Role of oxidative stress in calcific aortic valve disease and its therapeutic implications

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Abstract

Calcific aortic valve disease (CAVD) is the end result of active cellular processes that lead to the progressive fibrosis and calcification of aortic valve leaflets. In western populations, CAVD is a significant cause of cardiovascular morbidity and mortality, and in the absence of effective drugs, it will likely represent an increasing disease burden as populations age. As there are currently no pharmacological therapies available for preventing, treating, or slowing the development of CAVD, understanding the mechanisms underlying the initiation and progression of the disease is important for identifying novel therapeutic targets. Recent evidence has emerged of an important causative role for reactive oxygen species (ROS)-mediated oxidative stress in the pathophysiology of CAVD, inducing the differentiation of valve interstitial cells into myofibroblasts and then osteoblasts. In this review, we focus on the roles and sources of ROS driving CAVD and consider their potential as novel therapeutic targets for this debilitating condition.

Keywords
Aortic valve • Calcification • Reactive oxygen species • Oxidative stress • NADPH oxidases

1. Introduction

Calcific aortic valve disease (CAVD) is a progressive condition in which a normal tricuspid aortic valve or a congenitally abnormal bicuspid aortic valve becomes thickened, fibrosed, and calcified. The nature of this remodelling results in a spectrum of disease ranging from mild aortic sclerosis to severe aortic stenosis (AS). CAVD is currently the third most prevalent cardiovascular disease after coronary artery disease and hypertension.¹ The prevalence of CAVD increases with age. Aortic valve sclerosis affects a quarter of over 65 years old,¹ and progression from sclerosis to stenosis occurs in ~2% of individuals per year.²–⁴ Most patients with established AS then develop moderate-to-severe symptoms requiring treatment.¹,⁵ It is expected that in the absence of any preventative therapy, CAVD will represent an increasing disease burden as populations in developed countries age⁵ and as the prevalence of the associated cardiometabolic risk factors for CAVD namely obesity, type 2 diabetes, dyslipidaemia, and hypertension increase.⁶,⁷ Consequently, understanding the mechanisms underlying the initiation and progression of CAVD is vital for developing novel therapeutic targets.⁸

Amongst the various reported mediators of fibrocalcific valvular changes, there is emerging evidence of a critical, causative role for reactive oxygen species (ROS).⁹–¹³ This review will focus on the specific sources of ROS or ROS-mediated stress signalling driving CAVD, and examine their potential as novel therapeutic targets for this debilitating disease.

2. ROS in normal physiology

ROS are a group of highly reactive chemical forms of molecular oxygen, divided into free radical species (with at least one free electron) and non-radical (two electron) species.¹⁴ The most important examples of these are superoxide and hydrogen peroxide, respectively. Superoxide anions derive from the reduction of molecular oxygen in a reaction mediated by a variety of different enzymes at the cell membrane, cytoplasm, and in organelles, such as mitochondria, peroxisome, and endoplasmic reticulum.¹⁵,¹⁶ Subsequent spontaneous dismutation of superoxide or dismutation by superoxide dismutase (SOD) enzymes produces
hydrogen peroxide. In turn, catalase converts hydrogen peroxide to water, although when partially reduced, hydrogen peroxide is converted to hydroxide ion and hydroxyl radical.

At physiological concentrations, ROS regulate cell growth, differentiation, senescence, migration, apoptosis, and autophagy.16–18 They also modulate a variety of metabolic processes including glycolysis, oxidative phosphorylation, and fatty acid synthesis.15,19 These responses are predominantly mediated via the oxidation of cysteine thiolate groups by hydrogen peroxide and of iron–sulphur clusters by superoxide on a wide-range of target proteins.20,21 These modifications regulate protein localization and function, as well as intermolecular and protein–protein interactions.22 Cysteine thiolate oxidation, in particular, leads to disulfide formation, cysteine persulfidation, and glutathionylation, which serve as important modulators of protein activity.22

Several mechanisms maintain ROS at physiological concentrations, including subcellular compartmentalization, SOD and catalase, peroxiredoxins, and the thioredoxin and glutathione systems which reverse the aforementioned cysteine residue modifications.23,24 The NRF2–KEAP1 system is also an important oxidant sensor in which oxidation of residues on KEAP1 leads to the up-regulation of NRF2 nuclear translocation where it acts as a transcription factor for a series of antioxidant proteins.25

3. ROS in cardiovascular diseases
Numerous cardiovascular disorders and diseases including endothelial dysfunction, hypertension, vascular calcification, atherosclerosis, cardiac remodelling, stroke, and diabetes are associated with an oxidative stress state in which ROS-producing enzyme activity and expression levels are up-regulated, whilst the expression and activity of antioxidant mechanisms are down-regulated.26–28 Within the cardiovascular system, three major sources of ROS are uncoupled nitric oxide synthases (NOS), reduced nicotinamide adenine dinucleotide phosphate oxidases 2 proteins, and mitochondria.29–31 The roles of these sources of ROS in cardiovascular diseases, extensively reviewed elsewhere,15,26–28,30,32,33 are briefly described here.

3.1 Uncoupled NOS
NOS function as dimers which catalyse the transformation of L-arginine and molecular oxygen to nitric oxide (NO) and L-citrulline, requiring NADPH-derived electrons. NOS uncoupling occurs when there is a depletion of tetrahydrobiopterin (BHH), an obligatory co-factor that actions as an auxiliary electron donor in the above reaction.34,35 Uncoupling results in the production of superoxide as the enzyme switches from its classical NO synthase function to that of an NADPH-dependent oxidase.36 In turn, superoxide combines with NO to produce peroxynitrite, thereby reducing the bioavailability of NO.11,34,37,38 In the case of the endothelial NOS isoform (eNOS), in particular, this leads to impaired endothelial-derived NO-mediated relaxation of vascular smooth muscle with an associated increase in systemic vascular resistance and hypertension.39,40 Reduced NO bioavailability also results in detrimental vascular remodelling, impaired platelet aggregation, and leukocyte adhesion.37 Endothelial dysfunction secondary to BH4 deficiency is an early marker of, and a critical step in the development of atherosclerosis, as well as in diabetic micro- and macrovascular disease.15,41–49 Superoxide release from uncoupled eNOS and nNOS (neuronal NOS) has also been implicated in pressure-overload left ventricular hypertrophy and diastolic dysfunction.42,50–53

