Published in final edited form as:

Hamostaseologie. 2016 May 10; 36(2): 77–88. doi:10.5482/HAMO-14-11-0076.

Oxidative Stress, NADPH Oxidases, and Arteries

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Summary

Atherosclerosis and its major complications –myocardial infarction and stroke– remain major causes of death and disability in the United States and world-wide. Indeed, with dramatic increases in obesity and diabetes mellitus, the prevalence and public health impact of cardiovascular diseases (CVD) will likely remain high. Major advances have been made in development of new therapies to reduce the incidence of atherosclerosis and CVD, in particular for treatment of hypercholesterolemia and hypertension. Oxidative stress is the common mechanistic link for many CVD risk factors. However, only recently have the tools existed to study the interface between oxidative stress and CVD in animal models. The most important source of reactive oxygen species (and hence oxidative stress) in vascular cells are the multiple forms of enzymes nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase). Recently published and emerging studies now clearly establish that: 1) NADPH oxidases are of critical importance in atherosclerosis and hypertension in animal models; 2) given the tissue-specific expression of key components of NADPH oxidase, it may be possible to target vascular oxidative stress for prevention of CVD.

Keywords

Oxidative stress; atherosclerosis; hypertension; diabetes

Cardiovascular diseases (CVD) caused one death every 40 seconds (784,750 of 2,515,458 deaths) in the United States in 2010 (1). Coronary heart disease (CHD) alone caused 379,559 deaths in 2010, while 40.6% of CVD mortality is attributed to hypertension. Only 54% of hypertensive patients using antihypertensive medications attain target levels of blood pressure. Although multiple risk factors lead to CHD, statins reduce morbidity and mortality risk more than any other preventive approach (2). Statins reduce LDL-cholesterol (3) and exert anti-inflammatory effects (4). However, statins are not well tolerated and uniformly effective in all patients (5,6), necessitating alternative pharmacological approaches for the treatment of CHD and hypertension.

Strong evidence suggests that altered redox signaling caused by increased bioavailability of reactive oxygen species (ROS) is a major contributor to the onset and/or progression of CVD, including atherosclerosis and hypertension (7,8). ROS include free radicals such as superoxide ($O^{2\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), and nonradicals such as hydrogen peroxide

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The authors have no conflicts of interest to disclose.

(H_2O_2). Atherosclerosis, an inflammatory disease, is the common cause of CVD and ROS play a critical role in the processes involved in atherogenesis. An important initial event in atherogenesis is increased endothelial permeability at sites of disturbed flow in the vasculature, which allows transcytosis of LDL into the subendothelial space of the arterial wall (9). The disturbed flow-triggered ROS generation causes endothelial cell activation and increases expression of cell surface adhesion molecules and cytokines, enabling the recruitment, adhesion, and transmigration of leukocytes into the subendothelial space (10). LDL is oxidized by ROS produced by all the activated major cells in the arterial wall, including endothelial cells, smooth muscle cells, and macrophages (11). Activated aortic wall cells also produce proinflammatory secretory phospholipase A_2 which hydrolyzes phospholipids in LDL, increasing its affinity to arterial proteoglycans and causing lipoprotein aggregation and accumulation (12,13). Oxidized LDL is ingested by macrophages forming foam cells which combine with leukocytes, generating the fatty streaks that develop into plaques over time.

In addition, ROS-induced endothelial dysfunction affects CVD by decreasing endothelium-dependent vasodilation. Oxidative stress plays a major role in endothelial dysfunction as superoxide can react with nitric oxide ($^{\bullet}$NO), forming peroxynitrite and reducing the bioavailability of $^{\bullet}$NO which has anti-inflammatory and vasodilatory functions. Peroxynitrite, a potent oxidant itself, can oxidize small-molecule antioxidants such as glutathione and tetrahydrobiopterin (14,15). Decreased bioavailability of tetrahydrobiopterin, an essential cofactor for endothelial nitric oxide synthase, makes the enzyme transfer electrons from NADPH to oxygen instead of its substrate L-arginine, causing eNOS uncoupling and producing superoxide instead of NO. Uncoupling of eNOS is an important contributor to hypertension (16,17). Furthermore, peroxynitrite also oxidizes the enzyme dimethylarginine dimethylaminohydrolase, which metabolizes asymmetric dimethylarginine, an endogenous inhibitor of eNOS, resulting in elevated levels of the inhibitor and decreased $^{\bullet}$NO synthesis (18).

