels of D_3/ETB receptor coimmunoprecipitation were greater in RPT cells from WKY rats than in those from SHRs. Stimulation of D_3 receptor inhibited Na^+-K^+-ATPase activity, which was augmented by the pretreatment with the ETB receptor agonist BQ3020 in WKY RPT cells, but not in SHR RPT cells. ***Conclusion:*** ETB receptors regulate and physically interact with D_3 receptors differently in WKY rats and SHRs. The impaired natriuretic effect in SHRs may be, in part, related to impaired ETB and D_3 receptor interactions.

Introduction

Dopamine has been recognized as an important modulator of central as well as peripheral physiologic functions in both humans and animals, which produces its biologic effects through five genetically distinct dopamine receptor subtypes: D_1, D_2, D_3, D_4, and D_5. These receptors are categorized into two groups known as D_1-like (D_1 and D_5, whose rat homologs are D_{1A} and D_{1B}) and D_2-like (D_2, D_3, and D_4) dopamine receptors based on their ability to stimulate and inhibit adenylyl cyclase, re-

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spectively [1–3]. Dopamine receptors have been identified in a number of organs and tissues, which include kidney [4–6]. Within the kidney, dopamine receptors are present along the nephron, with highest density on renal proximal tubule (RPT) cells, where activation of dopamine receptor, mainly via D_1 and D_3 receptor, decreases sodium transport [1–3]. Under euvolemic conditions and magnified during moderate volume expansion, dopamine acts to increase sodium excretion and decrease blood pressure [1–3].

Endothelin, like dopamine, is produced by renal tubules where it can regulate sodium transport in an autocrine or paracrine manner [7]. Endothelin regulates epithelial sodium transport via two receptor subtypes (ETA and ETB). The effect of endothelin on sodium transport is complicated; there are both stimulatory and inhibitory effects on sodium reabsorption. Endothelin increases sodium reabsorption [8, 9]. Whereas there are also reports that endothelin-1 inhibits Na^+-K^+-ATPase activity in this nephron segment [10], our previous study showed that activation of ETB receptor inhibits Na^+-K^+-ATPase activity in RPT cells from Wistar-Kyoto (WKY) rats [11]. ETB receptors can lower blood pressure by decreasing endothelin-1 levels, and promoting renal loss of sodium and water [12–14]. 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 [12, 14]. Naturally occurring or induced deletion of the ETB receptor gene and chronic pharmacological blockade of ETB receptors result in salt-sensitive hypertension in rats [15].

Disruption of the D_3 receptor gene in mice produces hypertension that is associated with a decreased ability to excrete a sodium load [16]. Our previous studies found that activation of renal D_3 receptor induces natriuresis and diuresis in WKY rats; in the presence of ETB receptor antagonist, the natriuretic effect of D_3 receptor is reduced [17]. The mechanisms underlying the D_3 and ETB receptor interaction are not known. We hypothesize that ETB receptor activation may regulate D_3 receptor expression in RPT cells from WKY rats, which is impaired in RPT cells from SHRs. The interaction between D_3 and ETB receptors was studied further in RPT cells from WKY rats and SHRs, which have characteristics similar to freshly obtained RPT brush border membranes and RPTs, at least with regard to D_1 receptors and responses to G protein stimulation [18–21]. We now report that activation of ETB receptor increased D_3 receptor expression in RPT cells from WKY rats. In contrast, BQ3020 no longer affected D_3 receptor expression in SHR cells. D_3/ETB receptors coimmunoprecipitated in both WKY and SHR cells. BQ3020 treatment increased D_3/ETB receptor coimmunoprecipitation in WKY cells but had no effect in SHR cells, and basal D_3/ETB receptor coimmunoprecipitation was higher in WKY cells than in SHR cells. Stimulation of D_3 receptor inhibited Na^+-K^+-ATPase activity, which was augmented by the pretreatment with ETB receptor agonist, BQ3020, in WKY RPT cells, but not in SHR RPT cells.

Methods

Cell Culture

Immortalized RPT cells from 4- to 8-week-old SHR and WKY rats were cultured at 37 °C in 95% air/5% CO_2 atmosphere in DMEM/F-12 culture media as previously described [22, 23]. The cells (80% confluence) were extracted in ice-cold lysis buffer (phosphate-buffered saline with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), sonicated, placed on ice for 1 h, and centrifuged at 16,000 *g* for 30 min. The supernatants were stored at –70° until used for immunoblotting and/or immunoprecipitation.

