A novel adipokine CTRP1 stimulates aldosterone production

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ABSTRACT **Complement-C1q TNF-related protein 1 (CTRP1), a member of the CTRP superfamily, is expressed at high levels in adipose tissues of obese Zucker diabetic fatty (*fa/fa*) rats, and CTRP1 expression is induced by proinflammatory cytokines, including TNF-α and IL-1β. In the present study, we investigated stimulation of aldosterone production by CTRP1, since it was observed that CTRP1 was specifically expressed in the zona glomerulosa of the adrenal cortex, where aldosterone is produced. Increased aldosterone production by CTRP1 in cells of the human adrenal cortical cell line H295R was dose-dependent. Expression levels of aldosterone synthase CYP11B2 were examined to investigate the molecular mechanisms by which CTRP1 enhances the production of aldosterone. The expression of CYP11B2 was greatly increased by treatment with CTRP1, as was the expression of the transcription factors NGFIB and NURR1, which play critical roles in stimulation of CYP11B2 gene expression. It was also revealed that angiotensin II-induced aldosterone production is, at least in part, mediated by the stimulation of CTRP1 secretion, not by the increase of CTRP1 mRNA transcription. In addition, the levels of CTRP1 were significantly up-regulated in hypertensive patients' serum. As CTRP1 was highly expressed in obese subjects as well as up-regulated in hypertensive patients, CTRP1 may be a newly identified molecular link between obesity and hypertension. Jeon, J. H., Kim, K., Kim, J. H., Baek, A., Cho, H., Lee, Y. H., Kim, J. W., Kim, D., Han, S. H., Lim, J.-S., Kim, K.-I., Yoon, D. Y., Kim, S.-Y., Oh, G. T., Kim, E., Yang, Y. A novel adipokine CTRP1 stimulates aldosterone production. *FASEB J.* 22, 1502–1511 (2008)**

Key Words: hypertension · adipocytes · obesity · CYP11B2

ADIPONECTIN HAS STRUCTURAL FEATURES that include an N-terminal collagen-like domain and a C-terminal C1q globular domain (1). Proteins containing the latter are termed complement-C1q TNF-related proteins (CTRPs). Among this family, CTRP3 (also known as collagenous repeat-containing sequence of the 26-kea protein; CORES-26) has been reported to play an important role in regulating both chondrogenesis and cartilage development (2) and to have anti-inflammatory properties (3). CTRP2 is known to increase glycogen accumulation and fatty acid oxidation by the activation of AMP-activated protein kinase (4). Mutation of CTRP5 leads to insufficient levels of secreted CTRP5, causing late-onset retinal macular degeneration (5, 6). CTRP1 is reported to inhibit collagen-induced platelet aggregation by specifically blocking binding of the von Willebrand factor to collagen (7). In a previous study, we observed that the expression of CTRP1 is greatly increased in adipose tissues of *db/db* mice and Zucker diabetic fatty (*fa/fa*) rats (8).

The steroid hormone aldosterone, synthesized in the zona glomerulosa of the adrenal cortex, plays a critical role in the regulation of sodium and water balance. These effects are mediated by the binding of aldosterone to the mineralocorticoid receptor in epithelial cells in the kidneys, colon, and salivary glands (9). Through osmotic effects, aldosterone maintains constant circulating blood volume by causing sodium retention when plasma volume is insufficient and by decreasing sodium retention when plasma volume is ample (10, 11). The most important stimuli of aldosterone secretion are angiotensin II and extracellular potassium.

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The renin-angiotensin-aldosterone system (RAAS) plays a key role in the production of aldosterone. Kidney-derived renin cleaves liver-derived angiotensinogen to form angiotensin I (Ang I) in circulating blood. Angiotensin-converting enzyme (ACE), located on the luminal side of the endothelium, subsequently converts Ang I to Ang II. Ang II exerts its effects *via* stimulation of Ang II type 1 (AT1) and Ang II type 2 (AT2) receptors. While the AT1 receptor (12) mediates the classical physiological effects of Ang II, including vasoconstriction, aldosterone and vasopressin release, and sodium and water retention, signaling through the AT2 receptor regulates complex biological programs, such as embryonic development, cell differentiation, tissue repair, and programmed cell death (13, 14). The production of aldosterone mediated by the AT1 receptor is regulated at two critical enzyme steps: the formation of pregnenolone from cholesterol by the mitochondrial enzyme *P*-450scc (side-chain cleavage) (1), and the conversion of corticosterone to aldosterone by cytochrome *P*-450 11β-hydroxylase 2 (CYP11B2) (2).

It is well known that obesity is the leading cause of hypertension. However, the mechanisms linking obesity and hypertension are not well understood. A growing body of evidence suggests that activation of oxidative stress, the sympathetic nervous system, and RAAS are related to obesity-associated hypertension (15). Human studies have demonstrated that circulating levels of all components of RAAS (including angiotensinogen, renin, and aldosterone), and ACE activity are augmented in obese compared to lean individuals (16, 17). In the present study, we show that CTRP1 is expressed in the zona glomerulosa of the adrenal cortex, stimulating aldosterone production through induction of CYP11B2 gene expression. Therefore, CTRP1 could be a molecular link between obesity and hypertension.

MATERIALS AND METHODS

Cell cultures

H295R human adrenocortical carcinoma cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 15 mM HEPES, 0.00625 mg/ml insulin, 0.00625 mg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml bovine serum albumin (BSA), 0.00535 mg/ml linoleic acid, and 2.5% Nu-Serum I. The insulin, transferrin, selenium, BSA and linoleic acid (ITS+ Universal Culture Supplement Premix), and Nu-Serum were purchased from BD Biosciences (Bedford, MA, USA). Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Animals

Male Sprague-Dawley (SD) rats (Dae Han Bio Link, Seoul, Korea) 5 and 16 wk of age were allowed to acclimate to handling for 4 days under standard conditions (12 h light/dark). Food and water were provided *ad libitum*.

Northern blot analysis

Total RNA from SD rat tissues was prepared using RNAzol B (TEL-TEST, Friendswood, TX, USA), electrophoresed in a 3% formaldehyde gel, and transferred to a nylon membrane using a turbo blotter (Whatman Bioscience, Cambridge, UK). To estimate the RNA loading, the transferred membrane was photographed. ^{32}P-labeled cDNA probes were prepared from the coding region of rat CTRP1 cDNA using a High Prime DNA labeling kit (Roche Applied Science, Indianapolis, IN, USA) and used in the sequential hybridizations. After each hybridization, the membrane was washed with 0.1 × SSC, 0.05% sodium dodecyl sulfate (SDS) at 50°C and exposed to X-ray film for 18 h at −80°C with an intensifying screen.

