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Cinacalcet suppresses calcification of the aorta and heart in uremic rats

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High serum parathyroid hormone levels are associated with vascular calcification. Cinacalcet is a calcimimetic agent that inhibits parathyroid hormone secretion and is used to treat patients with secondary hyperparathyroidism. Here we measured the effects of oral cinacalcet on calcification of the aorta and heart in rats with a remnant kidney (5/6 nephrectomy) model of uremia that were fed a high-phosphate diet containing lactose to accelerate the process of aortic calcification. Alizarin red staining showed that the smooth muscle in the aortic arch of rats with a remnant kidney was calcified. The tissue levels of calcium and phosphorus in the aorta and hearts of these rats were significantly increased compared to sham-operated rats. Expression of the osteoblastic lineage genes osteocalcin, osteopontin and runt-related gene 2 were also increased in the aorta of these rats. Cinacalcet suppressed these calcification-related changes by reducing serum parathyroid hormone, calcium, phosphorus, and the calcium-phosphorus product. Parathyroidectomy also suppressed calcification in this model. We suggest that cinacalcet inhibits calcification of the aorta and heart in uremic patients with secondary hyperparathyroidism by decreasing serum parathyroid hormone levels.

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KEYWORDS: calcification; calcium; calcium receptor; cinacalcet; parathyroid hormone; phosphorus

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Aortic calcification is associated with increased morbidity and mortality in patients with chronic kidney disease.[1–6] Elevation of serum calcium (Ca), phosphorus (P), and Ca × P product are considered the main risk factors for aortic calcification.[7,8] Secondary hyperparathyroidism (2HTP) is also often observed in patients with advanced chronic kidney disease and in dialysis patients. Whether parathyroid hormone (PTH) has beneficial or detrimental effects on vascular calcification is still the subject of some debate. High serum PTH levels, which promote elevation of serum Ca, P, and Ca × P product, have been associated with higher mortality in 2HPT patients.[9] It has been reported that high serum PTH levels are associated with severity of coronary Ca deposits in hemodialysis patients.[10] Neves *et al.*[11] also reported that high PTH levels induce both high bone turnover and aortic calcification independently of uremia. In contrast, emerging evidence suggests a protective effect of PTH against vascular calcification: a bioactive fragment of PTH, 1–34 PTH, has also been demonstrated to have inhibitory effects on calcification in low-density lipoprotein receptor knockout mice.[12]

Cinacalcet HCl (cinacalcet) is a novel therapeutic agent for the treatment of dialysis patients diagnosed with 2HPT. Cinacalcet is a calcimimetic compound and inhibits PTH secretion from the parathyroid cells by activation of the Ca receptor.[13,14] We have previously demonstrated that cinacalcet has direct suppressive effects on PTH secretion in cultured human parathyroid cells derived from patients with 2HPT.[15] In previous clinical studies, cinacalcet also has been reported to suppress serum PTH, Ca, P, and Ca × P product in patients with 2HPT.[16–18] It has been further reported that cinacalcet does not induce aortic calcification in nephrectomized (NX) rats.[19] Recently, Lopez *et al.*[20] reported that the calcimimetic compound AMG 641 diminished 1,25-dihydroxyvitamin D3 ($1,25(OH)_2VD$)-induced aortic calcification in 5/6 NX rats. However, no prior study has examined whether cinacalcet can suppress aortic calcification that is caused by the progression of 2HPT.

In the present study, we examined the effects of cinacalcet on the incidence of aortic and heart calcification in NX uremic rats fed high-phosphate chow containing 20% lactose (HP-lactose chow). In addition, the effects of parathyroidectomy (PTX) were examined in this animal model to

further elucidate the role of PTH during the development of both aortic calcification and heart calcification.

RESULTS

Serum biochemistry

During the treatment period of this study, three rats died in the NX-vehicle-treated group, but no deaths occurred in the sham-operated group. One animal died in the NX-cinacalcet-treated group (15 mg/kg); the cause was thought to be due to asphyxia by endotracheal administration failure. No deaths related to treatment with cinacalcet were observed. At the end of the current study time frame, body weights were found to have decreased significantly in NX rats compared with sham-operated rats (data not shown). There were no differences in body weights of NX-vehicle rats and those treated with cinacalcet.

Table 1 shows the results of serum biochemical analyses at the end of the study period on day 41 or 42 post-treatment. Compared with the sham-operated rats, NX rats developed uremia, as evidenced by significant increases in serum creatinine (Cr) and blood urea nitrogen (BUN) levels. The serum Cr and BUN levels tended to be maintained at low levels following cinacalcet treatment, compared with those of vehicle controls, but the differences were not statistically significant. Serum Ca levels decreased, whereas serum phosphate levels increased in the NX-vehicle-treated group compared with the sham-operated group. The serum Ca × P product, which is thought to be a risk factor for aortic calcification, also increased in the NX-vehicle-treated group.