Immunoblotting

The antibodies were polyclonal, purified, and antipeptides. The rat ETB receptor-immunizing peptide (298-CEMLRKKSGMQIALND-314; Alomone Labs, Jerusalem, Israel) [24, 25] and the rat D_3 receptor-immunizing peptide (288-QPP SPG QTH GGL KRY YSI C-306; Alpha Diagnostic International, San Antonio, Tex., USA) [26–28] were located on the third extracellular loop of their corresponding receptors. The specificity of these antibodies had been reported [24–28]. RPT cells were treated with vehicle (dH_2O), BQ3020 (Sigma, Co., St. Louis, Mo., USA) [29, 30], or an ETB receptor antagonist (BQ788; Sigma) [29, 30] at the indicated concentrations and times. Immunoblotting was performed as reported, except that the transblots were probed with ETB (1:300) or D_3 receptor antibodies (1:250) [24–28].

Immunoprecipitation

RPT cells were incubated with vehicle or BQ3020 (10^{-8} M) for 30 min as described above. The cells were lysed with ice-cold lysis buffer for 1 h and centrifuged at 16,000 *g* for 30 min. Equal amounts of lysates, except as indicated (200 μg protein/ml supernatant for WKY RPT cells and 800 μg protein/ml supernatant for SHR RPT cells) were incubated with the anti-ETB receptor antibody (1 μg/ml) for 1 h, and protein-G agarose at 4°C for 12 h. The immunoprecipitates were pelleted and washed four times with lysis buffer. The pellets were suspended in sample buffer, boiled for 10 min, and subjected to immunoblotting with anti-D_3 receptor antibody. In order to determine the specificity of the bands, normal rabbit IgG (negative control) and D_3 receptor antibody (positive control) were used as the immunoprecipitants instead of the ETB receptor antibody [22–24].

Na^+-K^+-ATPase Activity Assay

Na^+-K^+-ATPase activity was determined as the rate of inorganic phosphate released in the presence or absence of ouabain [31–33]. To prepare membranes for Na^+-K^+-ATPase activity assay, RPT cells cultured in 21-cm^2 plastic culture dishes were washed twice with 5 ml chilled phosphate-free modified Krebs buffer (118 mM NaCl, 4 mM KCl, 27.2 mM $NaHCO_3$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM Hepes and 0.25 mM $CaCl_2 \cdot 2H_2O$), and centrifuged at 3,000 *g* for 10 min. The cells were then placed on ice and lysed in 2 ml of lysis buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$ and 5 mM $MgCl_2$). 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 M sodium iodide, and the mixture was centrifuged at 48,000 *g* for 25 min. The pellet (membrane fraction) was washed 2 times and suspended in 10 mM Tris and 1 mM EDTA (pH 7.4). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif., USA.) and adjusted to 1 mg/ml. The membranes were stored at –70°C until further use. To measure Na^+-K^+-ATPase activity, 100-μl aliquots of membrane fraction were added to a 800-μl reaction mixture (75 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 6 mM sodium azide, 1 mM Na_4EGTA, 37.5 mM imidazole, 75 mM Tris HCl, and 30 mM histidine; pH 7.4) with or without 1 mM 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 mM) and terminated after 15 min of incubation at 37°C by adding 50 μl of 50% trichloracetate. 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 + ferrous sulfate) was added to the reaction mixture. The mixture was then combined thoroughly and centrifuged at 3,000 *g* for 10 min. Formation of phosphomolybdate was determined spectrophotometrically at 740 nm against a standard curve prepared from K_2HPO_4. Na^+-K^+-ATPase activity was estimated as the difference between total and ouabain-insensitive ATPase activity, and results in this experiment were expressed as μmol phosphate released per mg protein per min.

To eliminate the effect of proteases on the results, we added protease inhibitor (1 mM phenylmethylsulfonyl fluoride, 10 mg/ml each leupeptin and aprotinin) in all solutions in this experiment [34].

Statistical Analysis

The data are expressed as mean ± SEM. Comparison within groups was made by repeated-measures ANOVA, and comparison among groups was made by factorial ANOVA with Duncan's test. A value of $p < 0.05$ was considered significant.

