Regression of Medial Elastocalcinosis in Rat Aorta
A New Vascular Function for Carbonic Anhydrase

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Background—We sought to determine whether carbonic anhydrase (CA), which plays an important role in bone resorption, contributes to vascular mineral loss induced by an endothelin receptor antagonist.

Methods and Results—Wistar rats were compared with rats receiving warfarin and vitamin K\(_1\) (WVK) for 8 weeks alone or in association with the endothelin receptor antagonist darusentan (30 mg/kg per day), the CA inhibitor acetazolamide (100 mg/kg per day), or both for the last 4 weeks. Rats were also treated with WVK for 5 or 6 weeks, and darusentan was added for the last week or last 2 weeks of treatment, respectively. Treatment with WVK produced medial elastocalcinosis in the aorta and carotid arteries. Immunohistochemistry revealed that CA II was already abundant in the adventitia and in calcified areas of aortic sections from WVK-treated rats. Darusentan did not significantly modify its abundance or distribution. In contrast, CA IV immunostaining, which was weak in WVK-treated rats, became apparent after 1 week of darusentan treatment and declined toward basal levels thereafter. These findings were confirmed by a parallel increase in CA IV protein abundance and activity in the aorta. The mineral loss induced by darusentan was blunted by acetazolamide treatment, confirming the functional relevance of the biochemical findings. Moreover, CA IV immunostaining was enhanced much later in the carotids, where darusentan did not cause regression of elastocalcinosis.

Conclusions—Vascular mineral loss induced by the blockade of endothelin receptors seems dependent on the activation of membrane-bound CA IV, suggesting that mineral loss may proceed via local changes in pH similar to that seen in bone resorption. (Circulation. 2005;112:1628-1635.)

Key Words: aging ■ carbonic anhydrases ■ endothelin ■ vasculature ■ calcification

Arterial medial elastocalcinosis (or medial arterial calcification [MEC]) is characterized by the deposition of calcium-phosphate mineral within elastic lamellae of arteries. This ubiquitous process generally appears after 50 years of age\(^1\) but is accelerated by hypertension,\(^2\) diabetes,\(^3\) and end-stage renal disease.\(^4\) Vascular MEC contributes to the increase in rigidity of large arteries observed with aging and thus to the general process of arteriosclerosis. Arterial stiffening leads to an elevation of systolic blood pressure (SBP) and pulse pressure (PP) and contributes to the development of arterial stiffening.\(^5\) Epidemiological studies have shown SBP and PP to be strong predictors of cardiovascular events, especially in patients aged >55 years.\(^7\) Although the control of diastolic blood pressure (DBP) can be obtained in large populations, the control of SBP and PP is achieved to a much lesser extent.\(^8\) We postulate that drugs devised to reduce MEC could be of therapeutic value by reducing arterial stiffness.

The pathological process of MEC is still poorly understood, and, until recently, elastocalcinosis was considered to be a passive process that occurred with time and was associated with damaged elastic fibers. However, substantial new evidence now shows that vascular calcification is an active phenomenon that is controlled by the expression of growth factors, matrix, proteins, and bone-related proteins.\(^9\)–\(^11\) As an example, mice that lack matrix Gla protein (MGP), a noncollagenous matrix protein secreted by vascular smooth muscle cells, develop to term but die within 2 months as a result of massive elastocalcinosis that leads to aortic rupture,\(^12\) indicating that MGP is an important endogenous inhibitor of vascular calcification. Interestingly, warfarin inhibits the vitamin K-dependent gamma carboxylation of MGP, and chronic treatment with warfarin leads to elastocalcinosis,\(^13\) arterial stiffening, and PP elevation in rats.\(^14\) In this model of ISH, we have previously shown that treatment with the endothelin receptor antagonist darusentan induced mineral loss in calcified arteries.\(^15\) Others have also demonstrated that endothelin could be involved in vascular calcification.\(^16\)

