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## Angiotensin II type 2 receptor inhibits expression and function of insulin receptor in rat renal proximal tubule cells

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### Abstract

Both renin–angiotensin systems and insulin participate in kidney-involved blood pressure regulation. Activation of angiotensin II type 2 receptor (AT<sub>2</sub>R) decreases sodium reabsorption in renal proximal tubule (RPT) cells, whereas insulin produces the opposite effect. We presume that AT<sub>2</sub>R has an inhibitory effect on insulin receptor expression in RPT cells, which may affect renal sodium transport and therefore be of physiological or pathological significance. Our present study found that activation of AT<sub>2</sub>R inhibited insulin receptor expression in a concentration and time-dependent manner in RPT cells from Wistar-Kyoto (WKY) rats. In the presence of a protein kinase C (PKC) inhibitor (PKC inhibitor peptide 19–31, 10<sup>-6</sup> mol/L) or a phosphatidylinositol 3 kinase inhibitor (wortmannin, 10<sup>-6</sup> mol/L), the inhibitory effect of AT<sub>2</sub>R on insulin receptor was blocked, indicating that both PKC and phosphatidylinositol 3 kinase were involved in the signaling pathway. There was a linkage between AT<sub>2</sub>R and insulin receptor which was determined by both laser confocal microscopy and coimmunoprecipitation. However, the effect of AT<sub>2</sub>R activation on insulin receptor expression was different in RPT cells from spontaneously hypertensive rats (SHRs). Being contrary to the effect in WKY RPT cells, AT<sub>2</sub>R stimulation increased insulin receptor in SHR RPT cells. Insulin (10<sup>-7</sup> mol/L, 15 minutes) enhanced Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in both WKY and SHR RPT cells. Pretreatment with CGP42112 decreased the stimulatory effect of insulin on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in WKY RPT cells, whereas pretreatment with CGP42112 increased it in SHR RPT cells. It is suggested that activation of AT<sub>2</sub>R inhibits insulin receptor

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Supplementary Data

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expression and function in RPT cells. The lost inhibitory effect of AT<sub>2</sub>R on insulin receptor expression may contribute to the pathophysiology of hypertension.

## Keywords

Angiotensin II type 2 receptor; hypertension; insulin receptor; renal proximal tubule cells; kidney

## Introduction

Kidney contributes importantly to the long-term blood pressure control via regulating sodium homeostasis.<sup>1-3</sup> Renal proximal tubule (RPT) is one of the most critical segments for sodium reabsorption within kidney and therefore involved in blood pressure adjustment. This process is regulated by numerous hormones and humoral factors, including renin-angiotensin system (RAS) and insulin/insulin receptor.<sup>4-6</sup>

Angiotensin II (Ang II), as the major effector peptide of RAS, regulates sodium balance via two main receptor sub-types, angiotensin II type 1 receptor (AT<sub>1</sub>R) and angiotensin II type 2 receptor (AT<sub>2</sub>R).<sup>7,8</sup> AT<sub>1</sub>R and AT<sub>2</sub>R are expressed throughout the vascular and tubule sites in adult kidney. Stimulation of AT<sub>1</sub>R increases sodium (Na<sup>+</sup>) reabsorption in RPT and induces antinatriuresis. AT<sub>2</sub>R, as an antagonist of AT<sub>1</sub>R, can oppose or offset this action.<sup>9-11</sup> Activation of AT<sub>2</sub>R decreases sodium reabsorption, induces natriuresis, maintains negative Na<sup>+</sup> balance, and lowers blood pressure chronically in angiotensin-dependent hypertension via reducing Na<sup>+</sup> reabsorption in RPT.<sup>1</sup>

Insulin/insulin receptor is another critical signaling pathway involved in sodium balance in RPT. Insulin receptor was expressed throughout the nephron.<sup>12,13</sup> Studies have demonstrated that insulin/insulin receptor has the potential of increasing sodium retention through stimulating sodium transporters Na<sup>+</sup>-H<sup>+</sup>-exchanger and Na<sup>+</sup>-K<sup>+</sup>-ATPase in RPT.<sup>14-16</sup>

Some researches have been performed to elucidate the interaction between AT<sub>2</sub>R and insulin in different aspects. AT<sub>2</sub>R can impact the  $\beta$ -cell to  $\alpha$ -cell ratio of the neonate islets, insulin secretory function, and the glucose tolerance in pups.<sup>17</sup> Activation of AT<sub>2</sub>R leads to inhibition of insulin-induced extracellular signal-regulated protein kinase 2 (ERK2) activity and cell proliferation in transfected Chinese hamster ovary cells.<sup>18</sup> In rat pheochromocytoma cell line, AT<sub>2</sub>R inhibits insulin-associated phosphoinositide 3-kinase and Akt phosphorylation and induces cell apoptosis.<sup>19</sup> Although relationship between AT<sub>2</sub>R and insulin receptor has been revealed, the detailed mechanisms remain unclear. Both AT<sub>2</sub>R and insulin receptor exist in RPT and they execute the opposite functions in sodium metabolism,<sup>5,6,20</sup> we presume that activation of AT<sub>2</sub>R inhibits insulin receptor in RPT. To confirm this hypothesis, we explored the interaction between AT<sub>2</sub>R and insulin receptor in RPT cells from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). We found that activation of AT<sub>2</sub>R was capable of decreasing insulin receptor expression and function in RPT cells from WKY rats, whereas this inhibitory effect was disturbed in RPT cells from SHRs. We suggested that an aberrant interaction between AT<sub>2</sub>R and insulin receptor may play an important role in the abnormal regulation of sodium excretion and consequently be implicated in hypertension.

## Methods

### Cell Culture

Immortalized RPT cells from WKY rats and SHR<sub>s</sub> were cultured as previously described.<sup>4</sup> Briefly, cells were cultured at 37°C in 95% O<sub>2</sub> and 5% CO<sub>2</sub> atmosphere in Dulbecco's Modified Eagle Medium/F-12 containing 10% fetal bovine serum, 5 µg/mL transferrin, 5 µg/mL insulin, 4 µg/mL dexamethasone, and 10 ng/mL epidermal growth factor. The cells were diluted to 1 × 10<sup>6</sup> cells/mL and plated in different culture dishes according to the specific experimental requirements. Before the treatment of cells, the medium was replaced with serum-free medium for 2 hours.