Results

Activation of ETB Receptor Increases D_3 Receptor Expression in Rat RPT Cells from WKY Rats, but Not from SHRs

An ETB receptor agonist, BQ3020, increased D_3 receptor expression in a concentration- and time-dependent manner in RPT cells from WKY rats. The stimulatory effect was evident at 10^{-10} M (fig. 1a). The stimulatory effect of BQ3020 (10^{-8} M) was noted as early as 8 h and maintained for at least 30 h (fig. 1b). The specificity of BQ3020 as an ETB receptor agonist was also determined by studying the effect of the ETB receptor antagonist BQ788. Consistent with the studies shown in figure 1a and b, BQ3020 (10^{-8} M/24 h) increased D_3 receptor expression. The ETB receptor antagonist BQ788 (10^{-8} M) by itself had no effect on D_3 receptor expression, but reversed the stimulatory effect of BQ3020 on D_3 receptor expression (fig. 1c).

In contrast, in RPT cells from SHRs, BQ3020 (10^{-8} M) had no effect on D_3 receptor expression (fig. 1d). To determine whether higher concentrations of BQ3020 could have any effect on D_3 receptor expression, SHR cells were treated with varying concentrations of BQ3020 for 24 h. Consistent with the results from figure 1d, 10^{-7}–10^{-11} M BQ3020 had no effect on D_3 receptor protein expression (fig. 1e).

ETB Receptor Coimmunoprecipitates with the D_3 Receptor in Rat RPT Cells

To determine whether there is a physical interaction between the ETB and the D_3 receptor, additional experiments were performed. ETB receptors were first immunoprecipitated with anti-ETB receptor antibodies and then probed with anti-D_3 receptor antibodies. As shown in figure 2, the 45-kDa band representing the coimmunoprecipitated ETB and D_3 receptors, was increased by treatment with BQ3020 (10^{-8} M) in RPT cells from WKY rats for 30 min; however, BQ3020 had no effect on ETB/D_3 receptor coimmunoprecipitation in SHR cells, the basal level of D_3/ETB receptor coimmunoprecipitation was higher in WKY cells than in SHR cells.

Pretreatment with BQ3020 Increases the Inhibitory Effect of D_3 Receptor on Na^+-K^+-ATPase Activity in WKY Cells, but Not in SHR Cells

To investigate the physiological significance of the ETB/D_3 receptor interaction, the effects of D_3 or/and ETB receptor stimulation on Na^+-K^+-ATPase activity were determined in WKY and SHR cells. Stimulation of D_3 receptors by PD128907 (10^{-10}–10^{-5} M) for 15 min decreased Na^+-K^+-ATPase activities in a concentration-dependent manner in WKY and SHR cells (fig. 3). However, the basal levels of Na^+-K^+-ATPase activity were higher in SHR cells than in WKY cells. Pretreatment with BQ3020 (10^{-8} M) for 24 h augmented the inhibitory effect of D_3 (10^{-7} M/15 min) on Na^+-K^+-ATPase activity in WKY cells, but not in SHR cells (fig. 4). The cells pretreated with BQ3020 for 24 h were washed three times (15 min/wash)