In bone, dissolution of mineral (during resorption) is a physiological function performed by osteoclasts, which are specialized cells producing a polarized secretion of H\(^+\) to
release calcium and phosphate from the inorganic mineral phase (hydroxyapatite).\(^{17}\) Proteins are derived from carbonic acid, generated by the abundant carbonic anhydrase (CA) isoenzyme II catalyzing the reversible hydration of CO\(_2\): CO\(_2\) + H\(_2\)O ↔ H\(_2\)CO\(_3\) ↔ HCO\(_3\)\(^-\) + H\(^+\).\(^{18}\) In a previous study we were unable to detect the presence of macrophages or osteoclast-like cells within the calcification areas.\(^{19}\) In vascular smooth muscle cells, CA II (cytoplasmic) and IV (membrane bound) are constitutively present, and their presence and activity have been proposed to explain the hypotensive effect of thiazide diuretics.\(^{20}\) The aim of the present study was to determine whether the regression of MEC induced by an endothelin receptor antagonist is mediated by the local activation of CA.

### Methods

#### Animals and Experimental Protocol

Age-matched control male Wistar rats (initial weight of 175 to 200 g or 6 to 7 weeks of age; Charles River Canada, St-Constant, Quebec, Canada) were compared with rats receiving warfarin (20 mg/kg per day in the drinking water) and vitamin K\(_\text{I}\) (phyloquinone, 15 mg/kg per day SC; ICN Biomedicals Inc) (WKV) for 4 or 8 weeks. Darusentan (an endothelin receptor antagonist, 30 mg/kg per day mixed in the chow; Knoll AG), acetazolamide (CA inhibitor, 100 mg/kg per day mixed in the chow), or both were administered from weeks 5 to 8 in WKV-treated animals (groups +DAR(5–8), +ACTZ(5–8), +DAR+ACTZ(5–8), respectively). Additional WKV-treated rats were administered darusentan for 1 (DAR(5) group) or 2 weeks (DAR(5–6) group). Each group contained at least 8 rats (Table). Finally, in an additional 16 rats, the WKV treatment was stopped after 4 weeks, and animals were killed 4 [placebo(4–8) group] or 18 weeks later [placebo(4–22) group]. Animal experiments were approved by the Animal Care and Use Committee of Université de Montréal.

#### Hemodynamic Parameters

At the end of the treatments, animals were anesthetized (pentobarbital 65 mg/kg IP), and a short PE-50 catheter was inserted into the left femoral artery and pushed into the distal abdominal aorta. The catheter was connected to a pressure transducer to allow the measurement of DBP and SBP and heart rate. Mean arterial pressure and PP were calculated from these values.

#### Immunohistochemistry and Histology

Animals were killed by decapitation under general anesthesia, and the aorta and carotid arteries were harvested. A portion of thoracic aorta and the left carotid were frozen at −80°C for subsequent calcium, protein expression (Western blot), and CA activity (only for aorta) evaluation. Another portion of thoracic aorta (5 mm long) and the right carotid artery were immediately fixed in 4% cacodylate-buffered paraformaldehyde and embedded in paraffin blocks. Seven-micrometer-thick aortic and carotid artery sections were mounted on glass slides and deparaffinized with xylene, followed by hydration in graded concentrations of ethanol (from 100% to 50%). Each slide-mounted tissue section was incubated overnight at room temperature with primary antibody against CA II (AB1828, Chemicon International) or CA IV (N-16, Santa Cruz Biotechnology Inc). Antibodies were used at a dilution of 1:200 (CA II) or 1:50 (CA IV) in 0.2% bovine serum albumin Tween-20/saline buffered saline containing 0.5% of appropriate normal serum (goat or rabbit). After washing, primary antibodies were detected with appropriate secondary antibodies conjugated to biotin. Biotin was detected with the use of a Vectastain ABC-AP Kit (Vector Laboratories Inc), and color development was achieved with Fast Red TR/Naphthol AS-MX phosphate alkaline phosphatase substrate with 1 mmol/L levamisole. The sections were counterstained with warm (57°C) filtered methyl green (Vector Laboratories) for 5 minutes. Adjacent 4-μm-thick slices were stained with von Kossa reagent to localize mineral deposition. Slides from each group were used as negative controls by omitting the primary antibody. Mandibular incisor and molar teeth from untreated rats were used as positive controls. Coverslips were mounted onto glass slides with the use of Kaiser’s glycerol jelly mounting medium.

