Review Article

Atherosclerosis and NADPH Oxidase

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Abstract

Vascular diseases including coronary artery disease, cerebrovascular and peripheral vascular diseases are the largest cause of mortality and morbidity. Reactive oxygen species (ROS), especially superoxide ($O_2^{\bullet-}$) have been implicated in the pathogenesis of virtually every stage of vascular lesion formation in atherosclerosis. Atherosclerosis is a disease affecting arterial blood vessels. It is a chronic inflammatory response in the walls of arteries, in large part to the deposition of lipoproteins (plasma proteins that carry cholesterol and triglycerides). Oxidation of low density lipoprotein (LDL) was injurious to artery wall cells and suggested that LDL oxidation might be important in atherogenesis. Among the potential sources of ROS, the NADPH oxidases appear to be especially important for redox signalling and indeed possess several biochemical properties that make them well suited for involvement in signal transduction. The NADPH oxidase system plays a key role in generating ROS, including $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2) in phagocytic cells, fibroblasts, vascular smooth muscle cells, and endothelial cells. The NADPH oxidase system is regulated systemically in veins and arteries, which strengthens the importance of the molecular regulation of the enzyme in cardiovascular disease (CVD), especially atherosclerosis.

***Key Words*:** Atherosclerosis; NADPH oxidase; ROS

Vascular diseases including coronary artery disease, cerebrovascular and peripheral vascular diseases are the largest cause of mortality and morbidity in industrialised countries. Many common risk factors for vascular disease, such as hypertension and diabetes, remain prevalent in Western and other populations, suggesting that vascular disease will continue to impose a substantial burden on health care resources throughout the next generation. The earliest detectable changes in vascular disease states are abnormalities of the endothelium, resulting in loss of the endothelium's normal homeostatic functions that normally act to inhibit disease-related processes such as inflammation and thrombosis (Channon and Guzik, 2002).

Not long ago, superoxide ($O_2^{\bullet-}$) was considered simply a destructive by-product of cellular reactions. However, it is becoming clear that $O_2^{\bullet-}$ and other reactive oxygen species (ROS) play a role in maintaining cellular homeostasis and cell signaling. $O_2^{\bullet-}$ is generated by virtually every cell type within the vascular wall. Furthermore, experimental animal models and clinical studies of atherosclerosis,

hypertension, and diabetes have demonstrated increased vascular $O_2^{\bullet-}$ and/or ROS production (Loomis et al., 2005).

Oxidative stress is defined as the imbalanced redox state in which pro-oxidants overwhelm antioxidant capacity, resulting in increased production of ROS. ROS have been implicated in the pathogenesis of virtually every stage of vascular lesion formation in atherosclerosis (Fortuño et al., 2005). Oxidative stress plays an important role in the pathogenesis of atherosclerosis. Recently, two large trials in humans showed that systemic oxidative stress level is correlated with cardiovascular disease (CVD) and its various risk factors (Park et al., 2005).

Atherosclerosis

Atherosclerosis is a disease affecting arterial blood vessels. It is a chronic inflammatory response in the walls of arteries, in large part to the deposition of lipoproteins (plasma proteins that carry cholesterol and triglycerides). It is commonly referred to as a "hardening" or "furring" of the arteries. It is caused by the formation of multiple plaques within the arteries. Two decade ago, atherosclerosis was considered to be a degenerative process because of the accumulation of lipid and necrotic debris in the advanced lesions. Now it is recognized that it is a multifactorial process which, if it leads to clinical sequelae, requires extensive proliferation of smooth muscle cells within the intima of the affected artery. The form and content of the advanced lesions of atherosclerosis demonstrate the results of three fundamental biological process. These are: proliferation of intimal smooth muscle cells together with variable numbers of accumulated macrophages; formation by the proliferated smooth muscle cells of large amounts of connective tissue matrix, including collagen, elastic fibers and proteoglycan; and accumulation of lipid, principally in the form of cholesteryl ester and free cholesterol within the cells as well as in the surrounding connective tissues (Russell, 1988).

Various hypotheses have been postulated in an attempt to explain the development of the process of atherosclerosis. There are three major hypotheses of atherosclerosis and they include the "response to injury" hypothesis, "response to retention" hypothesis and the "oxidation hypothesis". According to the "response to injury" hypothesis, endothelial injury is a key event which initiates the inflammatory mechanisms associated with atherosclerosis, starting from the endothelial damage, smooth muscle cell migration and proliferation, deposition of the intracellular and extracellular lipid, and accumulation of extracellular matrix. According to the "response to retention" hypothesis, the central atherogenic process is the sub-endothelial retention and accumulation of lipoproteins by extracellular matrix molecules, such as proteoglycans. According to the "oxidation hypothesis", the central component of the atherogenic process is the oxidative modification of LDL, which acts as an immunogenic stimulus for monocyte recruitment to the vessel wall and phagocytic uptake of oxidized LDL by macrophages (Figure 1) (Micić, 2006).

