Angiotensin II type 1 receptor blocker inhibits arterial calcification in a pre-clinical model

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Aims
Arterial calcification is a common complication of several disorders and is a strong predictor of mortality. The mechanism underlying arterial calcification is not fully understood and as such, no pharmaceutical therapies are currently available which impede its progression. The aim of this study was to investigate the effects of an angiotensin II (AngII) type 1 receptor blocker (ARB) on arterial calcification.

Methods and results
Male New Zealand White rabbits were fed an atherogenic diet to induce atherosclerosis and arterial calcification over a period of 12 weeks, with an ARB administered in the final 4 weeks. Using clinically relevant micro-computed tomography, we found that animals fed the atherogenic diet displayed extensive arterial calcification when compared with control. In contrast, administration of the ARB completely inhibited calcification (2.80 ± 1.17 vs. 0.01 ± 0.01% calcified tissue in cholesterol and ARB-treated, respectively; n = 6 and 5; P < 0.05). Calcified regions were characterized by up-regulation of bone morphogenetic protein 2, osteocalcin, and the AngII type 1 receptor and concomitant down-regulation of α-smooth muscle actin, consistent with a phenotypic switch from vascular to osteoblast-like cells.

Conclusion
These data provide the first evidence that angiotensin receptor blockade can inhibit arterial calcification by disrupting vascular osteogenesis and suggest that ARBs may be a novel treatment option for patients suffering from vascular calcification.

Keywords
Arterial calcification • Angiotensin receptor blocker • Pre-clinical model • Vascular smooth muscle cells

1. Introduction
Arterial calcification, also known as Mönckeberg sclerosis, involves mineralization of the internal elastic lamina (IEL) and elastic fibres within the medial layer, resulting in hardened arteries and increased pulse pressure. Commonly associated with advanced age, chronic kidney disease, diabetes mellitus, and atherosclerosis, arterial calcification is closely related to cardiovascular morbidity and mortality.1–3 In the past, it was considered a passive, degenerative, and, most importantly, irreversible process.4 More recently, it has become clear that this process is highly regulated, involving a number of pro- and anti-calcification mediators, and resembles natural bone formation.5 Indeed, a recent study by Speer et al.6 has shown that vascular smooth muscle cells (SMCs) are capable of osteoblast transdifferentiation in calcifying arteries.

This transdifferentiation was associated with down-regulation of SMC markers and concomitant up-regulation of the osteoblast transcription factor Runx2/Cbfa1. Although tremendous progress has been made in this area, the molecular mechanisms underlying this process remain to be fully defined.

There is growing evidence that vasoactive agents are important modulators of vascular calcification. Naturally existing peptides such as endothelin-1 and urotensin II can promote arterial calcification, whereas others—adrenomedullin and C-type natriuretic peptide—act to inhibit its progression.7–9 However, the role of the renin–angiotensin–aldosterone system (RAAS) and its vasoactive agent, angiotensin II (AngII), has not been investigated. AngII plays a number of roles in vascular pathology and is thought to exert its effects by inducing NAD(P)H oxidase and increasing cellular reactive oxygen species (ROS).10 In turn, ROS stimulate the expression of...
bone morphogenetic protein 2 (BMP2) and the osteoblast transcription factor Runx2/Cbfa1, thereby inducing osteoblast transdifferentiation. The blockade of RAAS has been shown to reduce morbidity and mortality in hypertension, atherosclerosis, heart failure, stroke, diabetes, and chronic kidney disease, often independent of changes in blood pressure.

Here, we test the hypothesis that an AngII type 1 receptor blocker (ARB) can inhibit arterial calcification in vivo. We also further characterize the mechanism by which vascular calcification occurs using immunohistochemical methods.

2. Methods

2.1 Pre-clinical model

Male New Zealand White rabbits (1.6–2.0 kg, Charles River Laboratories, St-Constant, QC, Canada) were fed either regular chow (control; n = 9) or an atherogenic diet (cholesterol; n = 9) consisting of 0.5% cholesterol and 10 000 IU/day Vitamin D2 for 12 weeks to rapidly induce atherosclerosis and arterial calcification. The treatment group (ARB; n = 6) received the atherogenic diet for 12 weeks with the ARB olmesartan medoxomil (1 mg/kg/day) in the final 4 weeks. Olmesartan medoxomil, suspended in 60% molasses, was administered daily via oral gavage.