3.2 Nox proteins
NOXs generate ROS as their primary function, catalysing electron transfer from NADPH to O2.54–56 There are seven mammalian NOX homologues (NOX1 to NOX5, Duox1, and Duox2) with NOX2 (also termed gp91phox) and NOX4 most widely expressed within the cardiovascular system.31,55 NOX2 is acutely activated by agonists such as angiotensin II, mechanical stimulation, and metabolic factors in a process that requires intracellular association between the transmembrane NOX2-p22phox complex and the cytosolic subunits p47phox, p67phox, p40phox, and Rac1 to generate ROS.54,57 By contrast, NOX4 is constitutively active and regulated mainly by its own expression level,58–61 whilst it may also be activated by mechanical stretch and agonists such as TGF-β.62,63 Moreover, while NOX2 generates superoxide, NOX4 predominantly generates H2O2.31,61,64,65

Overwhelming evidence indicates that the pathophysiological roles of the NOXs are isofrom and cell-type specific. NOX2 mediates the development of adverse cardiac fibrosis, cardiomyocyte hypertrophy, contractile dysfunction, and cardiomyocyte death induced by angiotensin II, pressure overload, or myocardial infarction.30,31,35,66–75 In the vasculature, NOX2 may also have important roles driving the initiation and progression of atherosclerosis.15,32,76–81

In contrast to the largely deleterious roles of NOX2, NOX4 may mediate protective signalling in the heart and vasculature.60,82–87 NOX4 protects against pressure overload-induced cardiac remodelling and dysfunction through paracrine preservation of myocardial capillary density,60,88 NRF2-dependent modulation of redox state,89 and enhancement of the integrated stress response.90 Cardiomyocyte NOX4 also maintains optimal mitochondrial function and cardiac performance during physiological exercise.91 Endothelial NOX4 protects against chronic pressure-overload induced cardiac remodelling,84,86 and AngII-stimulated myocardial fibrosis.92 In the vasculature, NOX4 protects against endothelial dysfunction, leukocyte adhesion, inflammation, and atherosclerosis.82,87 However, up-regulated vascular smooth muscle cell (VSMC) NOX4 correlates with VSMC dysfunction and plaque instability, whilst VSMC NOX4 deletion attenuates western-diet-induced atherosclerosis.93,94

3.3 Mitochondria
Mitochondria generate ROS (mitoROS) as natural by-products of oxidative phosphorylation and basal metabolic activity.95,96 MitoROS are tightly regulated by a number of mechanisms including the glutaredoxin, glutathione, and thioredoxin systems which support thiol redox
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4.1 Initiation of CAVD

The progressive processes of leaflet fibrosis and calcification are initiated by damage to the endothelial cells lining the aortic valve. This damage, triggered by diverse risk factors including ageing, obesity and hypertension, systemic inflammation, and mechanical and shear stress, permits the infiltration, deposition, retention, and subsequent oxidation of lipoproteins, such as low-density lipoprotein (LDL) and lipoprotein(a) (Lp(a)). These events result in a chronic inflammatory response mediated by innate and adaptive immune responses, and in increased mitoROS scavenging, genetic inhibition of mitoROS, or mitotargeted catalase each attenuate lesion progression and reduce inflammatory signalling and immune cell infiltration.

4.2 Propagation of CAVD

In the propagation phase of the disease fibrosis and calcification become the driving forces. Cytokines secreted from macrophages and T cells, such as TNFα, IL-1β, IL-6, IL-8x, insulin-like growth factor-1, and TGF-β, induce the transition of the predominant aortic valve cell type, namely valve interstitial cells (VICs), into myofibroblasts and osteoblasts. VICs are a heterogeneous population of fibroblast-like cells which are important physiological regulators of valve structure. Cytokines induce VIC differentiation by up-regulating the expression of several osteogenesis pathway genes including runt-related transcription factor 2 (Runx2), low-density lipoprotein receptor-related protein 5 (Lrp5), distal-less homeobox 5 (Dlx5), SRY-box 9 (SOX9), and muscle homeobox protein MSX2, as well as bone morphogenic protein 2 (BMP2)—a potent osteogenic differentiation factor, and the osteoblast marker proteins osteopontin (OPN), osteocalcin, and osteonectin. Direct activation of Toll-like receptors 2 and 4 by oxidized LDL also induces VIC BMP2 expression.

Osteogenic VIC transformation is then promoted further by the down-regulation of and/or mutations in NOTCH1, which impair the ability of NOTCH1 to suppress Runx2 and SOX9 expression. Furthermore, up-regulated WNT/β-catenin signaling, reductions in anti-osteogenic microRNAs, such as miRNA-30b, and increased receptor activator of nuclear factor kappa B (RANK)/RANK ligand interactions all serve to promote the differentiation of VICs into osteoblast-like cells by up-regulating the expression of osteoblast-related genes.

Differentiated VICs then secrete microvesicles containing ectonucleotidases, promoting calcium phosphate nucleation within valve leaflets. VIC overexpression of ectonucleotidases, such as Ectonucleotide pyrophosphatase 1 (ENPP1), alkaline phosphatase (ALP) and 5'-nucleotidase also drive valvular mineralization by generating inorganic phosphate and adenosine, and in the case of ALP, by hydrolyzing inorganic pyrophosphate and de-phosphorylating OPN, natural inhibitors of calcium phosphate deposition.

Apoptotic bodies released from VICs driven to programmed cell death by inflammatory cytokines, such as TGF-β1 and ENPP1-mediated depletion of the key-cell survival signal ATP, also act as a nidus for calcium and phosphorous crystal deposition. In a subset of patients, heterotopic ossification mediated by the recruitment of bone-marrow-derived circulating osteogenic progenitor cells and endothelial progenitor cells occurs resulting in the formation of lamellar bone within the aortic valve leaflets.

