Cinacalcet attenuates the renal endothelial-to-mesenchymal transition in rats with adenine-induced renal failure

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Wu M, Tang R, Liu H, Xu M, Pan M, Liu B. Cinacalcet attenuates the renal endothelial-to-mesenchymal transition in rats with adenine-induced chronic renal failure. Am J Physiol Renal Physiol 306: F138–F146, 2014. First published October 23, 2013; doi:10.1152/ajprenal.00375.2013.—Elevated serum parathyroid hormone (PTH) is an important complicated phenomenon in patients with chronic kidney disease (CKD). Emerging evidence indicates the involvement of PTH in organ fibrosis, and suppression of PTH by cinacalcet (CINA) ameliorates the progression of fibrotic disorders. However, the underlying mechanisms are largely unknown. The endothelial-to-mesenchymal transition (EndMT) has been shown to be an important mechanism involved in renal fibrosis. The present study aimed to investigate whether CINA treatment attenuated renal EndMT in rats with adenine-induced chronic renal failure (CRF). Compared with the control group, serum PTH was significantly higher in the CRF group and was suppressed after CINA treatment. Serum calcium, phosphorus, and calcium × phosphorus product levels were similar in the CRF group and CINA-treated CRF group. Renal collagen accumulation was significantly increased in the CRF group, which was markedly ameliorated by CINA treatment. Expression of the endothelial marker CD31 was significantly downregulated in rats with CRF, whereas expression of the mesenchymal markers fibroblast specific-protein 1 and α-smooth muscle actin was markedly upregulated. These changes were inhibited by CINA treatment. The protein levels of these EndMT-related markers were strongly correlated with serum PTH concentrations. Furthermore, in the in vitro study showed that PTH could significantly increase the expression of fibroblast specific-protein 1 and α-smooth muscle actin and decrease CD31 in mRNA and protein levels in a concentration- and time-dependent manner. In conclusion, our study suggests that reducing serum PTH by CINA treatment could attenuate renal fibrosis via suppression of EndMT in the adenine-induced CRF rat model.

Cinacalcet; parathyroid hormone; endothelial-to-mesenchymal transition; chronic kidney disease; renal fibrosis

SECONDARY HYPERPARATHYROIDISM frequently contributes to the natural progression of chronic kidney disease (CKD) (6,13). Although the effect of elevated parathyroid hormone (PTH) levels on mineral metabolism has been well recognized, recent evidence has demonstrated the involvement of PTH in fibrotic disorders (5,8,14,35). The beneficial effects of strategies lowering PTH, such as vitamin D analog treatment, on the progression of renal fibrosis have also been documented (37). As a therapeutic alternative, the calcimimetic compound cinacalcet (CINA) has been reported to effectively suppress serum PTH levels in CKD (2,22). Recently, in vivo studies (17,30) have further suggested the antifibrotic role of CINA treatment in uremic rats, but the underlying mechanisms are still largely unknown.

Endothelial tissues have been recognized as a target of PTH action. Studies (33,34) have elucidated that PTH could directly induce endothelial injury through PTH receptors expressed on the cell membrane. Recently, the endothelial-to-mesenchymal transition (EndMT) has been demonstrated to be an important mechanism contributing to endothelial impairment and the development of fibrotic disorders (16,20,38). In addition, inhibition of EndMT could preserve the endothelial phenotype and prevent organ fibrosis (21,39,41). Thus, whether the antifibrotic effect of CINA is mediated by the blockage of EndMT represents an interesting question for research. In the present study, we investigated the effect of CINA treatment on renal fibrosis and EndMT.

