NIH Public Access
Author Manuscript
J Am Coll Cardiol. Author manuscript; available in PMC 2009 September 2.

Published in final edited form as:
J Am Coll Cardiol. 2008 September 2; 52(10): 851–854. doi:10.1016/j.jacc.2008.05.044.

Oxidation, Inflammation, and Aortic Valve Calcification: *Peroxide Paves An Osteogenic Path*

Dwight A. Towler, MD, PhD
Center for Cardiovascular Research, Internal Medicine – Bone & Mineral Diseases, Washington University in St. Louis, St. Louis, MO 63110

Arterial calcification increasingly afflicts our aging populace(1). Approximately 2% of individuals over age 65 will require aortic valve replacement (AVR) for calcific aortic stenosis (1). Based upon recent epidemiologic studies (2), the increasing prevalence of metabolic syndrome and type II diabetes (T2DM) will further increase the need for AVR – unless strategies are identified and implemented that prevent or reverse valve calcification. Similar concerns exist for two 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 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 world-wide 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 drive (a) osteogenic vascular matrix remodeling(8); and (b) locally neutralize paracrine and systemic inhibitors of calcium deposition (9).

In this issue of the *Journal*, Miller, Heistad, and colleagues (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) to the pathobiology of most forms of arterial mineralization. Using dihydroethidium (DHE) staining and lucigenin chemiluminescence, the authors identified increased superoxide levels in stenotic calcified valves vs. normal human heart valves. DHE staining spatially resolved a gradient of oxidative stress within calcifying aortic valves, with highest levels localizing to regions possessing extensive calcium deposition(10). DCF (dichlorodihydrofluorescein) staining for hydrogen peroxide – the more durable ROS product of dismutation that propagates intracellular signals and iron-catalyzed oxidative damage (Figure 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, since SOD isoforms and activities were down-regulated. More

Please address correspondence to: Dwight A. Towler MD, PhD, Professor of Medicine, Internal Medicine -- Bone & Mineral Diseases, Washington University in St. Louis, Campus Box 8301, 660 South Euclid Avenue, St. Louis, MO 63110, ph: 314.454.7434 fax: 314.454.8434, email: dtowler@dom.wustl.edu.

Disclosure: Dr. Towler has served as a paid consultant on musculoskeletal disease therapeutics for Wyeth, GlaxoSmithKline, and Eli Lilly. He receives funding for his research from the National Institutes of Health, and from the Barnes-Jewish Hospital Foundation.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

importantly, for reasons to be discussed, *Catalase* expression was reduced in both calcified and non-calcified segments of diseased valves as compared to normal valves. Thus, increases in ROS "tone" in aortic valves undergoing calcification are accompanied by reductions in defenses that remove several reactive oxygen species(10) -- including the second messenger, hydrogen peroxide(11).

NADPH Oxidases: The Road Not Taken

NADPH oxidase / 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 evaluated whether *Nox* subunits were increased at venues of aortic valve calcification and oxidative stress (10). 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),. DPI (diphenyliodonium) -- an inhibitor of flavoenzymes such as Nox, xanthine oxidase, and nitric oxidase 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 utilize molecular oxygen -- rather than arginine – as the terminal electron recipient in the NOS NADPH/flavin/iron relay(15) (Figure 1). Therefore, the authors astutely examined the impact of selective NOS inhibition on valve superoxide, implementing the antagonistic arginine analog, L-NAME. L-NAME reduced superoxide production, indicating the contribution of NOS uncoupling to calcified aortic valve ROS generation(10). Had valvular NOS been in coupled, L-NAME treatment would have increased superoxide accrual – since NOS-dependent nitric oxide production scavanges superoxide via peroxynitrite formation (15) (Figure 1). Thus, Miller et al. demonstrate that calcifying aortic valves generate a surfeit of superoxide and peroxide via uncoupled NOS activity in the setting of impaired antioxidant defenses – viz., valvular catalase deficiency and reduced NO production(10) (Figure 1, asterisks).