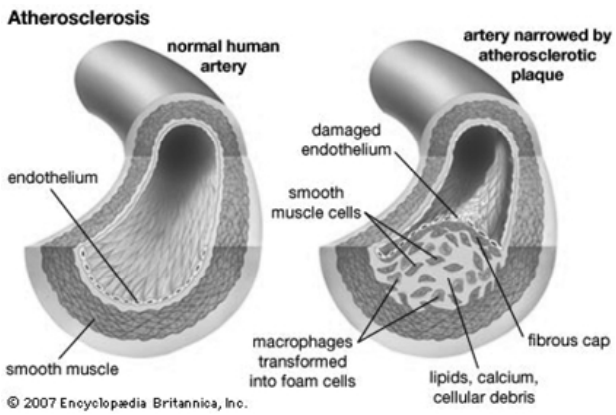

Figure 1 Atherosclerosis: comparison of arteries (http://www.britannica.com/eb/article-9010075/atherosclerosis)

The fatty streak, the first visible lesion of atherosclerosis, is the "foam cell". This cell, loaded with droplets rich in cholesteryl esters, is derived mainly from arterial wall macrophages, which originate from circulating monocytes that have penetrated into the subendothelial space. Smooth muscle cells and endothelial cells in lesions also can and do accumulate lipid droplets but monocyte-derived macrophage foam cells predominate. This being the case, an understanding of just how arterial macrophages take up and store their load of cholesterol should shed light on the mechanisms that initiate atherogenes (Figure 2) (Steinberg, 2009).

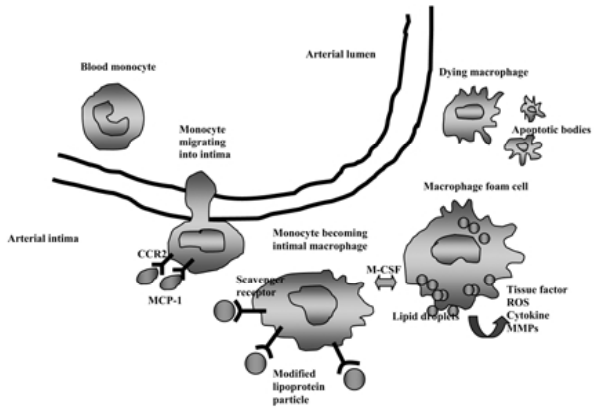

Figure 2 Mononuclear phagocytes in atherosclerosis (Adapted from Libby, 2002).

LDL oxidation and artery wall cell cytotoxicity

In 1979, Chisolm and colleagues reported that oxidation of LDL was injurious to artery wall cells and suggested that low density lipoprotein (LDL) oxidation may be important in atherogenesis. Chisolm and colleagues also demonstrated that HDL inhibited the LDL-induced cytotoxicity. Over the ensuing two decades this group elucidated the basis for these observations and established the important role of oxidized cholesterol products especially cholesterol hydroperoxides (Navab et al., 2004).

Atherosclerosis and its complications lead to half of all adult deaths in the United States. The lesion, or atheroma, is an inflammatory site where a variety of cells, cell products, and lipoproteins interact to promote injury and disease. An important consequence of these interactions is the cellular oxidation of LDL, which alters the lipoprotein to a highly atherogenic form. A variety of cell types present in atherosclerotic lesions, including monocytes/ macrophages, smooth muscle cells, and endothelial cells, can oxidize LDL. In turn, oxidized LDL promotes cell injury, smooth muscle cell proliferation, foam cell formation, chemotaxis of leukocytes, cellular secretion of inflammatory mediators, and other events that modulate atheroma biology. Oxidized LDL has been detected in atheromas of rabbits and humans, and antioxidant therapy may decrease cardiovascular events and mortality, perhaps by inhibiting the oxidation of lipoproteins. Therefore, oxidation of LDL, a biologically plausible mechanism of LDL modification, may explain why high plasma levels of native LDL are a major risk factor for coronary artery disease (Kalayoglu et al., 1999).