Cinacalcet treatment was found to decrease serum Ca levels significantly after 90 min at both dosages. The serum P levels and Ca × P product showed a trend towards a decrease following cinacalcet treatment at both 90 min and 24 h after administration. The serum PTH and osteocalcin levels were found to be elevated in the NX-vehicle-treated group compared with the sham-operated group. In contrast, serum PTH levels were significantly decreased at 90 min after administration in both cinacalcet-treated groups compared with the NX-vehicle-treated group. The serum osteocalcin levels showed a trend towards a dose-dependent decrease by cinacalcet treatment. Although there was no significant difference, the serum $1,25(OH)_2VD$ levels also showed a trend towards a dose-dependent decrease by cinacalcet treatment.

Tissue Ca and P content

In the NX-vehicle-treated group, tissue Ca and P content in the aorta was significantly increased compared with the baseline values measured in the NX group (Figure 1). At the end of the study period, the Ca and P levels in the aorta were found to have increased approximately 80- and 10-fold,

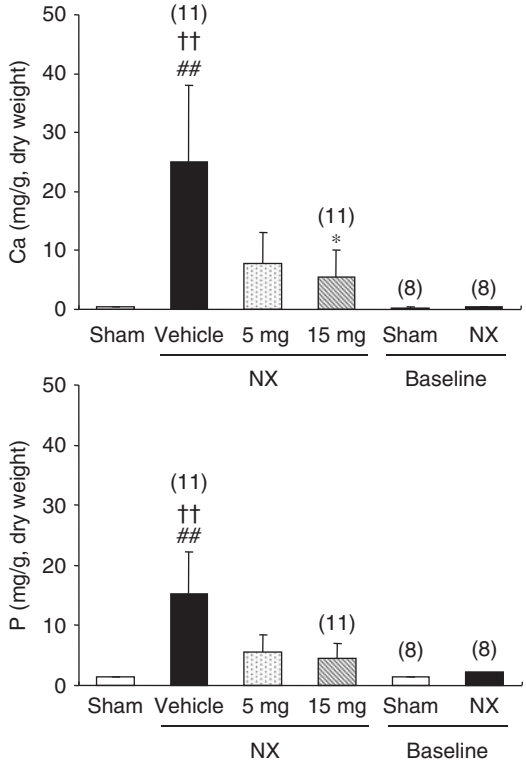

Figure 1 | Aortic mineral content. Thoracic–abdominal aortae were dissected and incinerated. The calcium (Ca) and phosphorus (P) content of the resulting ash samples was then measured and expressed as the weight of Ca or P per dry weight of tissue. The aortic Ca and P content was found to have increased significantly in nephrectomized (NX) rats. However, cinacalcet treatment suppressed these increases. The values shown are the mean ± s.e. ($n = 8$–12). $^{††}P < 0.01$ vs the NX baseline; $^{\#\#}P < 0.01$ vs the sham-operated group; $^{*}P < 0.05$ vs the NX-vehicle-treated group.

Table 1 | Serum biochemical measurements (for cinacalcet study)

	Sham		NX control		NX+cinacalcet (5 mg/kg)		NX+cinacalcet (15 mg/kg)	
	90 min	24 h	90 min	24 h	90 min	24 h	90 min	24 h
Ca (mg/100 ml)	9.2 ± 0.2	9.5 ± 0.2	8.4 ± 0.3[a]	8.9 ± 0.3[b]	7.4 ± 0.1[c]	8.8 ± 0.2	6.9 ± 0.3[d]	8.3 ± 0.2
P (mg/100 ml)	6.9 ± 0.2	6.9 ± 0.2	13.9 ± 2.1[b]	15.7 ± 2.5[a]	11.8 ± 1.4	13.5 ± 1.4	12.3 ± 0.8	14.5 ± 0.8
Ca × P ($mg^2/(100\,ml)^2$)	63 ± 3	66 ± 3	114 ± 15[b]	135 ± 16[a]	87 ± 10	118 ± 11	86 ± 8	121 ± 8
PTH (pg/ml)	113 ± 12	126 ± 18	1584 ± 335[a]	1731 ± 309[a]	545 ± 169[c]	988 ± 185	251 ± 67[d]	793 ± 62
BUN (mg/100 ml)	ND	17 ± 1	ND	103 ± 38[a]	ND	69 ± 7	ND	70 ± 4
Cr (mg/100 ml)	ND	0.5 ± 0.0	ND	1.6 ± 0.4[a]	ND	1.2 ± 0.2	ND	1.0 ± 0.1
$1,25(OH)_2VD$ (pg/ml)	ND	48 ± 12	ND	101 ± 27	ND	79 ± 16	ND	40 ± 7
Osteocalcin (ng/ml)	ND	24 ± 2	ND	215 ± 42[a]	ND	165 ± 29	ND	136 ± 10

$1,25(OH)_2VD$, 1,25-dihydroxyvitamin D3; BUN, blood urea nitrogen; Cr, creatinine; Ca, calcium; ND, not done; NX, nephrectomized; P, phosphorus; PTH, parathyroid hormone. Blood was collected at 90 min and 24 h after the administration of cinacalcet at the end of the treatment period on day 41. Values are mean ± s.e. ($n = 11$–12).
[a]$P < 0.01$; [b]$P < 0.05$ vs the sham-operated group; [c]$P < 0.05$; [d]$P < 0.01$ vs the NX-vehicle-treated group.