Alongside this mineralization process, leaflet thickening and fibrosis occur. This is mediated by the differentiation of VICs into myofibroblasts that induce extracellular matrix (ECM) remodelling by secreting excess collagen and increasing their expression of matrix metalloproteinases. These changes form a vital scaffold upon which hydroxyapatite nucleation and progressive amorphous deposition occur. Fibrotic changes are driven, in particular, by RANK/RANKL stimulation and by angiotensin II which is produced by chymase released from mast cells and by ACE delivered via LDL infiltration. The resulting extracellular matrix has relatively decreased elastin content and excessive disorganized collagen content which significantly alters leaflet biomechanics, leading to a higher mechanical load that in itself directly encourages further myofibroblastic differentiation in a positive feedback loop.

4.3 Systemic inflammation and oxidative stress in CAVD

Analysis of plasma from patients with known AS reveals evidence of a systemic pro-inflammatory state within which oxidative stress occurs. Accordingly, reduced levels of C4 indicating active complement pathway activation are observed alongside increased circulating pyroglutamic acid, an intermediate only generated when levels of the antioxidant glutathione are depleted.

In a recent cohort study using thiobarbituric acid to assess systemic lipid peroxidation and 2,4-dinitrophenylhydrazine to assess for the oxidative modification of plasma proteins, elevated oxidative stress was observed to correlate with the severity of AS, as reflected by mean aortic valve area and mean and maximum aortic gradients. Levels of oxidative stress also correlate with impaired systemic fibrinolysis, again
| Experimental models/tissues | In vitro/in vivo/ex vivo | Observations | Sources of ROS | Inhibitors tested | ROS assay | Ref |
|----------------------------|--------------------------|--------------|----------------|------------------|-----------|-----|
| **Animal studies**         |                          |              |                |                  |           |     |
| LDLr\(^{-}/\)ApoB\(^{100/100}\) mice fed normal chow | In vivo and in vitro | Hypercholesterolaemia induces CAVD in a subset of mice. Stenotic valves in hypercholesterolaemic mice demonstrate increased superoxide levels. | –                | –                | DHE       | 9   |
| LDLr\(^{-}/\)ApoB\(^{100/100}\) mice fed a western-type diet | In vivo and in vitro | Western-type diet induces hypercholesterolaemia and aortic valve lipid infiltration and deposition, and apoptosis leading to CAVD. | NOX2            | Pioglitazone     | –         | 223 |
| Chronic Ang II infusion mice fed a hypercholesterolaemic diet | In vivo and in vitro | Chronic infusion of Ang II and hypercholesterolaemia induce oxidative stress within AVs, resulting in leaflet thickening, and ECM remodelling. | –                | MnBuOE           | –         | 205 |
| Cultured porcine aortic valve interstitial cells | In vitro | TGF-β1 induces ROS production and calcium nodule formation via Smad and MAPK pathways. | –                | –                | DCF       | 203 |
| Cultured porcine aortic valve interstitial cells | In vitro | TGF-β1 induces superoxide production which partly mediates in vitro calcification. Co-application with NO donors scavenges superoxide, reducing calcification. | –                | DETA-NONOate SNP peg-SOD | DHE       | 247 |
| Cultured porcine aortic valve interstitial cells | In vitro | Application of osteogenic medium induces valvular fibrosis and calcification mediated by ROS, collagen deposition, and up-regulation of fibronectin, OPN and Runx2, β-catenin accumulation. | NOX2            | Celastrol        | DHE       | 13  |
| Cultured porcine aortic VECs and porcine aortic valve tissue | Ex vivo and in vivo | TNFα increases VEC intracellular oxidative stress, as well as superoxide and H₂O₂ synthesis. TNFα or H₂O₂ decrease nitric oxide synthase by reducing eNOS expression. This results in myofibroblastic activation, calcification and changes in ECM composition and structure. | Uncoupled NOS and NOX2 | L-NAME BH4 | DCF       | 209 |
| Rabbits fed high cholesterol diet + vitamin D | In vivo and in vitro | Superoxide and H₂O₂ levels are increased around calcifying foci and potentiates the progression of AV calcification. AV calcification improves by reducing H₂O₂ levels with lipoic acid. | NOX2, NOX4 Lipic acid | Tempol | DHE       | 10  |
| Rabbits fed high cholesterol diet + vitamin D | In vivo and in vitro | High cholesterol + vitamin D induces celastrol-sensitive increases in ROS which drives the development of CAVD and maladaptive cardiac remodelling. | NOX2            | Celastrol        | DHE       | 13  |
| Cultured bovine aortic VICS | In vitro | Application of LPS induces oxidative stress, ALP overexpression, VIC calcification and ECM remodelling. | XOS             | Allopurinol L-arginine | –         | 236 |

**Human studies**

Continued
| Experimental models/tissues | In vitro/in vivo | Observations | Sources of ROS | Inhibitors tested | ROS assay | Ref |
|----------------------------|-----------------|--------------|----------------|-------------------|-----------|-----|
| AV tissue from patients undergoing valve replacement surgery for symptomatic AS | In vitro | Superoxide and $H_2O_2$ levels are increased near calcified regions. SOD activity, expression of all 3 SOD isoforms, and catalase are significantly decreased in pericalcific regions. | Uncoupled NOS | L-NAME | DHE | 11 |
| AV tissues from patients with stenosis or sclerosis collected at surgery or autopsy | In vitro | Increases in ROS production are noted around calcifying foci in human sclerotic or stenotic AV. | NOX2 | peg-SOD, peg-Catalase | DHE | 10 |
| AV tissue and isolated cultured human VICs from patients with CAVD | In vitro | CAVD tissue demonstrates nitrotyrosine accumulation and increased peroxynitrite levels. SOD and catalase expression and activity are down-regulated. $H_2O_2$ induces impaired DNA-damage responses, profibrotic and pro-osteogenic signalling leading to osteogenic differentiation and calcification in isolated VICs | – | Adenoviral delivery of SOD/Catalase | – | 12 |
| AVs and isolated VICs from patients undergoing heart transplantation, valve replacement, or from deceased donor hearts. | In vitro | In sclerotic and stenotic valves, nitrotyrosine is diffusely distributed with areas of higher intensity. Dityrosine is only observed in stenotic tissue. TGF-$\beta$ induces $\alpha$-SMA up-regulation and aortic valve remodelling. | – | MnBuOE | – | 205 |
| Human VICs obtained from a normal healthy donor and two donor patients with calcified aortic valves | In vitro | In isolated human VICs reduction in antioxidant enzymes results in $H_2O_2$-induced increases in RUNX2 and OPN mRNA expression. | – | CNPs | DCF | 204 |
| Calcified human aortic valves obtained from adults undergoing valve replacement surgery | Ex vivo | Superoxide accumulates in calcified regions of the valves and in the fibrosa endothelium. Fibrosa-specific loss of SOD1 expression confers the fibrosa as the preferential site of superoxide accumulation. | – | – | DHE | 209 |
| Isolated aortic valve endothelial cells isolated from bicuspid valves explanted from patients with AS undergoing valve replacement | In vitro | Reduced expression of the antioxidants GPX3 and SRXN1 are observed in ECs isolated from BAVs leading to increased oxidative stress susceptibility. | – | – | – | 206 |