NADPH oxidase

ROS are characterized by high chemical reactivity and have emerged to be an important factor in many pathophysiological processes. ROS include free radicals, such as $O_2^{\bullet-}$ and hydroxyl ($OH^{\bullet}$), and non-radical species such as hydrogen peroxide (H_2O_2). Their biological effects depend upon the specific moiety generated, its localization and the relative balance between levels generated and the activity of antioxidant systems that reduce ROS levels. Three general types of ROS-dependent biological action may be considered. Firstly, in settings of redox imbalance (oxidative stress) where large amounts of radicals are generated, these may induce oxidation and damage of macromolecules, membranes and DNA and thus be detrimental for cellular function and viability. Secondly, the ROS superoxide interacts with the signalling molecule NO (nitric oxide), resulting in a reduction in NO bioavailability and the generation of another reactive species, peroxynitrite, which itself has biological activity. ROS-mediated reduction in

NO bioavailability is a key mechanism that contributes to the development of endothelial dysfunction. Thirdly, it is recognized that tightly regulated ROS production modulates the activity of diverse intracellular molecules and signalling pathways, thereby inducing highly specific acute and chronic changes in cell phenotype (commonly termed 'redox signalling'). In most cases, the main ROS involved in redox signalling is probably H_2O_2, which is generated upon dismutation of superoxide (a process catalysed by superoxide dismutases). There are several potential sources of ROS in most cells, including the mitochondria, NADPH oxidases (NOX), cytochrome P450-based enzymes, xanthine oxidase and uncoupled NO synthases. Among these, the NADPH oxidases appear to be especially important for redox signalling and indeed possess several biochemical properties that make them well suited for involvement in signal transduction (Dworakowski et al, 2006).

NADPH oxidase diversity, structure and isoforms

The NADPH oxidase enzyme complex was originally discovered in neutrophils, where it is a potent source of millimolar quantities of superoxide ($O_2^{\bullet-}$) during phagocytosis and plays a vital role in non-specific host defense against ingested pathogen (Dworakowski et al., 2006; Li and Shah M., 2003). The NADPH oxidase system plays a key role in generating ROS, including $O_2^{\bullet-}$ and H_2O_2 in phagocytic cells, fibroblasts, vascular smooth muscle cells, and endothelial cells. The NADPH oxidase system is regulated systemically in veins and arteries, which strengthens the importance of the molecular regulation of the enzyme in CVD (Park et al., 2005).

NADPH oxidase is a multiple-subunit electron transport system. The best-characterized NADPH oxidase is that found in phagocytes, where this enzyme is also the major inducible source of $O_2^{\bullet-}$ and where it plays a role in immune protection owing to its bactericidal activity. The phagocytic oxidase consists of a membrane-associated cytochrome that comprises a large subunit, $gp91^{phox}$ ('phox' being derived from phagocytic oxidase), and a small one, $p22^{phox}$. Besides these, there are at least three cytosolic subunits ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) and a low-molecular-weight G protein (Rac1 or Rac2). $p47^{phox}$, $p67^{phox}$ and $gp91^{phox}$ (NOX2) present on phagocytic NADPH oxidase have been identified in vascular cells. However, several studies have confirmed that $p22^{phox}$ is present in all NADPH oxidase systems and that this subunit is essential for the functionality of the enzyme. Upon cell stimulation, $p47^{phox}$ becomes phosphorylated, and the cytosolic subunits form a complex which migrates to the membrane where it binds to the cytochrome. Then electrons are transferred from the substrate, NADPH, to $O_2^{\bullet-}$, leading to $O_2^{\bullet-}$ generation (Fortuño et al., 2005; Xia et al., 2006).

Broadly comparable enzymes have been reported to exist in numerous non-phagocytic cell types, including endothelial cells, vascular smooth muscle (VSM), cardiomyocytes and fibroblasts, where they appear to subserve other functions. The molecular composition of these non-phagocytic enzymes has begun to be clarified in the last 5-6 years. Initially, several homologues of the $gp91^{phox}$ catalytic subunit were identified, each encoded for by separate genes. These homologues are now designated NOXs (NADPH oxidases), with $gp91^{phox}$ also called NOX2. NOX2 is the β-subunit of cytochrome b_{558} and is the key catalytic subunit of the NADPH oxidase. Several homologues of $gp91^{phox}$, have recently been reported to be expressed in nonphagocytic cells. Other members of the NOX family comprise of NOX1, NOX3, NOX4 and NOX5, as well as larger and more complex homologues termed DUOX1 and DUOX2 (Li and Shah, 2003). NOX 1 to 5 are 65-kDa core proteins, whereas DUOX 1 and 2 are 175- to 180-kDa proteins that have a domain homologous to $gp91^{phox}$ as well as an additional peroxidase domain. Using this new terminology, NOX2 represents the neutrophil $gp91^{phox}$. The first homologue of $gp91^{phox}$, namely NOX1, was found to have significant

pro-proliferative activity and was also therefore known by the alternative term mitogenic oxidase or MOX-1. NOX1 shares 56% sequence homology with neutrophil gp91^{phox}. NOX3 is known only from its genomic sequence. A homologue of gp91^{phox} termed NOX4 (or Renox) has been cloned in the kidney. The predicted NOX4 protein consists of 578 amino acids with 39% homology to neutrophil gp91^{phox} and has been found to be a renal source of ROS production. NOX4 expression was found to be abundant in human distal tubular cells and suggested to function as an oxygen sensor for erythropoietin synthesis. NOX4 was also found abundantly expressed in fetal kidney, an organ that is not considered to produce high amount of erythropoietin; it might be involved in the regulation of renal cell growth and death (Ray and Shah, 2005).

