The calcimimetic AMG 641 accelerates regression of extraosseous calcification in uremic rats

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1Departamento de Medicina y Cirugia Animal, Universidad de Cordoba, and 2Unidad de Investigacion y Servicio de Nefrologı ´a, Hospital Universitario Reina Sofia, Cordoba, Spain; and 3Department of Metabolic Disorders, Amgen, Incorporated, Thousand Oaks, California

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Lopez I, Mendoza FJ, Guerrero F, Almaden Y, Henley C, Aguilera-Tejero E, Rodriguez M. The calcimimetic AMG 641 accelerates regression of extraosseous calcifications in uremic rats. Am J Physiol Renal Physiol 296: F1376–F1385, 2009. First published March 25, 2009; doi:10.1152/ajprenal.90737.2008.—The purpose of the present study was to test the hypothesis that extraskeletal calcification regresses in uremic rats after reduction in phosphorus intake and treatment with calcimetics. Extraosseous calcification was induced in five to six nephrectomized rats fed a high-phosphorus (1.2%) diet who received calcitriol (80 ng/kg ip) every other day for a period of 14 days. Next, dietary phosphorus was reduced to 0.6%, and rats were treated with vehicle (n = 20), calcitriol [80 ng/kg ip/48 h (n = 20)], or the calcimimetic AMG 641 [1.5 mg/kg sc/48 h (n = 20)]. Aortic and soft-tissue calcium and phosphorus content was evaluated after 14 and 28 days. At 28 days, reduction of phosphorus intake resulted in a significant decrease in tissue mineral content in vehicle- and AMG 641-treated rats but not in rats receiving calcitriol. Aortic calcium and phosphorus was lower in rats treated with AMG 641 (96.7 ± 26.4 mg/g) than in rats receiving vehicle (178.3 ± 38.6 mg/g). An infiltrate of phagocytic cells expressing the calcium-sensing receptor was identified in areas surrounding foci of calcification. Additional studies in parathyroidectomized rats demonstrated that AMG 641 increased the urinary excretion of calcium (6.2 ± 0.6 vs. 3.1 ± 0.5 mg/day, vehicle) (P < 0.001). In conclusion, experimentally induced extraosseous calcification in uremic rats can be partially resolved by reducing phosphorus intake; the addition of calcimimetics may accelerate the regression process through mechanisms potentially involving a direct stimulatory effect on mineral phagocytic cells plus an increase in urinary calcium excretion.

Calcimimetics suppress PTH synthesis and secretion and, in contrast to most vitamin D sterols, do not induce hypercalcemia (6, 21). Moreover, it has been demonstrated that calcimetics prevent the development of extraosseous calcification in uremic rats (24, 25). Clinical studies in uremic patients (4) and experimental work (19) have shown that high extracellular phosphorus levels is a key factor in the development of VC, and thus a significant reduction in extraosseous calcification is likely to require that extracellular phosphorus levels are controlled.

The reversibility of extraskeletal calcification in patients with CKD is an important clinical issue; however, little information regarding the potential maneuvers directed to achieve regression of calcifications is available. There are only a few clinical reports documenting effective resolution of calcification (mostly calciphylaxis) in several patients with chronic kidney disease (7, 10, 12, 15, 26, 28, 35, 36, 40). A more recent report demonstrated resolution of soft tissue calcification in a hemodialysis patient receiving the calcimimetic cinacalcet (41). Previous work from our laboratory has demonstrated that extraosseous calcification can be partially reversed in rats with normal renal function in a relatively short period after suppressing the calcifying stimulus (calcitriol administration) (3).

We hypothesize that extraskeletal calcification will also regress in uremic animals after eliminating the main calcifying stimulus (dietary phosphate load) and that calcimetics may help to reduce soft tissue calcification, including VC.

Therefore, the objective of this study was to investigate whether there is a decrease in extraosseous calcification in uremic rats after reduction of phosphorus intake and to evaluate to what extent calcitriol and the calcimimetic AMG 641 may affect a potential regression of calcification. The primary endpoint of the study was to determine the decrease in extraskeletal calcification in uremic rats by measuring changes in the vascular and soft tissue content of calcium and phosphorus. In addition, histological and radiological studies were conducted to complement the quantification of calcium and phosphate.