**Table I Continued**
| Experimental models/tissues | In vitro/in vivo/ex vivo | Observations | Sources of ROS | Inhibitors tested | ROS assay | Ref |
|-----------------------------|--------------------------|--------------|----------------|------------------|-----------|-----|
| Human aortic VICs isolated from patients undergoing AVR | In vitro | VICs incubated with Lp(a) develop increased ROS formation and undergo significant calcium deposition. | Mitochondria | – | DHE mitoSOX™ Red | 225 |
| Primary and cultured human VICs isolated from stenotic AVs from patients undergoing valve replacement | In vitro | DRP1 overexpression, indicative of mitochondrial dysfunction, is observed, mediating OGM-induced VIC calcification. | Mitochondria | – | – | 228 |
| Myocardial biopsies from patients with AS undergoing elective valve replacement | Ex vivo | Myocardial biopsies demonstrate increased markers of the mitochondrial UPR, potentially indicating the presence of oxidative stress. | Mitochondria | – | – | 226 |
| Isolated human VICs obtained from valves explanted from patients undergoing aortic valve replacement | In vitro | Application of Inorganic phosphate induces calcification of isolated human VICs. | SSAO | LJP1586 | – | 234 |
suggesting that the oxidative stress in CAVD patients might not simply reflect a localized phenomenon. Evidence from animal models of CAVD also supports a putative role for systemic inflammation in CAVD. In both wild-type mice and in the atherosclerotic ApoE*3Leiden mouse model, intraperitoneal LPS induces AV thickening, but not calcification, suggesting a role for systemic inflammation in the early stages of CAVD. There are conflicting data on the association between serum levels of C-reactive protein (CRP), a marker of systemic inflammation, and CAVD. Several relatively small cohort studies (n ≤ 141) have identified a correlation between CRP levels and the presence and, in some studies, the severity of CAVD. However, a significantly larger cohort study (n = 5621) performed over a 5-year period, found that C-reactive protein is in fact not associated with baseline incidence of AS, progression to aortic sclerosis, or progression to AS.

4.4 Localized valvular ROS signalling in CAVD

The majority of the evidence demonstrating roles for oxidative stress in CAVD derives from studies using aortic valve tissue and isolated VICs that together indicate a localized inflammatory response within which ROS signalling occurs. Indeed, a study that used computed tomography–positron emission tomography on patients with varying degrees of CAVD, observed localized valvular uptake of the tracers 18F-sodium fluoride and 18F-fluorodeoxyglucose that assessed for valvular calcification and inflammation, respectively. The degree of local valvular tracer uptake was observed to be correlated with the severity of disease. Pre-clinical and human studies reportedly demonstrate increased local ROS levels in CAVD. Many of these use fluorescent dyes to indicate ROS accumulation which possess varying degrees of specificity. Thus, whilst dihydroethidium (DHE) is a relatively specific indicator of...
superoxide, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) reacts with hydrogen peroxide and itself induces superoxide production, dismuta-
tion of which leads to self-amplification of DCF fluorescence. Caution is, therefore, required when interpreting results using this assay. The spec-
cificity of the commonly used chemiluminescence-based techniques, such as lucigenin-enhanced chemiluminescence for superoxide, have also been questioned, although when low concentrations are used, concerns regarding redox cycling in which lucigenin can react with oxygen to produce superoxide, are mitigated. The variety of assays used by the studies reviewed in this article are summarized in Table 1.

Around 30% of hypercholesterolaemic LDLr-/-ApoB100/100 mice fed a normal diet develop AS, with elevated aortic valve superoxide present both prior to CAVD developing, and more abundant in mice that subsequently develop AS. In isolated cultured porcine VICs, TGF-β1 induces ROS production subsequently leading to calcium nodule formation in a signalling cascade involving P38 MAPK and MEK1/2/ERK1/2 pathways. These findings are replicated by exogenous ROS application, which promotes VC calcium precipitation by up-regulating fibrotic and osteogenic gene expression, indicating that oxidative stress may precede the differentiation of VICs to an osteoblastic phenotype. In rabbits fed a high cholesterol diet, both superoxide and H2O2 levels are increased in and around calcify-
ing aortic valve, with AV calcification reversed by reducing H2O2 levels with lipoic acid.

Importantly, ROS have also been implicated in human CAVD. In explanted valves from patients with established AS, superoxide and H2O2 levels are markedly increased near the calcified regions of the valve. This accumulation is in part due to a marked reduction in the peri-calcific activity and expression of all three SOD isoforms as well as catalase. Along similar lines, reduction in antioxidant enzymes results in hydrogen peroxide-induced increases in Runx2 and OPN mRNA ex-
pression in isolated human VICs.