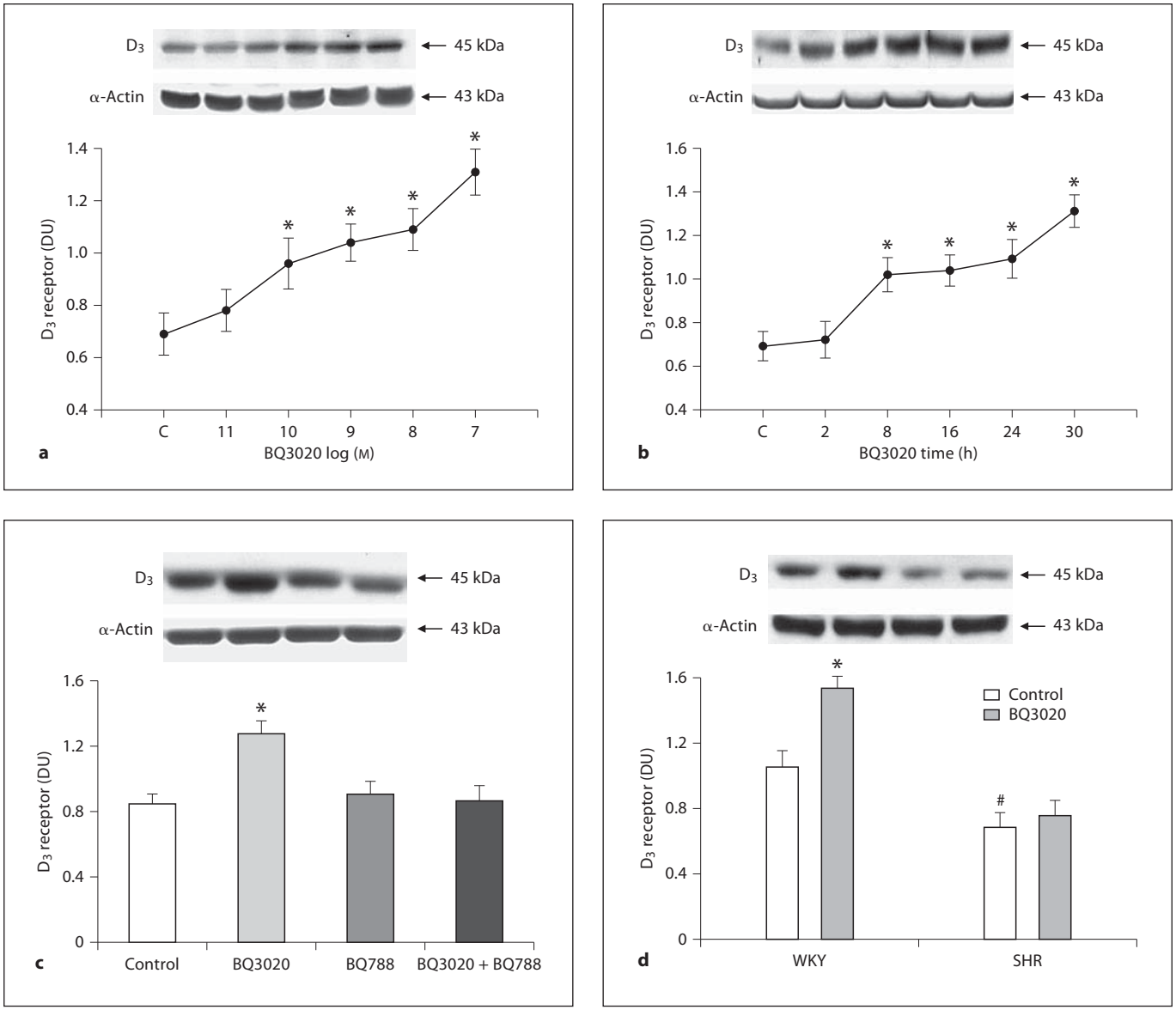

Fig. 1. Effect of BQ3020 on D_3 receptor expression in RPT cells from SHRs and WKY rats. **a** Concentration-response of D_3 receptor expression in RPT cells from WKY rats treated with BQ3020. Immunoreactive D_3 receptor expression was determined after 24-hour incubation with the indicated concentrations of BQ3020. Results are expressed as the ratio of D_3 receptor to α-actin densities [n = 6, * $p < 0.05$ vs. control (C), ANOVA, Duncan's test]. DU = Density units. **b** Time course of D_3 receptor expression in RPT cells from WKY rats treated with BQ3020. The cells were incubated for the indicated times with 10^{-8} M BQ3020. Results are expressed as the ratio of D_3 receptor to α-actin densities [n = 7, * $p < 0.05$ vs. control (0 time), ANOVA, Duncan's test]. **c** Effect of BQ3020 and an ETB antagonist (BQ788) on D_3 receptor expression in RPT cells from WKY rats. The cells were incubated with the indicated reagents (BQ3020, 10^{-8} M; BQ788, 10^{-8} M) for 24 h. Results are expressed as the ratio of D_3 receptor to α-actin densities (n = 6, * $p < 0.05$ vs. others, ANOVA, Duncan's test). **d** Differential effects of BQ3020 (10^{-8} M/24 h) on D_3 receptor expression in RPT cells from both WKY rats and SHRs. Results are expressed as the ratio of D_3 receptor to α-actin densities (n = 10, * $p < 0.05$ vs. control, # $p < 0.05$ vs. WKY control, ANOVA, Duncan's test).