METHODS

Animals. Male Wistar rats 9–10 wk old, weighing 250–300 g (Animal Breeding Facility of the University of Cordoba, Cordoba, Spain) were housed using a 12:12-h light-dark cycle and given ad libitum access to a standard diet (calcium = 0.9%, phosphorus = 0.6%). All experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba. %Nephrectomy. Uremia was induced by % nephrectomy (% Nx), a two-step procedure that reduces the original functional renal mass by five-sixths. In the first step, animals were anesthetized using xylazine (5 mg/kg ip) and ketamine (80 mg/kg ip), and a 5- to 8-mm incision was made on the left mediolateral surface of the abdomen.

ELEVATIONS OF PLASMA calcium, phosphorus, and Ca × P product observed in chronic kidney disease (CKD) patients with secondary hyperparathyroidism (HPT) are associated with vascular calcification (VC) and increased risk of cardiovascular morbidity and mortality (14, 34). In the clinical setting, the development of extraosseous calcification in uremic patients is largely related to abnormalities in mineral metabolism and to management of secondary HPT (4, 5, 14, 27, 34, 38, 39). Current strategies to control secondary HPT, including the use of vitamin D sterols, phosphate binders, and calcimetics (23), have differing effects on these minerals. Calcitriol, while effective in controlling parathyroid hormone (PTH) levels, has been shown to induce VC in vivo and in vitro (16, 25).

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The left kidney was exposed, and the two poles (2/3 of renal mass) were ablated. The kidney was inspected and, when any hemorrhage was noted, it was returned to an anatomic position within the peritoneal cavity. The abdominal wall and skin incisions were closed with sutures, and the rat was placed back into its home cage. After 1 wk of recovery, in the second step, the animal was reanesthetized and a 5- to 8-mm incision was made on the right mediolateral surface of the abdomen. The right kidney was exposed and unencapsulated, the renal pedicle was clamped and ligated, and the kidney was removed. The ligated pedicle was returned to a neutral anatomical position and the abdomen and skin incisions closed with sutures. The animal was allowed to recover in its home cage. An additional group (n = 6) of nonuremic sham-operated rats that underwent the same surgical manipulation without renal ablation was also studied.

**Experimental design.** The experimental schedule is shown in Fig. 1. One day after the second surgery, the diet was changed to 0.6% calcium and 1.2% phosphorus (Altromin), and the animals received calcitriol (80 ng/kg ip/48 h, Calcijex, Abbott, Madrid, Spain) for 14 days to control secondary HPT. At day 14, 15 animals were killed to quantify calcification in the aorta and other soft tissues (time 0). The remaining rats were switched to the standard diet (calcium 0.9%, phosphorus 0.6%) and randomly assigned to three treatment groups: % Nx+vehicle (0.9% NaCl, n = 20); % Nx+calcitriol (80 ng/kg ip/48 h, n = 20); or % Nx+AMG 641 (1.5 mg/kg sc/48 h, n = 20).

At day 14, one-half of the animals were killed; the other half of the animal population was killed at day 28. Death was carried out by aortic puncture and exsanguination under general anesthesia (thiopental sodium ip) 24 h after the last dose of the drug. Following death, the thoracic aorta, the lungs, and the stomach were dissected.

**In vivo assessment of vascular and soft tissue calcification.** Radiology was used to ensure that all rats exhibited calcification at the end of the induction period and to compare the evolution of extraosseous calcification from time 0 to the end of the regression period (28 days). Radiological examination was performed under sedation (xylazine, 5 mg/kg; ketamine 80 mg/kg ip). A laterolateral radiograph of the entire animal was obtained using a high-frequency X-ray system (exposure parameters: 41 KV, 100 mAs, and 0.02 s, Siemens, Madrid, Spain) with digital processing (Fuji Computed Radiographs).

