Original Article

Cinacalcet ameliorates aortic calcification in uremic rats via suppression of endothelial-to-mesenchymal transition

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Aim: Experimental studies found that cinacalcet (CINA) markedly attenuated vascular calcification in uremic rats, but its underlying mechanisms are still largely unknown. Recent evidence have demonstrated that endothelial cells (ECs) participate in ectopic calcification in part by mediating endothelial-to-mesenchymal transition (EndMT). In this study, we investigated whether CINA ameliorated aortic calcification in uremic rats via suppression of EndMT.

Methods: Uremia was induced in rats by feeding rats a 0.75% adenine diet for 4 weeks. After adenine withdrawal, the rats were maintained on a 1.03% phosphorus diet for next 8 weeks. At initiation of the adenine diet, rats were orally administered CINA (10 mg/kg one day) for 12 weeks. The aortic expression of EndMT- and chondrocyte-markers was examined. The effect of elevated PTH on EndMT was also studied in aortic ECs.

Results: In uremic rats, CINA treatment significantly decreased the serum PTH concentrations, but did not affect the elevated levels of serum calcium (Ca), phosphorus (P) and Ca×P product. Besides, CINA significantly attenuated aortic calcification, and inhibited the expression of chondrocyte markers (SOX9 and COL2A1) and chondrocyte proteoglycan in uremic aortas. Moreover, CINA treatment largely abolished the up-regulation of mesenchymal markers (FSP1 and α -SMA) and down-regulation of the endothelial marker (CD31), which accompanied aortic calcification in uremic aorta samples. *In vitro*, PTH increased the expression of EndMT-markers in a concentration- and time-dependent manner.

Conclusion: These findings suggest that strategies aiming at reducing serum PTH might prevent uremic aortic calcification by abrogating EndMT.

Keywords: cinacalcet; uremia; aortic calcification; endothelial cells; parathyroid hormone; endothelial-to-mesenchymal transition

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Introduction

Cardiovascular disease is the primary cause of mortality in patients with chronic kidney disease (CKD)^[1, 2]. Numerous studies have demonstrated that vascular calcification (VC) is a common feature in CKD patients and that it contributes to the development of cardiovascular events^[3]. As a pathologic process, VC could be mediated by mechanical, neurohormonal, inflammatory and/or oxidative stress-associated factors^[4, 5]. Secondary hyperparathyroidism, characterized by elevated serum parathyroid hormone (PTH) concentrations, is a frequent component of the natural progression of CKD^[6]. Although the effect of PTH on mineral metabolism has been well established, experimental and clinical evidence indicates that PTH is also causally involved in the pathogenesis of VC

in CKD^[7-9].

Considering the detrimental effects of elevated PTH, strategies aimed at lowering serum PTH levels are frequently prescribed in patients with CKD. Cinacalcet (CINA), a calcimimetic agent, reduces PTH levels without increasing circulating levels of calcium and phosphorus^[10-12]. Meanwhile, emerging studies suggested that suppression of PTH by CINA markedly attenuated vascular remodeling and calcification in uremia^[13-16]. However, the underlying mechanisms of this action are still largely unknown.

Studies using transgenic lineage-tracing techniques have demonstrated that the endothelial cells (ECs) contribute to calcified lesions via the endothelial-to-mesenchymal transition (EndMT)^[17-20]. These results suggest that blockade of EndMT might be a novel therapeutic target for VC^[19, 20]. However, whether suppression of serum PTH concentrations by CINA could ameliorate EndMT in vascular calcification is still unknown. In the present study, we investigated the effect of

CINA on aortic EndMT in uremia.

Materials and methods Cell culture

Primary human aortic endothelial cells (HAECs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured as previously described^[21]. Briefly, cells were grown in endothelial culture medium (No 1001, ScienCell) containing 5% fetal bovine serum (FBS) (No 0025, ScienCell), 1% endothelial cell growth supplement (No 1052, ScienCell) and 1% penicillin/streptomycin solution (No 0503, ScienCell) in 5% CO₂ at 37 °C. HAECs between passages 3–5 were expanded in monolayers in culture flasks or dishes. At approximately 80% confluence, the culture medium was changed to a serum-free solution for 24 h prior to use of the HAECs in all experiments. Human recombinant PTH fragment 1–34 (P3796, Sigma, USA) was then added to the serumfree culture medium.