Vascular Oxidative Stress Presages Osteochondrogenic Programming

The authors then s 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 identified that Runx2/Cbfa1 and Msx2 were indeed expressed in calcifying human aortic valves(10), confirming the contribution of active osteochondrogenic regulatory programs to valve calcium accrual(1). Once again, however, another surprise emerged. While 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 RT-qPCR analysis(10) The segregation of Msx2 and Runx2/Cbfa1 expression into distinct domains within diseased valves may reflect the actions of the paracrine *Wnt* signaling milieu that programs osteogenesis (17). Via the cell-surface receptors LRP5 and LPR6, canonical Wnt ligands induce dimerization with co-receptors that activate nuclear β-catenin signals necessary for osteogenic differentiation (reviewed in (17)). Conversely, these pathways are inhibited by antagonistic ligands such as Dkk1(17,20). Since Msx2-positive cells elaborate canonical Wnt ligands

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(Wnt3a and Wnt7a) -- but express very little if any Dkk1(20) --- cells in the adjacent vicinity may upregulate 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 has clearly shown that Wnt3a, LRP5, and β-catenin are upregulated in calcifying human aortic valves as compared to non-calcifying specimens (1)

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 (H_2O_2) in calcifying valve segments(10). H_2O_2 is a pro-inflammatory second messenger(11). In preclinical models of diabetic arterial diseases, H_2O_2 is initially generated via TNFα-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 has shown that H_2O_2 can also upregulate *Runx2/Cbfa1* expression and promote osteogenic mineralization of vascular smooth muscle (24). Thus, Miller's insightful molecular study of human aortic valve calcification (10) converges with accumulating pre-clinical data to highlight the fundamental contributions of peroxide signaling to vascular calcification (Figure 1). Aortic valve H_2O_2 accumulates in part due to valvular catalase deficiency(10). Since perturbed expression of *Gpx1* is associated with increased coronary calcification in T2DM(25), futures studies may 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 evaluated; eNOS plays an important role in valve morphogenesis, and deficiency predisposes to biscuspid valve calcification (1,16). The reasons for aortic valve NOS uncoupling remain to be determined(15) – and may differ during disease initiation and progression. In addition to tetrahydrobiopterin deficiency, oxidative stress itself can uncouple NOS(15). This has clinical implications, since once vascular mineral is deposited, it induces further inflammation and oxidative stresses (26). Pro-active nutritional and pharmacologic strategies that reduce NOS uncoupling and enhance valve peroxidase activities may help prevent aortic valve calcification (Figure 1) (1). The mechanisms of acquired catalase deficiency -- and relative contributions of *Catalase* vs. *Gpx* isoforms to aortic valve peroxide tone -- remain to be determined (Figure 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. Nox4 is critical to maintenance of the vascular myofibroblast phenotype(28). Thus, down-regulation of valvular *Nox4* (10) may permit osteogenic "trans-differentiation" of valve myofibroblasts. Oxysterols, bioactive components of oxidized LDL, also upregulate Runx2-dependent transcription(29). Thus, deficiencies in the enzymes that reduce lipoprotein oxidation may 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 may 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 pro-inflammatory 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

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

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).

Acknowledgements

Supported by NIH grants HL69229, HL81138, HL88651, AR43731, and the Barnes-Jewish Hospital Foundation.

LITERATURE CITED

1. Rajamannan NM, Bonow RO, Rahimtoola SH. Calcific aortic stenosis: an update. Nat Clin Pract Cardiovasc Med 2007;4:254–62. [PubMed: 17457349]
2. Katz R, Wong ND, Kronmal R, et al. Features of the metabolic syndrome and diabetes mellitus as predictors of aortic valve calcification in the Multi-Ethnic Study of Atherosclerosis. Circulation 2006;113:2113–9. [PubMed: 16636166]
3. Abedin M, Tintut Y, Demer LL. Vascular calcification: mechanisms and clinical ramifications. Arterioscler Thromb Vasc Biol 2004;24:1161–70. [PubMed: 15155384]
4. Lehto S, Niskanen L, Suhonen M, Ronnemaa T, Laakso M. Medial artery calcification. A neglected harbinger of cardiovascular complications in non-insulin-dependent diabetes mellitus. Arterioscler Thromb Vasc Biol 1996;16:978–83. [PubMed: 8696962]
5. Shao JS, Cai J, Towler DA. Molecular mechanisms of vascular calcification: lessons learned from the aorta. Arterioscler Thromb Vasc Biol 2006;26:1423–30. [PubMed: 16601233]
6. Burke AP, Kolodgie FD, Farb A, Weber D, Virmani R. Morphological predictors of arterial remodeling in coronary atherosclerosis. Circulation 2002;105:297–303. [PubMed: 11804983]
7. Ehara S, Kobayashi Y, Yoshiyama M, et al. Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study. Circulation 2004;110:3424–9. [PubMed: 15557374]
8. Aikawa E, Nahrendorf M, Figueiredo JL, et al. Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo. Circulation 2007;116:2841–50. [PubMed: 18040026]
9. Johnson RC, Leopold JA, Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. Circ Res 2006;99:1044–59. [PubMed: 17095733]
10. Miller JD, Chu Y, Brooks RM, Richenbacher WE, Pena-Silva R, Heistad DD. Dysregulation of anti-oxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans. J Am Coll Cardiol. 2008In Press
11. Haurani MJ, Pagano PJ. Adventitial fibroblast reactive oxygen species as autacrine and paracrine mediators of remodeling: bellwether for vascular disease? Cardiovasc Res 2007;75:679–89. [PubMed: 17689510]
12. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 2007;87:245–313. [PubMed: 17237347]
13. Liu J, Ormsby A, Oja-Tebbe N, Pagano PJ. Gene transfer of NAD(P)H oxidase inhibitor to the vascular adventitia attenuates medial smooth muscle hypertrophy. Circ Res 2004;95:587–94. [PubMed: 15308582]
14. Dikalova A, Clempus R, Lassegue B, et al. Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. Circulation 2005;112:2668–76. [PubMed: 16230485]
15. Munzel T, Daiber A, Ullrich V, Mulsch A. Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. Arterioscler Thromb Vasc Biol 2005;25:1551–7. [PubMed: 15879305]
16. O'Brien KD. Pathogenesis of calcific aortic valve disease: a disease process comes of age (and a good deal more). Arterioscler Thromb Vasc Biol 2006;26:1721–8. [PubMed: 16709942]
17. Johnson ML, Rajamannan N. Diseases of Wnt signaling. Rev Endocr Metab Disord 2006;7:41–9. [PubMed: 16944325]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