In situ hybridization

For *in situ* hybridization the adrenal gland tissues were isolated from 5- and 16-wk-old SD rats. A specimen from a normal rat adrenal gland was fixed in 4% paraformaldehyde solution. *In situ* hybridization was performed with digoxigenin-labeled rat CTRP1 riboprobes. Rat CTRP1 antisense and sense riboprobes were synthesized from rat cDNA fragments. Frozen sections of each tissue were prepared and collected in phosphate buffered saline (PBS) in a 24-well plate. The tissues were treated with proteinase K, acetylated with 0.25% acetic anhydride, prehybridized, hybridized with probes at a concentration of 0.5 μg/ml, treated with RNase A, and washed. The washed tissues were incubated with antidigoxigenin alkaline phosphate-conjugated serum (Roche Applied Science). The final coloring reaction was performed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution. For microscopy, each sample section was mounted under a coverslip on a gelatin-coated slide.

Aldosterone measurement

H295R cells were plated at a concentration of 5×10^5 cells/ml in 24-well plates and incubated for 24 h. Prior to experiments, the cells were starved with serum-free DMEM (Dulbecco's modified Eagle's medium)/Ham's F12 medium 24 h. The serum-starved cells were treated for various times in different concentrations of CTRP1, and culture supernatants were harvested at each time point. The levels of aldosterone in the culture supernatants were determined using a human aldosterone ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA). Recombinant human CTRP1 was produced using the baculovirus system and purified in the laboratory (8).

Intracellular calcium measurement

For intracellular calcium measurement, 8×10^6 cells/ml of H295R cells were trypsinized and suspended in Krebs buffer (136 mM NaCl, 1.2 mM KH_2PO_4, 1.8 mM KCl, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, and 5.5 mM D-glucose, buffered to pH 7.4 with 20 mM HEPES) supplemented with 1% BSA. Cells were incubated in the dark for 30 min at 37°C in the presence of 5 μM Fura-2/AM (Molecular Probes, Eugene, OR, USA). The cells were washed twice with calcium-free Krebs medium for measurements without external calcium, and resuspended to a final concentration of 2×10^6 cells/ml. After 2 min equilibration time with nontreated control cells, stimulators were directly added in the tube. All measurements were recorded at 37°C under constant stirring. Fura-2/AM fluorescence (alternative excitation at 340 and 380 nm, and emission at 510 nm) was detected using a RF-5301PC fluorometer (Shimadzu, Columbia, MD, USA).

Calcium concentrations were determined from fluorescence ratios using software provided with SUPERCAP.

siRNA preparation and transfection

Small interference RNA (siRNA) oligonucleotides were obtained from Samchully Pharmaceuticals (Seoul, South Korea). The following sequences were used for construction of human CTRP1 siRNA (siRNA-1: forward 5′-GUAAACCGUGGAGGACAAA-3′, reverse 5′-UUUGUCCUCCACGGUUUAC-3′; siRNA-2: forward 5′-CAUGCACAGCAACCACUAC-3′, reverse 5′-GUAGUGGUUGCUGUGCAU G-3′). Green fluorescent protein (GFP) siRNA was used as a control for CTRP1 siRNA. The synthesized siRNA oligonucleotides were annealed and transfected into H295R cells using Amaxa Nucleofection Technology (Amaxa, Cologne, Germany). Briefly, 5×10^6 cells and 2.5 mM duplex siRNA were mixed in 100 μl of R -type buffer and transferred to the cuvettes provided. Mixtures were nucleofected using a T-020 pulsing parameter with an Amaxa Nucleofector apparatus and immediately transferred to prewarmed culture medium at 37°C in 6-well plates, and incubated for 18 h.

Real-time RT-PCR analysis

Total RNA from H295R cells prepared as above was isolated using RNAzolB, and 3 μg of total RNA was reverse transcribed using M-MuLV reverse transcriptase (Promega, Madison, WI, USA) at 37°C for 1 h. PCR for the amplification of various target genes was then performed. The following primers were used for real time PCR (human CYP11B1: forward 5′-AGGAGACCTTGCCGCTCTAC-3′, reverse 5′-GGACGTGCCCGGCCGCAATA-3′; human CYP11B2: forward 5′-CTGGGACATTGGTGCGC-3′, reverse 5′-GTGTTTCAGCACATGGT-3′). PCR was performed in a Rotor-Gene 3000 real-time cycler (Corbett Research, Sydney, Australia), with amplification cycles of 10 s of denaturation at 94°C, 15 s of annealing at 55°C, and 20 s of extension at 72°C. Total number of cycles was 40. The amplification efficiency of the target was equal to that of the endogenous reference. β-Actin was used as the endogenous reference in the comparative cycle threshold (C_T) method. RT-PCR analysis was performed for the amplification of steroidogenic acute regulatory protein (StAR) and CYP11A1 genes. The following primers were used for RT-PCR (human CYP11A1: forward 5′-GACTCCTGGGAGACAGCAAG-3′, reverse 5′-GAATGTGGTGCCCACATCGCTG-3′; human StAR: forward 5′- GTGAGCGTGCGCTGTGCCAAG-3′, reverse 5′-CAGGACCTGGTTGATGATGC′). RT-PCR was performed with 26 cycles of 10 s of denaturation at 94°C, 15 s of annealing at 55°C, and 20 s of extension at 72°C.

Immunoprecipitation

Human serum samples were obtained from 15 men and 5 women with hypertension, and 15 healthy male and 5 female volunteers. Human serum samples were suspended in an equal volume of GST-IP buffer (20 mM Tris-HCl, pH 7.9, 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40, protease inhibitors). Goat anti-human CTRP1 polyclonal antibody (R&D System, Minneapolis, MN, USA) was added, and the mixture incubated for 2 h at 4°C, then immunoprecipitated with Protein G-agarose beads (Roche Applied Science) for 2 h at 4°C. After washing the beads five times with GST-IP buffer, the bound proteins were recovered by boiling in SDS-PAGE sample buffer. The eluted proteins were separated on 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Marlborough, MA, USA). The membrane was blocked with 5% skim milk in TBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl) for 30 min, then incubated for 2 h with mouse anti-human CTRP1 monoclonal antibody (E21H7), which produced in our laboratory. After washing three times with TBST (0.05% Tween-20, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl) for 15 min, the membrane was incubated with the anti-mouse IgG secondary antibody conjugated with horseradish peroxidase. The signal was detected using the Amersham enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Uppsala, Sweden) and analyzed with a LAS3000 luminescence image analyzer (Fujifilm, Valhalla, NY, USA).