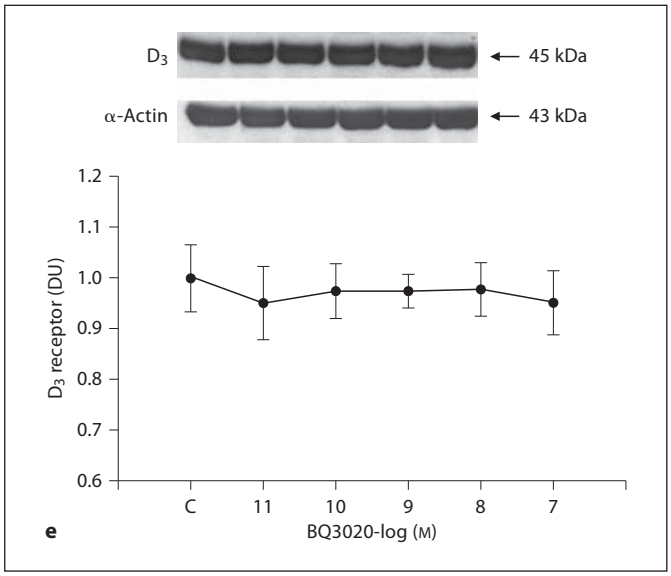

Fig. 1. Effect of BQ3020 on D_3 receptor expression in RPT cells from SHRs and WKY rats. **e** Concentration-response of D_3 receptor expression in SHR RPT cells treated with BQ3020. Immunoreactive D_3 receptor expression was determined after 24-hour incubation with the indicated concentrations of BQ3020. Results are expressed as the ratio of D_3 receptor to α-actin densities (n = 5, p = NS vs. control, ANOVA, Duncan's test).

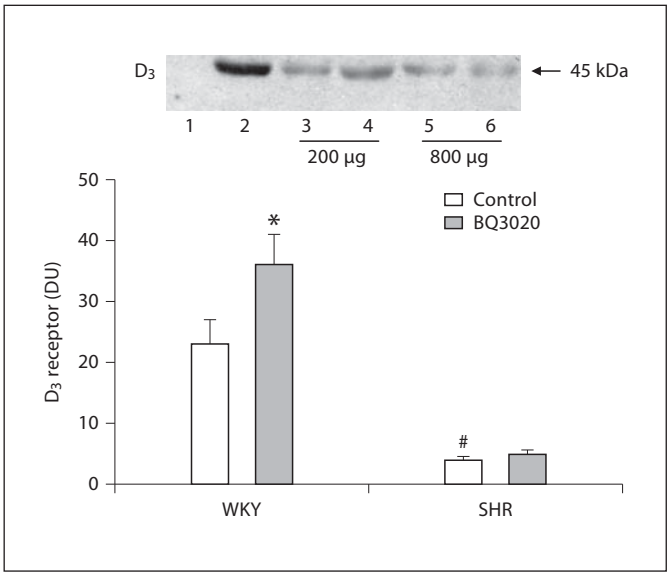

Fig. 2. Effect of BQ3020 on coimmunoprecipitation of D_3 and ETB receptors in rat RPT cells. The cells were incubated with BQ3020 (10^{-8} M) for 30 min. Thereafter, the samples were immunoprecipitated with anti-ETB antibodies and immunoblotted with anti-D_3 antibodies (* $p < 0.05$ vs. control, # $p < 0.05$ vs. WKY rats, n = 8, ANOVA, Duncan's test). One immunoblot (45 kDa) is depicted in the **inset** (lane 1 = negative control, lane 2 = positive control, lane 3 = vehicle-treated RPT cell of WKY rats, lane 4 = BQ3020-treated RPT cell of WKY rats, lane 5 = vehicle-treated RPT cell of SHRs, lane 6 = BQ3020-treated RPT cell of SHRs).