### Immunoblotting

The RPT cells were treated with different reagents or the vehicle (dH<sub>2</sub>O) at the indicated concentrations and times according to the specific experimental requirements.

Immunoblotting was performed as previously reported except that the transblots were probed with the rabbit anti-insulin receptor antibody (1:400, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), rabbit anti-phospho-ERK1/2 antibody, or rabbit anti-ERK1/2 antibody (1:400, Cell Signaling Technology, Beverly, MA).<sup>4,21</sup> The amount of protein transferred onto the membranes was verified by immunoblotting for α-actin (Santa Cruz Biotechnology Inc) and used for the normalization of the receptor densities.

### RNA Isolation, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA from RPT cells was extracted using Trizol re-agent, and 1 ng RNA was used to synthesize cDNA following the manufacturer's instructions. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the forward primer was 5' - CAGGGCTGCCTTCTCTTGTTG-3' and the reverse primer was 5' - GGTGATGGGTTTCCCGTTGA-3' (GeneBank Accession No.: NM\_017008.4). For insulin receptor, the forward primer was 5' -TTCAGGAAGACCTTCGAGGATTACCTGCAC-3' and the reverse primer was 5' -AGGCCAGAGATGACAAGTGACTCCTTGTT-3' (GeneBank Accession No.: NM\_017071.2). Insulin receptor mRNA expression was normalized for GAPDH mRNA.

### Analysis of the Second Messengers Involved in the Regulation of AT<sub>2</sub>R on Insulin Receptor Expression

To determine the second messenger(s) involved in the AT<sub>2</sub>R-mediated regulation of insulin receptor expression in RPT cells from WKY rats and SHR<sub>s</sub>, several inhibitors or agonists were used: protein kinase C (PKC) inhibitor (19-31, 10<sup>-6</sup> mol/L), PKC activator (phorbol 12-myristate 13-acetate, PMA, 10<sup>-7</sup> mol/L), protein kinase A (PKA) inhibitor (14-22, 10<sup>-6</sup> mol/L), PKA activator (Sp-cAMP-S, 10<sup>-7</sup> mol/L), calcium channel blocker (nicardipine, 10<sup>-6</sup> mol/L), calcium channel agonist (BAY-K8644, 10<sup>-6</sup> mol/L), and phosphatidylinositol 3 kinase (PI3K) inhibitor (wortmannin, 10<sup>-6</sup> mol/L). These reagents were added into the incubation medium 15 minutes before the addition of the AT<sub>2</sub>R agonist CGP42112 (10<sup>-6</sup> mol/L). The PKC inhibitor 19-31, PMA, Sp-cAMPs, BAY-K8644, nicardipine, and CGP42112 were purchased from Sigma-Aldrich (St. Louis, MO). PKA inhibitor 14-22 was

purchased from Calbiochem Company (Darmstadt, Germany). Wortmannin was purchased from Millipore (Billerica, MA).

### Immunoprecipitation

RPT cells were cultured with vehicle or CGP42112 ( $10^{-6}$  mol/L) for 24 hours and then were extracted with ice-cold lysis buffer for 1 hour, centrifuged at 16,000g for 30 minutes. Equal amounts of lysates were incubated with rabbit anti-AT<sub>2</sub>R antibody (Santa Cruz Biotechnology Inc, 1.0 µg/mL) for 24 hours at 4°C and G-protein agarose for 1 hour at room temperature. The immunoprecipitates were pelleted and washed four times with lysis buffer. The pellets were suspended in sample buffer, boiled for 10 minutes, and subjected to immunoblotting with the rabbit anti-insulin receptor antibody. The bands were quantified by densitometry through Quantiscan. To determine the specificity of the bands found on the immunoblots, IgG was used instead of AT<sub>2</sub>R antibody as the immunoprecipitant (negative control). Moreover, we also exchanged the antibodies for immunoprecipitation and immunoblotting to confirm the results. The specificity of AT<sub>2</sub>R antibody has been checked in our previous published paper.<sup>22</sup>

### Confocal Microscopy of Double-Stained Kidney Sections and RPT Cells

Fixing with 4% paraformaldehyde for 24 hours, kidneys from WKY rats were embedded in paraffin, sectioned and mounted on slides. Then, sections were deparaffinized, rehydrated, and subjected to antigen retrieval. After incubating with goat anti-AT<sub>2</sub>R and rabbit anti-insulin receptor antibodies (1: 100 dilution, Santa Cruz Biotechnology Inc) overnight at 4°C, sections were stained with Alexa Fluor 568-rabbit antigoat IgG antibody (1:100 dilution, red; Molecular Probes, Eugene, OR) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:100 dilution, green; Molecular Probes). Immunofluorescence images were acquired at excitation wavelengths of 480 nm and 560 nm; emission was detected at 535 and 645 nm (Olympus AX70 laser confocal microscopy).

To determine the colocalization of AT<sub>2</sub>R and insulin receptor in cells, RPT cells were fixed (3% paraformaldehyde) for 30 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. The subsequent immunofluorescence experiment was the same as above.

### Na<sup>+</sup>-K<sup>+</sup>-ATPase Activity Assay

RPT cells were treated with vehicle or indicated re-agents, and then, the membranes were obtained as previously reported method.<sup>5</sup> To measure Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, 100 µL aliquots of the membrane fraction was added to 800 µL reaction mixture (75 mM NaCl, 5 mM KCl, 5 mM MgCl<sub>2</sub>, 6 mM sodium azide, 1 mM Na<sub>4</sub>EGTA, 37.5 mM imidazole, 75 mM Tris HCl, and 30 mM histidine; pH 7.4) with or without 1 mM ouabain to make the final volume to 1 mL. After preincubating the mixtures for 5 minutes at 37°C in a water bath, reactions were initiated by adding 4 mM Tris-ATP and terminated after 15 minutes by adding 50 µL of 50% trichloroacetic acid at 37 °C. For the determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures with ouabain. The amount of phosphate produced was quantified by adding 1-mL coloring reagent (10% ammonium molybdate in 10N sulfuric acid and ferrous sulfate). After mixture and centrifugation (3000g, 10 minutes), the amount of phosphomolybdate in the supernatant

was quantified spectrophotometrically at 740 nm against a standard curve prepared from  $K_2HPO_4$ . The difference between total and ouabain-insensitive ATPase activity was used for  $Na^+-K^+$ -ATPase activity.