Increased ROS levels are also seen in valves from patients with aortic sclerosis. Here, peroxynitrite and nitrogen dioxide-generated nitroty-
sine levels are increased alongside superoxide and hydrogen peroxide. Together, these induce DNA damage, trigger dysfunctional DNA-repair mechanisms, and promote early VIC phenotypic alteration via up-regulation of AKT signalling leading to Runx2 and MSX2 overexpression as well as in vitro calcification. These changes are reversed with adenosine delivery of superoxide dismutase and catalase. In explanted AV from patients with CAVD, nitrotyrosine is distributed throughout the sclerotic leaflets, with localized areas of high intensity also noted. In contrast, dityrosine is only observed in stenotic valves indicating more advanced oxidation occurring as disease progresses.

Finally, in bicuspid aortic valves (BAV) from patients with AS undergo-
ing surgical replacement, application of hydrogen peroxide triggers DNA damage and apoptosis in isolated valvular endothelial cells (ECs). Interestingly, increased levels of DNA damage and sustained apoptosis signalling are observed in bicuspid compared with tricuspid valves, sec-
ondary to molecular differences in oxidative stress susceptibility with re-
duced expression of the antioxidants glutathione peroxidase 3 and sulfiredoxin noted in BAV ECs.

### 4.5 NOS-derived ROS in CAVD

Since uncoupled NOS primarily generate superoxide, it is feasible that the pro-inflammatory milieu in the initiation of CAVD may drive NOS uncoupling and thereby contribute to increases in superoxide levels ob-
served in CAVD. Indeed, proteomic analyses of calcified aortic valves reveal decreased expression of HSP90, part of a complex with endothelial NOS, the dissociation of which can cause uncoupling of eNOS, leading to the production of ROS and endothelial dysfunction.

In support of this hypothesis, the arginine analogue L-NAME, a NOS inhibitor, reduces superoxide production by over 50% in calcified human aortic valves. Data from animal models likewise support a role for uncoupled NOS-derived ROS in CAVD. In cultured porcine aortic valve ECs and in porcine aortic valve leaflets, exogenous TNFα and H2O2 in-
duce eNOS uncoupling leading to increases in superoxide and H2O2 lev-
els. Application of TNFα and H2O2 also reduces eNOS expression resulting in a reduction in NO synthesis as measured using the Griess as-
say. This in turn drives disorganization of the extracellular matrix and val-
cular calcification. These changes are partially reversed in the presence of the NOS inhibitor L-NAME, NOX inhibitor apocynin, or PEG-SOD and are fully reversed by the eNOS co-factor BH4, confirming NO uncoupling as a key mediator in this model of CAVD.

Reduced NO bioavailability within diseased valve leaflets further increases the burden of ROS by decreasing NO-mediated quenching of superoxide. Indeed, alongside eNOS uncoupling, several other mecha-

isms are responsible for reducing NO synthesis in CAVD including me-

chano-stress-induced down-regulation of eNOS expression, and vascular endothelial-mesenchymal transition which occurs as a result of TGF-β1 stimulation, the constituent elements of the ECM, shear and mechanical stress, and protein S-glutathionylation—the consequence of an imbalance between reduced and oxidized glutathione.

Hypercholesterolaemia also decreases the expression and activity of eNOS, and therefore taken together these mechanisms reduce the availability of NO, resulting in increased superoxide levels driving val-

ular myofibroblast proliferation and extracellular matrix produc-

tion. Moreover, enhanced levels of superoxide from other sources such as NOX2 (described in detail below) result in increased peroxynitrite formation as it reacts with residual NO. In a positive feedback loop, this induces further eNOS uncoupling via peroxynitrite-mediated oxidation of BH4 and NOX4. Peroxynitrite is also implicated in evoking DNA damage and dysregulated DNA repair responses within cultured human aortic VICs leading to the up-regulated expression of Runx2 and MSX2 and subsequent in vitro calcification.

### 4.6 NOX-derived ROS in CAVD

Recent evidence has emerged that NOX-derived ROS are critically in-
volved in the development of CAVD. This is particularly so in animal models of CAVD, although conflicting evidence emerges with regards to the specific isoforms involved. In hypercholesterolemic mice fed a Western-type diet in order to induce CAVD, increases in NOX2 and p22phox mRNA levels are observed within harvested valve leaflets, whilst no changes are observed for NOX4, eNOS, catalase, or SOD.

Our recent studies with cultured primary porcine aortic VICs reveal that application of osteogenic medium (OGM) results in marked fibrosis and calcification associated with increased expression of NOX2, collagen, fibronectin, pro-osteogenic β-catenin accumulation, OPN, and Runx2. Additionally, in vivo evidence for NOX2-mediated CAVD is also shown in a rabbit model of CAVD fed cholesterol-enriched chow and vitamin D (HC + VitD). HC + VitD-treated rabbits develop thick-

ened and fibrosed valve leaflets with increased calcium deposits. Phenotypically, this translates into significant decreases in AV area ac-
companied by enhanced transvalvular peak and mean jet velocity, ven-
icular dilatation, and contractile impairment as well as exaggerated cardiac hypertrophy.

NOX2, p22phox, and its regulator protein disul-
fide isomerase are strongly expressed around calcification foci in HC+VitD rabbit aortic valves, coincident with the sites of highest
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4.7 Mitochondrial ROS in CAVD

Recent evidence indicates a potential role for mitoROS in the development of CAVD. In isolated cultured human VICs, application of Lp(a) generates superoxide production from mitochondria as indicated by an increase in mitoSOX™ Red fluorescence, with Lp(a) application subsequently inducing in vitro VIC calcification. Notably, MitoSOX™ Red fluorescence transiently increases after 1 h incubation with Lp(a), and then normalizes at 4 h. Thus, the significance of this observation is unclear given the temporary nature of the finding, although mitoROS might be important in the initiation of VIC calcification. Moreover, whether inhibiting mitoROS abrogates Lp(a)-induced VIC calcium deposition was not examined.

