Overexpression of C1q/Tumor Necrosis Factor–Related Protein-3 Promotes Phosphate-Induced Vascular Smooth Muscle Cell Calcification Both In Vivo and In Vitro

Yun Zhou,* Jin-Yu Wang,* Han Feng,* Cheng Wang, Li Li, Dan Wu, Hong Lei, Hao Li, Li-Ling Wu

Objective—Vascular calcification is highly correlated with increased cardiovascular morbidity and mortality. C1q/tumor necrosis factor–related protein-3 (CTRP3) is a newly identified adipokine that plays important roles in cardiovascular system. Here, we investigated the role of CTRP3 in vascular calcification and its underlying mechanism.

Approach and Results—Adipokines are involved in the regulation of vascular calcification. Leptin enhances the osteoblastic differentiation of vascular smooth muscle cells (VSMCs) through reactive oxygen species–extracellular signal–regulated kinase 1/2–Runx2 pathway. CTRP3 increased extracellular signal–regulated kinase 1/2 phosphorylation and reactive oxygen species production. Preincubation with U0126, an extracellular signal–regulated kinase 1/2 upstream kinase inhibitor, had no effect on CTRP3-induced reactive oxygen species production. However, pretreatment with N-acetyl-l-cysteine, a reactive oxygen species scavenger, suppressed CTRP3-induced extracellular signal–regulated kinase 1/2 phosphorylation. Both N-acetyl-l-cysteine and U0126 significantly inhibited CTRP3-induced upregulation of Runx2 and calcified nodule formation.


Key Words: C1QTNF3 protein  ■  Runx2 protein, rat  ■  vascular calcification

Vascular calcification is a chronic condition and is associated with high morbidity and mortality of cardiovascular diseases. An imbalance between inducers and inhibitors has been suggested to play a critical role in the pathological process of vascular calcification.

Adipokines, predominantly secreted by adipose tissue, are a series of factors controlling energy metabolism, inflammation, and cardiovascular function. Accumulating evidence has suggested that adipokines are involved in the regulation of vascular calcification. Leptin enhances the osteoblastic differentiation and calcification in cultured cells as well as apolipoprotein E–deficient mice. In contrast, several adipokines, including adiponectin, omentin, and apelin, can play a protective role against vascular calcification by inhibiting osteoblastic differentiation of vascular smooth muscle cells (VSMCs). However, the precise roles of adipokines in vascular calcification regulation and the underlying mechanisms still require further investigation.

C1q/tumor necrosis factor–related protein-3 (CTRP3) is a newly identified adipokine with important roles in regulation of inflammation and metabolism. CTRP3 is ubiquitously expressed in adipose, kidney, cartilage, fibroblasts, chondrocytes, monocytes, and VSMCs. CTRP3 reduces glucose output in hepatocytes and exerts anti-inflammatory effect in adipocytes and monocytes. A recent study shows that CTRP3 also has antiapoptotic, proangiogenic, and cardioprotective roles.
Because CTRP3 promotes proliferation of endothelial cells, VSMCs, chondrogenic precursors, and chondrocytes,\textsuperscript{12,16,17} we hypothesized that CTRP3 might be involved in the regulation of vascular calcification. In this study, we investigated the effects of CTRP3 on vascular calcification in chronic renal failure (CRF) rat, arterial ring, and primary cultured VSMCs. We also explored the underlying mechanism of CTRP3-mediated osteogenic transition and calcification of VSMCs.

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

### Results
Changes in Blood Biochemical Parameters
Rat CRF was induced by adenine diet to mimic the process of arterial medial calcification. The adenine-fed rats had severe renal failure, with a significant increase in blood urea nitrogen and creatinine at 4 weeks ($P<0.01$) and even higher at 6 weeks ($P<0.01$), similar to a previous report.\textsuperscript{18} Serum calcium level of CRF rats did not change, whereas serum phosphorus increased by 41.4\% at 4 weeks and 79.6\% at 6 weeks ($P<0.01$) compared with the age-matched controls. Body weight and abdominal fat weight were decreased in CRF rats (Table in the online-only Data Supplement). In addition, blood biochemical parameters were not affected by periadventitial delivery of recombinant adenovirus carrying full-length CTRP3 (Ad-CTRP3) or green fluorescent protein (Ad-GFP) to abdominal aortas, compared with the age-matched CRF rats.

