Altered AT₁ Receptor Regulation of ETB Receptors in Renal Proximal Tubule Cells of Spontaneously Hypertensive Rats

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Abstract—The renin-angiotensin and endothelin systems regulate blood pressure, in part, by affecting renal tubular sodium transport. In rodents, ETB receptors decrease proximal tubular reabsorption, whereas AT₁ receptors produce the opposite effect. We hypothesize that ETB and AT₁ receptors interact at the receptor level, and that the interaction is altered in spontaneously hypertensive rats (SHRs). In immortalized renal proximal tubule (RPT) cells from Wistar-Kyoto (WKY) rats, angiotensin II, via AT₁ receptors, increased ETB receptor protein in a time- and concentration-dependent manner. In contrast, in SHR RPT cells, angiotensin II (10⁻⁸ M/24 hours) had no effect on ETB receptor protein. AT₁/ETB receptors colocalized and co-immunoprecipitated in both rat strains but long-term angiotensin II (10⁻⁸ M/24 hours) treatment increased AT₁/ETB co-immunoprecipitation in WKY but not in SHR cells. Short-term angiotensin II (10⁻⁸ M/15 minutes) treatment decreased ETB receptor phosphorylation in both WKY and SHR cells, and increased ETB receptors in RPT cell surface membranes of RPT cells in WKY but not SHRs. Basal cell surface membrane ETB receptor expression was also higher in WKY than in SHRs. We conclude that AT₁ receptors regulate ETB receptors by receptor interaction and modulation of receptor expression. The altered AT₁ receptor regulation of ETB receptors in SHRs may play a role in the pathogenesis of hypertension. (Hypertension. 2005;46[part 2]:926-931.)

Key Words: angiotensin II ■ endothelin ■ hypertension, essential ■ kidney ■ phosphorylation

Endothelins are a family of isopeptides (ET₁, ET₂, and ET₃), with at least 2 receptor subtypes (ETA and ETB).¹² Renal tissue expresses both endothelin receptors, and endothelin is secreted by renal tubules,¹² where it can regulate sodium transport in an autocrine/paracrine manner.³–⁵ ETA receptors contribute to the pathogenesis of hypertension²–⁴ by stimulating vasopressor areas in the brain, increasing the secretion of aldosterone and the release of catecholamines, regulating renal sodium transport, and increasing growth factors and vascular smooth muscle contractility. The vasoconstrictor effects of endothelin are mediated by both ETA and ETB receptors.² However, ETB receptors can also relax vascular smooth muscle by releasing endothelial vasodilators,² resulting in a decrease in blood pressure.²,⁷,⁸ Naturally occurring or induced deletion of the ETB receptor gene and pharmacological blockade of ETB receptors results in salt-sensitive hypertension in rats.⁴,⁵ ETB receptors are also involved in the hypertension in DOCA/salt hypertensive and spontaneously hypertensive rats (SHRs), and in humans with essential hypertension.⁵,⁷,⁸

The renin-angiotensin system may exert some of its regulatory effects on blood pressure and renal sodium handling via its interaction with the endothelin system.³–¹¹ Angiotensin II regulates endothelin synthesis in the kidney¹⁰ and the hypertension associated with chronic angiotensin II infusion can be attenuated by an ETA/ETB receptor antagonist.¹,¹¹ Although the ETB receptor may exert its natriuretic effect by actions in the distal nephron,²,¹² it may also inhibit sodium transport in the proximal tubule cells,¹³ opposite of the effect of AT₁ receptor activation.⁹ We hypothesize that ETB and AT₁ receptors interact at the receptor level, and that the interaction is aberrant in SHRs. We examined the interaction between AT₁ and ETB receptors in immortalized rat renal proximal tubule (RPT) cells that have characteristics similar to freshly obtained RPT brush border membranes and RPTs, at least with regard to D₁ receptors and responses to G protein stimulation.¹⁴–¹⁷

Methods

Cell Culture
Immortalized RPT cells from 4- to 8-week-old Wistar-Kyoto (WKY) and SHRs were cultured at 37°C in 95% air/5% CO₂ atmosphere in DMEM/F-12 culture media as previously described.¹⁴–¹⁶,¹⁸–²¹ The cells (80% confluence) were lysed in ice-cold lysis buffer, sonicated, placed on ice for 1 hour, and centrifuged at 16 000g for 30 minutes. The supernatants were stored at −70°C until use for immunoblotting and/or immunoprecipitation.
Immunoblotting

