see commentary on page 377

Metabolic acidosis inhibits soft tissue calcification in uremic rats

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Metabolic acidosis is common in patients with chronic kidney disease, which is known to affect bone metabolism. We examined the effect of metabolic acidosis on the development of vascular and other soft-tissue calcifications in uremic rats treated with calcitriol. Extraskeletal calcification was measured *in vivo*, in control rats and rats with a remnant kidney model of uremia with or without ammonium chloride-induced acidosis. Soft-tissue calcification was assessed histologically, by measurement of the expression of the sodium-dependent phosphate cotransporter Pit-1 and by quantification of tissue calcium and phosphorus. Calcitriol administration to uremic rats resulted in significant deposition of material positive for von Kossa stain in the aorta, stomach, and kidney, elevated aortic calcium and phosphorus, increased aortic Pit-1 expression, and high mortality. Calcitriol-treated uremic rats with acidosis did not develop aortic or soft-tissue calcification, did not increase aortic Pit-1 expression, and had significantly lower mortality. Additionally, an acidotic environment prevented calcification of vascular smooth muscle cells *in vitro*. Our study shows that metabolic acidosis inhibits extraskeletal calcification.

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Extraosseous calcifications, particularly vascular calcifications (VCs), are frequently observed in patients with chronic kidney disease (CKD) and represent an important risk factor for cardiovascular death.[1,2] In addition to ischemic heart disease, VCs are also related to uremic peripheral vasculopathy.[3] CKD patients are affected by both intimal calcification (atherosclerosis) and medial calcification (arteriosclerosis), but the latter seems to be more prevalent.[4]

Medial calcifications are associated to treatment with calcitriol and other vitamin D derivatives, which are used for management of secondary hyperparathyroidism (HPT).[5–8] The effects of calcitriol on VC are both indirect (mediated through an increase in blood calcium (Ca) and phosphorus (P)) and direct. Vascular smooth muscle cells (VSMCs) express vitamin D receptors and thus are responsive to calcitriol.[9–11]

Metabolic acidosis is also a common complication in patients with CKD, which is known to have deleterious effects on bone metabolism.[12] Acidosis has been shown to promote physicochemical bone dissolution, to activate osteoclast-mediated calcium release from bone, and to inhibit bone formation by osteoblasts.[13,14] The effect of acidosis on the development of VC is unclear. Medial calcification involves transdifferentiation of VSMCs to osteoblast-like cells.[15] Therefore, it can be hypothesized that the effects of acidosis on bone could also be extrapolated to VC. This contention is supported by recent *in vitro* data, which suggest that a decrease in pH prevents calcium deposition in cultured rat aortas.[16] However, *in vivo*, acidosis is also known to induce vascular wall damage,[17] which could initiate or aggravate the calcification process.

The current study was conducted to investigate the *in vivo* effect of metabolic acidosis on the development of vascular and other soft-tissue calcifications in a rodent model of uremia.

RESULTS

Plasma biochemistry is shown in Table 1. Plasma creatinine was significantly ($P<0.001$) increased in all 5/6 Nx groups. Nephrectomy alone did not influence the acid–base balance of the rats. However, administration of NH_4Cl resulted in decreased pH and bicarbonate in all groups when compared

Table 1 | Plasma biochemistry

	Sham (n=12)	Sham+AC 0.75% (n=13)	5/6 Nx (n=14)	5/6 Nx+AC 0.5% (n=13)	5/6 Nx+CTR (n=17)	5/6 Nx+CTR+AC 0.5% (n=12)	5/6 Nx+CTR+AC 0.3% (n=11)
Creatinine (mg per 100 ml)	$0.56 \pm 0.02^{a,b,c,d}$	$0.55 \pm 0.02^{a,b,c,d}$	0.93 ± 0.02	0.99 ± 0.06	1.04 ± 0.07	1.14 ± 0.89^{a}	1.14 ± 0.10^{a}
pH	$7.42 \pm 0.01^{c,d}$	$7.31 \pm 0.01^{a,b,c}$	7.44 ± 0.01	$7.20 \pm 0.03^{a,b,c,d}$	7.46 ± 0.02	$7.06 \pm 0.07^{a,b}$	$7.31 \pm 0.02^{a,b,c}$
Bicarbonate (mmol l^{-1})	$28.2 \pm 1.8^{c,d}$	$21.1 \pm 0.9^{a,b,c,d}$	29.2 ± 1.1	$15.8 \pm 1.4^{a,b,c}$	28.1 ± 0.7	$11.0 \pm 1.2^{a,b}$	$15.1 \pm 0.8^{a,b,c}$
Ca^{2+} (mmol l^{-1})	$1.21 \pm 0.01^{c,d}$	$1.23 \pm 0.02^{c,d}$	1.19 ± 0.01	$1.22 \pm 0.01^{b,c,d}$	1.27 ± 0.02^{a}	$1.48 \pm 0.03^{a,b}$	$1.35 \pm 0.02^{a,b,c}$
Phosphorus (mg per 100 ml)	$6.3 \pm 0.4^{b,c,d}$	$6.4 \pm 0.5^{b,c,d}$	6.8 ± 0.4	$6.9 \pm 0.3^{b,c,d}$	9.7 ± 0.7^{a}	$8.4 \pm 0.2^{a,b}$	$10.1 \pm 0.6^{a,c}$
PTH (pg ml^{-1})	28.5 ± 5.6^{a}	16.0 ± 6.1^{a}	115.5 ± 11.4	$80.1 \pm 23.5^{a,b,c,d}$	37.0 ± 13.9^{a}	$4.7 \pm 1.2^{a,b}$	10.1 ± 1.8^{a}

