## Material and Methods

Materials and Methods are available in the online-only Data Supplement.

## Reagents

Recombinant human globular domain of C1q/tumor necrosis factor-related protein-3 (CTRP3) was purchased from Aviscera Bioscience (00082-01-100, Santa Clara, CA). Antibody for CTRP3 was from Abcam (ab36870, Cambridge, MA). Antibodies for phospho-extracellular signal-regulated kinase 1/2 (sc-7383, p-ERK1/2), phospho-p38MAPK (sc-166182, p-p38MAPK), phospho-c-Jun N-terminal kinase (sc-6254, p-JNK), ERK1/2 (sc-292838), p38MAPK (sc-7149), JNK (sc-7345), and actin (sc-1616) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for runt related transcription factor 2 (BS8734, Runx2), osteopontin (BS1264, OPN), sex determining region Y-box 9 (BS1597, Sox9）and collagen type II alpha 1(BS1071, COL2A1) were from Bioworld Technology (Minneapolis, MN). Antibodies for bone morphogenetic protein 2 (18933-1-AP, BMP2), smooth muscle-*α*-actin (14395-1-AP, SMA), smooth muscle 22*α* (10493-1-AP, SM22*α*) and alkaline phosphatase (11187-1-AP, ALP) were from ProteinTech Group (Chicago, IL). Adenine (V900471), PD98059 (P215), U0126 (U120), and N-Acetyl-L-cysteine (A9165, NAC) were from Sigma-Aldrich (St. Louis, MO). Trypsin, Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA).

## Recombinant adenovirus construction

Recombinant adenovirus encoding full-length human CTRP3 (Pubmed No. NM_030945.2) (Ad-CTRP3) was constructed and amplified according to the manufacturer's protocol (BD Biosciences Clontech, CA). Recombinant adenovirus carrying the gene for green fluorescent protein (Ad-GFP) was used as a negative control. For in vivo studies, after anesthetization (ketamine/xylazine, 80/10 mg/kg, intraperitoneal), a single exposure of $6\times10^8$ plaque forming units (pfu) of Ad-CTRP3 or Ad-GFP dissolved in 30% pluronic gel solution was periadventitially delivered to the rat abdominal or common carotid arteries.

## Animal model of chronic renal failure

Eight-week-old male Wistar rats were randomly divided into six groups: control, chronic renal failure (CRF), CRF+Ad-GFP, and CRF+Ad-CTRP3, n=6-8 per group. All animals received humane care in compliance with the Institutional Authority for Laboratory Animal Care of Peking University Health Science Center. Control rats were fed standard CE-2 chow (containing 1.2% calcium and 0.6% phosphorus), while CRF rats were fed CE-2 chow containing 0.75% adenine for 4 or 6 weeks as previously described.[1] In adenovirus transfected groups, Ad-GFP or Ad-CTRP3 were periadventitially delivered to the rat abdominal arteries before feeding them with adenine diet. After 6 weeks, the

rats were euthanized and blood was collected to measure blood urea nitrogen (BUN), creatinine (Cr), calcium, and phosphate by an autoanalyzer (Hitachi7180, Hitachi, Tokyo, Japan). Serum level of CTRP3 was determined with a commercial ELISA kit from Aviscera Bioscience (SK00082-07, Santa Clara, CA). The abdominal arteries were excised for further analysis.

**Arterial ring organ calcification**

Arterial ring organ calcification was induced as described previously.[2] Briefly, 3 days after adenovirus infection, common carotid arteries were removed from rats in a sterile manner. After removing the adventitia and endothelium, the vessels were cut into 2- to 3-mm rings and placed in regular DMEM containing 10% FBS with or without high-Pi (3.8 mmol/L $PO_4^{3-}$) at 37°C in 5% $CO_2$ for 6 days, with medium changed every 2 days. After 6 days, the arterial rings were prepared for quantitative analysis of calcium content by use of a QuantiChrom Calcium Assay Kit (Biosino Bio-Technology and Science, Beijing) according to the manufacturers' instructions. The sections from each common carotid artery (6 μm) were processed for alizarin red staining and immunohistochemical analysis.