Western blot analysis

Total cell lysates were prepared at the indicated time point after treatment of CTRP1 40 ng/ml or Ang II 100 μM, separated on a 12% SDS-polyacrylamide gel, and then transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% skim milk in TBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl) for 30 min, and then incubated with anti-CYP11B2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-NGFIB and anti-NURR1 antibodies (Perseus Protemoics, Tokyo, Japan) overnight. After washing three times with TBST (0.05% Tween 20, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl) for 15 min, the membrane was incubated with the anti-mouse IgG or the donkey anti-goat IgG secondary antibody conjugated with horseradish peroxidase. The signal was detected using the Amersham ECL system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

Subjects

The study population included 20 patients with essential hypertension and 20 healthy volunteers, all aged between 30 and 80 yr. The body mass index (BMI) of patients group was 22.83 ± 3.38. Among the 20 patients, there were 8 cases of Type II diabetes mellitus and 10 cases of cardiovascular diseases. Hypertension was defined as a diastolic blood pressure (BP) > 90 mmHg or a systolic BP > 140 mmHg. The study protocol was approved by the Internal Regulatory Board in the College of Medicine, Dankook University. All of the patients were aware of the nature, purpose, and potential risks of the study and gave written consent to involvement.

RESULTS

CTRP1 mRNA is expressed in the adrenal gland

Obesity is considered as a risk factor for various diseases, including diabetes, cardiovascular diseases, hypertension, and cancer. We previously showed that CTRP1 was greatly increased in adipose tissue in animal models, including *db/db* mice and obese Zucker diabetic rats. This finding suggested that CTRP1 may be a molecular link between obesity and related diseases. To test this possibility, we performed Northern blot analysis to examine expression patterns of CTRP1 in various rat tissues, including adrenal glands, adipose tissues, heart, muscle, and kidney. Interestingly, CTRP1 was highly expressed in the adrenal gland of SD rats (**Fig. 1*A***), and fat and heart tissues also showed CTRP1 bands at the longer exposure times (data not shown). To determine whether CTRP1 is also expressed in the

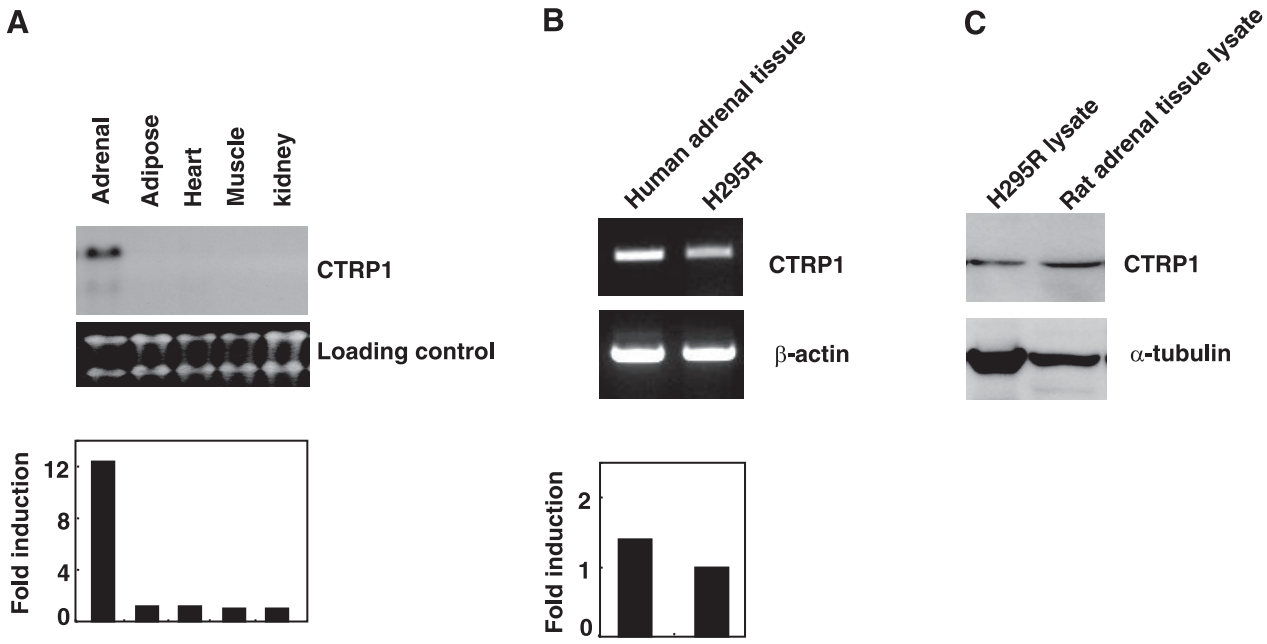


Figure 1. Distribution of CTRP1 mRNA expression in tissues. *A*) Total RNA from various tissues of SD rats was isolated, and a rat tissue blot was prepared. The blot was hybridized with a rat CTRP1 probe. *B*) Total RNA from human adrenal tissue and H295R cells was reverse transcribed, and PCR was performed with a pair of human CTRP1 primers. Quantification of CTRP1 bands was performed to measure the intensity of bands using DNR Bioimaging system *C*) H295R cell and rat adrenal tissue lysates were subjected to Western blot analysis using anti-CTRP1 antibody.

human adrenal gland, CTRP1 expression was assessed using RT-PCR analysis of human adrenal gland tissue and human adrenocortical carcinoma cell line H295R. Both showed strong expression of CTRP1 mRNA (Fig. 1*B*). To examine protein level of CTRP1 in H295R cell and rat adrenal tissue lysates, Western blot analysis was performed using anti-CTRP1 antibody. Level of CTRP1 protein in H295R cell lysate was similar to rat adrenal tissue lysate (Fig. 1*C*). *In situ* hybridization showed that expression of CTRP1 mRNA was highly specific to the zona glomerulosa of the adrenal cortex, and no expression was observed in the medulla region (**Fig. 2**). This pattern of expression was similar in both 5-wk-old and 16-wk-old SD rats.