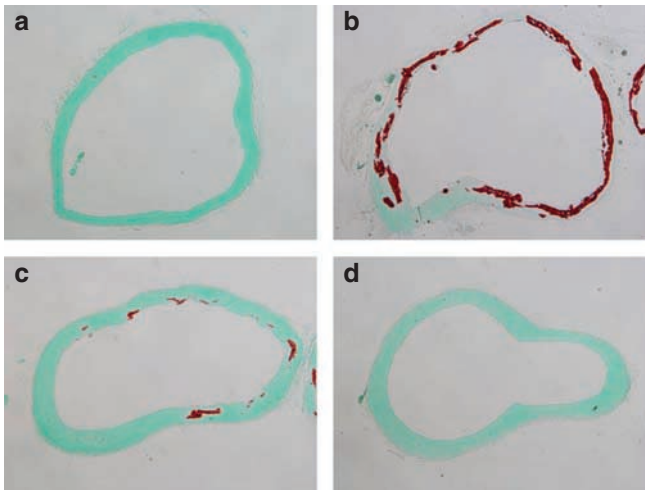

Figure 2 | Alizarin red staining of the aortic arch. (**a**) Sham-operated rat; (**b**) NX-vehicle-treated rat; (**c**) cinacalcet-treated rat (5 mg/kg); (**d**) cinacalcet-treated rat (15 mg/kg). Aortic arches were stained with alizarin red. As indicated by the red staining, the sections from nephrectomized (NX) rats showed calcification in the medial smooth muscle cells, indicating the presence of Mönckeberg-type arterial calcification. No calcium deposits were observed in the aortic tissues from animals treated with 15 mg/kg cinacalcet (**d**) and only slight calcification was found in rats treated with 5 mg/kg cinacalcet (**c**) (Original magnification, × 40.)

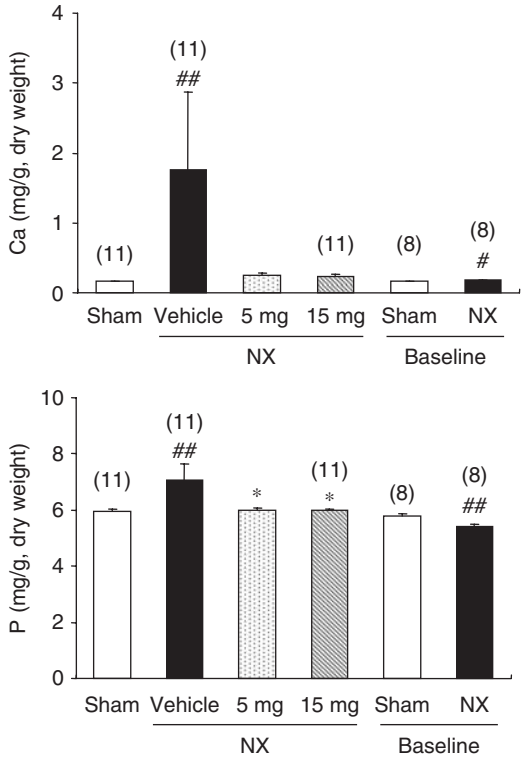

Figure 3 | Heart mineral content. Dissected hearts were incinerated, and the levels of calcium (Ca) and phosphorus (P) content were then measured as described in Figure 1. In the nephrectomized (NX)-vehicle-treated group, the heart Ca and P content increased significantly, but this increase was suppressed in both cases by cinacalcet treatment. The values shown are the mean ± s.e. ($n = 8$–12). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs the sham-operated group; $^{*}P < 0.05$ vs the NX-vehicle-treated group.

respectively, in the NX-vehicle-treated group compared with the sham-operated group. However, cinacalcet treatment reduced both the Ca and P levels in these animals (Figure 1). Alizarin red staining showed calcification of the aortic arch in the NX-vehicle-treated group, which displayed Mönckeberg arterial calcification, characterized by Ca deposition in the medial smooth muscle cells (Figure 2). Cinacalcet treatment suppressed Ca deposition around the medial smooth muscle cells (Figure 2).

The tissue Ca and P content in the heart was also found to be significantly increased in the NX-vehicle-treated group compared with the sham-operated group (Figure 3). However, cinacalcet treatment reduced the Ca and P content in the heart (Figure 3). By alizarin red staining, calcification of the aortic root was evident in the NX-vehicle-treated group but was suppressed by cinacalcet treatment (Figure 4). No distinct Ca deposits could be observed in the cardiac muscle cells by alizarin red staining.

