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High calcium diet down-regulates kidney angiotensin-converting enzyme in experimental renal failure

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High calcium diet down-regulates kidney angiotensin-converting enzyme in experimental renal failure.

Background. Calcium salts are used as phosphate binders in renal failure, while high calcium diet also improves vasorelaxation and enhances natriuresis. The influences of calcium intake on renal renin-angiotensin system (RAS) are largely unknown.

Methods. Four weeks after NTX, rats were put on 3.0% or 0.3% calcium diet for 8 weeks (12-week study). In additional experiments, 15 weeks after NTX, rats were put on similar diets for 12 weeks (27-week study). Appropriate blood, urine, and kidney samples were taken. Renal angiotensin-converting enzyme (ACE) and angiotensin II receptors (AT_1, AT_2) were examined using autoradiography, ACE also using Western blotting, and connective tissue growth factor (CTGF) using immunohistochemistry.

Results. In the 12-week study, albuminuria increased 5-fold in NTX rats, but only 2-fold in calcium NTX rats on 3.0% calcium. In the 27-week study, high calcium intake decreased blood pressure, retarded progression of renal failure, reduced glomerulosclerosis, interstitial damage, and aortic calcifications, and improved survival from 50% to 92% in NTX rats. In both experiments plasma parathyroid hormone and phosphate were elevated after NTX, and suppressed by high calcium diet, while kidney ACE was down-regulated by 40% or more after increased calcium intake. In the 27-week study renal CTGF was decreased and cortical AT_1 receptor density reduced after high calcium diet.

Conclusion. High calcium diet down-regulated kidney ACE, reduced albuminuria and blood pressure, and favorably influenced kidney morphology in experimental renal failure. These findings suggest a link between calcium metabolism and kidney ACE expression, which may play a role in the progression of renal damage.

Key words: angiotensin-converting enzyme, calcium diet, phosphate, parathyroid hormone, renal failure.

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Chronic renal failure (CRF) is associated with increased cardiovascular morbidity and mortality, the underlying causes of which include anemia, acidosis, hypertension, volume overload, and accumulation of uremic toxins [1, 2]. Changes in calcium-phosphate balance, especially hyperphosphatemia, are also closely associated with the decline of kidney function, ectopic calcification, and reduced survival in renal patients. Disturbed calcium-phosphorus balance is a major cause for cardiovascular complications during impaired kidney function [1, 2].

Phosphate retention, hypocalcemia, and reduced vitamin D levels lead to the development of secondary hyperparathyroidism in CRF [3]. Elevated plasma phosphate and parathyroid hormone (PTH) can be suppressed by dietary phosphate restriction, but oral phosphate binders, most commonly calcium salts, are usually required to manage hyperphosphatemia and hyperparathyroidism during chronic kidney disease [3].

In experimental hypertension high calcium diet has consistently reduced blood pressure [4, 5]. High calcium intake has also decreased plasma renin activity (PRA) [6], reduced angiotensin II (Ang II) binding sites in apical brush-border membrane of renal cortex [7], and increased sodium excretion in spontaneously hypertensive rats [8, 9]. The mechanisms of the increased natriuresis by high calcium intake are not well established, but in addition to suppression of plasma renin, stimulation of calcium-sensing receptors in nephrons may play a role, with the subsequent reduction in sodium reabsorption [10]. Recently, high dietary calcium intake was reported to normalize the disturbances in calcium homeostasis due to vitamin D deficiency by regulating the expression of renal calcium transport protein genes, but increased calcium intake also influenced the expression of several other genes that were not playing a role in calcium handling [11].

The renin-angiotensin system (RAS) is a major regulator of sodium metabolism, renal function, arterial tone,

and blood pressure. Expression of human angiotensinogen in the kidneys of mice results in hypertension in the absence of changes in systemic Ang II [12]. Local intrarenal RAS is considered to play an important role in the progression of kidney diseases [13].

The influences of increased calcium intake on blood pressure, vascular tone, and natriuresis in experimental animals resemble those effects that are obtained by inhibition of the actions of RAS. However, limited data is available about the effects of high calcium diet on RAS and progression of renal damage. In this study, we tested the hypothesis whether increased calcium intake could beneficially influence the renal components of RAS in rats subjected to NTX. The present results show that, in addition to controlling plasma phosphate and PTH, increased calcium intake down-regulates kidney angiotensin-converting enzyme (ACE) in experimental CRF.

METHODS

Animals and experimental design

Male Sprague-Dawley rats were used ($N = 76$) with free access to water and food pellets (Lactamin R34; AnalyCen, Lindköping, Sweden). The chow contained 0.9% calcium, 0.8% phosphorus, 0.27% sodium, 0.2% magnesium, 0.6% potassium, 12,550 kJ/kg energy, 16.5% protein, 4.0% fat, 58% nitrogen-free extract, 3.5% fiber, 6.0% ash, and 10% water. The vitamin contents were (IU/kg) 1500 vitamin D, 12,000 vitamin A, and (rest in mg/kg) 63 vitamin E, 0.25 vitamin K_1, 10 vitamin K_3, 3 vitamin B_1, 12 vitamin B_2, 4 vitamin B_6, 0.02 vitamin B_{12}, 10 pantotenic acid, 1000 choline chloride, and 40 niacin. The trace elements were (mg/kg) 30 copper, 190 iron, 100 manganese, 110 zinc, 2 iodine, and 1 cobalt. Surgery was performed at 8 weeks of age; NTX was carried out by removal of upper and lower poles of the left kidney, and the whole right kidney [14, 15], while sham-operation was performed by kidney decapsulation. Anesthesia, antibiotics, postoperative pain relief, and measurement of systolic blood pressure by tail-cuff were as previously reported [14, 15].

In the 12-week study, 4 weeks after NTX (rat age 12 weeks), the animals were divided into four groups so that systolic blood pressures and body weights in the sham and calcium-sham, and NTX and calcium-NTX groups, respectively ($N = 9$ to 10 in each) were similar. The sham and NTX rats were switched to chow containing 0.3% calcium, while calcium-sham and calcium-NTX rats continued on 3% calcium. Extra calcium was supplied as carbonate (AnalyCen).

In the 27-week study, 15 weeks after NTX (rat age 23 weeks), the rats were divided into two groups (NTX and calcium-NTX) so that systolic blood pressures, body weights, plasma creatinine (tail vein sampling at week 13), and 24-hour urine volumes were similar ($N = 13$ to 14). Eleven sham-operated rats served as controls. The sham and NTX rats continued on 0.3%, and calcium-NTX rats on 3% calcium (AnalyCen). These calcium diets were chosen, since we have observed clear differences in calcium metabolism after 0.3% versus 3.0% calcium intake in rats, in the absence of adverse effects that would indicate calcium deficiency or excess [14].

High calcium diets continued for 8 weeks (12-week study) or 12 weeks (27-week study), and 24-hour fluid consumption and urine output were measured during the last study weeks. The rats were anaesthetized (urethane 1.3 g/kg), and blood samples from cannulated carotid artery were drawn with ethylenediaminetetraacetic acid (EDTA) and heparin as anticoagulants, as appropriate. The hearts and the kidneys were removed and weighed. In the 12-week study, a kidney half from four rats per group, and in the 27-week study a kidney half and a standard section of the thoracic aorta from all rats, were fixed in 4% formaldehyde for 24 hours, and embedded in paraffin. The remaining kidney pieces were snap-frozen in isopentane at -40°C and stored at -80°C. The experimental design of the study was approved by the Animal Experimentation Committee of the University of Tampere, and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland. The investigation conforms to the Guiding Principles for Research Involving Animals.

In vitro autoradiography of renal ACE and Ang II receptors

Quantitative in vitro autoradiography of ACE and Ang II receptors (AT_1 and AT_2) was performed on 20 μm thick tissue sections with the radioligands [^{125}I]-MK351A and [^{125}I]-Sar1, Ile8-Ang II, respectively, as described earlier [16, 17]. The density of AT_1 receptors was determined in the presence of the AT_2 antagonist PD 123,313 (10 μmol/L), and the density of AT_2 receptors in the presence of the AT_1 antagonist losartan (10 μmol/L). The optical densities were quantified by image analyzing system (AIDA two-dimensional densitometry) coupled to the FujiFilm BAS-5000 phosphoimager (Tamro, Finland).

