Vitamin E protection of obesity-enhanced vascular calcification in uremic rats

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Peralta-Ramírez A, Montes de Oca A, Raya AI, Pineda C, López I, Guerrero F, Diez E, Muñoz-Castañeda JR, Martínez J, Almaden Y, Rodríguez M, Aguilera-Tejero E. Vitamin E protection of obesity-enhanced vascular calcification in uremic rats. Am J Physiol Renal Physiol 306: F422–F429, 2014. First published December 26, 2013; doi:10.1152/ajprenal.00355.2013.—This study aimed to determine the extent of extraskeletal calcification in uremic Zucker rats, by comparing obese and lean phenotypes, and to evaluate the influence of vitamin E (VitE) on the development of calcifications in both uremic rats and human vascular smooth muscle cells (HVSVMCs) cultured in vitro. Zucker rats of lean and obese phenotypes with normal renal function [control (C); C-lean and C-obese groups] and with uremia [5/6 nephrectomy (Nx); Nx-lean and Nx-obese groups] and uremic rats treated with VitE (Nx-lean + VitE and Nx-obese + VitE groups) were studied. Uremic groups were subjected to Nx, fed a 0.9% phosphorus diet, and treated with calcitriol (80 ng/kg ip). The aortic calcium concentration was significantly higher (P < 0.05) in Nx-obese rats (10.0 ± 2.1 mg/g tissue) than in Nx-lean rats (3.6 ± 1.3 mg/g tissue). A decrease in plasma glutathione peroxidase activity was observed in Nx-obese rats compared with Nx-lean rats (217.2 ± 18.2 vs. 382.3 ± 15.5 nmol·min⁻¹·ml⁻¹, P < 0.05). Treatment with VitE restored glutathione peroxidase activity and reduced the aortic calcium concentration to 4.6 ± 1.3 mg/g tissue. The differences in mineral deposition between Nx-lean, Nx-obese, Nx-lean + VitE, and Nx-obese + VitE rats were also evidenced in other soft tissues. In HVSMCs incubated with high phosphate, VitE also prevented oxidative stress and reduced calcium content, bone alkaline phosphatase, and gene expression of core-binding factor-α1. In conclusion, uremic obese rats develop more severe calcifications than uremic lean rats and VitE reduces oxidative stress and vascular calcifications in both rats and cultures of HVSVMCs.

Obesity; rat; vascular calcification; vitamin E

Obesity and metabolic syndrome are becoming two of the largest health problems in Western society (33) and are reaching epidemic proportions in some developing countries (28, 17). Obesity represents a major risk factor for chronic kidney disease (CKD) (10, 18). The influence of obesity on the development of CKD is commonly explained through obesity-associated metabolic derangements, namely, type II diabetes and hypertension (7, 14). In addition, obesity itself can influence the progression of CKD due to its direct effects on renal hemodynamics: hyperfiltration, increased glomerular capillary wall tension, and podocyte changes (5, 6). Moreover, a high caloric intake may also promote CKD through downregulation of the sirtuin-1- adiponectin axis (34).

Extraskeletal calcification is a common feature in patients with CKD. Vascular calcification represents an important contributor to the high rate of cardiovascular mortality associated with CKD (3). In uremic patients, vascular calcification is caused in part by deranged mineral metabolism, including hyperphosphatemia, hypercalcemia, and abnormal parathyroid hormone (PTH) levels (8).

Although some features of the metabolic syndrome (type II diabetes, hypertension, dyslipidemia, and proinflammatory status) are well-known risk factors for vascular calcification (19), to our knowledge, the influence of obesity on vascular calcification has not been explored in experimental models of uremia. Moreover, the influence of oxidative stress, a consistent feature of metabolic syndrome, on extraskeletal calcifications and the usefulness of antioxidants to prevent the development of calcifications remain largely unexplored.

The purpose of this study was to determine the extent of extraskeletal calcifications in uremic Zucker rats, by comparing obese and lean phenotypes. Furthermore, we evaluated the influence of vitamin E (VitE), a well-known antioxidant, on the development of calcifications in both uremic rats and human vascular smooth muscle cells (HVSVMCs) cultured in vitro. We hypothesized that uremic obese rats with metabolic syndrome would develop more severe extraskeletal calcifications than their lean counterparts and that controlling oxidative stress with the antioxidant VitE would decrease the extent of extraskeletal calcifications.

