Nox and Inflammation in the Vascular Adventitia

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For nearly half a century, strong associations have been made linking early adventitial activation in disease with endothelial dysfunction,[1,2] thus challenging the notion that the luminal endothelium is the initial sensor and propagator of cardiovascular disease. Over the past 2 decades, perturbational studies have established a causal role of adventitial factors in this regard. This review-based update acts as a springboard for a revitalized debate of the adventitia's role in vascular disease.

From a pathological perspective, the adventitia reportedly plays a deleterious role via production of large amounts of nicotinamide adenine dinucleotide phosphate oxidase (Nox)-derived reactive oxygen species (ROS) in response to vascular injury and disease.[3,4] A combined adventitial fibroblast and inflammatory cell infiltration facilitated by the vasa vasorum (VV) is expected to synergize and propagate vessel inflammation.[5] In fact, Noxes are highly activated by inflammatory cytokines, hormones, lipids, and angiotensin II (Ang II).[6] In the adventitia, Nox-derived ROS is the dominant culprit for intracellular redox signaling cascade activation, either in an autocrine or in a paracrine manner, leading to subsequent vascular cell activation, proliferation, hypertrophy, or apoptosis and thereby promoting vascular diseases including hypertension and atherosclerosis.[7] Despite these discoveries, studies delving into the role of the adventitia in disease propagation or progression remain limited. This gap in knowledge presents a relatively untapped opportunity to study vascular disease pathogenesis in a renewed light. This focused mini-review addresses the role of adventitial Nox-derived ROS from a perivascular viewpoint looking inwards to promoting vascular inflammation and disease.

The Adventitia, Adventitial Fibroblasts, and ROS: Instigators of Vessel Inflammation

Forming the outermost layer of the vessel wall, the adventitia is emerging as a prominent channel for the progression of vascular remodeling. Within the adventitial milieu, adventitial fibroblasts/myofibroblasts, lymphocytes, stem cell–like vascular and hematopoietic lineage progenitors, and endothelial cells reside.[8–10] To date, the scientific maxim has been that vascular inflammation is initiated at the main luminal endothelial surface and progresses through the media toward the adventitia. However, this rigid hematocentric or "inside-out" view of the vasculature is slowly being debunked by another perspective of the adventitia as an integral tissue of sorts, in its own right, influencing vessel function.[11] Importantly, a plethora of evidence details the direct effects of adventitia-derived ROS on medial growth as well as ROS-dependent vascular wall inflammation in hypertension, aortic aneurysms (AAs), and atherosclerosis.[12] By extension, the adventitia has profound implications for vessel tone, with adventitial fibroblasts producing substantial amounts of Nox-derived ROS, which augment constriction.[13,14] In addition, adventitial fibroblast cell-derived cytokines (ie, interleukin [IL]-1β, IL-6, and monocyte chemoattractant protein-1) are implicated in fibroblast activation and recruitment of inflammatory cells to the adventitial layer, which impairs vascular function.[15] Therefore, the directionality of "inside-out" vascular disease hypothesis requires careful reconsideration. Gaining traction within the literature is the notion of an adventitious (from the Latin for coming from without) or "outside-in" paradigm, in which the nexus of vascular wall ROS and inflammation propagates in the adventitia and spreads through the media inward toward the intima.[16,17] Hallmarks of the "outside-in" hypothesis include increased activation and migration of fibroblasts and myofibroblasts, increased VV neovascularization, a relatively high adventitial infiltration of leukocytes, and enhanced adventitia-derived ROS levels.[5,8,9,18] Together, these processes seem to exacerbate attraction and delivery of inflammatory cells to the adventitia and subsequently throughout the vessel wall. Indeed, a complex interaction between vascular cells and different classes of leukocytes (T lymphocytes, neutrophils, and monocytes/macrophages) exists and supports the vascular inflammation phenotype.[19] Importantly, adventitial hyperplasia precedes medial hypertrophy and neointimal hyperplasia, and even endothelial dysfunction, in various manifestations of cardiovascular disease.[1,2,20,21] These findings appear to suggest that the adventitia is an initial-sensor and a prominent motivator of vascular phenotype change (Figure).

Nox: The Nexus of Adventitial Signaling?