The amino acid sequence for the immunogenic peptide (rabbit anti-human AT1 receptor antibody) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) is QDCCPKAGRHC (amino acids 15 to 24). The amino acid sequence for the immunogenic peptide (rabbit anti-rat ETB receptor antibody) (Alomone Labs, Jerusalem, Israel) is CEM-LRKKSGQIALND (amino acids 298 to 314). The specificity of these antibodies has been described.20–25 After starvating the RPT cells in serum-free media for 2 hours, they were treated with vehicle (dH2O), angiotensin II, or an AT1 receptor antagonist (losartan) (E. I. Du Pont De Nemours & Co, Wilmington, Del) at the indicated concentrations and times. Immunoblotting was performed as reported,14–16,18–21 except that the transblots were probed with ETB (1:300) or AT1 receptor antibodies (1:400). The amount of protein transferred onto the membranes was determined by Ponceau-S staining and immunoblotting for α-actin.14–16,18–21

Immunoprecipitation

RPT cells were incubated with vehicle or angiotensin II (10^{-8} M) for 24 hours (AT/ETB receptor co-immunoprecipitation) or 15 minutes (ETB receptor phosphorylation), as described. Equal amounts of lysates (500 μg protein/mL, supernatant from RPT cells of WKY and SHRs) were incubated with affinity-purified anti-ETB receptor antibody (AT/ETB receptor co-immunoprecipitation) or polyclonal antiphosphoserine antibody (Zymed Laboratory, San Francisco, Calif) (ETB receptor phosphorylation) (1 μg/mL) for 1 hour and protein-G agarose at 4°C for 12 hours.

The immunoprecipitates were pelleted and washed 4 times with lysis buffer. The pellets were suspended in sample buffer, boiled for 5 minutes before adding detergent (dH2O), angiotensin II, or an AT1 receptor antagonist, losartan (E. I. Du Pont De Nemours & Co, Wilmington, Del) at the indicated concentrations and times. Immunoblotting was performed as reported,14–16,18–21 except that the transblots were probed with ETB (1:300) or AT1 receptor antibodies (1:400). The amount of protein transferred onto the membranes was determined by Ponceau-S staining and immunoblotting for α-actin.14–16,18–21

Cell Surface ETB Receptor Expression

RPT cells were cultured in serum-free medium for 2 hours, and then treated with angiotensin II (10^{-9} M) for 15 minutes. Sulfo-NHS-LC-biotin (final concentration 250 μg/mL; Pierce) was added into the medium 5 minutes before adding angiotensin II. The cells were washed 3 times with ice-cold phosphate-buffered saline, lysed, sonicated, and placed on ice for 1 hour. Supernatants from the cell lysates were immunoprecipitated with the anti-ETB receptor antibody, followed by immunoblotting. After blocking (10% milk in wash buffer), the membranes were incubated with peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratory, Inc, West Grove, Pa) in wash buffer at 1/5000 dilution for 30 minutes. The biotinylated protein bands were visualized by enhanced chemiluminescence (Western Blotting Detection Kit, Amersham, Arlington Heights, Ill). The intensity of the band represented ETB receptors on the cell surface membrane.26

Statistical Analysis

The data are expressed as mean±SEM. Comparison within groups was made by repeated measures ANOVA (or paired t test when only 2 groups were compared), and comparison among groups (or t test when only 2 groups were compared) was made by factorial ANOVA with Holm-Sidak test. A value of P<0.05 was considered significant.

Results

Specificity of Receptor Antibodies

The band for the ETB receptor (=45 kDa) was found in RPT cells, as previously reported.23–25 The band was specific because it was completely blocked when the antibodies were reabsorbed by the immunizing peptide (Figure 1).

AT1 Receptors Increase ETB Receptor Expression in RPT Cells From WKY Rats but not From SHRs

Angiotensin II increased ETB receptor expression in a concentration- and time-dependent manner in RPT cells from WKY rats. The increase occurred at 10^{-11} to 10^{-7} M (24-hour incubation) (Figure 2A). The stimulatory effect of angiotensin II (10^{-8} M) was noted as early as 8 hours and maintained for at least 30 hours (Figure 2B). In RPT cells from SHRs, angiotensin II had no effect on ETB receptor expression (WKY: control=0.9±0.04, angiotensin II [10^{-8} M/24 hours]=1.4±0.09; SHR: control=0.9±0.04, angiotensin II [10^{-8} M/24 hours]=1±0.1 density units [DU]; n=7/group) (Figure 2C).