In isolated porcine aortic VICs, application of TNFα induces acute, pegan-SOD-sensitive increases in mitochondrial ROS indicated by an increase in MitoSOX Red fluorescence, with the peak effect observed at 30 min. Moreover, in ex vivo porcine aortic valve leaflets, 21-day treatment with TNFα likewise induces pegan-SOD-sensitive increases in mitoROS production in VECs located in the ventricular endothelium. Importantly, this study did not produce any direct experimental evidence implicating mitoROS as a propagator of CAVD and thus the observed increases in mitoROS may simply represent associations.

Myocardial biopsies from patients with AS demonstrate increased markers of the mitochondrial unfolded protein response, indicating a dysfunctional mitochondrial protein-folding environment. This environment is likely the result of cellular stress conditions, including up-regulated ROS and oxidative stress that results from and contributes to mitochondrial dysfunction in CAVD. Myocardial biopsies from patients with AS also demonstrate reduced expression of fatty acid translocase—a key enzyme involved in fatty acid oxidation, and the increased expression of glucose transporters 1 and 4. Decreases are also observed in the expression of the fatty acid binding proteins FABP and H-FABP, the β-oxidation protein medium chain acyl-coenzyme A dehydrogenase, the Krebs cycle protein α-ketoglutarate dehydrogenase, and the oxidative phosphorylation protein ATP synthase. Complex I of the electron transport chain is also down-regulated. Together these changes suggest a metabolic shift from fatty acid to glucose utilization, and, more generally, suggest further evidence of general mitochondrial dysfunction in patients with CAVD. However, the extent to which this metabolic shift results from and/or contributes to mitoROS in initiating CAVD remains unclear with no direct data linking the two. Moreover, it is important to point out that these data derive from myocardial tissue alone and thus whether similar abnormalities occur in the aortic valve tissue of these patients likewise requires further investigation.

In primary human VICs obtained from patients with established AS undergoing valve replacement surgery, DRP1 immunoreactivity is detected in calcified valve tissue. Similarly, in cultured human VICs treated with OGM containing inorganic phosphate and L-ascorbic acid for three weeks, DRP1 mRNA is significantly increased. DRP1 siRNA reduces human osteogenic medium-induced VIC calcification, indicating an important role for this mitochondrial regulator protein in regulating VIC calcification in vitro. These findings together intimate that mitochondrial dysfunction is an important feature of CAVD.
4.8 Other sources of ROS in CAVD

Several studies have identified semicarbazide-sensitive amine oxidase (SSAO), also known as vascular adhesion protein-1 as an additional deleterious source of ROS in CAVD. SSAO generates H2O2 from endogenous amines such as histamine and dopamine, with their activity and expression up-regulated in atherosclerosis, obesity, and diabetes.232-234 SSAO expression and activity are also increased in human CAVD where serum and valvular expression levels correlate with disease severity.232,233 Here, enzyme activity is up to seven times higher than it is in healthy parts of the same valves as assessed by H2O2 production following the application of the SSAO substrate benzylamine.234 In addition, a significant correlation of SSAO expression with oxidative stress is observed, where the enzyme is co-localized with calcified regions of aortic valve tissue. SSAO mRNA levels are positively correlated with mRNA levels of the NOX subunit p22phox and the nuclear enzyme poly(ADP-ribose) polymerase which is activated following DNA damage and oxidative stress.235 In addition, inhibition of SSAO activity with LJP1586 attenuates calcification induced by high concentrations of phosphate in primary cultures of aortic VICs isolated from aortic valves of CAVD patients.234

In bovine aortic VICs, application of LPS induces increased ALP expression and VIC calcification.236 This is associated with increased expression of xanthine oxidase (XOS), which, under oxidative stress, produces superoxide.237 Co-application of allopurinol which inhibits XOS, reverses LPS-induced ALP overexpression.236

5. Targeting ROS in CAVD

There is a major unmet clinical need for novel pharmacological treatments capable of preventing or slowing the progression of CAVD. Several avenues using existing therapies have failed to show benefit in large randomized controlled trials (RCTs) including treatment with statins, antihypertensives, and drugs targeting phosphate and calcium metabolism.113,117,244-246 This failure partially results from the fact that by the time patients present with CAVD, the multifactorial, and self-perpetuating cellular mechanisms driving the disease have already been set in motion.113,117,244-246 As described above, specific ROS sources and ROS-mediated signalling may represent novel therapeutic targets given their roles in the initiation and propagation of the cellular mechanisms driving CAVD. Proposed strategies for inhibiting ROS-induced oxidative stress either systemically or locally in CAVD are therefore outlined below and summarized in Table 1 and Figure 3.

5.1 Impairing lipid oxidation

As described, lipid oxidation is a key trigger of inflammation in the initiation of CAVD.248 Oxidized LDLs are increased in valves removed from patients with AS, with an association between the level of oxidized LDL and the extent and pace of aortic valve fibrosis and calcification.189,245,249-251 As carriers of oxidized phospholipids (OxPL), Lp(a) also have an important role in the initiation of inflammation seen in CAVD. Approximately one-third of patients with AS have elevated plasma Lp(a), with large genetic and cohort studies revealing that the higher the level, the faster CAVD progresses, with marked increases in the risk of requiring valve replacement or death from the disease.189,141,225,245,252,253

In vitro studies have demonstrated that LDL and Lp(a) oxidation promote the expression of osteogenic differentiation genes but also ROS-mediated VIC calcification. Isolated human aortic VICs cells incubated with either LDL or Lp(a) demonstrate calcification, with those treated with Lp(a) displaying a higher burden of calcium deposition and ROS formation.225 Moreover, progression of CAVD is significantly impaired in transgenic Ldlr-/- mice that express a single-chain variable fragment of E06, a natural antibody which binds to the phosphocholine headgroup of OxPL and blocks the uptake of oxidized low-density lipoprotein by macrophages.234

These data, therefore, suggest that diminishing the extent of lipid/phospholipid oxidation by lowering plasma LDL and/or Lp(a) levels might represent a therapeutic avenue for treating CAVD. Indeed, in Ldlr-/-ApoB100/100 mice, inactivation of the mttp gene significantly impairs hypercholesterolaemia-induced oxidative stress, thereby reducing aortic valve lipid deposition, osteogenic signalling, and valvular calcification.225 Nevertheless, despite promising associations between statin use and lower prevalence of CAVD in observational studies,256-262 RCT data demonstrate no benefit for statin use in CAVD.240,241,244-246,263-266 Randomization to statin use is not only of no benefit in halting the
progression of established CAVD but also does not reduce the incidence of CAVD in patients with no established diagnosis at the start of the clinical trials. In addition, as yet there are no RCT data on specific Lp(a)-lowering therapy, although an antisense oligonucleotide that specifically lowers Lp(a) levels is currently under investigation.