Adventitial fibroblast Nox has been implicated in fibroblast function,[22] as well as being the major contributor to vascular ROS in cardiovascular disease.[23] Both superoxide and its metabolite hydrogen peroxide (H_2O_2), generated from the former by spontaneous dismutation or facilitated by the enzyme superoxide dismutase, have emerged as important players

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(*Hypertension*. 2016;67:14-19. DOI: 10.1161/HYPERTENSIONAHA.115.03622.)

Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.115.03622

in vascular pathophysiology because of their signaling roles in a range of cellular processes, including cell proliferation, migration, hypertrophy, and apoptosis.[24] Essentially, Nox-derived ROS remain front runners of adventitial redox signaling as other ROS generating enzymes (eg, mitochondrial complex I and xanthine oxidases) are yet to be implicated in this response.

The Nox family of enzymes consists of 7 isozymes, namely Nox1-5 and DUOX1-2[25] with varying expression patterns throughout adventitial-resident cells. Early studies in our laboratory demonstrated abundant colocalization of Nox2, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ in aortic vascular adventitia and enrichment of $p67^{phox}$-dependent Nox activity in cultured adventitial fibroblasts of the rabbit aorta.[3] With regard to the other Nox isoforms and cytosolic subunits, reports illustrate expression of the adventitial fibroblast Nox1 and Nox4 oxidase systems.[26,27] Notably, Nox4-derived H_2O_2 is documented to be protective in many vascular and heart disease models.[28,29] Irrespective of the Nox isoform, fibroblast Nox activity is enhanced by a wide variety of stimuli, including hypoxia, cytokines, hormones, metabolic factors, and mechanical injury.[6] As expected, adventitial leukocytes exhibit high Nox2 expression[30] and this increased leukocyte–Nox–ROS is predicted to contribute to an overall increase in local oxidant level after adventitial leukocyte accumulation, a potential result of the chronic sterile inflammasome.[31] In terms of adventitial stem cells, a potential influential role of Nox expression/activation is yet to be explored. That notwithstanding, these findings collectively predict a complex disarray of ROS-mediated signaling from the adventitia in response to various stimuli in vascular disease.

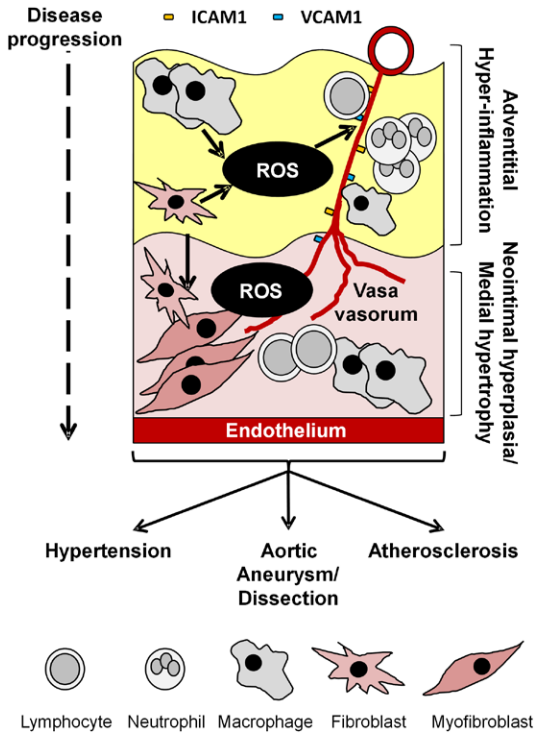

Figure. Schematic representation of the adventitious "outside-in" paradigm in cardiovascular disease. In the "outside-in" model, adventitial reactive oxygen species (ROS) from multiple cell types propagate a rapidly growing and leaky vasa vasorum, which provides a gateway for inflammatory cells to enter the media and intima. There leukocytes and myofibroblasts accumulate and cause local smooth muscle cells to become hypertrophic or proliferate depending on the disease milieu: hypertension, aortic aneurysm/dissection, and atherosclerosis. ICAM indicates intercellular adhesion molecule; and VCAM, vascular cell adhesion molecule.

The VV: The New Front Door to Medial Inflammation?