PTH, parathyroid hormone.
Plasma biochemistry in sham-operated rats (Sham) and uremic rats treated with vehicle (5/6 Nx) or calcitriol (5/6 Nx+CTR), with normal pH or receiving NH_4Cl in drinking water at 0.75% (Sham+AC 0.75%), 0.5% (5/6 Nx+AC 0.5% and 5/6 Nx+CTR+AC 0.5%), or 0.3% (5/6 Nx+CTR+AC 0.3%).
[a]$P<0.05$ vs 5/6 Nx.
[b]$P<0.05$ vs 5/6 Nx+CTR.
[c]$P<0.05$ vs 5/6 Nx+CTR+AC 0.5%.
[d]$P<0.05$ vs 5/6 Nx+CTR+AC 0.3%.

with their nonacidotic control (in 5/6 Nx rats, pH decreased from 7.44 ± 0.01 to 7.20 ± 0.03 and bicarbonate, from 29.2 ± 1.1 to 15.8 ± 1.4 mmol l^{-1}). Calcitriol-treated rats showed more marked ($P<0.001$ vs 5/6 Nx + acidosis) decrease in pH and bicarbonate after drinking 0.5% NH_4Cl (7.06 ± 0.07 and 11.0 ± 1.2 mmol l^{-1}, respectively). Ionized calcium levels were not different in sham (1.21 ± 0.01 mmol l^{-1}) and 5/6 Nx (1.19 ± 0.01 mmol l^{-1}) rats. Treatment with calcitriol increased Ca^{2+} in 5/6 Nx rats (1.27 ± 0.02 mmol l^{-1}, $P=0.01$ vs 5/6 Nx), and hypercalcemia was further aggravated by acidosis (1.48 ± 0.03 mmol l^{-1}, $P<0.001$ vs all other groups). Calcitriol-treated rats also showed higher P levels than the other experimental groups. Plasma parathyroid hormone (PTH) concentrations were higher in 5/6 Nx than in sham rats (115.5 ± 11.4 vs 28.5 ± 5.6 pg ml^{-1}, $P<0.001$). Acidosis did not influence PTH in the sham group but resulted in a slight decrease in PTH in the 5/6 Nx rats to 80.1 ± 23.5 pg ml^{-1} ($P<0.05$). Treatment with calcitriol significantly decreased PTH levels (37.0 ± 13.9 pg ml^{-1}, $P<0.001$ vs 5/6 Nx), and the decrease was more marked in the acidotic rats treated with calcitriol (4.7 ± 1.2 pg ml^{-1}). Uremic rats treated with calcitriol that received 0.3% NH_4Cl showed pH (7.31 ± 0.02) and bicarbonate (15.1 ± 0.8 mmol l^{-1}) values that were not different from the 5/6 Nx + acidosis group. These rats were hypercalcemic (1.35 ± 0.02 mmol l^{-1}), hyperphosphatemic (10.1 ± 0.6 mg per 100 ml), and had decreased PTH (10.1 ± 1.8 pg ml^{-1}).

Aortic Ca content was low in sham-operated rats (2.1 ± 0.2 mg per g of tissue). Neither acidosis nor nephrectomy modified aortic Ca content. However, treatment with calcitriol significantly increased aortic Ca content in 5/6 Nx rats (4.4 ± 0.9 mg per g of tissue, $P=0.001$ vs 5/6 Nx). Interestingly, acidotic rats treated with calcitriol did not increase aortic Ca content: 1.8 ± 0.2 mg per g of tissue (NH_4Cl, 0.5%) and 1.4 ± 0.1 mg per g of tissue (NH_4Cl, 0.3%), $P<0.001$ vs 5/6 Nx + calcitriol and NS vs all other groups (Figure 1a). Changes in aortic phosphorus mirrored those of aortic calcium. A significant increase in aortic P level was detected in 5/6 Nx rats treated with calcitriol (2.2 ± 0.7 mg per g of tissue, $P<0.05$ vs all the other groups), and again, acidosis prevented the calcitriol-induced increase in aortic phosphorus:

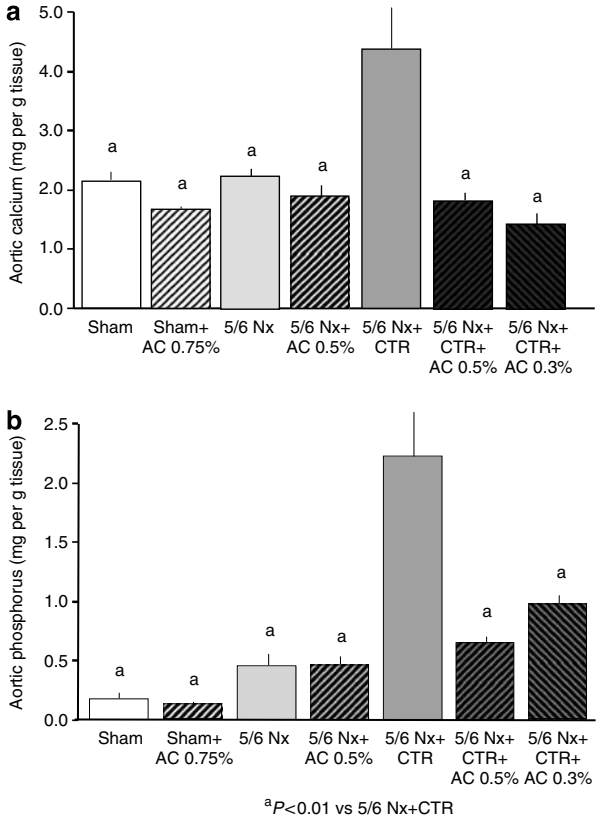


Figure 1 | Mineral content ((a) calcium, (b) phosphorus) of the aorta in sham-operated rats (Sham) and uremic rats treated with vehicle (5/6 Nx) or with calcitriol 80 ng kg^{-1} i.p. q.o.d. for 14 days (5/6 Nx + CTR). All groups are shown with normal pH and with acidosis, which was induced by administration of NH_4Cl in drinking water at 0.75% (Sham + AC 0.75%), 0.5% (5/6 Nx + AC 0.5% and 5/6 Nx + CTR + AC 0.5%), or 0.3% (5/6 Nx + CTR + AC 0.3%). [a]$P<0.01$ vs 5/6 Nx + CTR.

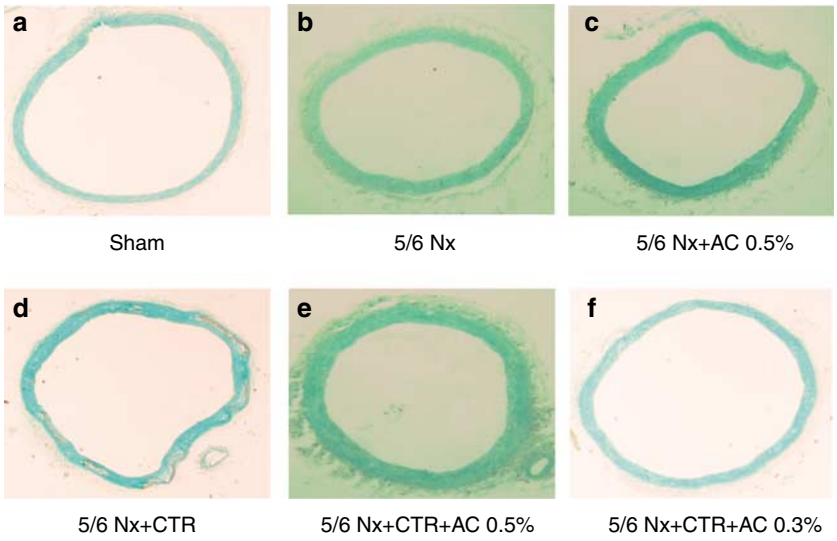
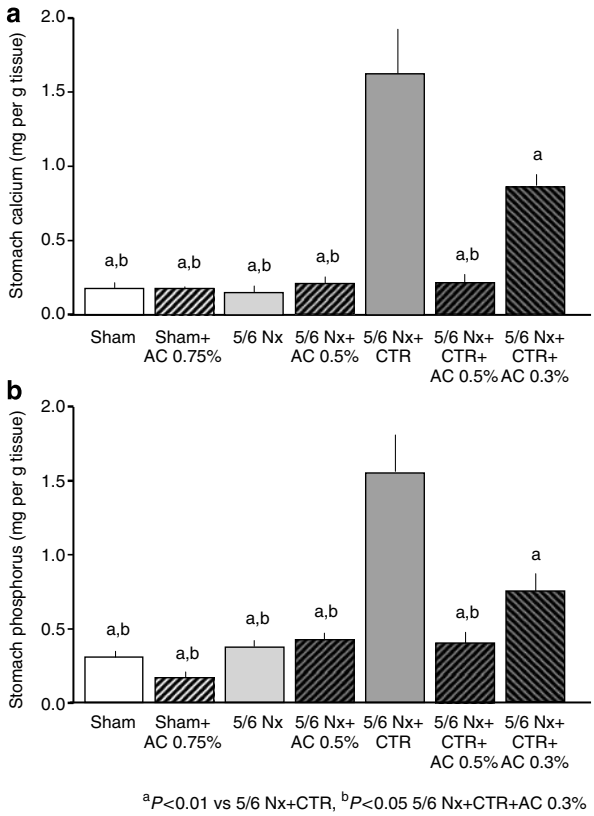