CTRP1 protein increases production of aldosterone

The adrenal gland is the main site of production of steroid hormones, including cortisol, androgen, and aldosterone. It is well known that the zona glomerulosa is important in aldosterone secretion. To assess whether CTRP1 protein is able to increase production of aldosterone in H295R cells, we treated the cells with

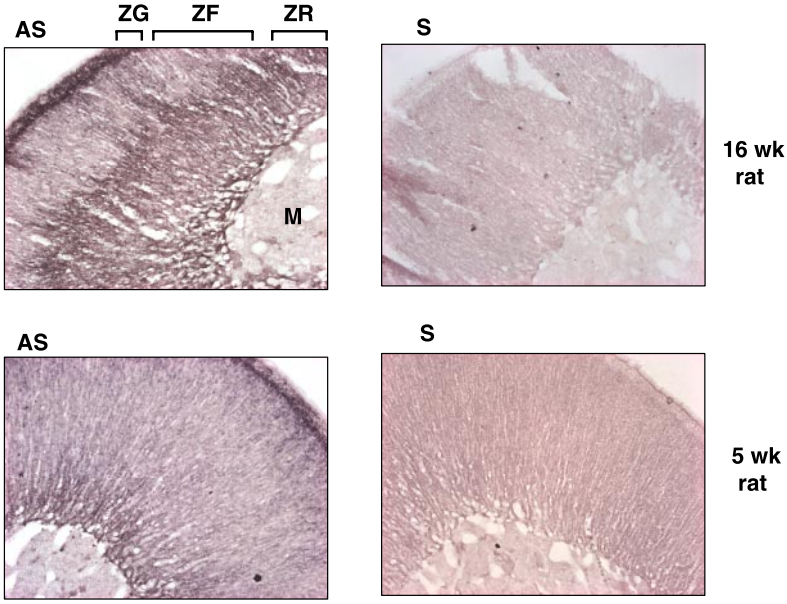


Figure 2. CTRP1 mRNA expressed in the zona glomerulosa region of the adrenal cortex. Adrenal glands were isolated from 5- and 16-wk-old SD rats and subjected to *in situ* hybridization. Sections of adrenal glands were hybridized with digoxigenin-labeled CTRP1 antisense and sense riboprobes, prepared by using rat CTRP1 cDNA. The final coloring reaction was performed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate solution. AS, antisense probe; S, sense probe; ZG, zona glomerulosa; ZF, zona fasiculata; ZR, zona reticulate; M, medulla.

different doses of recombinant human CTRP1. CTRP1 markedly stimulated the production of aldosterone, with its concentration peaking at more than 40 ng/ml (**Fig. 3*A***). To know whether this concentration is in the physiological concentration range, the concentration of CTRP1 in the human serum was measured by Western blot analysis. Its concentration range was 6–26 ng/ml. It indicates that the aldosterone-producing concentration of CTRP1 is in the physiological range. The time kinetics of aldosterone production by CTRP1 was examined by treating with CTRP1 for various time intervals at a concentration of 40 ng/ml. The level of aldosterone production peaked at 12 h and decreased after 24 h (Fig. 3*B*). We next tested whether cotreatment of CTRP1 with Ang II could enhance the aldosterone production because both CTRP1 and Ang II were able to increase the production of aldosterone. CYP11B2 mRNA expression was analyzed using real-time PCR 12 h after cotreatment of CTRP1 with Ang II. The CYP11B2 expression was additively increased (Fig. 3*C*). To examine whether the increase of CYP11B2 gene expression is able to lead to an increase of aldosterone production, level of aldosterone was measured 24 h after the cotreatment. The aldosterone production was also additively increased by the cotreatment of CTRP1 with angiotensin II (Fig. 3*C*).

Levels of CTRP1 in hypertensive patients are increased

We assessed whether the level of CTRP1 is increased in the blood of hypertensive patients, as aldosterone production was stimulated by CTRP1. Serum samples from hypertensive patients were immunoprecipitated with anti-CTRP1 antibody and subjected to Western blot analysis. The densities of CTRP1 bands were calculated by Multi-Gauge V 3.0 image analysis software. The CTRP1 levels were significantly increased in hypertensive patients compared with healthy controls (**Fig. 4*A***), and the BMI of patients group was 22.83 ± 3.38. Thus, we could exclude the possibility that the high serum levels of CTRP1 in patients group might be due to obesity because CTRP1 was produced in adipose tissue. To further confirm this fact, adiponectin levels were also measured. The serum levels of adiponectin were lower than control group, but it was not statistically significant (Fig. 4*B*).

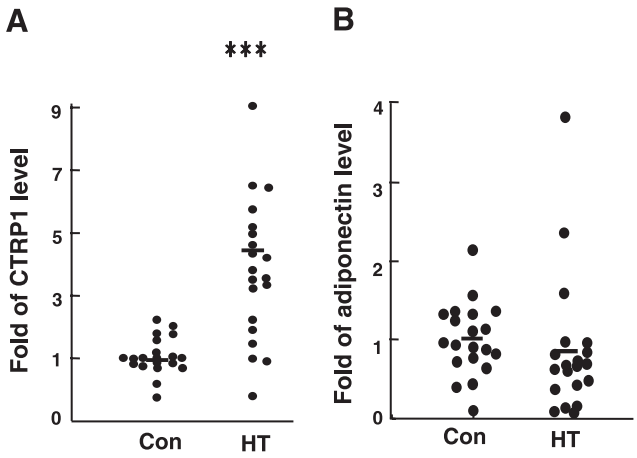


Figure 4. Levels of CTRP1 are increased in hypertensive patients. *A*) Serum samples from various patients were immunoprecipitated with anti-CTRP1 antibody and subjected to Western blot analysis. The bands were detected with the monoclonal anti-CTRP1 antibody, E21H7. The density of CTRP1 bands were calculated using Multi-Gauge V 3.0 image analysis software. ***$P < 0.001$ *vs.* control. *B*) The level of adiponectin in serum samples from various patients was measured by Western blot analysis using antiadiponectin antibody.