### Statistical Analysis

The data were analyzed individually and expressed as mean  $\pm$  standard error of the mean. Student unpaired *t* test was used to compare two independent groups, and one-way analysis of variance was used for a comparison of 3 groups. GraphPad Prism 5.0 and SPSS 17.0 were used to perform the statistical analyses. A value of *P* less than .05 was considered significant.

## Results

### Activation of $AT_2R$ Decreases Insulin Receptor Expression in WKY RPT Cells but Increases It in SHR RPT Cells

Administration of an  $AT_2R$  agonist CGP42112 decreased insulin receptor expression in a concentration- and time-dependent manner in WKY RPT cells. The inhibition effect was found not only at protein level, but also at mRNA level (Figure 1A–D).

To confirm the specificity of CGP42112 as an  $AT_2R$  agonist, PD123319 (Sigma-Aldrich), the  $AT_2R$  antagonist was used. Consistent with the previous results, PD123319 ( $10^{-6}$  mol/L), by itself, did not affect insulin receptor expression but reversed the inhibitory effect of CGP42112 on insulin receptor (Figure 1E, F).

In contrast to the inhibition effect of  $AT_2R$  on insulin receptor in RPT cells from WKY rats,  $AT_2R$  activation increased both mRNA and protein expression of insulin receptor in SHR RPT cells (Figure 2).

### Roles of PKC and PI3K in the Inhibition of $AT_2R$ on Insulin Receptor in WKY RPT Cells

Treating RPT cells from WKY rats with PKC inhibitor (19–31,  $10^{-6}$  mol/L) or PI3K inhibitor wortmannin ( $10^{-6}$  mol/L) blocked the inhibitory effect of  $AT_2R$  on insulin receptor expression (Figure 3A, B), which had no effect on insulin receptor expression by themselves, indicating that both PKC and PI3K were involved in the  $AT_2R$ -mediated signal transduction pathway. Some other key cell signaling proteins, including PKC activator (phorbol 12-myristate 13-acetate, PMA,  $10^{-7}$  mol/L), PKA inhibitor (14–22,  $10^{-6}$  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), have also been tested in the present study. None of these reagents blocked effect on  $AT_2R$  on insulin receptor expression (data not shown). To determine whether or not PKC and PI3K were involved in the upregulation of  $AT_2R$  on insulin receptor expression in SHR cells, PKC inhibitor (19–31,  $10^{-6}$  mol/L) or wortmannin ( $10^{-6}$  mol/L) was used. It resulted that neither PKC inhibitor nor wortmannin could blocked the upregulation of  $AT_2R$  on insulin receptor expression in SHR cells (Figure S1).

### AT<sub>2</sub>R Colocalizes and Interacts With Insulin Receptor

To investigate whether or not AT<sub>2</sub>R and insulin receptor can interact directly, immunofluorescence laser confocal microscopy and coimmunoprecipitation were performed. Immunofluorescence staining showed that AT<sub>2</sub>R and insulin receptor colocalized in kidney and cells from WKY rats (Figure 4A, B). What's more, stimulation of CGP42112 (10<sup>-6</sup> mol/L per 24 hours) increased AT<sub>2</sub>R/insulin receptor colocalization, especially at plasma membrane (Figure 4B). Coimmunoprecipitation study indicated there was a linkage between AT<sub>2</sub>R and insulin receptor at the basal state, which was increased by CGP42112 (10<sup>-6</sup> mol/L 24 hours) in WKY RPT cells (Figure 4C, D). Although there was AT<sub>2</sub>R/insulin receptor linkage in SHR RPT cells as well, CGP42112 (10<sup>-6</sup> mol/L per 24 hours) had not effect on AT<sub>2</sub>R/insulin receptor coimmunoprecipitation and colocalization (Figure S2A, B).

### Pretreatment With AT<sub>2</sub>R Agonist Decreases the Stimulatory Effect of Insulin Receptor on Na<sup>+</sup>-K<sup>+</sup>-ATPase Activity in WKY RPT Cells but not in SHR RPT Cells

To further explore the effect of AT<sub>2</sub>R on insulin receptor function, the Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in RPT cells from WKY rats and SHRs was checked with or without pretreatment with the AT<sub>2</sub>R agonist CGP42112. Activity of Na<sup>+</sup>-K<sup>+</sup>-ATPase was higher in SHR than WKY RPT cells at the basal level. Insulin (10<sup>-7</sup> mol/L 15 minutes) increased Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in both WKY and SHR RPT cells. However, pretreatment with CGP42112 inhibited the insulin-mediated stimulatory effect on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in WKY RPT cells but increased it in SHR RPT cells (Figure 5A, B), which was consistent with the regulation of AT<sub>2</sub>R on insulin receptor expression.

Besides of Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, we also checked the signaling of insulin, that is, ERK1/2 phosphorylation (p-ERK). Consistent with the result regarding to Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, insulin (10<sup>-7</sup> mol/L, 15 min) increased p-ERK in both WKY and SHR RPT cells. Pretreatment with CGP42112 (10<sup>-6</sup> mol/L) for 24 hours inhibited the stimulatory effect of insulin on ERK1/2 activity in WKY cells, but enhanced insulin induced ERK1/2 phosphorylation in SHR RPT cells (Figure 6A, B).

## Discussion

The present study for the first time demonstrated that there is a link between AT<sub>2</sub>R and insulin receptor in RPT cells. AT<sub>2</sub>R is capable of inhibiting insulin receptor expression via PI3K and PKC pathways in RPT cells from WKY rats. Correspondingly, activation of AT<sub>2</sub>R in WKY cells reduces the insulin-mediated stimulatory effect on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, whereas this repression effect of AT<sub>2</sub>R on insulin receptor was aberrant in SHR cells. Altered interaction between AT<sub>2</sub>R and insulin receptor may contribute to the pathogenesis of hypertension.

