Arterial Calcification Is Driven by RAGE in *Enpp1*^{–/–} Mice

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Key Words

Artery calcification • Receptor for advanced glycation end-products • Calgranulin • ENPP1 • Pit-1

Abstract

Background/Aims: Ectopic osteochondral differentiation, driven by ENPP1-catalyzed generation of the chondrogenesis and calcification inhibitor inorganic pyrophosphate (PP_i), promotes generalized arterial calcification of infancy. The multiligand receptor for advanced glycation end-products (RAGE), which promotes atherosclerosis and diabetic cardiovascular and renal complications, also mediates chondrocyte differentiation in response to RAGE ligand calgranulins such as S100A11. Here, we tested RAGE involvement in ENPP1 deficiency-associated arterial calcification. **Methods:** Because ectopic artery calcification in *Enpp1*^{–/–} mice is P_i-dependent and mediated by PP_i deficiency, in vitro studies on effects of S100A11 and RAGE on mouse aortic explants were conducted using exogenous P_i, as well as alkaline phosphatase to hydrolyze ambient PP_i. **Results:** S100A11 induced cartilage-specific collagen IX/XI expression and calcification dependent on RAGE in mouse aortic explants that was inhibited by the endogenous RAGE signaling inhibitor soluble RAGE (sRAGE). *Enpp1*^{–/–} aortic explants demonstrated decreased P_i-stimulated release of sRAGE, and increased calcification and type IX/XI collagen expression that were sup-

pressed by exogenous sRAGE and by *Rage* knockout. Last, *Rage* knockout suppressed spontaneous aortic calcification in situ in *Enpp1*^{–/–} mice. **Conclusion:** Cultured *Enpp1*^{–/–} aortic explants have decreased P_i-stimulated release of sRAGE, and RAGE promotes ectopic chondrogenic differentiation and arterial calcification in *Enpp1*^{–/–} mice.

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Introduction

Pathologic arterial calcification commonly develops in the intima of atherosclerotic lesions, and in artery media in diabetes, hyperphosphatemic renal failure, aging, and generalized arterial calcification of infancy (GACI) [1–4]. Calcification decreases artery wall compliance [5]. Moreover, hydroxyapatite crystals can stimulate inflammation and may promote smooth muscle cell (SMC) apoptosis [6], thereby potentially modulating atherosclerotic plaque stability. Artery media calcification in diabetes and chronic kidney disease promotes tissue ischemia, and intimal calcification is a biomarker for more extensive disease and increased mortality in atherosclerosis [5].

Arterial calcification appears to be actively initiated and organized by osteochondral differentiation of intra-arterial stem cells, pericytes, SMCs, and adventitial myofibroblasts [1–3, 7, 8]. Deficiency of physiologic inhibitors

of chondro-osseous differentiation such as the BMP-2 inhibitor matrix GLA protein (MGP) [9] can be compounded by lesion excess of BMP-2, and other inducers of chondro-osseous commitment and maturation [1–3]. Among factors promoting artery calcification are inorganic pyrophosphate (P_i) generation [10] and expression of the sodium-dependent P_i cotransporter Pit-1 in SMCs [11], as well as signaling stimulated by transglutaminase 2 and by the wnt β -catenin axis [7, 8].

Deficient ENPP1-catalyzed generation of the chondrogenesis and matrix calcification inhibitor PP_i [10, 12] promotes both GACI and the spontaneous artery calcification in *Enpp1*^{−/−} mice that serve to model GACI [4, 13, 14]. Importantly, artery calcification in *Enpp1*^{−/−} mice is associated with intra-arterial chondrogenic differentiation, and cultured ENPP1-deficient artery SMCs undergo accelerated chondrogenic trans-differentiation upon provision of a source of P_i [13]. A high-phosphate diet increases artery calcification in *Enpp1*^{−/−} mice and hypophosphatemia attenuates artery media calcification in *Enpp1*^{−/−} (as well as *Mgp*^{−/−}) mice [10]. Furthermore, survival in GACI has been linked with development of decreased serum phosphate [14], linked to phosphaturia that reflects the newly recognized physiologic role of ENPP1 in renal tubular phosphate handling [15, 16].