As a result of medial hypertrophy or hyperplasia, pO_2 levels plummet at the vessel wall core setting in motion hypoxia-dependent and hypoxia-independent signaling, which include fibroblast and myofibroblast proliferation, increased ROS levels and neovascularization. Consequently, as a microvascular network of vessels luminally lined with endothelial cells and surrounded by smooth muscle cells and pericytes, the VV serves to supply oxygen and nutrients to the walls of large vessels.[32] As a result, these new microvessels are expected to facilitate chemoattraction and delivery of inflammatory cells from the adventitia across the external laminae and into the media eliciting noxious effects.[11,33]

As observed in hypertension[34] and atherosclerosis,[35] the VV becomes densely arborized and extends from the adventitial layer into the media. Indeed in atherosclerosis, the nascent VV is considered to be an immature and leaky network, therein promoting vascular inflammation and plaque progression by facilitating extravasation of leukocytes into the medial layer.[36] Moreover, many experimental studies in animal models directly link VV neovascularization with neointimal thickening.[37,38] Whether neointimal thickening in this setting is the result of direct accumulation of leukocytes in the intima or inflammation-induced smooth muscle migration is still unclear. Interestingly, a niche of adventitial-resident endothelial progenitor cells and multipotent pericytes putatively provide the building blocks for neovascularization.[9,39] However, mechanisms by which these stem cell–like progenitors are mobilized from their adventitial niches and pervade other vessel layers are incompletely understood. Although yet unsubstantiated, a key role of the VV in facilitating migration of these progenitor cells to vascular sites of inflammation and injury is expected. Indeed, as vascular inflammation is known to involve chemotaxis, chemokinesis, and transmigration[33] of adventitial cells, greater consideration of the "outside-in" theory, concordant with a burgeoning VV, is required to fully comprehend neointimal hyperplasia in hypertension and atherosclerosis.

Currently, methods to visualize the VV in vivo are limited to end stage disease using either 3-dimensional microcomputed tomography,[40] fluorescence confocal image stacking,[38] or multiphoton microscopy.[41] Using microcomputed tomography, Herrmann et al[20] observed increased density of the VV penetrating the tunica adventitia and media of large vessels as early hallmarks of vascular inflammatory disease. As alluded to above, the initiating factor for increasing VV density purportedly involves decreased medial O_2 levels, in turn, eliciting hypoxia-inducible factor-1α[34,42] activation and fibroblast growth factor-2–dependent VV stabilization.[43] Moreover, Nox-derived ROS play a central role in hypoxia-inducible factor-1α activation.[44] In this regard, Khatri et al[45] identified

transgene overexpression of vascular smooth muscle cell p22^{phox} in mice triggered oxidative stress-induced hypoxia-inducible factor-1α and enhanced atheroma progression via angiogenic switching. However, although this angiogenic response may seem fortuitous in this context, it is expected to enhance plaque progression via extravasation of leukocytes after endothelial activation. Other reports identify proinflammatory cytokines IL-1β and tumor necrosis factor-α involvement in VCAM-1 expression via nuclear factor κB.[46] Indeed, ROS is known to participate in endothelial adhesion molecule ICAM-1, VCAM-1, and E-selectin expression[47] and compared with wild-type mice, tumor necrosis factor-α stimulation does not induce expression of VCAM-1 in coronary microvascular endothelial cells from p47^{phox} null mice, implicating a role of Nox2-derived ROS in this process.[48] Moreover, treatment of endothelial cells with superoxide dismutase and catalase inhibited ROS-mediated activation of nuclear factor κB.[49] Taking these findings together, one might expect a positive feedback loop involving Nox-derived ROS leading to an exaggerated accumulation of leukocytes in the adventitia and outer media. The abovementioned findings are supported by work by Cheng et al[50] who demonstrated that plaque vulnerability and growth correlated with expression of Est2 (a potent transactivator of endothelial proangiogenic receptors), and neovascularization. In still another study, Langheinrich et al[51] correlated neovascularization with extravasation of leukocytes and plaque progression. That being said, the existence of a feed-forward interaction between leukocyte-derived and adventitial parenchymal cell-derived ROS propagating VV adhesion molecule expression has not yet been confirmed. Thus, there is still much to be learned about adventitial fibroblast ROS, oxidative stress, and VV neovascularization cross-talk.