Western blotting of renal ACE

Frozen tissues (100 mg) were lysed in 1 mL of sodium dodecyl sulfate (SDS) buffer, pH 7.4, containing 10 mmol/L Tris-HCl, 2% SDS, and protein inhibitors (Complete™ Mini EDTA-free; Roche Diagnostics, Mannheim, Germany). Debris was removed by centrifugation (10,000*g*, 15 minutes), and protein concentrations were determined (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Aliquots containing 50 μg of protein in loading buffer (10% glycerol, 2% SDS,

60 mmol/L Tris-HCl, pH 6.8, 0.01% bromophenol blue, and 100 mmol/L dithiothreitol) were boiled for 5 minutes before electrophoresis on 12% SDS-polyacrylamide gels. The proteins were electrophoretically transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) in 25 mmol/L Tris-HCl, pH 8.0, 192 mmol/L glycine, and 20% methanol at 50 V overnight. After washing in H_2O and Tris-buffered saline with Tween (TBS-T) (20 mmol/L Tris-HCl, pH 7.6, 136 mmol/L NaCl, and 0.3% Tween-20), membranes were blocked in 5% nonfat milk powder in TBS-T at room temperature for 1 hour, and incubated for 3 hours with goat polyclonal antibody against rat ACE (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:200 in 5% milk in TBS-T buffer. After washing with 2.5% milk/TBS-T buffer, membranes were incubated with 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG for 1 hour (Sigma-Aldrich Co., St. Louis, MO, USA). Antibody binding was detected by chemiluminescense (WB Chemiluminescent Reagent Plus; NEN, Inc., Boston, MA, USA), and the autoradiograph was analyzed with Image Gauge 3.3 software (Fuji Photo Film Co., Tokyo, Japan).

Kidney and aortic morphology, connective tissue growth factor (CTGF) immunohistochemistry

Five micrometer-thick kidney sections were stained with hematoxylin-eosin and periodic acid-Schiff (PAS), von Kossa, or immonohistochemistry, and processed for light microscopic evaluation. An expert who was blinded to the treatments quantified all kidney tissue histology.

Glomerulosclerosis (hematoxylin-eosin and PAS stain). A score for each animal was derived as the mean of 100 systematically examined glomeruli at a magnification of ×400: 0, normal; 1, mesangial expansion or thickening of basement membrane; 2, segmental sclerosis involving <50% of the tuft; 3, diffuse sclerosis involving >50% of the tuft; and 4, diffuse glomerulosclerosis, total tuft obliteration and collapse [18].

Tubulointerstitial damage (hematoxylin-eosin and PAS stain). A scoring system was applied (from 0 to 4), in which tubular atrophy, dilation, casts, interstitial inflammation, and fibrosis were assessed in 10 kidney fields at a magnification of ×100: 0, normal; 1, lesions in <25% of the area; 2, lesions in 25% to 50% of the area; 3, lesions in > 50% of the area; and 4, lesions involving the entire area [18].

Calcification (von Kossa stain). Calcifications were counted from 10 random kidney sections at ×200 magnification. Each field was divided into 100 grids, and each grid containing foci of calcification denoted one score. Total scores of each field were counted. The index of calcification was determined for each rat by the mean score of the calculated 10 fields. Calcifications were also measured from aortic sections at ×200 magnification. The total area of each aortic section, and area of calcification, was measured by a computerized interactive system (Scion Image Beta 4.02, Frederick, MD, USA). The index of calcification for each rat was expressed as percentage of the calcified area related to the total area of the aortic cross-section.

Immunohistochemistry of CTGF. Five micrometer-thick kidney samples were processed as described previously [19]. The samples were incubated in blocking serum, and primary polyclonal antibody against mouse CTGF that cross-reacts with rat CTGF (ab6992, 1:400) (Abcam, Cambridge, UK) was applied for 60 minutes at room temperature. Then the slides were incubated for 30 minutes with biotinylated secondary antibody (antirabbit IgG; Vector Laboratories, Burlingame, CA, USA), and for 30 minutes with peroxidase-labeled biotin-avidin-complex using a commercial Elite ABC kit (Vector Laboratories). The color reaction was developed by incubation for 15 minutes in a 3-amino-9-ethyl carbazole solution containing hydrogen peroxide. Finally, the sections were counterstained with Mayer's hemalum and mounted. Negative controls were treated with blocking serum with and without nonspecific IgG instead of the primary antibody. Positive CTGF label in tissue was scored from 0 to 3 using light microscope [19].

PRA, electrolytes, phosphate, creatinine, urea nitrogen, protein, hemoglobin, $1,25(OH)_2D_3$, PTH, creatinine clearance, and urine albumin

PRA was determined by radioimmunoassay (Ang I RIA Kit; Diasorin S.p.a., Saluggia, Italy) according to the manufacturer's instructions. Urine albumin was determined by nephelometry (Behring Nephelometer 100 analyzer; Behringwerke, Marburg, Germany) using rabbit antirat albumin antibodies (Cappel Cochranville, Philadelphia, PA, USA) as reported earlier [20]. All other determinations were carried out as previously described in detail [14, 15].

Data presentation and analysis of results

The amounts of ACE and AT_1 receptors in renal tissue were depicted in relation to the mean value of the sham group. Statistics were by one-way and two-way analyses of variance (ANOVA), and the least significant difference test was used for post hoc analyses (SPSS 9.0, SPSS, Inc., Chicago, IL, USA). If the distribution of the variables was skewed, the Kruskal-Wallis test was applied, and post hoc analyses were performed with the Mann-Whitney *U* test, the *P* values being corrected with the Bonferroni equation. Spearman's two-tailed correlation coefficient (r) was used in the correlation analyses and the Kaplan-Meier method in the survival analyses, in which the difference between survivals was assessed using the

Table 1. The 12-week study: Experimental group data; 0.3% or 3.0% calcium diet during weeks 4 to 12

	Sham	Calcium-sham	NTX	Calcium-NTX
Body weight *g*	465 ± 7	465 ± 13	462 ± 7	459 ± 6
Heart weight/body weight *g/kg*	3.37 ± 0.06	3.33 ± 0.05	3.51 ± 0.10	3.52 ± 0.14
Renal tissue weight *g*	3.07 ± 0.08	2.83 ± 0.11	2.18 ± 0.13[a]	1.90 ± 0.09[a]
Renal tissue weight/body weight *g/kg*	6.56 ± 0.13	6.15 ± 0.22	4.67 ± 0.23[a]	4.18 ± 0.22[a]
Fluid intake *mL*	24.2 ± 3.3	28.2 ± 3.5	49.2 ± 6.3[a]	50.1 ± 4.8[a]
Urine volume *mL*	17.0 ± 1.8	21.4 ± 3.4	38.7 ± 2.9[a]	39.2 ± 4.1[a]
Systolic blood pressure *mm Hg*				
Week 4	133 ± 4	132 ± 5	140 ± 4	138 ± 4
Week 12	138 ± 4	135 ± 4	154 ± 4[a,b]	148 ± 4[b]

Values are mean ± SEM ($N = 9$ to 10).
[a] $P < 0.05$ versus sham; [b] $P < 0.05$ NTX groups versus sham groups (150 ± 3 mm Hg versus 136 ± 3 mm Hg, two-way ANOVA).

