

EDITORIAL COMMENT

Oxidation, Inflammation, and Aortic Valve Calcification

Peroxide Paves an Osteogenic Path*

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Arterial calcification increasingly afflicts our aging populace (1). Approximately 2% of individuals over age 65 years will require aortic valve replacement (AVR) for calcific aortic stenosis (1). On the basis of recent epidemiologic studies (2), the increasing prevalence of metabolic syndrome and type II diabetes mellitus (T2DM) will further increase the need for AVR—unless strategies are identified and implemented that prevent or reverse valve calcification. Similar concerns exist for 2 other types of vascular mineral deposition: atherosclerotic intimal calcification and medial artery calcification (3). Medial calcification is a strong predictor of lower extremity amputation in T2DM (4), a debilitating and costly outcome. Perturbed Windkessel physiology and altered vascular autonomic responses lead to tissue ischemia (5). Microcalcifications of cholesterol-laden or fibrous

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components of coronary atherosclerotic plaques attend outward vascular remodeling (6)—harbingers of acute coronary syndrome (7). A better understanding of arterial calcification and vascular mineral metabolism is needed. Once considered only a passive process of dead and dying cells, data from laboratories worldwide have shown that vascular calcification is an actively regulated form of tissue biomineralization (3). In response to metabolic, mechanical, and inflammatory insults, vascular mesenchymal cells elaborate matrix vesicles and gene regulatory programs that: 1) drive osteogenic vascular matrix remodeling (8); and 2) locally neutralize paracrine and systemic inhibitors of calcium deposition (9).

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In this issue of the *Journal*, Miller et al. (10) present an enlightening study that not only reveals the mechanistic underpinnings of human aortic valve calcification but also highlights the critical role of reactive oxygen species (ROS) in the pathobiology of most forms of arterial mineralization. With dihydroethidium staining and lucigenin chemiluminescence, the authors identified increased superoxide levels in stenotic calcified valves versus normal human heart valves. Dihydroethidium staining spatially resolved a gradient of oxidative stress within calcifying aortic valves, with highest levels localizing to regions possessing extensive calcium deposition (10). Dichlorodihydrofluorescein staining for hydrogen peroxide—the more durable ROS product of dismutation that propagates intracellular signals and iron-catalyzed oxidative damage (Fig. 1)—is also increased in regions of valve calcification, notably at the leaflet base (10). This was not due to increased superoxide dismutase (SOD) expression, because SOD isoforms and activities were down-regulated. More importantly, for reasons to be discussed, catalase expression was reduced in both calcified and noncalcified segments of diseased valves compared with normal valves. Thus, increases in ROS “tone” in aortic valves undergoing calcification are accompanied by reductions in defenses that remove several ROS—including hydrogen peroxide, a second messenger (11).

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases: the road not taken. NADPH oxidase/nitric oxide scavenger (Nox) activities (12) figure prominently in arterial oxidative stress, arising from non-laminar flow, inflammatory cytokine signaling, and activation of the renin-angiotensin-aldosterone system (13,14). Nox1 and Nox2 play critical roles in the aortic remodeling entrained to angiotensin (13,14). Thus, Miller et al. (10) evaluated whether Nox subunits were increased at venues of aortic valve calcification and oxidative stress. Surprisingly, Nox isoforms were uniformly decreased in calcifying valve segments, and no significant differences in Nox-dependent superoxide generation were measured between normal and diseased valves (10). This was completely unexpected because of the contributions of Nox signaling to atherosclerosis and vascular remodeling (11). Diphenyliodonium—an inhibitor of flavoenzymes such as Nox, xanthine oxidase, and nitric oxide synthase (NOS) (12)—did inhibit superoxide elaborated by calcifying valvular cells, confirming an enzymatic contribution to the generation of valve ROS.

