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Age-associated arterial calcification: the current pursuit of aggravating and mitigating factors

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Abstract

Purpose of the review: The incidence of arterial calcification increases with age, can occur independently of atherosclerosis and hyperlipidemia, contributes to vessel stiffening, and is associated with adverse cardiovascular outcomes. Here, we provide an up-to-date review of how aging leads to arterial calcification and discuss potential therapies.

Recent findings: Recent research suggests that mitochondrial dysfunction (impaired efficiency of the respiratory chain, increased reactive oxygen species production and a high mutation rate of mtDNA), cellular senescence, ectonucleotidases, and extrinsic factors such as hyperglycemia promote age-determined calcification. We discuss the future potential impact of anti-lipidemics, senolytics, and poly(ADP-ribose)polymerases inhibitors on age-associated arterial calcification.

Summary: Understanding how mechanisms of aging lead to arterial calcification will allow us to pinpoint prospective strategies to mitigate arterial calcification, even after the effects of aging have already begun to occur.

Keywords

atherosclerosis; aging; calcification; senescence; ectonucleotidase

Introduction

Atherosclerosis is a multi-faceted pathophysiologic process that accrues over a lifetime and tends to accelerate later in life [1]*. Atherosclerosis underpins the most common cardiovascular causes of death in older patients, thrombo-embolic stroke, myocardial infarction, and cardiac arrest [2]. Arteries, whether plaque-laden or not, often accrue calcific deposits over a lifespan [3]. Calcification may occur within the atherosclerotic plaque itself, within the wall of the blood vessel, as nodules, or on valvular tissue [4]. The variety of morphologies that vascular calcification manifests belies the mechanistic complexity in the pathogenicity of calcium deposition [5]. Vascular calcification is both reactive (to mechanical strain, inflammation, reactive oxygen species, local metabolites) and proactive,

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in that vascular smooth muscle cells may acquire an osteogenic and calcigenic phenotype to perpetuate arterial ossification [6].

Here, we review recent, novel mechanistic studies which build upon prior work in the biology of aging and vascular calcification. We discuss the shortfalls of current therapies to reduce the progression of vascular calcification and propose several promising avenues for future study.

Mitochondria and age-associated vascular calcification

Mitochondrial dysfunction, specifically, impaired efficiency of the respiratory chain, increased reactive oxygen species (ROS) production and the high mutation rate of mtDNA a consequence of proximity to ROS generation and limitations in mtDNA repair contribute to vascular aging [7]. Aging has been shown to instigate mitochondrial dysfunction and mitophagy independent of concomitant hyperlipidemia [1]. Intuitively, obese, insulin-resistant (ob/ob) mice induced to develop aortic vascular calcification with Vitamin D₃ exhibit increased oxidative stress [8]. Specifically, aortas of ob/ob mice treated with Vitamin D₃ exhibit enhanced oxidative stress in contrast to wild type or untreated ob/ob mice. This study indicates both aging and vascular-extrinsic factors such as hyperglycemia promote vascular calcification [8].

The rare human condition Hutchinson Gilford Progeria Syndrome (HGPS) occurs as a result of accumulation of an immature form of the protein lamin A (Progerin) and results in deficient DNA-repair, premature aging, and death [9, 10]. Study of HGPS has contributed to the elucidation of mechanisms of vascular aging. Vascular calcification in this syndrome occurs due to defective extracellular pyrophosphate (an endogenous inhibitor of calcification) metabolism [11]. Progerin accumulation results in defective mitochondrial oxidative phosphorylation [12], leading to increased ROS formation, and impaired ATP synthesis [11]. This is compounded by impairment of the nuclear factor erythroid 2-related factor 2 (NRF2) antioxidant pathway by Progerin [13]. Studies of selective expression of Progerin in *in vivo* models suggest that Progerin accumulation in endothelium results in dysfunctional endothelium and impaired neorevascularization [14], but that the effect on vascular calcification is due to vascular smooth muscle cell Progerin accumulation [15]. Even in this model, plaque calcification occurred in only 2/9 *apoE*^{-/-} vascular-smooth muscle (SM22α) Progerin-expressing mice, highlighting the relative resistance of murine models to vascular calcification and a challenge in studying this manifestation of vascular aging in animal models [15, 16]. In this context, antioxidants such as sulforaphane, found in cruciferous vegetables, have showed promise in augmenting Progerin clearance by autophagy in HGPS fibroblasts [17]. Recently, CRISPR-Cas9-targeted disruption of laminA/Progerin was shown to increase murine lifespan in a HGPS model, although mice displayed late impaired colonic motility leading to death in some instances [18]. In this study, a single-dose of adeno-associated virus was used to deliver guide RNAs to reduce lamin A/Progerin while maintaining lamin C (sufficient for normal lifespan), alleviating degeneration of aortic medial-layer vascular smooth muscle cells in a murine model of HGPS and implicating gene therapy as potentially viable future treatment strategy [18]. Taken together, these studies suggest that anti-oxidant treatment and other approaches to preserve mitochondrial

function, number, and efficiency continue to hold promise as a strategy to mitigate age-associated vascular calcification.