Figure 2 | Aortic calcification. von Kossa-stained tissue sections of the aorta in sham-operated rats (Sham, **a**), in uremic rats with normal pH (5/6 Nx, **b**) and with acidosis (5/6 Nx + AC 0.5%, **c**), and in uremic rats treated with calcitriol, 80 ng kg^{-1} i.p. q.o.d. for 14 days with normal pH (5/6 Nx + CTR, **d**) and with acidosis: 0.5% NH_4Cl (5/6 Nx + CTR + AC 0.5%, **e**) and 0.3% NH_4Cl (5/6 Nx + CTR + AC 0.3%, **f**).

0.7 ± 0.1 mg per g of tissue (NH_4Cl, 0.5%) and 0.9 ± 0.1 mg per g of tissue (NH_4Cl, 0.3%), $P < 0.01$ vs 5/6 Nx + calcitriol (Figure 1b).

Aortic tissue sections from nephrectomized rats are shown in Figure 2. Neither sham operated, 5/6 Nx, nor 5/6 Nx + acidosis rats showed any mineral deposits in the aorta (Figure 2a–c). Moderate mineral deposition was detected by von Kossa staining in the media of the 5/6 Nx rats treated with calcitriol. Calcium deposits showed a patchy concentric pattern along the tunica media (Figure 2d). Interestingly, the 5/6 Nx rats that were treated with calcitriol and made acidotic did not show mineral deposits in their aortas (Figure 2e and f).

The soft-tissue mineral content in the rats that did not receive calcitriol had low-gastric content of calcium and phosphorus ranging between 0.1 and 0.2 mg per g of tissue and 0.4 and 0.5 mg per g of tissue, respectively (Figure 3). Accordingly, no von Kossa staining was detected in tissue sections from these groups. Nephrectomized rats treated with calcitriol showed significant ($P < 0.05$) increases in both gastric calcium (1.6 ± 0.7 mg per g of tissue) and phosphorus (1.5 ± 0.6 mg per g of tissue), which were readily observed in the von Kossa-stained tissue sections (Figure 4). However, the acidotic rats (0.5% NH_4Cl) treated with calcitriol did not increase the gastric mineral content (Ca = 0.2 ± 0.1 mg per g of tissue, P = 0.4 ± 0.1 mg per g of tissue, NS vs 5/6 Nx). The rats that received 0.3% NH_4Cl showed higher gastric Ca (0.8 ± 0.1 mg per g of tissue) and P (0.7 ± 0.1 mg per g of tissue) than the rats that drank 0.5% NH_4Cl, but this level of mineralization was still significantly lower ($P < 0.01$) than their normal pH controls (Figure 3). von Kossa staining was not detected in the soft tissues of acidotic rats (0.5% NH_4Cl) treated with calcitriol (Figure 4).



[a]$P < 0.01$ vs 5/6 Nx+CTR, [b]$P < 0.05$ 5/6 Nx+CTR+AC 0.3%

Figure 3 | Mineral content ((a) calcium, (b) phosphorus) of the stomach in sham-operated rats (Sham) and uremic rats treated with vehicle (5/6 Nx) or with calcitriol 80 ng kg^{-1} i.p. q.o.d. for 14 days (5/6 Nx + CTR). All groups are shown with normal pH and with acidosis, which was induced by administration of NH_4Cl in drinking water at 0.75% (Sham + AC 0.75%), 0.5% (5/6 Nx + AC 0.5% and 5/6 Nx + CTR + AC 0.5%), or 0.3% (5/6 Nx + CTR + AC 0.3%). [a]$P < 0.01$ vs 5/6 Nx + CTR, [b]$P < 0.05$ vs 5/6 Nx + CTR + AC 0.3%.

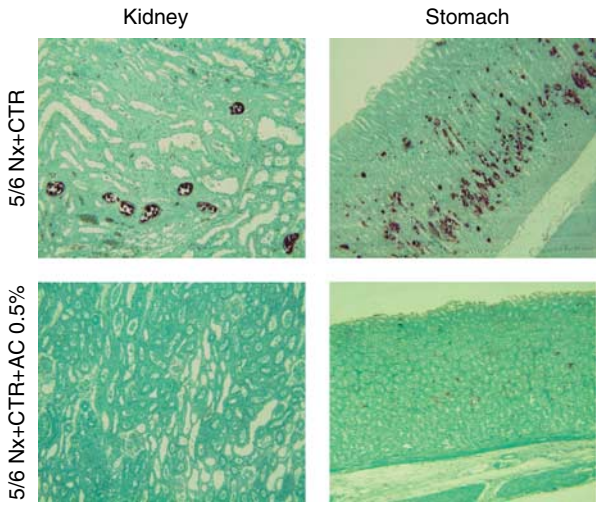


Figure 4 | von Kossa-stained tissue sections of the kidney and the stomach of uremic rats (5/6 Nx) treated with calcitriol, 80 ng kg^{-1} i.p. q.o.d. for 14 days with normal pH (5/6 Nx + CTR) and with acidosis, 0.5% NH_4Cl (5/6 Nx + CTR + AC 0.5%).

