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Artery calcification in uremic rats is increased by a low protein diet and prevented by treatment with ibandronate

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The present experiments investigate medial artery calcification in adult rats made uremic by feeding a synthetic diet containing 0.75% adenine for 4 weeks. Calcification was assessed by Alizarin red staining of intact aortas, by von Kossa staining of carotid artery sections, and by calcium and phosphate incorporated into the thoracic aorta. The major conclusions are as follows: Lowering the protein content of the diet from 25 to 2.5% dramatically increases the frequency and extent of medial artery calcification in uremic rats without significantly affecting the elevation in serum creatinine, phosphate, or parathyroid hormone. This observation suggests that low dietary protein intake could be a risk factor for medial artery calcification in uremic patients. Medial artery calcification in uremic rats is prevented by a dose of ibandronate that inhibits bone resorption. The observation suggests that bone resorption inhibitors could prevent artery calcification in uremic patients. Medial artery calcification in uremic rats correlates with increased serum bone Gla protein (BGP; osteocalcin), but not with serum matrix Gla protein or fetuin. This finding indicates that it could be of interest to examine the relation between serum BGP and artery calcification in uremic patients. Each of these conclusions lends support for our hypothesis that medial artery calcification is linked to bone resorption. Future investigations of the as yet unknown biochemical basis for this link will be facilitated by the present discovery that a synthetic, 2.5% protein diet containing 0.75% adenine produces consistent and dramatic medial calcification in adult rats within just 4 weeks.

Kidney International (2006) **70,** 1577–1583. doi:10.1038/sj.ki.5001841; published online 6 September 2006

KEYWORDS: medial artery calcification; chronic kidney disease; ibandronate; low protein diet; bone Gla protein (osteocalcin)

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Received 8 July 2006; revised 14 July 2006; accepted 25 July 2006; published online 6 September 2006

Our long-term goal is to understand the mechanisms that initiate calcification of the elastic lamellae of the artery media and the mechanisms that inhibit this calcification. In the course of our investigations, we have become intrigued with the evidence for an association between increased bone resorption and increased artery calcification seen in patients with postmenopausal osteoporosis (see Price *et al.*[1] for references), in rats treated with toxic doses of vitamin D,[2,3] and in the osteoprotegerin-deficient mouse.[4] The existence of this association has led us to propose that medial artery calcification is linked to bone resorption, and one prediction of this hypothesis is that inhibitors of bone resorption should inhibit artery calcification.[1] In previous studies, we tested this prediction using three different types of bone resorption inhibitors, each with an entirely different mode of action on the osteoclast: the amino bisphosphonates alendronate and ibandronate,[1,3,5,6] the cytokine osteoprotegerin,[6,7] and the V-H^+-ATPase inhibitor SB 242784.[8] Each bone resorption inhibitor proved to potently inhibit medial artery calcification in both of the rat models tested: rats in which the calcification-inhibitory activity of matrix Gla protein (MGP) was removed by treatment with warfarin and rats treated with high doses of vitamin D. Ibandronate, osteoprotegerin, and SB 242784 are each highly specific inhibitors of the osteoclast at the concentrations used in these studies, and have no known effects on vascular cells. Their ability to potently inhibit artery calcification, therefore, strongly supports the hypothesis that medial artery calcification is linked to bone resorption.

Calcification of the elastic lamellae of the artery media is common in patients with chronic kidney disease and causes adverse hemodynamic changes.[9] (Increased calcification of atherosclerotic plaques is also observed in uremic patients.[9]) Arterial medial calcification is also a strong prognostic marker of all-cause and cardiovascular mortality in hemodialysis patients, independent of classical atherogenic factors.[10] A continuing goal of our research has accordingly been to test the hypothesis that bone resorption inhibitors can prevent medial artery calcification in chronic kidney disease, and we have sought for a number of years to develop a rat model of uremia that causes consistent and extensive calcification of the artery media. We initially tried different

permutations of the 5/6 nephrectomy procedure in the rat, but the serum creatinine elevations achieved by this procedure were only about threefold above normal, artery calcification was observed in less than 20% of the animals, and the calcification was never extensive (personal observations). We next induced uremia by feeding rats for 4 weeks with a diet supplemented with 0.75% adenine, a procedure shown previously to elevate serum creatinine by about sevenfold and to induce medial artery calcification.[11,12] Because it proved difficult to import the natural diet used in these earlier studies, we developed a synthetic, adenine-supplemented diet that mimicked the composition of the natural diet used in these earlier studies. Rats fed for 4 weeks with this synthetic diet did have the expected increase in serum creatinine, but artery calcification was detected in only about 30% of the animals.