The immunoglobulin superfamily member receptor for advanced glycation end-products (RAGE) [17–22] is a widely expressed transmembrane protein that serves as a cognate receptor not only for the advanced glycation end-products (AGEs) that accumulate in diabetic and aging tissues, but also S100 calgranulins, HMGB1, β -amyloid, advanced oxidation protein products and the leukocyte integrin Mac-1 [17–22]. RAGE not only promotes atherosclerosis and diabetic cardiovascular and renal complications [17–22], but also mediates chondrocyte differentiation [23, 24]. Furthermore, recent studies have revealed the capacity of AGEs to induce calcification by cultured SMCs and pericytes mediated by RAGE signaling [25–27]. RAGE signaling can be suppressed via ligand sequestration by endogenous secretory and endoproteolytically released soluble RAGE (sRAGE) [18, 19, 28–30]. Moreover, decreased sRAGE levels have been reported in association with certain forms of chronic inflammation [18, 19] and in calcific aortic valve stenosis [31].

Here, we examined effects of RAGE deficiency on aortic calcification in *Enpp1*^{−/−} mice. As a model RAGE ligand for studies of cultured aortic explants, we focused on S100A11, a member of the S100/calgranulin family of small (about 10–14 kDa) polypeptides that function partly in cells by shuttling bound calcium and serving as mo-

lecular chaperones [17, 32, 33]. S100 calgranulins, secreted by SMCs and other cells activated by stressors [17, 32, 33], can exert inflammatory cytokine functions mediated by RAGE signaling and suppressed by sRAGE [23, 24, 34]. Expression of calgranulins is a biomarker of chondrogenic differentiation [35, 36], and S100A11 promotes chondrocyte maturation to hypertrophy, a procalcifying differentiation state [23, 24]. Our results reveal that S100A11 promotes P_i -driven artery explant calcification in the presence of PP_i deficiency and that RAGE drives arterial calcification in *Enpp1*^{−/−} mice.

Materials and Methods

Reagents

Unless otherwise indicated, chemical reagents were from Sigma-Aldrich. Polyclonal antibodies to S100A11 were described previously [23, 24], polyclonal antibodies to Pit-1 were from Alpha Diagnostic International, polyclonal antibodies to RAGE from Millipore, and polyclonal antibodies to collagen IX/XI from Calbiochem. Dr. A.M. Schmidt (Columbia University, N.Y., USA) generously provided sRAGE, validated for purity and freedom from endotoxin contamination as previously described [30].

Mouse Aorta Isolation, and Aortic Ring Organ Culture Studies and Calcification Studies

All animal procedures were humanely performed according to an institutionally reviewed protocol. We studied previously described *Enpp1*-deficient mice [13] and *Rage*^{−/−} mice (from Dr. A.M. Schmidt) (both established on a C57BL/6 background), with genotyping by PCR [23, 24]. Since *Enpp1*^{−/−} mice do not interbreed well and die prematurely due to the ‘tiptoe walking’ phenotype of severe peripheral joint fusion developing in the first few months of life [37], our breeding scheme was to first breed *Enpp1*^{−/+} mice with *Rage*^{−/−} and to interbreed F1 *Enpp1*^{−/+} and *Rage*^{−/+} mice, and then to maintain and interbreed F2 mice that were *Enpp1*^{−/+} and *Rage*^{−/−} in order to generate the double-knockout mice. For assessment of the *Enpp1*^{−/−} skeletal ankylosis (‘tiptoe walking’) phenotype, we analyzed 2-month-old animals for grip function in the forepaws and for decline in body weight characteristic of the poor self-feeding ability of this phenotype [37, 38]. We gently lifted mice by their tails to lift their rear paws to induce gripping of the cage with the forepaws, classifying effective forepaw ‘grip time’ of the cage as greater or less than 3 s.

For calcification assays, aortae were isolated and cut into 2- to 3-mm rings, then cultured in M231 medium (Cascade Biologics) supplemented with bFGF, EGF, insulin, and 5% FCS, as described [8], and explants were treated for 15 min with 0.2 mg/ml Collagenase I (Worthington) before adding 2.5 mmol/l sodium P_i and 7 U/ml alkaline phosphatase for 7 days. The explants were taken from multiple mice, and each aorta was cut into four pieces, then subjected to treatment, with the replicate value indicating how many mice were used. We measured calcification in samples decalcified in 0.6 N HCl for 24 h, via colorimetric free calcium determination using phenolsulfonphthalein (Bioassay Systems), and corrected for dry weight [8].

