Regulation of Superoxide Anion Production by NADPH Oxidase in Monocytes/Macrophages

Contributions to Atherosclerosis

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Abstract—Monocyte extravasation into the vessel wall has been shown to be a critical step in the development of atherosclerosis. Upon activation, monocytes produce a burst of superoxide anion due to activation of the NADPH oxidase enzyme complex. Monocyte-derived superoxide anion contributes to oxidant stress in inflammatory sites, is required for monocyte-mediated LDL oxidation, and alters basic cell functions such as adhesion and proliferation. We hypothesize that monocyte-derived superoxide anion production contributes to atherosclerotic lesion formation. In this brief review, we summarize our current understanding of the signal transduction pathways regulating NADPH oxidase activation and related superoxide anion production in activated human monocytes. Novel pathways are identified that may serve as future targets for therapeutic intervention in this pathogenic process. The contributions of superoxide anion and NADPH oxidase to atherogenesis are discussed. Future experiments are needed to clarify the exact role of NADPH oxidase-derived superoxide anion in atherogenesis, particularly that derived from monocytes. (Arterioscler Thromb Vasc Biol. 2004;24:23-28.)

Key Words: monocyte ■ macrophage ■ superoxide anion ■ NADPH oxidase ■ atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the vessel wall characterized by the archetypal chronic inflammatory cell infiltrate of macrophages. The protective versus pathologic role for monocytes/macrophages in disease progression has not been fully delineated, but the conversion of these cells into characteristic lipid-laden foam cells suggests that the macrophage is exposed to an overwhelming burden of lipid and cell debris that might paralyze any protective role that may have preceded. Evidence indicates that atherosclerotic lesion development is dramatically compromised if monocytes are prevented from entering the blood vessel wall and thus a pathologic contribution is indicated.1–3

When circulating in blood, monocytes are resting cells poised to respond to an activating stimulus. In response to such a stimulus, monocytes adhere to activated endothelial cells on the blood vessel wall and extravasate into the...
adjacent tissue. While residing in the tissue, they differentiate to monocyte-derived macrophages. One of the most immediate responses of monocytes to a variety of activating stimuli is the production of the potent oxygen free radical, superoxide anion. The enzyme complex primarily responsible for the production of this highly reactive oxygen species is the NADPH oxidase complex. Recent studies in mice rendered deficient in a central component of the oxidase have revealed that NADPH oxidase–deficient animals develop significantly less atherosclerosis, as assessed by measuring total atherosclerotic lesion area. Therefore, understanding the regulation of the activity of this important enzyme complex may lead to novel therapeutic interventions for preventing atherosclerosis.

Data clearly indicate that monocytes actively contribute to atherosclerotic lesion development. The production of superoxide anion, as outlined above, also significantly contributes to lesion development. Although many cells in the vascular wall have been shown to be able to produce superoxide anion, monocytes/macrophages are likely a significant source of NADPH oxidase–derived superoxide anion in atherosclerotic lesions. It is therefore reasonable to suggest that monocyte/macrophage-derived superoxide anion participates in the pathogenesis of atherosclerosis.

The regulation of the activity of NADPH oxidase in primary human monocyte/macrophages, the predominant inflammatory cell in atherosclerotic vessels, has been the focus of several years of research in our laboratory. This review summarizes our present understanding of the regulation of NADPH oxidase activity in monocyte/macrophages and contrasts the enzyme complex components and oxidase regulation to that in neutrophils and to the related enzyme oxidase complexes expressed in other arterial cells, such as vascular smooth muscle cells. For all of our research, we have activated the monocytic NADPH oxidase using opsonized zymosan, a mimic of a yeast pathogen and a potent activator of the oxidase complex in atherosclerosis, it is likely that monocyte/macrophage-derived superoxide anion participates in the pathogenesis of atherosclerosis.

Regulation of Monocyte NADPH Oxidase

Components of the monocyte/macrophage NADPH oxidase are shown in color-filled circles. The regulatory pathways, controlling NADPH oxidase assembly and activation, are also indicated. Upon monocyte activation by a physiological stimulus, for example opsonized zymosan (ZOP), a serum-coated yeast cell wall, the cytosolic components move to join the membrane components gp91phox and p21phox. The assembly and activation of the NADPH oxidase enzyme complex is regulated by calcium influx, calcium release from intracellular stores, PKC-dependent phosphorylation of cPLA2, and arachidonic acid (AA) production. AA regulates the translocation of p47phox and p67phox to the membrane. These latter 2 components will not translocate unless first phosphorylated. Recent studies indicate that these phosphorylation events are regulated by PKCδ. Rac1 is the predominant Rac isoform expressed in monocytes, in contrast to Rac2 in neutrophils. Rac1 dissociates from its inhibitor rhoGDI upon monocyte activation and also translocates to the membrane, where it participates in the fully assembled and active NADPH oxidase.

b558, consisting of gp91phox and p22phox. Cytosolic components include p47phox, p67phox, and Rac1 (Figure). 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, our 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.