Adventitial Nox-ROS and Vascular Disease

To date, the question of which vessel segment when activated, intima versus adventitia, is the predominant driver of vascular disease remains unanswered. The evidence referenced herein should leave little doubt that the adventitia plays a mounting role in vascular disease progression. That is, the inside-out hypothesis falls short of fully addressing the underpinnings for vessel remodeling and neointimal hyperplasia. Nonetheless, we consider it highly likely that both the intima and adventitia play prominent roles.

Hypertension

Hypertension affects 1:3 people in the United States, and, although therapies exist with the aim of reducing blood pressure, eg, angiotensin-converting enzyme inhibitors, β-blockers and diuretics,[52] hypertension-induced vascular inflammation remains an intractable problem. In Ang II, deoxycorticosterone acetate–salt and spontaneous hypertensive rat models, Nox2-derived ROS remain the main culprits for adventitial redox signaling.[53,54] Notably, adventitial fibroblast ROS is observed to play an active role in Ang II hypertension–associated vessel remodeling by affecting secretion of monocyte chemoattractant protein-1 and IL-6.[15,55] Significantly, adventitial leukocyte accumulation and vessel inflammation are observed in human hypertensive specimens and animal models[56–58] and adventitial macrophage infiltration occurs in conjunction with the development of vascular wall hypertrophy in models of Ang II–induced hypertension.[17] Intriguingly, this accumulation is acknowledged to occur in the absence of significant intimal macrophage migration and it remains to be determined whether this macrophage accumulation in hypertension is synergized by outside-in infiltration via the VV and resident macrophage progenitor cells.[39,59]

Our laboratory discovered that Ang II induces p67^{phox}-dependent adventitial ROS in the mouse[60] and this was recently corroborated by the Cowley laboratory, who showed that p67^{phox} knockout rats display significantly lower mean arterial pressures in a salt-sensitive hypertension model compared with wild-type rats.[61] Moreover, Nox1/2 inhibition, using the nonselective VasoPharm compounds, inhibited endothelial dysfunction in spontaneous hypertensive rat aortas, with an evident decrease in adventitial ROS and potential Nox2 expression.[62] Therefore, as adventitial Nox2 is activated in hypertension models, we hypothesized Nox2-derived ROS plays a paracrine role to induce medial hypertrophy.[63] Importantly, our group recently expanded on this notion by discovering that extracellular feed-forward ROS signaling promotes SMC hypertrophy via aquaporin 1 and Nox1.[64] Similarly, Ang II–induced vessel hypertrophy was reportedly attenuated with adventitial catalase gene transfer.[65] In aggregate, these studies implicate Nox 1 and 2 as potential therapeutic targets for inhibiting adventitial fibroblast activation, medial hypertrophy, and hypertension.

AA/Dissection

AAs are a chronic vascular degenerative disease characterized by the deterioration of the aortic wall architecture, leading to progressive segmental dilation and lethal risk of aortic rupture.[66] To date, limited therapeutic strategies exist for combatting AA outside of surgical repair, and much of the disease pathogenesis is unknown. A large proportion of AA is characterized by an inflammatory response within the aortic wall, which includes dramatic adventitial remodeling.[67] As with hypertension, AA pathogenesis commences with macrophage recruitment and accumulation in the adventitia; however, it differs in ensuing migration and redistribution throughout the media.[68] Moreover, reports identify that other leukocytes accumulate and populate the adventitia, including neutrophils and lymphocytes.[69] Therefore, the adventitia seems to act as an efficient gateway for leukocyte infiltration. The lethality of AA stems from acute rupture (aortic dissection) allowing luminal blood to permeate the media. In an acute model of aortic dissection, adventitial CXCL1/G-CSF expression after dissection triggered local neutrophil recruitment, which exaggerated the disease.[70] This finding alone is expected to stimulate a line of inquiry into a plausible role of neutrophils in exacerbated ROS generation and dissection of the vessel wall. Furthermore, Nox2 was recently identified as a central culprit for aortic dissection in an Ang II–infused ApoE-null mouse model.[71] Fan et al[71] elegantly demonstrate that increased endothelial Nox2 expression and ROS promote cyclophillin-A secretion and SMC-derived reactive species, leading to vascular inflammation and dissection. Although evidence of adventitial VV permeating the medial layer is clear in AA,[72] whether