Increased CTRP3 Is Associated With Vascular Calcification in CRF Rats
ELISA showed that the level of circulating CTRP3 increased to 783±48 ng/mL in 4-week and 791±41 ng/mL in 6-week CRF rats compared with 667±35 ng/mL in control (Figure 1A). The expression of CTRP3 mRNA in kidney and abdominal adipose tissue decreased significantly in CRF rats, whereas adenoviral delivery of CTRP3 or GFP did not change the local CTRP3 mRNA levels (Figure 1B and 1C).

The amplified CTRP3 sequence after reverse transcription-polymerase chain reaction was detected at the size of 272 bp, and CTRP3 protein was observed with a molecular mass of ≈ 36 kDa in VSMCs (Figure 1D). Adipose tissue obtained from control rats was used as a positive control. CTRP3 proteins increased in the abdominal aortas of 4-week ($P<0.05$) and further increased in 6-week CRF rats ($P<0.01$) compared with controls (Figure 1E). Histological assessment with alizarin red staining showed extensive linear calcification in the aortic media of 6-week CRF rats. Immunohistochemical staining showed that CTRP3 expression was slightly increased

### Figure 1
Expression of C1q/tumor necrosis factor-related protein-3 (CTRP3) in different tissues of control and chronic renal failure (CRF) rats. A, Serum CTRP3 level was determined by ELISA assay. $P<0.05$ vs control (n=6–8 per group). B and C, Expression of CTRP3 mRNA in kidney (B) and abdominal adipose tissue (C) was detected by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and normalized to that of $\beta$-actin. $P<0.05$, $**P<0.01$ vs control (n=6–8 per group). D, Expression of CTRP3 mRNA (top) and protein (bottom) in cultured vascular smooth muscle cells (VSMCs) harvested from control rat aortas was detected by RT-PCR and Western blot, respectively. E, CTRP3 protein expression in abdominal aortas was examined by Western blot and normalized to that of actin. $*P<0.05$, $**P<0.01$ vs control, $\#P<0.05$ vs 4-week CRF rats (n=6–8 per group). Ad indicates adenovirus; and GFP, green fluorescent protein.
transfected with Ad-GFP or without (FD) arteries were cut into small rings and cultured with (FD) week 0. P (pfu) Ad-CTRP3 for 1, 4, and 6 weeks (n=3 per group). ** rat abdominal aortas transfected with 6×10⁸ C P** in abdominal aortas. Values are mean±SEM (n=6–8 per group). μ aortas. Bar, 25 μm. Alizarin red staining and hematoxylin/eosin (HE) staining of abdominal arteries. Bar, 25 μm. B, Relative staining and quantitative analysis of calcium content. Control, Ad-GFP, Ad-CTRP3, after 6 weeks, the abdominal arteries were excised for alizarin red staining and hematoxylin/eosin (HE) staining of abdominal arteries. Bar, 25 μm. B, Quantitative fluorescence analysis of Ad-CTRP3 protein in CRF rat abdominal aortas. Values are mean±SEM (n=6 per group). **P<0.01 vs control. ##P<0.01 vs chronic renal failure (CRF). C, Quantitative fluorescence analysis of Ad-CTRP3 protein in CRF rat abdominal aortas transfected with 6×10⁶ plaque-forming units (pfu) Ad-CTRP3 for 1, 4, and 6 weeks (n=3 per group). **P<0.01 vs week 0. D to H, Rat common carotid arteries were periaortically transfected with Ad-GFP or Ad-CTRP3. After 3 days, the carotid arteries were cut into small rings and cultured with (D and E) or without (F–H) 3.8 mmol/L PO₄³⁻ for 6 days. D, Alizarin red staining and HE staining of carotid arteries. Bar, 25 μm. E, Quantitative analysis of calcium deposition in carotid arteries. Bar, 25 μm. F, Alizarin red staining and HE staining of carotid arteries. Bar, 25 μm. G, Quantitative analysis of calcium deposition in carotid arteries. Bar, 25 μm. H, Alizarin red staining and HE staining of carotid arteries. Bar, 25 μm.