RAS is one of the major regulatory systems in the modulation of cardiovascular and renal function.<sup>23,24</sup> Ang II, the primary RAS effector peptide hormone, executes multiple physiological or pathological roles via binding to different receptor subtypes, including AT<sub>1</sub>R and AT<sub>2</sub>R. Studies have shown that AT<sub>1</sub>R predominately mediates Ang II-induced vasoconstriction, sympathetic nervous system activation, inflammation, sodium reabsorption

in RPT, and aldosterone secretion.<sup>1,23</sup> AT<sub>2</sub>R is antagonistic to the above effects under most circumstances.<sup>25,26</sup> Kemp et al<sup>1</sup> have demonstrated that AT<sub>2</sub>R activation initiates its translocation to the RPT apical membrane. Intrarenal AT<sub>2</sub>R activation prevents Na<sup>+</sup> retention and lowers blood pressure in Ang II–dependent hypertension. Ali et al<sup>27</sup> found that AT<sub>2</sub>R activation shifts the abnormal regulation of AT<sub>1</sub>R and Angiotensin-converting enzyme 2 (ACE2) which contributes to obesity-related hypertension. AT<sub>2</sub>R activation also prevents salt-sensitive hypertension in the obese Zucker rat model of obesity and the metabolic syndrome.

Insulin is the central hormone involved in the control of glucose and lipid metabolism. Other nonmetabolic effects of insulin are intensively studied as well. Activation of insulin receptor not only promotes glucose uptake, protein synthesis, and inhibits lipid metabolism, but also is crucial for renal function in glomeruli and tubules.<sup>28,29</sup> Insulin receptor has been shown regulating sodium excretion in RPTs. Studies supported that renal-specific knockout of insulin receptor in mice results in impairment of sodium transport and increased blood pressure.<sup>30–32</sup> Our previous study has found that insulin increased Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in RPTs.<sup>5</sup> When signaling is aberrant here, as may occur in insulin-resistant states, it may be responsible for a number of important renal complications including hypertension.

As two pivotal regulators in the regulation network of blood pressure, the cross talk between RAS and insulin has been described in many models. For example, Ang II can decrease the number of insulin receptors in the membrane in bovine aortic endothelial cells.<sup>33</sup> Ang II leads to phosphorylation of insulin receptor substrates at Ser<sup>636/639</sup> through activating mTOR/p70S6K and inhibits the insulin-stimulated phosphorylation of eNOS via AT<sub>1</sub>R.<sup>34</sup> Insulin-induced capillary recruitment is abrogated, and glucose disposal is attenuated after the treatment with AT<sub>2</sub>R antagonist, PD123319.<sup>35</sup> However, correlation between AT<sub>2</sub>R and insulin receptor on sodium transport in kidney remains undetermined. Our current study showed that AT<sub>2</sub>R agonist CGP42112 decreased insulin receptor mRNA and protein expressions in a concentration- and time-dependent manner in WKY rats RPT cells. Pretreatment with the AT<sub>2</sub>R agonist decreased the stimulatory effect of insulin on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in RPT cells from WKY rats. Therefore, we deduced that AT<sub>2</sub>R depresses the function of insulin receptor via inhibiting insulin receptor expression. Balance between AT<sub>2</sub>R and insulin receptor is important to keep the steady state for sodium reabsorption in the proximal tubule. However, the antagonism of AT<sub>2</sub>R on insulin receptor is lost in RPT cells from SHRs. Early studies have proved that WKY and SHRs RPT cells own different ways of physiological regulation.<sup>5,6</sup> There are different responses even to the same treatment in WKY rats and SHRs. For example, intravenous infusion of losartan for 1 week significantly lowered systolic blood pressure and increased urine volume and sodium excretion in D<sub>4</sub> dopamine receptor agonist treated SHRs, but not in WKY rats.<sup>36</sup> We showed here that there was an aberrant regulation of AT<sub>2</sub>R on insulin receptor in SHRs which might be involved in the pathogenesis of hypertension.

The mechanism for the inhibitory effect of AT<sub>2</sub>R on insulin receptor expression in WKY rats was further investigated in this study. Both PKC and PI3K play important roles in the signal transduction in inhibition of AT<sub>2</sub>R on insulin receptor. Previous studies show that the signaling pathway for RAS and insulin involves the activation of PI3K and PKC. AT<sub>2</sub>R can

inhibit the phosphorylation and the signal transduction of PKC.<sup>37,38</sup> A previous study has found that inhibition of PKC prevents hepatic insulin resistance.<sup>39</sup> Similarly, mutation or knockout of PKC can change the insulin sensitivity or insulin receptor phosphorylation.<sup>40,41</sup> The present study showed that the inhibitory effect of AT<sub>2</sub>R on insulin receptor expression was blocked by PKC or PI3K inhibitor in WKY RPT cells, but not in SHR RPT cells, indicating that PKC and PI3K are involved in this signaling pathway in normotensive states, but not in hypertensive states. Besides insulin receptor protein expression, mRNA level was also decreased by stimulating AT<sub>2</sub>R in WKY RPT cells, indicating that the regulation may occur at the transcriptional level. However, the detailed mechanisms remain to be elucidated in the future.

