

Hypothesis

A central role for the endothelial NADPH oxidase in atherosclerosis

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Abstract An increasing body of evidence has demonstrated that NADPH oxidase plays a critical role in several early steps leading toward the development of atherosclerosis. These effects appear to be carried out by both the ability of O_2^- to act as a small second messenger molecule, and potentially the oxidation of low density lipoprotein by O_2^- . We describe a model for the initiation and development of atherosclerosis that suggests targeted inhibition of NADPH oxidase as a powerful site for prevention and treatment of this disease.

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Key words: Atherosclerosis; Low density lipoprotein; Endothelial cell; NADPH oxidase; Oxidized low density lipoprotein

1. Introduction

Despite continued research efforts, atherosclerosis with its progression to heart disease, stroke and peripheral vascular disease continues to be the leading cause of death in all western civilizations [1]. Leading risk factors for the development of atherosclerosis include a high serum level of low density lipoprotein (LDL), smoking, diabetes, obesity, gender, high blood pressure, increased serum homocysteine and a strong link to family history suggesting genetic factors as well [2–9]. Various studies have demonstrated that even in advanced cases of atherosclerosis, the endothelium remains intact and functional, yet it seems to reside in a state of increased transcytosis, termed hyperpermeable [10,11]. This increased transport across the endothelial cell (EC) layer is responsible for the continued deposition of LDL into the intima. ECs demonstrate increased rates of transport both in vivo and in vitro when exposed to atherogenic levels of LDL [12–14]. The increased cellular activity is typified by massive uptake of LDL, lipid accumulation in the extracellular matrix of the intima, oxidation of the accumulating LDL, proliferation of smooth muscle cells and increased expression of cell adhesion molecules characteristic of an atherosclerotic endothelium.

Atherogenic levels of LDL have also been shown to lead to a marked increase in NADPH oxidase generated reactive oxygen species (ROS) by the endothelium [15,16]. More significantly, the increase in ROS is both required and sufficient to generate the physiological changes that accompany the gener-

ation of an atherosclerotic endothelium. We describe here a hypothesis where superoxide (O_2^-) generated by an endothelial NADPH oxidase plays a central role in the development of atherosclerosis.

2. NADPH oxidase

NADPH oxidase is an inducible electron transport system found in cells that transfers reducing equivalents from NADPH to oxygen resulting in O_2^- generation. This electron transport system has been primarily studied in neutrophils [17–20] where it plays an important role in bacterial killing. The NADPH oxidase consists of a membrane-integrated *b*-type cytochrome, cytochrome *b*₅₅₈, which is composed of 91 and 22 kDa subunits (gp91phox and p22phox, respectively), and at least three cytosolic proteins (p47phox, p67phox and p21rac) [21]. NADPH oxidase is a highly regulated enzyme that exists in a dormant unassembled state in quiescent cells. It is converted to an active assembled form in response to a stimulus by translocation of cytosolic subunits (p47phox and p67phox) to the membrane and assembly of them with the gp91phox and p22phox subunits [21,22].

Recently, the presence of a functional NADPH oxidase in human ECs has been demonstrated [23]. In addition, components of the NADPH oxidase have also been found in the ECs of other species [24,25]. The endothelial NADPH oxidase appears to have EC-specific p47phox and p67phox subunits that remain antigenically related to those expressed in neutrophils. This suggests that there may be differences in regulation of the NADPH oxidase between ECs and neutrophils. This is expected since we postulate that low-level O_2^- production by ECs is acting mainly as a second messenger for the regulation of certain cellular processes. Tissue-specific expression of different NADPH oxidase components may confer differences in regulation.

Exaggerated generation of O_2^- from genetically distinct NADPH oxidase subunits that may assemble and become active at variable threshold levels may play a role on an individual basis. An underlying hypothesis is that members of the general public may have an overactive NADPH oxidase that accounts for a genetic predisposition to atherosclerosis. Variants of NADPH oxidase have been reported in cases of chronic granulomatous disease [26] where neutrophils are unable to generate sufficient O_2^- in response to infectious organisms. Indeed, variants with a partially functional NADPH oxidase may have sufficient activity to avoid immunological problems and may be highly resistant to the atherosclerotic process.

