Oxidant Generation Predominates Around Calcifying Foci and Enhances Progression of Aortic Valve Calcification

Marcel Liberman, Estêvão Bassi, Marina Kamla Martinatti, Fábio Cerqueira Lario, João Wosniak Jr, Pablo M.A. Pomerantzeff, Francisco R.M. Laurindo

Objective—We hypothesized that reactive oxygen species (ROS) contribute to progression of aortic valve (AV) calcification/stenosis.

Methods and Results—We investigated ROS production and effects of antioxidants tempol and lipoic acid (LA) in calcification progression in rabbits given 0.5% cholesterol diet +10⁴ IU/d Vit.D₂ for 12 weeks. Superoxide and H₂O₂ microfluorotopography and 3-nitrotyrosine immunoreactivity showed increased signals not only in macrophages but preferentially around calcifying foci, in cells expressing osteoblast/osteoclast, but not macrophage markers. Such cells also showed increased expression of NAD(P)H oxidase subunits Nox2, p22phox, and protein disulfide isomerase. Nox4, but not Nox1 mRNA, was increased. Tempol augmented whereas LA decreased H₂O₂ signals. Importantly, AV calcification, assessed by echocardiography and histomorphometry, decreased 43% to 70% with LA, but increased with tempol (P≤0.05). Tempol further enhanced apoptosis and Nox4 expression. In human sclerotic or stenotic AV, we found analogous increases in ROS production and NAD(P)H oxidase expression around calcifying foci. An in vitro vascular smooth muscle cell (VSMC) calcification model also exhibited increased, catalase-inhibitable, calcium deposit with tempol, but not with LA.

Conclusions—Our data provide evidence that ROS, particularly hydrogen peroxide, potentiate AV calcification progression. However, tempol exhibited a paradoxical effect, exacerbating AV/vascular calcification, likely because of its induced increase in peroxide generation. (Arterioscler Thromb Vasc Biol. 2008;28:000-000.)

Key Words: calcification ■ atherosclerosis ■ antioxidants ■ valves ■ free radicals

Degenerative aortic valve (AV) stenosis, the third most prevalent cardiovascular disease in the elderly, shares common risk factors and pathophysiological features with atherosclerosis.1-6 Although the role of oxidative stress in atherosclerosis is well explored,7,8 it is unclear whether redox processes contribute to progression of AV calcification.2,3,9-11,15,16 Scarce observations provide indirect support for this hypothesis.10 In vitro studies showed that exogenous superoxide, hydrogen peroxide, or other oxidants increase the number and activity of calcifying vascular cells (CVCs),11 referred to as a specific subpopulation of cells, derived from (de)differentiation of vascular smooth muscle cells,12 pericytes, or mesenchymal cells13 that can produce hydroxyapatite in the vascular wall.14 In addition, reactive oxygen species (ROS) mediate increase in BMP2 expression and signaling, favoring osteogenesis.2 On the other hand, calcium resorption by osteoclasts is dependent on ROS derived from its own NAD(P)H oxidase,15 whereas nitric oxide induces osteoclast detachment and inhibits calcium resorption.16 Recent data from an experimental mouse model of aortic sclerosis suggested locally increased superoxide generation.3 Observational clinical studies with statins indicated possible decrease in calcification progression in hypercholesterolemic patients,4 but a prospective clinical trial in normocholesterolemic subjects showed lack of effect.5 In the present study, we investigated the occurrence and microtopography of ROS generation in an experimental rabbit model of AV calcification and sclerosis and in specimens from human with AV sclerosis or stenosis. In addition, the role of redox processes in the progression of AV calcification was tested by assessing the effects of 2 antioxidants, tempol and lipoic acid (LA), both in a rabbit model17 and in an in vitro model of VSMC calcification.18

Methods

An extended Methods section is available as supplemental material (available online at http://atvb.ahajournals.org).

Human Aortic Valves

AV from patients with stenosis (n=5) or individuals with sclerosis (n=4), collected respectively at surgery or autopsy (<6hs postmortem), were analyzed for ROS microtopography, histology, and immunohistochemistry for NAD(P)H oxidase subunits and compared with young autopsied controls (n=6).

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Rabbit Model

The rabbit AV calcification model modified from Drolet et al. involved administration for 3 months of 0.5% cholesterol-enriched diet + vitamin D3 104 IU/d (HC + vitD rabbits, n = 34). Some HC + vitD rabbits concomitantly received tempol 100 μmol/kg/d (n = 15) or lipico acid (LA) 120 μmol/kg/d (n = 11) in drinking water. Controls received 0.5% cholesterol alone (n = 9) or normal chow (n = 32). Plasma cholesterol, calcium, phosphorus, and creatinine levels were analyzed.

After the 12th week, AV were collected and processed for morphometric analysis and collagen histomorphometry, von Kossa staining for calcium detection, immunohistochemistry for NAD(P)H oxidase subunits, protein disulfide isomerase (PDI), macrophage marker RAM11, nitrotyrosine, Ki67 and osteoblast markers, immuno-fluorescence for NAD(P)H oxidase subunits, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), and TRAP histochemistry for osteoclasts. Quantitative morphometric analysis was performed by Leica Quantimet software.

In Situ ROS Generation

In situ microfluorotopography of dihydroethidium (DHE) oxidation products was performed as described, with 3 μmol/L final DHE concentration. Slides were analyzed by confocal microscopy (Zeiss LSM510) with laser excitation at 488 nm and emission at 610 nm, with final DCF-DA concentration of 3 μmol/L. Dichlorofluorescein diacetate (DCF-DA), using a similar protocol, was performed as described,8 with 3 μmol/L final DCF-DA concentration of 3 μmol/L. Controls, performed through D) normalized for control values. Each bar represents mean ± SE of at least 3 different valves. *P<0.05 vs Control; #P<0.05 between specified groups.