Besides oxidizing LDL and inducing inflammation and endothelial dysfunction, ROS play a major role in vascular remodeling. A major contributor to vascular remodeling under oxidative stress conditions is the phenotypic modulation of vascular smooth muscle cells, which includes loss of contractility, increased proliferation and migration and enhanced production of extracellular components such as collagen and fibronectin (19,20). Extracellular matrix metalloproteinases, enzymes involved extracellular matrix turnover, are activated by ROS-induced oxidation (21). For example, activation of MMP-2 is correlated with elastic fiber fragmentation which contributes to the stiffening of the arterial vasculature (22). Whereas moderate levels of ROS promote VSMC proliferation excess ROS production induces VSMC apoptosis (20). We have shown that increase in collagen I expression, impaired elastic lamellae integrity, and increased medial VSMC apoptosis under chronic oxidative stress conditions cause aortic stiffening, an independent risk factor for CVD (23). Furthermore, oxidative stress-induced MMP activation and increased VSMC apoptosis may render atherosclerotic plaques more susceptible to rupture, increasing the risk of acute coronary syndromes (9,24).

The sources of increased ROS production include damaged or dysfunctional mitochondria and enhanced synthesis or activation of the enzymes nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), xanthine oxidase, lipoxygenases, myeloperoxidase, and cytochrome P450 oxidases and an uncoupled endothelial nitric oxide synthase. The NADPH oxidases generate ROS as the main function of their activity, whereas other enzymes generate ROS as byproducts of their catalytic function.

NADPH Oxidases

NADPH oxidases are a family of transmembrane proteins which are oxygen- and NADPH-dependent oxidoreductases that produce $O_2^{\bullet-}$ and/or H_2O_2 in various cell types and tissues, often in response to hormones, growth factors and immune mediators (25–27). The classical phagocytic enzyme has flavocytochrome b558, a transmembrane catalytic core, composed of Nox2 and p22phox proteins and the cytosolic regulatory proteins p47phox, p40phox, p67phox, and small G-protein Rac1 or Rac2 (Figure 1). In response to microbial exposure or inflammatory mediators, the cytosolic proteins assemble with the catalytic core in the membrane, in part mediated by phosphorylation of p47phox and GTP binding to Rac, activating the enzyme.

The mammalian NADPH oxidase family includes seven isoforms: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2 (26,27). Nox1-4 have similar predicted domain structure with six α-helical transmembrane domains in the N-terminus and a cytoplasmic C-terminus dehydrogenase domain containing conserved binding sites for FAD and NADPH. Nox4 is constitutively active as it does not possess cytosolic regulatory subunits and calcium-binding EF hands (Figure 1). NoxO1 and NoxA1, the homologs of p47phox and p67phox, respectively, and Rac1 are the cytosolic regulatory subunits for Nox1 (Figure 1). However, Nox1 activity involves interaction of p47phox with NoxA1 in mouse vascular smooth muscle cells (VSMC), indicating that NADPH oxidase subunit expression and composition may vary in various vascular beds and species (28,29). Only Nox1, Nox2, Nox3, and Nox4 require association with p22phox for enzyme activity (30). Nox5 is distinct from Nox1-4 by containing a calmodulin-like EF domain in the N-terminus with four Ca^{2+}-binding sites (Figure 1). The Duox (dual oxidase) enzymes are similar to Nox5 in possessing an EF domain, but also contain an additional N-terminus transmembrane α-helix, followed by an extended extracellular domain that shares ~20% identity to myeloperoxidase at the amino acid level (30). Nox5 and the Duox enzymes are constitutively active and do not require cytosolic proteins for activity. Furthermore, they are acutely activated by elevated cellular calcium levels via their EF domain in response to receptor-linked stimuli.

Nox isoform expression varies among different cell types of the systemic and renal vasculature, often with more than one isoform expressed in various cell types (7,27). Nox1 is mainly expressed in VSMCs whereas Nox2 is present in endothelial cells and fibroblasts of the arterial wall. However, expression of Nox1 in endothelial cells and fibroblasts and Nox2 in human resistance arteries was also reported (31). Nox4 is expressed in all the vascular wall cells—VSMCs, endothelial cells, and fibroblasts (26,32). Nox5 is present in human VSMCs and endothelial cells, but is absent in rodents (33). Duox1 expression was observed in the human aortic VSMCs.

In the kidney, Nox1 is expressed in the rat renal cortex (18), and glomerular mesangial cells (35). In addition, p22phox, p67phox, Nox2, and Nox4 protein expression was observed in rat renal cortex. Chabrashvili et al. (36) reported p22phox, Nox2, p67phox, and p47phox expression in afferent arterioles as well as in macula densa by immunochemical staining. While mesangial cells contain Nox4, p22phox, p47phox, and p67phox, podocytes express Nox2, p22phox, p47phox, and p67phox. Nox2, Nox4, p22phox, and p47phox expression was observed in human arcuate and interlobular arteries (37).