#### Western Blot Analysis

Aortic levels of CA II and IV (n=4 to 7 per group) were quantified by Western blot analysis with the use of extraction conditions for membrane proteins: 0.6% sodium dodecyl sulfate (SDS), 5% Triton X-100, 0.1% aprotinin, and 0.25% phenylmethylsulfonyl fluoride in phosphate-buffered saline (pH 7.4). Equal quantiles of proteins (20 μg for CA II and 40 μg for CA IV) were separated on 12% SDS-polyacrylamide gels. Proteins were detected with specific antibodies for CA II (2 μg/mL; clone M-14; Santa Cruz Biotechnology) and CA IV (2 μg/mL; clone N-16; Santa Cruz Biotechnology).
CA Activity Measurement
Soluble and membrane-bound CA (II and IV, respectively) were separated by a method described previously. In brief, aortas were homogenized in Sato’s buffer (25 mmol/L triethanolamine, pH 8.1, 59 mmol/L Na2SO4, 1 mmol/L benzamidine chloride), and samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatants were centrifuged at 28,000 rpm (141,000 m) for 90 minutes at 4°C to precipitate all membrane-bound CA. Supernatants were then used to measure cytosolic CA activity (n=6 to 10 per group). Optimal dissolution of the precipitates was obtained with a 1-hour incubation at room temperature in a mixed detergent solution (10% SDS and 0.2% saponin in Sato’s buffer) with constant agitation followed by centrifugation (13,000 rpm, 30 minutes, 15°C) to precipitate free proteins. These supernatants were used to measure membrane-bound CA activity (n=7 per group).

CA activity was measured by a colorimetric method with the use of imidazole-Tris buffer and ρ-nitrophenol as the indicator as described previously. One enzyme unit is the activity that halves the reaction time in 1-mL reaction vessel containing 500 μL buffer. For the present experiment, a microassay was performed, with 50 μL of sample and 50 μL of buffer. Therefore, the number of enzyme units was corrected for the smaller volume, as described by Maren. The CA activity was expressed as enzyme units per milligram of protein.

Calcium Determination
Aortas and carotid arteries were dried at 55°C in heating blocks and extracted with 10% formic acid (30 L/mg of dried tissue) overnight at 4°C. Calcium levels were determined by colorimetry through a reaction with α-cresolphthalein complexone (Sigma).

Micro-CT
Aortic pieces were submitted to micro-CT to visualize mineral deposition. X-ray scans of aorta were performed on a standard desktop micro-CT instrument from Skyscan (model 1072). This instrument has an 80-Kev sealed, air-cooled, microfocus x-ray source with a polychromatic beam derived from a tungsten target and has a spot size of <0.8 μm. For these analyses, the x-ray source was operated at maximum power (80 KeV) and at 100 μA. Images were captured with a 12-bit, cooled CCD camera (1024×1024 pixels) coupled by a fiber-optic taper to the scintillator.

Chemically fixed aortas were folded (to fit in the chamber) and wrapped in plastic film (to prevent drying) and scanned by micro-CT at a magnification resulting in a pixel size of 8.25 μm. With a rotation step of 0.9 degrees, the total scanning time was 35 minutes for each rotated aorta, after which ≈300 cross sections (slice-to-slice distance of 16.5 μm) were reconstructed to give a 3-dimensional distribution of the mineral in the aortas. Three-dimensional reconstructions were performed with the use of Skyscan tomography software based on triangular surface rendering.

Drugs and Statistical Analysis
All drugs were purchased from Sigma Chemical Co unless specified otherwise. Values are expressed as mean±SEM. Statistical analyses were performed by ANOVA, and, when significant (P<0.05), a Bonferroni post hoc test was done. Significance was obtained for P<0.05 per number of comparisons (eg, for 4 comparisons, the probability value for each comparison had to be <0.0125). When variance was not homogeneous among groups, ie, Bartlett test <0.05 (CA II and CA IV activity and carotid calcium content), or when sample size was too small (CA II and CA IV Western blot), a nonparametric Kruskal-Wallis test followed by a Dunn multiple comparison test was performed (P<0.05 was considered significant). For all tests, a priori selected comparisons were control versus WVK and WVK plus treatment versus WVK.