In summary, we have demonstrated that AT<sub>2</sub>R inhibits insulin receptor expression in WKY cells, while increases it in SHR cells. PKC and PI3K are signaling pathways involved in the regulation of AT<sub>2</sub>R on insulin receptor in WKY cells, but not in SHR cells. The regulation is pathophysiological significance because pretreatment with CGP42112 inhibited the insulin-mediated stimulatory effect on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity or ERK1/2 phosphorylation in WKY RPT cells but increased it in SHR RPT cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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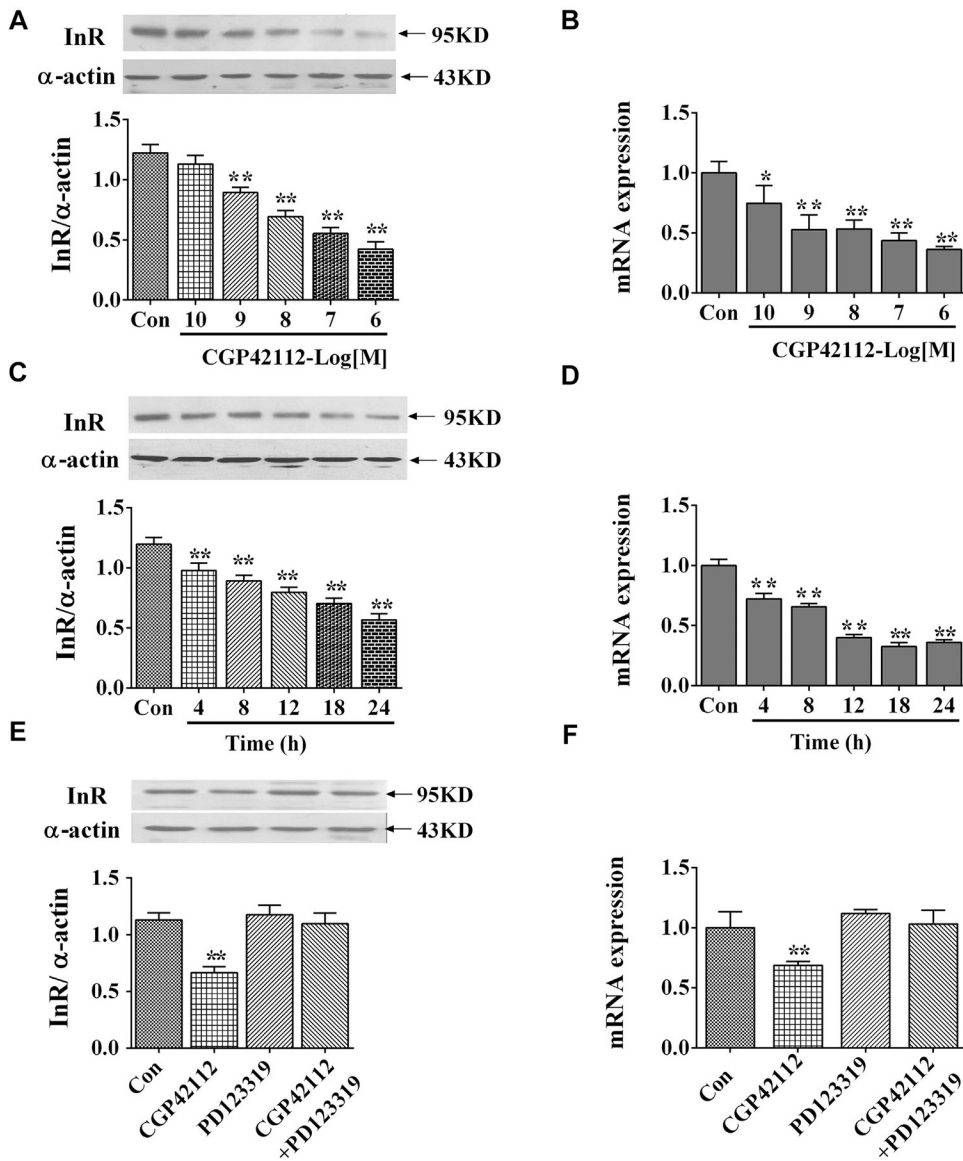
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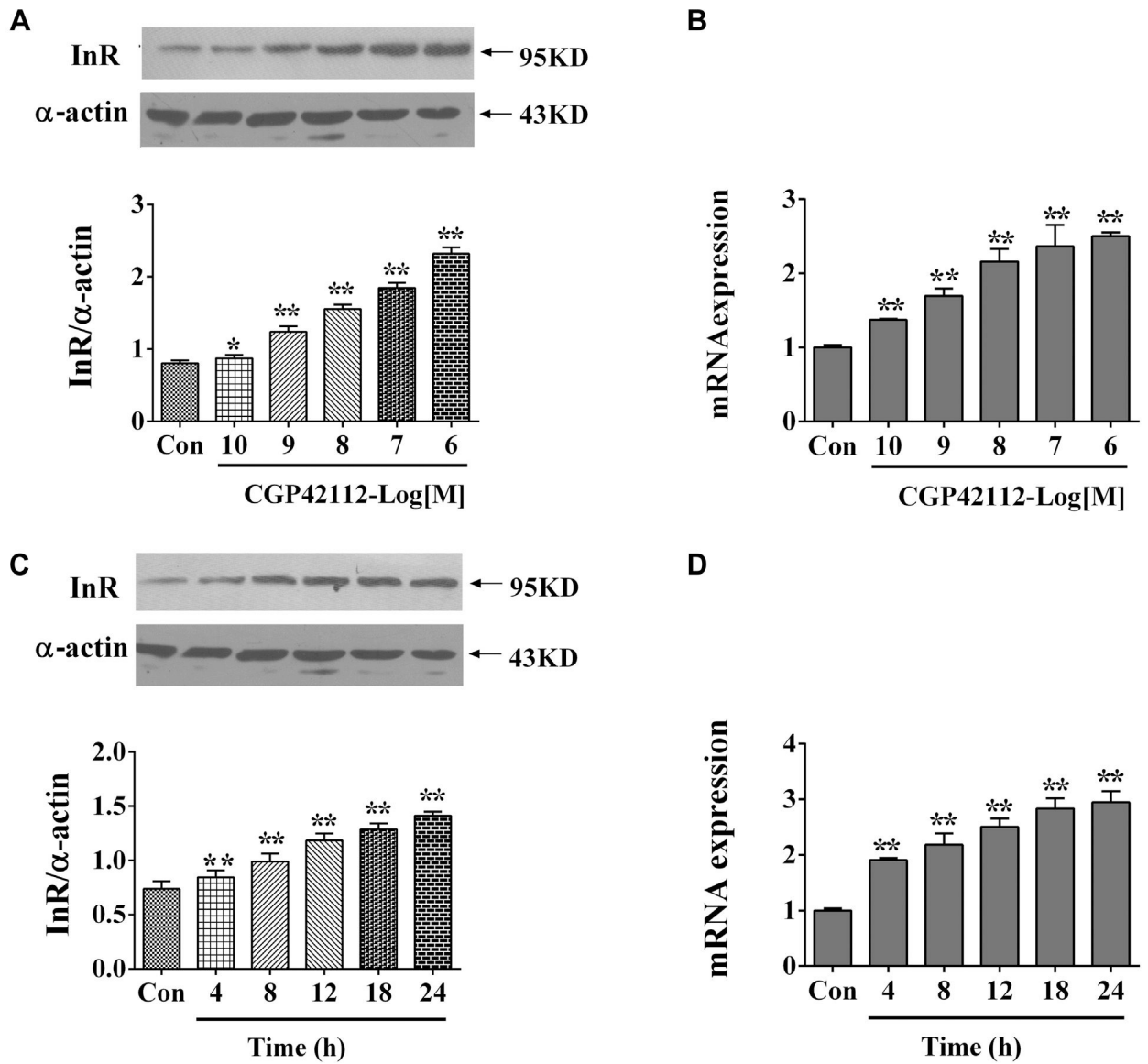