Activation of Nox1, Nox2, Nox3, and Nox5 results in increased $O^{2\bullet-}$ generation while Nox4 predominantly produces H_2O_2. NADPH oxidase-derived ROS generation could be extracellular and/or intracellular, depending on the subcellular localization of the Nox isoform (38). Subcellular localization at which Nox isoforms are expressed include plasma membrane, endosome, caveolae, endoplasmic reticulum, mitochondria, and nucleus.

NADPH Oxidase 1

Nox1 NADPH oxidase regulates proliferation and migration of VSMC, processes which potentiate atherogenesis by promoting vascular remodeling (39,40). Increase in Nox1 and p22phox expression was observed early after balloon injury of carotid artery (41), whereas Nox1 deficiency attenuated wire injury-induced neointima formation in femoral artery (39). Sheehan et al. (42) reported that Nox1 activation is an important contributor to experimental atherosclerosis as $ApoE^{-/-}/Nox1^{-/y}$ mice had significantly decreased aortic atherosclerotic lesion area and macrophage content in aortic sinus area compared with $ApoE^{-/-}$, when they were fed a high-fat diet (Figure 1, Table 1).

Our data support the important role of Nox1 NADPH oxidase in atherosclerosis as overexpression of Nox1 activator protein NoxA1 increased neointimal hyperplasia in injured mouse carotid arteries (28). In addition, aortas and atherosclerotic lesions of $ApoE^{-/-}$ mice and human carotid atherosclerotic lesions express increased NoxA1 protein level. Lending further support to the role of Nox1 in atherogenesis, GKT136901, a Nox1 and Nox4 inhibitor, decreased ROS generation and atherosclerosis and attenuated the expression of adhesion protein CD44 and its principal ligand hyaluronan in atherosclerotic lesions (43) (Figure 2).

Nox1 also plays a key role in diabetes-accelerated atherosclerosis. GKT137831, another Nox1/4 inhibitor, prevented diabetes-mediated increase in atherosclerotic lesion area in $ApoE^{-/-}$ mice by attenuating vascular T cell infiltration, ROS levels and markers of inflammation, and necrotic area (44) (Figure 1, Table 1). This effect is mediated through Nox1, but not Nox4, as only deletion of Nox1 decreased atherosclerosis, vascular ROS levels, expression of chemokines, proinflammatory and profibrotic markers, and infiltration of macrophages (45).

Evidence from experimental models of hypertension such as those induced by angiotensin II (Ang II) and deoxycorticosterone acetate (DOCA-salt), renovascular hypertension, and spontaneously hypertensive rats support the role of vascular NADPH oxidases in regulating blood pressure (7,8). Support for the role of Nox1 NADPH oxidase activity in Ang II-

induced hypertension is evident from the use of genetically altered mice. Increase in ROS levels and blood pressure in response to Ang II infusion were significantly blunted in Nox1$^{-/y}$ mice (46,47) (Figure1, Table 1). L-NAME, a nitric oxide synthase inhibitor, abolished the pressor response to Ang II in these mice, suggesting that preservation of the availability of $^{\bullet}$NO because of the depletion of Nox1-derived ROS is the underlying mechanism (47). Complementing the Nox1 deletion studies, Ang II infusion in transgenic mice overexpressing Nox1 in VSMCs increased vascular $O_2^{\bullet-}$ production, decreased $^{\bullet}$NO bioavailability, impaired vasorelaxation, and elevated systolic blood pressure (46,48).

NADPH Oxidase 2

Judkins et al. (49) reported increased Nox2 expression and ROS levels in the aortic endothelium of ApoE$^{-/-}$ mice before the appearance of atherosclerotic lesions. Complimenting this observation, ApoE$^{-/-}$/Nox2$^{-/y}$ on high-fat diet had decreased aortic ROS production with increased $^{\bullet}$NO bioavailability and a 50% reduction in aortic atherosclerotic lesion area compared with the ApoE$^{-/-}$ mice. Supporting the role of Nox2 in atherogenesis, Nox2$^{-/y}$ mice had decreased leukocyte infiltration and reduced neointima formation in response to arterial injury compared with the wild-type (50) (Figure 1). Nox2 NADPH oxidase is involved in endothelial dysfunction and the development of renovascular hypertension (51), and Nox2$^{-/y}$ mice had significantly decreased afferent arteriolar tone and reactivity to Ang II (52). Analogous to this, spontaneously hypertensive rats (SHR) had a 10-fold increase in Nox2 mRNA expression, a 3-fold increase in $O_2^{\bullet-}$ production, and strongly diminished response to acetylcholine (53). Nox2-dependent NADPH oxidase activity is the main source of $O_2^{\bullet-}$ production in human renal proximal resistance arteries which could impact long-term arterial pressure control (37).