In the last 18 years, many non-phagocytic cells have been found to contain NADPH oxidase type ROS-generating activity. In the cardiovascular system, these include VSMCs (vascular smooth-muscle cells), endothelial cells, adventitial and cardiac fibroblasts and cardiomyocytes. These cells usually constitutively generate low-level NADPH-dependent ROS but production is significantly augmented by various specific stimuli. Recently, it has been shown that there is in fact a family of non-phagocytic NADPH oxidases based on seven different isoforms of gp91^{phox} or NOX2, each encoded by different genes. The NOX family may be classified into three groups based on predicted domain structures: (i) NOX1– NOX4 have up to 60% homology and are predicted to contain six transmembrane α-helices and an NADPH-binding domain towards the C-terminus; (ii) NOX5 has the same basic structure as NOX1– NOX4 but with an additional N-terminal calmodulin-like Ca^{2+}-binding domain; (iii) DUOX1 and DUOX2 are similar to NOX5 but include an additional N-terminal peroxidase homology domain. NOX isoform expression varies in a cell-specific manner. NOX1 is expressed in many epithelia (e.g. colon) as well as in VSMCs. NOX2 is expressed in endothelial cells, cardiomyocytes and fibroblasts in addition to its classical expression in phagocytes. NOX3 is primarily expressed in foetal tissues and adult inner ear. NOX4 was first identified in kidney but is in fact widely expressed in many tissues including placenta, endothelial cells, VSMCs, cardiomyocytes, fibroblasts, ovary, testis and skeletal muscle. NOX5 is expressed in foetal tissues and adult testis, spleen, ovary, placenta and pancreas. It is of interest that several cell types can co-express more than one NOX subunit. For example, cultured VSMCs express both NOX1 and NOX4, while endothelial cells and cardiomyocytes co-express NOX2 and NOX4 (Dworakowski et al., 2006).

Despite a relatively high degree of conservation in the overall topology of the NOXs, studies to date indicate that they differ greatly in their tissue distribution and are also likely to be differentially activated and regulated. It is thought that all the NOX homologues may bind to p22^{phox} in a similar manner to the gp91^{phox}/p22^{phox} complex, although this needs to be definitively demonstrated. However, the requirement for other cytosolic subunits for an active oxidase complex may vary among the NOXs. In the case of NOX1, homologues of p47^{phox} and p67^{phox} (termed NOXO1 and NOXA1 respectively) have been found to be important for its activation. Interestingly, NOXO1 may exhibit significant differences in function compared with p47^{phox}. On the other hand, it is reported that NOX4 activation does not require either p47^{phox} and p67^{phox} or NOXO1 and NOXA1. The detailed mechanisms responsible for the activation and regulation of these homologues remain to be established (Ray and Shah, 2005).

NADPH oxidase protein subunits are present in human blood vessels including atherosclerotic coronary arteries, in saphenous veins and mammary arteries from patients with coronary artery disease, and in human vascular smooth muscle cells and endothelial cells in culture. Increased levels of the p22^{phox}, p47^{phox}, p67^{phox} and NOX subunits are present in human atherosclerotic coronary arteries, and in diabetic vessels, in association with increased

superoxide production. This suggests that upregulated gene expression and/or post-transcriptional increases in protein levels are important in mediating increased NADPH oxidase activity in human vascular disease. For example, Angiotensin II increases NADPH oxidase activity by transcriptional upregulation of subunit expression. However, it is clear that the cytosolic regulatory proteins $p47^{phox}$, $p67^{phox}$ and Rac also play an important part in regulating NADPH oxidase activity in cardiovascular disease states by acute activation of the enzyme complex, for example by phosphorylation and translocation of $p47^{phox}$ (Channon and Guzik, 2002).