Results
Localization, Quantification, and Activity of CA II and CA IV
Immunohistochemistry of CA II revealed that the enzyme was abundant in the adventitia of aortic and carotid artery sections but barely detectable in the media and intima (Figure 1A and 1B). In aortic sections from WVK-treated rats, CA II immunostaining was also observed in the calcified (undulated) areas (boxed regions), but this was not true in carotid arteries (Figure 1C and 1D). Treatments with darusentan did

Figure 1. Immunolocalization of CA II in control rats at 8 weeks [control(8)] (A and B); 8 weeks of warfarin/vitamin K1 treatment [WVK(8)] (C and D); and 8 weeks of WVK combined with darusentan treatment for the last 4 weeks [+DAR(5–8)] (E and F). Thoracic aortas (A, C, and E) and carotid arteries (B, D, and F) were fixed in aldehyde, embedded in paraffin, and analyzed immunohistochemically for CA II localization. Typical calcified areas are boxed (C and E). Lum indicates lumen; Med, media; and Adv, adventitia.
not modify this pattern of expression in aortic sections (Figure 1E) but enhanced CA II expression in the media of carotid arteries (Figure 1F).

CA IV had a low level of expression in aortic and carotid sections from control and WVK-treated rats (Figure 2A to 2D). In contrast, CA IV was abundant throughout the aortic sections of rats treated for 1 week with darusentan (Figure 2E) and gradually returned toward a basal level after 2 and 4 weeks of this treatment (Figure 2G and 2I). In contrast, the increased abundance of CA IV occurred only after 4 weeks of darusentan treatment in carotid arteries (Figure 2F, 2H, and 2J). Sections from paraffin-embedded hemimandibles containing both molar and incisor teeth were used as positive controls and showed the expected distribution of CA in the maturation-stage enamel organ (data not shown). In the enamel organ, cells positive for CA II and IV included maturation-stage ameloblasts and the cells of the papillary layer. As negative control, we used aortic sections from rats of all groups and omitted the primary antibody. All those sections were negative in terms of red staining (not shown).

The immunohistochemistry results were confirmed by Western blot analysis. The amount of CA II tended to increase by the WVK treatment (Figure 3A). Darusentan given for 1 or 2 weeks did not have a significant effect on CA II expression, although there was a tendency for an increase after 4 weeks of treatment ($P > 0.05$; Figure 3A). The WVK treatment had no effect on CA IV protein expression, but darusentan increased it significantly after 1 week of administration (Figure 3B). The amount of CA IV protein returned to basal level after 2 and 4 weeks of darusentan treatment.

Eight weeks of WVK treatment had no effect on cytosolic and membrane-bound CA activities compared with control rats (Figure 4A and 4B). The addition of darusentan had no significant effect on cytosolic CA activity (Figure 4A). Basal membrane-bound CA activity was lower than that of the soluble isoenzyme. There was a 4.4-fold increase of membrane-bound activity after 1 week of darusentan that gradually receded to a nonsignificant 1.8- and 1.4-fold after 2 and 4 weeks of treatment, respectively (Figure 4B).
Calcium Content in Aorta and Carotid Artery

As shown in Figure 5, treatment of rats with WVK for 4 weeks produced a maximal elevation of calcification that plateaued when the treatment was prolonged for an additional 4 weeks. The administration of darusentan for 4 weeks caused regression of the amount of calcium present in the vascular wall. However, at the earlier time points (1 and 2 weeks), no changes in calcium content were observed. Although the CA inhibitor acetazolamide had no effect on calcification when used alone, it completely blunted the effect of darusentan (Figure 5). Rats given the 4-week WVK treatment followed by no treatment for 4 or 18 weeks showed a very slow regression trend for a decrease to aortic calcium content: WVK(4), 12.7 ± 1.7 μg/mg of proteins; placebo(5–8), 12.2 ± 3.1; placebo(5–22), 6.7 ± 1.1 [P < 0.05 versus WVK(4)].