Table 2. The 12-week study: Laboratory findings and proportions of angiotensin II (Ang II) type 2 (AT_2) receptors; 0.3% or 3.0% calcium diet during weeks 4 to 12

	Sham	Calcium-sham	NTX	Calcium-NTX
Plasma				
Renin activity *ng/mL/hour Ang I*	42.9 ± 4.2	45.9 ± 1.1	16.8 ± 6.2[a]	21.7 ± 4.2[a]
Urea nitrogen *mmol/L*	6.01 ± 0.23	6.65 ± 0.25	9.94 ± 0.41[a]	11.06 ± 0.97[a]
Creatinine *μmol/L*	63.8 ± 2.1	66.9 ± 2.2	100.7 ± 2.9[a]	95.9 ± 5.4[a]
Ionized calcium *mmol/L*	1.35 ± 0.02	1.47 ± 0.02[a]	1.27 ± 0.01[a]	1.47 ± 0.02[a,b]
Phosphate *mmol/L*	1.44 ± 0.05	1.07 ± 0.07[a]	1.75 ± 0.10[a]	1.01 ± 0.10[a,b]
Ca x Pi product	2.03 ± 0.07	1.58 ± 0.10[a]	2.19 ± 0.12	1.55 ± 0.14[a,b]
Parathyroid hormone *pg/mL*	146.2 ± 22.0	39.8 ± 2.1	554.3 ± 79.7[a]	48.2 ± 5.4[a,b]
$1,25(OH)_2D_3$ *pmol/L*	91.6 ± 8.6	95.0 ± 4.9	74.9 ± 5.6[c]	81.1 ± 8.8[c]
Hemoglobin *g/L*	187.5 ± 3.2	182.1 ± 2.8	163.8 ± 5.4[a]	168.7 ± 1.8[a]
Sodium *mmol/L*	138.5 ± 0.4	137.5 ± 0.4	138.8 ± 0.5	138.5 ± 0.5
Potassium *mmol/L*	3.95 ± 0.12	3.65 ± 0.15	3.81 ± 0.12	4.10 ± 0.25
pH	7.46 ± 0.02	7.44 ± 0.02	7.45 ± 0.02	7.46 ± 0.02
Urine calcium *μmol/24 hours*	11.7 ± 2.1	398.9 ± 41.0[a]	40.6 ± 7.5[a]	452.7 ± 40.0[a,b]
Creatinine clearance *μL/min/100 g body weight*	296.0 ± 8.3	289.6 ± 7.2	244.7 ± 7.0[a]	261.8 ± 14.6[a]
Proportion of Ang II receptors %				
AT_2 receptors in renal cortex	0.65 ± 0.24	2.23 ± 1.15	1.45 ± 0.43	2.01 ± 0.59
AT_2 receptors in renal medulla	0.93 ± 0.26	0.97 ± 0.19	1.45 ± 0.43	1.36 ± 0.37

Values are mean ± SEM ($N = 9$ to 10 for all groups).
[a] $P < 0.05$ compared with the sham group; [b] $P < 0.05$ compared with the NTX group; [c] $P < 0.05$ NTX groups versus sham groups (78.0 ± 5.0 pmol/L versus 93.3 ± 5.1 pmol/L, two-way ANOVA).

log rank test. Results were expressed as mean ± SEM, and differences were considered significant when $P < 0.05$.

RESULTS

Animal data in the 12-week study

No differences in body weights or heart weights were detected between the groups, while renal mass in both NTX groups was about 30% lower than in the sham group (Table 1). Fluid intake and urine output were higher in the NTX and calcium-NTX groups when compared with sham rats. Systolic blood pressure was modestly elevated 12 weeks after NTX, and was not affected by increased calcium intake (Table 1).

Laboratory findings in the 12-week study

PRA was clearly lower in both NTX groups than in the sham-operated controls, and was not affected by increased calcium ingestion. Plasma concentrations of urea nitrogen and creatinine were 1.6-fold higher in the NTX group when compared with sham, while no differences between the NTX and calcium-NTX groups were observed in these variables (Table 2). Plasma phosphate, calcium x phosphorus (Ca x Pi) product, and PTH were increased, while ionized Ca^{2+}, proteins and hemoglobin were decreased in the NTX group when compared with sham. High calcium diet elevated plasma ionized calcium, and suppressed plasma phosphate, Ca x Pi product, and PTH levels, in both NTX and sham rats. Although plasma levels of active vitamin D in individual study groups did not differ (NTX group vs. sham group, $P = 0.056$), analyses by two-way ANOVA showed that plasma $1,25(OH)_2D_3$ was lower in the two NTX groups than the sham groups (78.0 ± 5.0 pmol/mL versus 93.3 ± 5.1 pmol/mL, respectively, $P = 0.043$). The NTX group showed a modest elevation in urinary calcium excretion, while both groups on high calcium diet showed a marked increase in calcium excretion. Creatinine clearance was reduced in the NTX rats (Table 2).

Table 3. The 27-week study: Experimental group data; 0.3% or 3.0% calcium diet during weeks 15 to 27

	Sham	NTX	Calcium-NTX
Body weight *g*			
Week 15	499 ± 8	475 ± 9	479 ± 9
Week 27	557 ± 7	498 ± 38[a]	488 ± 13[a]
Heart weight/body weight *g/kg*	3.21 ± 0.03	4.19 ± 0.43[a]	4.03 ± 0.13[a]
Renal tissue weight *g*	3.34 ± 0.08	2.94 ± 0.34	2.37 ± 0.09[a,b]
Renal tissue weight/body weight *g/kg*	5.94 ± 0.09	5.93 ± 0.61	4.92 ± 0.16[a]
Fluid intake *mL*	27.7 ± 1.6	55.7 ± 5.4[a]	57.9 ± 4.9[a]
Urine volume *mL*	20.3 ± 1.4	40.7 ± 3.3[a]	43.6 ± 4.3[a]
Systolic blood pressure *mm Hg*			
Week 15	133 ± 3	151 ± 5[a]	152 ± 4[a]
Week 27	129 ± 2	173 ± 4[a]	145 ± 3[a,b]
Rat number			
Week 15	11	14	13
Week 27	11	7[a]	12[b]
Renal histology			
Glomerulosclerosis index	0.50 ± 0.04	1.91 ± 0.12[a]	1.39 ± 0.12[a,b]
Interstitial damage index	0.54 ± 0.05	2.93 ± 0.28[a]	1.92 ± 0.09[a,b]
Calcification index	0 ± 0	99.3 ± 65.6[a]	3.6 ± 3.6[b]
Aortic calcification % *of cross-section*	0 ± 0	5.8 ± 2.3[a]	0 ± 0[b]

Values are mean ± SEM ($N = 7$ to 14).
[a] $P < 0.05$ versus sham; [b] $P < 0.05$ versus NTX.

Table 4. The 27-week study: Laboratory findings and proportions of angiotensin II (Ang II) type 2 (AT_2) receptors; 0.3% or 3.0% calcium diet during weeks 15 to 27

	Sham	NTX	Calcium-NTX
Plasma			
Creatinine at week 13 *μmol/L*	62.7 ± 1.6	90.1 ± 3.5[a]	91.0 ± 3.7[a]
Creatinine at week 27 *μmol/L*	64.6 ± 2.0	186.0 ± 34.2[a]	118.0 ± 6.7[a,b]
Urea nitrogen *mmol/L*	5.2 ± 0.2	23.7 ± 7.6[a]	12.8 ± 1.0[a,b]
Ionized calcium *mmol/L*	1.36 ± 0.06	1.34 ± 0.03	1.55 ± 0.03[a,b]
Phosphate *mmol/L*	1.16 ± 0.06	2.52 ± 0.46[a]	0.81 ± 0.08[a,b]
Ca x Pi product	1.55 ± 0.07	3.48 ± 0.59[a]	1.18 ± 0.11[a,b]
Parathyroid hormone *pg/mL*	88.3 ± 12.5	1171.0 ± 320.2[a]	3.7 ± 0.4[a,b]
Proteins *g/L*	64.3 ± 0.7	59.5 ± 1.2[a]	62.1 ± 0.5[a,b]
Hemoglobin *g/L*	174.8 ± 1.9	148.3 ± 10.7[a]	154.6 ± 1.8[a]
Sodium *mmol/L*	137.9 ± 0.6	138.6 ± 1.4	136.5 ± 0.4
Potassium *mmol/L*	3.52 ± 0.11	4.52 ± 0.57[a]	3.56 ± 0.06[b]
pH	7.41 ± 0.02	7.28 ± 0.06[a]	7.40 ± 0.02[b]
Urine calcium *μmol/24 hours*	22.2 ± 5.5	64.7 ± 8.6[a]	525.3 ± 37.4[a,b]
Creatinine clearance *μL/ min/100 g body weight*	353.4 ± 15.9	143.5 ± 24.7[a]	187.2 ± 10.9[a]
Proportion of Ang II receptors%			
AT_2 receptors in renal cortex	0.26 ± 0.17	0.32 ± 0.28	0.22 ± 0.11
AT_2 receptors in renal medulla	0.34 ± 0.18	0.16 ± 0.11	0.12 ± 0.09

Values are mean ± SEM ($N = 7$ to 14 for all groups).
[a] $P < 0.05$ versus sham; [b] $P < 0.05$ versus NTX.