We reasoned that consistent artery calcification might require a longer period of feeding an adenine-rich diet. In an attempt to reduce the nitrogen excretion load and so enable rats to thrive on the adenine-rich diet for periods longer than 4 weeks, we designed a diet that contained 2.5% protein rather than the 25% protein in the original synthetic diet. Previous studies have shown that rats fed a 2.5% protein diet can maintain body mass but cannot grow.[13,14] In pilot studies, we found that all rats fed the diet containing 2.5% protein and 0.75% adenine unexpectedly had extensive medial calcification within just 4 weeks; longer periods of feeding this diet were therefore not pursued.

The goal of the present experiments was to further investigate the effect of protein content on medial artery calcification in rats fed an adenine-rich diet for 4 weeks, and to determine the effect of treatment with a bone resorption inhibitor on this calcification. Arteries were examined for calcification by Alizarin red staining of the aortas and associated arteries, by von Kossa staining of carotid artery sections, and by extraction and quantitative analysis of calcium and phosphate incorporated into the artery wall. Serum from these rats was analyzed for typical measures of uremia as well as for three proteins whose serum levels are known to be associated with bone metabolism or artery calcification: bone Gla protein (BGP; osteocalcin), MGP, and fetuin (called α2-HS glycoprotein in humans). BGP is the most abundant non-collagenous bone protein and increased serum BGP levels are a marker for increased bone turnover in patients with the high bone turnover type of renal osteodystrophy.[15,16] MGP is a calcification inhibitor secreted by vascular and other cell types and deficiencies in MGP are associated with medial artery calcification in mice,[17] rats,[18] and humans.[19] There is evidence that increased serum MGP levels may be a marker for increased medial artery calcification in rats treated with high doses of vitamin D.[3] Fetuin is a major serum inhibitor of apatite mineral formation,[20–22] and there is evidence that decreased serum fetuin may be a marker for increased artery calcification in rats treated with high doses of vitamin D[6] and in patients with chronic kidney disease.[23–25]

RESULTS

Effect of dietary protein content on medial artery calcification in uremic rats

In order to assess the effect of dietary protein on medial artery calcification in uremic rats, 13-week-old adult rats were fed for 4 weeks with a diet that contained 0.75% adenine and either 25% protein (11 rats) or 2.5% protein (13 rats). Over the course of this 4-week experiment, the rats fed the 25% protein lost 27.6±4.9% of their initial weight and the rats fed the 2.5% protein lost 31.0±3.1% of their initial weight; the difference in weight lost between the two treatment groups was not statistically significant. A similar weight loss has been seen previously in rats fed a diet containing 0.75% adenine for 4 weeks.[26]

The results of this experiment show that the protein content of the diet has a major impact on artery calcification in rats fed a 0.75% adenine diet for 4 weeks. Figure 1 shows