**Postmortem assessment of vascular and soft tissue calcification.** Vascular and soft-tissue calcification was studied postmortem on days 14 and 28 by histology and measurement of tissue calcium and phosphorus content. A sample of the thoracic aorta, stomach, and right lung were fixed in 10% buffered formalin and subsequently sectioned and stained by the von Kossa method to evaluate mineralization. The extent of aortic calcification (von Kossa staining) was blindly evaluated by three independent observers using a semiquantitative score (range 0–4). Another portion of the aorta was demineralized in 10% formic acid, and the tissue calcium and phosphorus content was measured as previously described by Price et al. (32). Stomach and lung mineral content was measured by a second method previously described by Price et al. (33). Briefly, the stomach and the left lung from each animal were placed into separate 30-ml tubes, after which 20 ml of 150 mM HCl was added. The tubes were mixed by inversion for 24 h at room temperature, and calcium and phosphorus were measured in the acid extract. The lung and the stomach were selected as target organs to study mineralization based on the results obtained in previous studies, which demonstrated that mineral deposition occurs at early stages in these tissues (24, 25, 29).

**Histology and immunohistochemistry.** Fresh tissue was fixed in 10% buffered formalin, embedded in paraffin, and cut into 3-μm sections. Paraffin-embedded sections of the aorta, stomach, and lung were stained with hematoxylin and eosin for examination of the cell populations associated with soft-tissue calcification. The avidin-biotin-peroxidase (ABC) method (Vector Laboratories, Burlingame, CA) was used for the immunohistochemical examination of calcimimetic receptor (CaSR) expression in the aortic wall. Endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide in methanol for 30 min. After an additional 30-min incubation in 10% normal goat serum in phosphate buffered saline (PBS), pH 7.6, tissue sections were incubated with the primary antibodies against the CaSR overnight at 4°C (Acris Antibodies, Hiddenhausen, Germany). After three 10-min rinses in PBS, tissue sections were incubated with the secondary antibody (biotinylated goat anti-rabbit immunoglobulins) for 30 min (Dako, Glostrup, Denmark). After two 10-min rinses in PBS, tissue sections were incubated with ABC diluted in PBS for 1 h. After three 10-min rinses in PBS, tissue sections were incubated for several seconds in Vector NovaRed (Vector Laboratories), rinsed in tap water, lightly counterstained with Mayer’s hematoxylin, dehydrated, and mounted.

**Studies on urinary excretion of calcium and phosphorus.** At the time of death (14 days), a urine sample was obtained by puncturing the urinary bladder. Fractional excretion of calcium and phosphorus...
was calculated by measuring both minerals and creatinine in the urine sample and in a plasma sample.

An additional group of parathyroidectomized rats (n = 7) was used to study the influence of AMG 641 on urinary calcium excretion. Rats were parathyroidectomized under general anesthesia (xylazine, 5 mg/kg; ketamine 80 mg/kg ip). The following week they were introduced in metabolic cages to obtain 24-h urine and were randomly treated with either AMG 641 (3 mg/kg sc, n = 4) or vehicle (n = 3). After 7 days of washout, the rats that had been treated with AMG 641 received vehicle (n = 4) and the rats that had been treated with vehicle received AMG 641 (3 mg/kg sc, n = 3), and their 24-h urine was obtained. The entire procedure was repeated twice to obtain two values from each rat with AMG 641 and vehicle treatment. The urine collected over 24 h was centrifuged (1,500 rpm, 5 min), the volume was measured, and an aliquot was saved for measurement of calcium and phosphorus.

Blood and urine chemistries. Blood for chemical analyses was obtained from the abdominal aorta at the time of death. Blood for measurements of ionized calcium levels was collected in heparinized syringes and immediately analyzed using a Ciba-Corning 634 ISE.