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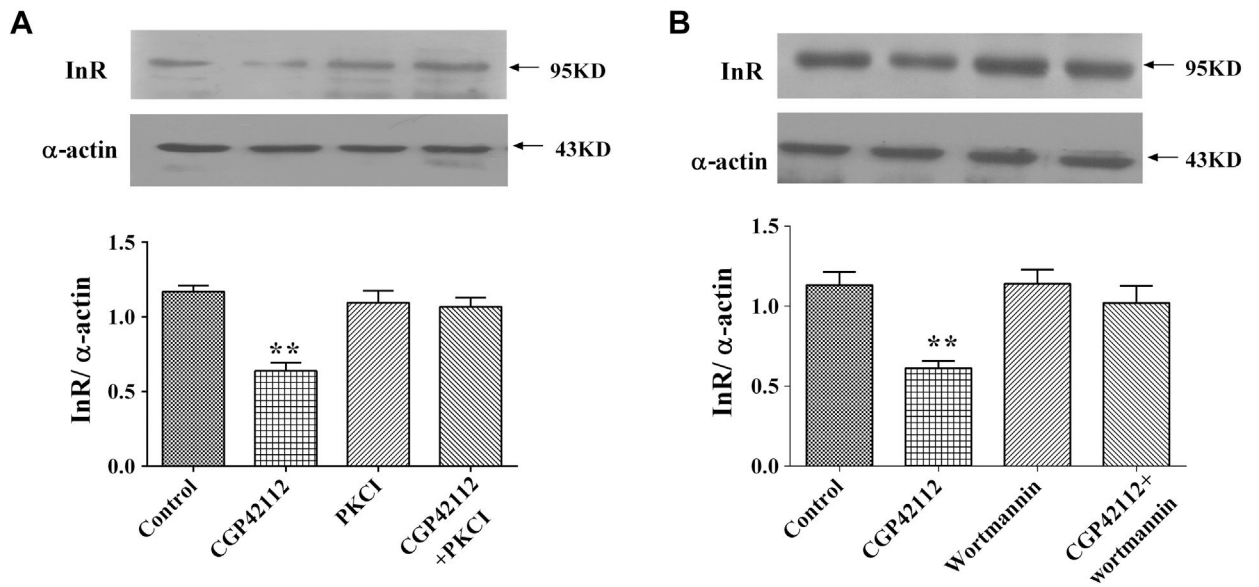
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**Figure 1.** Effect of AT<sub>2</sub>R agonist, CGP42112, on insulin receptor expression in RPT cells from WKY rats. Concentration response of insulin receptor protein expression (A) and mRNA expression (B) in RPT cells after treatment with CGP42112 for 24 hours. Results are expressed as the ratio of insulin receptor and  $\alpha$ -actin densities ( $n = 6$ ; \* $P < .05$ ; \*\* $P < .01$  vs. control). Time course of insulin receptor protein expression (C) and mRNA expression (D) in RPT cells after treatment with CGP42112 ( $10^{-6}$  mol/L) for varying time periods. Results are expressed as the ratio of insulin receptor and  $\alpha$ -actin densities ( $n = 6$ ; \*\* $P < .01$  vs. control [0 time point]). (E and F) Effect of the AT<sub>2</sub>R agonist, CGP42112 ( $10^{-6}$  mol/L), and antagonist, PD123319 ( $10^{-6}$  mol/L), on insulin receptor protein (E) and mRNA expression (F). The cells were incubated for 24 hours. Results are expressed as the ratio of insulin receptor and  $\alpha$ -actin densities ( $n = 6$ ; \*\* $P < .01$  vs. others). AT<sub>2</sub>R, angiotensin II type 2 receptor; RPT, renal proximal tubule; WKY, Wistar-Kyoto.