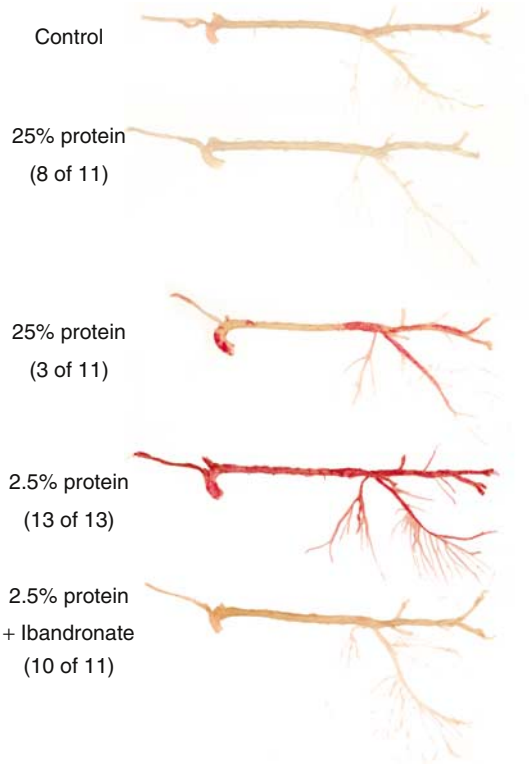

Figure 1 | Evidence that a 2.5% protein diet exacerbates artery calcification in uremic rats and that treatment with ibandronate prevents this calcification: Alizarin red staining of aortas. Thirteen-week-old adult male rats were fed a synthetic diet containing 0.75% adenine for 4 weeks in order to induce uremia. The diet for 11 rats contained 25% protein, whereas the diet for 24 rats contained 2.5% protein. Eleven animals in the 2.5% protein group were injected daily with the bone resorption inhibitor ibandronate at a dose of 0.25 mg/kg (see Materials and Methods). Twelve rats of the same age served as controls. The figure shows the Alizarin red S staining of the aorta from a representative animal in each treatment group, with the aorta oriented so that the femoral bifurcation is on the right, a carotid artery on the left, and the celiac and mesenteric arteries on the bottom. Note the complete absence of Alizarin red staining in the arteries of rats fed the adenine diet with 2.5% protein and treated daily with ibandronate.

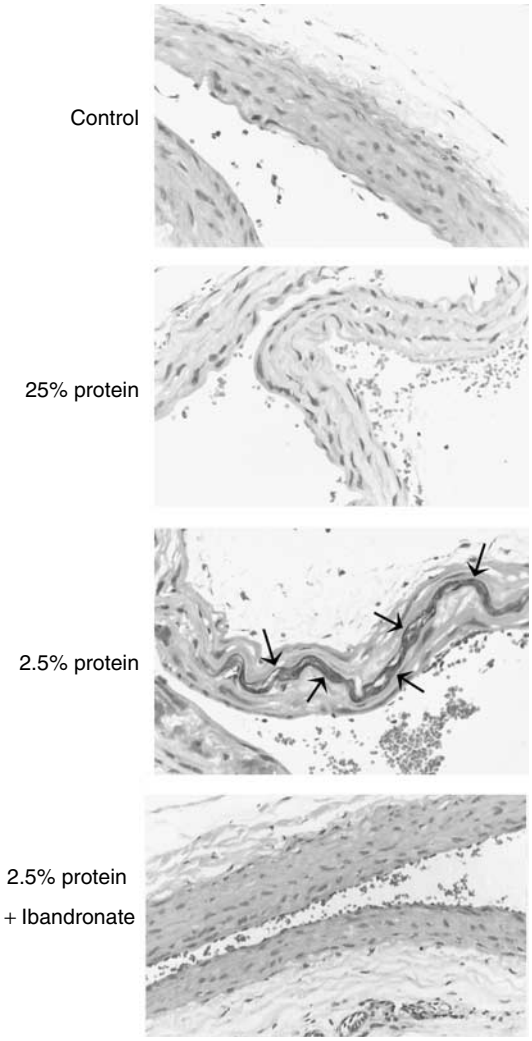

Figure 2 | Evidence that a 2.5% protein diet exacerbates artery calcification in uremic rats and that treatment with ibandronate prevents this calcification: von Kossa staining of carotid arteries. In the experiment described in Figure 1, one carotid artery from each rat was removed immediately after the rats were killed, fixed in 10% buffered formalin, and cross-sections of each artery were stained for calcification with von Kossa (stains calcification brown to black) and counter-stained with nuclear fast red. The panels illustrate the typical level of medial calcification seen in the carotid artery of the 13 uremic animals fed the 2.5% protein diet, and the absence of calcification in the carotid artery of the 12 control animals, in the artery of eight of the 11 uremic animals fed a 25% protein diet, and in the artery of the 11 uremic animals fed the 2.5% protein diet and treated with ibandronate. The three uremic rats fed the 25% protein diet whose arteries stained with Alizarin red (Figure 1) also had von Kossa staining for calcification in the carotid artery (not shown). The arrows indicate sites of calcification of the elastic lamellae in the uremic rats fed the 2.5% protein diet.