Fig. 1. Effects of S100A11, RAGE and sRAGE on calcification and chondrogenic type IX/XI collagen expression in cultured aortic explants. **a** Studies were done only in wild-type mouse artery explants; S100A11 (100 ng/ml) or buffer was added. In both **a** and **b**, calcification was measured in mouse aortic explants of the indicated genotypes treated with collagenase for 15 min and then cultured for 7 days with sodium P_i and alkaline phosphatase, with or

without S100A11 (100 ng/ml) and 10-fold excess sRAGE (at a dose of 1 μ g/ml) (**a** n = 12–14, **b** n = 14–20). **c** Aortic explants, treated as above, were analyzed for chondrocyte-specific type IX/XI collagen expression (detected as brown staining, with artery media staining as blue) by immunohistochemistry at 7 days. Statistical analyses here and elsewhere were done by 2-way ANOVA.

Quantitative Real-Time RT-PCR

Total RNA was isolated from aortic rings using Trizol reagent after the adventitia was removed, and reverse transcription and quantitative real-time RT-PCR were performed using the Light-Cycler 2.0 (Roche Diagnostics), as described [8]. *Scl20a1* mRNA encoding Pit-1 was detected using primers: forward 5'-CGTGCCAAAGAAGGTGAAC-3' and reverse 5'-CACTCATGTCCATCTCAGACT-3', and mouse *S100A11* mRNA primers were: forward 5'-AGCTGGACCTCAACTGT-3', and reverse 5'-GTAGGTGTGCTGGGCTC-3', and mouse *Rage* extracellular domain primers: forward 5'-CTGCAGGGACCCTTAGC-3', and 5'-TGGTGGTTCCTCCTTGG-3', with relative quantification of target gene and reference (GAPDH) analysis determined the normalized target gene: GAPDH mRNA copy ratios using LightCycler Software (Version 4.0).

Immunohistochemistry

Sections (10 μ m) of aortic ring cultures were fixed for 10 min in 90% methanol and 3% hydrogen peroxide and then permeabilized in 0.1% Triton X-100 for 5 min and blocked in 1% BSA and 1% casein for 30 min. Primary antibodies were diluted 1:100 in blocking buffer and incubated at 37°C for 1 h, and 3-amino-9-ethylcarbazole was used to detect positive antigen staining via a Histostain Plus kit (Invitrogen). All light microscopy images were visualized on a Nikon microscope, and Nikon digital camera images were captured using ACT-2U software.

SDS-PAGE/Western blotting and sRAGE ELISA

Western blotting analyzed aliquots of 30 μ g protein obtained from sonicated whole aortas or precipitated from conditioned medium using 15% trichloroacetic acid, followed by separation via 10% SDS-PAGE. Horseradish peroxidase-conjugated anti-

Fig. 2. Effects of S100A11 on expression of the artery calcification mediator Pit-1 in cultured aortic explants. **a** *Scl20a1* mRNA was analyzed by qPCR ($n = 11-14$, $* p < 0.05$) at 3 days. **b** Pit-1 was analyzed by SDS-PAGE/Western blotting at 7 days (representative of 3 experiments) in aortic explants treated, as above, with sodium P_i and alkaline phosphatase.

rabbit IgG (0.11 $\mu\text{g}/\text{ml}$) was from Pierce. Immunoreactive products were detected by enhanced chemiluminescence [24]. Quantification of conditioned medium sRAGE employed an ELISA from R&D Systems according to the manufacturer's protocol.

Statistical Analyses

Error bars, unless otherwise indicated, represent SEM. Statistical analyses were performed by 2-way ANOVA.