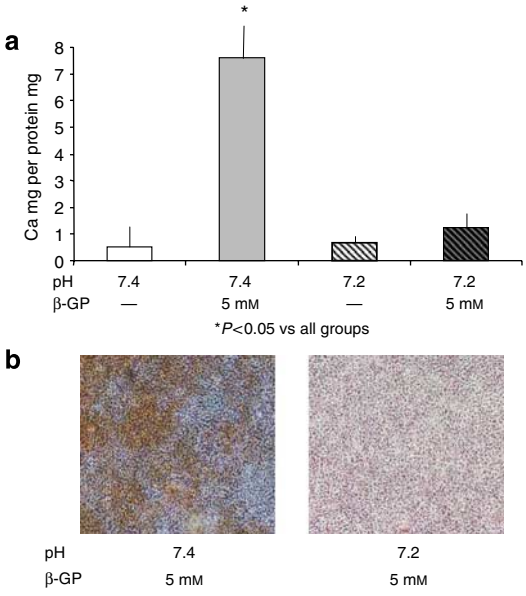


Figure 5 | *In vitro* studies of calcification using bovine VSMC incubated at normal (7.4) or low (7.2) pH with or without β-glycerophosphate (5 mM). (**a**) Calcium content; (**b**) Alizarin red stain.

No deaths were recorded during the first 15 days of the study in any experimental group. At 30 days, 4 out of the 10 (4/10) 5/6 Nx rats treated with calcitriol died. By contrast, only one (1/10) of the 5/6 Nx rats receiving 0.3% NH_4Cl + calcitriol died.

An increase in Pit-1 (mRNA Pit-1 vs mRNA β-actin) expression was detected in the calcified aortas of calcitriol-treated rats. Pit-1 was significantly ($P<0.05$) higher in the 5/6 Nx + calcitriol group (1.70 ± 0.38) than in the 5/6 Nx group (1.05 ± 0.23). Expression of Pit-1 in aortic tissue was lower ($P<0.05$ vs 5/6 Nx + calcitriol) in the 5/6 Nx + acidosis (0.87 ± 0.07) and 5/6 Nx + acidosis + calcitriol (0.77 ± 0.08) groups of rats.

Results of the *in vitro* studies are shown in Figure 5. Bovine VSMCs cultured at normal pH (7.4) with β-glycerophosphate (5 mM) showed extensive Alizarin red staining and high calcium levels (7.6 ± 0.7 mg Ca per mg protein). By contrast, when these cells were incubated at low pH (7.2), no staining was detected and the calcium levels (1.2 ± 0.1 mg Ca per mg protein) were not different from their control levels without β-glycerophosphate (0.7 ± 0.5 mg Ca per mg of protein).

DISCUSSION

The study reported here investigated the influence of metabolic acidosis on the development of calcitriol-induced extraosseous calcifications in a rat model of uremia. Our results show that acidosis caused by ingestion of NH_4Cl prevents the development of extraskeletal calcifications in uremic rats treated with calcitriol, even though the plasma Ca and P were increased in these rats.

Acidosis is a common complication of uremia that can contribute to the development of arterial wall inflammation.[17] Acidosis has been reported to increase the production of IL-6 and the chemokine RANTES from smooth muscle cells *in vitro*.[18] *In vivo*, Pickering *et al.*[19] reported a decrease in TNF plasma levels after correction of acidosis in patients receiving chronic ambulatory peritoneal dialysis. Since arterial wall inflammation is a well-known stimulus for VC, it can be speculated that acidosis could promote VC.

Medial calcifications (arteriosclerosis) are the more prevalent form of VC in uremic patients.[4] In medial calcifications, there is a transdifferentiation of VSMCs to osteoblastic cells, which ultimately can lead to osteoid tissue formation in the arterial wall.[15] The effect of acidosis on bone has been extensively studied, and the available data indicate that acidosis promotes bone dissolution through a series of physicochemical and cellular mechanisms.[12–14] Thus, it was hypothesized that acidosis could also act on extraskeletal calcifications in a manner similar to bone. This contention is supported by *in vitro* data demonstrating decreased mineral deposition in rat aortas cultured at low pH.[16] *In vivo*, acidosis has also been associated with decreased calcium deposition in the kidneys of azotemic rats.[20] Furthermore, clinical studies also suggest that acidifying agents may be useful in the treatment of extraosseous calcifications.[21]

To determine the influence of acidosis on the development of extraskeletal calcifications, we have used a model of calcitriol-induced calcifications in 5/6 Nx rats. This model, which has been previously reported,[22] has the advantage of resembling the clinical situation in patients with CKD, since many of these patients are treated with vitamin D derivatives for the control of secondary hyperparathyroidism. In this study, calcitriol has been administered at the dose that is required to normalize PTH levels in 5/6 Nx rats.[22] In addition, 5/6 Nx rats do not experience significant

changes in pH or bicarbonate, thus representing a good control group for 5/6 Nx acid-treated rats.