When uncoupled by tetrahydrobiopterin deficiency or inflammation that precludes homodimer formation, NOS monomers use molecular oxygen—rather than arginine—as the terminal electron recipient in the NOS NADPH/flavin/iron relay (15) (Fig. 1). Therefore, the authors astutely examined the impact of selective NOS inhibition on valve superoxide, implementing the antagonistic arginine analog, nitro-L-arginine methyl ester (L-NAME). L-NAME reduced superoxide production, indicating the contribution of NOS uncoupling to calcified aortic valve ROS generation

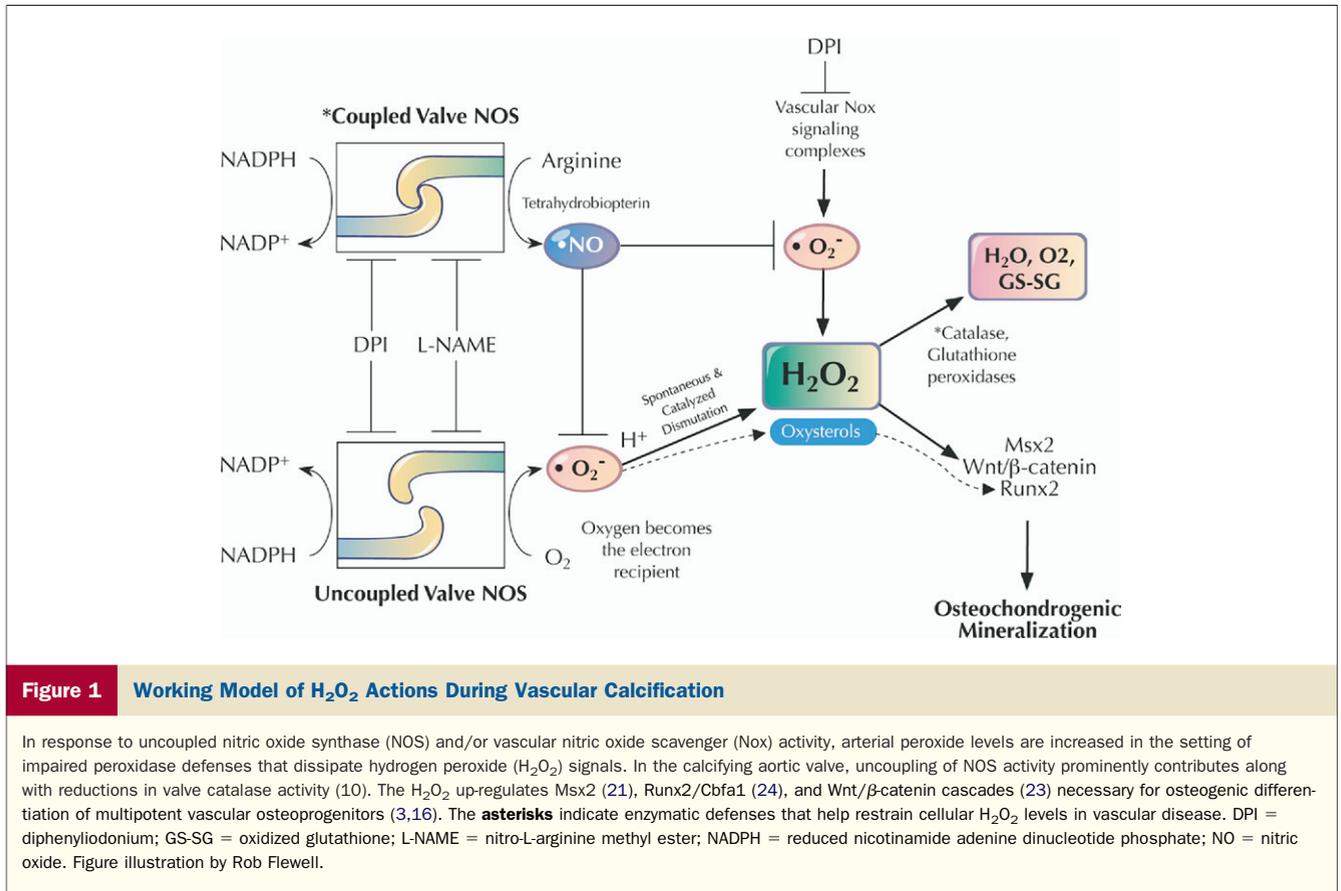


Figure 1 Working Model of H₂O₂ Actions During Vascular Calcification

In response to uncoupled nitric oxide synthase (NOS) and/or vascular nitric oxide scavenger (Nox) activity, arterial peroxide levels are increased in the setting of impaired peroxidase defenses that dissipate hydrogen peroxide (H₂O₂) signals. In the calcifying aortic valve, uncoupling of NOS activity prominently contributes along with reductions in valve catalase activity (10). The H₂O₂ up-regulates Msx2 (21), Runx2/Cbfa1 (24), and Wnt/β-catenin cascades (23) necessary for osteogenic differentiation of multipotent vascular osteoprogenitors (3,16). The **asterisks** indicate enzymatic defenses that help restrain cellular H₂O₂ levels in vascular disease. DPI = diphenyliodonium; GS-SG = oxidized glutathione; L-NAME = nitro-L-arginine methyl ester; NADPH = reduced nicotinamide adenine dinucleotide phosphate; NO = nitric oxide. Figure illustration by Rob Flewell.

(10). Had valvular NOS been coupled, L-NAME treatment would have increased superoxide accrual, because NOS-dependent nitric oxide production scavenges superoxide via peroxynitrite formation (15) (Fig. 1). Thus, Miller et al. (10) demonstrate that calcifying aortic valves generate a surfeit of superoxide and peroxide via uncoupled NOS activity in the setting of impaired antioxidant defenses—namely, valvular catalase deficiency and reduced nitric oxide production (Fig. 1, asterisks).