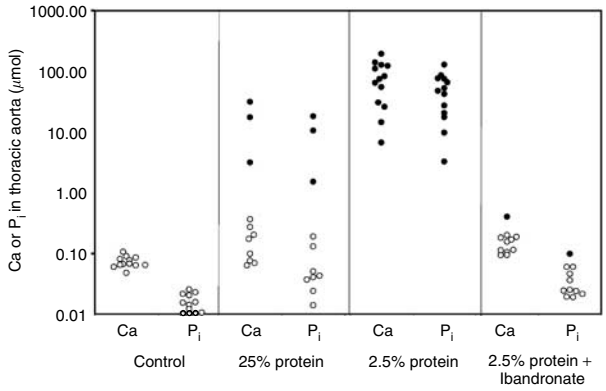

Figure 3 | Evidence that a 2.5% protein diet exacerbates artery calcification in uremic rats and that treatment with ibandronate prevents this calcification: calcium and phosphate levels in thoracic aortas. A 4 cm segment of the thoracic aorta from each animal in the experiment described in Figure 1 was analyzed for calcium and phosphate (see Materials and Methods). Each point in the figure shows the level of calcium or phosphate found in a single thoracic segment. Closed symbols (●) denote values obtained from thoracic aortas that stained with Alizarin red; open symbols (○) denote values obtained from thoracic aortas that did not stain.

that all 13 rats fed the 2.5% protein diet had uniform Alizarin red staining in the aorta and in the associated arteries. In contrast, only three of the 11 rats fed the 25% protein diet had evidence of Alizarin red staining for calcification, and in these rats staining involved no more than half of the artery wall (Figure 1). Figure 2 shows that the rats fed the 2.5% protein diet also had consistent von Kossa staining in the elastic lamellae of the carotid artery media. In contrast, only the three rats in the 25% protein group that had Alizarin red staining also had von Kossa staining in the carotid artery media (not shown). Figure 3 shows that the rats fed the 2.5% protein diet all have an approximately 1000-fold elevation of calcium and phosphate in the artery wall compared to age-matched control rats ($P<0.001$). In contrast, only the three rats in the 25% protein group that had Alizarin red staining had statistically significant elevations of calcium and phosphate in the artery wall (Figure 3). Taken together, these data demonstrate that a 2.5% protein diet dramatically increases the frequency with which artery calcification is observed in uremic rats compared to the calcification frequency seen in rats fed the 25% protein diet.

Serum measures of uremia in rats fed a diet containing adenine and either 2.5 or 25% protein are shown in Table 1. Both diets produced a comparable increase in serum levels of phosphate, creatinine, and parathyroid hormone (PTH), and a comparable decrease in serum albumin. Both diets also dramatically elevated serum blood urea nitrogen, but the extent of elevation was greater in the rats fed 25% protein, as expected. Total and corrected serum calcium levels were normal in the rats fed the diet containing adenine and 25% protein, and slightly reduced in the rats fed the diet containing adenine and 2.5% protein ($P\leqslant0.01$).

Three serum markers of bone metabolism and/or artery calcification were measured to determine if any marker correlated with the extent of artery calcification. As seen in Table 2, serum BGP correlates with artery calcification in the uremic rat, with a twofold elevation in the rats fed the 25% protein diet compared with an eightfold elevation in rats fed the 2.5% diet. Serum fetuin and MGP levels were both dramatically affected by uremia and by the protein content of

Table 1 | Serum biochemical measurements

Serum measurement	Control (n=12)	25% Protein (n=11)	2.5% Protein (n=13)	2.5% Protein+ibandronate (n=11)
Calcium (mM)	2.6±0.1	2.4±0.2[a]	2.0±0.3[b,c]	1.7±0.2[b,c,d]
Albumin (g/dl)	3.9±0.3	3.5±0.3[b]	3.2±0.4[b]	3.4±0.3[b]
Total protein (g/dl)	5.9±0.5	5.6±0.5	5.0±0.7[b,e]	5.3±0.6[f]
Corrected Ca (mM)	2.6±0.2	2.5±0.2	2.2±0.2[b,c]	1.9±0.2[b,c,g]
Phosphate (mM)	2.5±0.2	4.8±0.8[b]	5.6±1.1[b]	5.8±1.1[b,e]
Creatinine (mg/dl)	0.4±0.1	3.7±0.8[b]	4.2±0.8[b]	4.1±0.7[b]
BUN (mg/dl)	9.4±5.5	187.8±23.8[b]	130.7±40.5[b,c]	105.1±35.4[b,c]
PTH (pg/ml)	64.7±11.6	1777.0±667.9[b]	2218.2±267.1[b]	2554.8±1054.0[b]