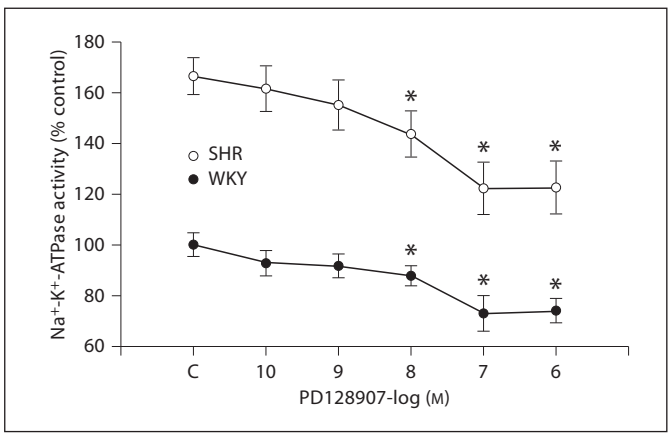

Fig. 3. Effect of the D_3 receptor on Na^+-K^+-ATPase activity in RPT cells from WKY rats and SHRs (n = 6). The RPT cells were treated with the indicated concentrations of the D_3 receptor agonist PD128907 (10^{-10}–10^{-6} M) for 15 min. Results in this experiment are expressed as percent change from control [* $p < 0.01$ vs. control (C), ANOVA, Duncan's test].

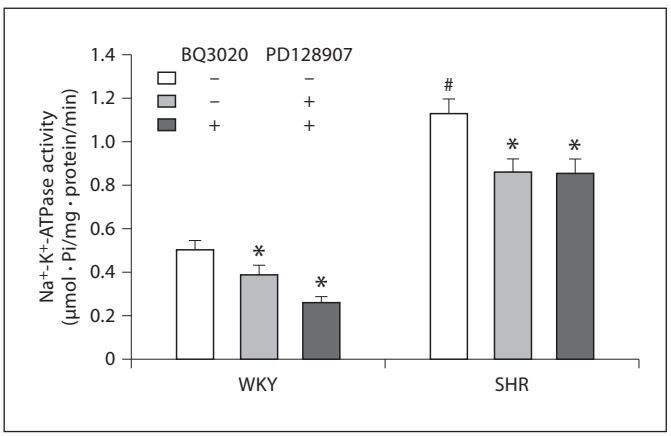

Fig. 4. Effect of pretreatment with the ETB receptor agonist BQ3020 on inhibitory effect of D_3 receptor on Na^+-K^+-ATPase activity in WKY and SHR RPT cells. The cells were pretreated with BQ3020 (10^{-8} M) or vehicle (dH_2O) for 24 h. After washing for 15 min, the cells were treated with the D_3 receptor agonist PD128907 (10^{-7} M) for 15 min. Results in this experiment are expressed as μmol phosphate released per mg protein per min (* $p < 0.05$ vs. control, # $p < 0.05$ vs. WKY rats, n = 12, ANOVA, Duncan's test).

with serum-free culture medium to remove all the added BQ3020, kept in serum-free culture medium for 2 h, and then treated with vehicle or PD128907 for 15 min.

Discussion

There are several novel observations in our study. First, we showed that an ETB receptor agonist, BQ3020, increased D_3 receptor protein expression in rat WKY RPT cells, but not in SHR cells. This effect was clearly exerted at the ETB receptor because an ETB receptor antagonist, BQ788, blocked the effect of the ETB receptor agonist. Second, we demonstrated that ETB receptors coimmunoprecipitated with D_3 receptors in rat RPT cells. ETB receptor stimulation with BQ3020 increased ETB/D_3 receptor coimmunoprecipitation in WKY cells, but not in SHR cells, and the basal levels of ETB/D_3 receptor coimmunoprecipitation was lower in SHR cells than in WKY cells. Third, stimulation of D_3 receptor inhibited Na^+-K^+-ATPase activity, which was augmented by the pretreatment with ETB receptor agonist BQ3020 in WKY RPT cells, but not in SHR RPT cells.