Vascular oxidative stress presages osteochondrogenic programming. Miller et al. (10) then related spatial patterns of aortic valve oxidative stress to the elaboration of osteochondrogenic transcription factors known to program biomineralization (16). Runx2/Cbfa1, Msx1, and Msx2 play critical roles in osteogenic mineralization (17). Runx2/Cbfa1 and Msx2 had been previously identified in calcifying human arteries (18); moreover, in a model of diabetic aortic calcification, Msx2 participates in a signaling relay that entrains osteogenic Wnt/β-catenin signaling to vascular inflammation (19,20). Miller et al. (10) identified that Runx2/Cbfa1 and Msx2 were indeed expressed in calcifying human aortic valves, confirming the contribution of active osteochondrogenic regulatory programs to valve calcium accrual (1). Once again, however, another surprise emerged. Whereas the expression of Msx2 was tightly entrained to regions of valve biomineralization, Runx2/Cbfa1 expression was visualized most robustly in adjacent diseased valve

segments—confirmed by reverse transcriptase-polymerase chain reaction analysis (10). The segregation of Msx2 and Runx2/Cbfa1 expression into distinct domains within diseased valves might reflect the actions of the paracrine Wnt signaling milieu that programs osteogenesis (17). Via the cell-surface receptors LRP5 and LRP6, canonical Wnt ligands induce dimerization with coreceptors that activate nuclear β-catenin signals necessary for osteogenic differentiation (reviewed in Johnson et al. [17]). Conversely, these pathways are inhibited by antagonistic ligands such as Dkk1 (17,20). Because Msx2-positive cells elaborate canonical Wnt ligands (Wnt3a and Wnt7a)—but express very little if any Dkk1 (20)—cells in the adjacent vicinity might up-regulate Runx2/Cbfa1 expression, a target of Wnt signaling in bone (17). However, the relationship of nuclear β-catenin accumulation to the spatial patterns of Msx2 and Runx2/Cbfa1 expression in calcifying valves has yet to be assessed. Of note, Rajamannan et al. (1) have clearly shown that Wnt3a, LRP5, and β-catenin are up-regulated in calcifying human aortic valves as compared with noncalcifying specimens.