Cellular senescence and vascular calcification

Cells that are no longer dividing (senescent) have been shown to promote atherogenesis via a senescence-associated secretory phenotype (SASP), influencing the microenvironment and conferring relative resistance to apoptosis. Telomere (repetitive nucleotide sequences that preserve chromosome stability) shortening triggers a DNA damage checkpoint, inducing senescence; in addition, environmental cues can initiate a senescence phenotype.

Observational reports suggest that telomere length is associated with coronary artery calcium deposition in humans [19]. Recent data utilizing a primate, baboon model demonstrate the importance of diet in accelerating telomere shortening. In this model, prolonged exposure to a high cholesterol, high fat diet accelerated leukocyte telomere shortening at early stage atherosclerosis [20].

Insights from the study of HGPS indicate that aging vascular smooth muscle cells also display disrupted lamin A processing, leading to DNA damage and senescence [21]. Furthermore, dysfunctional lamin A processing leads to osteogenic differentiation and senescence (indicated by p16 expression and a senescence-associated secretory phenotype) of vascular smooth muscle cells [22]. This study shows that inhibiting the DNA damage response kinases ataxia-telangiectasia mutated/ataxia-telangiectasia- and Rad3-related effectively inhibited vascular smooth muscle cell osteogenic differentiation [22].

Recent studies suggest a role for long noncoding RNAs (lncRNAs) in regulating senescence and vascular calcification [23, 24]. Long noncoding RNAs are genes with a variety of biological functions, including functioning as signals, decoys, guides, and scaffolds [25]. Haemmig, et al. recently performed RNA sequencing to profile lncRNAs derived from the aortic tissue of *Ldlr*^{-/-} mice prone to atherosclerosis [23]*. They found an inverse relationship between the lncRNA small nucleolar host gene-1 (*SNHG12*) atherosclerosis, DNA damage and senescence (measured by gene expression of senescence markers p16, p21, and p27). The study also found that *SNHG12* interacts with DNA-PK, a regulator of the DNA damage response [23]*. Lin, et al. suggest a role for microRNA MiR-34c-5p and lncRNA-ES3 in regulating calcification and senescence, as indicated by alkaline phosphatase, osteocalcin, Runx2, and p16 and p21 expression in vascular smooth muscle cells [24]. In summary, avoidance of extrinsic accelerators of telomere shortening (including dietary fat intake) and inhibition of the DNA damage response through lncRNA or other means are currently of interest in the study of arterial calcification and aging.

Ectonucleotidases and vascular aging

Mounting evidence suggests that ectonucleotidases may play a role in vascular calcification and in particular age-related vascular calcification (Figure 1) [16, 26, 27]. Ectonucleotidases are transmembrane enzymes found on the cellular surface of vascular and immune cells that are responsible for phosphohydrolyzing extracellular ATP and ADP, generating extracellular AMP and adenosine [28]. Regulation of the extracellular purinergic axis has important

downstream consequences on thrombosis and the inflammatory response to injury [29-31]. Humans lacking functional CD73 (ecto-5'-nucleotidase or ecto-5'-NT), which converts extracellular AMP to adenosine, develop profound peripheral vascular, particularly lower extremity, arterial calcification [32]. CD73 activity resulted in reduced adenosine production, which would otherwise inhibit tissue nonspecific alkaline phosphatase (TNAP) [32]. Without CD73, TNAP metabolized pyrophosphate, an endogenous inhibitor of vascular smooth muscle cell calcification. Absent CD73 and exogenous AMP accumulation resulted in FOXO1 nuclear localization, increased alkaline phosphatase activity, and subsequent metabolism of pyrophosphate, resulting in unhindered mineralization [33]**. However, the human vascular calcification phenotype has not been recapitulated in animal models, possibly due to the relative resistance of the murine species to developing vascular calcification in the absence of another stimulus [26, 34]. Paradoxically, aged *apoE*^{-/-} mice with functional CD73 developed more atherosclerosis and aortic root calcification (at the base of the plaque) when compared with *apoE*^{-/-}/*cd73*^{-/-} mice [26]*. It is possible that in the setting of hyperlipidemia, CD73 may allow for the accumulation of atherosclerotic plaque generation via adenosine generation, which has predominantly anti-inflammatory and vasodilatory effects [26]*. Similarly, in another murine *apoE*^{-/-} model, CD73 also promoted atherosclerosis and regulated vascular smooth muscle cell phenotype [35]. The observations from humans and animal models are both striking and challenging to reconcile.