MATERIALS AND METHODS

Animals. Study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Southeast University (Nanjing, China). A rat model of chronic renal failure (CRF) and secondary hyperparathyroidism was established as previously described (11). In brief, 8-wk-old male Wistar rats (SLAC Laboratory Animal) were randomly divided into the following three groups: a control group (CTL group; n = 6), a vehicle-treated CRF group (CRF group; n = 8), and a CINA-treated CRF group (CINA + CRF group; n = 8). CRF was induced by feeding rats a 0.75% adenine diet for 4 wk. Rats fed the diet without adenine served as controls. After adenine withdrawal, all animals were maintained on a 1.03% phosphorus diet for the next 8 wk. All diets were provided by XieTong Biotechnology. At the initiation of the adenine diet, rats in the CRF + CINA group were administered CINA (sc-207438, Santa Cruz Biotechnology) orally once daily (10 mg/kg) until the time of euthanization. Based on exposure levels, the equivalent dose to 10 mg/kg is 60 mg, which was used clinically (15).

Cell culture. Primary human renal glomerular endothelial cells (ECs) were purchased from Sciencell Research Laboratories (Carlsbad, CA) and cultured as previously described. Briefly, cells were grown in endothelial culture medium (ScienCell) containing 5% FBS (11). ECs were expanded in monolayers in culture flasks. At ~80% confluence, the culture medium was changed to serum-free solution for 24 h before the use of cells in all experiments. Human recombinant PTH fragment 1–34 [PTH (1–34); P3796, Sigma] was then added to the serum-free culture medium.

Serum and urine biochemistry. Serum creatinine, blood urea nitrogen, and total calcium and phosphorus concentrations were measured at the Southeast University-affiliated Zhongda Hospital using an autoanalyzer system (Hitachi). Serum PTH determinations were performed with ELISA (Lengton). Urinary protein levels were measured with a protein assay kit using the modified Bradford method (KeyGen).

Histology and immunohistochemical staining. Paraﬁn-embedded rat kidney sections (3 μm thick) were prepared by a routine procedure. Sections were stained with periodic acid–Schiff reagent according to a standard protocol. Kidney sections were also subjected to Masson trichrome staining to assess fibrotic lesions. Immunohistochemical staining was performed using the routine protocol. The antibodies used were collagen type I (ab-292, Abcam), fibroblast specific-protein...
1 (FSP1; ab-27957, Abcam), and α-smooth muscle actin (α-SMA; ab-5694, Abcam). For negative controls, the specific primary antibodies were replaced with PBS. For semiquantitative analysis, immunostaining intensity was computed as integrated optical density using Image-Pro Plus software as previously described (28).

**Immunofluorescence staining.** Cryosections (5 μm thick) or coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 10% BSA in PBS at room temperature. Slides were then immunostained with primary antibodies against CD31 (sc-1506) and α-SMA (ab-5694) at 4°C overnight. After an incubation with a mixture of the secondary antibodies for 1 h in the dark at room temperature, images were captured using a laser scanning confocal microscope (LSM 510 META, Zeiss).

**Real-time PCR.** Total RNA was extracted using RNAiso Plus according to the manufacturer’s directions (TAKARA). RNA concentrations and purity were confirmed with a Nanodrop 2000 (Thermo). Samples with a relative absorbance ratio at 260/280 nm between 1.8 and 2.0 were used. All RNA samples were reverse transcribed (Applied Biosystems). The quantification of specific mRNAs was performed using an ABI Prism 7300 and a Sequence Detection System (Applied Biosystems) with the SYBR green Real-time PCR Kit (TAKARA). Primer sequences are shown in Table 1. Relative mRNA values were normalized to β-actin and calculated using the comparative cycle threshold (ΔΔCt) method.

**Western blot analysis.** Equal amounts of protein obtained from each lysate were electrophoresed on a 4–20% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Pall) using electroblotting. Blots were incubated overnight with primary antibodies against CD31 (sc-1506), FSP1 (ab-27957), α-SMA (ab-5694), and collagen type I (ab-292) followed by horseradish peroxidase-labeled secondary IgG (Santa Cruz Biotechnology). Signals were detected using an advanced ECL system (GE Healthcare), and the absorbance value at 260/280 nm was used to evaluate the correlation between the serum PTH concentration and protein expression. P values of <0.05 were considered to be statistically significant.