Animal data in the 27-week study

There were no differences in body weights at week 15, but body weights were lower in both NTX groups when compared with sham at week 27 (Table 3). The heart/body weight ratio was higher in the NTX and calcium-NTX groups when compared with sham. In the NTX group the kidneys appeared macroscopically swollen whereby the renal tissue/body weight ratio was similar to sham, while the calcium-NTX group showed lower renal tissue weights. Fluid intake and urine output were higher in both NTX groups than in sham rats. Corresponding to the 12-week experiment, both NTX groups exhibited mild elevation of systolic blood pressure at study week 15. During the follow-up period blood pressure increased in the NTX group, whereas the high calcium diet showed a significant antihypertensive effect (Table 3). At the end of the study, only 7 of the initial 14 rats survived in the NTX group, while survival was significantly improved in the calcium-NTX group (Table 3) (Fig. 2A).

Laboratory findings in the 27-week study

Plasma concentration of creatinine was similarly elevated in the NTX groups before the dietary interventions at study week 13 (Table 4). At week 27, plasma creatinine and urea nitrogen were further increased in the NTX group, while both of these values were lower in the calcium-NTX than the NTX group. Plasma phosphate, Ca x Pi product, and PTH were increased in the NTX group, while all of these values were lower in the calcium-NTX than the sham group. Plasma-ionized calcium did not differ from sham in the NTX group, but was increased in calcium-NTX rats (Table 4).

Hemoglobin and plasma proteins were lower in both NTX groups than in sham rats, while proteins were higher in the calcium-NTX than the NTX group. Plasma pH was decreased and potassium increased in the NTX group, whereas these variables in the calcium-NTX group were not different from sham. Daily urinary calcium excretion was moderately increased in the NTX group, whereas calcium-NTX rats showed a marked increase in urinary calcium. Creatinine clearance was lower in the NTX groups when compared with the sham group (Table 4).

Renal and aortic histology

In the 12-week study, the indices of glomerulosclerosis and interstitial damage (arbitrary scale from 0 to 4), and

number of calcifications (deposits/cm^2) in kidney tissue, were higher in the NTX group than the calcium-NTX group (0.70 ± 0.11 versus 0.11 ± 0.01, 1.30 ± 0.18 versus 0.38 ± 0.03, and 12.5 ± 3.0 versus 2.0 ± 1.3, respectively, $P < 0.05$ for all comparisons). No differences in tissue histology were detected between the sham and calcium-sham groups (not shown).

In the 27-week study the indices of glomerulosclerosis, tubulointerstitial damage, and kidney tissue calcification were clearly increased in the NTX group (Table 3). All of these indices were lower in the calcium-NTX than the NTX group. High calcium diet also significantly reduced calcifications in the thoracic aorta (Table 3).

Renal components of RAS and albumin excretion in the 12-week study

When analyzed using quantitative in vitro autoradiography, which measures binding to the active site of ACE [21], the renal tissue ACE content was approximately 40% lower in the calcium-sham and calcium-NTX groups when compared with the sham and NTX groups (Fig. 1A and B). The distribution of ACE was also different between NTX and sham rats (Fig. 1A). The highest ACE signal was detected in a circular fashion in the inner cortex and outer medulla in the sham group, whereas ACE was more widely distributed throughout the remnant kidney in the NTX group. The outcome of ACE protein determination by Western blotting well paralleled the results of autoradiography: renal ACE was lower in the calcium-NTX group when compared with the NTX and sham groups (Fig. 1C). ACE did not differ between NTX and sham groups (Fig. 1A to C).

AT_1 receptor density in kidney cortex was slightly reduced in the NTX and calcium-sham groups when compared with the sham group, while no significant differences in cortical AT_1 receptor density were detected between the NTX and calcium-NTX groups (Fig. 1E). AT_1 density in renal medulla (Fig. 1F), and AT_2 density in cortex and medulla (not shown), was similar in all study groups. The average proportion of AT_2 of all Ang II receptors in the kidney varied 0.65% to 2.23%, with no significant differences between the groups (Table 2).

The scatter plots depicting the variables of calcium metabolism in relation to renal tissue ACE, determined by autoradiography, showed clustering of data points in separate subgroups in the NTX and calcium-NTX groups (Fig. 2A to D). In the NTX groups, renal tissue ACE content correlated to urinary albumin excretion ($r = 0.512$, $P = 0.025$), and inversely correlated to cortical AT_2 receptor density ($r = -0.574$, $P = 0.010$). Both of these correlations were significant if the NTX group alone was included in the analyses ($r = 0.733$, $P = 0.025$ and $r = -0.705$, $P = 0.034$, respectively).

The level of daily urinary albumin excretion was increased in NTX rats, but was lower in the calcium-NTX than the NTX group (Fig. 1D).

Renal components of RAS and CTGF in the 27-week study

Measurements using autoradiography in vitro showed that kidney tissue ACE content was lower in the calcium-NTX group when compared with the sham and NTX groups (Fig. 3C). The outcome of Western blotting confirmed that renal ACE protein was significantly reduced in the calcium-NTX group when compared with the NTX group, while ACE protein was increased in the NTX group when compared with sham (Fig. 3D).

AT_1 density in renal cortex (autoradiography) was lower in the calcium-NTX than the NTX group (Fig. 2E). No differences in renal cortical AT_1 density between the NTX and sham groups, and renal medullary AT_1 density between any of the study groups, were observed (Fig. 2F). The proportion of cortical and medullary AT_2 was less than 0.34% of all Ang II receptors, with no significant differences between the study groups (Table 4).

Since CTGF has been suggested to be a mediator of Ang II–induced tissue damage [22, 23], we measured renal CTGF content in the study groups using immunohistochemistry. Kidney CTGF score was markedly increased in the NTX group when compared with sham, whereas the CTGF score was significantly lower in the calcium-NTX than the NTX group (Fig. 3B).