Expression of calcification-related factors

The mRNA expression of osteoblastic markers, osteocalcin, osteopontin, and runt-related gene 2 (*Runx2*) increased in the aortae of NX-vehicle-treated rats compared with sham-operated rats (Figure 5). The mRNA expression levels of these proteins in NX-cinacalcet-treated rats were similar to those in sham-operated rats. Expression of the osteocalcin and osteopontin proteins was also increased in aortae of NX-vehicle-treated rats, whereas no marked expression was observed in aortae of NX-cinacalcet-treated rats (Figure 6).

The mRNA expression level of matrix Gla protein (MGP), a calcification inhibitor, was also increased in NX rats

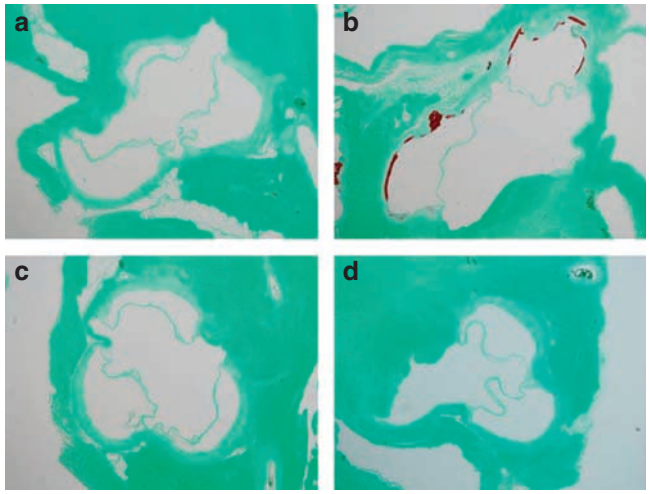

Figure 4 | Alizarin red staining of the aortic valve. Dissected aortic root tissues were stained with alizarin red. Sections from nephrectomized (NX) rats show calcification, indicated by red staining. No calcium deposits were detectable, however, in the same tissues from animals treated with cinacalcet. (**a**) Sham-operated rat; (**b**) NX-vehicle-treated rat; (**c**) cinacalcet-treated rat (5 mg/kg); (**d**) cinacalcet-treated rat (15 mg/kg). (Original magnification, × 40.)

compared with sham-operated rat (Figure 5). There was no increase in MGP expression in the cinacalcet-treated NX rats. The mRNA expression level of osteoprotegerin (OPG) was similar among the groups (Figure 5).

Effects of PTX on tissue calcification

In NX-PTX rats, the body weights showed an even greater decrease compared with NX-sham rats (data not shown). Table 2 shows the results of serum parameter measurements at day 69 after PTX. In the NX-sham rats, serum PTH, P, Ca × P product, and osteocalcin levels were elevated, whereas the serum Ca levels decreased significantly compared with the sham control rats. Serum PTH levels in NX-PTX animals were below the detection limit. Serum levels of Ca × P product, $1,25(OH)_2VD$, and osteocalcin were also decreased in NX-PTX rats compared with NX-sham rats. Moreover, the serum Ca and P levels tended to show decreases following PTX. The Ca and P content in the aorta increased significantly in NX-sham rats compared with sham control rats (Figure 7). In addition, compared with NX-sham rats, the aortic Ca and P content in NX-PTX rats was at markedly lower levels (Figure 7).

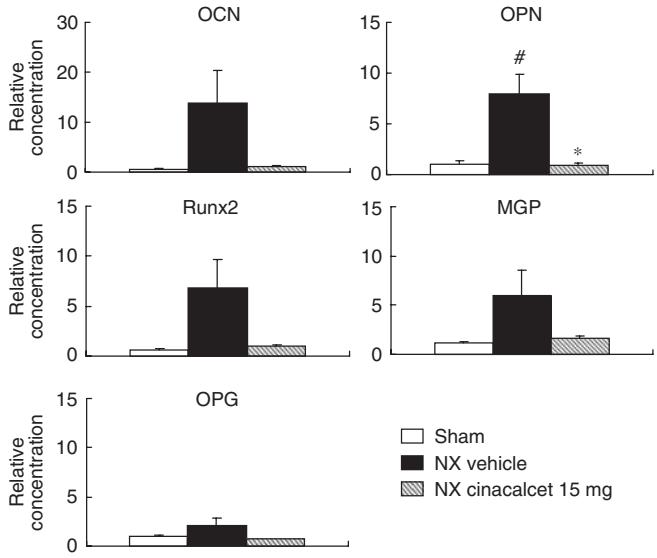

Figure 5 | The mRNA expression of calcification-related factors. Total RNA was isolated from rat aortae and reverse transcribed to cDNA. The cDNA was quantified for osteocalcin (OCN), osteopontin (OPN), runt-related gene 2 (*Runx2*), matrix Gla protein (MGP), and osteoprotegerin (OPG). The aortic mRNA expression levels of osteocalcin, osteopontin, Runx2, and MGP were increased in the NX-vehicle-treated rats compared with the sham-operated rats. Their expression levels in NX-cinacalcet-treated rats were similar to those in sham-operated rats. The values shown are the mean ± s.e. ($n = 5$). $^{\#}P < 0.05$ vs the sham-operated group; $^{*}P < 0.05$ vs the NX-vehicle-treated group.