**Figure 2.** Overexpression of C1q/tumor necrosis factor-related protein-3 (CTRP3) promotes phosphate-induced vascular calcification. A and B, Rat abdominal aortas were transfected periaortically with adenovirus carrying green fluorescent protein (Ad-GFP) or Ad-CTRP3 and then fed a diet with or without 0.75% adenine. After 6 weeks, the abdominal arteries were excised for alizarin red staining and quantitative analysis of calcium content. A, Alizarin red staining and hematoxylin/eosin (HE) staining of abdominal aortas. Bar, 25 μm. B, Quantitative analysis of calcium deposition in abdominal aortas. Values are mean±SEM (n=6–8 per group). **P<0.01 vs control. ##P<0.01 vs chronic renal failure (CRF). C, Quantitative fluorescence analysis of Ad-CTRP3 protein in CRF rat abdominal aortas transfected with 6×10⁶ plaque-forming units (pfu) Ad-CTRP3 for 1, 4, and 6 weeks (n=3 per group). **P<0.01 vs week 0. D to H, Rat common carotid arteries were periaortically transfected with Ad-GFP or Ad-CTRP3. After 3 days, the carotid arteries were cut into small rings and cultured with (D and E) or without (F–H) 3.8 mmol/L PO₄³⁻ for 6 days. D, Alizarin red staining and HE staining of carotid arteries. Bar, 25 μm. E, Quantitative analysis of calcium deposition in carotid arteries. Bar, 25 μm. F, Alizarin red staining and HE staining of carotid arteries. Bar, 25 μm. G, Quantitative analysis of calcium deposition in carotid arteries. Bar, 25 μm. H, Alizarin red staining and HE staining of carotid arteries. Bar, 25 μm.

**Figure 2 (Continued).** Analysis of calcium deposition in carotid arteries cultured with high phosphate (n=6 per group). **P<0.01 vs control. #P<0.01 vs Pi. F, Expression of alkaline phosphatase (ALP) in carotid arteries cultured without phosphate was examined. **P<0.01 vs control. G, Quantitative analysis of calcium deposition in carotid arteries cultured without phosphate was examined. **P<0.01 vs control. H, Alizarin red staining and HE staining of carotid arteries. Bar, 25 μm.

**Overexpression of CTRP3 Promotes Vascular Calcification in CRF Rats and in High-Phosphate Cultured Carotid Arterial Rings**

To further explore the role of CTRP3 in vascular calcification, we periaortically delivered Ad-CTRP3 or Ad-GFP to rats to induce phosphate-induced vascular calcification. Calcification of abdominal aortas was induced by adenine diet after 6 weeks, as demonstrated by alizarin red staining and calcium deposition (Figure 2A and 2B). Ad-GFP transfection did not change calcium deposition in the abdominal aortas as compared with CRF rats. In contrast, Ad-CTRP3 markedly increased calcium deposition and calcified nodule formation. Quantitative fluorescence analysis showed that the expression of Ad-CTRP3 in CRF rat aortas was markedly increased at 1 week and returned to control level after 6 weeks (Figure 2C). We further evaluated the effect of CTRP3 on vascular calcification in a rat carotid ring organ culture model. Rat common carotid arteries were removed 3 days after Ad-GFP or Ad-CTRP3 transfection, cut into 2- to 3-mm rings, and were cultured in regular DMEM with or without high phosphate (3.8 mmol/L PO₄³⁻) for 6 days. Ad-CTRP3-transfected carotid explants showed much more calcium deposition than Ad-GFP-transfected explants in high-phosphate medium (Figure 2D and 2E). Although overexpression of CTRP3 increased alkaline phosphatase (ALP) protein expression, it was not sufficient to induce calcium deposition calcified nodule formation (Figure 2F–2H) when carotid explants were cultured without high phosphate. These results, therefore, suggested that overexpression of CTRP3 was sufficient to induce calcium deposition calcified nodule formation and that CTRP3 might be involved in aortic medial calcification of CRF rats.