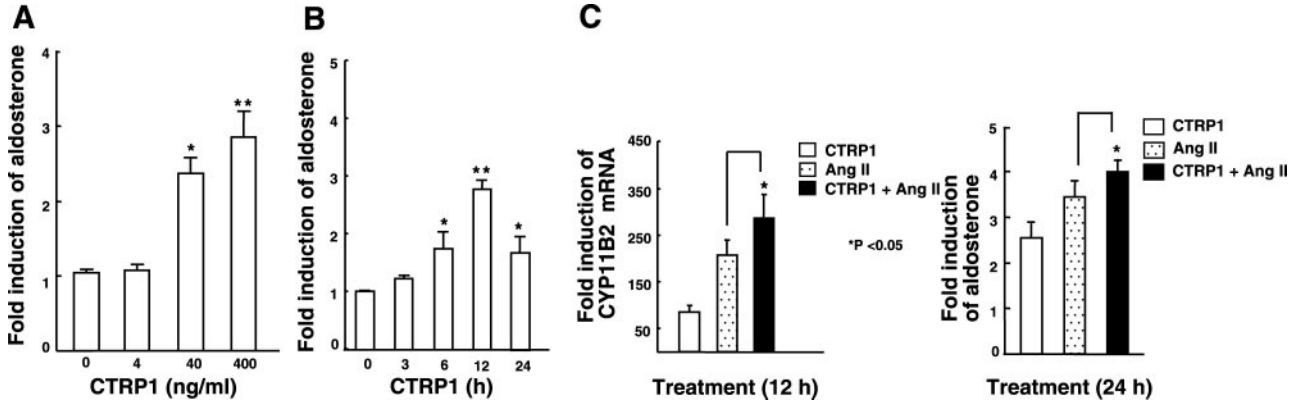


Figure 3. CTRP1 increases production of aldosterone. *A*) H295R cells were treated with the indicated concentrations of CTRP1 for 24 h. Culture medium was harvested, and the levels of aldosterone were determined using a human aldosterone ELISA kit. The experiments were performed in triplicate. *B*) H295R cells were treated with 40 ng/ml of CTRP1. The culture medium was harvested at the indicated time points, and the levels of aldosterone were determined using a human aldosterone ELISA kit. The experiments were performed three separate times in triplicate. Representative results are shown from three independent results. *$P < 0.05$; **$P < 0.01$ *vs.* control *C*). H295R cells were treated with 20 ng/ml of CTRP1 and 500 nM of Ang II. CYP11B2 mRNA expression was analyzed using real-time PCR 12 h after the cotreatment (left) and levels of aldosterone were determined using a human aldosterone ELISA kit 24 h after the cotreatment (right). Representative results are shown from two independent results. Each experiment was performed in triplicate. *$P < 0.05$ *vs.* Ang II treatment.

CTRP1 increases expression of CYP11B2 mRNA

To find a molecular pathway by which CTRP1 increases the production of aldosterone, we examined the expression of a number of steroidogenic enzymes, including *P*-450scc (CYP11A1), StAR, P45011beta (CYP11B1), and P450aldo (CYP11B2). H295R cells were treated with CTRP1 for various time intervals, and RT-PCR analysis was performed using isolated total RNA. CTRP1 strongly induced transcription of CYP11B2 mRNA, which is a rate-limiting enzyme for aldosterone production (**Fig. 5*A***). Induction of CYP11B2 began 6 h after CTRP1 treatment, peaked at 12 h, and diminished at 24 h. Ang II also showed an increase in CYP11B2 gene expression, and the level of induction was 2-fold higher than CYP11B2 gene induction by CTRP1. To further confirm this result at the protein level, we performed Western blot analysis using anti-CYP11B2 antibody. The protein level was also increased by the treatment of CTRP1 and Ang II (Fig. 5*B*). CTRP1 did not increase the transcription of CYP11B1 whereas Ang II increased transcription of CYP11B1 and StAR, consistent with previous reports (18, 19). However, CYP11A1 transcription was not significantly changed by CTRP1 or Ang II treatment. To further confirm CYP11B2 induction by CTRP1, real-time PCR analysis was performed. The results were consistent with those of RT-PCR analysis.

CTRP1 increases intracellular calcium concentration

Since an increase in intracellular calcium concentration plays a critical role in the induction of CYP11B2 mRNA expression, we assessed whether CTRP1 is able to increase intracellular calcium concentration for aldosterone production. H295R cells were labeled with Fura-2 AM, and then changes in calcium concentration were measured after the CTRP1 treatment. The exposure of H295R cells to CTRP1 caused a rapid transient increase in intracellular calcium concentration (**Fig. 6**).

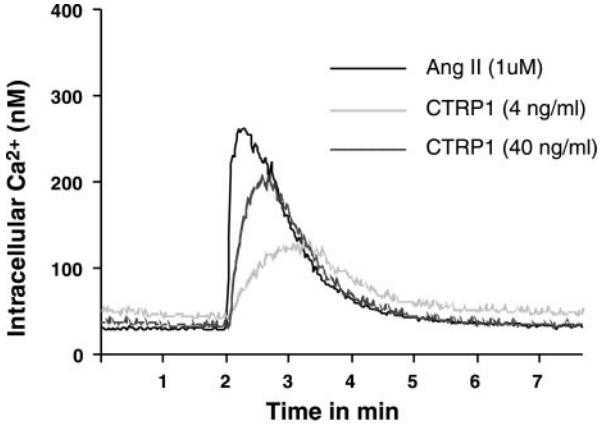
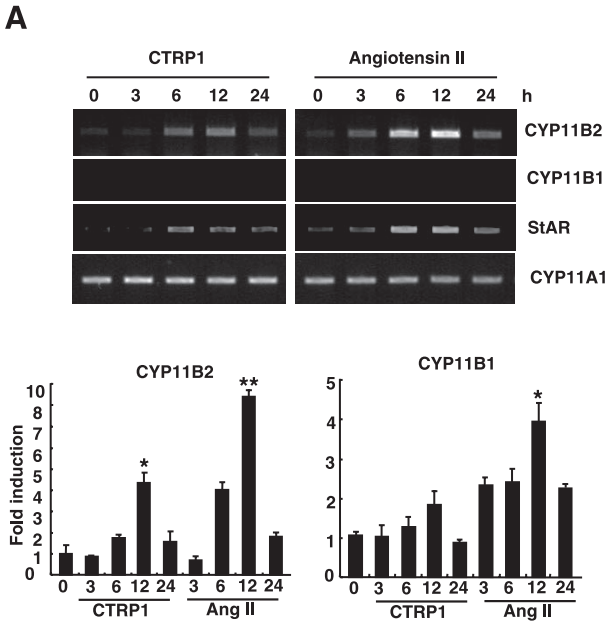


Figure 6. CTRP1 stimulates the release of calcium in H295R cells. H295R cells were loaded with Fura-2 AM; baseline or resting calcium concentrations were monitored for 2 min before the addition of CTRP1 or Ang II, and fluorometric recordings were continued for an additional 6 min. The data were converted to nanomole calcium concentrations. Representative trace recordings from experiments that were repeated four times are shown.