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3. Superoxide as a second messenger

Several studies have identified O_2^- as a second messenger in a variety of cellular processes [27]. Superoxide has been demonstrated to be involved in the promotion of apoptosis [43], the constriction of the vascular smooth muscle despite the presence of nitric oxide (NO) or superoxide dismutase [28,29], and a number of metabolic changes in EC. Most notable is an increase in endocytotic activity in the presence of atherosclerotic levels of LDL [15]. Besides increasing the level of EC transport, NADPH oxidase-generated O_2^- is accountable for several other initiating events in the process of atherosclerosis. It is involved in monocyte attachment to the EC [30], platelet aggregation [31], the nuclear localization of the transcription factor nuclear factor kappa B (NF- κ B) [30,32], increased expression of ELAM-1 mRNA in EC [33], smooth muscle cell proliferation [34], stress fiber formation [35], responsiveness of EC to pulsatile stretch [36], maintenance of vascular tone [37] and oxidation of LDL in macrophages [38]. O_2^- has also been suggested to further activate NADPH oxidase in a positive feedback mechanism by acting through phospholipase A_2 and the release of arachidonic acid (AA) (Fig. 1) [39].

4. Activation of NADPH oxidase by LDL

High concentrations of LDL have been shown to lead to the activation of NADPH oxidase and the subsequent generation of O_2^- . Initially, high concentrations of LDL would be expected to lead to increased binding and uptake by cell surface LDL receptors (Fig. 1). LDL-bound receptor may lead to the activation of a signal transduction pathway responsible for the direct activation of NADPH oxidase. This pathway is expected to be dependent on the activity of phospholipase A_2 and the release of AA, a direct activator of NADPH oxidase [40] (Fig. 1). Alternatively, LDL degraded in the lysosome might be expected to transiently increase the cytoplasmic concentration of AA.

Cytoplasmic free AA, at a threshold level, converts an inactive NADPH oxidase to its active form. NADPH oxidase generates increasing amounts of O_2^- that can promote phospholipase A_2 activation and further release of AA [41]. Liberated AA, and its metabolites, markedly escalates NADPH oxidase cytosolic component (p47phox and p67phox) recruitment and complex assembly generating increased levels of O_2^- . This O_2^- acts as a second messenger molecule to increase the rate of bulk phase transendothelial transport. This is based on a coupling of endocytosis to exocytosis, or transcytosis [42]. The increased transcellular transport via bulk phase transcytosis results in increased deposition of LDL on the abluminal side of the EC (Fig. 1). The exact site of O_2^- action is currently unknown, but may be similar to the effect of NO on cGMP synthase.

5. Oxidation of LDL by NADPH oxidase

The subendothelial accumulation of foam cells is an important indicator of the initiation of atherosclerosis. These foam cells are derived from the unchecked uptake of LDL by monocytes and macrophages [43,44]. Incubation of LDL with macrophages does not lead to the production of foam cells since increased intracellular cholesterol concentrations