ETB receptor has been found to have a natriuretic effect in renal medullary collecting ducts and medullary thick ascending limb of Henle [35–37]. However, both inhibitory and stimulatory effects have been reported in the proximal tubules [10, 36–39]. There is evidence that endothelin-1 inhibits Na^+-K^+-ATPase activity in this nephron segment [10]; our previous study showed that activation of ETB receptor inhibits Na^+-K^+-ATPase activity in WKY RPT cells [11]. Short-term stimulation of ETB receptors in opossum kidney cells, a proximal tubular cell line, activates the sodium hydrogen exchanger 3 (NHE3) [39]. However, chronic treatment of the same opossum kidney cells by endothelin has an opposite effect on NHE3 activity. Thus, a 6-hour exposure of opossum kidney cells to endothelin-1 inhibits NHE3 expression and activity [36]. It is of interest that the ability of the ETB receptor agonist to increase D_3 receptor expression also occurs within the same time frame, as the enhanced ability of ETB to inhibit NHE3 expression and activity.

As a major subtype of D_2-like receptor, the natriuretic effect of D_3 receptor is reported [16]. Our previous study found that D_1-like and D_2-like receptors synergistically increase sodium excretion in WKY rats [28, 40]; and this synergistic effect occurs between D_1 and D_3 receptors [23]. Homozygous D_3 receptor null mice develop hypertension with an impaired ability to excrete a sodium load, indicating a natriuretic effect of D_3 receptor in the kidney [16]. This effect is confirmed in salt-sensitive Dahl rats, WKY rats and SHRs [41, 42], which may contribute the inhibitory effect of D_3 receptor on Na^+-K^+-ATPase activity in RPTs [43, 44]. Our present study found that stimulation of D_3 receptor inhibits Na^+-K^+-ATPase activity in RPT cells.

An increasing body of evidence shows dopamine and endothelin systems can interact with each other. Dopamine and ETB receptors have been found in brain and spinal regions known to control cardiovascular function [45]. In the rat striatum, a decrease in dopamine production decreases endothelin receptor, while endothelin via ETB receptors induces the release of dopamine [46]. In the peripheral system, an ETB receptor blocker for both ETB_1 and ETB_2 receptors decreases, whereas a selective ETB_1 blocker increases blood pressure in D_2 dopamine receptor knockout mice, but not in D_2 receptor wild-type control mice. ETB receptor expression is greater in D_2 receptor knockout mice than in D_2 receptor wild-type mice [47]. This study found activation of ETB receptor increases D_3 receptor expression and D_3/ETB receptor coimmunoprecipitation, and those effects are lost in SHR cells, supporting the interaction between dopamine and endothelin system in the kidney. It indicates that impaired natriuretic effect in SHRs may be, in part, related to impaired D_3 and ETB receptor interactions. Our present study found that pretreatment with BQ3020 for 24 h augments the inhibitory effect of D_3 receptor on Na^+-K^+-ATPase activity in WKY cells, but not in SHR cells. The aberrant interaction between D_3 and ETB receptor might be involved in the pathogenesis of hypertension.

The mechanism of the increase in ETB receptors caused by D_3 receptors was not studied. The D_2 receptor has been reported to regulate expression of other receptors [48]; it is possible that D_3 receptors regulate ETB receptor expression by the similar mechanism(s). We also found that D_3 and ETB receptors can physically interact, as determined by their coimmunoprecipitation. Moreover, ETB receptor stimulation results in an increase in the interaction between D_3 and ETB receptors in WKY rats. The increase of D_3/ETB receptor coimmunoprecipitation in RPT cells following ETB receptor agonist stimulation could have been caused by the physical interaction between the D_3 and ETB receptors because the stimulation period is only 30 min, which is too short to change the protein expressions. In the basal state, there was less D_3/ETB receptor coimmunoprecipitation in SHRs compared to WKY rats. This could be due to decreased D_3 expression in SHRs relative to WKY rats, but could not be caused by differences in ETB expression, in agreement with a previous study.

In summary, we have demonstrated that ETB receptors positively regulate the expression of D_3 receptors in rat RPT cells. Furthermore, D_3 and ETB receptors coimmunoprecipitate in RPT cells. Activation of ETB receptor increases D_3/ETB receptor coimmunoprecipitation in WKY cells, but has no effect in SHRs. Stimulation of D_3 receptor inhibited Na^+-K^+-ATPase activity, which was augmented by the pretreatment with ETB receptor agonist BQ3020 in WKY RPT cells, but not in SHR RPT cells.

We conclude that ETB receptors regulate D_3 receptors by direct physical receptor interaction and receptor expression. The impaired natriuretic effect in SHRs may be, in part, related to impaired ETB receptor regulation of D_3 receptors.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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