CTRP1 induces Nurr1 and NGFIB mRNA expression

It has been reported that nerve growth factor-induced clone B (NGFIB) (NR4A1) and Nur-related factor 1 (NURRI) (NR4A2) play critical roles in the induction of CYP11B2 expression (20). We assessed whether CTRP1 is able to regulate the expression of NGFIB and NURRI by investigating NGFIB and NURRI mRNA expression in H295R cells stimulated with CTRP1 or Ang II for the indicated time period. Both NGFIB and NURRI mRNA expression increased 3 h after treatment with CTRP1 and returned to the basal level 12 h after



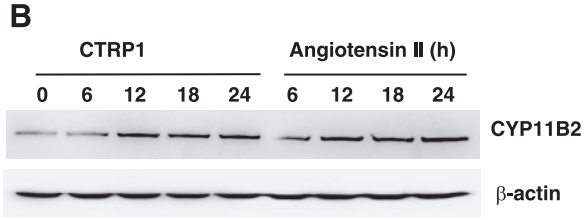


Figure 5. CTRP1 increases expression of CYP11B2 mRNA in H295R cells. *A*) H295R cells were treated with 40 ng/ml of CTRP1 or 100 μM of Ang II. Total RNA was isolated at the indicated time points and RT-PCR was performed using CYP11B2, CYP11B1, StAR, and CYP11A1 primer pairs. Real-time quantitative PCR (bottom) was also carried out using CYP11B2 and CYP11B1. The experiments were performed in triplicate. $*P < 0.05$; $**P < 0.01$ *vs.* control. *B*) H295R cells were treated with 40 ng/ml of CTRP1 or 100 μM of Ang II for the indicated time. Total cellular protein was separated by SDS-PAGE, followed by Western blot analysis using anti-CYP11B2 antibody.

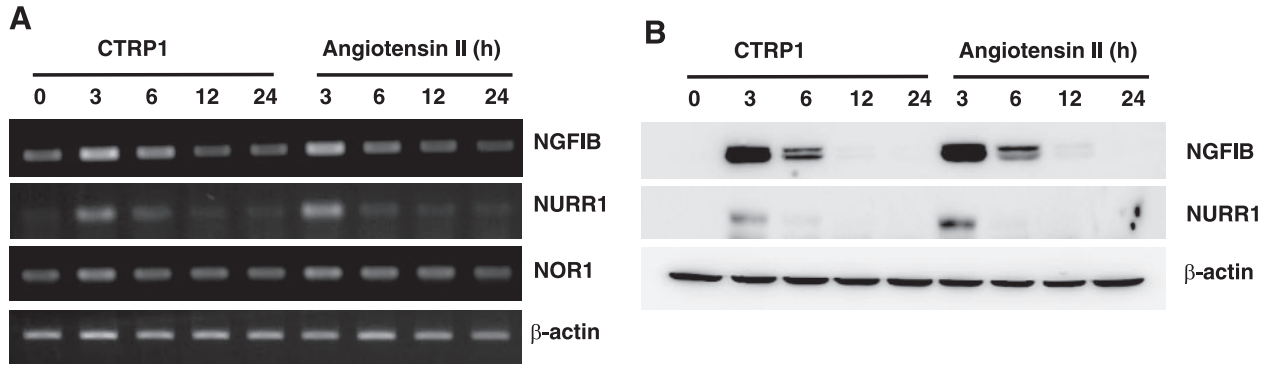


Figure 7. CTRP1 increases expression of NGFIB and NURR1 mRNA in H295R cells. *A*) H295R cells were treated with 40 ng/ml of CTRP1 or 100 μM of Ang II. Total RNA was isolated at the indicated time points, and RT-PCR was performed using NGF1B, NURR1, and NOR1 primer pairs. A pair of β-actin primers was used as a control. *B*) H295R cells were treated with 40 ng/ml of CTRP1 or 100 μM of Ang II for the indicated time. Total cellular protein was separated, followed by Western blot analysis using anti-NGFIB and anti-NURR1 antibodies.

treatment (**Fig. 7*A***). Both CTRP1 and Ang II showed a similar pattern of gene induction, suggesting that the induction of NGFIB and NURRI mRNA expression is probably a common mechanism for CTRP1- and Ang II-mediated aldosterone production in H295R cells. To further confirm this result at the protein level, we performed Western blot analysis using anti-NGFIB antibody and anti-NURR1 antibody. The protein levels were also increased by the treatment of CTRP1 and Ang II (Fig. 7*B*).

Human CTRP1 uses its own receptor for aldosterone production

Now we wondered whether CTRP1 might bind to the AT1 receptor and transmit intracellular signals because CTRP1 and Ang II shared signal pathways, including calcium increase, CYP11B2, Nurr1, and NGFIB mRNA induction. To assess this possibility, H295R cells were pretreated with losartan to block AT1 receptor prior to incubation with CTRP1 for 12 h. The CYP11B2 mRNA level was determined using RT-PCR. Although Ang II-induced CYP11B2 mRNA expression was completely inhibited by the losartan treatment, CTRP1-induced CYP11B2 mRNA expression was not blocked by losartan (**Fig. 8*A***). To further confirm this result, levels of aldosterone were measured 24 h after the treatment. The CTRP1-induced aldosterone production was also not blocked by losartan treatment, while Ang II-induced aldosterone was greatly blocked (Fig. 8*B*), indicating that CTRP1 binds to its own receptor for signal transduction rather than Ang II receptor.

Ang II stimulates the secretion of CTRP1 protein

Both Ang II and CTRP1 stimulated the production of aldosterone. Therefore, it is possible that Ang II may be able to increase aldosterone production through the induction of CTRP1 production. To assess this possibility, H295R cells were treated with Ang II and CTRP1 gene expression was examined using RT-PCR analysis. No change of CTRP1 gene expression was observed (**Fig. 9*A***). Although Ang II did not increase CTRP1 mRNA transcription, it is possible that Ang II could change the secretion rate of CTRP1 protein. To test this possibility, H295R cells were treated with Ang II and levels of CTRP1 were measured using Western blot analysis. In the culture media, CTRP1 production was increased by the Ang II treatment and in the cytosol of H295R cells, CTRP1 was gradually decreased. This result indicates that Ang II-induced aldosterone production is, at least in part, mediated by the stimulation of CTRP1 secretion, not by the increase of CTRP1 mRNA transcription (Fig. 9*B*). Thus, we wondered whether Ang II could stimulate production of aldosterone in the absence of CTRP1. To knock down CTRP1 expression, a CTRP1 siRNA construct was designed, and its efficiency was determined by Western blot analysis. CTRP1 protein expression was greatly suppressed by CTRP1 siRNA compared to the control GFP siRNA (Fig. 9*C*). After the confirmation of CTRP1 siRNA efficiency, H295R cells were transfected with the CTRP1 siRNA construct and treated with Ang II. Interestingly, both the basal level and Ang II-induced aldosterone production was significantly reduced in the absence of CTRP1 expression (Fig. 9*D*), indicating that Ang II-induced aldosterone production is, at least in part, mediated by the CTRP1 protein, although it is not completely dependent on the presence of CTRP1 protein.