Components and NADPH oxidase activation

The NADPH oxidase system of neutrophils and other phagocytic leukocytes is composed of multiple membrane-associated (cytochrome b_{558}) and cytosolic components (Rac, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ (Figure 3)). The active, fully assembled oxidase catalyzes the one electron reduction of oxygen to produce superoxide anion using NADPH as substrate. When the leukocyte is activated through the action of inflammatory mediators (soluble chemoattractants, chemokines, or phagocytic particles), the cytosolic oxidase component $p47^{phox}$ becomes phosphorylated on multiple sites through the action of several kinases. The phosphorylation of $p47^{phox}$ is thought to lead to the disruption of an inhibitory intramolecular interaction that exposes SH3 domains in $p47^{phox}$ for binding to proline-rich regions of other NADPH oxidase components. Cytosolic $p47^{phox}$ exists both in a free form and in complexes with the cytosolic oxidase components, $p67^{phox}$ and $p40^{phox}$. The phosphorylation-induced conformational change(s) in $p47^{phox}$ results in translocation of a $p47^{phox}$/p67*phox* complex to the membrane, where it interacts via multiple binding sites with the integral membrane protein, flavocytochrome b_{558} (cytochrome b [cyt b]) to form an active enzyme complex. The active oxidase also requires the translocation of Rac GTPase, which occurs simultaneously, but dissociably, from the translocation of the $p47^{phox}$/$p67^{phox}$ complex. Cyt b possesses 2 subunits, $gp91^{phox}$ and $p22^{phox}$, with the larger subunit containing an NADPH binding site, 2 heme groups, and bound flavin adenine dinucleotide (FAD). The formation of this minimal protein complex allows electrons to flow via a 2-step mechanism from NADPH to FAD (step 1) and then from FAD to the heme of cyt b and finally to molecular oxygen (step 2), whose reduction leads to the formation of superoxide anion. The cytosolic component $p40^{phox}$ may play a role in regulating the response of the system to phosphatidylinositol-3-phosphate *in vivo* but is not required for NADPH oxidase activity in the cell-free system (Bokoch and Dieboid, 2002). NADPH oxidase activity can also be regulated at the gene level of oxidase subunits. It has been shown that NADPH oxidase activity is significantly elevated in atherosclerotic lesion, leading to increased superoxide anion production. The activation of PKC (protein kinase C) is associated with increased production of superoxide anions in phagocytes as well as in other vascular cells (Siow et al., 2006)

Endothelial sources of $O_2^{\cdot-}$ and H_2O_2

Oxygen radicals are produced endogenously under normal conditions, and the levels are increased under conditions of oxidative stress. The most common oxygen radicals are superoxide, hydrogen peroxide, and hydroxyl radical. Whereas the anions superoxide and hydroxyl radical are more reactive, H_2O_2, which does not possess the chemical structure of a radical, is more membrane permeable. Several enzymes located throughout the cell, including in the plasma membrane, cytosol, mitochrondria, and peroxisomes, generate oxygen radicals. Superoxide is produced during normal mitochondrial respiration and by NADH oxidase, NADPH oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, and cytochrome P-450. Under conditions where tetrahydrobiopterin is limited, superoxide can also be produced from nitric oxide synthase (NOS)

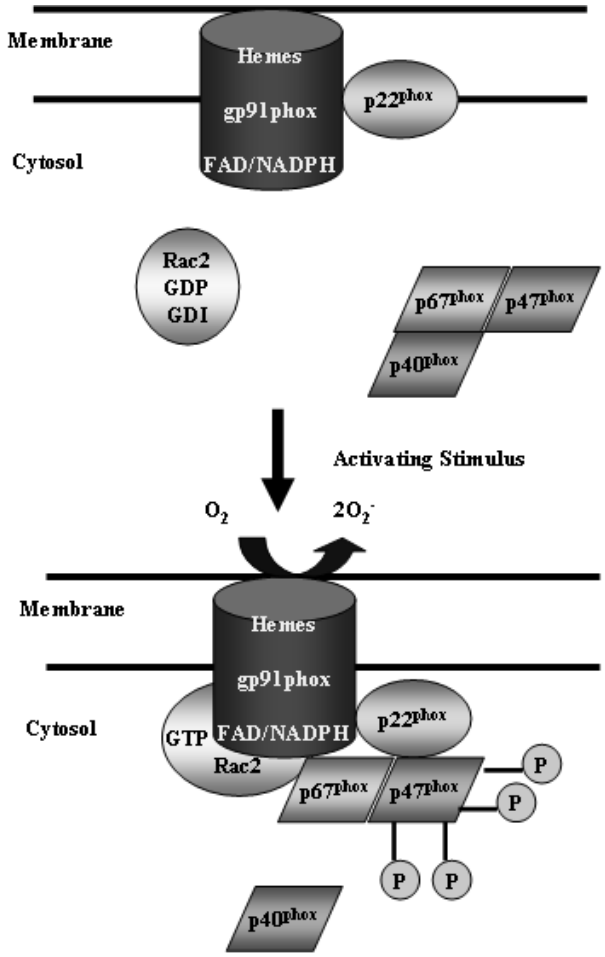

Figure 3 Activation and assembly of the phagocyte NADPH oxidase (Adapted from Bokoch and Dieboid, 2002).

(Schnackenberg, 2002). Several potential sources of $O_2^{\bullet-}$ are implicated in endothelial physiology and pathophysiology, including the mitochondrial electron transport chain, xanthine oxidase, cytochrome P-450 enzymes, uncoupled NOSs, the phagocytic myeloperoxidase system and NADPH oxidases (Ray and Shah, 2005).