MATERIALS AND METHODS

In Vivo Experiments

Animals and surgical procedures. Two strains of rats were used in this study: Zucker rats with the obese phenotype (n = 32) and Zucker rats with the lean phenotype (n = 30). Rats were aged 3 mo at the start of the experiments. Animals (Harlan Laboratories Models, Barcelona, Spain) were housed with a 12:12-h light-dark cycle and given ad libitum access to standard diet (calcium: 0.6% and phosphorus: 0.6%). Uremia was induced by 5/6 nephrectomy (Nx), a two-step procedure that reduces the original renal mass by five-sixths. After the second surgery, the mineral content of the diet was changed to 0.6% calcium and 0.9% phosphorus, and rats were treated with calcitriol (80 ng/kg ip, Calcijex, Abbot, Madrid, Spain) administered every other day (3 times/wk) to control secondary hyperparathyroidism. Euthanasia was performed 24 h after animals received the last dose of calcitriol. All

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experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the University of Cordoba (Cordoba, Spain).

**Experimental design.** Before the experiments began, all rats were maintained for 2 wk on the standard diet. At 12 wk of age, rats from each strain were randomly allocated to the following groups: control and Nx. Control rats (C-obese, n = 8; C-lean, n = 8) were euthanized to obtain a nonuremic blood biochemical profile and a negative control for calcification. The remaining rats were subjected to Nx (Nx-obese, n = 24; Nx-lean, n = 22) as described above. Uremic obese and lean rats were allocated to the following four groups: 1) Nx-obese rats (n = 12) that received a high-phosphate diet, as described above, containing the minimum requirement of VitE (27 mg/kg), 2) Nx-lean (n = 12) that received a high-phosphate diet containing the minimum requirement of VitE (27 mg/kg), 3) Nx-obese rats supplemented with VitE (30,000 mg/kg) in the diet (Nx-obese + VitE; n = 12), and 4) Nx-lean rats supplemented with VitE (30,000 mg/kg) in the diet (Nx-lean + VitE; n = 10). All groups were euthanized after 4 wk.

**Assessment of vascular calcification.** After euthanization, the thoracic aorta and stomach were dissected and processed to study mineral content. Calcification was studied by measuring the aortic and gastric calcium and phosphorus content. Aortic and gastric tissues were demineralized in 10% formic acid and 150 mM HCl, respectively. The calcium and phosphorus content was measured in the supernatant according to previously described methods. Fresh tissue was also fixed in 10% buffered formalin, embedded in paraffin, and cut into 3-μm sections. Paraffin-embedded sections of the aorta and stomach were stained by the von Kossa method to evaluate mineralization.

**Evaluation of oxidative stress.** Oxidative stress was assessed by measuring the activity of glutathione peroxidase (GPx) in both plasma and cardiovascular tissue (the aorta and heart) as well as advanced glycation end products (AGEs) in plasma. Tissue samples of the aorta and heart were rinsed with physiological saline solution, frozen, and stored at −80°C until processed. For analysis, tissue samples were thawed and homogenized in 5 ml of cold buffer [50 mM Tris·HCl (pH 7.5), 5 mM EDTA, and 1 mM DTT] per gram of tissue using a Polytron tissuemizer (Kinematica, Luzernerstrasse, Switzerland). Homogenates were centrifuged at 10,000 g for 15 min at 4°C. The supernatant was removed for assay and stored on ice. GPx activity was quantified in plasma and tissue using a GPx assay kit (no. 703102, Cayman Chemical, Ann Arbor, MI). In tissue samples, a portion of the homogenized supernatant was used for the measurement of protein concentration by the Bradford method, and the enzyme activity was normalized for total protein. AGEs were measured in plasma by ELISA (Oxiselect AGE ELISA kit, Cell Biolabs, San Diego, CA) according to the manufacturer’s instructions.

**Blood chemistry.** Blood for chemistry 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 Ca2+/pH analyzer (Ciba-Corning, Essex, UK). Afterward, plasma was separated by centrifugation and stored at −80°C until assayed. PTH levels were quantified according to the vendor’s instructions using a rat PTH 1-84 immunoradiometric assay kit (Immunotopics, San Clemente, CA). Plasma creatinine, phosphorus, glucose, total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, and triglycerides were measured by spectrophotometry (BioSystems, Barcelona, Spain). ELISA tests were used to quantify plasma TNF-α (Biosource, Invitrogen, Carlsbad, CA). A radioimmunoassay was used in plasma samples to determine insulin, leptin, and adiponectin (Millipore, St. Charles, MO). Vitamin D activity was evaluated by measuring plasma concentrations of 1,25-dihydroxyvitamin D (calcitriol) using a radioreceptor assay (IDS kit, Boldon, UK).