Table 1. Plasma biochemistry in nephrectomized rats

<table>
<thead>
<tr>
<th>Induction/Treatment</th>
<th>Creatinine, mg/dl</th>
<th>Ionized Calcium, mM</th>
<th>Phosphorus, mg/dl</th>
<th>PTH, pg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 0</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>% Nx + Vit D</td>
<td>2.03±0.21</td>
<td>0.84±0.04</td>
<td>20.0±1.8</td>
<td>120.7±26.3</td>
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<tr>
<td>% Nx + vehicle</td>
<td>1.50±0.16*</td>
<td>1.32±0.06*</td>
<td>11.0±2.1*</td>
<td>14.7±7.4*</td>
</tr>
<tr>
<td>% Nx + AMG 641</td>
<td>1.40±0.10*</td>
<td>1.12±0.04*†</td>
<td>5.7±0.4*</td>
<td>20.0±6.7*</td>
</tr>
<tr>
<td>14 days</td>
<td>1.25±0.08*</td>
<td>1.08±0.04*†</td>
<td>5.8±0.2*†</td>
<td>7.8±3.9*</td>
</tr>
<tr>
<td>% Nx + Vit D</td>
<td>1.42±0.10*</td>
<td>1.32±0.04*</td>
<td>6.7±0.6*†</td>
<td>1.3±0.1*</td>
</tr>
<tr>
<td>% Nx + vehicle</td>
<td>1.54±0.11*</td>
<td>1.07±0.03*†</td>
<td>5.6±0.4*</td>
<td>30.4±7.3*</td>
</tr>
<tr>
<td>% Nx + AMG 641</td>
<td>1.56±0.07*‡</td>
<td>1.07±0.01*†</td>
<td>6.2±0.4*</td>
<td>22.9±8.0*</td>
</tr>
<tr>
<td>28 days</td>
<td></td>
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</table>

Values are means ± SE. Results are for nephrectomized (% Nx) rats at the end of the induction of calcification (time 0) and in % Nx rats treated with calcitriol (80 ng/kg ip/48 h (% Nx + Vit D)), vehicle (% Nx + vehicle), or calcimimetic AMG 641 (1.5 mg/kg sc/48 h (% Nx + AMG 641)) at 14 and 28 days during the regression phase. Biochemical values in sham-operated (nonuremic) rats are creatinine = 0.55 ± 0.2 mg/dl, ionized calcium = 1.22 ± 0.1 mmol/l, P = 6.5 ± 0.7 mg/dl, parathyroid hormone (PTH) = 38.1 ± 7.4 pg/dl. *P < 0.05 vs. time 0. †P < 0.05 vs. % Nx + Vit D. ‡P < 0.05 vs. the same group at 14 days.

Fig. 2. Calcium (A) and phosphorus (B) content of the aorta in % Nx rats at the end of the protocol for induction of calcification (time 0) and values obtained at 14 and 28 days during the regression period in rats treated with Vit D (80 ng/kg on alternate days), vehicle (saline solution), and AMG 641 (1.5 mg/kg on alternate days). Sham-operated (nonuremic) rats (n = 6) had means ± SE aortic calcium = 2.03 ± 0.15 mg/g tissue and aortic phosphorus = 0.12 ± 0.04 mg/g tissue.

AJP-Renal Physiol • VOL 296 • JUNE 2009 • www.ajprenal.org
Ca²⁺/pH Analyzer (Ciba-Corning, Essex, UK). Afterward, plasma was separated by centrifugation and stored at −20°C until assayed. PTH levels were quantified according to the vendor’s instructions using a rat PTH₁₋₃₄ immunoradiometric assay kit (Immunotopics, San Clemente, CA). Plasma creatinine, phosphorous, and total calcium were measured by spectrophotometry (Biosystems, Barcelona, Spain). Urine was obtained as described above for measurement of urinary calcium and phosphorus excretion. The urinary concentration of calcium, phosphorus, and creatinine was measured by spectrophotometry (Biosystems).

Statistics. Values are expressed as means ± SE. The difference between means for two different groups was determined by t-test; the difference between means for three or more groups was assessed by ANOVA. Fisher’s least significant difference test was used as a post hoc procedure. *P < 0.05 was considered significant.

RESULTS

All control data (values in rats without the 5⁄6 Nx) are presented in legends for figures and tables.