DISCUSSION

The results of our present study indicate that cinacalcet treatment inhibits aortic calcification in NX rats that are maintained on an HP-lactose chow. In addition, PTX also suppressed aortic calcification in this animal model. Hence, we postulate that cinacalcet can suppress aortic calcification by decreasing the PTH level. We further found in the present analyses that bone turnover was elevated in the NX-vehicle-

Table 2 | Serum biochemical measurements (for PTX study)

	Sham	NX-sham	NX-PTX
Ca (mg/100 ml)	8.8 ± 0.1	7.0 ± 0.5[a]	6.1 ± 0.3
P (mg/100 ml)	5.8 ± 0.1	16.7 ± 3.4[b]	12.6 ± 1.2
Ca × P ($mg^2/(100\,ml)^2$)	52 ± 1	111 ± 19[b]	77 ± 8
PTH (pg/ml)	85 ± 7	3216 ± 925[a]	9 ± 1[c]
BUN (mg/100 ml)	15 ± 1	104 ± 17[a]	63 ± 10
Cr (mg/100 ml)	0.4 ± 0.0	2.0 ± 0.4[a]	1.2 ± 0.2
$1,25(OH)_2VD$ (pg/ml)	57 ± 13	43 ± 10	4 ± 1[c]
Osteocalcin (ng/ml)	21 ± 2	489 ± 83[a]	79 ± 7[c]

$1,25(OH)_2VD$, 1,25-dihydroxyvitamin D3; BUN, blood urea nitrogen; Cr, creatinine; Ca, calcium; NX, nephrectomized; PTX, parathyroidectomy; P, phosphorus; PTH, parathyroid hormone.
Blood was collected at both 90 min and 24 h after treatment on day 68 post-PTX. Values are the mean ± s.e. (n=6).
$^{a}P < 0.01$; $^{b}P < 0.05$ vs the sham-control group; $^{c}P < 0.01$ vs the NX-sham group.

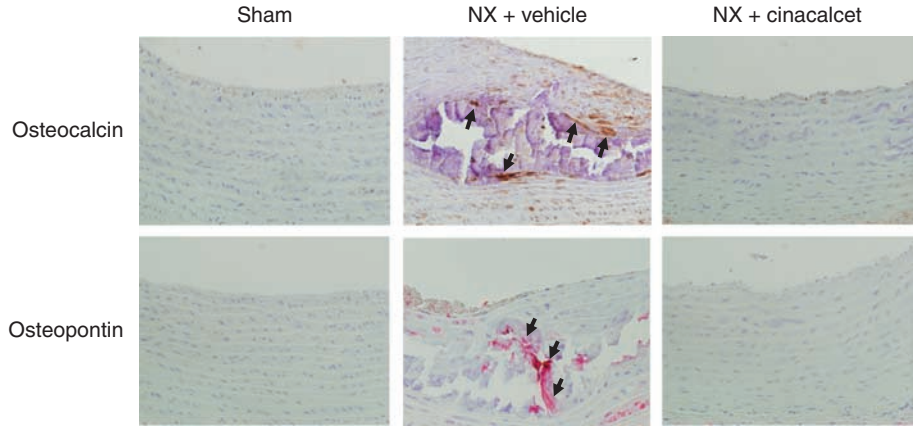

Figure 6 | Osteocalcin and osteopontin protein expression in aortae. Histological sections of aortic arches were used for immunostaining of osteocalcin and osteopontin. The protein expression of both osteocalcin and osteopontin was increased in the aortae of NX-vehicle-treated rats (arrows), whereas no marked expression was observed in the aortae of NX-cinacalcet-treated rats. In each photograph, the top side is intima and the bottom side is adventitia. (Original magnification, × 400.)