Our results clearly show that acidosis prevents the development of extraosseous calcifications in uremic rats treated with calcitriol. Two mechanisms are commonly implicated on the calcifying effect of calcitriol and other vitamin D analogs: a direct effect on VSMC, which have vitamin D receptors;[9–11] and an indirect effect mediated through the elevations of extracellular Ca and P.[6] It is interesting to note that the acidotic rats treated with calcitriol experienced more severe hypercalcemia than their normal pH controls (5/6 Nx + calcitriol). Phosphorus was significantly reduced in the 5/6 Nx + calcitriol group that received 0.5% NH_4Cl, and this could also have played a role in preventing calcifications. Nonetheless, the Ca × P product was increased in both acid-treated groups (0.3 and 0.5% NH_4Cl). Also of interest is the fact that acidosis tended to decrease PTH concentrations. We have previously demonstrated an acute direct stimulatory effect of acidosis on PTH secretion[23] and have also shown that this effect is counteracted by acidosis-induced hypercalcemia.[24] Therefore, the decreased PTH levels detected in acidotic rats are likely to be secondary to the elevations in plasma calcium. In conclusion, the data of this study indicate that the preventive effect of acidosis on calcifications is evident even in the face of high plasma Ca × P product.

Since metabolic acidosis had a significant effect on PTH and phosphate levels, which are known to affect calcification, *in vitro* studies were conducted to isolate the effect of acidosis from other changes in mineral metabolism. Our results, which are in agreement with previous *in vitro* studies,[16] show that acidosis also prevented the development of calcifications in cultures of bovine VSMC, where PTH was not present and phosphate levels were controlled. Thus, although *in vivo* PTH and phosphate may have some modulatory effect on the anticalcifying effect of acidosis, the *in vitro* experiments demonstrate that acidosis by itself can prevent calcifications.

Two direct mechanisms could potentially be involved in the protective effect of acidosis on extraskeletal calcifications: (a) a physicochemical mechanism; and (b) a cellular mechanism. The physicochemical mechanism is based on the well-known fact that acidosis decreases Ca and P deposition by increasing their solubility. It has been reported that the calcium and phosphate solubility for hydroxyapatite increases two- and fourfold, respectively, when pH is reduced from 7.40 to 6.90.[25] The cellular mechanisms are more complex; in the process of medial calcification, calcifying VSMC, a subpopulation of VSMC, experience phenotypic changes and are transformed into cells that are very similar if not the same as osteoblasts.[15] This phenotypic transformation is known to be triggered by vitamin D analogs, such as calcitriol. Metabolic acidosis has been reported to inhibit the production of collagen and other matrix components by mouse osteoblasts.[26,27] More recent studies have shown a downregulation of alkaline phosphatase and an upregulation of matrix-gla protein by metabolic acidosis in cultured rat osteoblasts.[28] Thus, it is likely that even if calcifying VSMC are activated by calcitriol to be transdifferentiated into osteoblasts, at low pH, they may be ineffective in producing osteogenic proteins.

Phosphate uptake through the sodium-dependent phosphate cotransporter (Pit-1) has been reported to be essential for VSMC calcification.[29] Our results show that VC was associated with increased Pit-1 expression in rat aortic tissue and that acidosis prevented the upregulation of Pit-1. Thus, in calcitriol-treated rats, acidosis may in fact limit cellular phosphate uptake by preventing upregulation of Pit-1.

In a previous study, we have demonstrated that cellular mechanisms affecting cells of the monocytic–macrophagic lineage are involved in regression of VCs.[30] These cells, which migrate from the vascular lumen to the calcifying foci and phagocyte mineral, behave in a manner similar to osteoclasts. Acidosis has been shown to stimulate osteoclast function. Thus, an attractive hypothesis on the role of acidosis would be an increase in the mechanisms of mineral clearance at the arterial wall. However, the microscopic study did not show mononuclear phagocyting cells either at the arterial wall or in other soft tissues.

The group of nephrectomized rats that were treated with calcitriol and received 0.5% NH_4Cl showed more marked metabolic acidosis than their nontreated controls (5/6 Nx + acidosis), as demonstrated by the lower pH and bicarbonate values. This finding was unexpected and could be due to several factors: (1) calcitriol treatment may deteriorate renal function (as shown by the increased creatinine values and renal von Kossa staining) and impair the renal handling of the added acid; (2) the lower PTH values in calcitriol-treated rats could impair bone buffering of the acid load;[31] (3) calcitriol-induced hypercalcemia may cause polyuria/polydipsia and thus increase water (and NH_4Cl) consumption. Water consumption was measured in the different groups to test the latter hypothesis. Calcitriol-treated rats (5/6 Nx + calcitriol + acidosis) drank $4.0 \pm 0.3\ ml\ h^{-1}$, which is about twice the water consumption of the 5/6 Nx group ($2.3 \pm 0.3\ ml\ h^{-1}$, $P < 0.001$). Thus, an increase in acid intake, probably secondary to hypercalcemia-induced polyuria/polydipsia, seems to be the more likely explanation for the lower pH observed in the calcitriol-treated rats.