18. Tyson KL, Reynolds JL, McNair R, Zhang Q, Weissberg PL, Shanahan CM. Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification. Arterioscler Thromb Vasc Biol 2003;23:489–94. [PubMed: 12615658]

19. Al-Aly Z, Shao JS, Lai CF, et al. Aortic Msx2-Wnt calcification cascade is regulated by TNF-alpha-dependent signals in diabetic Ldlr-/- mice. Arterioscler Thromb Vasc Biol 2007;27:2589–96. [PubMed: 17932314]

20. Shao JS, Cheng SL, Pingsterhaus JM, Charlton-Kachigian N, Loewy AP, Towler DA. Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals. J Clin Invest 2005;115:1210–20. [PubMed: 15841209]

21. Shao JS, Aly ZA, Lai CF, et al. Vascular Bmp Msx2 Wnt signaling and oxidative stress in arterial calcification. Ann N Y Acad Sci 2007;1117:40–50. [PubMed: 18056036]

22. Lai-Huang CF, Shal JS, Al-Aly Z, Cheng SL, Towler DA. TNF-alpha Dependent Redox Signals Upregulate Msx2 Gene Transcription via Stress Activated Protein Kinases in Arterial Myofibroblasts. J Bone Miner Res 2007;22:Abstract T165.

23. Funato Y, Michiue T, Asashima M, Miki H. The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt-beta-catenin signalling through dishevelled. Nat Cell Biol 2006;8:501–8. [PubMed: 16604061]

24. Byon CH, Javed A, Dai Q, et al. Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor runx-2 by akt signaling. J Biol Chem. 2008

25. Nemoto M, Nishimura R, Sasaki T, et al. Genetic association of glutathione peroxidase-1 with coronary artery calcification in type 2 diabetes: a case control study with multi-slice computed tomography. Cardiovasc Diabetol 2007;6:23. [PubMed: 17825092]

26. Shanahan CM. Inflammation ushers in calcification: a cycle of damage and protection? Circulation 2007;116:2782–5. [PubMed: 18071088]

27. Mohler ER 3rd, Gannon F, Reynolds C, Zimmerman R, Keane MG, Kaplan FS. Bone formation and inflammation in cardiac valves. Circulation 2001;103:1522–8. [PubMed: 11257079]

28. Cucoranu I, Clempus R, Dikalova A, et al. NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. Circ Res 2005;97:900–7. [PubMed: 16179589]

29. Richardson JA, Amantea CM, Kianmahd B, et al. Oxysterol-induced osteoblastic differentiation of pluripotent mesenchymal cells is mediated through a PKC- and PKA-dependent pathway. J Cell Biochem 2007;100:1131–45. [PubMed: 17031848]

30. Laird DF, Mucalo MR, Yokogawa Y. Growth of calcium hydroxyapatite (Ca-HAp) on cholesterol and cholestanol crystals from a simulated body fluid: A possible insight into the pathological calcifications associated with atherosclerosis. J Colloid Interface Sci 2006;295:348–63. [PubMed: 16229855]