BUN, blood urea nitrogen; PTH, parathyroid hormone.
Blood was obtained from rats in each of the four indicated treatment groups 4 weeks after rats had been placed on to an adenine-containing diet in order to induce uremia (see Figure 1). Blood was allowed to clot for 30 min at room temperature; serum was obtained by centrifugation, and stored at −70°C until analyzed. The values presented are the mean ±s.d. For the measurement of PTH, n=6 for the control group, n=5 for the 25% protein group, n=6 for the 2.5% protein group, and n=5 for the 2.5% protein+ibandronate group. Corrected calcium is calcium normalized for differences in serum albumin.[27,28]
[a]$P<0.01$ compared to value for control.
[b]$P<0.001$ compared to value for control.
[c]$P<0.001$ compared to value for 25% protein.
[d]$P<0.01$ compared to value for 2.5% protein.
[e]$P<0.05$ compared to value for 25% protein.
[f]$P<0.025$ compared to value for control.
[g]$P<0.001$ compared to value for 2.5% protein.

Table 2 | Levels of calcium and phosphate in thoracic aorta segments and serum levels of bone-associated proteins

Measurement	Control (n=12)	25% Protein (n=11)	2.5% Protein (n=13)	2.5% Protein+ibandronate (n=11)
Thoracic aorta Ca (μmol)	0.07±0.02	4.83±10.16	80.59±53.69[a,b]	0.17±0.09[a,c]
Thoracic aorta PO_4 (μmol)	0.02±0.01	2.79±5.96	49.99±34.24[a,b]	0.04±0.02[c,d]
Serum BGP (ng/ml)	185.6±44.8	530.9±281.9[a]	1620.2±993.9[a,b]	266.6±130.5[c,e]
Serum MGP (ng/ml)	198.8±25.4	1216.0±185.2[a]	926.4±120.7[a,b]	1882.6±404.3[a,b,c]
Serum fetuin (mg/ml)	0.89±0.06	0.14±0.06[a]	0.38±0.07[a,b]	0.12±0.05[a,c]

BGP, bone Gla protein; MGP, matrix Gla protein.
Blood was obtained from rats in each of the four indicated treatment groups 4 weeks after rats had been placed on to an adenine-containing diet in order to induce uremia (see Figure 1). Blood was allowed to clot for 30 min at room temperature; serum was obtained by centrifugation, diluted into RIA diluent, and stored at −70°C until analyzed. The values presented are the mean ±s.d. For the measurement of fetuin, n=6 for the control group, n=5 for the 25% protein group, n=6 for the 2.5% protein group, and n=5 for the 2.5%+ibandronate group. For the measurement of serum MGP, n=6 for the control group.
[a]$P<0.001$ compared to value for control.
[b]$P<0.001$ compared to value for 25% protein.
[c]$P<0.001$ compared to value for 2.5% protein.
[d]$P<0.01$ compared to value for control.
[e]$P<0.025$ compared to value for 25% protein.

the diet, but these changes did not correlate with the extent of artery calcification.

To further evaluate the possible association between serum BGP and artery calcification in uremic rats, serum BGP levels in each of the 13 rats fed adenine and 2.5% protein were plotted against the calcium level found in the thoracic aorta of the same rat. As can be seen in Figure 4, there is a strong positive correlation between serum BGP levels measured at the time of death and the level of calcium in the artery wall ($r=0.81$; $P<0.001$). A strong, positive correlation was also found between serum BGP and the level of phosphate in the artery wall (not shown).