Animals

Study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Southeast University (Nanjing, China). The rat model of uremia and secondary hyperparathyroidism was established as previously described^[22]. Briefly, 8-week-old male Wistar rats (SLAC Laboratory Animal Co Ltd, China) were randomly divided into three groups: the control group (CTL, n=6), the uremia group (U, n=8) and the CINA-treated uremia group (U+CINA, n=8). Uremia was induced by feeding rats a 0.75% adenine diet for 4 weeks. Rats that were fed the diet without adenine served as controls. After adenine withdrawl, all animals were maintained on a 1.03% phosphorus diet for next 8 weeks. At initiation of the adenine diet, rats in the U+CINA group were administered CINA (sc-207438, Santa Cruz Biotechnology, USA) orally once daily (10 mg/kg) until the time of killing. Based on exposure levels, the dose equivalent to 10 mg/kg is 60 mg, which is the clinically used dosage^[23]. Blood samples were taken to assess biochemical parameters, and aorta samples were used for histology.

Serum biochemistry

Serum total calcium (Ca) and phosphorus (P) concentrations were measured at Zhongda Hospital, Southeast University School of Medicine using an autoanalyzer system (Hitachi, Japan). Serum parathyroid hormone (PTH) levels were determined using ELISA assays (Lengton, China).

Histology and immunohistochemical staining

Paraffin-embedded rat aorta sections (3-µm thick) were prepared using a routine procedure. The sections were subjected to von kossa staining for assessing calcified lesions. Immunohistochemical staining was performed according to a routine protocol. The antibodies used were as follows: CD31 (sc-1506, Santa Cruz, USA), FSP1 (ab-27957, Abcam, Hong Kong, China), SOX9 (ab185230, Abcam) and COL2A1 (sc-52658, Santa Cruz, USA). For the negative controls, the specific primary antibody was replaced with phosphate-buffered saline.

Immunofluorescence staining

Cryosections (5-µm thick) or coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and blocked with 10% bovine serum albumin (BSA) in phosphate-buffered saline at room temperature. The slides were then immunostained with primary antibodies against CD31 (sc-1506) and FSP1 (ab-27957) at 4°C overnight. After incubation with a mixture of the two secondary antibodies for 1 h in the dark at room temperature, images were captured using a laser scanning confocal microscope (LSM 510 META, Zeiss, Germany).

Real-time PCR

Total RNA was extracted using RNAiso Plus according to the manufacturer's instructions (TAKARA, China). The RNA concentration and purity were confirmed with a Nanodrop 2000 (Thermo, USA). Samples with a relative absorbance ratio at 260/280 between 1.8 and 2.0 were used. All RNA samples were reverse transcribed (Applied Biosystems, USA). The quantification of specific mRNAs was performed using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, USA) with the SYBR Green Real-time PCR Kit (TAKARA, China). The relative amount of mRNA was normalized to β -actin and calculated using the standard curve method. Briefly, the pre-PCR product of each gene was used as the standard. The standard curve was established with a 10-fold serial dilution of the product and was included in all PCR runs. The ratio of target gene abundance/housekeeping gene abundance was used to evaluate the expression level of each gene. Controls containing ddH2O were confirmed to be negative in all runs.

Western blot analysis

Equal amounts of the protein obtained from each lysate were electrophoresed in a 4%–20% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Pall, USA) by electroblotting. The blots were incubated overnight with primary antibodies against CD31 (sc-1506), FSP1 (ab-27957), α -SMA (ab5694), SOX9 (ab185230) and COL2A1 (sc-52658) followed by incubation with a horseradish peroxidase-labeled secondary IgG antibody (Santa Cruz). The signals were detected using an advanced ECL system (GE Healthcare, UK). β -Actin was used as an internal control.

Statistical analysis

Data are expressed as mean±standard deviation (SD). Statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Bonferroni correction using SPSS 16.0 statistical software. A value of P<0.05 was considered to be statistically significant.

Results

Physical and metabolic parameters of animals

As shown in Figure 1, serum Ca levels decreased, whereas



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Figure 1. Analysis of (A) serum PTH concentrations, (B) Ca, (C) P and (D) Ca×P product in the study groups. The data are expressed as the mean±SD (*n*=6 for each group). **P*<0.05 vs the CTL group. **P*<0.05 vs the U group. CTL, control; U, uremia; CINA, cinacalcet.

serum P levels and the Ca×P product increased in the uremia (U) group compared with controls (CTL). Following CINA treatment, the serum Ca, P and Ca×P product levels in rats with uremia were maintained at low levels, but the differences were not statistically significant (Figure 1B-1D). Compared with the control group, serum PTH concentrations were markedly elevated in uremic rats, which were significantly attenuated by the administration of CINA (Figure 1A).