**RESULTS**

**Physical and metabolic parameters of animals.** As shown in Table 2, despite a lower body weight, kidney weight indexes (left kidney weight/body weight) of CRF rats were significantly lower than those of control rats; these levels were reversed by CINA treatment. Compared with control rats, rats in the CRF group developed severe renal dysfunction, as evidenced by significant increases in their serum creatinine, blood urea nitrogen, and urinary protein levels. The administration of CINA largely prevented kidney dysfunction and proteinuria in this model. Serum calcium levels decreased, whereas levels of serum phosphorus levels and the calcium × phosphorus product increased in the CRF group compared with the control group. After CINA treatment, serum calcium, phosphorus, and calcium × phosphorus product levels tended to be maintained at low levels, but the differences were not statistically significant. Serum PTH concentrations were found to be markedly elevated in the CRF group compared with the control group. The administration of CINA significantly decreased serum PTH levels in rats with CRF.

**CINA treatment ameliorated renal fibrosis in CRF rat models.** Kidney histology clearly revealed visible nephropathy in CRF rat models (Fig. 1A). The area of renal fibrosis in the CRF group was significantly increased compared with the control (Fig. 1B).

### Table 1. Primers sequence used for real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Organism</th>
<th>Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>Rattus norvegicus</td>
<td>NM_031591.1</td>
<td>5'-TCTCCAGCGCCAGTACGATG-3'</td>
<td>5'-GATTTTCCCTCTGCGAATA-3'</td>
</tr>
<tr>
<td>Fibroblast specific-protein 1</td>
<td>Rattus norvegicus</td>
<td>NM_013112.2</td>
<td>5'-AGCTACTAGAGACGAGGTG-3'</td>
<td>5'-CGTAATGGATGACTTCTTAC-3'</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>Rattus norvegicus</td>
<td>NM_031002.4</td>
<td>5'-TGGTATGGAGATGTGGCCGA-3'</td>
<td>5'-TGTGGGGATAGAGGTGTACCC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Homo sapiens</td>
<td>NM_01414945.1</td>
<td>5'-GAATGGCTTGGGCTTGGTAA-3'</td>
<td>5'-ATGCAGATGATCTGGATCTTCA-3'</td>
</tr>
<tr>
<td>CD31</td>
<td>Homo sapiens</td>
<td>NM_000442.4</td>
<td>5'-CATTCCAGCGCCAGTACGATG-3'</td>
<td>5'-TGACAAATGATCAGTCTTCTC-3'</td>
</tr>
<tr>
<td>Fibroblast specific-protein 1</td>
<td>Homo sapiens</td>
<td>NM_002961.2</td>
<td>5'-GTCCACCTCTCCAGAATTGAC-3'</td>
<td>5'-TGCAGAACTCTGGATCTGACG-3'</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>Homo sapiens</td>
<td>NM_001101.3</td>
<td>5'-CTGGAAGGTGCGACGGAAG-3'</td>
<td>5'-TGACGTGGACATCAGAAG-3'</td>
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### Table 2. Physical and biochemical parameters of animal groups

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CTL Group</th>
<th>CRF Group</th>
<th>CRF + CINA Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>523.54 ± 19.71</td>
<td>352.49 ± 48.45*</td>
<td>400.19 ± 38.19**</td>
</tr>
<tr>
<td>Kidney weight index, mg/g</td>
<td>3.66 ± 0.13</td>
<td>3.14 ± 0.29*</td>
<td>4.22 ± 0.47†</td>
</tr>
<tr>
<td>Serum sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine, mmol/l</td>
<td>35.25 ± 4.72</td>
<td>366.6 ± 134.73*</td>
<td>126 ± 41.66†</td>
</tr>
<tr>
<td>Blood urea nitrogen, mmol/l</td>
<td>67.3 ± 0.53</td>
<td>94.7 ± 24.28*</td>
<td>27.95 ± 6.41†</td>
</tr>
<tr>
<td>Parathyroid hormone, pg/ml</td>
<td>78.46 ± 12.51</td>
<td>440.64 ± 43.25*</td>
<td>326.54 ± 47.86**</td>
</tr>
<tr>
<td>Phosphorus, mmol/l</td>
<td>2.40 ± 0.16</td>
<td>3.86 ± 0.50*</td>
<td>3.01 ± 0.94</td>
</tr>
<tr>
<td>Calcium, mmol/l</td>
<td>2.23 ± 0.04</td>
<td>1.86 ± 0.22*</td>
<td>1.81 ± 0.12*</td>
</tr>
<tr>
<td>Calcium × Phosphorus product</td>
<td>5.35 ± 0.39</td>
<td>7.18 ± 0.92</td>
<td>5.45 ± 1.71</td>
</tr>
<tr>
<td>Urine sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>10.52 ± 3.79</td>
<td>220.26 ± 23.07†</td>
<td>127.53 ± 45.53†</td>
</tr>
</tbody>
</table>