Real-Time Polymerase Chain Reaction

Quantitative expression of Nox1 or Nox4 mRNA was performed in aortic tissue as detailed in supplement, with primers designed according to rabbit sequences.

Echocardiography

Valve area, left ventricular mass and dimensions, peak and mean aortic valve flow velocity were assessed by ultrasound/doppler as detailed elsewhere1 and in supplement, with 12-MHZ phased-array probe and Philips Sonos 5500 system. Exams were performed at beginning of protocol and after 3 months of intervention.

Calcification Analysis

A semi-quantitative index (range 0 to 3) based on opacity and valve motility was used for estimating AV echogenicity, an index of valve thickening and calcification, as follows: 0-absent, 1-mild; 2-moderate, with preserved motility/opening; 3-severe, with decreased motility/opening. Data were analyzed on a blinded fashion. Histomorphometry of calcium conglomerates was performed with Leica Quantimet software in von Kossa and hematoxylin-rosin stained sections. Ex vivo 64-row computed tomography of aortic specimens was performed as detailed in supplement.

In Vitro VSMC Calcification Model

The model was described by Shiioi et al.18 Rabbit aortic vascular smooth muscle cells cultured in DMEM with FBS10%, at 70% confluence and 4th passage, were supplemented during 14 days with 6 mmol/L calcium chloride, alone or in combination with 10 mmol/L β-glycerophosphate, or both in combination with 0.1 μmol/L vitamin-D3. The latter combination was supplied in the absence or presence of 100 μmol/L tempol or 100 μmol/L LA for 14 days, changing medium every 3 days. Exogenous hydrogen peroxide (50 μmol/L), catalase (500U/mL), or the NO donor NOC-18 (30 μmol/L) were added in specific experiments, changing medium every 3 days. We used either primary cells or cells from an immortalized line.19

Results

Human Aortic Valves

Human stenotic and to a lesser degree sclerotic AV exhibited thickening, fibrosis, elastolysis, and calcification (not shown). Microtopography of superoxide production showed increased fluorescence signals in sclerotic, and specially in stenotic AV, observed mainly around calcifying foci. Signals decreased on specimen incubation with Peg-SOD (Figure 1A through 1D). Expression of p22phox and Nox2 NAD(P)H oxidase activity through lucigenin (5 μmol/L) chemiluminescence.

Figure 1. Microfluorotopography of DHE oxidation products in human aortic valves (AV) showing increased fluorescence signals vs Control (A) in sclerotic (B), and specially in stenotic AV (C), observed mainly around calcifying foci (Ca2+), which decreased after incubating slides with 500U/mL Peg-SOD (D). NAD(P)H oxidase subunit expression was also increased around such foci (Ca2+), shown by both p22phox (E) and Nox2 (F) peroxidase-immunostaining. Below, graph representing total fluorescence intensity (area units [AU], corresponding to panels A through D) normalized for control values. Each bar represents mean ± SE of at least 3 different valves. *P<0.05 vs Control; #P<0.05 between specified groups.

CVC layer calcium content was assessed by colorimetric assay, as detailed in supplement. Superoxide production was assessed through high-performance liquid chromatography (HPLC) analysis of DHE oxidation products as described20 and membrane fraction NAD(P)H oxidase activity through lucigenin (5 μmol/L) chemiluminescence.

Statistics

Data are mean±SE. Comparisons were tested through 1-way ANOVA plus Student–Newman-Keuls or Dunnet post-hoc tests, at a 0.05 significance level.
oxidase subunits was increased around such foci (Figure 1E and 1F; supplemental Figure I).

Rabbit Model
Preliminary experiments with serial echocardiograms showed progressive increases in AV calcification in HC vitD rabbits, with accelerated progression after 8 weeks.

Histological Characterization
AV calcification accompanied the increased plasma levels of cholesterol and calcium-phosphorus product, mainly because of phosphate increase, and minor increase in serum creatinine levels (supplemental Table I). AV from HC vitD rabbits showed early stages of cusp mineralization (supplemental Figures II and III): thickening caused by collagen infiltration and increased cellularity, massive macrophage infiltration in the aortic face, and several conglomerate foci of subendothelial calcification. In 3 intact rabbits fed regular chow, tempol was given at 100 μmol/kg/d for 3 months. There were no changes in blood biochemical parameters and AV calcification, showing that tempol alone had no effects in the study variables (not shown).

Echocardiographic Data
Significant increase in echogenicity occurred in HC+vitD rabbits (supplemental Figure III). Within the time frame of study, the absolute AV area and aortic jet/left ventricular outflow tract velocity ratio did not differ among groups (supplemental Figure IV). Final AV area decreased versus baseline in all HC+vitD rabbits, but such decrease reached statistical significance only in HC+vitD rabbits given tempol. Concomitantly, there was increase in posterior wall thickness (PWT), revealing concentric ventricular remodeling in all HC+vitD rabbits (supplemental Figure IV). Thus, such animal model shows changes consistent with AV sclerosis.

Reactive Oxygen Species Generation
DHE fluorescence microtopography (Figure 2) showed increased superoxide signals corresponding to macrophage infiltration and an even more pronounced increase, ~6-fold above normal, in cells surrounding calcifying foci, which, as shown below, did not stain for macrophage markers. Signals were importantly reduced after specimen incubation with Peg-SOD. Superoxide signals were decreased in LA- and specially tempol-treated rabbits (Figure 2G and 2H). Overall
Effects of Antioxidants on AV Calcification and Cell Infiltration

In HC+vitD rabbits, nitro/oxidative stress preferentially occurred around calcifying foci, and to a lesser extent in macrophage staining area, as shown from increased 3-nitrotyrosine immunostaining (Figure 4), which was decreased with LA and particularly with tempol (supplemental Figure V).