DISCUSSION

The present study for the first time demonstrated that high calcium diet reduces kidney ACE content in rats with CRF, with an associated decrease in albuminuria and beneficial influence on kidney morphology. Two methods were applied to assess renal ACE: autoradiography that measures binding to the active site of ACE, and Western blotting that measures ACE protein. Previously, a linear relationship between radioligand binding to ACE in tissue sections, as determined by quantitative in vitro autoradiography, and ACE activity in tissue membrane suspensions, as measured by enzymatic assay, has been reported [21]. Here we examined the effects of calcium supplementation on renal ACE and Ang II receptors, because high calcium diet has reduced blood pressure [4], enhanced vasorelaxation [5], increased sodium excretion [9], and decreased PRA in experimental hypertension [6]. Such effects of increased calcium intake resemble those that are achieved by inhibition of the actions of RAS. Furthermore, infusion of the N-terminal 34 amino acid PTH peptide increases renin release in dogs [24], whereas increased calcium intake results in suppression of plasma PTH levels [3]. An inverse relationship between the levels

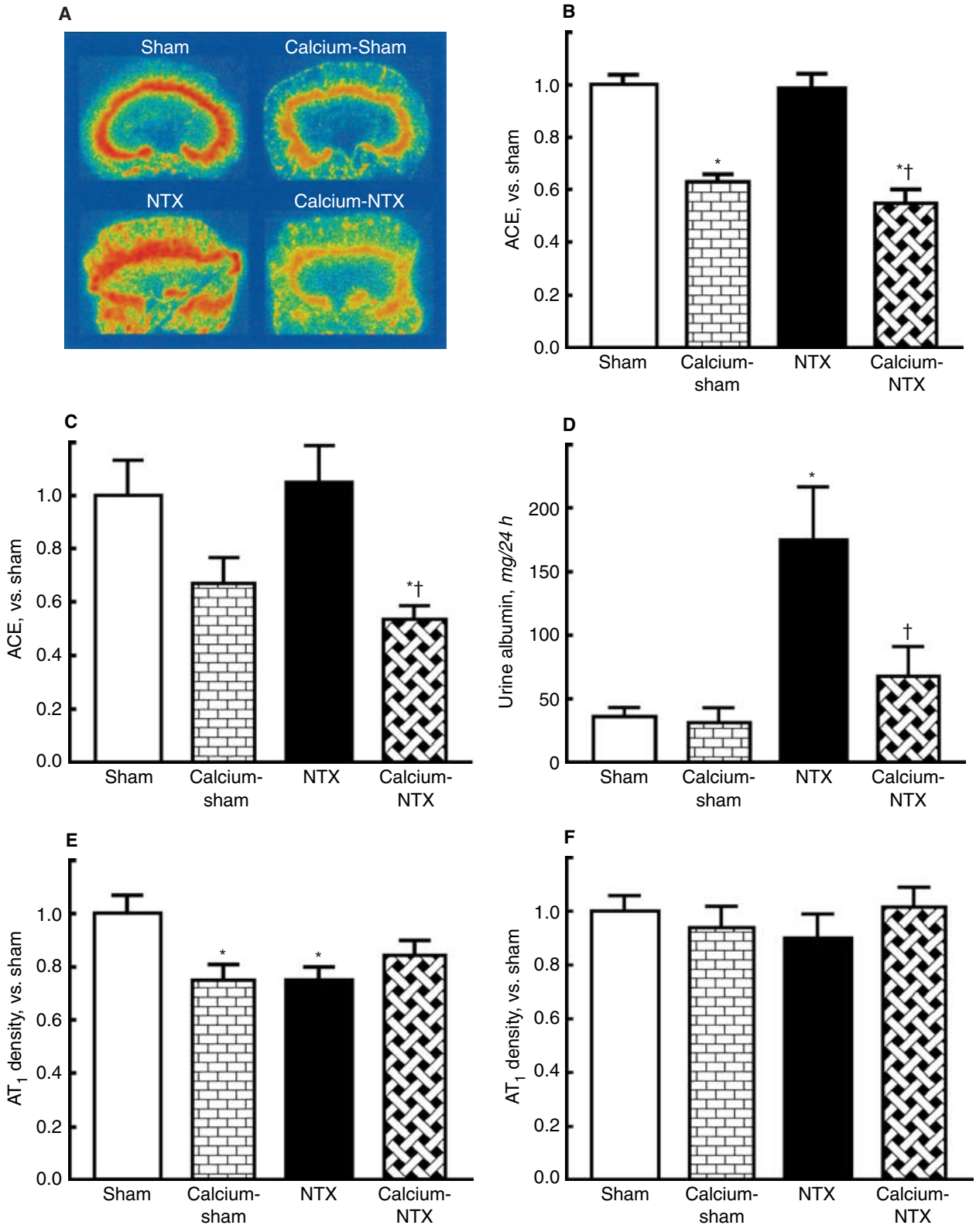


Fig. 1. The 12-week study. Renal tissue angiotensin-converting enzyme (ACE) and angiotensin II type 1 (AT_1) receptor density (related to the mean value of the sham group), and urinary albumin excretion. Representative original tracings (intensity of red coloring reflects active binding to tissue ACE (*A*), and bar graphs (*B*) ($N = 9$ to 10) of ACE determined by quantitative in vitro autoradiography; ACE protein determined by Western blotting (*C*) ($N = 9$ to 10 except for $N = 5$ in the calcium-sham group); urinary albumin excretion (*D*) ($N = 9$ to 10); AT_1 receptors in the kidney cortex (*E*) and medulla (*F*) determined by quantitative in vitro autoradiography. Mean ± SEM. $^*P < 0.05$ versus sham; $^\dagger P < 0.05$ versus NTX.