Plasma biochemical parameters. Subtotal nephrectomized (5⁄6 Nx) rats receiving a high-phosphorus diet for 14 days were uremic (elevated plasma creatinine 2.0 ± 0.2 mg/dl), and their biochemical parameters indicated development of secondary HPT (Table 1, time 0: decreased ionized calcium = 0.84 ± 0.04 mmol/l, increased phosphorus = 20 ± 1.8 mg/dl, and increased PTH = 121 ± 26 pg/ml).

When dietary phosphorus was reduced from 1.2 to 0.6% (regression phase), a significant (*P < 0.05) decrease in creatinine was detected in all groups at 14 days (range: 1.3–1.5 mg/dl). Ionized calcium increased significantly in all groups compared with time 0, and the rats receiving calcitriol had higher values than AMG 641- and vehicle-treated rats (1.32 ± 0.06 mmol/l, *P < 0.05). A significant decrease in phosphorus was identified in all groups during the regression period, but the rats receiving calcitriol showed higher phosphorus levels (11.0 ± 2.1 mg/dl) than the other two groups (*P < 0.05). Finally, all groups experienced a significant decrease in PTH (range 8–20 pg/ml). Biochemical values at day 28 were very similar to those recorded at day 14. The main differences were found in the calcitriol-treated rats that had completely suppressed PTH (1.3 ± 0.1 pg/ml) and lower phosphorus (6.7 ± 0.6 mg/dl) than at day 14 (Table 1).

Aortic and soft tissue mineral content. Aortic calcium and phosphorus were very high (13.8 ± 0.8 and 20.5 ± 1.7 mg/g of tissue, respectively), demonstrating a high degree of VC in 5⁄6 Nx rats at the end of the high-phosphate diet induction period (time 0) (Fig. 2). During the initial part of the regression
phase (low-phosphate diet ± various treatments for 14 days), no significant changes in aortic calcium or phosphorus were detected. However, by the end of the regression period (following 28 days of the low-phosphate diet ± treatment), AMG 641- and vehicle-treated rats had lower aortic calcium ($P < 0.05$) compared with that observed at the end of the induction period ($time 0$). While rats receiving AMG 641 had the lowest mean aortic calcium levels (7.4 ± 0.9 mg/g of tissue) of all groups, the tissue content of calcium was not different from the vehicle group. However, aortic phosphorus was significantly ($P < 0.05$) decreased only in rats receiving AMG 641 (10.9 ± 2.7 mg/g of tissue) (Fig. 2). Thus the calcium $\times$ phosphorus product in the aorta was reduced in the AMG 641 group (96.7 ± 26.4 mg/g, day 28) compared with all other groups (vitamin D = 215.4 ± 63.2 mg/g, vehicle = 178.3 ± 38.6 mg/g, day 28) by the end of the regression period.

Gastric mineral content decreased relatively quickly during the regression phase (Fig. 3). During the initial part of the regression phase (14 days), significant ($P < 0.05$) differences from $time 0$ (2.2 ± 0.1 mg/g of tissue) were detected in the rats receiving vehicle (1.4 ± 0.4 mg/g of tissue) or AMG 641 (1.3 ± 0.3 mg/g of tissue), but not in calcitriol-treated rats (2.7 ± 0.1 mg/g of tissue). After 28 days, the rats treated with AMG 641 showed a further decrease in the calcium content of the stomach (1.0 ± 0.2 mg/g of tissue), which was significantly lower than in the vehicle-treated rats (1.7 ± 0.3 mg/g of tissue, $P < 0.05$). The results obtained for gastric phosphorus content were similar to calcium content (Fig. 3).

Mineral content of the lung (Fig. 4) decreased significantly by 14 days into the regression period in rats treated with AMG 641 (calcium = 0.7 ± 0.4 mg/g of tissue, phosphorus = 0.5 ± 0.2 mg/g of tissue, $P < 0.05$ vs. $time 0$). Significant reductions in both calcium and phosphorus were also observed at 28 days in rats treated with AMG 641. At this time point, the vehicle-treated rats showed a decrease in pulmonary phosphorus (1.0 ± 0.3, $P < 0.05$ vs. $time 0$) but not in calcium (Fig. 4).