**CTRP3 Promotes β-Glycerophosphate–Induced VSMC Calcification In Vitro**

We further examined the in vitro effects of CTRP3 on arterial calcification in cultured primary VSMCs induced by β-glycerophosphate (βGP). Compared with VSMCs treated with βGP alone, calcium concentration was significantly increased in the presence of various concentrations of CTRP3 (1, 2, and 4 μg/mL) as early as day 3 and further increased at day 6 and 12 (Figure 3A). Similarly, ALP activity was also elevated in the presence of various concentrations of CTRP3 (Figure 3B). However, CTRP3 alone did not increase calcium concentration and calcified nodule formation when VSMCs were cultured without βGP, consistent with in vivo finding that CTRP3 was not sufficient to...
induce calcification. The intensity of alizarin red staining was significantly higher in βGP+CTRP3 (2 μg/mL) group compared with βGP group. Knockdown of CTRP3 expression by small interfering RNA reduced βGP-induced calcified nodule formation, compared with scramble small interfering RNA control (Figure 3C–3F). The data indicated that CTRP3 promoted βGP-induced VSMCs calcification in vitro.

CTRP3 Promotes Phosphate-Induced Osteogenic Transition of VSMCs

VSMC phenotypic transition from contractile to osteogenic phenotype plays a crucial role in vascular calcification. We evaluated the effect of CTRP3 on VSMC transition both in vivo and in vitro. As shown in Figure 4A and 4B, the expression of contractile markers smooth muscle α-actin (SMA) and smooth muscle 22α in the abdominal aorta of 6-week CRF rats was significantly decreased by 31.9% and 27.3%, respectively, compared with the control group (P<0.01). On Ad-CTRP3 transfection, the contractile marker expression further decreased by 53.9% and 52.7% (P<0.01) compared with CRF rats. Ad-GFP transfection did not alter the expression of contractile marker genes. On the contrary, the osteogenic markers runt-related transcription factor 2 (Runx2) and bone morphogenetic protein 2 were significantly increased in CRF rats (P<0.01) and were further elevated in Ad-CTRP3–transfected group (P<0.01; Figure 4C and 4D). The observation was also verified by immunohistochemical staining of SMA and Runx2 (Figure II in the online-only Data Supplement).

Similarly, the expression of SMA and smooth muscle 22α at both mRNA and protein levels was decreased in VSMCs treated with βGP+CTRP3 (2 μg/mL) for 48 hours (Figure 5A–5D). In parallel, the levels of Runx2, bone morphogenetic protein 2, and osteopontin mRNA (Figure 5A and 5B) and protein (Figure 5E–5G) were markedly increased in βGP+CTRP3–treated VSMCs. CTRP3 alone upregulated the expression of Runx2, bone morphogenetic protein 2, osteopontin, sex-determining region Y-box 9, and collagen type II α1. However, it did not affect SMA level and even increased smooth muscle 22α level slightly, when VSMCs were incubated without βGP for 48 hours (Figure 5H–5N). These results indicated that CTRP3 promoted phosphate-induced osteogenic transition of VSMCs both in vivo and in vitro.