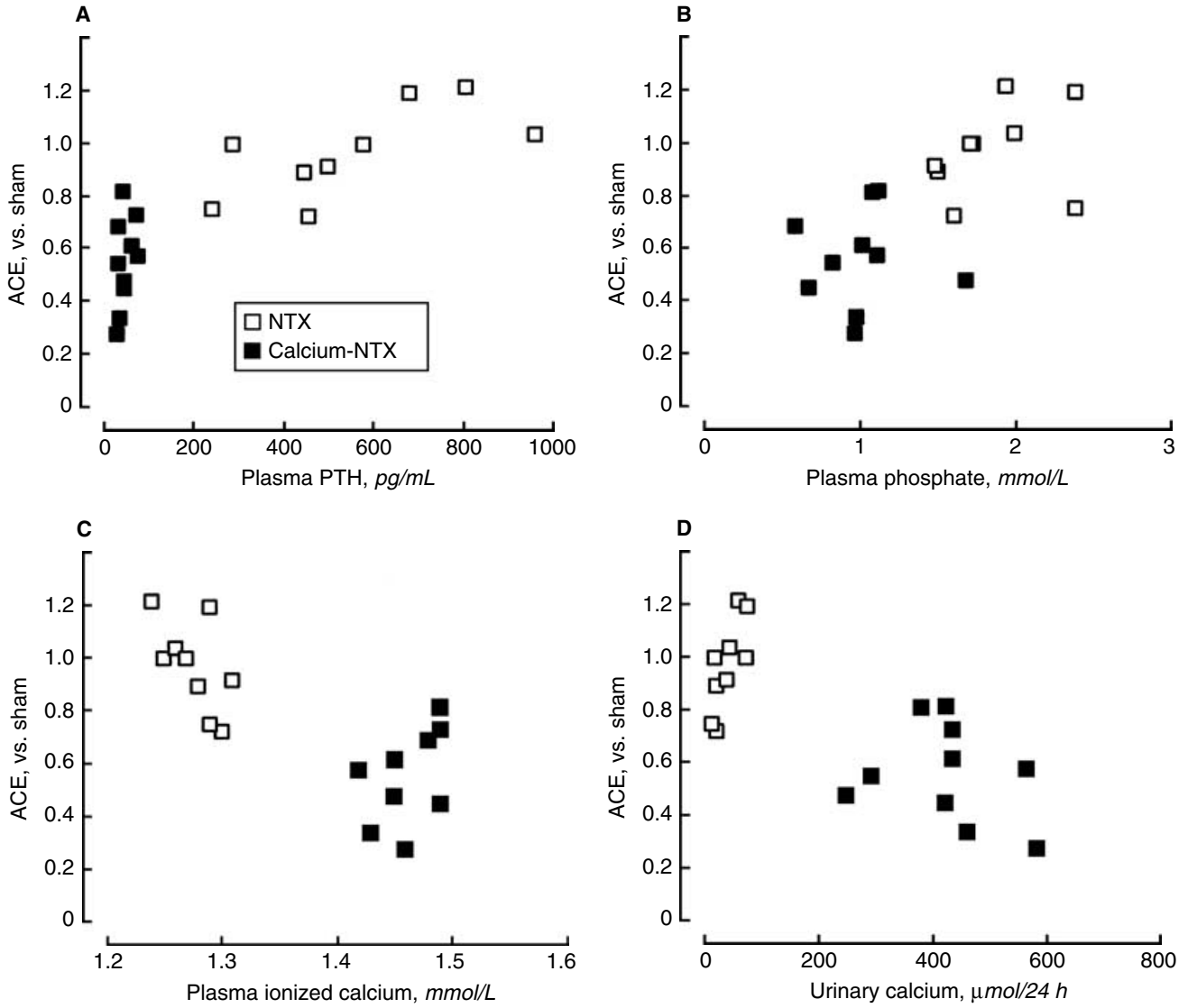


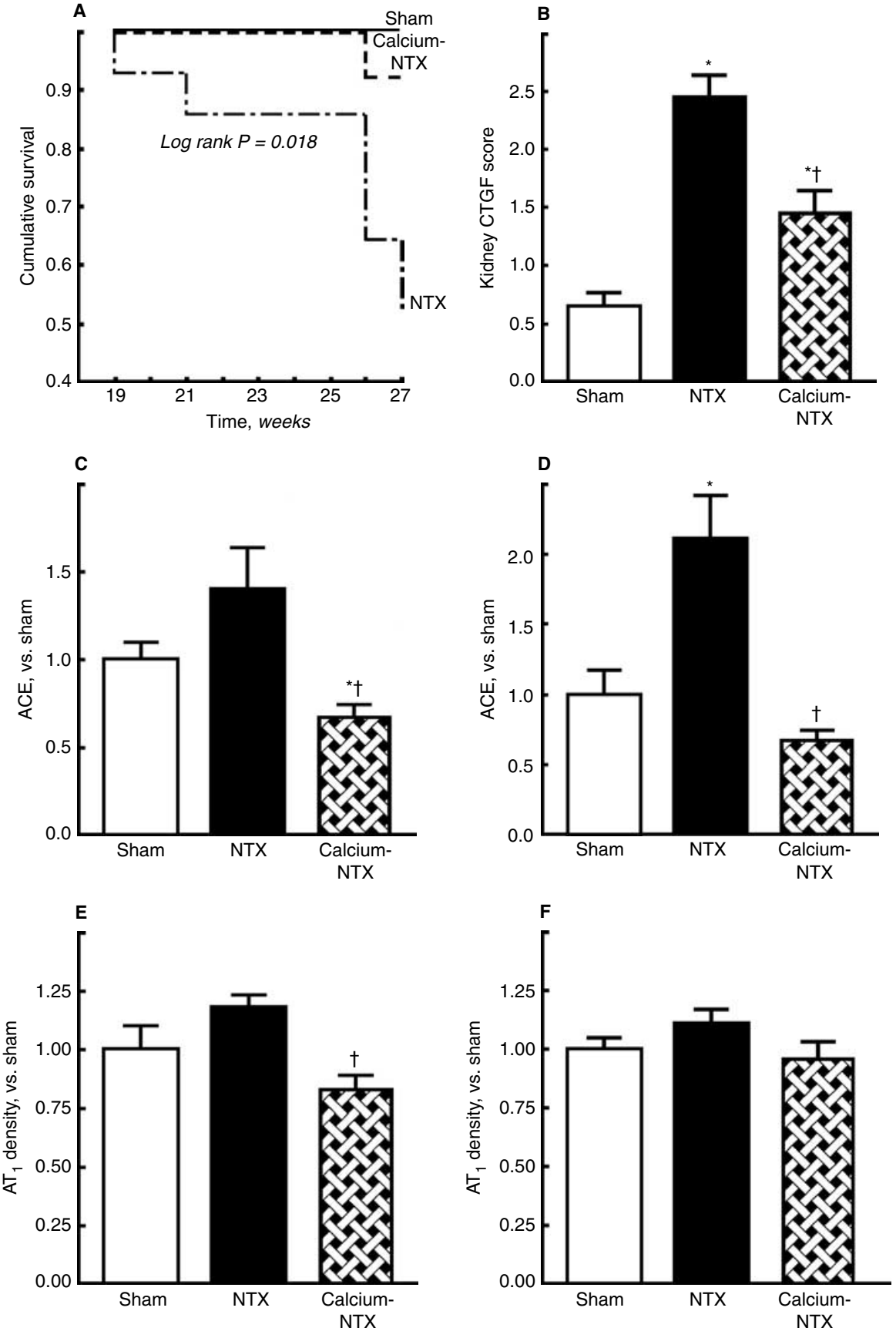
Fig. 2. The 12-week study. Scatter plots of plasma parathyroid hormone (PTH) (*A*), phosphate (*B*), ionized calcium (*C*), and urinary calcium excretion (*D*) proportionate to renal tissue angiotensin-converting enzyme (ACE) content in the NTX and calcium-NTX groups; quantities of ACE were related to the mean value of the sham group ($N = 9$ to 10 in each group).

of calcium-regulating hormones and activity of circulating RAS has also been reported in essential hypertension [25, 26].

In this study, renal failure was induced by surgical 5/6 renal ablation, and the NTX rats showed several characteristic findings of CRF (Tables 1 to 4), which well correspond to previously published results [15, 27]. Blood pressure was modestly elevated 12 weeks after NTX, and further increased during the 27-week follow-up. Corresponding to the reports in experimental hypertension [15, 27], the high calcium diet showed a clear antihypertensive effect in the 27-week study. The finding that Sprague-Dawley rats do not readily develop overt hypertension after surgical 5/6 renal mass reduction has been attributed to low activity of circulating RAS in this model [27]. This view is supported by the present results, since PRA was clearly lower in the NTX rats than in sham rats.

Secondary hyperparathyroidism is a common complication of CRF, and the causes for increased PTH secretion are high plasma phosphate, low ionized calcium, and low

Fig. 3. The 27-week study. Cumulative survival (*A*), immunohistochemistry of kidney tissue connective tissue growth factor (CTGF) (*B*), renal tissue angiotensin-converting enzyme (ACE) determined by autoradiography (*C*) and Western blotting (*D*), and angiotensin II type 1 (AT_1) receptor density in renal cortex (*E*) and medulla (*F*); quantities of ACE and AT_1 receptors were related to the mean value of the sham group. Mean ± SEM ($N = 7$ to 11 in each group). $^*P < 0.05$ versus sham; $^\dagger P < 0.05$ versus NTX.


A
Sham
Calcium-NTX
NTX
Log rank P = 0.018
Cumulative survival
0.9
0.8
0.7
0.6
0.5
0.4
19
21
23
25
27
Time, weeks
B
Kidney CTGF score
2.5
2.0
1.5
1.0
0.5
0.0
*
*†
Sham
NTX
Calcium-NTX
C
ACE, vs. sham
1.5
1.0
0.5
0.0
*†
Sham
NTX
Calcium-NTX
D
ACE, vs. sham
2.0
1.5
1.0
0.5
0.0
*
†
Sham
NTX
Calcium-NTX
E
AT1 density, vs. sham
1.25
1.00
0.75
0.50
0.25
0.00
†
Sham
NTX
Calcium-NTX
F
AT1 density, vs. sham
1.25
1.00
0.75
0.50
0.25
0.00
Sham
NTX
Calcium-NTX

calcitriol [3]. Successful treatment of high phosphate can ameliorate the progression of secondary hyperparathyroidism and reduce cardiovascular complications in CRF [2, 3]. High phosphate and PTH levels increase the risk of ectopic calcifications [3], and elevated phosphate may directly induce phenotypic changes in vascular smooth muscle that predispose the vessel wall to calcification [28]. Because of increased ingestion of calcium salts to control phosphate and PTH, renal patients are subjected to a substantial calcium burden, which may predispose to the accumulation of calcium salts in extraskeletal tissues [3]. However, in experimental CRF increased intake of calcium carbonate has actually reduced kidney calcification, the probable mechanism being the lowering of plasma phosphate [29].