In Vitro Experiments

**Vascular smooth muscle cell culture and quantification of calcification.** Human aortic smooth muscle cells (HASMCS) were obtained from Clonetics. Cells were cultured in DMEM supplemented with FBS (20%, Bio Whittaker, Verviers, Belgium), Na pyruvate (1 mmol/l), glutamine (4.5g/l), penicillin (100 U/ml), streptomycin (100 mg/ml), and HEPES (20 mmol/l) at 37°C in a humidified atmosphere with 5% CO2. Cells were used after the fifth passage. HASMCS at 80% confluence were incubated with the medium described above supplemented with phosphate salts (Na2HPO4 and NaH2PO4) at a 1:2 proportion (Sigma-Aldrich, St. Louis, MO) to reach a final concentration of 3.3 mmol/l phosphate. After the incubation period, cells were decalcified by 24 h of incubation in HCl (0.6 mol/l). The amount of calcium in the supernatant was determined using the phenolsulphonphthalein method (QuantiChrom Calcium Assay Kit, BioAssay Systems). Cells were washed three times with PBS (Sigma-Aldrich) and solubilized in 0.1 mol/l NaOH and 0.1% SDS. Cell protein content was measured by a Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). The calcium content was normalized for total protein.

**Antioxidant treatment.** VitE (α-tocopherol succinate, Sigma-Aldrich) was added to the culture at doses of 4 and 40 μmol/l to provide antioxidant treatment. These doses were chosen after preliminary studies in which a dose-response curve was obtained.

**Bone alkaline phosphatase.** For bone alkaline phosphatase measurements, cells were washed three times with PBS and solubilized with 1% Triton X-100 in 0.9% NaCl. Bone alkaline phosphatase was determined by an ELISA kit (human bone alkaline phosphatase ELISA kit, Mybiosource, San Diego, CA) according to the manufacturer’s instructions. Measurements of bone alkaline phosphatase were normalized to total protein determined with the Bio-Rad protein assay solution (Bio-Rad Laboratories).

**Real-time PCR.** Total RNA was isolated from HASMCs using a RNA extraction kit (Rneasy, Qiagen). cDNA was synthesized with a first-strand cDNA synthesis kit (Roche) from 0.5 μg of total RNA in the presence of random hexamers in a final volume of 20 μl. The primers used for PCR amplification were as follows: human core-binding factor-α1 (Cbfa-1), forward 5′-GCAGTTCCCAGCATTTCAT-3′ and reverse 5′-CGGACAATACCGAGGACAT-3′; and human β-actin, forward 5′-GACCTTCTCCAGGCTTCTT-3′ and reverse 5′-ATCCA-CATCTGTTGGAAGGT-3′. Real-time PCR was performed in duplicate with QuantiTect SYBR green PCR (Qiagen) according to the manufacturer’s protocol. All PCR amplifications were carried out using Lightcycler (Roche Molecular Biochemicals). The expression of target genes was normalized to the expression of β-actin. All primers were used equally efficiently.

**Measurement of AGEs and lipid peroxidation.** HASMCs were washed three times and resuspended in PBS. Cells were lysed by sonication. AGEs were measured in lysates by ELISA (Oxiselect AGE ELISA kit, Cell Biolabs, San Diego, CA) according to the manufacturer’s instructions.

**Statistics.** Values are expressed as mean ± SE. Sample size and the power of a contrast of hypothesis were calculated with statistical software (GRANMO, IMIM, Barcelona, Spain). Accepting an α-risk of 0.05 and a β-risk of 0.2 in a one-sided test, a minimum of 10 subjects/group were considered necessary to recognize as statistically significant a difference greater than or equal to 3.5 units. The common SD was assumed to be three, and a drop out rate of 1% was anticipated. 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. A Fisher least-significant-difference test was used as a post hoc procedure. A correlation study...
was carried out using the Pearson test. *P* values of <0.05 was considered significant.

**RESULTS**

**In Vivo Experiments**

C-obese rats ate approximately twice as much and their body weight duplicated that of the C-lean rats. Interestingly, while Nx-lean rats maintained their food intake unchanged, there was a greater reduction in food intake in Nx-obese rats, which was also reflected in their body weight. Plasma creatinine was higher in Nx groups than in control groups. The plasma creatinine concentration was slightly higher in Nx-obese rats (1.27 ± 0.20 mg/dl) than in Nx-lean rats (1.11 ± 0.07 mg/dl), although the differences were not significant (*P* = 0.375). Treatment with VitE did not influence renal function or body weight in either Nx-lean or Nx-obese rats. However, food intake was significantly increased in Nx-obese rats treated with VitE (Table 1).