The specificity of the effect of angiotensin II at the AT1 receptor was also determined by studying the effect of an AT1 receptor antagonist, losartan, in WKY RPT cells. Consistent with the studies shown in Figure 2A, 2B, and 2C, angiotensin II (10^{-8} M/24 hours), increased ETB receptor expression (control=0.9±0.09, angiotensin II=1.5±0.2; n=6, P<0.05). Losartan (10^{-8} M/24 hours), by itself, had no effect on ETB receptor expression (0.9±0.1) but reversed the stimulatory effect of angiotensin II on ETB receptor expression (0.8±0.2) in WKY cells (Figure 2D).

AT1 Receptors Co-Immunoprecipitate With ETB Receptors in Rat RPT Cells

To determine whether there is an interaction between the ETB and the AT1 receptor, ETB receptors were first immunoprecipitated with anti-ETB receptor antibodies and then probed with anti-AT1 receptor antibodies. The density of the 45-kDa band, representing the co-immunoprecipitated ETB and AT1 receptors, was similar in WKY and SHRs in the basal state (Figure 3). However, co-immunoprecipitation of ETB...
and AT1 receptors was increased by angiotensin II (10^{-8} M/24 hours) in WKY (control=23±4 DU; angiotensin II=44±6 DU; n=9, P<0.05) but not in SHRs (control=20±4 DU, angiotensin II=14±3 DU; n=9).

**Angiotensin II Increases Cell Surface ETB Receptor Expression in RPT Cells From WKY Rats, not From SHRs**

To determine the short-term effect of angiotensin II (10^{-8} M) on the ETB receptor, RPT cells were treated with angiotensin II for 15 minutes. Cell surface proteins were labeled with the cell impermeable, noncleavable sulfo-NHS-LC-biotin, and then immunoprecipitated with rabbit anti-rat ETB receptor antibody. Angiotensin II increased cell surface membrane ETB receptors in cells from WKY but not in those from SHRs (WKY: control=29±3 DU, angiotensin II=48±7 DU; SHR: control=13±5 DU, angiotensin II=11±5 DU; n=5). The basal level of cell surface membrane ETB receptor was also greater in WKY than in SHR cells (Figure 4).

**Angiotensin II Decreases ETB Receptor Phosphorylation in RPT Cells From Both SHR and WKY Rats**

Because serine phosphorylation reduces the function of ETB receptors, we investigated the effect of angiotensin II on ETB receptor phosphorylation. Basal ETB phosphorylation was similar in WKY and SHRs (Figure 5). Angiotensin II (10^{-8} M/15 minutes) decreased ETB receptor phosphorylation in both WKY and SHRs (WKY: control=29±2 DU,
angiotensin II=22±1 DU; SHR: control=29±2 DU, angiotensin II=20±2 DU; n=11, P<0.05).

Discussion
The current study shows that angiotensin II chronically increases total ETB cellular receptor expression, as well as

ETB, as well as ETA, receptors have been previously demonstrated in the kidney. Both of them colocalize in glomeruli and vessels, whereas the ETB receptor seems to be the main subtype in tubules. The presence and the type of endothelin receptor subtypes expressed in renal proximal tubules are not yet settled. Functional ETB receptors are present in renal proximal tubules and our immunoblotting studies support this notion in cells derived from S1 segments of rat renal proximal tubules. The ETB polyclonal antibody used in our study is specific for the ETB receptor; the band is blocked by its immunizing peptide. A monoclonal ETB antibody gives the same results. The use of proximal tubule segments other than the S1 segment may explain the negative results of others.

The mechanism of the increase in ETB receptors caused by AT1 receptors was not studied. However, the D2 receptor has

Figure 3. Effect of angiotensin II (10^{-8} M/24 hours) on the co-immunoprecipitation of AT1 and ETB receptors in RPT cells. The cell samples were prepared as indicated in the Methods. (*P<0.05 vs control, n=9, ANOVA, Holm-Sidak test). One immunoblot (45 kDa) is depicted in the inset: (lane 1=positive control, lane 2=negative control, lane 3=vehicle-treated WKY RPT cells, lane 4=angiotensin II-treated WKY RPT cells, lane 5=vehicle-treated SHR RPT cells, lane 6=angiotensin II-treated SHR RPT cells).

Figure 4. Effect of angiotensin II on cell surface ETB receptor expression in RPT cells from WKY and SHRs. The cells were incubated with angiotensin II (10^{-8} M/15 minutes), and labeled with sulfo-NHS-LC-biotin. Thereafter, the samples were immunoprecipitated with anti-ETB receptor antibody and immunoblotted with avidin-conjugated peroxidase. Results are expressed as density units (DU) (*P<0.05 vs control, #P<0.05 vs WKY, n=5).