Changes in mineral content during the regression phase were also assessed by radiographic studies and by the examination of von Kossa-stained sections. Radiological findings in a representative rat at $time 0$ and a rat from each treatment group at 28 days are depicted in Fig. 5. Extraosseous calcification was readily observed at $time 0$ in the stomach, remnant kidney, and intrathoracic vessels. In contrast, by day 28, the amount of calcification was substantially decreased in the vehicle- and AMG 641-treated groups, and in the latter the calcification was barely apparent.

**Fig. 4.** Calcium ($A$) and phosphorus ($B$) content of the lungs in % Nx rats at the end of the protocol for induction of calcification ($time 0$) and values obtained at 14 and 28 days during the regression period in rats treated with Vit D (80 ng/kg on alternate days), vehicle (saline solution), and AMG 641 (1.5 mg/kg on alternate days). Sham-operated (nonuremic) rats ($n = 6$) had means ± SE pulmonary calcium = 0.24 ± 0.04 mg/g tissue and pulmonary phosphorus = 0.25 ± 0.04 mg/g tissue.
Marked mineral deposition (von Kossa staining) was observed at time 0 in the aorta, stomach (Fig. 6), and lung (not shown). The extent of von Kossa-stained areas decreased during the regression phase in rats receiving vehicle and calcimimetic; the decrease was more evident in the AMG 641-treated rats. Thus, at 28 days, semiquantitative scores for aortic calcification were 2.8 ± 0.3 in vehicle-treated rats, 3.7 ± 0.1 in rats receiving calcitriol, and 2.1 ± 0.3 in AMG 641-treated rats. It is important to note that the severity of calcification is determined by the extent of the affected areas rather than the intensity of the staining. In fact, less calcified arteries tend to show darker staining because the vascular architecture is better preserved.

Histology and immunohistochemistry. Histopathological examination of the aortic wall using hematoxylin-eosin-stained slides showed the presence of numerous cells (morphologically identified as monocytes-macrophages) around the calcification foci (Fig. 7A). Some of these cells, which were oriented toward the mineral deposits, appeared to be multinucleated. Immunohistochemistry of the vascular wall revealed the presence of the CaSR in vascular smooth muscle cells; in addition, CaSR was also expressed on the surface of the phagocytic cells situated around the calcified foci (Fig. 7B).

Studies on urinary excretion of calcium and phosphorus. The mean ± SE fractional excretion of calcium and phosphorus was similar in the uremic rats treated with vehicle (5.9 ± 1.7 and 29.9 ± 7.5%, respectively) and with AMG 641 (5.8 ± 1.0 and 27.1 ± 5.2%, respectively). However, rats treated with calcitriol showed a higher excretion of calcium (7.6 ± 1.9%) and phosphorus (49.9 ± 5.2%), and the latter was significantly (P < 0.05) different from both vehicle and AMG 641 (Table 2).

Additional groups of parathyroidectomized rats treated with vehicle (n = 7) or AMG 641 (n = 7) were used to study the influence of renal CaSR activation by AMG 641 on urinary calcium excretion. Parathyroidectomized rats had undetectable PTH levels, lower plasma calcium than normal rats (total calcium = 7.3 ± 0.3 mg/dl, ionized calcium = 0.92 ± 0.03 mmol/l), and normal phosphorus (4.3 ± 0.2 mg/dl). Rats treated with vehicle did not change their plasma calcium or phosphorus levels. The 24-h mineral urinary excretion in vehicle-treated rats was calcium = 3.1 ± 0.5 mg and phosphorus = 0.4 ± 0.1 mg. Treatment with AMG 641 resulted in a moderate but significant decrease in plasma calcium (total calcium = 6.5 ± 0.2 mg/dl, ionized calcium = 0.84 ± 0.04 mmol/l) and an increase in plasma phosphorus (5.0 ± 0.2 mg/dl). A significant increase in urinary excretion of calcium (6.2 ± 0.6 mg/24 h, P < 0.001 vs. vehicle) was observed after treatment with AMG 641; however, the urinary phosphorus excretion was unchanged after AMG 641 administration (0.4 ± 0.1 mg/24 h, not significant vs. vehicle) (Table 2).