Results

S100A11 Induces Chondrocyte-Specific Collagen IX/XI Expression and Calcification in Aortic Explants Dependent on RAGE

Because ectopic artery calcification in *Enpp1*^{-/-} mice is P_i -dependent and mediated by PP_i deficiency, all in vitro studies on effects of S100A11 and RAGE on mouse aortic explants were conducted using exogenous P_i as well as alkaline phosphatase to hydrolyze ambient PP_i . S100A11 increased the calcification of P_i /alkaline phosphatase-treated mouse wild-type aortic explants (fig. 1a). The capacity of S100A11 to increase mouse aortic explant calcification was inhibited by exogenous sRAGE (at 10-fold excess to S100A11) (fig. 1a) in wild-type explants. In contrast, S100A11 failed to increase calcification in *Rage*^{-/-} aortic explants (fig. 1b). Under these conditions, there was no significant difference between basal calcification (i.e. without added S100A11) in wild-type and *Rage*^{-/-} artery explants (fig. 1b). S100A11 stimulated expression of chondrocyte-specific type IX/XI collagen in wild-type but not *Rage*^{-/-} aortic explants (fig. 1c). S100A11 also induced *Scl20a1* mRNA [8] and expression

of its encoded protein, the sodium-dependent P_i co-transporter Pit-1 [39] (a promoter of artery calcification) [11], in aortic explants (fig. 2a, b).

Calcification and Type IX/XI Collagen Expression Are Inhibited in vitro and in vivo by RAGE Knockout in Enpp1^{-/-} Aortic Explants

Aortae from 20- to 21-week-old *Enpp1*^{-/-} mice manifest spontaneous calcification and features of ectopic chondrogenic differentiation in situ [13]. Here, we analyzed cultured aortic explants of 2-month-old mice. We cross-bred *Rage*⁻ and *Enpp1*-deficient mice and observed that increased calcification and type IX/XI collagen expression in cultured P_i - and alkaline phosphatase-treated *Enpp1*^{-/-}/*Rage*^{+/+} aortic explants were not shared in *Enpp1*^{-/-}/*Rage*^{-/-} explants, which demonstrated responses comparable to those in *Enpp1*^{+/+}/*Rage*^{+/+} and *Enpp1*^{+/+}/*Rage*^{-/-} explants (fig. 3a, b).

Similar to the in vitro results in P_i /alkaline phosphatase-treated aortic explants, spontaneous calcification in situ in 5-month-old *Enpp1*^{-/-}/*Rage*^{+/+} aortae was markedly decreased by *Rage* knockout in age-matched *Enpp1*^{-/-} aortae (fig. 3c). Nevertheless, calcification was higher ($p < 0.05$) in *Enpp1*^{-/-}/*Rage*^{-/-} aortae in situ than in *Enpp1*^{+/+} aortae with or without *Rage* knockout (fig. 3c). Moreover, *Rage* knockout did not correct the gross skeletal phenotype and associated failure to thrive of *Enpp1*^{-/-} mice, as assessed by development within months of the tiptoe-walking phenotype evidenced by significantly decreased grip strength, and significant weight loss in *Enpp1*^{-/-} mice irrespective of *Rage* genotype (table 1).

Fig. 3. Effects of RAGE on calcification in aortic samples that lack or express ENPP1. **a** Aortic explants from 2-month-old donors of the indicated genotypes were treated with sodium P_i and alkaline phosphatase as above, and calcification was quantified at 7 days (n = 8–20). **b** Type IX/XI collagen was assessed by immunohisto-

chemistry at 7 days. **c** Calcification in situ was analyzed in aortae removed from 5 month-old mice (n = 11) without further treatment. Calcification was markedly reduced by *Rage* knockout in *Enpp1*^{-/-} mice though remaining elevated (p < 0.05) compared to *Enpp1*^{+/+} mice with or without *Rage* knockout.

Table 1. Body weights (±SD) and grip time (measured as described in the Methods) at 2 months of age in *Enpp1*^{-/-} mice with and without *Rage* knockout

Genotype	<i>Enpp1</i> ^{+/+} / <i>Rage</i> ^{+/+}	<i>Enpp1</i> ^{+/+} / <i>Rage</i> ^{-/-}	<i>Enpp1</i> ^{-/-} / <i>Rage</i> ^{+/+}	<i>Enpp1</i> ^{-/-} / <i>Rage</i> ^{-/-}
Weight, g	32.3 ± 1.5	33.6 ± 5.2	25.8 ± 0.3*	27.3 ± 1.9*
Grip time				
<3 s	0/108	0/82	46/46	70/70
>3 s	108/108*	82/82*	0/46*	0/70*

* p < 0.05.