Expression of NAD(P)H Oxidase Subunits

NAD(P)H oxidase subunits p22phox, Nox2, and particularly its newly-described regulator protein disulfide isomerase were strongly expressed around calcification, coincident with the site of RAM11-negative cells and highest ROS production (Figure 4). Both Nox2 and protein disulfide isomerase colocalized with p22phox in such cells (supplemental Figure VI). Analysis of Nox1 and Nox4 mRNA expression by real-time PCR showed increase in Nox4 expression in HC+vitD rabbits, reflected in increased Nox4/Nox1 ratio, which was especially increased with tempol (Figure 5).

Phenotypic Markers of CVC/Osteoclasts

Several markers were analyzed for assessing the phenotype of cells surrounding calcifying foci. Importantly, staining for macrophage marker RAM11 was absent around calcifying foci (Figure 2A; supplemental Figure II). There was increased immunoreactivity for the osteoblast differentiation markers osteopontin and Cbfa-1 in HC+vitD rabbits (supplemental Figure VIIE and VIIF). Osteopontin mRNA was also increased and was unchanged by antioxidants (supplemental Figure VIIIC). In addition, osteoclasts were identified by positive TRAP histochemistry (supplemental Figure VIIID). This implicates ROS generation in cellular events related to calcification process.

Figure 3. A through F, Microfluorotopography of DCF-DA oxidation products in rabbit AV. A, Normal control; B, Cholesterol-only control rabbit; C, HC+vitD; D, HC+vitD+in vitro Peg-Catalase; E, HC+vitD+tempol; F, HC+vitD+LA. Fluorescence was increased in HC+vitD (C) and particularly pronounced with tempol (E), decreasing after in vitro incubation with 400 U/mL Peg-catalase (D). Below, Graph representing total fluorescence intensity normalized for Normal control values. Each bar represents mean±SEM of at least 3 different rabbits. Letters below each bar correspond to the above panels, and H is HC+vitD+tempol+in vitro Peg-catalase (figure not shown). *P<0.05 vs Normal; #P<0.05 between specified groups.

Figure 2A through F, Microfluorotopography of DCF-DA oxidation products in rabbit AV. A, Normal control; B, Cholesterol-only control rabbit; C, HC+vitD; D, HC+vitD+in vitro Peg-Catalase; E, HC+vitD+tempol; F, HC+vitD+LA. Fluorescence was increased in HC+vitD (C) and particularly pronounced with tempol (E), decreasing after in vitro incubation with 400 U/mL Peg-catalase (D). Below, Graph representing total fluorescence intensity normalized for Normal control values. Each bar represents mean±SEM of at least 3 different rabbits. Letters below each bar correspond to the above panels, and H is HC+vitD+tempol+in vitro Peg-catalase (figure not shown). *P<0.05 vs Normal; #P<0.05 between specified groups.
In cultured CVCs, calcium deposition increased in proportion with strength of calcifying stimuli during 14 days. Such deposition was further enhanced with tempol, but not with LA (Figure 6). CVC incubation with exogenous 50 μmol/L hydrogen peroxide amplified mineralization response irrespective of antioxidant coincubation. Incubation with catalase did not change basal calcification, but completely prevented increased calcification induced by tempol. Incubation with NO donor NOC-18 (30 μmol/L) for 14 days did not change CVC calcium deposition (not shown). Thus, while exogenous oxidants increased cell calcification, the mechanism of calcification appears to differ in vitro (versus in vivo) enough that baseline CVC calcification is redox-insensitive. Superoxide production, as assessed by HPLC analysis of 2-hidroxyethidium (Figure 6), as well as membrane fraction NAD(P)H oxidase activity (supplemental Figure IX) was increased with calcifying stimuli, partially reduced with tempol and strongly decreased with LA.

Discussion

Convergence between ROS and valve calcification is usually regarded in a chronic inflammation context. Thus, in our rabbit model, the observed topography of ROS production around calcifying foci from cells displaying phenotypic markers of osteoblasts or osteoclasts, but not of macrophages, is an important novel finding, although contribution of other phenotypes cannot be excluded. Analogous patterns occurred in human AV sclerosis or stenosis, despite advanced disease in the latter. The rabbit model—mimicking earlier human disease—displayed massive AV infiltration of macrophage foam cells exhibiting increased superoxide signals versus control, but not as marked as in cells around calcification. This probably reflects the role of ROS as signaling intermediates of cellular metabolism or developmental processes occurring during CVC or osteoclast formation and activity. Moreover, these data stress that even with the likely occurrence of substantial lipoprotein oxidation, cellular signaling still seems of prime or at least comparable importance as ROS source. Thus, our data show that oxidant stress pattern in valve calcification shares partial resemblance with atherosclerosis, and extends beyond vascular or inflammatory cell activation. Of note, tempol and LA decreased superoxide levels without changes in macrophage density (supplemental Figure VIIID).