CINA treatment ameliorated aortic calcification and the expression of chondrocyte markers in uremic rat models

As determined by von Kossa staining, the area of calcified lesions in the aortas of the U group was significantly increased compared to controls, but was decreased by treatment with CINA (Figure 2). As determined by alcian blue staining, levels of chondrocyte proteoglycans were markedly elevated in rats from the U group compared with the CTL group, whereas this induction was partially abolished by CINA treatment (Figure 3A). Calcium accumulation, a late chondrogenic marker, increased in the uremic aortas compared to the controls as detected by alizarin red staining, which was largely inhibited by CINA treatment (Figure 3B). These results were consistent with the results obtained by von Kossa staining (Figure 2). As shown in Figure 3C and 3D, illustrated by real-time PCR and Western blot, mRNA and protein levels of chondrocyte specific markers were markedly up-regulated in the rats with uremia. This induction was suppressed by the treatment with CINA.



Figure 2. CINA ameliorates aortic calcification in rats with uremia. (A) Representative micrographs demonstrate calcified lesions in aorta samples from different groups of rats, as indicated. Aorta sections were subjected to von Kossa staining. (B) Semi-quantitative determination of aortic calcification in the different groups. The data are expressed as the mean \pm SD (*n*=6 for each group). **P*<0.05 vs the CTL group. #*P*<0.05 vs the U group. CTL, control; U, uremia; CINA, cinacalcet.



Figure 3. CINA decreases aortic expression of chondrocyte markers in uremic rats. (A) Aortic accumulation of chondrocyte matrix in the different groups of rats (original magnification is ×100). Aorta sections were subjected to alcian blue staining. (B) Representative micrographs demonstrate aortic accumulation of calcified matrix in the different groups of rats (original magnification is ×100). Aorta sections were subjected to alcian blue staining. (C) Real-time PCR results showing aortic mRNA expression of chondrocyte markers SOX9, COL2A1, and Aggrecan in the different groups of rats, as indicated. (D, E) Western blot analyses showing aortic protein expression of SOX9 and COL2A1 in the different groups of rats, as indicated. Representative Western blots (D) and quantitative determination of protein levels (E) are presented. The data are expressed as the mean \pm SD (*n*=6 for each group). **P*<0.05 vs the CTL group. **P*<0.05 vs the U group. CTL, control; U, uremia; CINA, cinacalcet.

CINA treatment ameliorated aortic EndMT in uremic rat models

As determined by real-time PCR and Western blot analysis, mRNA and protein levels of the endothelial cell marker CD31 were dramatically decreased in aorta tissues from the U group compared with controls, but CD31 expression was partially restored by CINA treatment (Figure 4A-4C). By contrast, aortic FSP1 expression was significantly augmented in the U group compared to controls, an effect that was largely inhibited by CINA treatment (Figure 4A-4C). Moreover, confocal microscopy revealed an increased co-localization of CD31 and FSP1 (yellow) in aortas from the U group compared with controls, and administration of CINA abrogated this change (Figure 4D).

PTH induced EndMT in cultured HAECs

Considering that similar serum Ca and P levels were observed in the U and U+CINA groups, the different histological

findings in the aorta of these two groups can most likely be attributed to differences in PTH exposure. To further confirm the effect of elevated PTH on inducing EndMT, we next performed in vitro experiments using HAECs. As PTH (1-34) is the shortest fragment with the same biological effect as the intact PTH, commercially available PTH (1-34) was used to treat cultured HAECs^[24]. The results of real-time PCR showed that PTH incubation led to a significant decrease in the mRNA expression of CD31 and an increase in the mRNA levels of FSP1 and α -SMA at 48 h post-treatment (Figure 5A–5C). In addition, HAECs were treated with 10-8 mol/L PTH for various periods of time, and the results indicated that PTH caused a significant decrease in CD31 mRNA expression and an increase in FSP1 and a-SMA mRNA levels from 0-48 h posttreatment (Figure 5D-5F). As determined by Western blot, PTH caused a significant decrease in the protein level of CD31, as well as subsequent increases in the protein expression levels



Figure 4. CINA attenuates the expression of EndMT-related markers in aortas of uremic rats. (A) Real-time PCR results showing aortic mRNA expression of CD31 and FSP1 in the different groups of rats, as indicated. (B, C) Western blot analyses showing cardiac protein expression of CD31 and FSP1 in the different groups of rats, as indicated. Representative Western blots (B) and quantitative determination of protein levels (C) are presented. (D) Representative micrographs showing aortic double-labeling of CD31 and FSP1 in the different groups, as indicated (Scale bar, 20 μ m). **P*<0.05 vs the CTL group. **P*<0.05 vs the U group. CTL, control; U, uremia; CINA, cinacalcet.

of FSP1 and α -SMA, in a concentration- and time-dependent manner (Figure 6). Confocal microscopy detected that cells treated with 10⁻⁸ mol/L PTH for 48 h acquired FSP1 staining and lost CD31 staining compared with the control cells (Figure 7A). As observed by inverted microscopy, some PTH-treated cells showed a distinct change from a cobblestone-like to a spindle-shaped morphology (Figure 7B). These data suggest that PTH induces EndMT in cultured HAECs.