Knockdown of Runx2 Inhibits CTRP3-Promoted VSMC Calcification In Vitro

Among various inducers of vascular calcification, Runx2 is an important transcription factor to accelerate osteogenic transition of VSMCs. CTRP3 increased the expression of Runx2 in the abdominal aortas of CRF rats and in cultured VSMCs as described above. To explore whether Runx2 mediates the VSMC calcification promoted by CTRP3, VSMCs were transfected with small interfering RNA against Runx2 for 48 hours (Figure 6A and 6B). The calcium concentration and alkaline phosphatase (ALP) activity were measured and normalized to cellular protein content for quantitative analysis. **P<0.01 vs untreated. #P<0.05 vs βGP, §P<0.05 vs βGP+siRNA scramble (n=3).
Angiotensin II (100 nmol/L) was used as a positive control.23

In VSMCs, suggesting that reactive oxygen species (ROS) production was significantly increased by N-acetyl-l-cysteine (20 μmol/L), PD98059 (an ERK1/2 upstream kinase inhibitor; 20 nmol/L), or U0126 (10 nmol/L) pretreatment in the presence of βGP (Figure 6E). In addition, CTRP3-enhanced calcified nodule formation in the presence of βGP was suppressed by ROS scavenger or ERK1/2 kinase inhibitors (Figure 6F and 6G). Even in the absence of βGP, the induction of Runx2 expression by CTRP3 could be inhibited by N-acetyl-l-cysteine (20 μmol/L), PD98059 (20 nmol/L), or U0126 (10 nmol/L) pretreatment (Figure 6H and 6I). These results indicated that CTRP3 promoted ROS production, which in turn activated ERK1/2–Runx2 signaling pathway to enhance phosphate-induced osteogenic transition of VSMCs.

Discussion

There are 3 major new findings presented by our study. First, we demonstrated for the first time that CTRP3 promoted phosphate-induced vascular calcification in vivo, ex vivo, and in vitro. Second, we revealed that CTRP3 accelerated βGP-induced osteogenic transition of VSMCs by downregulating contractile markers and upregulating osteogenic markers. Third, although Runx2 was an essential mediator of CTRP3 response, increased ROS production and ERK1/2 activation also facilitate the CTRP3-induced Runx2 upregulation and VSMC calcification. Our results provide new insights into the important roles of CTRP3 in cardiovascular system and characterize a novel mechanism of adipokine-modulated vascular calcification.