In the present study, we found that plasma phosphate and PTH were effectively reduced by the high calcium diet. The elevated ionized calcium levels and suppressed PTH levels indicate that increased calcium intake actually resulted in mild hypercalcemia in the 27-week study. In spite of this, the increase in renal and aortic tissue calcifications was prevented by elevated calcium ingestion in NTX rats (Table 3), probably because of the effective suppression of plasma phosphate and Ca x Pi product. The reduced mortality in the NTX rats ingesting the high calcium diet (Fig. 3A) argues against the view that increased calcium intake would be toxic to the animals in this model of CRF. Furthermore, since the samples of severely affected rats in the 27-week study were lost due to the reduced survival in the NTX group, the present evaluation probably underestimated the extent of tissue damage and decline of renal function in this group. Altogether the present results suggest that especially hyperphosphatemia, but not mild hypercalcemia alone, is detrimental to the kidneys and vasculature in CRF.

A decrease in renal calcification with a high calcium diet seems to contradict with the opinion that such a diet may be deleterious to patients with advanced CRF. This view is based on clinical reports, which showed an association between soft tissue calcifications and high oral calcium supplements in hemodialysis patients [30, 31]. Most studies have reported that high concurrent circulating levels of phosphorus and calcium, or high Ca x Pi product, are risk factors for calcification, but have not given detailed information about dietary calcium intake in these patients [32, 33]. Thus, calcium-based phosphate binders may be associated with progressive vascular calcification if hyperphosphatemia is not adequately controlled [31]. However, all clinical reports have not shown that increased oral calcium load would be a risk factor for soft tissue calcification in dialysis patients [34]. A recent analysis stressed the importance of examining combinations of parameters of calcium-phosphorus metabolism, and not single variables alone, when assessing cardiovascular risk in hemodialysis patients [35]. A thorough analysis of dietary calcium intake, with reliable information about patient compliance, should be included in such an approach.

The NTX group showed higher albumin excretion (12-week study) and increased indices of glomerulosclerosis and interstitial damage (12-week and 27-week studies), while high calcium intake was associated beneficial influence on albuminuria and kidney morphology. These findings may be due to the observed changes in calcium metabolism. Another putative explanation would be down-regulation of components of RAS in the kidney, since renal tissue ACE content was significantly reduced after increased calcium ingestion in NTX rats. In the 12-week study, no differences in renal AT_1 density were observed between the NTX and calcium-NTX groups, but a significant decrease in cortical AT_1 density was detected in the calcium-supplemented sham-operated rats. In the 27-week study, high calcium intake reduced cortical AT_1 receptor density in NTX rats. Subsequently, the net effect of lower ACE without changes, or a concurrent decrease, in cortical AT_1 receptor density, could result in reduced Ang II action at the tissue level, and provide an explanation to the observed beneficial results on morphology and albumin excretion.

Local intrarenal RAS is an important determinant of tissue injury, inflammation, and progression of renal disease, while inhibition of RAS can preserve renal function in kidney diseases [13, 27, 36]. After subtotal nephrectomy, pathologic expression of renin and Ang II has been reported in rat kidneys [36]. In the 27-week study we found that renal tissue ACE was increased in the NTX group when determined by Western blotting, while only an insignificant trend was observed using autoradiography. This discrepancy can be explained by the patchy and wider distribution of ACE in the remnant versus sham-operated kidneys (Fig. 1A), which somewhat interferes with the autoradiography analysis when comparing normal and remnant kidney tissue. However, reliable comparisons between the NTX and calcium-NTX groups could be performed, as evidenced by the parallel outcomes of the autoradiographs and Western blotting in these rats.

Previously, Ang II has been reported to up-regulate several proinflammatory mediators in the kidney [13]. Especially the prosclerotic cytokine CTGF has been suggested as a mediator of the Ang II–induced fibrosis and tissue damage [22, 23]. Therefore, we measured CTGF content in the kidney by the use of immunohistochemistry. The results showed that CTGF score was markedly increased in the NTX group, whereas the score was significantly lower in the calcium-NTX than the NTX group (Fig. 3B). This finding supports the view that Ang II action at the tissue level was reduced by the high calcium

diet. In experimental CRF, disturbed calcium balance and hyperphosphatemia have been previously linked with increased tissue fibrosis and thickening of arterial wall [37], the findings of which resemble the structural changes that are associated with long-term activation of RAS at the tissue level [13, 36].

The regulation of ACE in tissues is not well understood. In the kidney intense proteinuria up-regulates ACE [38], while in cultured endothelial cells ACE expression is induced by vascular endothelial growth factor [39] and down-regulated by tumor necrosis factor-α [40]. The present results showed that alterations in calcium-phosphate balance contribute to the regulation of ACE in the kidney. In addition to its other physiologic roles, extracellular calcium ion functions like a hormone by regulating cellular processes through specific calcium receptor–mediated mechanisms [10]. In the nephron, high calcium delivery stimulates calcium-sensing receptors, the mechanism of which plays a role in the enhanced natriuresis induced by high calcium ingestion [10]. In vascular tissue, elevated extracellular calcium concentration can increase the synthesis of proteins that inhibit soft tissue calcification, via a mechanism that is functionally related to the calcium-sensing receptor [41]. Some of the proteins that regulate calcification belong to the transforming growth factor-β family of cytokines [42]. Whether high renal calcium delivery influences the synthesis of calcification-regulation proteins, and whether these proteins in turn influence the expression of renal components of RAS, remain to be studied in the future. Interestingly, AT_2 receptor activation has been reported to decrease ACE activity, which may partially underlie AT_2's attenuation of AT_1-mediated actions [43]. Our findings suggested an inverse correlation between renal ACE and AT_2 receptors, thus supporting a link between the regulations of these two components of RAS in kidney tissue. It is also noteworthy that increased calcium intake down-regulated renal ACE without a compensatory increase in circulating PRA.

In addition to the changes in calcium-phosphate balance, a multitude of mechanisms could explain the effects of high calcium diet on renal tissue ACE. As the dietary intake of calcium changes, so do levels of calcitonin, calcitonin gene-related peptide, natriuretic peptides, sympathetic nervous system activity, metabolism of other electrolytes, calcium-binding proteins, intracellular free calcium levels, membrane-bound calcium, and cellular electrolyte fluxes [4]. Any of these changes, or their combination, could be the source of altered ACE, and the precise molecular mechanism of the down-regulation of renal ACE expression by increased calcium intake is a subject for further studies. In concert with the view that calcium supplementation can modulate gene expression in the kidney, increased dietary calcium intake was recently found to normalize the disturbances in calcium homeostasis in mice in which the 25OH D_3-1α-hydroxylase gene was inactivated [11]. In these mice high calcium diet was shown to regulate the expression of renal calcium transport protein genes, but also the expression of a large number of other genes that were not previously identified as playing a role in renal calcium handling [11].

CONCLUSION

Our results showed that high calcium diet not only suppressed plasma phosphate and PTH, and increased plasma ionized calcium, but also reduced albuminuria, favorably influenced kidney morphology, and down-regulated kidney ACE in experimental renal failure. These findings suggest a link between calcium metabolism and ACE expression in kidney tissue that could also be important in the progression of renal damage.