Aortic p22phox expression and NADPH oxidase activity were upregulated in rats infused with Ang II (54). Antihypertensive agents losartan and hydralazine inhibited increase in p22phox expression and NADPH oxidase activity, whereas infusion of recombinant heparin-binding superoxide dismutase decreased both blood pressure and p22phox expression, suggesting that activation of NADPH oxidase system plays a key role in hypertension (Table 1). Congruent with this, Chabrashvilli et al. (55) reported that Ang II infusion increases oxidative stress via Ang II type 1 receptor by upregulating the expression of p22phox and Nox1 in the renal cortex.

We showed decreased $O_2^{\bullet-}$ production and proliferative response to growth factors in p47phox$^{-/-}$ VSMC compared with the wild-type cells (56). Furthermore, ApoE$^{-/-}$/p47phox$^{-/-}$ had significantly less atherosclerosis than ApoE$^{-/-}$ mice, both on standard chow and high-fat diet (56,57) (Figure 3). The decrease in aortic atherosclerotic burden and diminished neointimal hyperplasia in response to arterial injury in the ApoE$^{-/-}$/p47phox$^{-/-}$ mice is associated with reduced CD44 adhesion molecule expression (57). Using allogenic, sex-mismatched bone marrow transplantation, we also showed that the atheroprotective effect of p47phox deletion in ApoE$^{-/-}$ mice is caused by the inhibition of NADPH oxidase activity in monocytes/macrophages as well as vascular wall cells (58).

Experimental models also support the role of p47phox-dependent NADPH oxidase in hypertension. In the kidney of SHR rat, p47phox mRNA and protein expression were significantly increased in the vasculature, macula densa, and distal nephron, preceding hypertension (36). Hypertensive response to Ang II infusion and vascular $O_2^{\bullet-}$ production were markedly blunted in p47phox$^{-/-}$ compared with the wild-type mice (59). Consistent with the decrease in Ang II-induced mean arterial pressure, afferent arterioles in p47phox$^{-/-}$ mice had significantly decreased ROS levels and myogenic contraction response to Ang II (60) (Figure 4).

Underscoring the clinical relevance of Nox2 NADPH oxidase in human atherosclerosis, Sorescu et al. (61) reported increased $O_2^{\bullet-}$ generation and Nox2 and p22phox expression in the shoulder region of atherosclerotic plaque, which were associated with the severity of atherosclerosis. Interestingly, Nox4 expression is upregulated in atheromas containing an abundance of VSMC, whereas it is downregulated in more advanced plaques characterized by fibrosis and reduction in intimal SMC. Furthermore, p22phox expression and ROS generation were increased with atherosclerosis progression in coronary arteries and were significantly higher in unstable angina pectoris compared with stable angina pectoris (62,63). Simultaneous intravascular ultrasound and immunohistochemistry analyses indicate that p22phox-dependent NADPH oxidase-derived ROS significantly contribute to coronary atherogenesis and arterial remodeling associated with plaque vulnerability (64).

NADPH Oxidase 4

Using ApoE$^{-/-}$/LDLR$^{-/-}$ mice, Xu et al. (65) showed that Nox4 expression was increased in advanced aortic atherosclerosis lesions, which is associated with increased ROS generation, cell cycle arrest, senescence, and increased susceptibility to apoptosis in SMC. Furthermore, Nox4 overexpression in aortic SMC recapitulated SMC phenotype seen in advanced atherosclerotic lesions, suggesting that increased Nox4 expression in advanced lesions may cause plaque instability. Strong experimental evidence is lacking for a role of Nox4 NADPH oxidase in hypertension. However, Shah and colleagues reported that transgenic mice with endothelial-specific Nox4 overexpression have greater acetylcholine-induced vasodilation and significantly lower basal systemic blood pressure than the wild-type littermates (66) (Table 1). The increased vasodilatory response was attributed to increased H_2O_2 production and H_2O_2-induced hyperpolarization.

NADPH Oxidase 5

Growth factor and hormone induced Nox5 activation increases human endothelial cell and aortic SMC proliferation by modulating redox-sensitive mitogenic signaling pathways (67–69). Supporting this data, Guzik et al. (70) reported significantly increased Nox5 mRNA and protein expression and increased ROS generation in the coronary arteries of CAD patients. Nox5 expression was increased in the endothelium in the early lesions and in VSMC in the advanced lesions (Figure 1, Table 1). The beneficial effect of calcium channel antagonists in the treatment of angina and CAD was attributed to diminished Nox5 activation in cells harboring L-type calcium channels, including VSMC in lesions.

Conclusions

In conclusion, accumulating data from experimental and NADPH oxidase deficiency animal models and human studies strongly support a role for NADPH oxidases in vascular homeostasis and disease. Evolving consensus suggests that decreasing oxidative stress by targeting specific sources of ROS such as NADPH oxidases might yield new therapies for the treatment of atherosclerosis and hypertension. The tissue-specific variations in the composition of various NADPH oxidases could provide an opportunity to develop specific small molecule inhibitors of these enzymes to treat CVD, with fewer off-target effects. Advances in drug delivery vehicles and vascular imaging techniques along with the availability of specific small molecule inhibitors of NADPH oxidase isoforms may transform the treatment of atherosclerosis and hypertension.