VV Nox2 or intimal Nox2 is the culpable endothelial source requires further inquiry. A potential caveat to these findings is the knowledge that global Nox2 deficiency exacerbated Ang II–induced AAs[73] via increased macrophage IL-1β and matrix metalloproteinase activity. Conversely, Meng et al[74] recently demonstrated that regulatory T lymphocytes (T regs) prevent Ang II–induced abdominal aneurysms in an ApoE-null mouse model. In particular, T regs were able to decrease aortic expression of monocyte chemoattractant protein-1, IL-6, and ICAM-1, as well as reduce matrix metalloproteinase-2 and matrix metalloproteinase-9 expression. In fact, redox mechanisms are linked with expression of monocyte chemoattractant protein-1 and ICAM-1, and activation of matrix metalloproteinases. Therefore, a T reg–mediated dampening of immune response is likely to involve suppression of Nox/ROS. Further studies are thus warranted to fully appreciate the role of the adventitial VV as well as the specific classes of leukocytes, which ostensibly dictate AA disease progression.

Atherosclerosis

Atherosclerosis is a multifactorial, multicellular vascular inflammatory disease. Current therapeutic strategies involve statins (lipid-lowering agents) and dietary control, in addition to surgical stenting. However, modulators of vascular inflammation are conspicuously absent. Not surprisingly, therefore, increased VV vascularity is a hallmark of the disease. At this juncture, it should be clear that an attendant leukocyte infiltration would promote atheromatous plaque progression.[75,76] In animal models, endothelial activation portends chronic inflammation and plaque progression[77] and the role of Nox2 in endothelial cell activation and dysfunction is well defined.[6] Therefore, an unrestrained neovascularization and endothelial activation in atherosclerosis is consistent with global Nox2 deficiency reducing early plaque burden and atherosclerosis.[78] That said, cell-specific Nox2 knockouts would be useful in addressing the relative and temporal contribution of endothelial dysfunction versus sterile inflammasome response in the disease.[79] The role of adventitial fibroblasts in atherosclerosis has been documented, where they are described as early activators in the disease[80] and migrate through the vessel wall as differentiated myofibroblasts. Finally, a cross-talk between adventitial cells (including progenitor cells) and SMCs in the propagation of plaques is expected to be the basis of many intriguing studies in coming years.

Concluding Remarks and Personal Perspective

A chronic increase in adventitial activation and Nox-derived ROS has emerged as a catalyst for vascular hypertrophy and hyperplasia in vascular disease. In this regard, our perspective as vascular biologists requires an alternate and complementary view of the typical hematocentric paradigm associated with disease initiation and progression. Instead, a greater focus on the adventitia from a perivascular perspective is required to fully investigate the relative contribution of adventitial fibroblasts, vascular stem cell–like progenitors, and inflammatory cells to disease progression. Inevitably, this vantage point is expected to illuminate our appreciation of the adventitial VV as a prominent gateway responsible for overall vascular inflammation. In closing, surprisingly few studies have emerged during the past 5 years in *Hypertension*, *Circulation*, or *Circulation Research*, which explore the adventitia in disease aside from key discoveries documenting pools of adventitial stem cell progenitors. Thus, further investigation of the role of the adventitia will greatly improve our knowledge of vascular disease progression and, importantly, accelerate basic research to better understand the sequence of events in vascular remodeling. From a preclinical perspective, inhibitory agents targeting specific Nox enzymes are expected to permit dissection of the precise roles of Nox-derived ROS and form the basis for new drug therapies treating vascular inflammation.

Sources of Funding

This work was supported by National Institutes of Health grant, P01HL103455-04, R01HL112914 and by the Institute for Transfusion Medicine and the Hemophilia Center of Western Pennsylvania.

Disclosures

None.

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Hypertension
Journal of the American Heart Association

Nox and Inflammation in the Vascular Adventitia

Daniel N. Meijles and Patrick J. Pagano

Hypertension. 2016;67:14-19; originally published online November 9, 2015;
doi: 10.1161/HYPERTENSIONAHA.115.03622
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563