Decreased calcification progression with LA in our rabbit model indicates that ROS potentiate AV calcification. The large effect of LA verified at echogenicity analysis contrasts with the smaller effect evident at histomorphometry. Echogenicity reflects AV thickening and microcalcification, suggesting LA effects in halting early onset of calcium conglomerates, because macrophage and collagen densities were unchanged (supplemental FigureVIIIC and VIIID). Contrarily, histology preferentially shows larger mineral conglomerates. This could indicate that once a threshold conglomerate size is reached, further growth occurs rapidly and overrides...
antioxidant effects, an explanation in line with known exponential growth rate of calcification. This could also reflect a limitation of our models, related to abnormally high calcification stimulus attributable to induced increase in calcium-phosphorus product, which can activate CVC differentiation via phosphate cotransporter Pit-1.21 Although such increase is found in some diseases, degenerative AV calcification usually occurs under normal calcium-phosphorus product. In fact, in our in vitro model, LA or catalase showed little effect in basal calcification, whereas exogenous peroxide clearly increased calcium deposits. Another methodological caveat is that total DHE fluorescence analysis, used because calcium deposits and small rabbit AV size limited use of other methods, lacks specificity for superoxide.20 Thus, whereas Peg-SOD controls indicate that superoxide contributes to DHE fluorescence, other oxidants, heme or peroxidases might play additional roles.20 Analogous observations apply to in situ DCF studies regarding hydrogen peroxide detection. On the other hand, 2-hydroxyethidium measurements in CVC in vitro provide more specific evidence for superoxide.20 The NAD(P)H oxidase complex exhibited dynamic expression changes during calcification, with upregulation of Nox4 and Nox2, but not Nox1. ROS topography around calcifying foci coincided with increased expression of subunits p22phox and Nox2 and enhanced 3-nitrotyrosine staining. Protein disulfide isomerase, recently described by us to assist NAD(P)H oxidase activity,19 was strongly upregulated at this location. This pattern is in line with known roles of Nox4 in cell differentiation/apoptosis and of Nox2 in inflammation.22 The precise correlation between each Nox isoform and cell type remains undefined, considering lack of available information about Nox subtypes in CVCs/osteoblasts. In osteoclasts, Nox2 is prominent, whereas Nox4 is upregulated on Nox2 knockout, both supporting bone resorption.23 Together, our observations implicate hydrogen peroxide as a species contributing to vascular/AV calcification. Tempol, which increased hydrogen peroxide production, promoted enhanced calcification in vivo or in vitro, contrary to LA, which promoted hydrogen peroxide decrease and halted calcification progression in vivo. Of note, both antioxidants promoted comparable superoxide decrease. Moreover, CVC incubation with exogenous hydrogen peroxide promoted enhanced calcification, whereas analogous incubation with nitric oxide donor was without effect. In addition, catalase prevented tempol-associated increase in CVC calcification.

The importance of hydrogen peroxide is in line with observed increase in Nox4, which may preferentially generate hydrogen peroxide.22 Indeed, in our CVC, Nox4/Nox1 ratio increased 1.6-fold versus control VSMCs, whereas 14-day exposure to hydrogen peroxide (see Methods) further enhanced such ratio by 4- to 5-fold in both cases (not shown). Mechanisms whereby ROS enhance vascular/calciﬁcation are yet poorly understood. ROS upregulate expression of BMP family proteins such as BMP2 and BMP4,24 as well as Cbfα-1 and alkaline phosphatase.2,11 Oscillatory shear stress (to which AV is exposed) induces BMP4 via p47phox/Nox1-dependent NAD(P)H oxidase, further inducing intercellular adhesion molecule-1 (ICAM-1)
expression and monocyte adhesion. ROS may also trigger apoptosis, a potential seed for calcification. Conversely, calcium resorption is enhanced by osteoclast Nox2 or Nox4-derived ROS and inhibited by nitric oxide.

Although antioxidant LA effects have been reported in several preparations and in humans, precise mechanisms of LA effects are unclear. LA is thought to react with peroxynitrite only at low rates and probably does not scavenge hydrogen peroxide directly. Although LA might owe its antioxidant activity to the reduced form dihydrolipoic acid, LA itself can also scavenge species such as hypochlorous acid thanks to the unique reactivity of its 2,3-dithiolane ring. Moreover, LA shares with distinct antioxidants such as resveratrol, but not /H9251-tocopherol or ascorbic acid, the capacity to induce heme oxygenase-1 via Nrf-2.

Tempol-mediated increase in calcification and hydrogen peroxide levels provide further evidence that ROS accelerate AV calcification in vivo. Central to this question is the mechanism of tempol effects in our system. Similarly to other reports, tempol decreased superoxide levels and nitrotyrosine staining, in line with its proposed mechanisms of action, namely SOD-mimetic activity and shift of nitroxidative stress toward nitric oxide. Tempol-induced hydrogen peroxide increase was previously reported in endothelial or tumor cells and increased at mmol/L tempol concentrations or high glucose levels. Our EPR assessment of plasma tempol concentration yielded values well within or even below usual. Explanation for those effects is yet unclear, but it is unlikely that hydrogen peroxide increase is the usual outcome of possible SOD-like tempol effect, which in itself is debatable. Mitochondrial function impairment attributable to tempol was reported. In addition, while reacting poorly with superoxide anion radical, tempol reactivity strongly enhances at lower pHs via direct reaction with hydroperoxyl radical (OOH), the protonated uncharged form of superoxide. Thus, at least in our in vivo model, an unusually strong SOD-like effect might have occurred because of decreased pH caused by the carbonic anhydrase activity of osteoclast, which promotes intra- and extracellular acidification. This is consistent with lack of toxicity of high tempol concentrations in intact cells and with tempol-induced oxidant stress in the acidic renal medulla. Also, we observed higher cell loss around calcifying foci with tempol. Increased progression of valve calcification with tempol may be clinically relevant, considering that this nitroxide antioxidant is already undergoing prospective clinical studies for several conditions.

Collectively, our observations point to a role of redox processes, particularly those resulting in hydrogen peroxide increase, possibly related to NAD(P)H oxidase activity, in the progression rate of AV calcification. These results further a link between pathogenesis of AV stenosis and atherosclerosis, and pave the way to clinically effective interventions.
capable of slowing such progression, as suggested by protective LA effects.

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Disclosures
None.