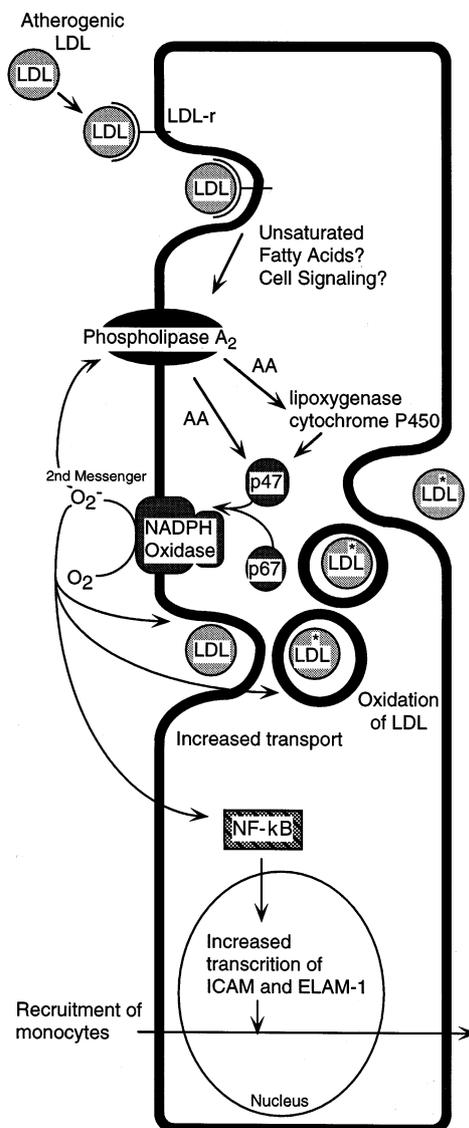


Fig. 1. Model for the central involvement of NADPH oxidase in generation of an atherogenic EC. Atherogenic levels of LDL cause a release of intracellular AA in a phospholipase A_2 dependent manner. The migration of the cytosolic NADPH oxidase subunits (p47phox and p67phox) to the membrane-bound gp91phox/p22phox complex is promoted directly by AA and its metabolites. The result is a production of O_2^- by the EC-specific NADPH oxidase. Acting as a second messenger of cellular signaling, the O_2^- causes a further increase in phospholipase A_2 activity and further release of AA, and continued activation and assembly of NADPH oxidase in a positive feedback manner. The increased levels of O_2^- also cause a translocation of the transcription factor NF- κ B to the nucleus and the up-regulation of a number of genes associated with the initial stages of atherosclerosis such as ICAM and ELAM-1. In addition, O_2^- causes an increase in the trafficking across the EC layer accounting for the increase in the passage of LDL through the EC. During transit, the exposure of LDL to high levels of O_2^- may account for a generation of ox-LDL (*LDL) on the abluminal side of the EC. The ox-LDL released from the EC will be taken up by monocytes/macrophages attracted by the increased number of cell adhesion molecules, accounting for the production of foam cells typically observed at the onset of atherosclerosis.

lead to down-regulation of the LDL receptor [43]. However, macrophages and monocytes readily take chemically modified or oxidized LDL (ox-LDL) up through a scavenger pathway that is not down-regulated. Monocytes, macrophages, smooth

muscle cells and ECs are all capable of oxidizing LDL *in vitro* [36,44,45]. Oxidation of LDL by macrophages has been shown to be dependent on NADPH oxidase [36] and this may also be the case in other cell types.

The finding that ECs have their own functionally active NADPH oxidase raises the possibility that LDL can be oxidized as it traverses the EC layer of the blood vessel. This modification would likely occur when LDL crosses the EC barrier by transcytosis. LDL invaginated into endocytotic vesicles would be expected to come into contact with O_2^- generated by the EC NADPH oxidase. Presumably, accessory factors required for the oxidation of LDL would be present (i.e. Cu^{2+} , etc.). This process would result in ox-LDL being released to the extracellular matrix on the abluminal side of the EC. The EC-generated ox-LDL could be the major source of ox-LDL taken up by foam cells. Thus, the EC plays a central role in the delivery of LDL to the abluminal side and potentially in the generation of ox-LDL in the vessel wall.

6. NADPH oxidase inhibitors

Apocynin, a methoxy-substituted catechol, is a well-characterized inhibitor of NADPH oxidase [46]. It is naturally occurring and has been used by primitive cultures for centuries in the treatment of inflammatory diseases. Apocynin has been shown to be extremely effective at eliminating the increase in O_2^- production and increase in transcytosis in ECs associated with high levels of LDL [23,40]. Since apocynin has been shown to impede the assembly of the p47phox subunit with the membrane complex, it likely strikes NADPH oxidase at its most vulnerable point to limit the production of O_2^- [46]. This mechanism was first described in neutrophils [46], and has now been demonstrated in ECs as well [23].

It is apparent from other studies that apocynin may have further anti-atherosclerosis benefits as well. Inhibition of macrophage NADPH oxidase with apocynin reduces cell-mediated oxidation of LDL by up to 89% [38]. Monocyte adhesion to ECs perturbed by substances that elicit EC ROS production results from the induction and cell surface expression of various adhesion molecules [30]. The mechanism for this response likely involves ROS acting as a signal-transducing molecule in the activation of the transcription factor NF- κ B [32]. NADPH oxidase inhibition with apocynin decreases the cell surface expression of adhesion molecules and significantly reduces monocyte adhesion to the EC surface [30]. Further, the strategy of using an NADPH oxidase inhibitor has been shown to be effective at inhibiting ROS generation by ECs despite an atherogenic level of serum LDL [15]. Perhaps this direction of atherosclerosis treatment will someday rival the administration of HMG-CoA reductase inhibitors, since in rabbits, NADPH oxidase inhibition has been shown to be effective at suppressing atherogenesis *in vivo* in spite of highly elevated serum LDL levels [46].

In addition to apocynin, other vanillins have been shown to be effective inhibitors of NADPH oxidase including protocatechuic, vanillic and caffeic acids [47,48]. Indeed, naturally occurring break down products of various catechols may have a natural role in controlling NADPH oxidase. Considered together, the points at which the inhibition of NADPH oxidase, and the resultant production of O_2^- , would help curb this disease are many.

7. Conclusions

At the very least, NADPH oxidase, as it is expressed in the human endothelium, is a major player in the beginning steps toward atherosclerosis. The production of ROS by NADPH oxidase has detrimental effects on the normal physiology of ECs by causing increased transcytosis of LDL, possible oxidation of LDL in the ECs, and other vascular changes. These include increased macrophage adhesion and foam cell accumulation initiating the cellular transformation typified by fatty streaks and plaque formation. Apocynin has been shown both *in vitro* and *in vivo* to be effective at protecting the endothelium from the initiating events of atherosclerosis. The future treatment of this disease should encompass the use of apocynin, its active form, or another member of the vanillin family as an agent against NADPH oxidase and its generation of O_2^- .

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