Vitamin D2 and its analogues have been used extensively to induce vascular calcification in animal models, and there is controversy as to whether or not it contributes to vascular calcification in humans. After 8 weeks, a subset of the control (n = 3) and cholesterol fed (n = 3) groups were euthanized to assess lesion progression at the time of pharmacological intervention. At 12 weeks, animals were euthanized via intravenous ketamine injection and perfused with Hanks’ balanced salt solution and heparin (1 U/mL). Immediately upon dissection, the thoracic aorta was fixed in 10% neutral-buffered formalin. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal protocols were approved by the University of Western Ontario Animal Use Subcommittee (reference number 2007-023).

2.2 Plasma chemistry

Blood samples were obtained for the measurement of total cholesterol and inorganic phosphate. EDTA was used in the isolation of plasma, preventing analysis of calcium. Total cholesterol was measured at weeks 0, 4, 8, 10, and 12 (endpoint) using a WAKO Cholesterol E Kit, according to the manufacturer’s instructions. Inorganic phosphate was examined at endpoint using standard autoanalyzer methods at

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Figure 1  Treatment with the ARB inhibited arterial calcification. (A) Representative maximum intensity projections, derived from micro-CT scans, and corresponding quantitation of control (n = 6), cholesterol (n = 6), and ARB (n = 5) animals reveals a significant increase in arterial calcification after 12 weeks on the atherogenic diet, except in ARB-treated animals where no significant calcification was detected. Scale bar = 4 mm. The Kruskal–Wallis test with Dunn’s post hoc test: *P < 0.01 vs. control. †P < 0.05 vs. cholesterol. (B) Histological examination of calcium (Alizarin Red S, top) and calcium salts (von Kossa, bottom) reveals that they are localized primarily to the IEL and medial layer, typical of arterial calcification. Scale bar = 500 μm. ND, none detected. Arrows indicate calcification.
the London Health Sciences Centre Core Laboratory (London, ON, Canada).

2.3 Micro-computed tomography

Thoracic aortae were placed in a humidified chamber to prevent dehydration and scanned with an eXplore Locus micro-computed tomography (micro-CT) scanner (General Electric Medical Systems, London, ON, Canada). Scans were acquired at 80 kVp and 130 mAs and reconstructed with isotropic voxel spacing of 90 μm. Images were analysed for the presence of calcium using MicroView analysis software (V2.2, GE Medical Systems, London, ON, Canada). Specifically, the total volume of all voxels containing calcified tissue (identified by setting a threshold level) was calculated and expressed as a per cent of total vessel volume.

2.4 Histological and immunohistochemical analysis

Thoracic aortae were embedded in Tissue-Tek O.C.T. compound and frozen in liquid nitrogen-cooled isopentane. Frozen sections (10 μm) were taken as cross-sections through the aorta distal to the first intercostal ostia. Serial sections were stained with Alizarin Red S and von Kossa for calcium deposition. Immunohistochemical studies were performed using the following primary antibodies: mouse anti-α-smooth muscle actin (α-SMA; clone 1A4), mouse anti-AngII type 1 receptor (AT1R; clone 1E10-1A9), mouse anti-BMP2 (clone 1A11), mouse anti-osteocalcin (clone OC4-30), mouse anti-osteopontin (clone MPIIIB10), and mouse anti-rabbit activated macrophage (clone RAM11). Sections were subjected to single-label immunohistochemistry using an Alkaline Phosphatase Substrate Kit (Vector Laboratories) and secondary antibody horse anti-mouse IgG (H + L) Alkaline Phosphatase conjugate according to the manufacturer’s instructions. Negative controls which omitted the primary antibody were routinely employed.

2.5 Statistical analysis

Data are expressed as mean ± SEM. All analyses were performed using GraphPad Prism (V4.0, GraphPad Software, La Jolla, CA, USA). Statistical analyses were performed using a Kruskal–Wallis test with Dunn’s post hoc test, or one-way repeated-measures ANOVA with Tukey’s post hoc test, as appropriate. A value of P < 0.05 was considered statistically significant.