References
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Supplemental Material

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Supplemental material consists of:

- Expanded methods section
- References
- Table I
- Legends for supplemental figures
- Supplemental figures I to X
Methods (expanded version)

Reagents

All chemicals, including α-lipoic-acid, tempol, cholesterol, PEG-superoxide dismutase (SOD), PEG-Catalase were from Sigma Chemical, except when specified; DCF-DA and DHE were from Invitrogen; OCT Tissue Tek embedding compound was from Fisher Scientific. For immunohistochemistry, the following commercial primary antibodies and dilutions were used: RAM11 1:200 (Dako Cytomation), Nox2 1:200, p22phox 1:200, osteopontin 1:200 and Cbfa-1 1:100 (Santa Cruz Biotechnology), nitrotyrosine 1:100 (Upstate Cell Signaling), PDI 1:32000 (Stressgen), Ki-67 1:200 (Novocstra LTD). Non-commercial antibodies were: osteopontin 1:50 (Hybridoma Bank-University of Iowa) and Nox2 clone 54.1 1:1600 (kind gift of Dr. J Burritt, University of Montana). Secondary antibodies were anti-mouse or anti-goat from Vectastain Elite ABC System (Vectastain, Vector Technologies, Burlingame, CA) for immunohistochemistry. For immunofluorescence, secondary antibodies were anti-mouse or anti-goat (Calbiochem) conjugated to rhodamine or FITC. For assessing cell loss in AV tissue samples, we used “in-situ cell death detection kit” from Roche Applied Science and DNase from Promega Corporation. Culture medium DMEM, penicillin (100mg/mL), streptomycin (100 mg/ml) and trypsin were from Gibco BRL-Life Technologies (Grand Island); FBS was from Cultilab, collagenase and elastase were from Worthington Biochemical Corporation. For cell culture calcium measurement, we used “Calcium Reagent Set” from Teco Diagnostics. Trizol, PCR, Reverse Transcriptase kits and Sybr Green were from Invitrogen Corporation.

Ethics

This study was approved by an internal scientific institutional Committe and by the Ethics Committee from Hospital das Clínicas – University of São Paulo Medical School, and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, rev. 1996).
**Human aortic valves**

Human stenotic AV were collected from patients with isolated tricuspid AV stenosis (n=5) during surgical replacement; control (n=6) and sclerotic (n=4) valves were acquired from corpses undergoing autopsy (not after 6 hours after death). We considered control valve a tricuspid and thin valve without evidence of subaortic or aortic arch disease from patients <35 years old. Sclerotic AV were collected from individuals >65 years old, revealing tricuspid valve thickening, initial calcifying foci, no evidence of rheumatic disease, any other valve dysfunction and no evidence of aortic dissection or aneurism. All sclerotic or stenotic AV were analyzed for ROS microtopography, histology and immunohistochemistry for NADPH oxidase subunits, as described below.

**Rabbit AV calcification model**

Male NZW rabbits weighing 2500-3000g were housed in individual cages in a controlled room under 12h light-dark cycle at temperature (25°C). After initial quarantine, rabbits were submitted to a 12-week protocol consisting of supplementation of diet with cholesterol 0.5% + Vitamin D$_2$ 10$^4$ IU/day (herein referred to as HC+vitD rabbits, n=34). In specific experiments, tempol 100 µmol/kg/day (n=15) or lipoic acid (LA) 120µmol/kg/day (n=11) were administered continuously in drinking water. As controls, some rabbits received cholesterol 0.5% only (HC, n=9) or regular chow (Normal, n=32). Tempol was diluted in water and LA in ethanol. Eight normal controls received equivalent ethanol only and did not differ from the other normal controls.

**Biochemical blood analysis**

Blood was collected from rabbits for analysis of total cholesterol, calcium, phosphorus and creatinine, which were measured from plasma by standard methods at baseline and after 3 months of intervention.

**Tissue processing**

Rabbit AV were collected after a lethal dose of pentobarbital sodium, rapidly dissected, fixed in 4% PBS-formaldehyde for 24 hours, and included in paraffin for morphology and immunohistochemistry, whereas another section was freshly frozen in
OCT compound for \textit{in situ} ROS detection. Histology was analyzed after hematoxylin and eosin or Verhoeff-Van Gieson stainings. Histomorphometry was performed using a Leica Quantimet software using specific stainings for collagen (Masson’s Trichrome), calcium (Von Kossa) and immunohistochemistry for macrophage infiltration (RAM11 antibody).

**Immunohistochemistry and immunofluorescence**

Briefly, AV were were cut in 3µm-thick sections in silanized slides for immunohistochemical staining. Sections were deparaffinized, tripsinized for antigen recovery and primary antibodies were applied for 12hs. Then, Vectastain Elite ABC System was used for development, with secondary anti-mouse or anti-goat antibodies conjugated to streptavidin–biotin complex. Final staining was performed through reaction with 3,3’-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and counterstained with Harris hematoxylin. For immunofluorescence, anti-mouse or anti-goat secondary antibodies conjugated to rhodamine or FITC were applied. Positive and negative (fetal bovine serum without primary Ab) controls were used in all cases and stained simultaneously. For total cell counting employing nuclei staining, DAPI (4’-6-diamidino-2-phenylindole) was used as a fluorescent dye using excitation-emission wavelengths of 365nm-395nm.

**TUNEL staining**

An estimate of apoptotic cells was performed by positive TUNEL staining using “in-situ cell death detection kit” from Roche, as detailed elsewhere\textsuperscript{1}. Some sections were pre-incubated with DNase as positive controls.

**TRAP**

Osteoclasts were identified by histochemical staining for TRAP\textsuperscript{2}. Sections (3-µm-thick) were deparaffinized, washed in deionized water for 5 min. and preincubated in 0.2M acetate buffer (sodium acetate 0.2 M and L(+) tartaric acid 50mM), pH 5.0, for 20 min. Following 5-min. wash in water, the sections were incubated in the same buffer with added naphthol AS-MX phosphate (0.5 mg/ml) and Fast Red TR (1.1 mg/ml) for up to 6 hours at 37°C, monitoring until osteoclasts were turn bright red. After rinsing in distilled water, the
sections were counterstained in methyl green and mounted in Keiser’s solution. For positive controls we used human regenerating bone fracture sections.