DISCUSSION

The present study was designed to test the hypothesis that extraskeletal calcification, including VC, may regress in uremic animals after reduction of dietary phosphorus combined with the administration of calcimimetics. Here we report that extraskeletal calcification in uremic rats with secondary HPT significantly regressed one month after reduction of dietary phosphorus. Moreover, the calcimimetic AMG 641 accelerated this regression through mechanisms that may involve a direct stimulatory effect on mineral-phagocytic cells combined with reductions in plasma calcium and calcium × phosphorus.

In an attempt to mimic the clinical situation of uremic patients (in which calcification is closely related to hyperphosphatemia) (2, 4, 9, 13, 30), calcification was induced using a previously reported protocol that includes the use of a high-phosphorus diet to achieve elevated extracellular phosphorus levels (6, 16, 21, 24, 25).

Radiographic studies were conducted to ensure that at the beginning of the regression period (time 0), all rats showed an equivalent extent of calcification and to allow an individual follow-up for calcification by noninvasive methods.
This is the first study that demonstrates that experimentally induced calcification can regress in a relatively short period in uremic rats. These data are in line with previous findings from our laboratory showing a rapid regression of calcitriol-induced VC in rats with normal renal function (3). It is important to note that in this study regression was facilitated by reducing phosphorus intake; the reduction of phosphate load is recommended in uremic patients as a therapeutic measure to prevent development of vascular calcification (18, 22, 23, 31).

In addition, this study further demonstrated that treatment with the calcimimetic AMG 641 enhanced the regression of soft tissue calcification in uremic animals. This effect may be partially explained by the phosphorus-lowering effect of AMG 641. However, in phosphate-restricted rats, administration of AMG 641 further reduces extraosseous calcification. This suggests that, as advanced in other publications (1, 17, 20), calcimimetics may also have a direct effect (possibly mediated by CaSR activation) on the vascular wall that helps to reduce calcification. Although the regression of vascular calcification obtained with the calcimimetic may appear to be relatively small, compared with what is accomplished by phosphorus restriction, we believe that it is significant because in the clinical setting it is very difficult to achieve adequate phosphorus control and thus any additional measures are relevant.

There are several mechanisms by which calcimimetics could potentially influence the course of extraosseous calcification. In previous work, we identified changes in mineral metabolism (e.g., decreased calcium × phosphorus product) as a major
factor in the protective effect of calcimimetics against calcification (24). However, the regression process is likely to be significantly different from calcification induction and may require an active process to regress already formed calcification. In a study of calcitriol-induced VC in rats with normal renal function, the resolution of VC seemed to be an active cellular process in which cells from the monocyte-macrophage lineage migrated from the vascular lumen to the calcifying foci to phagocytize mineral particles (3). In the present study, mono- and multinuclear phagocytic cells were also identified in the wall of the calcified arteries. Since the only known mechanism of action of calcimimetics involves interaction with the CaSR, we investigated the presence of the CaSR on the phagocytic cells in the arterial wall. Our results clearly demonstrate that these cells express CaSR, thus providing a possible mechanism for an effect of calcimimetics on cells that eliminate mineral deposits. Calcimimetics have already been shown to increase CaSR expression in the arterial wall (20), to prevent vascular remodeling in uremia (20), and to retard uremia-associated VC (17); however, further studies will be necessary to demonstrate a CaSR-mediated stimulatory effect of calcimimetics on the phagocytic cells that appear in calcified arteries.