Discussion

Serum PTH concentrations are progressively increased as renal function declines, and this elevation of PTH plays an important role in both the pathogenesis of bone mineral disease and in the development of various complications in uremia^[25, 26]. Recent studies have demonstrated the involvement of PTH in the development of calcified lesions, and strategies lowering PTH levels, including calcimimetics and parathyroidectomy, attenuate the progression of vascular calcification in CKD patients and uremic animal models^[27-30]. Given the adverse effects of PTH on VC, treatment that reduces PTH concentrations should protect against ectopic calcification in CKD. CINA, a calcimimetic agent, has been found to effectively inhibit PTH secretion^[10-12]. A number of studies have shown that suppression of PTH by CINA can significantly ameliorate VC in CKD^[13-16]. As expected, the result of present study showed here that CINA treatment could markedly reduce serum PTH concentrations and attenuate aortic calcification in uremic rats.

It is known that calcified lesion is a fundamental component of the adverse structural remodeling of vasculature present in uremia^[31]. Currently, uremic VC is recognized to be a cellregulated process with similarities to cartilage formation driven by chondrogenic cells^[32, 33]. Recently, evidence from clinical and experimental investigations revealed the involvement of EndMT in the development of ectopic calcification in pathological settings, including diabetes, prostate tumor and fibrodysplasia ossificans progressive (FOP)^[17, 18, 34]. These studies suggest that ECs directly contribute to VC via EndMT. In the present study, our data showed that CINA treatment attenuated aortic EndMT in the adenine-induced uremic rat model. Meanwhile, biochemical analysis indicated PTH concentrations were markedly suppressed by CINA in rats with



Figure 5. Effect of PTH on mRNA expression of CD31, FSP1 and α -SMA in HAECs. (A–C) HAECs were incubated for 48 h with increasing concentrations of PTH. (D–F) HAECs were incubated with PTH (10⁻⁸ mol/L) for different periods, as indicated. The mRNA expression levels were determined by real-time PCR. The data are expressed as the mean±SD (*n*=3 to 5). **P*<0.05 vs 0 group.

uremia. Moreover, *in vitro* study further showed that elevated PTH levels induced the expression of EndMT-related markers in a concentration- and time-dependent manner. Therefore, we propose that the beneficial efficacy of CINA in improving VC and EndMT could most likely be attributed to decrease on PTH exposure. This result was consistent with the work by Jung *et al*, which demonstrated that the effect of CINA on reducing VC was attributed to its suppression of PTH levels^[15].

In conclusion, elevation of PTH induces EndMT and contributes to aortic calcification in uremic rats, which could be prevented by CINA treatment. These results suggest that strategies aimed at lowering PTH levels might exert vascular protective effects through an anti-EndMT mechanism.

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Author contribution

Min WU, Ri-ning TANG, and Bi-cheng LIU designed research; Min WU, Ming-ming PAN, and Hong LIU conducted experiments; Min WU, Ri-ming TANG, and Hong LIU performed data analysis; Min WU, Ri-ning TANG, and Bi-cheng LIU wrote the manuscript.

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Figure 6. Effect of PTH on protein expression of CD31, FSP1 and α -SMA in HAECs. (A–C) HAECs were incubated for 48 h with increasing concentrations of PTH. (D–F) HAECs were incubated with PTH (10⁻⁸ mol/L) for different periods, as indicated. The protein expression levels were determined by Western blot. The data are expressed as the mean±SD (*n*=3 to 5). **P*<0.05 vs 0 group.

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Figure 7. Representative micrographs showing immunofluorescent staining (A) and cellular morphology (B) of CD31 and FSP1 in HAECs. HAECs were incubated with 10^{-8} mol/L PTH for 48 h. (A) Immunofluorescence of CD31 and FSP1 was observed by confocal microscopy. Scale bar, 50 µm. (B) Cell morphology was detected using inverted microscopy (original magnification is ×200).

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