3. Results

3.1 Animals and plasma chemistry

A subset of control (n = 3) and cholesterol (n = 3) animals were euthanized after 8 weeks to assess the extent of disease before administration of the ARB (Supplementary material online, Figure S1). A single ARB-treated rabbit died due to unknown causes after a week of treatment (9 weeks total) and was not included in the analysis. To assess systemic effects of ARB treatment, we examined plasma levels of total cholesterol and inorganic phosphate. Although total cholesterol levels were significantly increased in cholesterol animals when compared with control (1394.00 ± 352.16 vs. 15.49 ± 6.31 mg/dL in cholesterol and control, respectively; n = 6/group; P < 0.001), there was no significant effect of ARB treatment on cholesterol levels.

3.2 Arterial calcification is abolished after treatment with the ARB

To evaluate the effects of ARB treatment on arterial calcification, we administered olmesartan medoxomil for the final 4 weeks of the 12-week protocol. Calcification was significantly increased in cholesterol animals (non-detectable vs. 2.80 ± 1.17% calcified tissue in control and cholesterol, respectively; n = 6/group; P < 0.01) and, in contrast, was completely inhibited in all but one ARB-treated animal (0.01 ± 0.01% calcified tissue in ARB; n = 5; P < 0.05 vs. cholesterol; Figure 1A). Calcification, when present, was primarily localized to the IEL and media (Figure 1B). Some animals (three of six cholesterol and two of five ARB) exhibited micro-calcifications within atherosclerotic plaques (Figure 2) either in addition to or independent of IEL/media calcification. It should be noted that the calcification found in the ARB-treated animals was minor (<10 μm in size) and was localized to the plaque. These calcifications were too small to be detected by micro-CT because their signal was masked by the surrounding tissue, which occupied the majority of the 90 μm³ voxel.

![Figure 2](https://example.com/image2.png)

**Figure 2** Arterial calcification vs. micro-calcification of atherosclerotic plaques. Animals fed the atherogenic diet primarily displayed calcification of the IEL and medial layer (arrows). However, histological examination revealed that a number of animals (three of six cholesterol and two of five ARB) exhibited micro-calcifications within atherosclerotic plaques (arrowheads). It is important to note that these are generally considered distinct processes and that the minor calcification displayed in the ARB animals was localized to the plaque. Scale bar = 500 and 100 μm (inset). Abbreviations as in Figure 1.
3.3 Calcified regions express the bone-related proteins BMP2, osteopontin, and osteocalcin and dramatically increase expression of the AT1R

To gain insight into the mechanisms underlying this calcification process, we characterized the calcified regions using immunohistochemical methods. Adjacent sections acted as negative controls (omission of primary antibody) and showed no positive staining (data not shown). Calcified areas, and the tissue immediately surrounding them, showed expression of the osteogenic growth factor BMP2 (Figure 3). Calcified areas also exhibited increased expression of the bone protein and osteoblast-specific marker osteocalcin (Figure 3). In addition, calcified areas display dramatic up-regulation of the AT1R (Figure 3), which has been shown to increase in expression as osteoblasts mature. Calcified areas also showed limited expression of the calcium-binding protein osteopontin (data not shown). Corresponding low power images can be found in Supplementary material online, Figure S3. Taken together, this strongly suggests the presence of osteoblasts within areas of calcification.

3.4 Calcified areas of the media are not associated with SMCs or macrophages

To eliminate other possible cell types within calcified areas, we examined the expression of α-SMA, a marker of SMCs, and RAM11 as a marker of macrophages. Calcified areas were associated with a down-regulation of α-SMA (Figure 4). In addition, some areas not associated with calcification also displayed down-regulation of α-SMA (see Supplementary material online, Figure S4), possibly indicating initial progression towards an osteogenic phenotype. Interestingly, some of these areas also showed the up-regulation of BMP2 (data not shown). Macrophages were localized specifically to atherosclerotic lesions and were not observed within the medial layer (Figure 4). Corresponding low-power images can be found in Supplementary material online, Figure S4.

Figure 3 Characterization of calcified regions indicates an osteoblast-like phenotype. Immunohistochemical characterization of calcified regions (arrows) and adjacent sections reveals colocalized expression of the osteogenic growth factor BMP2, the bone protein and osteoblast-specific marker osteocalcin (OCN), and dramatic up-regulation of the AT1R. These data suggest an osteoblast-like phenotype within calcified areas. Scale bar = 500 μm.
material online, Figure S4. Taken together, this suggests a phenotypic switch from vascular to osteoblast-like cells.