CTRP3 is ubiquitously expressed in adipose and nonadipose tissues, and circulating levels of CTRP3 vary according to mammalian species and determining methods. Plasma CTRP3 concentration is ~1000±300 ng/mL in mice by immunoblot analysis.13 A recent report indicates that plasma CTRP3 levels in normal subjects are 226.2 to 335.5 ng/mL.24 In addition, circulating CTRP3 levels is affected by food intake and disease types. Circulating CTRP3 in fasted mice increases compared with fasted/refed or ad libitum fed mice. Although CTRP3 is reduced in diet-induced obese mice with high leptin levels, it is increased in leptin-deficient obese mice.13 In patients with glucose metabolism disorder, plasma CTRP3 level elevates significantly.24 A 3-month combined exercise decreases CTRP3 level from 444.3 to 374.4 ng/mL in obese Korean women.25 These results suggest that circulating CTRP3 level may serve as a novel biomarker candidate for metabolic-related diseases. Vascular calcification is a critical event in the development of vascular disease and frequently observed in patients with CRF.26 However, circulating levels of CTRP3 in patients with CRF or CRF animals are unknown. Here, with a CTRP3-specific ELISA kit, we showed that serum CTRP3 concentration in control rats was 667±35 ng/mL. Although CTRP3 levels in serum and calcified vasculature increased in CRF rats, the expression of CTRP3 mRNA decreased in kidney and abdominal adipose tissue. Because VSMC is not the main source of CTRP3,11 one possible explanation for elevated serum CTRP3 was attributable to decreased renal function characterized by increased blood urea nitrogen and creatinine, which was in accordance with the finding by Choi et al.24 Moreover, elevation
Figure 5. C1q/tumor necrosis factor–related protein-3 (CTRP3) accelerates β-glycerophosphate (βGP)–induced osteogenic transition of vascular smooth muscle cells (VSMCs) and knockdown of runt-related transcription factor 2 (Runx2) inhibits CTRP3-promoted VSMC calcification in vitro. A and B, VSMCs were incubated with 2 μg/mL CTRP3 and 10 mmol/L βGP for 3 days and then mRNA expression of contractile and osteogenic marker genes were determined by reverse transcription-polymerase chain reaction and normalized to that of β-actin. *P<0.05 vs βGP (n=3–5). C to G, VSMCs were incubated with 2 μg/mL CTRP3 and βGP for 6 to 48 hours, and the protein expression of smooth muscle α-actin (SMA; C), smooth muscle 22α (SM22α; D), Runx2 (E), bone morphogenetic protein 2 (BMP2; F), and osteopontin (OPN; G) was examined by Western blot and normalized to that of actin. *P<0.05 vs untreated. #P<0.05 vs βGP alone (n=5). H to N, VSMCs were incubated in the presence or absence of 2 μg/mL CTRP3 or 10 mmol/L βGP for 48 hours, and the protein expression of SMA (H), SM22α (I), Runx2 (J), BMP2 (K), osteopontin (L), sex-determining region Y-box 9 (Sox9; M) and collagen type II α1 (COL2A1; N) was examined by Western blot and normalized to that of actin. *P<0.05, **P<0.01 vs untreated. #P<0.05 vs βGP (n=3). O and P, VSMCs were transfected with scramble small interfering RNA (siRNA) or Runx2 siRNA for 48 hours and then incubated with 2 μg/mL CTRP3 in the presence of 10 mmol/L βGP for an additional 12 days. O, VSMCs were stained for mineralization with alizarin red. Bar=100 μm. P, Quantitative analysis of calcified nodule formation. *P<0.05 vs βGP. #P<0.05 vs βGP+CTRP3 (n=5).
of CTRP3 in serum and vasculature appeared before calcified nodule formation, implying that CTRP3 might be involved in the development of vascular calcification.

CTRP3 is a multifunctional adipokine in regulating cell metabolism and function, and its biological effect may vary depending on cell type, physiological, and pathological stimuli. By using periadventitial adenoviral delivery of CTRP3 both in vivo and ex vivo, we showed increased vascular calcification in CTRP3-overexpressed CRF rats. Our results in cultured VSMCs further confirmed the effects of CTRP3 on phosphate-induced vascular calcification. Knockdown of CTRP3 in VSMCs eliminated βGP-induced calcified nodule formation. We also noticed that, without βGP, CTRP3 alone was not sufficient to induce calcification in VSMCs. These results suggested that high phosphate was an independent risk of vascular calcification, and CTRP3 acted synergistically with phosphate to promote the progression of VSMC calcification.

Vascular calcification is an active and cell-mediated process. Many studies have revealed that phenotypic transition of VSMCs from contractile to osteogenic phenotype contributes to adipokine-participated vascular calcification. Treatment of calcifying VSMCs with leptin causes a significant increase in ALP activity, an early marker of osteogenic transition of VSMCs. Adiponectin inhibits ALP activity, osteocalcin secretion, and...
Runx2 expression of calcifying VSMCs.6,8 Omentin and apelin inhibit ALP activity and osteocalcin production during the osteoblastic differentiation of calcifying VSMCs.7,8 In the present study, we found that expressions of SMA and smooth muscle 22α were significantly decreased in the abdominal aortas of CRF rats, and overexpression of CTRP3 further reduced the expression of contractile markers. In contrast, increased osteogenic markers in CRF rats were further upregulated in CTRP3-overexpressed vasculature. In cultured VSMCs, CTRP3 alone increased the expression of osteogenic markers, but did not decrease the expression of contractile markers. Moreover, CTRP3 promoted βGP-induced osteogenic transition. These results further indicated that CTRP3 acted as a promoter instead of an inducer of VSMCs calcification.