Carotid arteries were affected differently than were aortas by the WVK treatment (Table). Calcium content continued to rise from week 4 to week 8, to reach values 4 times higher than in the aorta. In addition, darusentan did not induce any mineral loss when administered from the beginning of week 5 to week 8. The von Kossa staining showed that calcification in both the aorta and the carotid arteries was located in the media. In the aorta, calcification was limited to elastic lamellae close to the adventitia, which displayed large undulations, suggesting an altered vascular architecture (Figure 6A). In contrast, carotid arteries exhibited calcification throughout the media without large undulations (Figure 6B). The micro-CT data confirmed that the abundance of calcification was greater in the carotid arteries and also revealed the patchy appearance of MEC (Figure 6C).

Hemodynamic Parameters

PP was significantly increased by the WVK treatment after 4 and 8 weeks, mainly attributable to an elevation of SBP (Table). All treatments reduced PP to levels halfway between control and WVK values, with the exception of the 2-week darusentan administration, because of greater variability in the data (Table).

Discussion

The major finding of this study, in which we used an experimental model of MEC, is the identification of a novel
functional role for membrane-bound CA, most likely CA IV, in the control of vascular calcification. Moreover, we have shown that the enzyme is regulated by the endogenous endothelin system.

Ectopic calcification (including MEC), once it occurs, has generally been considered an irreversible phenomenon, although many studies show that it can be prevented. Molecules that inhibit bone resorption, such as bisphosphonates, and antihypertensive drugs, including endothelin receptor antagonist, have been shown to prevent MEC. Moreover, glutaraldehyde-fixed aortic valve (GFAV) leaflets showed accelerated and 4- to 5-fold greater calcification after subcutaneous implantation into osteopontin-null and heterozygote mice compared with wild-type controls, suggesting that endogenous osteopontin normally inhibits calcification. In this last study, a time-dependent mineral dissolution of GFAV calcification was noted in heterozygotes. Binding of osteopontin to the mineral provided a recognition site for macrophages and giant cells leading to their accumulation at the implant site, to CA II upregulation, and to acidification of the surrounding milieu, contributing to the dissolution of mineral on GFAV. We have shown that chronic administration of an endothelin receptor antagonist also induced mineral loss in an experimental model of MEC. In accord with the valve implant study in osteopontin heterozygotes, we have also reported local osteopontin expression at sites of MEC in this model. Moreover, in the present study we found that CA II was more abundant in calcified regions of the aorta. Thus, there seem to be common players in both conditions of calcified cardiovascular tissues. However, the regression of calcification in the GFAV leaflet and the WVK models may follow distinct mechanisms. Indeed, ED-1 staining, a specific marker for macrophages, was negative in WVK-treated rats, and no other histological evidence was seen for the presence of these cells. In addition, the present experiments show that darusentan did not change the vascular activity or distribution of CA II. Moreover, although osteopontin is overexpressed in the calcified areas, spontaneous regression is not efficient, as suggested by results from the placebo groups. In our model, CA II and osteopontin could be involved in limiting the extent of aortic MEC, thus explaining the plateau obtained after 4 weeks of WVK treatment. In agreement with this, calcification continued to progress beyond the 4-week time point in carotid arteries, where CA II expression was not observed in the arterial media. However, the fact that acetazolamide did not significantly enhance aortic calcification when used alone argues against a role for CA II in limiting the progression of elastocalcinosis, suggesting that osteopontin may have a more important role in that respect.

While performing the CA activity assay, we made the unexpected observation that the membrane-bound activity was significantly enhanced after 1 week of darusentan treat-
ment. The major membrane-bound isoenzyme is CA IV, and its localization was thus examined. In contrast to CA II overexpression in calcified areas during WVK treatment, the CA IV isoform activity and localization were not modified during MEC. However, our results show that there was a striking transient upregulation of this isoenzyme during endothelin receptor blockade, as revealed by immunohistochemistry, Western blot, and activity measurement approaches. Furthermore, the distribution of CA IV was not limited to calcified areas, but the enzyme appeared throughout the vascular wall. This may explain the remarkable increase in activity detected because overexpression only in calcified areas would likely have resulted in much smaller increases given the patchy distribution of calcification found by micro-CT. Chronic administration of acetazolamide, a general inhibitor of CA, confirmed the involvement of CA in the regression as it prevented the mineral loss induced by darusentan. Unfortunately, the absence of specific CA IV inhibitors does not allow for a more precise determination of the isoenzyme involved in this regression process. On the other hand, CA II and CA IV may in fact cooperate to allow mineral dissolution, as suggested for different functions. Because CA isoforms are involved in the production of carbonic acid, it is indeed tempting to speculate that CA IV overexpression during endothelin receptor antagonism leads to an acidification of the extracellular milieu and a slow but gradual dissolution of the mineral phase in the arterial wall. Indeed, CA IV is membrane bound and will affect extracellular rather than intracellular pH. However, we have no direct proof of a local pH change to support this hypothesis. The difference in the kinetics of enhanced expression/activity of CA IV and in the reduction of vascular calcium content seems to indicate that prolonged CA IV hyperactivity is required to decrease the calcium content. The results obtained in carotid arteries also suggest a pivotal role for CA IV in inducing mineral loss. Indeed, CA IV overexpression appeared to be delayed by 2 to 3 weeks, and there was no regression of calcification in the carotid arteries with 4 weeks of darusentan treatment.