An additional group of nephrectomized rats treated with calcitriol, in which the NH_4Cl concentration in drinking water was reduced to 0.3%, was also studied to compare rats with a similar degree of metabolic acidosis. These rats, which experienced a reduction in pH and bicarbonate similar to the 5/6 Nx + acidosis group, did not develop aortic calcifications, and when compared with their normal pH controls, had significantly reduced calcium deposition in soft tissues. Thus, a moderate degree of metabolic acidosis seems to be sufficient to prevent extraskeletal calcifications. Also of importance is the fact that, in accordance with their reduced mineral levels, the number of dead rats was lower in the group that received acid treatment (0.3% NH_4Cl).

In conclusion, we have shown that vascular and soft-tissue calcifications, induced by calcitriol administration to uremic rats, can be prevented by metabolic acidosis, even though acidotic rats have a high plasma Ca × P product. Since acidosis prevents upregulation of vascular Pit-1 expression, a possible mechanism for its anticalcifying effect may be reduced cellular uptake of phosphate. Although more preclinical data are needed and any clinical application of these results should consider the potential negative effects of acidosis, the data reported here could contribute to improve the management of the CKD patient at high risk of suffering extraskeletal calcification.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250 g were purchased from the Animal Breeding Facility of the University of Cordoba (Spain). Rats were housed with a 12/12-h light/dark cycle and given *ad libitum* access to normal diet (calcium: 0.9%, phosphorus: 0.6%). The experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba (Spain), and all animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science.

5/6 nephrectomy

The rodent model of CKD used in these studies was induced by 5/6 nephrectomy (5/6 Nx), a two-step procedure that reduces the original functional renal mass by five-sixths (5/6). In the first step, animals were anesthetized using xylazine (5 mg kg^{-1}, i.p.) and ketamine (80 mg kg^{-1}, i.p.), an 8-mm incision was made on the left mediolateral surface of the abdomen, and the left kidney was exposed. The two renal poles were tightly ligated and ablated, thus leaving 1/3 of the original renal mass. After 1 week of recovery, the animal was reanesthetized, and an 8-mm incision was made on the right mediolateral surface of the abdomen. The right kidney was exposed and unencapsulated, the renal pedicle clamped and ligated, and the kidney was removed. Sham-operated animals underwent the same procedures without renal manipulation.

Experimental design

The experimental schedule is shown in Figure 6. After the second surgery, the diet was changed to slightly increased phosphorus (0.9%) content. The rats were randomly assigned (based on the normal distribution of baseline body weights) into six experimental groups: sham operated ($n=12$), sham operated + acidosis ($n=13$), 5/6 Nx ($n=14$), 5/6 Nx + acidosis ($n=13$), 5/6 Nx + calcitriol 80 ng kg^{-1} i.p. q 48 h (Calcijex, Abbot, Madrid, Spain) ($n=17$), and 5/6 Nx + calcitriol 80 ng kg^{-1} q 48 h + acidosis ($n=12$).

To induce metabolic acidosis, NH_4Cl was added to the drinking water to form a 0.75% solution of NH_4Cl in sham-operated rats and a 0.5% solution of NH_4Cl in Nx rats.[32] Since rats treated with calcitriol developed more severe acidosis than the other experimental groups, an additional group of 5/6 Nx + calcitriol 80 ng kg^{-1} q 48 h + acidosis rats ($n=11$), in which the concentration of NH_4Cl in drinking water was adjusted to 0.3%, was also studied.

Treatments were maintained for 14 days. At day 14, rats were killed by aortic puncture and exsanguination under general anesthesia (i.p. sodium thiopental). In the calcitriol-treated rats, euthanasia was performed 24 h after receiving the last dose of calcitriol.

Twenty additional rats that received the treatment protocol 5/6 Nx + calcitriol ($n=10$) and 5/6 Nx + calcitriol + acidosis (0.3% NH_4Cl) ($n=10$) were followed up to 30 days for death-rate assessment.

Blood and tissue sampling

Blood was obtained under anaerobic conditions from the aorta. The thoracic aortas were dissected and divided into three parts, which were used for (a) histology; (b) measurement of tissue calcium and phosphorus content; and (c) quantification of Pit-1 mRNA. In addition, the stomach and the remnant kidney were removed