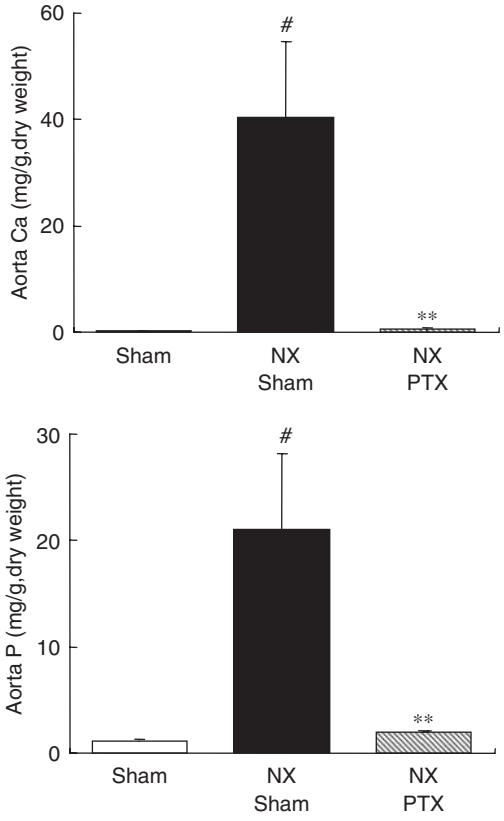

Figure 7 | Aortic mineral content in parathyroidectomized (PTX) rats. The calcium (Ca) and phosphorus (P) content in the aortae was found to increase significantly in nephrectomized (NX) rats; however, PTX inhibited these increases. The values shown are the mean ± s.e. ($n = 6$). $^{\#}P < 0.05$ vs the sham-operated group; $^{**}P < 0.01$ vs the NX group.

treated rats, as evidenced by increased serum osteocalcin levels. The evidence that cinacalcet decreases serum osteocalcin levels suggests that cinacalcet improves the deleterious effects of accelerated bone turnover during 2HPT by suppressing serum PTH levels. We speculate in this regard that cinacalcet-induced decreases in serum PTH levels inhibit the mobilization of Ca and P from the bone, thus suppressing the induction of aortic calcification.

Even though the link between the elevation of bone turnover and aortic calcification remains elusive, it is possible that PTH might at least partially induce this calcification process through such a mechanism.[11] Clinical data show that increased serum Ca, P, and Ca × P product are associated with an increased risk of cardiovascular disease.[7,8,21] In addition, vascular smooth muscle cells calcify when cultured *in vitro* in medium containing high Ca or P.[22,23] Findings from previous studies as well as from our own suggest that aortic calcification is accelerated by elevated serum Ca × P product, which is the result of an accelerated bone turnover in 2HPT. In the present study, decreases in serum Ca, P, and Ca × P product were observed following both cinacalcet treatment and PTX. These decreases, accompanied by a reduction in serum PTH level, might contribute to the suppression of aortic calcification.

It was reported previously that serum P levels tended to increase with cinacalcet treatment in an NX rat model.[19] On the contrary, it has also been reported that serum P is reduced by cinacalcet treatment in clinical studies.[24] In NX rats, it is thought that kidney function is maintained and that renal P absorption is increased by the suppressive effects of cinacalcet on serum PTH levels. In our present study, serum P levels showed a tendency to decrease following cinacalcet exposure. The inhibitory effects on the mobilization of P from bone apparently overtake the increased levels of renal P absorption by cinacalcet treatment.

The contribution of vitamin D to vascular calcification is a well-established characteristic of 2HPT. Shalhoub *et al.*[25] reported that $1,25(OH)_2VD$ induces mineral deposition on vascular smooth muscle cells *in vitro*. Thus, $1,25(OH)_2VD$ seems to have a direct effect on smooth muscle cells, causing ectopic calcification. In the present study, cinacalcet tended to decrease serum $1,25(OH)_2VD$ levels in NX rats. It is postulated that cinacalcet may inhibit aortic calcification, in part by decreasing serum $1,25(OH)_2VD$ levels.

There were significant increases in *Runx2*, osteocalcin, and osteopontin gene expression in the NX rats; however, these increased expressions were blocked in NX rats treated with cinacalcet compared with sham-operated rats. It has been reported that P and $1,25(OH)_2VD$ induce Runx2 expression and initiate mineralization of vascular smooth muscle cells. RunX2 increases the expression of both osteocalcin and osteopontin.[23,25,26] The suppressive effect of cinacalcet on the expression of these factors related to osteoblastic lineage, as demonstrated in this study, may be linked to the result of lowering of serum P and $1,25(OH)_2VD$ level. It has been reported that Ca receptor is expressed in both aortic smooth muscle cells and endothelial cells.[27] It should be further elucidated whether the direct effect of cinacalcet on smooth muscle cells might be to suppress the initiation of smooth muscle cell calcification.

MGP and OPG are considered calcification inhibitors. Further, it has been reported that MGP is increased by Ca in vascular smooth muscle cells.[28] However, in the present study, the increased expression of MGP was associated with calcified aortae of NX-vehicle-treated rats, suggesting that this increased MGP expression might be the compensatory action. In contrast to the NX-vehicle-treated rats, there was no increase in the expression of MGP in NX-cinacalcet-treated rats compared with sham-operated rats. There was also no change in the expression of OPG in rats treated with NX-vehicle or NX-cinacalcet compared with sham-operated rats. Thus, it may be considered that these proteins, which inhibit ectopic calcification, were not important in our study.