Effect of ibandronate treatment on medial artery calcification in uremic rats

In order to assess the effect of inhibiting bone resorption on medial artery calcification in uremic rats, 11 rats were fed for 4 weeks with a diet that contained 2.5% protein and 0.75% adenine and treated daily with ibandronate at a dose previously shown to inhibit bone resorption and prevent artery calcification in other rat models.[1,3,5,6] As noted above, all rats in the group that did not receive ibandronate had extensive Alizarin red and von Kossa staining for calcification (Figures 1 and 2) and a dramatic, 1000-fold increase in calcium and phosphate in the artery wall (Figure 3). In contrast, 10 of the 11 rats treated with ibandronate had no evidence of Alizarin red staining for calcification (Figure 1), while one rat had a small region of Alizarin red staining in the thoracic aorta (not shown). None of the 11 rats treated with ibandronate had evidence of von Kossa staining in carotid artery sections (Figure 2), and all of the rats treated with ibandronate had a 100- to 1000-fold reduction in calcium and phosphate in the artery wall compared to uremic rats that were not treated with ibandronate (Figure 3). These data show that ibandronate treatment prevents medial artery calcification in 10 of 11 rats, and dramatically reduces calcification in one rat.

The effect of ibandronate treatment on serum measures of uremia are shown in Table 1. Ibandronate treatment did not significantly affect the increase in serum levels of phosphate, creatinine, blood urea nitrogen, or PTH in the uremic rat. Ibandronate treatment did, however, reduce serum calcium

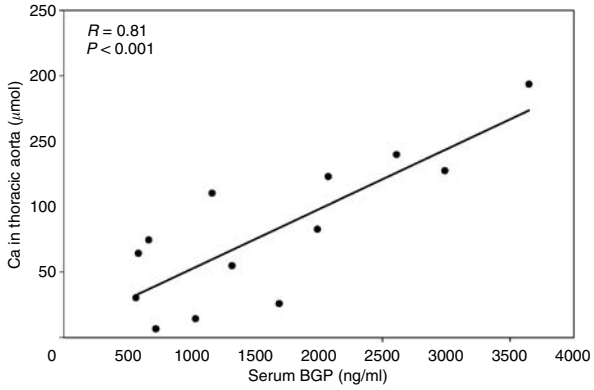

Figure 4 | Correlation between thoracic aorta calcium levels and serum BGP levels in uremic rats. Serum samples obtained from each of the 13 uremic rats that were fed the 2.5% protein diet but were not treated with ibandronate (see Figure 1) were assayed for BGP by radioimmunoassay (see Materials and Methods). Serum BGP is plotted against the calcium level found in the thoracic aorta of the same rat.

by about 15% ($P \leqslant 0.01$). The effect of ibandronate treatment on three serum markers of bone metabolism and/or artery calcification were measured to determine if any marker correlated with the extent of artery calcification. As seen in Table 2, only serum BGP levels correlate with artery calcification in rats treated with ibandronate.

DISCUSSION

The data presented here support three conclusions concerning medial artery calcification in the adenine-fed rat model of end-stage renal disease: (1) Lowering the protein content of the diet from 25 to 2.5% dramatically increases the frequency and extent of medial artery calcification in uremic rats without significantly affecting the elevation in serum creatinine, phosphate, or PTH. (2) Medial artery calcification in rats fed adenine and a 2.5% protein diet is prevented by a dose of ibandronate that has been previously shown to inhibit bone resorption and medial artery calcification in two other rat artery calcification models.[1,3,5,6] (3) Elevated serum levels of BGP, a marker for bone turnover in uremic patients,[15,16] are strongly correlated with medial artery calcification in rats fed adenine and a 2.5% protein diet.