The synthetic compound probucol, purported to inhibit LDL oxidation, has also been proposed as a novel treatment for CAVD. Indeed, probucol has been clinically proven to arrest the progression of atherosclerosis in the vasculature and in coronary artery restenosis following angioplasty. However, it is now clear that probucol likely mediates most of its effects via the induction of HO-1, rather than through direct inhibition of lipid oxidation.

5.2 Antioxidants

Broad ROS scavenging with natural and synthetic ‘antioxidant’ compounds has been attempted by several groups for attenuating atherosclerosis and vascular calcification. These studies, reviewed extensively elsewhere, have investigated a variety of compounds, such as diosgenin, vitamin A–E, quercetin, 10-DHGD, and curcumin, each of which have been shown in vitro to scavenge ROS and/or up-regulate endogenous antioxidant mechanisms, subsequently attenuating inflammation and vascular calcification.

Moreover, several large observational studies have suggested an inverse relationship between dietary ‘antioxidant’ intake such as α-tocopherol, β-carotene, and vitamins C and E, with cardiovascular morbidity and mortality. However, meta-analyses of randomized control trial data on the effects of vitamins B, C, E, and S or β-carotene have found identical rates of cardiovascular morbidity and mortality in the placebo and antioxidant groups.

A number of reasons for these disappointing findings have been proposed including the inadequate length of follow-up periods and the dosing of antioxidants tested. In addition, given that ROS also mediate important physiological processes, generalized scavenging of ROS is unlikely to prove beneficial if it interrupts cellular homeostasis and cardiovascular physiology such as endothelial-mediated control of vascular tone by superoxide and hydrogen peroxide as well as platelet aggregation, angiogenesis, and immune cell activity. It is, therefore, plausible to hypothesize that the failure of these compounds in atherosclerosis and vascular calcification, that they are unlikely to prove beneficial for those with CAVD.

5.3 Enhancing SOD and catalase activity

Reductions in antioxidant enzyme levels are observed in calcified aortic valve leaflets. Adenoviral delivery of SOD or catalase significantly reduces ROS-induced human VIC DNA damage, osteoblast differentiation, and valvular calcification. Similarly, application of the cell-permeable polyethylene glycol-SOD (peg-SOD) or peg-catalase significantly decreases superoxide and H2O2 levels respectively in human stenotic and vascular calcification, that they are unlikely to prove beneficial for those with CAVD.

5.4 Increasing NO bioavailability

Several studies have attempted to reduce the overall burden of ROS within the aortic valve by increasing the bioavailability of NO. Increasing NO bioavailability not only reduces the burden of ROS but also regulates Notch1 signalling and its nuclear localization to reduce the expression of osteogenic markers in VICs.

In isolated porcine VICS, application of TGF-β1 induces superoxide production which partly mediates valve calcification. However, co-application with the NO donors DETA-NONOate and sodium nitroprusside (SNP) scavenges superoxide, reducing in vitro calcification. DETA-NONOate also inhibits OGM-induced VIC differentiation and matrix calcification in isolated porcine VICS. Moreover, pre-treatment with L-arginine, the precursor for NO synthesis, significantly attenuates the osteogenic differentiation of bovine aortic VICs exposed to the endotoxin LPS. In the presence of L-arginine, LPS-induced ALP expression and subsequent matrix calcification are reduced, alongside reductions in LPS-induced TNF-alpha, IL-6, and IL-1β expression. Pre-treatment with L-arginine also reduces xanthine oxidase expression, and markers of ECM remodelling including ADAMTSL4, basigin, and COL3A1.

Finally, in calcified ex vivo human aortic valves exposed to TNFα, co-treatment with B-H4 mitigates eNOS uncoupling, increasing NO bioavailability which reduces superoxide levels, thereby attenuates the expression of osteogenic genes.

The above findings support, at least in principle, the proposal that increasing NO bioavailability might represent a novel approach for treating and/or slowing the progression of CAVD. Nevertheless, there is currently no clinical trial evidence on the effectiveness of this approach. In addition, part of the pleiotropic nature of statins is their ability to increase NO bioavailability via the stabilization of eNOS mRNA and in a rabbit model of CAVD, statins reduce AV calcification via this mechanism. Given the aforementioned failure of statin therapy in clinical trials, different approaches to increasing NO bioavailability require further investigation. These might include organic nitrate therapy, administration of L-arginine or B-H4, or using the KATP channel opener nicorandil, which also possesses a nitric oxide moity. Renin-angiotensin system inhibitors such as ACE inhibitors might also slow the progression of CAVD by increasing NO bioavailability via up-regulating eNOS expression, reducing bradykinin breakdown, and by suppressing NOX-generated superoxide.
5.5 Inhibiting NOX2

Specific inhibition of only the deleterious sources of ROS in CAVD represents a more nuanced approach than the broad ROS scavenging approaches described above. To this end, inhibitors of NOX2 signalling have been used in animal models of CAVD, but with varying results. In porcine aortic valves, apocynin, which blocks phosphorylation of the obligatory NOX2 cystolic component p47phox,\textsuperscript{58,295,296} but may also have non-specific antioxidant activity, only partly reduces TNFa-induced increases in superoxide and hydrogen peroxide.\textsuperscript{209} This is in contrast to isolated mice aortic myofibroblasts where apocynin markedly impairs TNF- and IL-1β-induced NOX2 ROS generation, a finding that is recapitulated with antisense oligonucleotides targeted against NOX2.\textsuperscript{137}