31. Scatena M, Liaw L, Giachelli CM. Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. Arterioscler Thromb Vasc Biol 2007;27:2302–9. [PubMed: 17717292]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

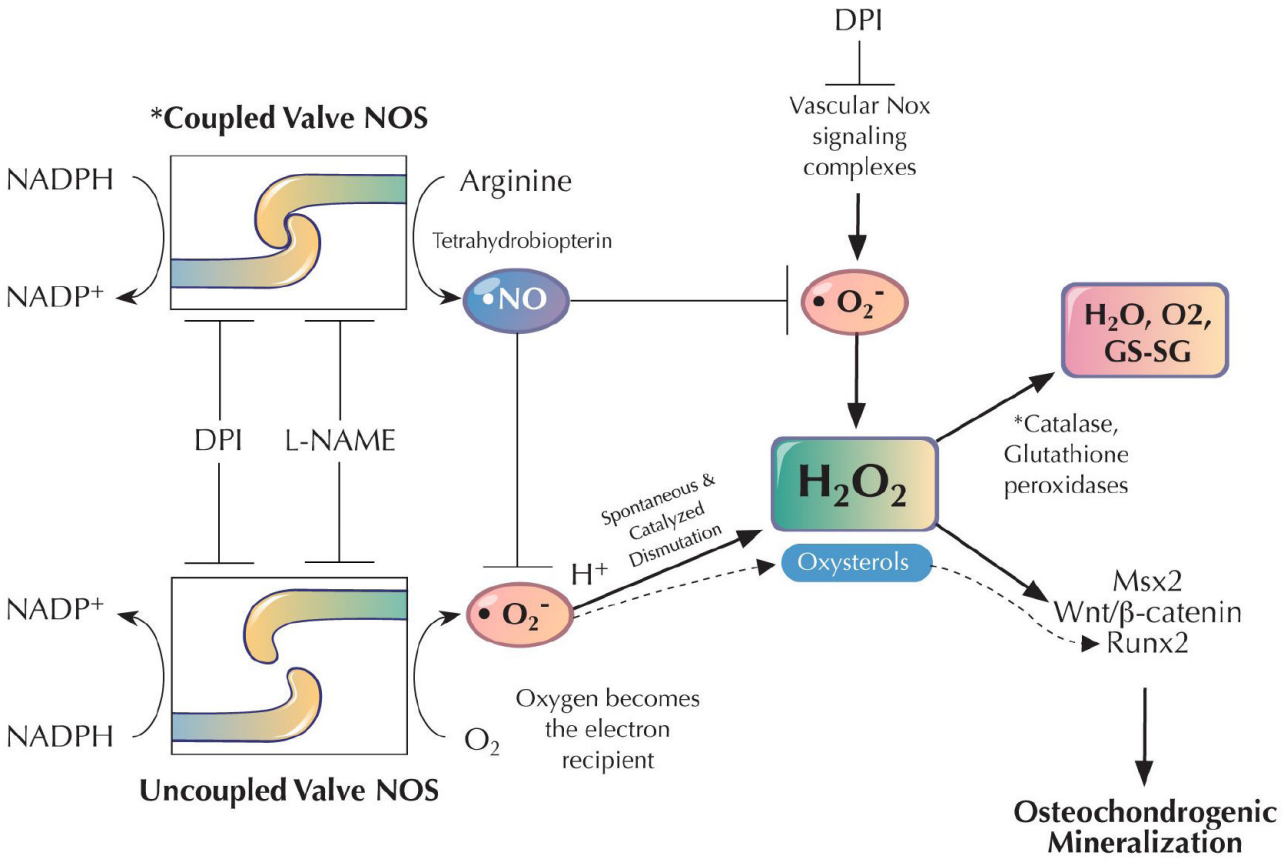

Figure 1. Working model of hydrogen peroxide actions during vascular calcification

In response to uncoupled NOS and/or vascular Nox activity, arterial peroxide levels are increased in the setting of impaired peroxidase defenses that dissipate H_2O_2 signals. In the calcifying aortic valve, uncoupling of NOS activity prominently contributes along with reductions in valve catalase activity(10). H_2O_2 upregulates Msx2 (21), Runx2/Cbfa1(24), and Wnt/β-catenin cascades (23) necessary for osteogenic differentiation of multipotent vascular osteoprogenitors(3,16). GS-SG, oxidized glutathione.