The mitochondrial electron transport chain can be a significant ROS source and increased mitochondrial ROS generation is implicated in diabetic vasculopathy and in ischaemia/reperfusion. Interestingly, mitochondria are quite susceptible to oxidative damage which can result in enhanced mitochondrial ROS production. Xanthine oxidase is expressed on the luminal surface of the endothelium in many organs and catalyses the conversion of hypoxanthine into urate in a process that generates $O_2^{\bullet-}$. The enzyme is normally present as xanthine dehydrogenase, which does not generate $O_2^{\bullet-}$, but is converted into xanthine oxidase either through oxidation or by proteolytic cleavage of a segment of xanthine dehydrogenase. The former mode of activation is notable in that it provides a mechanism for an increase in xanthine oxidase activity in response to oxidative stress, i.e. a mechanism that may potentially amplify oxidative stress originally arising from other sources. Increased xanthine-oxidase-derived $O_2^{\bullet-}$ production may be involved in ischaemia/reperfusion and in endothelial dysfunction in several diseases (Ray and Shah, 2005).

NADPH oxidase as a source of oxidative stress in the vessel wall

The vascular NADPH oxidase in endothelial and smooth muscle cells differs in several respects, although the details of precisely how it is activated are still emerging. Notwithstanding, several distinguishing characteristics of the vascular NADPH oxidase are clear. First, the vascular enzyme produces superoxide at lower levels over a longer period of time, and much of this is generated intracellularly where it is ideally placed for cell signaling roles. Second, the $gp91^{phox}$ catalytic subunit to which NADPH and oxygen bind is substituted by NOX1 or NOX4 homologues, particularly in smooth muscle. Third, whilst the NOX homologue and the membrane-bound $p22^{phox}$ subunit are essential to maintain a stable unit capable of supporting electron transfer for superoxide generation, it remains unclear what role the cytosolic components play in the vascular NADPH oxidase: this latter point has implications for the action and specificity of NADPH oxidase inhibitors. Some studied have shown that increased activity of NADPH oxidase makes an important contributation to the pathogenesis of experimental models of vascular disease including intimal hyperplasia

induced by periarterial collar and arterial balloon injury, cholesterol-induced atherosclerosis, vein graft intimal hyperplasia and hypertension. Gene disruption of the $p47^{phox}$ component has been shown to significantly reduced superoxide production by vascular smooth muscle cells and importantly to reduce the development of atherosclerosis lesions. Importantly, increased superoxide generation by NADPH oxidase in vessels has been linked to the clinical risk factors for atherosclerosis and impaired endothelial NO function in patients with coronary artery disease (Dusting et al., 2005).

Atherosclerosis and NADPH Oxidase

An increase in oxidative stress appears to be a major mechanism underlying the development of vascular endothelial dysfunction in a wide range of cardiovascular diseases (although other mechanisms such as reduced eNOS expression or activity may also contribute). In the last decade, numerous studies have found that major sources of ROS responsible for this increased oxidative stress are vascular NADPH oxidases. It is important to emphasize that, in many (if not most) cases, the increased NADPH oxidase activity emanates from several different cell types within the vessel wall, i.e. endothelial cells, VSM, adventitial fibroblasts and/or infiltrating inflammatory cells. One important question yet to be answered is whether the generation of ROS by different NADPH oxidase isoforms in these different cell types is a factor that can be used to therapeutic advantage. For example, NOX2-based ROS production is of major importance in the endothelium and adventitia, whereas NOX1 and/or NOX4 may be much more important in VSM (Ray and Shah, 2005).

Oxidative stress and cardiovascular disease

There is growing evidence that oxidative stress, meaning an excessive production of reactive oxygen and nitrogen species, underlies many forms of cardiovascular and other age-related diseases. Indeed, increased production in vascular tissues of reactive species (RS), particularly $O_2^{\bullet-}$, has been implicated as playing an important role in hypertension, vascular remodeling after angioplasty, atherosclerosis, myocardial infarction, and ischemic stroke. Excess superoxide or superoxide-derived RS have multiple pathophysiological actions in the artery wall. For example, RS, directly or indirectly, promote lipid peroxidation and LDL oxidation, which are necessary steps in subendothelial lipid accumulation and atherosclerotic lesion formation. Superoxide rapidly inactivates endothelium-derived nitric oxide (NO), the important endogenous vasodilator, thereby reducing NO bioavailability, elevating vascular resistance and promoting vasoconstriction. Moreover, impairment of NO function by superoxide may result in vascular smooth muscle cell proliferation and migration, as well as promoting leukocyte and platelet adhesion. RS are also known to act as intracellular messengers in proinflammatory signaling, leading to the activation of the redox sensitive transcription factor nuclear factor kappa B (NF-*k*B) and expression of adhesion molecules [such as selectins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)] and chemokines [monocyte chemoattractant protein-1 (MCP-1)] in the vascular endothelium, all of which have key roles in atherogenesis and restenosis (Dusting et al., 2005).