**In situ aortic valve ROS detection**

Dihydroethidium (DHE), a fluorescent dye freely permeable to cells, is trapped to DNA when oxidized to 2-hydroxyoxyethidium, mainly by superoxide, and ethidium, by other ROS, heme or peroxidases (ref. 20). Therefore, this probe allows identification of individual cells producing excess superoxide, although total fluorescence is not specific for the latter. Microtopography of superoxide is performed at confocal microscopy, with excitation at 488 nm and emission at 610 nm, detected with a 560nm longpass filter, as previously described (ref. 8). Longitudinal sections of human and rabbit AV were cut in the cryostat (30 µm thickness) and placed in glass slides. Samples were incubated with DHE 3µM, coverslipped and maintained in a light-protected humidified chamber at 37°C for 30 minutes. Images were obtained with a Zeiss LSM510 confocal laser scanning microscope and Axiovision software (Carl Zeiss MicroImaging GmbH, Germany). Simultaneous paralell acquisition of images was performed always for normal controls and other groups, using identical laser and software settings. To verify if fluorescence signal was mainly superoxide-derived, subsequent images was acquired after incubating the slide with 500 U/ml Peg-SOD for 30 min. We adapted this method to perform a novel analogous in situ detection of hydrogen peroxide using the fluorescent dye 2’,3’-dichlorofluorescein diacetate (DCF-DA). In this case, since the probe is mainly cytosolic and products do not bind to DNA, a diffuse staining is verified. The employed protocol was similar to that described above:similar protocol, with a final DCF-DA concentration of 3µM. Controls, performed by incubating slides for 30 min with Peg-catalase (400U/ml), indicated preferential detection of hydrogen peroxide.

**Morphometric image analysis**

For quantification of DHE and DCF fluorescense, TUNEL positive cells, number of nuclei (DAPI) and 3-nitrotyrosine staining area, we used Image J (version 1.37v; National Institutes of Health, Bethesda, MD) software (ref. 8).
**Echocardiography**

Transthoracic echocardiography was performed at baseline and at 12 weeks of follow-up. Animals were sedated with intramuscular injection of ketamine (30 mg/kg) and xylazine (3.5 mg/kg). The anterior thorax was shaved for better echo imaging. Images were acquired with a 12 MHZ phased-array probe connected to a Sonos 5500 imaging system (Philips Medical Imaging, Andover, Massachusetts). Parasternal long and short axis views were obtained by applying the transducer to right paraesternal location, and apical five-chamber view was obtained by applying the transducer to subcostal location. A comprehensive echocardiographic study was performed, including two-dimensional imaging, Doppler imaging and M-mode imaging. Images were saved in digital format (magneto-optical discs) for further blinded fashion one-operator analysis, as still frames (spectral Doppler and M-mode imaging) or loops (two-dimensional imaging). To minimize the inter and intra-assay variability, all imaging and analysis were carried out by the same investigator.

A parasternal short axis view M-mode imaging at mid left ventricular (LV) level was used to measure the following parameters (average of four cardiac cycles): LV end-diastolic (LVIDd) and end-systolic (LVIDs) dimensions and ventricular septum (VST) and posterior wall thickness (PWT). LV mass was obtained by the Devereux formula. Relative wall thickness (RWT) was calculated as RWT=2xPWT / LVIDd. Fractional shortening (FS=[LVIDd-LVIDs]/LVIDd) and LV ejection fraction by the Teichholz method were also calculated.

A parasternal long axis view was used to measure LV outflow tract mid-systolic dimension (LVOTd). Analysis of the aortic valve anatomy and motility was performed using paraesternal long axis, short axis and the apical five chambers. The latter view was used to estimate the peak and mean aortic valve flow velocities, determined by continuous wave Doppler imaging, averaging the values of four to five beats. The maximal instantaneous gradient and the mean gradient across the aortic valve were derived from aortic Doppler velocities by the modified Bernoulli equation. Left ventricular outflow tract (TVILVOT) was estimated by planimetry, using pulsed wave Doppler, with the volume sample (adjusted to 0.6 mm) positioned proximal to the aortic valve, as performed in humans.
Aortic valve area was calculated by continuity equation\(^5\):\(^7\):
\[
\pi \times (\text{LVOT}_d)^2 \times \text{TVI}_{\text{LVOT}}
\]
\[
\text{AVA} = \frac{4 \times \text{TVI}_{\text{AV}}}{\text{---------}}
\]

**Analysis of aortic valve calcification**

**Echogenicity analysis**

A semi-quantitative index (range 0-3) based on opacity and valve motility was used for estimating AV echogenicity, an index of valve thickening and calcification, as follows: 0-absent, 1-mild; 2-moderate, with preserved motility/opening; 3-severe, with decreased motility/opening. Data were analyzed on a blinded fashion.

**AV calcification area**

We measured the percentage of cuspid mineralization (total valve calcification/total valve area) as the mean of 2 tissue sections stained with Von Kossa staining\(^8\) and hematoxylin-eosin. Data were analyzed with Leica Quantimet software.

**Multidetector-row Computed Tomography (MDCT)**

Ex-vivo rabbit descending aorta segments were simultaneously scanned in a Toshiba Aquilion 64 multislice CT (Toshiba Otawara, Japan) and x-Ray attenuation in Hounsfield units was calculated using mean of 3 measurements for each segment. All measurements were performed in a Vitrea 2 Workstation (Vital Images Inc, Minnetonka, MN) by an investigator blinded to the experimental groups.