The plasma glucose concentration was similar in C-lean and C-obese rats; conversely, insulin concentrations were much higher in C-obese rats (10.8 ± 2.8 vs. 1.0 ± 0.1 ng/ml, *P* < 0.01). C-obese rats also had significantly higher levels of total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides than C-lean rats. All these differences between obese and lean rats were also observed in Nx groups. It is interesting to point out that, compared with C-lean rats, Nx-lean rats had higher plasma concentrations of insulin, cholesterol, and triglycerides. Also of interest is the fact that Nx-obese rats had significantly higher cholesterol (total, HDL, and LDL) and lower triglycerides than C-obese rats. Plasma levels of adipokines were higher in obese rats than in lean rats (in both control and Nx groups). Striking differences were found in the control groups in leptin (C-lean rats: 3.9 ± 0.2 ng/ml vs. C-obese rats: 51.9 ± 3.7 ng/ml, *P* < 0.01). These differences were also observed in the Nx groups; however, the changes between control and Nx groups were different in lean and obese rats. Whereas Nx-lean rats showed higher values of leptin and adiponectin than C-lean rats, Nx-obese rats only showed higher levels of leptin (86.4 ± 3.1 ng/ml) than their controls. TNF-α was not higher in obese rats than in lean rats, and after Nx, TNF-α only increased in lean rats (Nx-lean rats: 95.1 ± 6.0 pg/ml vs. C-lean rats: 82.6 ± 1.5 pg/ml, *P* < 0.05). Compared with nontreated rats, Nx-obese rats treated with VitE showed slightly lower concentrations of plasma glucose (185.8 ± 18.2 vs. 209.5 ± 40.6 mg/dl) and insulin (7.0 ± 0.9 vs. 8.6 ± 1.4 ng/ml), but the differences were not significant. TNF-α was significantly (*P* < 0.05) lower in both groups of rats treated with VitE (lean rats: 71.2 ± 4.5 vs. 95.1 ± 6.0 pg/ml and obese rats: 70.3 ± 1.8 vs. 82.1 ± 4.9 pg/ml; Table 2).

In the control groups, the plasma activity of GPx was lower in C-obese rats (800.8 ± 61.6 nmol·min⁻¹·ml⁻¹) than in C-lean rats (847.6 ± 11.1 nmol·min⁻¹·ml⁻¹), although the differences did not reach statistical significance. Along the same line, plasma AGES were slightly higher in C-obese rats (185.1 ± 22.2 μg/mg protein) than in C-lean rats (174.9 ± 8.9 μg/mg protein). The induction of uremia resulted in a decrease of plasma GPx activity (compared with control rats). Moreover, plasma GPx was significantly lower (*P* < 0.05) in Nx-obese rats (217.2 ± 18.2 nmol·min⁻¹·ml⁻¹) than in Nx-lean rats (382.3 ± 15.5 nmol·min⁻¹·ml⁻¹). After VitE administration, a high plasma activity of GPx was observed in Nx-obese rats, with values (388.3 ± 78.1 nmol·min⁻¹·ml⁻¹) that were not different from those found in Nx-lean rats (Fig. 1A). In addition, GPx activity was found to be higher in the aorta (124.7 ± 8.9 vs. 91.1 ± 11 nmol·min⁻¹·mg⁻¹ protein, *P* < 0.05) and in the heart (110.1 ± 6.9 vs. 20 ± 7.9 nmol·min⁻¹·mg⁻¹ protein, *P* < 0.001) of Nx-obese rats treated with VitE than in untreated Nx-obese rats. A nonsignificant increase in AGES was detected after Nx in uremic rats (Nx-obese rats: 245.1 ± 17.2 μg/mg protein and Nx-lean rats: 237.2 ± 14.9 μg/mg protein). Rats treated with VitE showed a tendency to decreased AGES that reached significance (*P* < 0.05) in obese rats (Nx-obese + VitE rats: 151.9 ± 25.1 μg/mg protein and Nx-lean + VitE rats: 146.8 ± 62 μg/mg protein).

**Table 1. Renal function, body weight, and food intake in the study groups**

<table>
<thead>
<tr>
<th></th>
<th>C-lean</th>
<th>Nx-lean</th>
<th>Nx-lean + VitE</th>
<th>C-obese</th>
<th>Nx-lean</th>
<th>Nx-obese</th>
<th>Nx-obese + VitE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.54 ± 0.04a</td>
<td>1.11 ± 0.07b</td>
<td>1.16 ± 0.2b</td>
<td>0.45 ± 0.02a</td>
<td>1.27 ± 0.20b</td>
<td>1.29 ± 0.08b</td>
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<tr>
<td>Body weight, g</td>
<td>251 ± 19a</td>
<td>286 ± 18a</td>
<td>238 ± 24a</td>
<td>503 ± 5b</td>
<td>440 ± 20b</td>
<td>452 ± 6b</td>
<td></td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>15.0 ± 1.1a</td>
<td>14.7 ± 1.2a</td>
<td>14.5 ± 3.0b</td>
<td>26.5 ± 0.4b</td>
<td>10.6 ± 1.6b</td>
<td>14.6 ± 0.9b</td>
<td></td>
</tr>
</tbody>
</table>

Data with different superscripts are significantly (*P* < 0.05) different.