Each of these three conclusions supports the hypothesis that there is a relationship between bone resorption and medial artery calcification in end-stage renal disease. (1) Low dietary protein has been demonstrated to increase bone resorption, leading to progressive loss of bone and augmenting bone loss in osteoporosis.[29] Low dietary protein would therefore be expected to augment the increase in bone resorption caused by elevated serum PTH in the uremic rat.[11] The fact that low dietary protein dramatically increases artery calcification therefore supports the existence of a link between bone resorption and medial artery calcification. (2) Amino bisphosphonates such as ibandronate bind to bone mineral, are taken up by the osteoclast during the process of bone resorption, and specifically inhibit farnesyl diphosphate synthase in the osteoclast, thereby inhibiting the bone resorption activity of the cell. The dose of ibandronate used in this study has been shown to inhibit bone resorption in the rat,[1,3,5] and there is also evidence that ibandronate treatment prevents hyperparathyroid bone changes in rats with mild renal failure.[30] The ability of ibandronate to prevent medial artery calcification in the uremic rat therefore supports a link between bone resorption and medial artery calcification. (Because of the low ibandronate doses used in these studies, it is unlikely that ibandronate prevents artery calcification by directly inhibiting mineralization (see Price *et al.*[1] and references therein), as pyrophosphate and etidronate are known to do. The ultimate test of mechanism will be to verify that other agents that inhibit bone resorption, such as osteoprotegerin, are also able to inhibit artery calcification in the uremic rat.) (3) Elevated serum BGP has been shown to correlate with increased bone turnover in patients with end-stage renal disease.[15,16] We believe that the elevation in serum BGP observed in the adenine-fed, uremic rat likewise arises from increased bone turnover, and that ibandronate treatment lowers serum BGP by inactivating osteoclastic bone resorption and thereby inhibiting bone turnover. It should be noted that the present study did not evaluate the effects of a low protein diet or of ibandronate on bone resorption, and that future studies will be needed to confirm that the predicted effects of these treatments on bone metabolism are actually observed in rats with adenine-induced uremia.

Our working hypothesis is that the impetus for medial artery calcification in the uremic rat begins with the elevation in serum phosphate. Genetic studies in mice have shown that increases in serum phosphate are associated with ectopic calcification in mice lacking MGP or Ank genes,[31] and our recent studies of the serum calcification factor that initiates the calcification of the elastic lamellae in devitalized arteries demonstrates that serum phosphate has a direct, biochemical effect on the ability of the serum calcification factor to initiate ectopic mineralization.[32] Elevated serum phosphate also elevates serum PTH in the uremic rat, and elevated serum PTH in turn accelerates bone resorption. In our view, this PTH-stimulated increase in bone resorption is a major driver of medial arterial calcification in the uremic rat.

The biochemical mechanism for the putative linkage between increased bone resorption and increased medial artery calcification in uremia is presently unclear. It is possible that an agent (or agents) responsible for calcification of the elastic lamellae of the artery media arises from sites of bone turnover, travels in blood, and initiates calcification within the artery media. This agent could directly initiate calcification in the artery, a hypothesis supported by the recent discovery that serum contains a macromolecular causative agent that initiates the calcification of the elastic lamellae of devitalized arteries.[32] Alternatively, this agent could be a signaling molecule that arises from sites of bone turnover, travels in blood, and initiates medial artery

calcification by virtue of a direct action on vascular smooth muscle cells in the artery wall.[33–35] These and other possible explanations for the apparent linkage between bone resorption and medial artery calcification in rats fed the adenine-rich, low protein diet merit future study. Many of these future investigations of medial artery calcification in the rat model of end-stage renal disease will be facilitated by the present discovery that a synthetic, 2.5% protein diet containing 0.75% adenine produces consistent and dramatic medial calcification in adult rats within 4 weeks.

Each of the three conclusions of this animal study have potential implications for the clinical management of uremic patients: (1) Low dietary protein intake could be a risk factor for medial artery calcification in the uremic patient. (2) Treatment with a bone resorption inhibitor such as the amino bisphosphonate ibandronate could prevent medial artery calcification in uremic patients. (It should be noted, however, that treatment of uremic patients with a closely related amino bisphosphonate, pamidronate, is associated with the development of adynamic bone disease,[36] and that there is a recent report that adynamic bone disease is associated with increased arterial calcification in uremic patients.[37]) (3) Increased serum levels of BGP, but not MGP or fetuin, may correlate with the initiation of medial artery calcification in the uremic patient, and reduction in serum BGP could provide a marker for monitoring treatment of uremic patients with ibandronate.