Three rats in the NX-vehicle group died during the course of our current experiments. In contrast, all the NX rats treated with cinacalcet survived and no deaths related to this treatment were observed. Cinacalcet was also found to suppress valvular and cardiac calcification. The progression of aortic valvular calcification is associated with the risk of cardiovascular disease and thus increases the risk of

mortality. Our finding that no deaths occurred in the cinacalcet treatment groups is encouraging because it further suggests cinacalcet's protective role during deleterious cardiovascular events. Ogata *et al.*[29] have reported that calcimimetic R-568 has a protective effect against the development of uremia and cardiovascular risk factors during renal failure. Our finding that BUN levels tended to decrease following both cinacalcet treatment and PTX suggests that their protective effects against renal calcification might also suppress development of uremia.

In summary, cinacalcet suppresses aortic calcification in an NX rat model. Our current findings thus indicate the potential of this drug for future treatments of aortic calcification in dialysis patients diagnosed with 2HPT.

MATERIALS AND METHODS

Animal protocols

The experimental protocols used for the treatment of the subject rats were approved by the Experimental Animal Ethical Committee of Kirin Brewery Co., Ltd. The experimental schedule is outlined in Figure 8. Sprague–Dawley rats (8-week old) were purchased from Charles River Japan (Tokyo, Japan) and fed a standard diet (CE-2; Clea Japan, Tokyo, Japan). These rats were allowed free access to food and water; after an acclimatization period of 10 days, they were divided into two groups that were matched with respect to body weight. The rats in the uremic group comprised 5/6 nephrectomized animals that were surgically treated under ether anesthesia. This procedure involved removal of the right kidney and ligature of branches of the left renal artery in a one-step procedure on day −15. Sham operations were performed on the control animals. Two weeks after the operations, the sham ($n=20$) and the NX rats ($n=44$) were divided into two and four groups, respectively, which were matched in terms of body weight and for serum Cr, Ca, P, PTH, and BUN levels. The rats in the baseline groups ($n=8$) were anesthetized with ether, and blood was collected by means of an aortic puncture on day 0. The aortic arch was then dissected and fixed in 10% formalin for subsequent histological studies. The thoracic–abdominal aorta was dissected and frozen for alizarin red staining to quantify the aortic calcification levels.

To accelerate the process of aortic calcification, we used a high-phosphate chow (Ca 1.0%, P 1.2%) containing 20% lactose (HP-lactose chow), which facilitates intestinal Ca absorption in a vitamin D-independent manner.[30] Beginning on day 0, all treatment groups ($n=12$) were fed the HP-lactose chow. Two NX groups were administrated cinacalcet orally once daily (5 or 15 mg/kg) for 41 days. Single sham and NX groups were treated with vehicle (0.5% methylcellulose solution in distilled water). On day 42, blood samples were collected 24 h after the last administration to measure serum levels of Ca, P, BUN, Cr, PTH, $1,25(OH)_2VD$, and osteocalcin. Serum levels of Ca, P, and PTH were also measured 90 min after the last administration on day 41. All rats were killed on day 42 under the same procedures described for the baseline groups. The aorta and heart from one NX rat in the vehicle-treated group and one in the cinacalcet (15 mg/kg) group could not be used in the study because the tissues were autolyzed.

PTX study

The experimental schedule for the PTX study is outlined in Figure 8. Two weeks after the nephrectomies were performed, the NX rats were divided into two groups matched by body weight, and by serum levels of Cr, Ca, P, and BUN. One of these NX groups underwent PTX, whereas the other NX group and a control group were sham operated. PTX was performed under anesthesia. Briefly, an incision was made in the neck region of the rats and the