DISCUSSION

In a previous study, we showed that CTRP1 is highly expressed in adipose tissue of obese Zucker fatty rats, an animal model for obesity and diabetes (8). Here, we have shown that a novel adipokine CTRP1 stimulated the production of aldosterone. Thus CTRP1 may be involved in blood pressure homeostasis through the regulation of aldosterone production. The pathogenesis of obesity-associated hypertension is multifactorial,

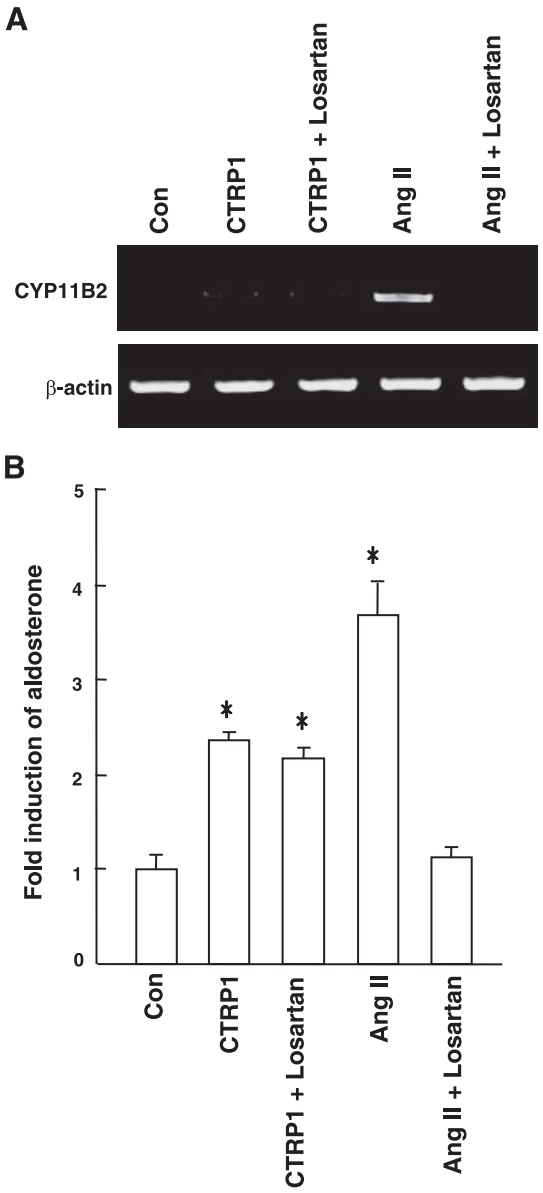


Figure 8. The signal transduction pathway of CTRP1 is not inhibited by the angiotensin II (Ang II) receptor blocker, losartan. *A*) H295R cells were incubated with 10 μM of losartan for 30 min then treated for 12 h with 40 ng/ml of CTRP1 or 100 μM Ang II. After treatment, total RNA was isolated and RT-PCR was performed using a CYP11B2 primer pair. A pair of β-actin primers was used as a control. Representative results are shown from two independent results. *B*) Levels of aldosterone were measured in the supernatants of H295R cells 24 h after the treatment. The experiments were performed in triplicate. $*P < 0.05$ *vs.* control.

involving several environmental and genetic factors. It is well known that oxidative stress, the sympathetic nervous system, and the RAAS are associated with obesity-associated hypertension, and all are augmented in obese compared to lean individuals (15). In this study, for the reason that CTRP1 is highly expressed in adipose depot of obese Zucker fatty rats and the zona glomerulosa region of the adrenal cortex of Sprague-Dawley rats, leading to an increase in the production of aldosterone, we suggest CTRP1 may be a critical protein associated with pathophysiological mechanisms in obesity-associated hypertension, Two important processes are involved in the regulation of aldosterone production. First, steroidogenic acute regulatory protein, which stimulates movement of cholesterol into the mitochondria, is responsible for acute production of aldosterone within minutes to hours of stimulation (21). Second, CYP11B2 stimulates the biosynthesis of aldosterone by catalyzing 18-oxidation of 18-hydroxycorticosterone hours to days after stimulation (22). In this study, the concentration of aldosterone began to increase 6 h after stimulation through induction of CYP11B2 expression by CTRP1 and peaked at 12 h. This implies that CTRP1 chronically increases the production of aldosterone. Well-known aldosterone inducers include ACTH, potassium, and Ang II. ACTH causes an acute increase in the production of aldosterone *in vivo* and in isolated cells, acts chronically to decrease plasma aldosterone levels in humans, and lowers adrenal expression of CYP11B2 in animal models (23–25). Using mice with a targeted deletion of the angiotensinogen gene, Okubo *et al.* (26) also demonstrated that potassium could increase adrenal CYP11B2 gene expression and thus substitute for Ang II. Potassium signaling in glomerulosa cells involves a depolarization of the membrane leading to an influx of calcium through T- and L-type channels. It remains to determine whether CTRP1 is able to activate T- and L-type channels for the induction of CYP11B2 gene. An *in vivo* study has provided strong evidence that sodium restriction increases rennin and Ang II levels, causing induction of adrenal CYP11B2 expression (27), a rate-limiting enzyme for aldosterone production. This Ang II-induced aldosterone synthesis was mediated by Ang II receptor AT1. We showed that CTRP1 can increase expression of transcription factors (NGFIB and NURR1) responsible for the induction of CYP11B2 gene expression. We also demonstrated that CTRP1 increases the concentration of intracellular calcium, which activates phosphorylation of NGFIB and NURR1 through CaM kinase activation (20), stimulating expression of the CYP11B2 gene. Although the signaling pathway involved in aldosterone production by CTRP1 is very similar to that involved in aldosterone production by Ang II, it is unlikely that CTRP1 binds to the AT1 receptor because the AT1 inhibitor, losartan, did not block CTRP1-induced CYP11B2 gene expression. On the other hand, we showed that Ang II stimulated the secretion of CTRP1 from H295R cells and that the secreted CTRP1 contributed to the increase of aldosterone production by Ang II. However, it seems unlikely that CTRP1 completely mediated Ang II-induced aldosterone production because CTRP1 siRNA failed to block the complete inhibition of Ang II effect on the aldosterone production. These results can explain the reason why CTRP1 and Ang II are using similar signal transduction pathways to increase aldosterone production. More detailed investigation is required to establish the exact molecular cross talk mechanism by which