Thus, inhibiting the endothelin system allows the expression of CA IV, which appears to contribute to the regression of MEC, potentially by altering the extracellular microenvironment. The relationship between CA isoenzymes and endothelin is currently unknown, but we have previously observed endothelin overexpression during WVK treatment. Depending on the assay system used, endothelin has been shown to either decrease or increase osteoblast alkaline phosphatase, osteocalcin, and osteopontin production as well as other markers of osteoblast differentiation. We have recently developed a vascular smooth muscle cell model of calcification, and it is our goal to investigate the mechanism by which endothelin regulates CA IV.

We have previously published that 50% ($r^2=0.51$) of the changes in PP can be accounted for by changes in aortic calcium content. In the present study, dissociation between calcium content and PP was observed, particularly in the acetazolamide-treated rats. It must be noted that other factors like ventricular ejection, pulse wave reflection, vascular smooth muscle cell contraction, fragmentation of elastic fibers, and an increase of rigid components (eg, collagen) can influence PP. In this regard, it has been shown that the vasodilatory effect of diuretics (including acetazolamide) is also mediated by a membrane-bound CA isoform, leading to cellular alkalization, potassium channel activation, and cellular hyperpolarization. Thus, this effect could account for the reduction in PP and could suggest yet another function for CA isoforms in the vascular wall.

In conclusion, our results suggest that the overexpression of endothelin is essential for the maintenance of WVK-induced elastocalcinosis. Inhibition of the endothelin system allows the expression of CA IV, a membrane-bound isoform that could reduce the pH of the extracellular milieu to allow the dissolution of minerals accumulated on the elastic lamellae. Thus, our results suggest a new functional role for vascular CA IV that can be induced by a specific pharmacological treatment in a condition of medial elastocalcinosis.

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Disclosure
Dr McKee has consulted for Enobia Pharma, Inc.

References

**CLINICAL PERSPECTIVE**

Elevation of diastolic blood pressure (DBP), which is found mainly in “young” hypertensives, is thought to be the major parameter to control to reduce cardiovascular complications. Accordingly, vasodilators were developed to reduce peripheral vascular resistance; however, with aging, increased rigidity in large conduit arteries leads to an elevation of systolic blood pressure (SBP) and pulse pressure (PP) and to the development of isolated systolic hypertension (ISH). This hemodynamic condition has been increasingly related to cardiovasculor morbidity and mortality. Many antihypertensive agents do not reduce arterial rigidity and therefore are not as effective in reducing SBP as DBP. Today the diagnosis and treatment of ISH is a clear mandate of national and international advisory committees on hypertension management, but adequate therapies have not been devised, owing in part to a poor understanding of the changes occurring in the aging arterial wall. We have previously reported that medial elastocalcinosis (calcification of elastic lamellae) of the aorta, which is very prominent in men aged >55 years, contributes to both vascular rigidity and the development of ISH in rats. Using this model, we now demonstrate that an endothelin receptor antagonist causes the regression of elastocalcinosis by activating carbonic anhydrase, a family of enzymes responsible for osteoclast-induced hydroxypatite dissolution in bone. By improving our understanding of mechanisms by which medial elastocalcinosis can be reduced, we can begin to design new therapeutic strategies to treat ISH.
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