**Table 2. Blood biochemistry in the study groups**

<table>
<thead>
<tr>
<th></th>
<th>C-lean</th>
<th>Nx-lean</th>
<th>Nx-lean + VitE</th>
<th>C-obese</th>
<th>Nx-lean</th>
<th>Nx-obese</th>
<th>Nx-obese + VitE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mg/dl</td>
<td>189.0 ± 3.7a</td>
<td>168.2 ± 7.6b</td>
<td>162.5 ± 8.5b</td>
<td>183.8 ± 12.5b</td>
<td>209.5 ± 40.6b</td>
<td>185.8 ± 18.2b</td>
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</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.0 ± 0.1a</td>
<td>2.4 ± 0.5a</td>
<td>1.9 ± 0.6a</td>
<td>10.8 ± 2.8b</td>
<td>8.6 ± 1.4a</td>
<td>7.0 ± 0.9a</td>
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<tr>
<td>Glucose/insulin</td>
<td>239.7 ± 30.5a</td>
<td>136.5 ± 48.8b</td>
<td>165.1 ± 59.0b</td>
<td>24.2 ± 4.1a</td>
<td>22.2 ± 3.4a</td>
<td>27.9 ± 3.9a</td>
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</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>67.3 ± 1.0a</td>
<td>90.4 ± 6.1b</td>
<td>66.0 ± 4.6b</td>
<td>113.0 ± 3.2a</td>
<td>199.8 ± 14.1b</td>
<td>222.8 ± 19.9b</td>
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<tr>
<td>High-density lipoprotein-cholesterol, mg/dl</td>
<td>26.0 ± 0.4a</td>
<td>32.4 ± 0.6b</td>
<td>28.0 ± 2.2b</td>
<td>42.6 ± 0.9b</td>
<td>56.2 ± 3.5d</td>
<td>65.2 ± 3.7d</td>
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<tr>
<td>Low-density lipoprotein-cholesterol, mg/dl</td>
<td>5.2 ± 0.4a</td>
<td>9.3 ± 0.8b</td>
<td>6.7 ± 1.6a</td>
<td>10.3 ± 1.3b</td>
<td>24.3 ± 3.4e</td>
<td>27.1 ± 4.2e</td>
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</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>49.7 ± 2.7a</td>
<td>60.4 ± 15.5a</td>
<td>88.5 ± 33.5a</td>
<td>709.4 ± 90.0b</td>
<td>236.2 ± 72.3a</td>
<td>296.7 ± 75.7a</td>
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<tr>
<td>Leptin, ng/ml</td>
<td>3.9 ± 0.2a</td>
<td>5.1 ± 0.5a</td>
<td>4.3 ± 0.7a</td>
<td>51.9 ± 3.7a</td>
<td>82.0 ± 3.9a</td>
<td>82.9 ± 4.3c</td>
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</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>2.2 ± 0.3a</td>
<td>6.9 ± 0.9b</td>
<td>6.9 ± 1.4a</td>
<td>5.5 ± 1.3b</td>
<td>3.9 ± 1.0b</td>
<td>5.2 ± 1.1b</td>
<td></td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>82.6 ± 1.5a</td>
<td>95.1 ± 6.0a</td>
<td>71.2 ± 4.5a</td>
<td>87.0 ± 5.3b</td>
<td>82.1 ± 4.9a</td>
<td>70.3 ± 1.8a</td>
<td></td>
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</tbody>
</table>

Data with different superscripts are significantly (*P* < 0.05) different.
calcium (correlation was found between plasma phosphate and either...calcium, PTH, and calcitriol) were very similar in...calcitriol). *P < 0.05 vs. Nx-obese rats.