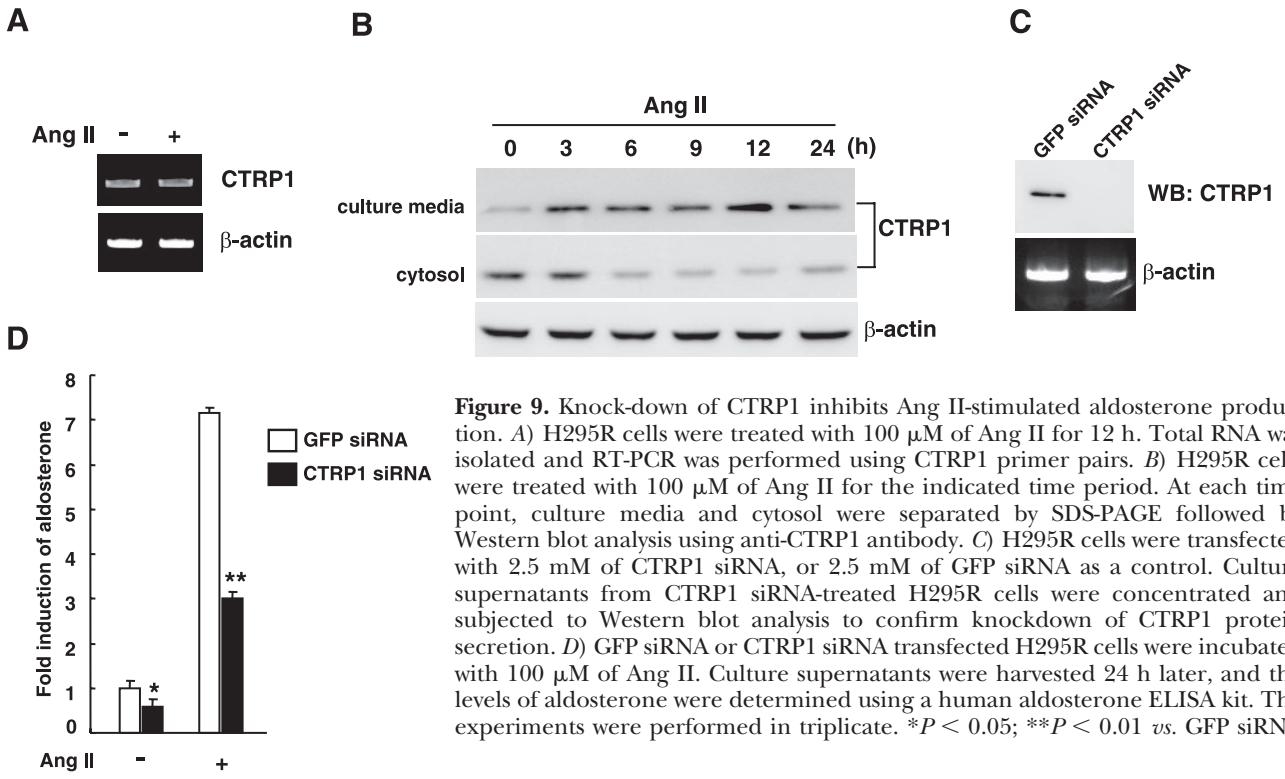


Figure 9. Knock-down of CTRP1 inhibits Ang II-stimulated aldosterone production. *A*) H295R cells were treated with 100 μM of Ang II for 12 h. Total RNA was isolated and RT-PCR was performed using CTRP1 primer pairs. *B*) H295R cells were treated with 100 μM of Ang II for the indicated time period. At each time point, culture media and cytosol were separated by SDS-PAGE followed by Western blot analysis using anti-CTRP1 antibody. *C*) H295R cells were transfected with 2.5 mM of CTRP1 siRNA, or 2.5 mM of GFP siRNA as a control. Culture supernatants from CTRP1 siRNA-treated H295R cells were concentrated and subjected to Western blot analysis to confirm knockdown of CTRP1 protein secretion. *D*) GFP siRNA or CTRP1 siRNA transfected H295R cells were incubated with 100 μM of Ang II. Culture supernatants were harvested 24 h later, and the levels of aldosterone were determined using a human aldosterone ELISA kit. The experiments were performed in triplicate. $*P < 0.05$; $**P < 0.01$ *vs.* GFP siRNA

CTRP1 and Ang II regulate the production of aldosterone.

Obesity, especially visceral obesity, is strongly associated with arterial hypertension and is characterized by hypersecretion of aldosterone (28, 29), which increases arterial blood pressure (28). Although the importance of obesity as a cause of hypertension is well established, and the accumulating evidence supports important roles for aldosterone in the pathogenesis of hypertension (30–33), the precise mechanisms of obesity-induced hypersecretion of aldosterone are not fully understood. However, there are two proposed molecular mechanisms: activation of RAAS and the role of secretory factors. Both human and rodent adipose tissues express functional RAAS including angiotensinogen, angiotensin-converting enzyme, angiotensin receptors, and renin (34–36). In adipose tissue, this contributes to the increase of serum aldosterone levels. The other proposed mechanism involves secretory factors from adipose tissues directly stimulating the adrenal cortex to produce aldosterone. In an effort to find adipocyte-derived factors that increase the production of aldosterone, the aldosterone-releasing factor was identified in human adipocyte culture medium (37), and stimulation of aldosterone and cortisol secretion by adipocyte-derived Wnt has also been reported (38). Although we do not know whether the previously reported aldosterone-releasing factor is CTRP1 (37), on the basis of this study, we clearly showed that CTRP1 is an aldosterone-stimulating factor secreted from obese adipose tissue and that it is expressed in the adrenal cortex where aldosterone is produced and secreted. In addition, increased levels of CTRP1 occurred in the blood of hypertensive patients.

In conclusion, this study has revealed that CTRP1 acts as an endogenous aldosterone-stimulating factor. Although the physiological role of CTRP1 needs to be elucidated, our data indicate that CTRP1 is expressed in the glomerulosa region of the adrenal cortex and enhances the production of aldosterone in H295R cells by enhancing synthesis of CYP11B2 aldosterone synthase. This study provides new insights into the pathophysiology of regulation of aldosterone production, as well as an explanation for the causal relation between hypertension and obesity. FJ

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