Acknowledgments

This work was supported in part by NIH grants AG024282, HL111664, and TR000083.

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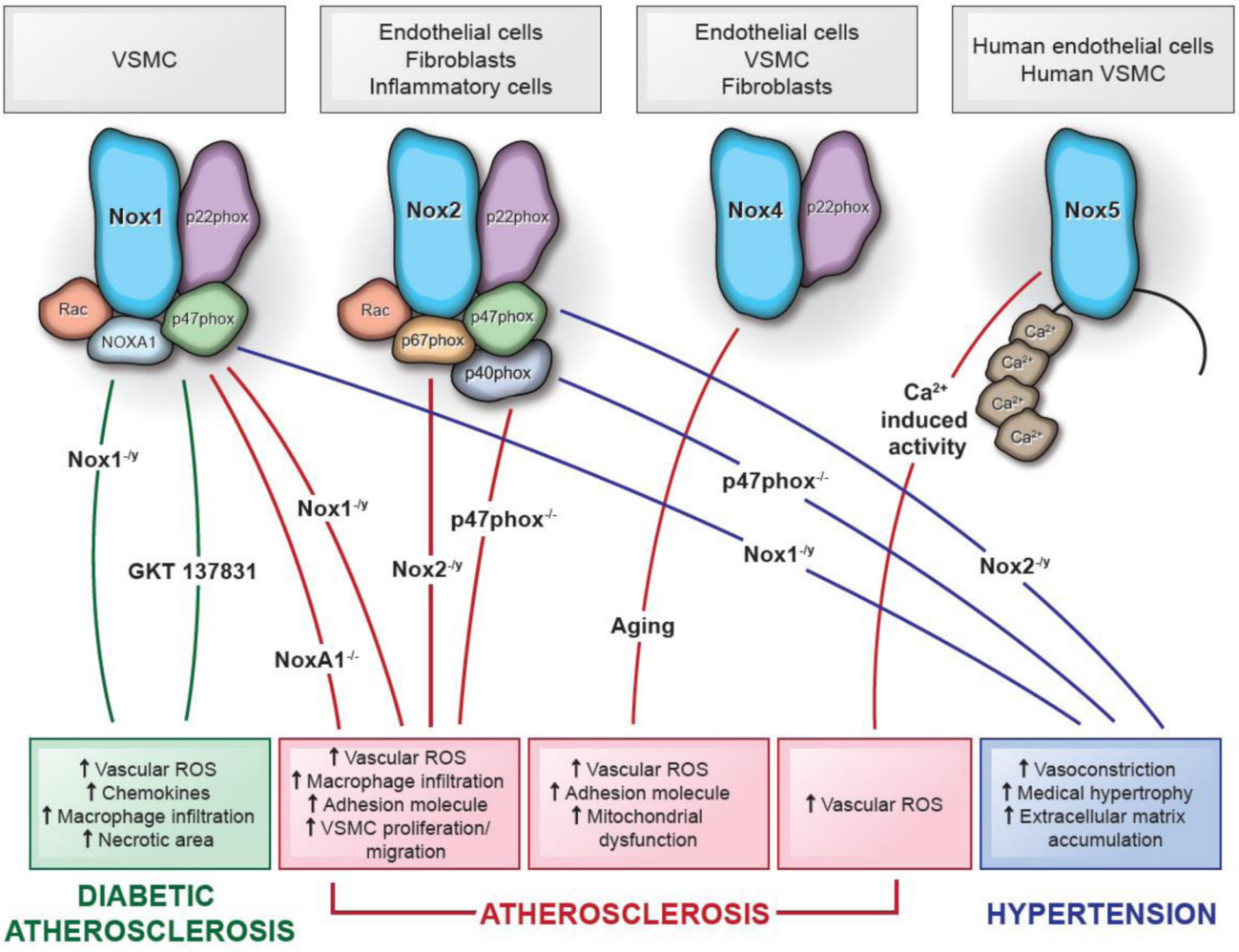

Figure 1.

The critical role of NADPH oxidases in atherosclerosis, diabetic atherosclerosis, and hypertension as evident from mouse models, NADPH oxidase inhibitors and human data.