Several key transcription factors such as Runx2, osterix, and Msx2 have been identified in calcified vascular lesions.23 Among them, Runx2 is thought to be a decisive factor and early marker of VSMC osteogenic transition and calcification.27,28 Compared with the trace expression in normal vasculature, Runx2 is significantly upregulated in the vascular lesions and seems to precede overt calcification.27 More importantly, Runx2 is closely associated with the effects of various adipokines in osteogenic transition of VSMCs. Adiponectin and apelin decrease Runx2 expression in cultured calcifying VSMCs,6,8 whereas tumor necrosis factor-α enhances the mRNA expression and DNA binding of Runx2.25,29 Here, we found that the expression of Runx2 increased significantly in the abdominal aortas of CRF rats. Overexpression of CTRP3 or treatment of VSMCs with CTRP3 further upregulated Runx2 expression. Knockdown of Runx2 reversed the effect of CTRP3 on VSMC calcification in vitro. These results suggested that Runx2 played a crucial role in CTRP3-promoted vascular calcification.

To gain further insight into the mechanisms by which CTRP3 promoted vascular calcification, we explored the intracellular signaling events in VSMCs. MAPK family activation plays a critical role in mediating the effects of adipokines on vascular calcification. However, the specific roles of major members of MAPK family, including ERK1/2, c-Jun N-terminal kinase, and p38MAPK, in vascular calcification depend on the stimuli and cell types. Adiponectin exerts its anticalcific effect through p38MAPK,8 whereas apelin plays a protective role against vascular calcification via ERK1/2 pathway.3 CTRP3 is reported to promote mouse VSMC proliferation via ERK1/2 and p38MAPK,12 and it also promotes the proliferation and migration of murine osteosarcoma cells and endothelial cells by activating ERK1/2.16,31 Here, we found that CTRP3 significantly increased phosphorylation of ERK1/2 but not c-Jun N-terminal kinase and p38MAPK in VSMCs, suggesting that ERK1/2 was required in CTRP3-promoted vascular calcification.

Oxidative stress plays an important role in the pathogenesis of vascular calcification.2 As a classical oxidative stressor and key mediator of intracellular signaling, hydrogen peroxide promotes osteogenic transition of VSMCs by upregulating Runx2.23 We found that CTRP3 significantly increased intracellular ROS production. N-acetyl-L-cysteine pretreatment inhibited CTRP3-induced Runx2 upregulation and calcified nodule formation, which suggested that ROS production was involved in CTRP3-mediated Runx2 upregulation and VSMC calcification. Many studies revealed ERK1/2 as a downstream effector of ROS, whereas a report by Lu and Cederbaum12 suggests a reciprocal relationship between ROS production and ERK activation. Here, we showed that ROS scavenger pretreatment significantly suppressed CTRP3-induced ERK1/2 phosphorylation, whereas inhibition of ERK1/2 did not affect CTRP3-induced ROS production, suggesting that ROS acted as an upstream molecule of ERK1/2 in CTRP3-treated VSMCs. Both ERK1/2 inhibitor and ROS scavenger reversed CTRP3-induced Runx2 upregulation and calcified nodule formation. Hence, CTRP3 promoted osteogenic transition and calcification of VSMCs through ROS–ERK1/2 signaling pathway.

Although adenine-induced CRF model is demonstrated to be a useful model for the study of vascular calcification, adenine diet causes weight loss and faster development of vascular calcification than that in humans.33,34 Whether the extensive medial calciﬁcation is attributable to weight loss or the adenine itself remains to be determined.33 More importantly, the effect of CTRP3 on vascular calcification in patients with CRF needs further investigation.

In summary, we have demonstrated that CTRP3 is a novel endogenous regulator of vascular calcification. CTRP3 promotes vascular calcification by accelerating phosphate-induced osteogenic transition of VSMCs through ROS–ERK1/2–Runx2 signaling pathway. These findings provide novel insight on the physiological and pathophysiological roles of CTRP3.

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Disclosures
None.