Decreased sRAGE Release by *Enpp1*^{-/-} Aortic Explants

Cultured *Enpp1*^{-/-} and wild-type aortic explants demonstrated comparable expression of *S100A11* mRNA (data not shown), RAGE protein (assessed by immunohistochemistry) (fig. 4a) and *Rage* mRNA (screened by qPCR

using primers for the RAGE extracellular domain shared by transmembrane RAGE, and endogenous secretory and proteolytically released forms of sRAGE) (data not shown). Wild-type aortic explants released significantly more sRAGE into conditioned media compared to the *Enpp1*^{-/-} samples cultured with or without sodium P_i and alkaline phosphatase (fig. 4b). Last, exogenous sRAGE inhibited calcification by cultured P_i and alkaline phosphatase-treated *Enpp1*^{-/-} explants from 2-month-old mice (fig. 4c).

Discussion

In this study, whose results are schematized (fig. 5), treatment of aortic explants with the RAGE ligand S100A11 increased P_i-mediated calcification in a RAGE-dependent manner in association with induction of Pit-1 and chondrocyte-specific type IX/XI collagen expression in vitro. Inflammation and stress-induced secretion of S100 calgranulins allow these proteins to exert inflammatory cytokine functions, mediated in large part by

Fig. 4. RAGE and sRAGE effects and expression in cultured aortic explants that express or lack ENPP1. **a** *Enpp1*^{-/-} and wild-type littermate aortic explants, treated with sodium P_i and alkaline phosphatase, were analyzed for RAGE expression by immunohistochemistry. **b** sRAGE in conditioned media by ELISA at 3 days (n = 8). **c** Explants were treated with 1 μg/ml sRAGE for 7 days and calcification quantified (n = 8–12).

RAGE signaling [17–19, 34, 35]. Hence, calgranulin release illustrates a paracrine effect of inflammation that can promote artery calcification. Other examples include ectopic osteochondral differentiation stimulated by oxidative stress, generation of pro-atherogenic oxidized lipids and TNFα [1–3, 5, 7, 40].

Rage knockout significantly decreased P_i donor-stimulated calcification in *Enpp1*^{-/-} aortic explants as well as aortic calcification in situ in *Enpp1*^{-/-} mice. With ENPP1 deficiency (and decreased function of the plasma membrane PP_i transporter ANK) markedly decreased extracellular PP_i mediates both increased chondrogenic potential of mesenchymal precursor cells and increased P_i donor-stimulated osteochondral trans-differentiation of cultured arterial SMCs [10, 12, 13]. This study demonstrated not only decreased sRAGE release by P_i donor-stimulated aortic *Enpp1*^{-/-} explants in vitro but also that exogenous sRAGE inhibited P_i donor-stimulated calcifi-

cation by *Enpp1*^{-/-} aortic explants in vitro. Decreased sRAGE release by *Enpp1*^{-/-} aortic explants was not associated with decreased RAGE expression. Calcium-regulated intramembrane proteolysis of RAGE is catalyzed by ADAM10 and γ-secretase [28, 29]. It is possible that PP_i metabolism, known to modulate expression of multiple genes [12, 13], influences expression of mediators of intramembrane RAGE proteolysis, and we have not yet directly assessed whether intramembrane RAGE proteolysis is altered in *Enpp1*^{-/-} aortae.

We observed dissociation between effects of *Rage* knockout on peripheral skeletal ankylosis (manifested by paw grip dysfunction and associated wasting) and the artery calcification phenotypic features of *Enpp1*^{-/-} mice in this study. Failure to thrive of *Enpp1* knockout mice, which is intimately linked to peripheral joint fusion, is not suspected to contribute to artery calcification, but there was no way to test this in the scope of this study. Similar dis-

Fig. 5. Schematic depicting the effects of ligand-induced RAGE signaling (e.g. by S100A11) and sRAGE on P_i -mediated artery calcification in wild-type and *Enpp1*^{−/−} mice. This model depicts the amplification loop by which the effects of RAGE and RAGE ligands such as S100A11 increase the capacity of the wild-type artery to calcify, in a P_i -mediated manner, and the P_i -mediated calcification pathway is known to be regulated by the sodium-dependent P_i co-transporter Pit-1 (which is induced by S100A11). The P_i -mediated calcification pathway in the artery is markedly enhanced by ENPP1 knockout-associated PP_i deficiency, in which a secondary deficiency of sRAGE compounds the diathesis for artery calcification, since sRAGE normally inhibits the capacity of RAGE ligands to stimulate artery calcification.