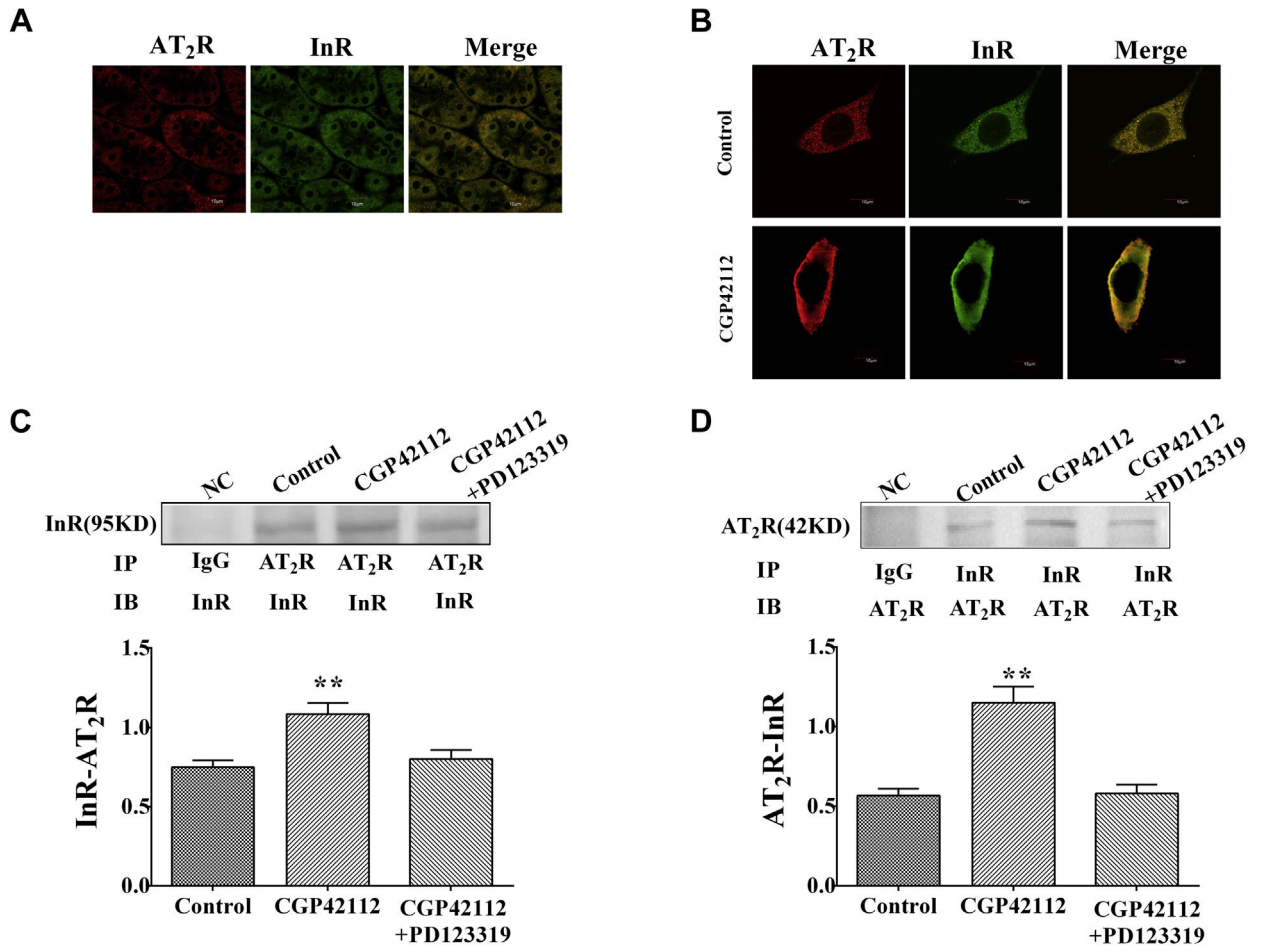


**Figure 2.** Effect of AT<sub>2</sub>R agonist, CGP42112, on insulin receptor expression in SHR RPT cells. Concentration response of insulin receptor protein expression (A) and mRNA expression (B) in RPT cells after treatment with CGP42112 for 24 hours. Results are expressed as the ratio of insulin receptor and  $\alpha$ -actin densities ( $n = 7$ ; \* $P < .05$ ; \*\* $P < .01$  vs. control). Time course of insulin receptor protein expression (C) and mRNA expression (D) in RPT cells after treatment with CGP42112 ( $10^{-6}$  mol/L) for varying time periods. Results are expressed as the ratio of insulin receptor and  $\alpha$ -actin densities ( $n = 7$ ; \*\* $P < .01$  vs. control [0 time point]). AT<sub>2</sub>R, angiotensin II type 2 receptor; RPT, renal proximal tubule; SHR, spontaneously hypertensive rats.



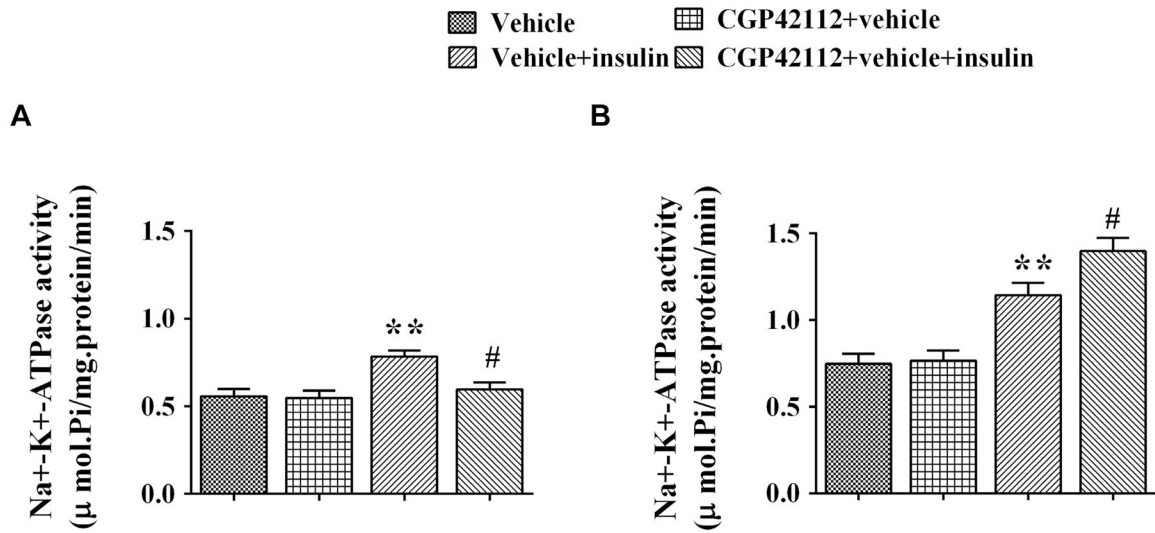
**Figure 3.**

Role of PKC and PI3K in the effect of AT<sub>2</sub>R on insulin receptor expression in RPT cells from WKY rats. (A) Effect of CGP42112 and the PKC inhibitor 19–31 on insulin receptor protein expression in RPT cells from WKY rats. The cells were incubated with CGP42112 (10<sup>-6</sup> mol/L) or PKC inhibitor 19–31 (10<sup>-6</sup> mol/L) for 24 hours. Results are expressed as the ratio of insulin receptor to α-actin densities (*n* = 5; \*\**P* < .01 vs. control). (B) Effect of CGP42112 and the PI3K inhibitor wortmannin on insulin receptor protein expression in RPT cells from WKY rats. The cells were incubated with CGP42112 (10<sup>-6</sup> mol/L) or wortmannin (10<sup>-6</sup> mol/L) for 24 hours. Results are expressed as the ratio of insulin receptor to α-actin densities (*n* = 5; \*\**P* < .01 vs. control). AT<sub>2</sub>R, angiotensin II type 2 receptor; RPT, renal proximal tubule; PI3K, phosphatidylinositol 3 kinase; PKC, protein kinase C; WKY, Wistar-Kyoto.