Figure 5. Effect of angiotensin II (10^{-8} M/15 minutes) on ETB receptor phosphorylation in RPT cells from WKY and SHRs. The RPT cellular lysate protein was immunoprecipitated with anti-phosphoserine antibody and immunoblotted with anti-ETB receptor antibody, as described in Methods. (*P<0.01 vs control, n=11, ANOVA, Holm-Sidak test).

AT1/ETB receptor co-immunoprecipitation in RPT cells from normotensive WKY rats. In contrast, in RPT cells from SHRs, angiotensin II has no such effects. Interestingly, short-term exposure to angiotensin II increases cell surface ETB receptor expression in RPT cells from WKY but not SHRs, but decreases the total cellular serine phosphorylation of ETB receptors to the same extent in both rat strains. We did not determine whether there is a difference in cell surface expression of phosphorylated ETB receptors between WKY and SHR cells. However, basal and angiotensin II-treated WKY cells have more cell surface membrane ETB receptors than in SHR cells. Therefore, a higher percentage of phosphorylated ETB receptors may exist in cell surface membranes in SHR than in WKY cells. This needs to be confirmed in the future. Moreover, any impairment of ETB receptor function caused by serine phosphorylation of ETB would be mitigated by the increase in cell surface localization and total ETB receptor expression in WKY rats. The net consequence is an impairment of ETB receptor function in RPT cells of SHRs.
been reported to change gene expression by translational and/or post-translational mechanisms. It is possible that AT₁ receptors regulate ETB receptor expression by similar mechanisms. We also found that AT₁ and ETB receptors can directly interact; AT₁ receptor stimulation increases the co-immunoprecipitation of AT₁ and ETB receptors. There are two possible explanations for this observation: increased ETB receptor expression, per se, or an increased interaction between AT₁ and ETB receptors. Further studies are needed to determine whether the increased interaction between these two receptors is a direct or an indirect mechanism, possibly mediated by an adaptor protein(s).

The physiological relevance of the interaction between AT₁ and ETB receptors was not evaluated in this study. However, the renin-angiotensin and endothelin systems interact to regulate blood pressure and renal sodium excretion. Thus, angiotensin II may exert some of its effects via its interaction with the endothelin system. ET-1, ET-2, and ET-3 have been reported to be synthesized in renal proximal tubules and angiotensin II can regulate ET-1 synthesis in the kidney. Endothelin potentiates the hypertrophic and mitogenic, as well as the vasoconstrictor effects of angiotensin II. We found that angiotensin II, via the AT₁ receptor, increases not only the co-immunoprecipitation of AT₁ and ETB receptors but also the expression of ETB receptors. It is possible that an increase in ETB receptor expression may serve to antagonize the stimulatory effect of angiotensin II on sodium reabsorption. ETB receptors, expressed in the RPT, medullary thick ascending limb of Henle, and collecting duct of the kidney, affect the reabsorption of sodium and water. The ETB receptor decreases sodium transport in the medullary thick ascending limb of Henle and collecting duct of the kidney, affects the reabsorption of sodium and water.1–5,12, 38–43 The ETB receptor decreases sodium transport in the medullary thick ascending limb of Henle and collecting duct of the kidney, affects the reabsorption of sodium and water.1–5,12, 38–43 These studies were supported in part by grants from the National Institutes of Health, HL23081, DK39308, HL68686, DK52612, HL62211, HL41618, HL704940, and National Natural Science Foundation of China, 30470728.

In conclusion, AT₁ receptors increase total and cell surface membrane expression of ETB receptors and their interaction in RPT cells from WKY but not SHRs. The role of the altered AT₁ receptor regulation of ETB receptors in hypertension and altered renal sodium handling in SHRs remains to be determined. However, these findings could be taken to indicate a relatively unopposed effect of angiotensin II in RPT cells of SHRs as compared with WKY rats.

**Perspectives**

ETB receptors decrease proximal tubular reabsorption, whereas low concentrations of angiotensin II, via AT₁ receptors, produce the opposite effect. This study shows that long-term activation of AT₁ receptor increases ETB receptor expression whereas short-term activation increases cell surface ETB receptor expression in WKY RPT cells; these stimulatory effects are not seen in SHR RPT cells. AT₁ and ETB receptors may modulate each other to maintain a normal salt balance in the normotensive state. The loss of this counter regulation, coupled with increased AT₁ receptor activity, may be another mechanism for the sodium retention and the increase in blood pressure in SHRs. Impaired cross-talk and counter-regulation between AT₁ receptors and other G protein-coupled receptors that decrease renal sodium transport may contribute to the high blood pressure in genetic hypertension.

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