None of the control groups (lean and obese) showed any degree of extraskeletal calcification. In contrast, vascular calcification was observed in the two groups of Nx rats, although the severity of calcification was very different in lean and obese rats. The aortic calcium concentration was significantly higher (P < 0.05) in Nx-obese rats (10.0 ± 2.1 mg/g tissue) than in Nx-lean rats (3.6 ± 1.3 mg/g tissue; Fig. 1B). Accordingly, a higher aortic phosphorus concentration was also observed in Nx-obese rats (16.4 ± 5.2 vs. 1.4 ± 0.9 mg/g tissue, P < 0.05). Vascular calcification was decreased in rats treated with VitE. Thus, aortic calcium in Nx-obese + VitE rats was 4.6 ± 1.3 mg/g tissue (P < 0.05 vs. untreated Nx-obese rats; Fig. 1B). Phosphorus was also lower in the aorta of rats treated with VitE (9.5 ± 3.8 mg/g of tissue) than in untreated rats (16.4 ± 5.2 mg/g tissue). Blood parameters of mineral metabolism (calcium, PTH, and calcitriol) were very similar in Nx-lean and Nx-obese rats. Plasma phosphate was significantly (P < 0.05) higher in Nx-obese rats (11.4 ± 0.7 mg/dl) than in Nx-lean rats (8.2 ± 0.4 mg/dl; Fig. 1C). When the data of Nx-obese and Nx-lean were pooled together, no significant correlation was found between plasma phosphate and either calcium (r = 0.393, not significant) or phosphorus (r = 0.268, not significant) content in the aorta. Interestingly, when the correlation study was done separated by groups, plasma phosphate was correlated with aortic calcium in Nx-lean rats (r = 0.620, P = 0.032) but not in Nx-obese rats (r = −0.037, P = 0.904). Treatment with VitE did not affect parameters of mineral metabolism.

The concentration of mineral deposits in other soft tissues was also significantly higher in Nx-obese rats than in Nx-lean rats. Thus, the stomach calcium content was 1.5 ± 0.2 mg/g tissue in Nx-obese rats versus 0.6 ± 0.1 mg/g tissue in Nx-lean rats (P = 0.003), and the gastric phosphorus concentration was 1.2 ± 0.2 mg/g tissue in Nx-obese rats versus 0.5 ± 0.1 mg/g tissue in Nx-lean rats (P = 0.018). Significantly lower calcium (0.9 ± 0.3 vs. 1.4 ± 0.2 mg/g tissue) and phosphorus (0.8 ± 0.1 vs. 1.3 ± 0.3 mg/g tissue) content were measured in the stomach of Nx-obese rats treated with VitE.

Differences in extraskeletal calcification between obese and lean rats were also evident by histology. Figure 2 shows von Kossa-stained sections of the aorta and stomach of Nx-obese and Nx-lean rats without and with VitE treatment. von Kossa-stained (brown) mineral deposits in the aorta correlated with the calcium content, as shown in Fig. 1. Thus, moderate mineral deposition was detected by von Kossa staining in the tunica media of the Nx-lean group, where calcium deposits showed a patchy concentric pattern along the entire medial circumference. Treatment with VitE reduced von Kossa staining in uremic rats, and the differences were much more evident in Nx-obese rats. In addition to the difference in the severity of mineral deposits, it is interesting to note that, in the vasculature, calcium was deposited almost exclusively in the tunica media. Similar differences in the severity of mineral deposits in stomach wall were also evident between groups by von Kossa staining (Fig. 2).

**In Vitro Experiments**

In vitro experiments were performed to elucidate the role of VitE on HVSMCs cultured with high phosphate and to isolate the effects of oxidative stress from other potential confounding factors that may be present in the in vivo experiments. Incubation in high-phosphate medium is known to increase oxidative stress and to induce transdifferentiation of HVSMCs to an osteogenic phenotype (30). As shown in Fig. 3, incubation of HVSMCs in a high-phosphate medium for 9 days resulted in calcification, increased expression of Cbfa-1, and increased bone alkaline phosphatase. Compared with the high-phosphate group, treatment with VitE decreased HVSMC calcification from 3.0 ± 0.5 to 0.4 ± 0.2 μg calcium/mg protein (4 μM VitE, P < 0.001) and 0.5 ± 0.2 μg calcium/mg protein (40 μM VitE, P < 0.001). Treatment with VitE also decreased Cbfa-1 and bone alkaline phosphatase expression in a dose-dependent manner. Thus, Cbfa-1 decreased from 2.8 ± 0.2 to 1.2 ± 0.1 mRNA Cbfa-1/β-actin (4 μM VitE, P < 0.05) and 0.7 ± 0.1 mRNA Cbfa-1/β-actin (40 μM VitE, P < 0.001) and bone alkaline phosphatase decreased from 3,358 ± 76 to 1,536 ± 132 ng/mg protein (4 μM VitE, P < 0.001) and 1,021 ± 98 ng/mg protein (40 μM VitE, P < 0.001; Fig. 3).