Involvement in endothelial activation

With regard to early atherogenesis, the endothelial expression of adhesion molecules and MCP-1is a key step in the process of monocyte adhesion and emigration to form macrophages and then foam cells. Atherosclerotic levels of LDL (low-density lipoprotein) stimulate NADPH-oxidase-generated $O_2^{\bullet-}$ which, in turn, may contribute to LDL oxidation; ox-LDL (oxidized LDL) acts as an especially potent stimulus for oxidase activation, both in the vasculature and in macrophages, thereby accentuating the process. A role for AT1 (angiotensin II type 1)-receptor-driven increases in NADPH oxidase activity has also been reported. It is therefore

clear that oxidase-derived ROS may be involved at multiple levels; indeed, studies in $p47^{phox-/-}$ mice crossed on to an ApoE (apolipoprotein E)$^{-/-}$ background have shown that NADPH oxidase deficiency retards the development of atherosclerotic lesions in the mouse aorta (Ray and Shah, 2005).

The traditional risk factors for the development of atherosclerotic disease, namely dyslipidaemia, hypertension, diabetes mellitus and cigarette smoking, are all associated with endothelial dysfunction that is, at least in part, attributable to increased ROS production. Several experimental studies have found that each of these factors is capable of increasing NADPH oxidase activity and thereby impairing endothelium-dependent (NO-dependent) vasodilatation. With regard to hypertension, this has been shown to be the case not only for angiotensin-dependent hypertension, but also genetic hypertension, renovascular hypertension and low renin [DOCA (deoxycorticosterone acetate)-salt] hypertension (Ray and Shah, 2005).

Production of reactive oxygen species

Traditionally, macrophages have been assumed to be the source of the ROS in the vessel wall, and there is no doubt that these cells play an important role in vessel pathology. However, it has become clear that virtually all cells in the vessel wall (endothelial, smooth muscle and adventitial cells) produce ROS, in varying amounts and in response to diverse stimuli, which can then act in an autocrine or paracrine fashion to modulate cellular function (Fortuño et al., 2005)

Monocyte/Macrophage NADPH Oxidase

In resting primary human monocytes, the NADPH oxidase complex is unassembled and its components are located in the cytosol and the membrane. Upon activation, the cytosolic components translocate to the membrane and associate with the membrane components, and the newly formed enzyme complex actively catalyzes the production of superoxide anion. The membrane components include the cytochrome b_{558}, consisting of $gp91^{phox}$ and $p22^{phox}$. Cytosolic components include $p47^{phox}$, $p67^{phox}$, and Rac1 (Figure 4). The NADPH oxidase components in neutrophils are similar to those in monocytes/macrophages with the exception of the latter protein. Instead of Rac1, Rac2, another Rho family member of small G-proteins, is a component of the neutrophil NADPH oxidase. This latter, recent finding may provide a novel target for therapy that might allow discrimination between therapies that target chronic versus acute inflammatory responses. Evidence indicates that the NADPH oxidase of monocytes resembles, but is regulated differently than, the well-studied NADPH oxidase enzyme complex of neutrophils. Some examples of these differences are that neutrophils, on stimulation, produce a more immediate respiratory burst (peaking at 2 to 10 minutes, depending on the stimulus), whereas monocytes gradually increase production of superoxide anion. They have peak production at ~1 hour that wanes over time but is still detectable after several hours. Furthermore, after stimulation of monocytes to activate NADPH oxidase, the cells can mount an additional response after sufficient recovery and restimulation. This process is not observed in neutrophils. These differences may contribute to the distinct roles of monocytes/macrophages and neutrophils in chronic versus acute inflammation. Additionally, agents that activate NADPH oxidase in neutrophils do not necessarily trigger the NADPH oxidase in monocytes/macrophages, indicating differential regulation likely through alternative signal transduction pathways. Thus, it is important to understand the unique pathways regulating NADPH oxidase activity in monocytes. Because most of what we know about NADPH oxidase regulation and activity has been derived from cell-free components of neutrophils or from intact neutrophils, the laboratory has focused on defining the regulation of NADPH oxidase assembly and activation in monocytes/macrophages. An additional key reason for focusing on understanding

this process in monocytes derives from the fact that whereas NADPH oxidase has been shown to promote atherogenesis, neutrophils are not present in the vessel wall either early or late in lesion development. Although other vascular cells express similar oxidases, monocytes/macrophages are a plausible source of superoxide anion in this disease (Cathcart, 2004).