Results are presented as means ± SD; n = 6 for the control (CTL) group, 8 for the chronic renal failure (CRF) group, and 8 for the cinacalcet (CINA)-treated CRF group. *P < 0.05 vs. the CTL group; †P < 0.05 vs. the CRF group.
control group, and it was decreased by the administration of CINA (Fig. 1, B and C). A quantitative determination of kidney fibrotic lesions showed decreased fibrotic percentages in the CINA-treated CRF group compared with the CRF group (Fig. 1C). Expression of collagen type I was markedly upregulated in the CRF group compared with the control group; its expression was downregulated by CINA treatment (Fig. 1, D and E). These results were consistent with the results revealed by periodic acid-Schiff and Masson trichrome staining (Fig. 1, A and C). Based on transmission electron microscopy analysis, rats in the control group displayed normal structure in the kidney. In contrast, rats from the uremia group presented endothelial disseminated protrusions and extracellular matrix accumulations (Fig. 1F). These changes were attenuated by CINA treatment.

**CINA treatment attenuated renal EndMT in CRF rat models.**

As determined by immunohistochemical analysis, FSP1 expression was significantly increased in the CRF group compared with the control group, and this induction was partially abolished by CINA treatment (Fig. 2).
α-SMA was also dramatically increased in the CRF group, whereas treatment with CINA significantly attenuated its expression (Figs. 2 and 3A). Confocal microscopy revealed increased colocalization of CD31 and α-SMA (yellow) in renal tissue from the CRF group compared with the control group, and the administration of CINA abrogated this change (Fig. 3B). As shown in Fig. 4, compared with normal control rats, the endothelial marker CD31 was substantially downregulated in the kidneys of CRF rats, as illustrated by real-time PCR and Western blot analysis. Its expression was restored after CINA treatment. Although mRNA and protein levels of FSP1 and α-SMA were markedly upregulated in rats with CRF, the administration of CINA also suppressed FSP1 and α-SMA expression.

Serum PTH concentration was correlated with expression of EndMT markers. As shown in Fig. 5, a significant negative correlation was observed between serum PTH concentrations and renal CD31 protein levels (r = 0.882, P < 0.01). In contrast, expressions of FSP1 and α-SMA were positively correlated with serum PTH concentrations (r = 0.889, P < 0.01, for FSP1; r = 0.853, P < 0.01, for α-SMA).

PTH induced EndMT in cultured renal ECs. To further confirm the effect of PTH on renal EndMT, we performed in vitro experiments using cultured renal ECs. As PTH (1–34) is the shortest fragment with the same biological effect as intact PTH, commercially available PTH (1–34) was used to treat ECs (33, 34). The results of real-time PCR showed that PTH incubation led to a significant decrease in mRNA expression of CD31 and an increase in mRNA levels of FSP1 and α-SMA at 48 h posttreatment (Fig. 6A). In addition, renal ECs were treated with 10−10 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 α-SMA mRNA levels from 0 to 48 h posttreatment (Fig. 6D). As determined by Western blot analysis, PTH caused a significant decrease in the protein level of CD31 as well as an increase in the protein expression of FSP1 and α-SMA in a concentration- and time-dependent manner (Fig. 6, B, C, E, and F). Confocal microscopy detected that cells treated with 10−10 mol/l PTH for 48 h acquired α-SMA staining and lost CD31 staining compared with control cells (Fig. 6G).