Providing additional evidence for a role of ectonucleotidases in vascular aging, in a murine model of HGPS, vascular smooth muscle cells demonstrated increased gene and protein expression of CD39 (eNTPD1) [11]. CD39 is an ectonucleotidase that converts extracellular ATP and ADP to AMP and acts upstream of CD73 [28]. The increased expression of CD39 was associated with adequate extracellular ATP metabolism but reduced pyrophosphate in this model (Figure 2) [11]. Subsequently, the administration of extracellular ATP, levamisole (TNAP inhibitor), and ARL67156 (CD39/eNTPD1 inhibitor) prevented vascular calcification and extended longevity by 12%, by increasing pyrophosphate in the murine HGPS model [27]**. Taken together, the role of purinergic signaling as modulated by ectonucleotidases remains unfully explored as a mechanism of vascular aging, and recent evidence suggests that strategic inhibition of ectonucleotidases could allow pyrophosphate to accumulate, inhibiting vascular calcification.

Inflammation and age-related calcification

There is a large body of evidence that chronic systemic inflammation, which occurs with aging, promotes both vascular calcification and atherosclerosis [36]. Additionally, arterial inhibition of endothelial transforming growth factor- β (TGF- β) reduces vascular inflammation and atherosclerotic plaque growth in hyperlipidemic *apoE*^{-/-} mice [37]. This emphasizes the importance of understanding the contribution of specific vascular cell types to plaque growth and stabilization of calcified plaques. Endothelial cells stimulated with tumor necrosis factor- α (TNF- α) release microparticles containing bone morphogenetic protein-2 (BMP-2), resulting in vascular smooth muscle cell osteogenesis [38]. Furthermore, C-reactive protein (CRP) aggravates phosphate-induced osteogenesis of human aortic vascular smooth muscle cells [39]. CRP-induced mineralization is suppressed by p38 MAPK inhibition (Figure 3) [39]. Novel systemic therapies aimed at reducing systemic

inflammation, such as an oral p38 inhibitor, which promoted macrophage inflammation resolution in aged humans, may potentially reduce vascular inflammation [40].

Aside from syndromes such as HGPS, vascular calcification can also occur early in life in children on dialysis, and recent evidence suggests that the vascular smooth muscle cells with an inflammatory, SASP contribute to this [41]. In this study, vascular smooth muscle cells from patients on dialysis had increased expression of SASP factors BMP2, osteoprotegerin (OPG), and IL-6, which are known to regulate calcification [41]. These studies suggest that the inflammation associated with aging (so called inflamm-aging) could be a target to reduce the acceleration of vascular calcification with time.

Hyperglycemia and age-related calcification

Diabetes is associated with increased arterial calcification, and hyperglycemia- and age-induced vascular calcification are mechanistically interwoven. Advanced glycation end products (AGEs) refer to glycated proteins or lipids, representing the biochemical consequence of hyperglycemia and accumulate over a lifetime. AGEs are implicated in vascular aging and calcification, arterial stiffness, and are counterbalanced by circulating soluble receptors for AGEs (sRAGE). sRAGE are hypothesized to play a protective role in vascular aging [42-44]. sRAGE has recently been shown in human studies to be inversely related to vascular calcification and stiffness, represented by both X-ray and pulse-wave velocity, supporting the theory that sRAGE may play a protective role in vascular aging [44, 45].

Additionally, there is heightened interest in the role of sirtuin proteins in mediating the calcifying effect of hyperglycemia. SIRT1 has been shown to delay senescence and reduce DNA damage [46]. Sirtuin proteins are histone deacetylases which protect against DNA damage and reduce vascular smooth muscle cell osteogenesis [47]. Hyperglycemia reduces SIRT1 expression, inducing vascular smooth muscle cell osteogenic differentiation [48]**. Recently, suppression of SIRT1 under diabetic conditions was mechanistically linked to RUNX2 signaling, a transcription factor critical for osteogenic cell transformation [48]**. Reemphasizing the role of the vascular smooth muscle cell microenvironment, endothelial cells stimulated with high glucose produce exosomes that consequently influence adjacent vascular smooth muscle cells to become senescent and calcify via mTOR signaling [49]. In summary, targeting AGEs and augmentation of SIRT1 are potential translational targets to reduce the impact of hyperglycemia-stimulated, age-associated vascular calcification.