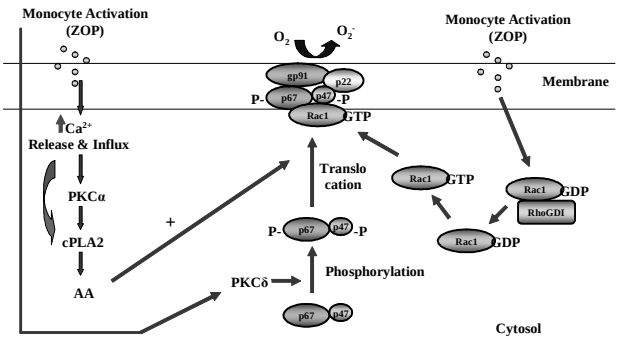

Figure 4 Regulation of monocyte NADPH oxidase (Adapted from Cathcart, 2004).

Superoxide anion, NADPH oxidase, and atherosclerosis

Superoxide anion, in addition to mediating LDL oxidation, may contribute to the pathogenesis of atherosclerosis in a variety of other ways. Among these, the production of reactive oxygen species induces stress responses that alter cell function, including adhesion, proliferation, and motility. Superoxide anion is also a very effective scavenger of nitric oxide and can thereby regulate endothelial relaxation and in the process also generate highly reactive peroxynitrite. Thus, superoxide anion has the potential to contribute to atherosclerosis in numerous ways. NADPH oxidase has been assessed for its contributions to the development of atherosclerosis in mice. Three studies have been performed in animals with gene knockouts of either of 2 central components of this enzyme complex ($p47^{phox}$ or $gp91^{phox}$). All 3 studies indicate that NADPH oxidase has little or no effect on the development of atherosclerosis in the aortic sinus. In contrast, however, 1 study evaluated total aortic lesions rather than those localized to the aortic sinus. In addition to the NADPH oxidases of monocytes and neutrophils, several other cell types have been reported to have lower-activity NADPH oxidases. It is believed that the lower production of superoxide anion may function as a second messenger, regulating basic cell functions such as cell growth. This is in striking contrast to the high-activity NADPH oxidase of phagocytes that, in addition to serving as intracellular signaling molecules, also is produced in sufficient quantities for killing microorganisms, mediating tissue injury, and oxidizing lipoproteins. This latter feature of monocyte-derived superoxide anion is believed to contribute to the pathogenesis of atherosclerosis (Cathcart, 2004).

Activation mechanisms of non-phagocytic NADPH oxidases

NADPHoxidase activity in non-phagocytic cells,such as cardiovascular cells, is acutely increased by diverse pathophysiological stimuli including: (i) G-protein-coupled receptor agonists, e.g. angiotensin II and endothelin-1; (ii) cytokines, e.g. TNF-α (tumour necrosis factor-α) and TGF-β (transforming growth factor-β); (iii) growth factors, e.g. thrombin, VEGF (vascular endothelial growth factor) and insulin; (iv) 'metabolic' factors, e.g. oxidized low-density lipoprotein, non-esterified ('free') fatty acids and glycated proteins; (v) hypoxia-reoxygenation or ischaemia-reperfusion; and (vi) mechanical stimuli, e.g. oscillatory shear. The molecular events at the level of the oxidase that are involved in its acute activation in cardiovascular cells are best characterized for the classical NOX2-containing oxidase and NOX1. In general, NOX2 oxidase activation in these cells involves a similar process to that in neutrophils, namely the association of cytosolic oxidase components ($p47^{phox}$, $p67^{phox}$ and Rac1) with cytochrome b_{558}. Binding of $p67^{phox}$ to an activation site on NOX2 initiates the electron transfer process but the key

post-translational modifications involved in oxidase activation are the phosphorylation of $p47^{phox}$ and Rac activation. Phosphorylation of $p47^{phox}$ allows its interaction with $p22^{phox}$ and facilitates the binding of $p67^{phox}$ to cytochrome b_{558} (Dworakowski et al., 2006).

Conclusion

In summary, it is likely that ROS such as superoxide anion functions at several different levels in contributing to the pathologic processes in atherosclerotic lesions. Superoxide anion likely participates in direct lipid and lipoprotein oxidation reactions, leading to foam cell formation, and also serves as a precursor for mediating myeloperoxidase and ceruloplasmin oxidation of lipids. Superoxide anion, either directly or indirectly, may alter vascular cell behavior, gene expression, and injury. Each of these roles could significantly contribute to lesion development. The NADPH oxidases are an important source of superoxide in human vessels; the activity of this enzyme system is increased in association with atherosclerotic risk factor profile and more marked endothelial dysfunction. Tightly regulated ROS production by a family of NADPH oxidases may be especially important in redox signalling. These enzymes appear to be involved in the pathophysiology of several cardiovascular (and other) diseases. NADPH oxidase has emerged as a major source of oxidative stress in the artery wall, particularly in artery disease including atherosclerosis.

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