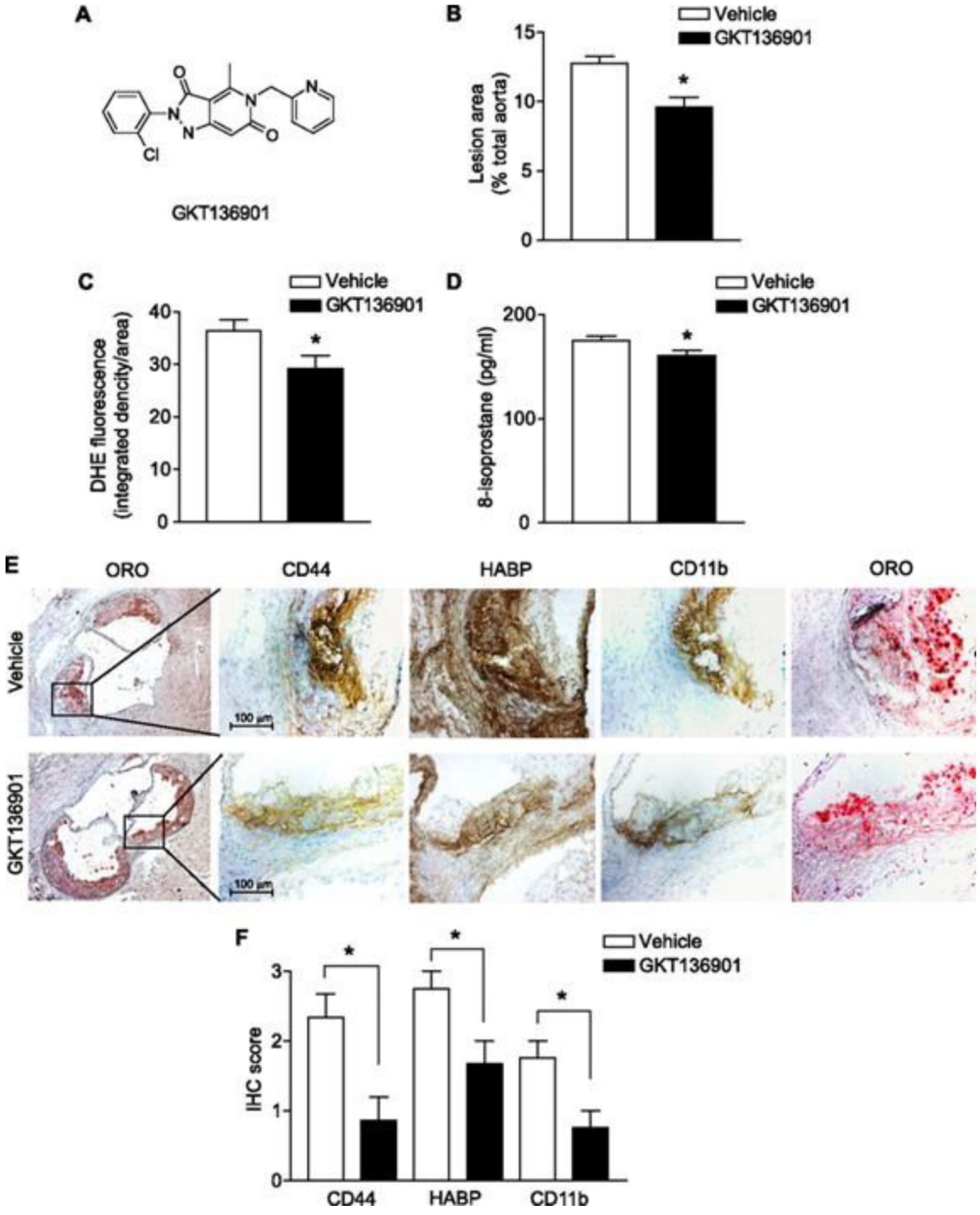

Figure 2.

GKT136901, a Nox1/Nox4 oxidase inhibitor, decreased atherosclerosis and attenuated ROS generation, plasma 8-isoprostane levels, and CD44 and hyaluronan expression in atherosclerotic lesions. Reprinted from reference 27, with permission from Journal of Biological Chemistry.