**Cell culture**

Rat vascular smooth muscle cells (VSMCs) were obtained by an explant method as previously described.[3] Briefly, medial tissue was separated from segments of rat aortas. Small pieces of tissue (1 to 2 $mm^3$) were placed in a 10-cm culture dish and cultured for several weeks in DMEM containing 4.5 g/L of glucose supplemented with 15% FBS, 10 mmol/L sodium pyruvate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (growing medium) at 37°C in a humidified atmosphere containing 5% $CO_2$. Cells that had migrated from the explants were collected and maintained in the growing medium. The cells up to passage 6 were used for experiments. For drug treatment experiments, 20 μmol/L NAC, 20 nmol/L PD98059, or 10 nmol/L U0126 were added into the incubation solution respectively for 30 minutes before treatment with 2 μg/mL CTRP3.

**In vitro calcification and quantification of VSMCs**

Calcification of VSMCs was induced as previously described.[3] Briefly, VSMCs were grown in 24-well plates and cultured with growing medium in the absence or presence of 10 mmol/L β-glycerophosphate (βGP calcification medium) for 12 days. The medium and reagents were replenished every 3 days. After washing with phosphate buffered saline (PBS), VSMCs were treated with 0.6 mol/L HCl overnight at 4°C. After removing the HCl supernatant, the remaining cell layers were then dissolved in 0.1 mol/L NaOH and 0.1% SDS for protein concentration analysis. The calcium content in the HCl supernatant was colorimetrically analyzed by use of a QuantiChrom Calcium Assay Kit and was normalized to protein content.[3]

**Alkaline phosphatase activity assay**

VSMCs were seeded in 24-well plates at $1\times10^4$/mL. Proteins were extracted from VSMCs at the required time points by freeze-thawing the cells in 0.1% Triton X-100 in PBS. Alkaline phosphatase (ALP) activity was measured colorimetrically as the hydrolysis of ρ-nitrophenyl phosphate with the use of ALP assay Kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturers' instructions. Results were normalized to the levels of total protein.

**Characterization of calcified nodules by alizarin red staining**

VSMCs in 24-well plates were washed with PBS 3 times and fixed in 4% formaldehyde for 10 minutes at room temperature. After washing with PBS, VSMCs were exposed to 2% alizarin red (aqueous, Sigma) for 5 minutes and washed with 0.2% acetic acid. Quantification of calcified nodule formation was analyzed as described previously[4].

**Immunohistochemical analysis**

Vascular specimens were fixed in 4% formaldehyde and embedded in Tissue-Tek O.C.T. Compound to be frozen in liquid nitrogen. Frozen sections (6 μm) were stained with the antibodies for CTRP3, SMA, and Runx2 overnight at 4°C, then with horseradish peroxidase-conjugated secondary antibody for 2 hours at 37°C followed by 3, 3-diaminobenzidine. Nuclei were stained with 4, 6-diamidino-2-phenylindole (Sigma). Negative controls which omitted the primary antibody were routinely employed. Fluorescence images were captured by use of the LeicaTCSSP5 confocal system.

**Western blot analysis**

Rat arteries or cultured VSMCs were homogenized with lysis buffer (containing 50 mM Tris-HCl, 0.1% sodium deoxycholate, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 40 mM NaF, 2.175 mM sodium orthovanadate, 0.1% SDS, 0.1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.2) by use of a polytron homogenizer. The homogenate was centrifuged at 1000 g for 10 minutes at 4°C, and the protein concentration of the supernatant was measured by the Bradford method. Equal amounts of proteins (40 μg) were separated on a 9% SDS-PAGE and transferred to polyvinylidene difluoride membrane as described previously.[5] The membranes were blocked with 5% non-fat milk for 1 hour at room temperature and then incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized by use of an enhanced chemiluminescence kit (Amersham Biosciences Inc., Piscataway, NJ). The densities of bands were quantified by use of the LEICA550IW image analysis system (Leica, Mannheim, Germany).