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REFERENCES

1. Luke RG: Chronic renal failure—A vasculopathic state. *N Engl J Med* 339:841–843, 1998
2. Rostand SG, Drüeke TB: Parathyroid hormone, vitamin D, and cardiovascular disease in chronic renal failure. *Kidney Int* 56:383–392, 1999
3. Slatopolsky E, Brown A, Dusso A: Role of phosphorus in the pathogenesis of secondary hyperparathyroidism. *Am J Kidney Dis* 37:S54–S57, 2001
4. Hatton DC, McCarron DA: Dietary calcium and blood pressure in experimental models of hypertension. A review. *Hypertension* 23:513–530, 1994
5. Mäkynen H, Kähönen M, Wu X, *et al*: Endothelial function in deoxycorticosterone-NaCl hypertension: effect of calcium supplementation. *Circulation* 93:1000–1008, 1996
6. Wuorela H, Arvola P, Pörsti I, *et al*: The effect of high calcium intake on Ca2+ ATPase and the tissue Na:K ratio in spontaneously hypertensive rats. *Naunyn Schmiedebergs Arch Pharmacol* 345:117–122, 1992
7. Levi M, Henrich WL: Dietary calcium modulates renal BBM angiotensin II binding and Na(+)-H+ antiporter activity in SHR. *Am J Physiol* 260:F657–F662, 1991
8. McCarron DA, Lucas PA, Shneidman RJ, *et al*: Blood pressure development of the spontaneously hypertensive rat after concurrent manipulations of dietary Ca^{2+} and Na^{+}. Relation to intestinal Ca^{2+} fluxes. *J Clin Invest* 76:1147–1154, 1985
9. Pörsti I, Wuorela H, Arvola P, *et al*: Effects of calcium supplementation and deoxycorticosterone on plasma atrial natriuretic peptide and electrolyte excretion in spontaneously hypertensive rats. *Acta Physiol Scand* 141:343–350, 1991

10. Brown EM: Physiology and pathophysiology of the extracellular calcium-sensing receptor. *Am J Med* 106:238–253, 1999
11. Hoenderop JG, Chon H, Gkika D, *et al*: Regulation of gene expression by dietary Ca2+ in kidneys of 25-hydroxyvitamin D3–1 alpha-hydroxylase knockout mice. *Kidney Int* 65:531–539, 2004
12. Sigmund CD: Genetic manipulation of the renin-angiotensin system: Targeted expression of the renin-angiotensin system in the kidney. *Am J Hypertens* 14:33S–37S, 2001
13. Ruiz-Ortega M, Ruperez M, Lorenzo O, *et al*: Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney. *Kidney Int* 62(Suppl):12–22, 2002
14. Jolma P, Kööbi P, Kalliovalkama J, *et al*: Treatment of secondary hyperparathyroidism by high calcium diet is associated with enhanced resistance artery relaxation in experimental renal failure. *Nephrol Dial Transplant* 18:2560–2569, 2003
15. Kööbi P, Kalliovalkama J, Jolma P, *et al*: AT1 receptor blockade improves vasorelaxation in experimental renal failure. *Hypertension* 41:1364–1371, 2003
16. Bäcklund T, Palojoki E, Grönholm T, *et al*: Dual inhibition of angiotensin converting enzyme and neutral endopeptidase by omapatrilat in rat in vivo. *Pharmacol Res* 44:411–418, 2001
17. Zhuo J, Ohishi M, Mendelsohn FA: Roles of AT_1 and AT_2 receptors in the hypertensive Ren-2 gene transgenic rat kidney. *Hypertension* 33:347–353, 1999
18. Schwarz U, Amann K, Orth SR, *et al*: Effect of 1,25 $(OH)_2$ vitamin D_3 on glomerulosclerosis in subtotally nephrectomized rats. *Kidney Int* 53:1696–1705, 1998
19. Inkinen K, Wolff H, Lindroos P, Ahonen J: Connective tissue growth factor and its correlation to other growth factors in experimental granulation tissue. *Connect Tissue Res* 44:19–29, 2003
20. Luimula P, Ahola H, Wang SX, *et al*: Nephrin in experimental glomerular disease. *Kidney Int* 58:1461–1468, 2000
21. Zhuo JL, Froomes P, Casley D, *et al*: Perindopril chronically inhibits angiotensin-converting enzyme in both the endothelium and adventitia of the internal mammary artery in patients with ischemic heart disease. *Circulation* 96:174–182, 1997
22. Finckenberg P, Inkinen K, Ahonen J, *et al*: Angiotensin II induces connective tissue growth factor gene expression via calcineurin-dependent pathways. *Am J Pathol* 163:355–366, 2003
23. Ruperez M, Ruiz-Ortega M, Esteban V, *et al*: Angiotensin II increases connective tissue growth factor in the kidney. *Am J Pathol* 163:1937–1947, 2003
24. Powell HR, McCredie DA, Rotenberg E: Renin release by parathyroid hormone in the dog. *Endocrinology* 103:985–989, 1978
25. Resnick LM: The role of dietary calcium in hypertension: a hierarchical overview. *Am J Hypertens* 12:99–112, 1999
26. Resnick LM, Muller FB, Laragh JH: Calcium-regulating hormones in essential hypertension. Relation to plasma renin activity and sodium metabolism. *Ann Intern Med* 105:649–654, 1986
27. Amann K, Simonaviciene A, Medwedewa T, *et al*: Blood pressure-independent additive effects of pharmacologic blockade of the renin-angiotensin and endothelin systems on progression in a low-renin model of renal damage. *J Am Soc Nephrol* 12:2572–2584, 2001
28. Jono S, McKee MD, Murry CE, *et al*: Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res* 87:E10–E17, 2000
29. Cozzolino M, Dusso AS, Liapis H, *et al*: The effects of sevelamer hydrochloride and calcium carbonate on kidney calcification in uremic rats. *J Am Soc Nephrol* 13:2299–2308, 2002
30. Goodman WG, Goldin J, Kuizon BD, *et al*: Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. *N Engl J Med* 342:1478–1483, 2000
31. Chertow GM, Raggi P, Chasan-Taber S, *et al*: Determinants of progressive vascular calcification in haemodialysis patients. *Nephrol Dial Transplant* 19:1489–1496, 2004
32. Kimura K, Saika Y, Otani H, *et al*: Factors associated with calcification of the abdominal aorta in hemodialysis patients. *Kidney Int* 56(Suppl 71):S238–S241, 1999
33. Raggi P, Boulay A, Chasan-Taber S, *et al*: Cardiac calcification in adult hemodialysis patients. A link between end-stage renal disease and cardiovascular disease? *J Am Coll Cardiol* 39:695–701, 2002
34. Moe SM, O'Neill KD, Fineberg N, *et al*: Assessment of vascular calcification in ESRD patients using spiral CT. *Nephrol Dial Transplant* 18:1152–1158, 2003
35. Stevens LA, Djurdjev O, Cardew S, *et al*: Calcium, phosphate, and parathyroid hormone levels in combination and as a function of dialysis duration predict mortality: Evidence for the complexity of the association between mineral metabolism and outcomes. *J Am Soc Nephrol* 15:770–779, 2004
36. Gilbert RE, Wu LL, Kelly DJ, *et al*: Pathological expression of renin and angiotensin II in the renal tubule after subtotal nephrectomy. Implications for the pathogenesis of tubulointerstitial fibrosis. *Am J Pathol* 155:429–440, 1999
37. Amann K, Tornig J, Kugel B, *et al*: Hyperphosphatemia aggravates cardiac fibrosis and microvascular disease in experimental uremia. *Kidney Int* 63:1296–1301, 2003
38. Largo R, Gomez-Garre D, Soto K, *et al*: Angiotensin-converting enzyme is upregulated in the proximal tubules of rats with intense proteinuria. *Hypertension* 33:732–739, 1999
39. Saijonmaa O, Nyman T, Kosonen R, Fyhrquist F: Upregulation of angiotensin-converting enzyme by vascular endothelial growth factor. *Am J Physiol Heart Circ Physiol* 280:H885–H891, 2001
40. Papapetropoulos A, Antonov A, Virmani R, *et al*: Monocyte- and cytokine-induced downregulation of angiotensin-converting enzyme in cultured human and porcine endothelial cells. *Circ Res* 79:512–523, 1996
41. Farzaneh-Far A, Proudfoot D, Weissberg PL, Shanahan CM: Matrix gla protein is regulated by a mechanism functionally related to the calcium-sensing receptor. *Biochem Biophys Res Commun* 277:736–740, 2000
42. Lund RJ, Davies MR, Hruska KA: Bone morphogenetic protein-7: An anti-fibrotic morphogenetic protein with therapeutic importance in renal disease. *Curr Opin Nephrol Hypertens* 11:31–36, 2002
43. Hunley TE, Tamura M, Stoneking BJ, *et al*: The angiotensin type II receptor tonically inhibits angiotensin-converting enzyme in AT2 null mutant mice. *Kidney Int* 57:570–577, 2000