The effect of high phosphate and VitE on oxidative stress was assessed in HVSMCs by measuring AGEs and LPO through the incubation period. An increase in AGEs and LPO was identified at the earlier stages of incubation. Later on, these...
parameters were progressively reduced and tended to normalize at the end of the incubation period (9 days). Treatment with VitE significantly reduced AGEs (17.7 ± 1.5 vs. 35.9 ± 2.3 µg/mg protein, *P* < 0.001) in HVSMCs cultured for 24 h and LPO (0.9 ± 0.2 vs 1.9 ± 0.2 µM MDA/mg protein, *P* < 0.001) in cells cultured for 48 h (Fig. 4).

DISCUSSION

This study was designed to investigate the effect of obesity/metabolic syndrome on extraosseous calcifications in a uremic rat model. Our results demonstrate that obese uremic rats, which are subjected to oxidative stress, develop more severe calcifications than lean uremic rats. Treatment with the antioxidant VitE significantly decreases calcifications in obese uremic rats. Moreover, in vitro VitE reduces oxidative stress and prevents calcification of HVSMCs.

Nx-obese rats had slightly higher plasma creatinine concentrations than Nx-lean rats. Thus, it could be argued that the more severe calcifications could be related to lesser renal function. We believe that this is not the case and that the higher creatinine levels are in fact a consequence of nephrocalcinosis (21). To test this hypothesis, two subgroups (*n* = 10 each) of rats with comparable creatinine levels (range: 0.8–1.3 mg/dl) were selected. Even though in these subgroups plasma creatinine was lower in Nx-obese rats (0.97 ± 0.07 mg/dl) than in Nx-lean rats (1.12 ± 0.05 mg/dl), aortic calcium was still significantly higher (*P* < 0.05) in Nx-obese rats (7.4 ± 1.3 mg/g tissue) than in Nx-lean rats (2.8 ± 1.1 mg/g tissue).

When parameters of mineral metabolism were studied, the only difference between Nx-lean and Nx-obese rats was a more elevated plasma phosphate in the latter group. The higher phosphate was likely related to the more impaired renal function secondary to calcium deposition in the remnant kidney. Again, when subgroups with similar phosphate levels were compared (obese rats: 9.1 ± 0.4 mg/dl vs. lean rats: 8.2 ± 0.4 mg/dl), aortic calcium was also higher in Nx-obese animals (8.1 ± 1.6 mg/g tissue) than in Nx-lean rats (3.6 ± 1.3 mg/g tissue). The lack of correlation between plasma phosphate and aortic mineral content provides further support to the contention that the slightly higher plasma phosphate in obese rats contributes only partially to the development of the severe calcifications found in this study. Moreover, whereas plasma phosphate correlated with calcification parameters in Nx-lean rats, it did not correlate at all in Nx-obese rats. Taken together, these data reinforce the concept that the metabolic derangements associated with obesity play an independent role in the development of soft tissue mineralization in Nx-obese rats.

The mechanisms responsible for the severe calcifications observed in obese rats are difficult to elucidate, and, most likely, these calcifications are the final result of many interrelated metabolic alterations. From the biochemical data gathered in this study, the most important differences in plasma biochemistry between obese and lean rats can be focused on leptin, insulin, the lipid profile, and GPx.

Leptin has been shown to promote osteoblast differentiation and mineralization of primary cultures of VSMCs and calcifying vascular cells (26). In vivo, leptin is thought to increase cardiovascular risk by promoting osteogenic differentiation and thus vascular calcification in apolipoprotein E-deficient mice (36). Moreover, in humans, plasma leptin levels have been found to be associated with coronary artery calcification (16). The obese Zucker rat has a mutation in the leptin receptor gene that results in reduced affinity for leptin and impaired signal transduction (9). Thus, even though it has been demonstrated that Zucker rats retain some ability to respond to leptin (24), the influence of hyperleptinemia on the calcifications found in the present study is likely small.

Although the clinical association between diabetes and vascular calcification is well established (20), the direct relationship between insulin and VSMC calcification is somewhat
controversial (25, 32). Recent evidence indicates that insulin promotes osteoblastic differentiation of VSMCs by increasing receptor activator of NF-κB ligand (RANKL) expression through ERK1/2 activation (35).

Dyslipidemia should have contributed to the severity of the calcifications in Nx-obese rats. It is interesting to note that the histological experiments clearly showed that the calcification is restricted to the tunica media (arteriosclerosis) without evidence of involvement of the tunica intima (atheromatosis). Patients with advanced CKD show both intimal and medial calcifications (2), although arteriosclerosis seems to occur earlier in the course of the disease without being associated with lipid or cholesterol deposition (11). High-fat diets associated with hyperinsulinemic diabetes have been reported to activate an aortic osteoblast transcriptional regulatory program that is independent of intimal atheroma formation (31).

Major differences between obese and lean rats were observed in oxidative stress. The decrease of plasma GPx activity found in obese rats indicates that these animals are subjected to severe oxidative stress. Increased oxidative stress is a well-known complication of obesity and metabolic syndrome (29).