**Real-Time Quantitative Reverse Transcriptase-PCR**

Total RNA was purified from descending rabbit aorta fragments, using Trizol. After checking RNA integrity, it was reverse-transcribed with *Superscript II* enzyme (Invitrogen) with random primers. Nox1 and Nox 4 quantification was performed by real-time RT-PCR. Primer sequences were designed from rabbit sequences for Nox1 and Nox4, kindly provided by Dr. Bernard Lassegue (Emory University, Atlanta): Nox1- *Forward*: CATCATGGAAGGAAGGAGA, *Reverse*: GCTTCCGGATAAACTCCACA; Nox4-
Forward: CCACAGACTTGGCTTTGGAT, Reverse: TACTGGCCAGGTCTTGCTTT. cDNA amplification was performed in a Corbett Rotor-Gene RG6000 thermocycler (Corbett Res. Pty Lim., Sidney, Australia), using SYBR green dye. Copy numbers were calculated from standard curves generated from Nox1 and Nox4 plasmid templates. Similar procedures were employed for analysis of osteopontin mRNA, using the following primer sequences: Forward: CTCCCGGTTAAACACGCTGATTC, Reverse: AGGATACTGGGCATTAGAGCG. GAPDH amplification was used for normalization in this case (Forward: TCACCATCTTCCAGGAGCGA, Reverse: CACAATGCCGAAGTGCTGTT).

**EPR analysis of plama tempol concentration**

Plasma EPR spectra for tempol detection were recorded at room temperature on a Bruker ER 200D-SRC upgraded to an EMX instrument, with the same parameters for a tempol standard solution and plasma samples.

**In vitro VSMC calcification model**

Aortas from intact rabbits were dissected and cleansed from adventitial tissue. Primary culture of vascular smooth muscle cells was established after digestion with collagenase IV (1 mg/mL), elastase (0.125 mg/mL), trypsin inhibitor (0.375 mg/mL) in DMEM at 37°C for 4 hours. Cells were seeded and grown in DMEM low glucose, with 10% fetal bovine serum (FBS) and essential amino acids in an incubator with 5% CO₂ at 37°C. Cells were used at the 4th passage. Calcification protocol began with cell confluence of 70%, the culture medium was supplemented with calcium chloride 6mM (CaCl₂), CaCl₂ 6mM+β-glycerophosphate 10mM or both in the presence of Vitamin D₃ 0.1µM (CaCl₂+β-GP+Vit.D₃). The latter combination was supplied in the absence or presence of either tempol 100µM or LA 100µM. Exogenous hydrogen peroxide (50µM) or catalase 500U/ml was added in specific experiments. The use of unmodified catalase was based on the fact that this enzyme is known to have access into smooth muscle cells after a 3-4 hour period⁹, which is adequate for the present experimental conditions. All incubations lasted for a total of 14 days, changing medium every 3 days.
**In vitro calcium measurement**

After 14 days of calcifying stimuli, cells were washed with PBS and incubated with HCl 0.6N 1ml/plate for 12hs at 4°C. The supernatant was centrifuged and total calcium was measured by colorimetric assay through reaction with o-cresolphthalein and reading in a spectrophotometer absorbing at 570nm after comparing with standard curve (Teco calcium measurement Kit) and normalized by total protein (Bradford method).

**NAD(P)H oxidase activity in membrane fraction**

VSMC membrane homogenates were obtained by sequential centrifugation as described previously (ref. 20). Protein concentration of total homogenate and membrane fraction was quantified by Bradford method. Membrane fraction (30µl/30µg protein) was diluted in 815µL PBS/ EDTA 10 µM solution and NADPH 300 µM was added at 37ºC. After baseline counting, lucigenin 5 µM was added into the scintillation tube and consecutive luminescence countings were registered in a Berthold 9505 luminometer (EG&Instruments GmbH, Germany). Baseline signal was subtracted from blank (PBS/EDTA 10 µM) and results normalized by protein concentration and expressed in counts per minute/mg.

**DHE oxidation products by HPLC analysis**

Cells with 80% confluence were rinsed twice with PBS/DTPA 0.1mM. All further procedures were performed in the dark or dim light. Cells were incubated with DHE 100 µM in PBS/DTPA 0.1 mM for 30 min at 37ºC, washed with cold PBS/DTPA 0.1 mM, extracted with acetonitrile (300 µL/well), scraped, sonicated (3 cycles at 4W for 10s) and centrifuged (12000g at 4ºC at 10 min). The supernatant was dried in vacuum (Speed Vac®Plus SC-110A, Thermo Savant) and kept at -20ºC. Samples were resuspended in 120 µL PBS/DTPA 0.1mM and injected (100 µL) in an HPLC system (Waters Corporation), as previously published by our laboratory (Fernandes et al, ref. 20). We used NovaPak C18 column (3.9x150 mm, with 5 µm particles), solution A (100% acetonitrile) and solution B (water; acetonitrile 10%; trichloroacetic acid 0.1%) as a mobile phase with a continuous flow of 0.4 mL/min. The procedure for analysis was initiated, loading solution A 0%, increasing progressively to 40% during the first 10 min, keeping this proportion for additional 10 min,
followed by injection of 100% of A for the next 5 min and 0% of A for the last 10 min. Simultaneous analysis of the remaining DHE and its derived products 2-hydroxyethidium and ethidium was performed, using respectively UV 245nm (photodiode array W2996 detector) and fluorescence (excitation length 510nm and emission 595nm, with fluorescence W2475 detector). Quantification was performed by comparing the area under the curve of the peak values obtained for the sample and a standard solution under identical chromatographic conditions. Results were expressed as the ratio between 2-hydroxyethidium or ethidium generated per DHE consumed (concentration difference between initial and remaining DHE).

Statistics

All data are reported as mean±SE. Comparisons were tested through one-way-ANOVA plus Student-Newman-Keuls (among all groups) or Dunnet (all groups vs. control) post-hoc tests, at a 0.05 significance level.

References for supplement


7- Garcia D, Dumensnil JG, Durand LG. Discrepancies between catheter and Doppler estimates of valve effective orifice area can be predicted from the pressure recovery phenomenon: practical implications with regard to quantification of aortic stenosis severity. *J Am Coll Cardiol.* 2003;41:435-442.