In hypercholesterolaemic LDLr\textsuperscript{-/-}/apoB\textsuperscript{100/100} mice fed a western diet, pioglitazone reduces aortic valve osteogenic signalling, aortic valve calcification, and improves cusp mobility, possibly due to a reduction of inflammation and oxidative stress that is associated with down-regulated NOX2 expression.\textsuperscript{223} The specific contribution of NOX2 to this improvement is not however clear, given that multiple other inflammatory mediators including TNF and IL-6 are also down-regulated by pioglitazone therapy.\textsuperscript{222}

Interestingly, in isolated porcine VICs, treatment with celastrol, a pentacyclic triterpene naturally extracted from the roots of Tripterygium wilfordii, and a potent NOX inhibitor with higher potency against NOX2,\textsuperscript{297} decreases NOX2 and Runx2 protein levels, VIC ROS levels, and reduces calcium deposition.\textsuperscript{13} Pro-osteogenic accumulation of β-catenin is also reversed with celastrol, impairing NOX2-mediated inactivation of GSK3β, which in turn enables β-catenin degradation. These findings are replicated when isolated aortic VICs are transfected with adenoviral vectors expressing a short hairpin sequence targeted against NOX2.\textsuperscript{223} An important observation given that NOX-independent effects of celastrol are also described in the cultured cell line PLB-985.\textsuperscript{298} Of note, celastrol may have other global beneficial effects, such as anti-obesity and anti-inflammation properties,\textsuperscript{299,300} which are comorbidities of CAVD likewise characterized by an increase in ROS production mediated, at least in part, by NOX2 activation.\textsuperscript{17}

Moreover, in a rabbit model of CAVD fed with HC + VitD, dietary celastrol markedly alleviates the degree of AS and improves cardiac dilatation, contractility, and function.\textsuperscript{13} Celastrol treatment also improves rabbit AV fibro-calcification, as indicated by decreases in collagen deposition, the expression of fibronectin, OPN, and the number of calcium deposits.\textsuperscript{13}

Recently, celastrol has also been shown to suppress Runx2 and OPN expression and reverse calcification in cultured porcine aortic VICs exposed to a medium containing high concentrations of calcium and phosphate.\textsuperscript{301} Here, celastrol reverses calcium-induced up-regulation of BMP2-BMPRII-Smad1/5 and Wnt/β-catenin signalling pathways, preventing their respective nuclear translocation and modulation of osteogenic gene expression. These findings are replicated in vivo by intraperitoneal injection of celastrol in mice fed adenine to induce CKD and intraperitoneal vitamin D to induce valvular calcification.\textsuperscript{301} However, NOX2 involvement in this model of CAVD remains to be investigated.\textsuperscript{301}

Together, these data suggest that NOX2 represents a novel therapeutic target in the treatment of CAVD with further work required to delineate its role in human CAVD. Given the role played by NOX2 in neutrophil function,\textsuperscript{302} future work targeting NOX2 in CAVD will need to consider whether this compromises innate immune responses, although previous work demonstrates that this only occurs with substantial NOX2 inhibition,\textsuperscript{303} thus safe therapeutic targeting of cardiovascular NOX2 could be feasible.

6. Conclusions and perspectives

CAVD is the end result of multiple active cellular and molecular mechanisms converging on the phenotypic change of aortic VICs. Oxidative stress is an important driver of these processes and targeted inhibition of the deleterious sources of ROS, particularly NOX2 and uncoupled eNOS, represent important avenues in the search for novel therapies. This targeted approach theoretically avoids interfering with the wide variety of normal cellular processes dependent on ROS signalling and may therefore yield better results for preventing or slowing the progression of CAVD than approaches non-specifically inhibiting redox signalling trialled thus far. Notably, in cancer medicine, a number of drugs targeting excessive ROS signalling that broadly up-regulate antioxidant mechanisms have thus far proved unsuccessful with unexpected toxic side effects often observed in normal tissues.\textsuperscript{304}

Presently there is a lack of in vivo and clinical data on human CAVD investigating roles and sources of adverse ROS signalling driving the disease. Specifically, there is a need for adequately powered cohort observational studies that might establish if there is a relationship between the extent of valvular oxidative stress, the pace of disease progression, and its relation to clinical severity. Novel imaging modalities that assess the in vivo burden of valvular oxidative stress using redox-sensitive probes may therefore represent important tools.\textsuperscript{305,306} In addition, larger scale in vitro studies examining roles and sources of ROS in human CAVD will also be necessary given the relatively small number of patients recruited to each of the studies described in this review. These in vitro studies may wish to make use of novel, and more-specific fluorescent ROS probes\textsuperscript{307} and ROS-targeting nanotechnologies,\textsuperscript{308,309} when exploring roles of ROS signalling in human CAVD.

Despite evidence of a role for mitoROS in a range of cardiovascular diseases,\textsuperscript{15,27,105} there remains no direct evidence of a pathophysiological role for these in CAVD. Future work might seek to identify whether mitoROS indeed play a role and whether targeting these ROS with recently developed site-specific mitochondrial ROS inhibitors, such as MitoQ effects the development and/or progression of the disease whilst maintaining the range of normal metabolic functions reliant on normal levels of mitoROS.\textsuperscript{310} Importantly, excessive ROS production by non-mitochondrial sources (such as uncoupled NOS enzymes or NOXs) can trigger mitochondrial dysfunction and further ROS production—sometimes termed ROS-induced ROS release.\textsuperscript{311,312} Therefore, further work is needed to explore the cross-talk between ROS at distinct subcellular compartment in aortic valve cells and its contribution to CAVD.

Finally, it would appear likely that any effective therapy preventing or slowing the progression of CAVD needs to be initiated early in the evolution of the disease, rather than at the stage where a patient presents with severe AS. Identifying and targeting pivotal molecular pathways essential for these in CAVD will prove challenging, and may require the use of state-of-the-art technologies, such as network medicine analysis and multi-omics mapping that generate transcriptional and protein expression signatures for the different stages of CAVD.\textsuperscript{113} Indeed, the feasibility of this approach has recently been demonstrated for CAVD,\textsuperscript{314} potentially an important step in characterizing the large number of cellular and molecular changes occurring as the disease develops and progresses.
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