DISCUSSION

CKD leading to end-stage kidney disease is associated with renal fibrosis, regardless of the initiating causes. Generally, renal fibrosis is considered to be a multifaceted process that is related to various pathological factors. Recently, increasing evidence has demonstrated a relationship between endothelial injury and renal disease. Ochodnicky et al. (29) identified that renal endothelial dysfunction preceded the development of kidney damage in a mouse model of spontaneous renal disease. In addition, the progression of renal fibrotic lesions was significantly accelerated in mice with endothelial dysfunction (18, 27, 42). Clinically, in renal biopsies of CKD patients, renal endothelial alterations were also strongly related with the
parameters of renal injury and fibrosis (10, 19). Additionally, recent studies (20, 40) using endothelial lineage tracing have elucidated that renal ECs undergo EndMT, contributing to the initiation of renal fibrosis, which indicates that EndMT is an important mechanism in the progression of renal fibrotic disorders. Our previous work (24, 38) also demonstrated that high glucose, angiotensin II, inflammatory stress, and hyperlipemia-associated EndMT contribute to the progression of fibrosis.

Meanwhile, inhibition of EndMT is able to ameliorate fibrotic disease (39). Thus, it has been proposed that inhibition of EndMT represents a potential therapeutic strategy against renal fibrosis. In the present study, our data showed that CINA treatment attenuated renal EndMT in the adenine-induced CRF rat model. These findings implied that CINA could prevent renal fibrosis by abrogating EndMT.

CINA, as a calcimimetic agent, increases the sensitivity of the calcium-sensing receptor to extracellular calcium and suppresses the synthesis and secretion of PTH (26). A number of clinical trials have demonstrated that CINA efficiently reduced PTH levels in patients with stage 3–5 CKD (2, 4, 7, 22). Meanwhile, the significant decrease in PTH was accompanied by hypocalcaemia in some patients (4, 7, 31). This undesirable effect of CINA is believed to be a result of the reduced bone resorption, improved bone mineralization, and increased urinary calcium excretion (1, 3, 12, 36). In the present study, serum calcium levels were similar in the CRF group and CINA-treated CRF group. The exact mechanism is not clear, but it might be due to differences between animal and human models. As our data shown, PTH concentrations were markedly suppressed by CINA in rats with CRF. In addition, an in vitro study has shown that elevated PTH levels induced the expression of EndMT-related markers in a concentration-
CINACALCET ATTENUATES RENAL EndMT

**A**

Relative mRNA levels

- CD31
- FSP1
- α-SMA

**B**

PTH: 0, 10^{-12}, 10^{-11}, 10^{-10} (mol/L)

- CD31
- FSP1
- α-SMA
- β-actin

**C**

Relative protein levels

- CD31
- FSP1
- α-SMA

**D**

Relative mRNA levels

- CD31
- FSP1
- α-SMA

**E**

PTH: 0, 12, 24, 48 (h)

- CD31
- FSP1
- α-SMA
- β-actin

**F**

Relative protein levels

- CD31
- FSP1
- α-SMA

**G**

CD31, α-SMA, DAPI, Merge

**CTL**

- CD31
- a-SMA
- DAPI
- Merge

**PTH**

- CD31
- a-SMA
- DAPI
- Merge
CINACALCET ATTENUATES RENAL EndMT

Fig. 6. Effect of PTH on the expression of endothelial-to-mesenchymal transition-related markers in renal endothelial cells (ECs). A–C: ECs were incubated for 48 h with increasing concentrations of PTH, as indicated. D–F: ECs were incubated with PTH (10⁻¹⁰ mol/l) for different periods, as indicated. mRNA expression levels were determined by real-time PCR. Protein expression levels were determined by Western blot. Data are expressed as means ± SD; n = 3–5. *P < 0.05 vs. the 0 group. G: representative micrographs showing immunofluorescent staining of CD31 and α-SMA in ECs. Cells were incubated with 10⁻¹⁰ mol/l PTH for 48 h. Scale bar = 50 μm.
CINACALCET ATTENUATES RENAL EndMT


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