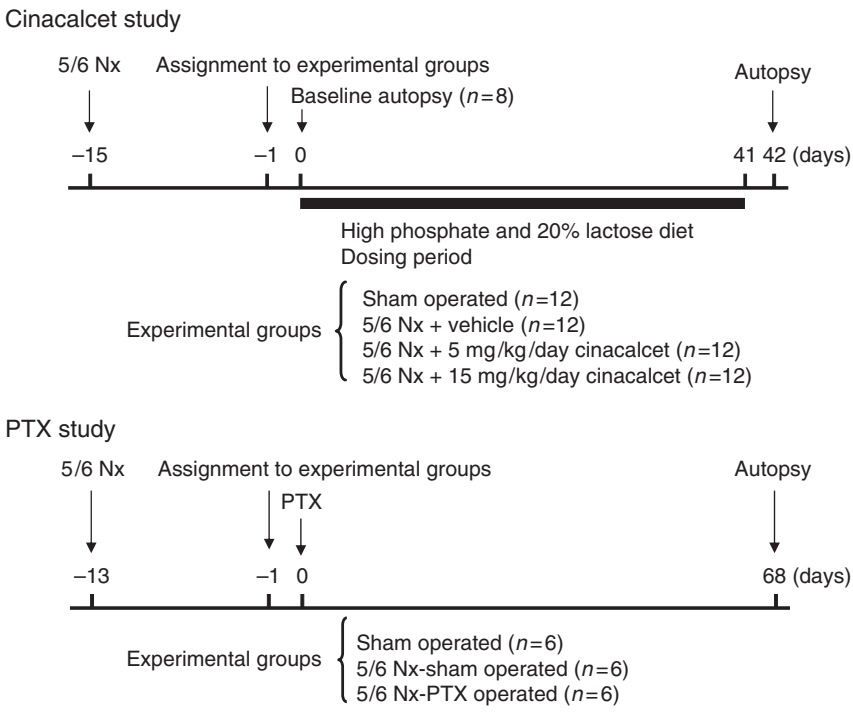

Figure 8 | Experimental design for the studies.

parathyroid–thyroid complex was exposed under microscopy. The parathyroid glands were then dissected out. The success of the PTX procedure was determined on the basis of a fall of serum PTH levels below the detection limit of a rat IRMA kit 1 day after the operation. After PTX, all animals were fed the HP-lactose chow diet for 68 days. After blood had been collected for serum parameter measurements, these rats were killed, and their heart and aorta were dissected in each case for the analysis of calcification.

Measurement of biochemical parameters

Serum levels of Ca, P, and BUN were measured with commercially available kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum Cr was measured with an enzymatic assay (CRE-EN; Kainos Laboratories, Tokyo, Japan). Serum PTH and osteocalcin levels were measured with a rat PTH IRMA kit and a Rat Osteocalcin IRMA kit (Immutopics, San Clemente, CA, USA), respectively. Serum $1,25(OH)_2VD$ was measured with a $1,25(OH)_2D$ RIA kit (TFB; Immunodiagnostic System, Ltd., Boldon, UK).

Examination of calcification in aorta and heart

Aortic arches and hearts were fixed in 10% formalin, and subsequently sectioned and stained with alizarin red to assess the extent of mineralization. These thoracic–abdominal aortae and hearts were dried and defatted in chloroform and methanol (2:1) for 48 h. The defatted samples were then dehydrated in acetone for 3 h and incinerated to ashes at 550 °C for 12 h in an electronic muffle furnace (KM-600; TOYOSEISAKUSYO Co., Ltd., Tokyo, Japan). The resulting ash samples were then dissolved in hydrochloric acid solution, diluted in distilled water, and subjected to measurement of Ca and P concentrations. The Ca and P content in the aorta and the heart was calculated as the weight of Ca and P per tissue dry weight in each case.

Immunohistochemical analysis

After inactivation of intrinsic peroxidase by incubation in 3% hydrogen peroxide diluted in methanol, the sections of aortic arches were treated with skim milk to prevent background staining and then incubated with anti-osteocalcin or anti-osteopontin rabbit antibody (COSMO BIO, LSL, Tokyo, Japan) overnight at 4 °C in a humidified chamber. After being rinsed, the sections were incubated with biotinylated secondary antibody and peroxidase-conjugated streptavidin, followed by visualization with vector-red or DAB substrate kits (Vector Laboratories Inc., Burlingame, CA, USA), and counterstained with hematoxylin.

Quantitative real-time PCR

Isogen reagent (Nippon Gene Co., Ltd., Tokyo, Japan) was used to isolate total RNA from frozen aortae. The TaqMan reverse transcription kit (Applied Biosystem, Foster City, CA, USA) was used to reverse transcribe cDNA from total RNA. The cDNA was quantified for octeocalcin, osteopontin, Runx2, MGP, and OPG. The primer sets for osteocalcin (primer ID: RA009113), osteopontin (primer ID: RA017345), MGP (primer ID: RA008628), and OPG (primer ID: RA023599) were all purchased from TAKARA Bio Inc., Otsu, Japan. The primer sets for Runx2 were designed as follows: 5′-ACTTCGTCAGCGTCCTATCAGTTC-3′ (forward), 5′-GCGTC AACACCATCATTCTGG-3′ (reverse). Samples were amplified in a 7900HT Fast Real-Time PCR system (Applied Biosystem), and the results were corrected by normalization with the corresponding levels of the internal control glyceraldehyde-3-phosphate dehydrogenase, which was amplified by using primers purchased from TAKARA Bio Inc. (primer ID: RA015380).

Statistical analysis

The differences between groups were compared by using the Mann–Whitney *U*-test, and multiple comparisons were performed with the Dunnett's test. Differences with *P*-values of <0.05 were accepted as statistically significant. All values are expressed as the mean ± s.e.

DISCLOSURE

Takehisa Kawata, Nobuo Nagano, Masaki Obi, Sonoe Miyata, Chika Koyama, Nami Kobayashi, Sachiko Wakita, and Michihito Wada are all employees of Kirin Pharma Co., Ltd.

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