4. Discussion

The present study is the first to show the dramatic inhibition of arterial calcification by an ARB. Using micro-CT and histology, we have shown that ARB administration, given after induction of disease, can robustly inhibit the calcification observed in animals not so treated. Although others have used doses of 25 000–50 000 IU/day Vitamin D₂,²¹,²² we achieved significant levels of vascular calcification in the same time period using 10 000 IU/day. The calcification we observed was localized to the IEL and medial layer, typical of Mönckeberg sclerosis as well as vascular calcification associated with chronic kidney disease and diabetes mellitus.²³,²⁴ In chronic kidney disease, up to 55% of deaths are cardiovascular related, and cardiovascular mortality is 10–100 times greater than that for age-matched populations.²⁵ In type 2 diabetes, arterial calcification is a strong predictor of mortality and lower-extremity amputation.²⁶,²⁷ Inhibiting the progression of arterial calcification remains the goal of several groups,⁶,²⁸–³² but it is generally thought to be irreversible. In contrast, at least one group has shown reversibility of vascular calcification in rats with normal renal function.³³ Here, we provide the first evidence that an ARB is capable of halting progression of arterial calcification.

Although the underlying pathologies (diabetes, chronic kidney disease, and advanced age) are distinct, the process ultimately leading to vascular calcification is related. Although vascular SMC transdifferentiation to osteoblasts is under intense investigation,³³–³⁷ the molecular mechanisms are not fully understood. The characterization of the calcific lesions we observed suggests that they result from such a transition. Expression of BMP2, an osteogenic growth factor, in the areas in and around calcification suggests that it is involved in directing this transition, as others have suggested.³⁸ Calcified areas display expression of the bone-associated proteins osteopontin and osteocalcin. Osteopontin can be expressed by a range of cell types, including bone cells and macrophages, and has been shown to contribute to arterial calcification.³⁹ Osteocalcin, an osteoblast-specific marker, is often used as a biomarker for bone formation and is also implicated in calcium ion homeostasis.²⁰ Calcified regions also displayed marked up-regulation of the AT1R. The AT1R is commonly expressed in the vasculature and is responsible for mediating signalling of the RAAS. Indeed, we observed diffuse staining for AT1R in aortae from all of our animals. However, recent work by Bandow et al.⁴⁰ has shown that osteoblasts, as they mature, greatly increase expression of the AT1R. We observed regional up-regulation of the AT1R in areas associated with calcification, again suggesting an osteoblast-like phenotype. Furthermore, calcified areas showed dramatic down-regulation of α-SMA, a smooth muscle marker, providing evidence for a phenotypic switch from vascular to osteoblast-like cells. Here, we provide the first evidence that the RAAS is involved in vascular osteogenesis and that an ARB is capable of modulating the process.

Despite the dramatic effect on calcification, ARB therapy had no effect on systemic disease indicators (hypercholesterolaemia and hyperphosphataemia), suggesting its effects are specific to the vascular system, rather than a result of secondary phenomena. As expected, ARB therapy did not normalize total cholesterol.⁴¹,⁴² There was also no effect on plasma levels of calcium or inorganic phosphate, either by the atherogenic diet or by ARB therapy. It is important to note that we were unable to reliably quantify changes in the extent of atherosclerosis, as extensive calcium deposition in cholesterol animals prevented both ultrasound examination and en face lipid staining.

Here, we show that angiotensin receptor blockade robustly inhibits the progression of arterial calcification. This form of calcification, commonly associated with advanced age, chronic kidney disease, and diabetes mellitus, is a result of osteoblast transdifferentiation of vascular cells,⁶ a process replicated here in a pre-clinical model. This study is the first to suggest ARB therapy as a novel treatment option for patients at risk for cardiovascular calcification.

Figure 4 Calcified regions of the media are not associated with SMCs or macrophages. Examination of α-SMA, a marker of SMCs, reveals dramatic down-regulation in areas of calcification. Furthermore, macrophages are localized specifically to atherosclerotic plaques and are not associated with areas of calcification. Scale bar = 500 μm.
Supplementary material

Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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