Peroxide paves the path of vascular osteogenesis. Why is this study so significant? In addition to identifying that it is NOS uncoupling—not Nox activation—that generates ROS in calcifying human aortic valves, the authors demonstrate increased accumulation of hydrogen peroxide (H₂O₂) in calcifying valve segments (10). The H₂O₂ is a pro-

inflammatory second messenger (11). In pre-clinical models of diabetic arterial diseases, H_2O_2 is initially generated via tumor necrosis factor- α -dependent Nox activation, upstream of arterial Msx2-Wnt expression (21,22). Furthermore, at low levels, H_2O_2 promotes nuclear β -catenin signaling by inhibiting nucleoredoxin (23). Recently, Chen et al. (24) have shown that H_2O_2 can also up-regulate Runx2/Cbfa1 expression and promote osteogenic mineralization of vascular smooth muscle. Thus, insightful molecular study by Miller et al. (10) of human aortic valve calcification converges with accumulating pre-clinical data to highlight the fundamental contributions of peroxide signaling to vascular calcification (Fig. 1). Aortic valve H_2O_2 accumulates in part due to valvular catalase deficiency (10). Because perturbed expression of glutathione peroxidase 1 is associated with increased coronary calcification in T2DM (25), future studies might address whether glutathione peroxidases also participate in maintaining aortic valve longevity in T2DM.

The opportunities: avoiding loss in translation. Certainly, much more remains to be done to translate these seminal observations into clinical practice. The specific NOS isoforms that contribute to aortic valve disease with aging remain to be evaluated; endothelial NOS plays an important role in valve morphogenesis, and deficiency predisposes to bicuspid valve calcification (1,16). The reasons for aortic valve NOS uncoupling remain to be determined (15) and might differ during disease initiation and progression. In addition to tetrahydrobiopterin deficiency, oxidative stress itself can uncouple NOS (15). This has clinical implications, because once vascular mineral is deposited, it induces further inflammation and oxidative stresses (26). Proactive nutritional and pharmacologic strategies that reduce NOS uncoupling and enhance valve peroxidase activities might help prevent aortic valve calcification (Fig. 1) (1). The mechanisms of acquired catalase deficiency—and relative contributions of catalase versus glutathione peroxidase isoforms to aortic valve peroxide tone—remain to be determined (Fig. 1). Interactions between ROS generation and the neoangiogenesis necessary for true “ossification”—seen in approximately 15% of calcified aortic valve specimens (27)—remain to be established. The absence of Nox subunit induction with disease progression does not mean that Nox signaling is unimportant for healthy aortic valves. The Nox4 is critical to maintenance of the vascular myofibroblast phenotype (28). Thus, down-regulation of valvular Nox4 (10) might permit osteogenic “transdifferentiation” of valve myofibroblasts. Oxysterols, bioactive components of oxidized low-density lipoprotein, also up-regulate Runx2-dependent transcription (29). Therefore, deficiencies in the enzymes that reduce lipoprotein oxidation might also contribute. Finally, once initiated, a substantial portion of aortic valve calcium accrual occurs via amorphous epitaxial mineral deposition that is independent of osteogenic cells (27) and might be enhanced by cholesterol (30). This represents a failure of local and

circulating tissue mineralization inhibitors such as fetuin (9) and osteopontin (31). Although osteopontin is increased in regions of calcification and inhibits mineral deposition, its bioactivity is regulated by processing that generates proinflammatory fragments (31). The impact of ROS on fetuin and osteopontin functions has not been studied. All in all, the study of Miller et al. (10) affords us a significantly improved understanding of aortic vascular calcification. It initiates a new era of investigation into the biology and pharmacology of calcific aortic stenosis, offering new hope for the prevention and medical treatment of an otherwise burgeoning clinical need (1,16).

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