Supplemental Table

Table I- Total serum cholesterol, calcium, phosphorus, CaxP product and creatinine at baseline (A) and after different interventions (B). *p<0.05 vs Normal; †p<0.05 vs HC e ‡p<0.05 vs HCD+Vit.D₂+tempol.

A

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<td>P (mg/dl)</td>
<td>CaxP product (mg²/dl²)</td>
<td>Creatinine (mg/dl)</td>
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B

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Supplemental Figure Legends

Figure I

Increased NAD(P)H oxidase expression around calcifying foci (Ca++) in stenotic human aortic valves, as shown by Nox2 immunostaining with an antibody distinct from Fig.4 (clone 59.1 Ab).

Figure II

Histological findings of rabbit AV, showing Normal rabbits (upper panels) and HC+VitD rabbits (lower panels). The latter exhibits findings analogous to human aortic stenosis: thickening, macrophage infiltration in the aortic face (asterisk mark), elastolysis and calcification (arrows). Stainings are: hematoxylin and eosin (left panels) and Verhoeff-Van Gieson, which also stains strongly for calcium deposits, in addition to elastic fibers (middle panels). The right panels depict peroxidase-immunostaining for rabbit macrophages (RAM11 Ab, 1:200 dilution).

Figure III

Echocardiographic images of Normal (left panels) and HC+Vit.D2 aortic valves (right panels), in diastole (above) and systole (below), depicting increased echogenicity in AV from HC+VitD rabbits, observed after 12 weeks of intervention.

Figure IV

Echocardiographic variables in the rabbit model. Panel A- absolute AV area after 3 months of intervention, showing little difference among groups; Panel B- increased loss of in final AV area vs. baseline AV area in all HC+vitD rabbits, which was statistically significant only with tempol (*p<0.05 vs Normal; #p<0.05 vs HC). HC controls showed no change vs. normal. Panel C - aortic jet/left ventricular outflow tract velocity ratio, which was unchanged among groups. Panel D - posterior wall thickness, which was increased in all HC+vitD rabbits irrespective of antioxidants, whereas HC controls showed minor non-significant increase.
**Figure V**

Effects of tempol or LA treatment on 3-nitrotyrosine immunostaining around AV calcifying foci (Ca\(^{++}\)). (A): Normal control; (B): HC+vitD; (C): HC+vitD +tempol; (D): HC+vitD +LA. Graph representing staining area (pixel\(^2\)). Letters below each bar correspond to the above panels. Each bar represents mean±SE of at least 3 different rabbits. *p<0.05 vs Normal; #p<0.05 between specified groups.

**Figure VI**

Confocal microscopy colocalization of p22phox and nox2 (superior panel) and PDI and p22phox (inferior panel) around calcifying foci in tissue slices from AV cusp of HC+VitD rabbit. Anti-mouse or anti-goat secondary antibodies were used, conjugated to rhodamine (red) or FITC (green) (Ca\(^{++}\) = calcifying foci). Dilution of primary antibodies was 1:200 (p22phox, goat), 1:1600 (Nox2, mouse) and 1:32000 (PDI, mouse).

**Figure VII**

Phenotypic markers of cells around calcifying foci in HC+VitD rabbits (right column), compared to normal controls (left column). Panels A,D: TRAP histochemistry showing osteoclast (arrow); Panels B,E: Cbfa-1 immunostaining (Ab dilution 1:100); Panels C,F: osteopontin immunostaining (Ab dilution 1:50). Inset: osteopontin RT-PCR, with lane 1=Normal; 2=HC+VitD rabbit; 3=HC+VitD +tempol; 4=HC+VitD +LA.

**Figure VIII**

Graphs depicting results of histomorphometric analysis in AV from Normal or cholesterol-only controls (HC) and HC+vitD rabbits treated or not with tempol or LA. Panel A: The bar graph represents the number of TUNEL-positive cells per field (AU). Increased staining in AV cusps from HC+VitD rabbits vs. Normal was observed, which was enhanced in tempol-treated rabbits. Panel B: The bar graph summarizes increased cell proliferation around calcifying foci in HC+VitD rabbits and its partial inhibition with tempol. Cholesterol-only (HC) control rabbits showed minor staining coinciding with macrophage infiltration area (Figure not shown). Panel C: Collagen accumulation, expressed as %area at Masson’s trichrome staining. Panel D: Quantification of RAM11
stained area depicting macrophage accumulation in AV. Macrophage infiltration was unchanged with antioxidants vs. HC+vitD rabbits and was particularly prominent in HC controls. Each bar represents mean±SEM of at least 3 different rabbits. *p<0.05 vs Normal; #p<0.05 between specified groups; †p<0.05 vs HC+Vit.D2, HC+Vit.D2+T and HC+Vit.D2+LA.

**Figure IX**

Measurement of NADPH oxidase activity in membrane fraction of cultured vascular smooth muscle cell incubated in vitro with calcifying stimuli for 14 days. Activity was assessed by lucigenin (5µM) chemiluminescence, as described in supplemental methods.

**Figure X**

EPR detection of tempol in plasma, yielding average concentration ~5µM after 100µmol/kg/day administration in drinking water. Blood was collected after 3 months of intervention, before killing the rabbit. Obtained characteristic spectrum was similar to exogenous standard, and absent in Normal rabbits, which exhibited only the ascorbyl radical (detail in upper right panel). Identical height of spectrum was obtained after incubation of plasma samples with ferrycyanide (not shown), indicating that tempol was not reduced by plasma ascorbate.
Suppl. Figure I

Aortic valve stenosis (negative control)  Control valve

Aortic valve stenosis  Aortic valve stenosis (400x)
Suppl. Figure II
Suppl. Figure III
Suppl. Figure IV
Suppl. Figure V
Suppl. Figure VI
Suppl. Figure VII
Suppl. Figure VIII
Suppl. Figure IX
Suppl. Figure X