Pharmacotherapy for vascular calcification

HMG-CoA reductase inhibitors

One of the most studied manifestations of arterial calcification is coronary artery calcification, which increases with age and is associated with adverse cardiovascular outcomes [50]. Inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), known collectively as statins, are a class of drugs with proven benefit in reducing major adverse coronary events [51], particularly in those with known coronary artery calcium [52]. It is therefore surprising that intravascular imaging of human coronary

arteries demonstrates that statins promote increased density and volume of coronary calcification [53]. Subsequent imaging studies have shown that while the volume of calcified coronary plaque increases with statin treatment, the volume of lipid-rich, non-calcified plaque stabilizes or decreases [54-56]. Observational studies have not demonstrated that statin-induced coronary calcification correlate with adverse outcomes [50]. Recently, a mechanism for this observation was suggested. Healy et al. reported increased activation of monocyte-derived Rac1 in statin-treated human subjects, and as a corollary, statin-fed mice demonstrated Rac1-dependent increased IL-1 β expression, and expression of osteogenic markers alkaline phosphatase and RUNX2 in atherosclerotic plaques despite similar lipid profiles [57]. While this study provides a plausible explanation for plaque ossification, it is counterintuitive that the mechanism involves plaque inflammation, since statins are widely held to have anti-inflammatory plaque properties [58]. Additional research would be helpful to clarify the mechanism by which statins stabilize and ossify atherosclerotic plaque.

PCSK9 inhibitors and ezetimibe

Even less is known how other anti-lipidemics such as proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors and the cholesterol absorption inhibitor, ezetimibe, impact coronary calcification. One recent study utilizing intravascular ultrasound of PCSK9 inhibitor-treated patients showed no change in calcium composition of plaque [59]. However, another recent study suggested that the addition of a PCSK9 inhibitor to statin therapy limited statin-associated accumulation of coronary calcification [60]. The mechanisms by which PCSK9 inhibition might influence statin-mediated coronary calcification remain unclear. Ezetimibe has not been shown to influence statin-induced plaque characteristics, as evaluated by optical coherence tomography [61].

Metformin and other senotherapeutics

Senolytics preferentially induce the apoptosis of senescent cells, while senostatic drugs slow or block cellular senescence. These drugs theoretically reduce senescence markers, the development of age-related diseases, and potentially improve life-expectancy. A variety of compounds are actively being studied at the bench and in clinical trials as potential senotherapeutics [62]. Given that vascular smooth muscle cell osteogenic differentiation is linked to senescence, the effect of senolytics and senostatics on vascular calcification is interesting. Metformin, a biguanide anti-diabetic drug, has a wide variety of anti-aging properties independent of its glucose metabolism [63]. Several studies have shown that metformin is associated with reduced coronary calcification in humans and metformin reduces calcification in rats [64-66]. Recently Ma et al. (2019) have shown that metformin, via AMP-activated protein kinase (AMPK) dependent mechanism, increases mitochondrial division and growth, increases mitophagy of defective mitochondria, and decreases expression of pyruvate dehydrogenase kinase 4 (PDK4), resulting in decreased oxidative stress [67]**. As a result, metformin decreases the transition of vascular smooth muscle cells (VSMCs) into an osteoblast-like phenotype. In humans, this was also manifested by metformin treatment being associated with reduced levels of osteoprotegerin, a protein associated with vascular calcification [68].

Little is known about the effects of other senolytics on vascular calcification. The combination of dasatinib, a tyrosine kinase inhibitor, and quercetin, a plant flavonoid, has been shown to reduce aortic calcification in mice [69]. Quercetin alone in rats was shown to inhibit vascular calcification in rats by reducing oxidative stress and mitochondrial fission [70]. Future clinical studies will be required to evaluate the role of senolytics in humans.