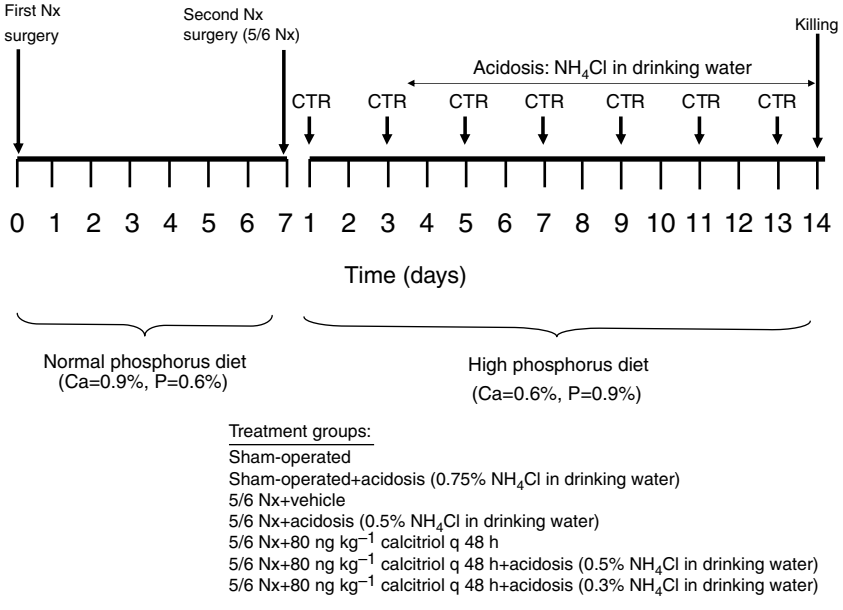


Figure 6 | Experimental design of the *in vivo* studies.

to study soft-tissue calcifications. Both histopathological studies (hematoxylin–eosin and von Kossa stains) and measurement of tissue calcium and phosphorus content were performed on tissue samples.

Quantification of aortic and gastric mineral content

For quantification of aortic Ca and P contents, the aortas were demineralized in a 10% formic acid solution. Subsequently, Ca and P contents were measured in the supernatant as described below.[33] Measurements of aortic Ca and P concentrations are reported as per dry weight. Quantification of organ mineral accumulation was carried out as previously reported.[34] Briefly, the stomach from each animal was placed into separate 30-ml tubes. Twenty milliliters of 150-mM HCl was added to each tube. The tubes were mixed end over end for 24 h at room temperature, and Ca and P concentrations were measured in the acid extract by spectrophotometry as described below.

Biochemical measurements

Ionized calcium, $PaCO_2$, and pH were measured using a selective electrode (model 634; Ciba Corning, Essex, England). Bicarbonate was calculated from pH and $PaCO_2$ values using the Henderson—Hasselbach equation. Intact PTH was quantified according to the vendor's instructions using the rat-specific IRMA assay (Scantibodies Laboratories Inc., Santee, CA, USA). Creatinine, phosphate, and total calcium were measured by spectrophotometry (BioSystems SA, Barcelona, Spain).

Pit-1 mRNA measurement (RNA isolation and real-time (RT)-PCR)

Fresh aortic tissue was dry-frozen in liquid nitrogen and stored at −80°C until RNA isolation. For RNA isolation, 1 ml of phenol—guanidine isothiocyanate solution (Tri-Reagent; Sigma, St Louis, MO, USA) was added to the aortic tissue. Tissue samples were ultrasonicated for 5 min at 4°C to allow for complete cell rupture. Thereafter, total RNA was extracted following a modification of the Chomczynski and Sacchi protocol[35] and dissolved in nuclease-free water (Promega, Madison, WI, USA). Total RNA was quantified by spectrophotometry. Pit-1 vs β-actin was amplified with an RT-PCR kit (QuantiTect SYBR green; Qiagen, Hilden, Germany) using specific primers and 100 ng of total RNA per sample. The following primers were used: Pit-1 (sense) CCGTCAGCAACCAGATCAACTC, (antisense) CCCATGCAGTCTCCCACCTTG; and β-actin (sense) TGTAACCAACTGGGACGATATGGA, (antisense) ACAATGCCAG TGGTACGACCAGA. DNA amplifications were processed by real-time PCR (LightCycler; Roche, Basel, Switzerland). Data were analyzed using LightCycler 3.5.28 software (Roche).

In vitro studies

Primary cultures of VSMC were obtained after enzymatic digestion of bovine aortas and were passaged six times in flasks. For the experiments, VSMCs were plated at 1×10^4 cells cm^{-2} and incubated at 36.5°C in a 5% CO_2 chamber. After confluence (3–4 days), cells were incubated in DMEM containing: Ca (1.8 mM), FCS (10%), glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids, and penicillin/streptomycin. The medium was changed every third day. Cultures were maintained at normal (7.4) or low (7.2) pH with or without β-glycerophosphate (5 mM). At the end of the incubation period (9 days), cells were washed with phosphate-buffered saline and calcification was quantified: (a) by spectrophotometry, using an OCPC Kit (Wako Diagnostics, Richmond, VA, USA); and (b) by histology, using 1% Alizarin red stain.

Statistics

Values are expressed as the mean ± s.e. The difference between means for the two groups was determined by *t*-test; the difference between means for three or more groups was assessed by ANOVA. $P < 0.05$ was considered significant.

DISCLOSURE

None of the authors have any relationship with companies that may have financial interest in the information contained in this paper.

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