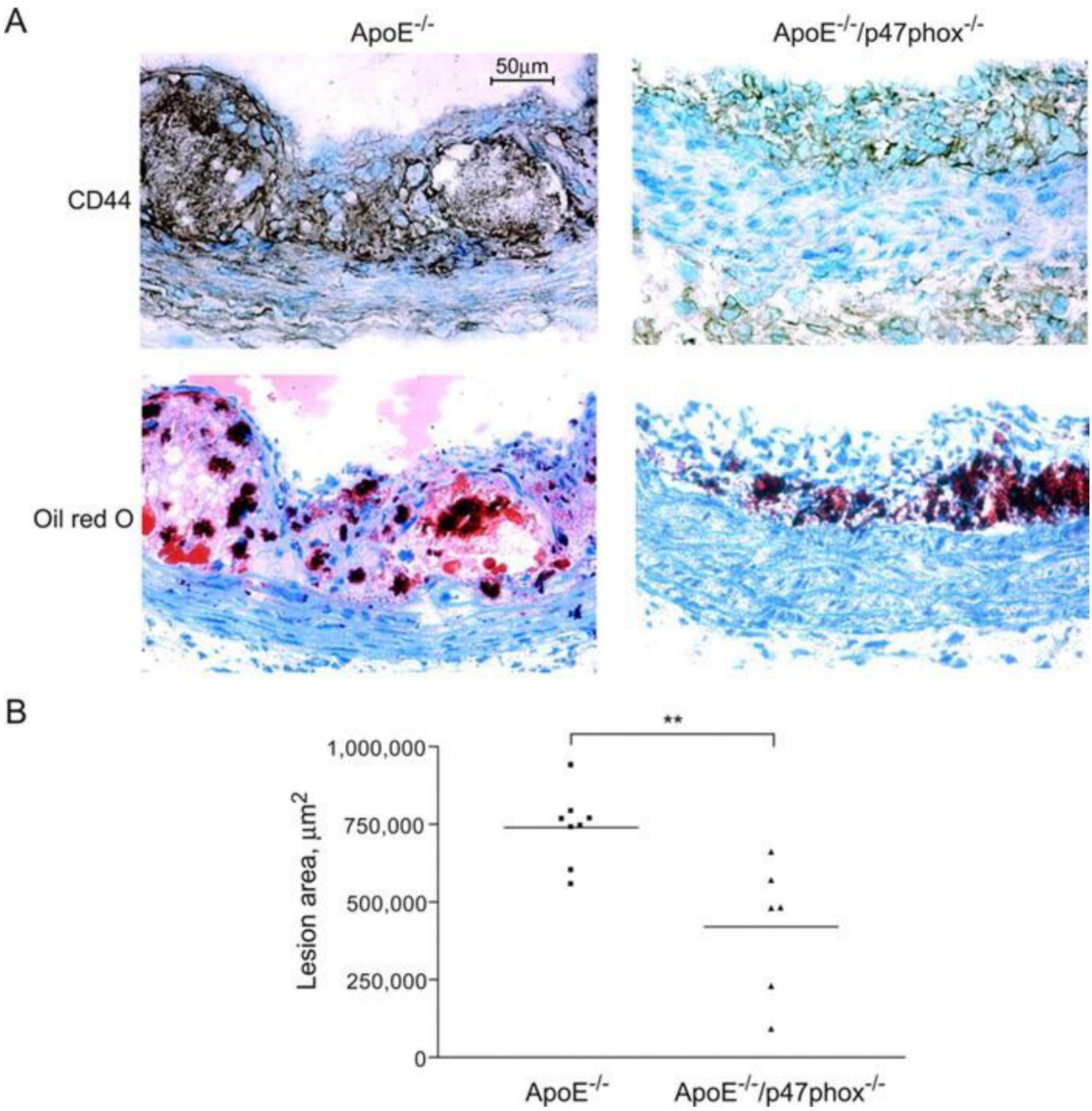

Figure 3.

$ApoE^{-/-}/p47phox^{-/-}$ express less CD44 in atherosclerotic lesions (A) and have decreased aortic root lesion area compared with $ApoE^{-/-}$ mice (B). Reprinted from reference 41, with permission from Circulation Research.