sociation between effects on artery calcification and peripheral skeletal ankylosis was previously observed with deficiency of the redox stress, inflammation, and chondrogenesis mediator *Vnn1* in *ank/ank* mice (whose extracellular PP_i deficiency state and phenotype are comparable to those of *Enpp1*^{−/−} mice) [12]. The periskeletal soft-tissue calcification of *ank/ank* mice appears to be driven more by ectopic osteoblastic than chondrocytic differentiation and function, whereas ectopic chondrogenic differentiation appears essential to artery media calcification in *ank/ank* mice [12]. In this context, RAGE supports maintenance of normal bone mass in mice by modulating osteoclast function, and *Rage*^{−/−} mice demonstrate increased bone mass [41, 42]. As such, in *Enpp1*^{−/−} mice, loss of contribution of RAGE signaling to osteoclast differentiation [41, 42] could support development of pathologic osteoblast-driven ectopic calcification in periskeletal soft tissues.

This study established direct effects of S100A11 and RAGE on calcification and chondrocyte-specific collagen expression in isolated aortic explants. However, one limitation of the work is that we did not rule out additional

effects of *Rage* knockout on the bone-vascular axis [43], via altered bone turnover, with the potential to impact on artery calcification. Other limitations included the focus on single models of in vitro and in vivo calcification. For example, we have not yet assessed the effects of RAGE in artery calcification driven by MGP deficiency, vitamin D excess, or atherosclerosis. In this light, diabetes-associated artery media calcification in mice and human atherosclerotic intimal calcification appear to be driven more by ectopic osteoblastic rather than chondrocytic differentiation [7]. Three recent studies have demonstrated that AGEs promote calcification by cultured vascular cells [25–27]. Here, we concentrated on artery explants, on mechanisms pertinent to GACI, and on a single RAGE ligand (S100A11), and not on potential effects of the multiple RAGE ligands (e.g. AGEs, HMGB1, and calgranulins other than S100A11) pertinent to arterial disease in aging, diabetes, atherosclerosis, and renal failure [17–22, 44–48]. We also have not yet determined whether alternative receptors for S100A11 such as CD36 [49] modulate artery calcification. Last, S100A11 stimulates chondrocyte-me-

diated extracellular matrix catabolism [23, 24], and we have not yet examined the potential role of RAGE signaling in arterial extracellular matrix catabolism, which mediates SMC differentiation and calcification [50, 51].

In conclusion, the RAGE ligand S100A11 promotes P_i donor-stimulated artery calcification in vitro. Moreover, RAGE is needed to drive full expression of pathologic artery calcification in an animal model of GACI, the *Enpp1*^{-/-} mouse, whose aortic explants demonstrate decreased sRAGE release in vitro. Our results suggest that factors that promote shedding of sRAGE could exert substantial effects on artery calcification. In this light, release of sRAGE is associated with (and a potential biomarker of) ongoing inflammation [28] and the RAGE ligand HMGB1 stimulates shedding of sRAGE [29]. Hypophosphatemia is linked with survival beyond infancy in GACI [14], and attenuates ectopic calcification in both extracellular PP_i-deficient and *Mgp*^{-/-} mice [10]. Nevertheless, deleterious systemic effects of hypophosphatemia impose limits on

therapeutic serum phosphate lowering to suppress GACI. Since RAGE deficiency promotes lung fibrosis [52], it is conceivable that *Rage* knockout could have adverse consequences in GACI, in which, not simply calcification at the internal elastic lamina, but also marked myointimal proliferation and arterial fibrosis, are found in occluded vessels [4]. Nevertheless, antagonism of cellular RAGE signaling, for example via increasing sRAGE levels, appears to merit investigation as a potential therapeutic approach to inhibition of artery media calcification in GACI. Furthermore, RAGE may be a molecular target in other diseases associated with pathologic arterial calcification.

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