Under physiological conditions, in patients with residual renal function, serum calcium levels are regulated in part through changes in urinary calcium excretion. However, the influence of calcimimetics on urinary calcium excretion is equivocal. Calcimimetics have been reported to increase urinary calcium excretion in healthy rats (11), have no influence or even decrease urinary calcium excretion in hypercalciuric stone-forming rats (8), and do not have an influence on urinary calcium excretion in patients with primary HPT (37). The disparate nature of these observations may potentially reside in the effect of calcimimetics in lowering PTH secretion. Moreover, the effect of calcimimetics on calcium and phosphorus excretion was also difficult to study in our model of uremia due to the confounding effect of the different PTH levels. In fact, in the uremic rats, no differences in the fractional excretion of calcium and phosphorus were observed between vehicle- and AMG 641-treated animals. Of note, the fractional excretion of both calcium and phosphorus was increased in the calcitriol-treated rats even though they experienced less regression, potentially resulting from an increase in the intestinal absorption and/or bone mobilization of calcium in these animals. To minimize the problems related to intergroup differences in PTH levels (which may also fluctuate in response to the drug throughout the day), parathyroidectomized rats (in which the influence of the calcimimetic on the parathyroid gland was absent) were used to assess the direct effect of AMG 641 on urinary calcium and phosphorus excretion. Our study demonstrated that administration of AMG 641 to parathyroidectomized rats enhanced urinary calcium excretion. Thus, in addition to its potential effect of promoting phagocytosis of mineral particles at the level of the arterial wall, calcimimetics seem to facilitate urinary excretion of the calcium eliminated from the calcified tissues.

The ability of AMG 641 to enhance the regression of existing vascular and soft tissue calcification is potentially of great importance for the treatment of patients with CKD. Calcimimetic therapy has been shown to decrease PTH, calcium, phosphorus, and calcium × phosphorus in the dialysis population (6, 21); however, the effects of calcimimetics on vascular calcification or cardiovascular mortality are currently being evaluated in controlled clinical trials. While the preclinical data presented here are not definitive for the clinical setting, they do suggest a positive effect of calcimimetic therapy on these outcomes, which we believe will be confirmed by the results of the ongoing ADVANCE and EVOLVE clinical trials.

### Table 2. Urinary excretion of calcium and phosphorus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FE_{Ca} %</th>
<th>FE_{P} %</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Nx + Vit D</td>
<td>7.6 ± 1.9</td>
<td>49.9 ± 5.2</td>
</tr>
<tr>
<td>%Nx + vehicle</td>
<td>5.9 ± 1.7</td>
<td>29.9 ± 7.5</td>
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<tr>
<td>%Nx + AMG 641</td>
<td>5.8 ± 1.0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>U_{Ca}, mg/24 h</th>
<th>U_{P}, mg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX + vehicle</td>
<td>3.1 ± 0.5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>PTX + AMG 641</td>
<td>6.2 ± 0.6‡</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. Fractional excretion of calcium (FE_{Ca}) and phosphorus (FE_{P}) in % Nx rats treated with calcitriol (80 ng/kg ip/48 h (% Nx + Vit D)), vehicle (% Nx + vehicle), or calcimimetic AMG 641 [1.5 mg/kg sc/48 h (% Nx + AMG 641)] during the regression phase and 24-h urinary calcium (U_{Ca}) and urinary phosphorus (U_{P}) excretion in parathyroidectomized rats (PTX) treated with vehicle (PTX + vehicle) or calcimimetic AMG 641 (3 mg/kg sc (PTX + AMG 641)) are shown. Mineral urinary excretion values in sham-operated (nonuremic) rats are FE_{Ca} = 0.6 ± 0.2%, FE_{P} = 6.2 ± 1.5%, U_{Ca} = 1.9 ± 0.2 mg/24 h, UP = 2.9 ± 1.2 mg/24 h.

*p < 0.05 vs. % Nx + Vit D; ‡p < 0.05 vs. PTX + vehicle.
Calcimimetic Regression of Extraosseous Calcification

In conclusion, the results of this study support the concept that experimentally induced extraosseous calcification can be partially reverted in uremic rats in a relatively short time frame and that the calcimimetic AMG 641 accelerates this regression process.

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DISCLOSURES

C. Henley is an employee of Amgen, Inc., which manufactures calcimimetics.

REFERENCES