MATERIALS AND METHODS

Materials

Ibandronate (Bondronat, Boehringer Mannheim, Germany) was purchased from Idis World Medicines (Surrey, UK); an ibandronate solution was prepared with 0.15 M NaCl and stored at 4°C. Adenine (Acros Organics, Geel, Belgium) was purchased from Fisher Scientific (Hampton, NH, USA). Four different isocaloric synthetic diets (350 kcal/100 g) were purchased from Harlan Teklad (Madison, WI, USA), each containing 4.7% fat, 5% cellulose, 1.06% calcium, and 0.92% phosphorus. One diet contained 2.5% protein (casein) and 75.3% carbohydrate (TD05030); a second contained 2.5% protein, 74.6% carbohydrate, and 0.75% adenine (TD05031); a third contained 50.3% carbohydrate and 25.4% protein (casein) (TD04512); and a fourth contained 49.6% carbohydrate, 25.4% protein, and 0.75% adenine (TD04513). Thirteen-week-old male rats (Sprague–Dawley derived) were purchased from Harlan Sprague–Dawley (San Diego, CA, USA).

Treatment of rats

Rats were fed the diets containing 0.75% adenine and either 2.5 or 25% protein for 4 weeks *ad libitum*. In order to assess the effect of ibandronate on vascular calcification, a subset of the rats fed the 2.5% protein diet received once daily subcutaneous injections of ibandronate at a dose of 0.25 mg/kg body weight/day. Ibandronate injections began 12 days after the start of the adenine diet, a time at which there was no detectable vascular calcification (personal observations). The average initial weight of the adenine-fed rats was 371.4 ± 8.4 g, and the weight loss over the 4-week experiment was: $27.6 \pm 4.9\%$ (adenine/25% protein); $31.0 \pm 3.1\%$ (adenine/2.5% protein); and $29.6 \pm 3.7\%$ (adenine/2.5% protein/ibandronate).

Animals were killed by exsanguination while under ether anesthetic and blood was allowed to clot for 30 min at room temperature. Serum was collected by centrifugation at 1400 *g* for 10 min. An aliquot of serum was immediately diluted into radioimmunoassay diluent[38] and stored at −70°C until analysis of BGP, MGP, and fetuin by radioimmunoassay. The remaining serum was stored in 0.5 ml aliquots at −70°C until use. The UCSD Animal Subjects Committee approved all animal experiments.

Methods

The right carotid artery, the thoracic and abdominal aortas, and portions of the pulmonary, mesenteric, hepatic, renal, celiac, and femoral arteries from each rat were dissected as a unit within 30 min of the animal's death, stained with 0.0016% (wt:vol) Alizarin red S in 0.5% KOH for 24 h, destained in 0.05% KOH, and photographed. For histological analyses, the left carotid artery was fixed in 10% buffered formalin for at least 1 day at room temperature, and San Diego Pathologists (San Diego, CA, USA), sectioned and von Kossa stained the artery. For quantitative assessment of calcification, a 4 cm segment of each thoracic aorta (beginning at the aortic arch and moving distally) was patted dry with a paper towel, placed in a 5 ml tube, and extracted by end-over-end mixing with 2 ml of 0.15 M HCl for 24 h at room temperature. Calcium levels in serum and in the acid extracts of tissues were determined colorimetrically using cresolphthalein complexone (JAS Diagnostics, Miami FL, USA) and phosphate levels were determined colorimetrically as described.[39]

Serum creatinine and blood urea nitrogen values were determined by the clinical laboratory at the UCSD Medical Center using a Beckman-Coulter LX-20 PRO serum/plasma blood analyzer. Serum albumin and total protein were determined by the Comparative Pathology Lab at UC Davis using a COBAS MIRA Plus analyzer (Roche Diagnostic Systems, Indianapolis, IN, USA). Serum samples were analyzed to determine the levels of BGP, MGP, and fetuin using specific radioimmunoassays developed in our laboratory.[38,40,41] Serum levels of PTH were determined using a rat intact PTH enzyme-linked immuno sorbent assay (ELISA) assay (Immutopics, San Clemente, CA, USA).

Statistical analysis

All quantitative data are presented as mean ± s.d. Differences between groups were analyzed by the Student's *t*-test. Differences with $P < 0.05$ were accepted as significant.

ACKNOWLEDGMENTS

We gratefully acknowledge Dawn Jolson's contributions to the discovery that low dietary protein increases artery calcification in uremic rats.

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