**Real-time quantitative RT-PCR (qRT-PCR) and conventional RT-PCR**

Total RNA was isolated from VSMCs using Trizol (Invitrogen, Carlsbad, CA) followed by cDNA synthesis using the First Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada). qRT-PCR was performed using the forward and reverse primers of sequences (rat): CTRP3 (F) GGAAAATCAGATACATCCAGCAACC, (R) TAGCTCACCTACAAATCGCCCTTAG; β-actin (F) TATCGGCAATGAGCGGTTC, (R) AGCACTGTGTTGGCATAGAG. Amplifications were performed in 35 cycles using an opticon continuous fluorescence detection system (MJ Research Inc., Waltham, MA, USA) with SYBR green fluorescence (Molecular Probes, Eugene, OR, USA). Each cycle consisted of a 45 s at 94°C, a 45 s at 56°C, and a 60 s at 72°C. All data were quantified by use of the comparative CT method, normalized to β-actin. For conventional RT-PCR, PCR was performed with Taq DNA polymerase (Invitrogen, Carlsbad, CA) with thermal cycles of 5 minutes 94°C, 30 cycles of 1 minute 94°C, 1 minute 57°C, 1 minute 72°C, finally followed by 10 minutes 72°C using the forward and reverse primers of sequences (rat): CTRP3 (F) ATTGCGTTCATGGCTTCTCTA, (R) GCATGGTTGCTGGATGTATCT; BMP2 (F) CAACACCGTGCTCAGCTTCC, (R) ATGTCCTTTACCGTCGTGGC; OPN (F) GGTTTGCTTTTGCCTGTTCG, (R) GTCCTCATCTGTGGCATCGG; Runx2 (F) GCAAGATGAGCGACGTGAGC, (R) ACGGTAACCACGGTCCCATC; SMA (F) AACTGGTATTGTGCTGGACTCTG, (R) CTGTTATAGGTGGTTTCGTGGAT; SM22α (F) ATCCAAGCCAGTGAAGGTGC, (R) GACTGTCTGTGAACTCCCTCTTA, respectively. The PCR products were then resolved by electrophoresis in a 1.5% agarose gel with ethidium bromide staining. Densitometry was determined in the GeneGenius Gel Imaging System (Syngene, Synoptics, Inc., Frederick, MD) and normalized to the internal control of β-actin. All PCR reactions were performed in triplicate.

**Small interfering RNA (siRNA) transfection**

VSMCs were cultured to 80% confluence and transfected with small interfering RNAs (siRNAs) of interest by use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously.[6] The potent siRNA for rat CTRP3 (catalog no. SASI_Rn02_00256341) and Runx2 (catalog no. SASI_Rn02_00297246) were from Sigma-Aldrich (St. Louis, MO). A scramble siRNA (Sigma-Aldrich) served as a negative control.

**Oxidative stress analysis**

Cellular oxidative stress in VSMCs treated with CTRP3 was detected using the cell permeable fluorogenic probe 2′7′-dichlorodihydrofluorescein diacetate (GENMED Scientifics, Shanghai, China) that emits green fluorescence upon oxidation by reactive oxygen species. Cells were examined by Leica TCS SP5 confocal system (Leica, Wetzlar, Germany).

**Statistical analysis**

All Data are presented as mean±standard error of the mean (SEM). Differences were analyzed by Student's *t* test for two groups or one-way ANOVA for multiple groups, followed by Tukey's multiple comparison post-hoc tests by use of GraphPad Prism 5.0 software. A value of $P<0.05$ was considered to be significant.

**References**


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