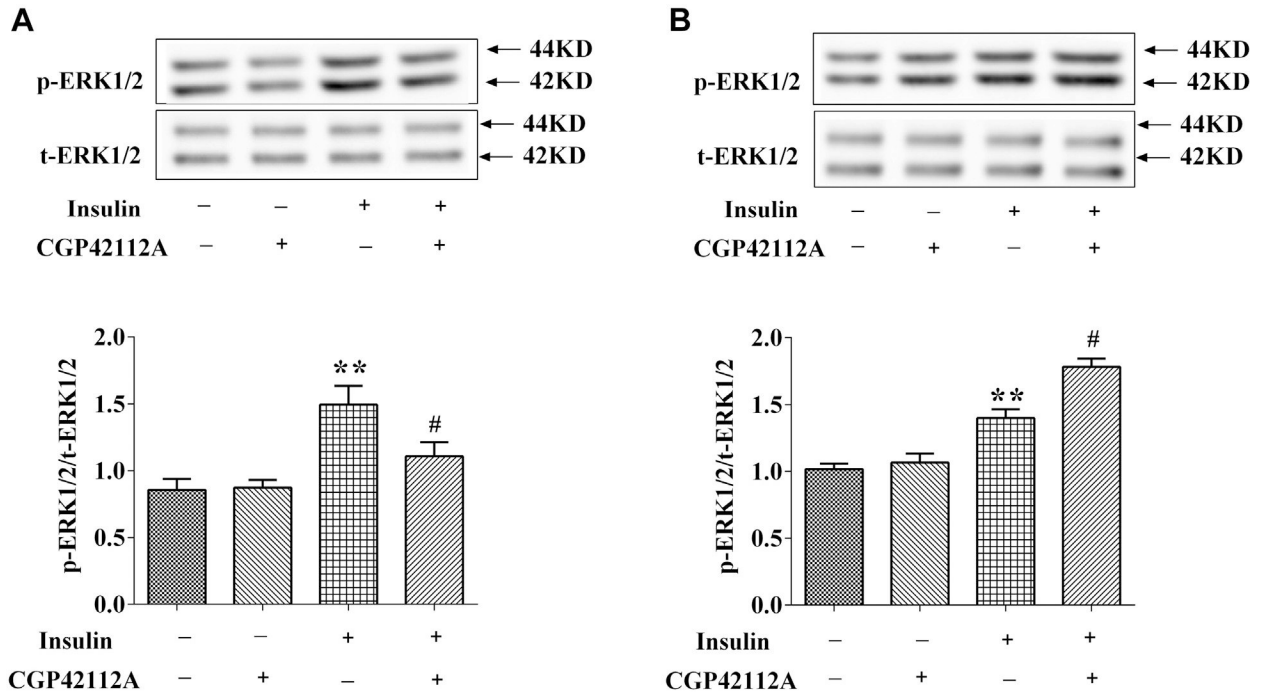


**Figure 4.**

The linkage between AT<sub>2</sub> and insulin receptors in kidney and RPT cells from WKY rats. (A and B) The colocalization of AT<sub>2</sub> and insulin receptors in kidney and RPT cells from WKY rats. The samples were washed, fixed, and immunostained for AT<sub>2</sub> and insulin receptors, as described in the Methods. Colocalization appears as yellow after merging the images of fluorescein isothiocyanate-tagged insulin receptor (green) and Alexa 568-tagged AT<sub>2</sub> receptor (red). As for WKY RPT cells, the cells were treated with or without CGP42112 (10<sup>-6</sup> mol/L) for 24 hours. (C and D) Effect of the AT<sub>2</sub>R agonist, CGP42112, on AT<sub>2</sub>/insulin receptor coimmunoprecipitation in RPT cells. After the cells were incubated with CGP42112 (10<sup>-6</sup> mol/L) for 24 hours, samples were immunoprecipitated with anti-AT<sub>2</sub>R antibody and immunoblotted with anti-insulin receptor antibody (C) or immunoprecipitated with anti-insulin receptor antibody and immunoblotted with anti-AT<sub>2</sub>R antibody (D). Results are expressed as relative density units (DUs) (*n* = 5, \*\**P* < .01 vs. control). AT<sub>2</sub>R, angiotensin II type 2 receptor; RPT, renal proximal tubule; WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rats.



**Figure 5.** Effect of pretreatment with AT<sub>2</sub>R agonist on the stimulatory effect of insulin on Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in WKY (A) and SHR (B) RPT cells. The cells were pretreated with AT<sub>2</sub>R agonist, CGP42112 (10<sup>-6</sup> mol/L), or vehicle (dH<sub>2</sub>O) for 24 hours after washing three times (15 minutes/times) with serum-free culture medium and then treated with insulin (10<sup>-7</sup> mol/L) for 15 minutes. Results are expressed as mmol phosphate released per mg protein per minute (*n* = 7, \*\**P* < .01 vs. control; #*P* < .05 vs. vehicle + insulin). AT<sub>2</sub>R, angiotensin II type 2 receptor; RPT, renal proximal tubule; SHRs, spontaneously hypertensive rats; WKY, Wistar-Kyoto.



**Figure 6.** Effect of insulin and AT<sub>2</sub>R agonists on phospho-ERK1/2 protein expression in RPT cells from WKY rats (A) and SHR rats (B). The cells were pretreated with AT<sub>2</sub>R agonist, CGP42112 (10<sup>-6</sup> mol/L), or vehicle (dH<sub>2</sub>O) for 24 hours after washing three times (15 minutes/times) with serum-free culture medium and then treated with insulin (10<sup>-7</sup> mol/L) for 15 minutes. The cell lysates were immunoblotted with antiphosphorylation (p)-ERK antibody and antitotal (t)-ERK antibody. P-ERK expression was corrected by t-ERK protein expression. (*n* = 3, \*\**P* < .01 vs. control; #*P* < .05 vs. insulin). AT<sub>2</sub>R, angiotensin II type 2 receptor; RPT, renal proximal tubule; WKY, Wistar-Kyoto.