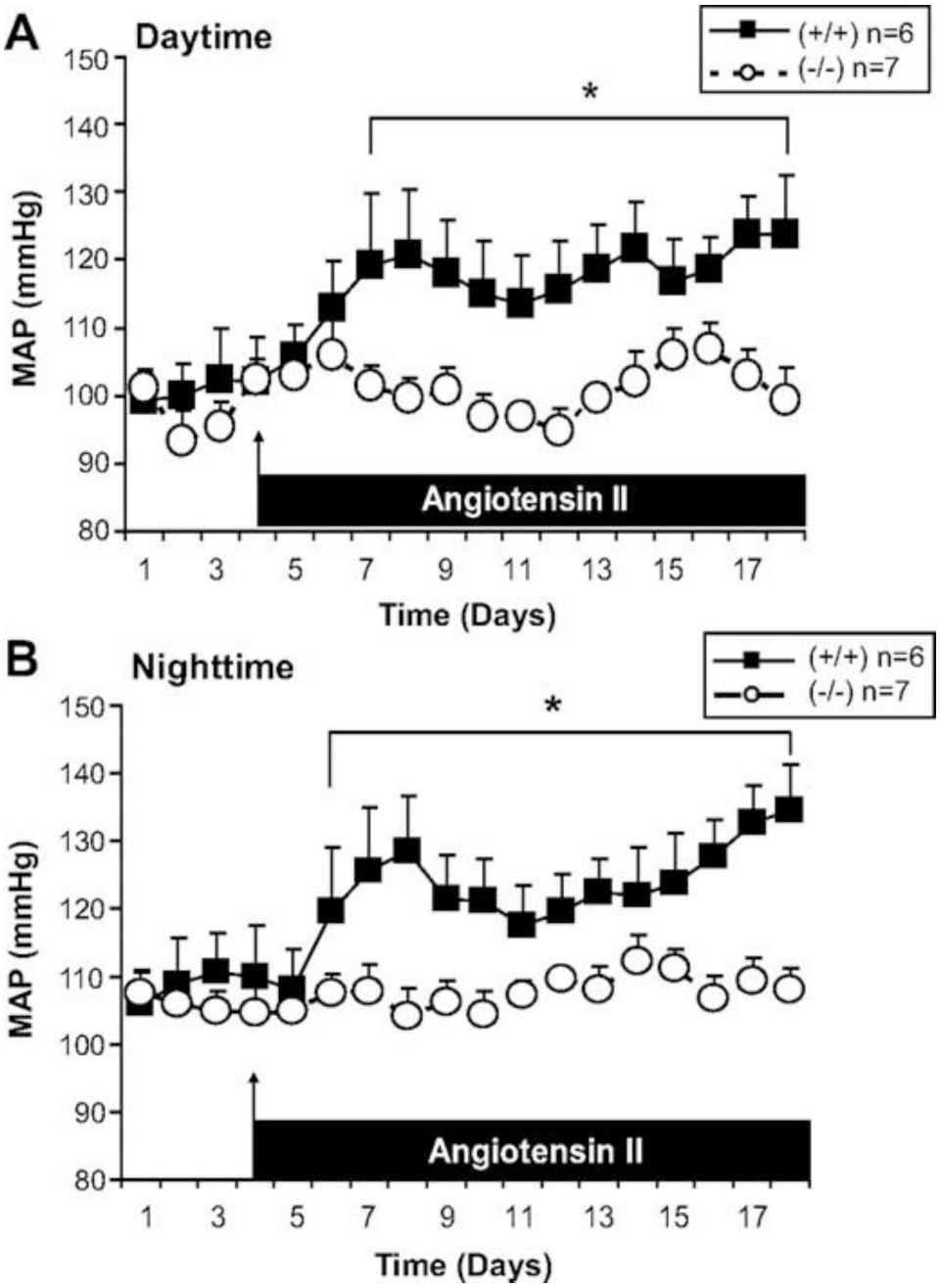

Figure 4.

Mean arterial pressure is decreased in p47phox$^{-/-}$ compared with the wild-type mice in response to Ang II infusion: daytime (asleep) A; nighttime (awake) B. Reprinted from reference 44, with permission from Hypertension.

Table 1

The role of NADPH oxidases in atherosclerosis, hypertension, and diabetes-accelerated atherosclerosis

Effector	Genetic model / pharmacologic agent	Phenotype	Ref.
Mouse/Rat model			
Nox1	$Nox1^{-/y}/ApoE^{-/-}$	Decreased atherosclerosis	42
	$Nox1^{-/y}$	Blunted BP elevation induced by Ang II	46,47
	$ApoE^{-/-}$, diabetes, GKT137831	Decreased diabetes-accelerated atherosclerosis	44,45
Nox1/Nox4	GKT136901	Decreased atherosclerosis and decreased CD44 expression and hyaluronan levels in atherosclerotic lesions	43
NoxA1	$ApoE^{-/-}$, NoxA1 overexpression	Increased atherosclerosis in $ApoE^{-/-}$ mice	28
Nox2	$Nox2^{-/y}/ApoE^{-/-}$	Decreased atherosclerosis	49
	$Nox2^{-/y}$	Decreased chronic Ang II infusion elevated blood pressure	52
	SHR rat	Increased Nox2 mRNA	53
p22phox	Rat	Ang II induced p22phox and Nox1 mRNA expression	55
	Rat	Ang II-induced hypertension	54
p47phox	$p47phox^{-/-}/ApoE^{-/-}$	Decreased CD44 and atherosclerosis	56,57
	SHR rat	Increased p47phox mRNA	36
	$p47phox^{-/-}$	Blocked Ang II-induced ROS and hypertension	59
	$p47phox^{-/-}$	Decreased the sensitivity of Ang II to induce BP response	60
Nox4	$ApoE^{-/-}/LDLR^{-/-}$, aged mice	Atherosclerotic plaque instability	65
	Endothelium-targeted Nox4 TG	Decreased systemic blood pressure	66
Clinical data			
Nox2	Human renal proximal resistance arteries	Decreased endothelium-dependent vasodilation	37
Nox2/p22phox	Human coronary artery	Severity of atherosclerosis	61
p22phox	Human coronary artery	Increased expression is correlated with atherosclerosis	62,64
Nox5	Human endothelial cells and VSMC	Elevated in endothelium of atherosclerotic aortas	69