Although oxidative stress has been linked to the pathogenesis of arterial diseases (13), its influence on vascular calcification has not been thoroughly explored. Oxidant agents, including some uremic toxins, have been reported to promote osteoblastic differentiation of VSMCs (22, 23). Thus, we hypothesized that oxidative stress would be a major contributor to the severe calcifications observed in obese uremic rats and that treatment with an antioxidant would reduce vascular calcifications. Our results demonstrate that administration of VitE, one of the best-known natural antioxidants, significantly reduces the degree of calcification in obese rats. Moreover, the decrease in calcification is accompanied by a simultaneous increase in GPx activity, not only in plasma but also in cardiovascular tissue. We theorize that the molecular mechanism linking VitE supplementation and increase in GPx is based on their synergistic effects to prevent peroxidative injury. Thus, while glutathione, through an interaction with GPx, can regenerate VitE, VitE prevents inactivation of GPx through lipid peroxyl radical scavenging.

Although based on these data it seems clear that the effect of VitE is linked to its antioxidant action, it is important to note that VitE also reduced TNF-α levels in uremic rats. We and others (1, 12) have shown that elevated TNF-α promotes vascular calcification (1, 12). Thus, an additional antiinflammatory effect of VitE, which would be beneficial to prevent calcification, cannot be excluded. Nonetheless, it should be noted that oxidative stress and inflammation are closely related and that they mutually amplify their actions within the vasculature (13).

Fig. 3. Human vascular smooth muscle cells (HVSMCs) cultured for 9 days with normal phosphate (control), high phosphate (HP; 3.3 mM), and HP plus VitE (4 and 40 μM). A: total calcium content. B: gene expression of core-binding factor-α1 (Cbfa-1). C: levels of bone alkaline phosphatase (BALP). Shown are the results of three experiments in triplicate. *P < 0.05 vs. control; †P < 0.05 vs. HP.

Fig. 4. HVSMCs cultured with normal phosphate (control), HP (3.3 mM), and HP plus VitE (40 μM). A: advanced glycation end products (AGEs) at 24 h. B: malondialdehyde (MDA) concentrations as an indicator of lipid peroxidation at 48 h. Shown are the results of two experiments in triplicate. *P < 0.05 vs. control; †P < 0.05 vs. HP.
We performed in vitro experiments to determine whether VitE, independent of its anti-inflammatory effect, was able to directly decrease calcification of VSMCs. These experiments revealed that the reversal of oxidative stress by addition of VitE has a beneficial effect on HVSMC calcification. Our results confirm that HVSMCs incubated in a high-phosphate medium are subjected to significant oxidative stress, as evidenced by the increased cellular content of AGEs and MDA. In vitro, these changes, which may be transient, arise before phenotype switching of TNFα to osteoblast-like cells, initiating a cascade of events leading to vascular calcification. The temporary sequencing of changes in oxidative stress pose an additional difficulty when investigating oxidative damage and may have influenced the different sensitivity of the parameters (GPx vs. AGEs) measured in the in vivo experiments.

Previous studies (27, 30, 37) have demonstrated the ability of high phosphate to induce the production of ROS in VSMCs. Furthermore, incubation of these cells with hydrogen peroxide (in the presence of basal phosphate concentration) promotes the switch of VSMCs from a contractile phenotype to an osteogenic phenotype (4). Byon et al. (4) reported that oxidative stress activates the Akt/phosphatidylinositol 3-kinase signaling pathway, inducing Cbfα-1 upregulation and VSMC mineralization. Sutra et al. (30) have shown that oxidative stress can activate Cbfα-1 via ERK 1/2 in VSMCs. As described above, insulin also promotes osteoblastic differentiation of VSMCs by increasing RANKL expression through ERK1/2 activation (35). Thus, oxidative stress appears as a common step in the calcification pathway including insulin resistance, lipid abnormalities, and inflammation. We found that treatment with VitE prevents the increase in markers of oxidative stress, osteoblastic transdifferentiation, and calcification in VSMCs. VitE has been related to the dephosphorylation of Akt, resulting in the inhibition of downstream signaling (15) implicated in the upregulation of Cbfα-1. Interestingly, in our study, VitE downregulated Cbfα-1 expression in VSMCs cultured with high phosphate. Taken together, these findings suggest that oxidative stress plays a key role in the early signaling of VSMC calcification.

In conclusion, our results demonstrate that uremic obese rats develop more severe calcifications than uremic lean rats and that VitE reduces oxidative stress and vascular calcifications in both obese rats and cultures of HVSMCs.

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AUTHOR CONTRIBUTIONS


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