PARP inhibitors

Poly(ADP-ribose)polymerases (PARPs) are a family of enzymes that attach polyADP-ribose (PAR) to proteins (termed PARylation). PARP1 in particular responds to DNA damage and targets histone and nuclear proteins for PARylation. PARP inhibition has previously been shown to inhibit atherosclerotic plaque development and increase plaque stability in animal models [71]. More recently, PARP1 has been shown to be relevant to vascular calcification. Specifically, PARP1 induced Runx2 expression, leading to the transition of vascular smooth muscle cells to an osteogenic phenotype and vascular calcification [72, 73]**. PARP1 deletion decreased vascular calcification while over-expression increased vascular calcification [73]**. In diabetic *apoE*^{-/-} mice, PARP1 increased Stat1 transcription, which binds to the Runx2 promoter, stimulating the cellular osteogenic phenotype [72].

PARP inhibition *in vitro* with the PARP specific inhibitor PJ34 or *in vivo* in rats with minocycline reduced vascular calcification [74]. Prior studies have shown that minocycline stabilizes plaque and reduces plaque size [75, 76]. Indeed, the tetracycline antibiotic, minocycline, was as effective as the specific PARP inhibitor, PJ34. This evidence suggests PARP inhibitors may be a promising future target to alleviate vascular calcification. Currently, Food and Drug Administration-approved PARP inhibitors (e.g. olaparib) are used for cancer treatment, and the effect of these inhibitors on vascular calcification in humans is unknown, although they reduce vascular calcification in animal models [74].

Conclusion

In summary, while exposure to environmental factors and genetics are key to the acceleration of arterial calcification over time, recent research suggests that targeting mitochondrial dysfunction and senescence through a variety of pharmacologic strategies may potentially curb the process. Modulation of the ectonucleotidase axis, quenching of AGEs, increasing SIRT1, and inhibition of the DNA damage responses are all additional, potential targets. Exciting developments in the field of PARP inhibitors and senolytics hold promise for the future. As the underlying drivers of vascular calcification may vary from individual to individual, similarly, we may one day require an individualized pharmacological approach to target each mechanism in order to prevent adverse cardiovascular outcomes.

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Key points:

- Anti-oxidant treatment and other approaches to preserve mitochondrial function, number, and efficiency continue to hold promise as a strategy to mitigate age-associated vascular calcification.
- Avoidance of extrinsic accelerators of telomere shortening (including dietary fat intake) and inhibition of the DNA damage response through long non-coding RNA or other means are currently of interest in the study of arterial calcification and aging.
- The role of purinergic signaling, as modulated by ectonucleotidases, is yet to be fully explored as a mechanism of vascular aging. Recent evidence suggests that strategic inhibition of ectonucleotidases could allow pyrophosphate to accumulate, inhibiting vascular calcification.
- Curbing inflammation associated with aging (so called inflamm-aging) could be a target to reduce the acceleration of vascular calcification with time.
- Targeting advanced glycation end products and augmentation of SIRT1 are potential translational targets to reduce the impact of hyperglycemia-stimulated, age-associated vascular calcification.

Pi = Inorganic phosphate → promotes mineralization

PPI = Pyrophosphate → inhibits mineralization

Figure 1. Ectonucleotidases, purinergic metabolism, and mineralization.

Cell surface ecto-enzymes such as CD39 (ectonucleoside triphosphate diphosphohydrolase-1 or ENTPD1) and CD73, found on the surface of endothelial, vascular smooth muscle cell, and circulating leukocytes, metabolize ATP and ADP to AMP and adenosine. ENPP1 (ectophosphodiesterase/nucleotide phosphohydrolase) metabolizes ATP to form pyrophosphate, which inhibits mineralization. Tissue nonspecific alkaline phosphatase (TNAP) promotes calcification by metabolizing pyrophosphate to inorganic phosphate.

Figure 2.

Vascular smooth muscle cells expressing progerin, as occurs in Hutchinson-Gilford progeria syndrome demonstrate increased ability to metabolize ATP to inorganic phosphate, promoting mineralization. Inhibition of CD39 (ectonucleoside triphosphate diphosphohydrolase-1 or ENTPD1) and Tissue nonspecific alkaline phosphatase (TNAP) allowed exogenous ATP to be preferentially metabolized to pyrophosphate by ectophosphodiesterase/nucleotide phosphohydrolase (ENPP1), reducing calcification in this model.

Figure 3. p38 MAPK in vascular calcification.

Phosphorylation/activation of p38 MAPK (mitogen-activated protein kinases) results in increased IL-6, Alkaline Phosphatase (ALPL), Core-binding factor alpha-1 (CBFA1), Runt-related transcription factor 2 (Runx2), tumor necrosis factor (TNF), and impaired inflammatory resolution (reduced T-cell immunoglobulin and mucin domain-containing molecule, or TIM4), all contributing to vascular aging and calcification.