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### Significance

Vascular calcification is a chronic condition and is associated with high morbidity and mortality of cardiovascular disease. Among various regulators of vascular calcification, adipokines have drawn more attention in recent years. C1q/tumor necrosis factor–related protein-3 (CTRP3), a newly identified adipokine, has been suggested to play important roles in inflammation and metabolism, but the effect of CTRP3 on vascular calcification is unknown. The current study provides compelling evidence that CTRP3 promotes phosphate-induced vascular calcification in vivo, ex vivo, and in vitro. We also demonstrate that CTRP3 promotes vascular calcification by accelerating phosphate-induced osteogenic transition of vascular smooth muscle cells through a reactive oxygen species–extracellular signal–regulated kinase 1/2–runt-related transcription factor 2 signaling pathway. Our findings provide new insights into the important roles of CTRP3 in cardiovascular system and characterize a novel mechanism of adipokine-modulated vascular calcification.
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Supplemental Figure I. Calcified nodule formation and CTRP3 expression in abdominal aortas of control and CRF rats. Representative alizarin red staining of calcified nodules and immunohistochemistry staining for CTRP3 in abdominal aortas of control and CRF rats. Bar=25 μm.

Supplemental Figure II. Expression of SMA and Runx2 in abdominal aortas of control and CRF rats. Representative immunohistochemistry staining for SMA or Runx2 in abdominal aortas of control and CRF rats. Bar=100 μm.
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 22α (10493-1-AP, SM22α) 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 6×10⁸ plaque forming units (pfu) of Ad-CTRP3 or Ad-GFP dissolved in 30% pluronic gel solution was periaventitially 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. In adenovirus transfected groups, Ad-GFP or Ad-CTRP3 were periaventitially 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 $1 \times 10^4$/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 p-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.

**Immunochemistry 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 Leica TCSSP5 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. 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) GGAAAATCAGATAATCCAGCAACC, (R) TAGCTCACCTACAAATCGCCCTTAG; β-actin (F) TATCGGCAATGAGCGGTTC, (R) AGCACTGTGGTTGGCATAGAG. 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) GCATGGTGCTGGATGTATCT; BMP2 (F) CAACACCGTGCTCAGCTTCC, (R) ATGTCCCTTACCCTGGTGCC; OPN (F) GGTTCGCTTTTTGCGCTTGTC; (R) GTCCTCATCTGTGGCATCGG; Runx2 (F) AGAATGAGCTTCCCTAGAG, (R) ACGTAAACCACCGTCCCATC; SMA (F) AACTGTATTGTGGCAGCTCC, (R) CTGTATAGGTGGTCTGGGAT; SM22α (F) ATCCAGCCAGTTAATGTTG, (R) GACTGTCTGTGAACCTCTCTTA, 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. 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
### Supplemental Table I. Serum biochemical parameters and body weight of rats

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<th>4 week</th>
<th>6 week</th>
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<tr>
<td></td>
<td>Control</td>
<td>CRF</td>
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<tr>
<td>BUN (mmol/L)</td>
<td>6.76±0.42</td>
<td>28.26±5.47**</td>
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<tr>
<td>Cr (μmol/L)</td>
<td>51.17±2.69</td>
<td>172.17±28.86**</td>
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<td>Calcium (mmol/L)</td>
<td>2.27±0.05</td>
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<td>Phosphorus (mmol/L)</td>
<td>2.27±0.10</td>
<td>3.21±0.28**</td>
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<td>Body Weight (g)</td>
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<td>250.00±35.99**</td>
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<tr>
<td>Abdominal fat weight (g)</td>
<td>5.09±0.36</td>
<td>1.71±0.36**</td>
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Rat serum levels of blood urea nitrogen (BUN), creatinine (Cr), calcium, and phosphorus were measured by an autoanalyzer. Values are mean ± SEM (n=6-8). **P<0.01 vs. the age-marched controls. #P<0.05, ##P<0.01 vs. CRF at 4 weeks.