Most of our studies have been conducted on peripheral blood monocytes, isolated by adherence to serum-coated plastic and studied within 24 hours. We therefore refer to the cells prepared in this fashion as monocytes/macrophages. We have confirmed most of our observations in elutriated monocytes and monocyte-derived macrophages (monocytes cultured for 7 to 10 days) and have as yet observed no differences in the regulation of NADPH oxidase activity between these related cells.

It should also be noted that we have focused our studies on the induction of NADPH oxidase activity during the first hour after exposure to the activator; thus, altered gene expression does not contribute to our observations. In contrast, expression of NADPH oxidase components can be induced by treatment with cytokines or growth factors in smooth muscle cells and monocytes/macrophages. This likely contributes to the magnitude of superoxide production by these cells but is not a factor in our studies.

Calcium Influx and Release Are Required
To begin to understand the regulation of this important enzyme complex in monocytes, we examined the role of intracellular calcium mobilization and calcium influx in monocyte-mediated superoxide anion production. Our studies indicated that both calcium influx through receptor-regulated channels and mobilization of intracellular calcium were crucial events for monocyte oxidation of lipids, a superoxide anion–dependent event. Because raising intracellular calcium can have profound effects on several other signal transduction pathways, particularly the calcium-dependent protein kinase C (PKC) and cytosolic phospholipase A2 (cPLA2) pathways, we next evaluated the involvement of these enzymes in regulating NADPH oxidase activity.

cPLA2 Activity Regulates Monocyte/Macrophage NADPH Oxidase
Phospholipases A2 (PLA2s) are enzymes that catalyze the hydrolysis of fatty acids from the sn-2 position of a wide variety of phospholipids. PLA2s have been shown to be important for supplying unsaturated fatty acids to lipid oxidation enzymes, eg, cyclooxygenases and lipoxygenases, as well as for generating lysophospholipids that also serve as potent regulatory molecules. Several different, molecularly unrelated PLA2 enzymes have been reported in the literature. Among these, cPLA2 is rapidly activated by increased concentrations of intracellular calcium (in the micromolar range), which causes translocation from the cytosol to the membrane/particulate fraction of the cells. We found that cPLA2 expression is barely detectable in unactivated monocytes. cPLA2 expression is substantially induced by activation of monocytes with opsonized zymosan. As cPLA2 protein expression is induced, cPLA2 phosphorylation and enzymatic activity are increased in parallel (Q. Li et al, unpublished data, 2003).

When cPLA2 activity was inhibited with pharmacologic inhibitors or cPLA2 protein expression was inhibited by treatment with specific antisense oligodeoxyribonucleotides (ODN), NADPH oxidase activity was impaired. Importantly, the addition of one of the major products of cPLA2 activity, arachidonic acid (AA), restored NADPH oxidase activity in the cPLA2-deficient monocytes. This result indicated that the production of AA by cPLA2 was an essential regulator of monocyte/macrophage NADPH oxidase activity (see the scheme in the Figure).

To additionally understand the role for cPLA2 and its product AA in regulating NADPH oxidase activity, we conducted a series of experiments exploring the effect of blocking cPLA2 expression on the phosphorylation and assembly of the central NADPH oxidase components p47phox and p67phox. These studies revealed that oxidase assembly was blocked when cPLA2 expression was inhibited by treatment with antisense ODN. Specifically, the cytosol to membrane translocation of p47phox and p67phox was prevented in cPLA2-deficient monocytes. The addition of AA to cPLA2-deficient monocytes restored translocation of both p47phox and p67phox, thus allowing the normal, activation-induced assembly of the oxidase and superoxide anion production. These results strongly support the conclusion that AA, derived from cPLA2 activity, contributes to the translocation of essential NADPH oxidase components, allowing for assembly of the active enzyme complex. Our laboratory is presently investigating the mechanisms whereby AA regulates the translocation of NADPH oxidase components (summarized in the Figure).

Regulation of NADPH Oxidase by Component Phosphorylation: Protein Kinase C Is Essential
Phosphorylation of p47phox and p67phox also regulates NADPH oxidase activity. p47phox and p67phox were both shown to be phosphorylated in response to activation of monocytes with opsonized zymosan. Both components also translocated to the membrane from the cytosol. Inhibition of phosphorylation with general serine/threonine kinase inhibitors blocked both the phosphorylation and translocation (E.A. Bey and M.K. Cathcart, unpublished data, 2003; X. Zhao and M.K. Cathcart, unpublished data, 2003). Phosphorylation of these central NADPH oxidase components was not affected by inhibition of cPLA2 expression or enzymatic activity. These findings suggest that phosphorylation precedes translocation and represents yet another level of control of this enzyme.
PKC comprises an enzyme family that mediates the phosphorylation of serine and threonine on proteins with particular amino acid motifs. The PKC family is comprised of several isoenzymes that can be distinguished from each other by amino acid sequence, calcium dependence, phospholipid regulation, substrate specificity, and intracellular localization. Members of this enzyme family serve as important mediators of signal transduction. The PKC family is comprised of several isoenzymes (presently 12 members). They can be divided into 3 subtypes: cPKC (classical PKC), nPKC (novel PKC), and aPKC (atypical PKC).26–28

Our early studies indicated a requirement for PKC activity in the activation of the NADPH oxidase. We showed that monocytes express several PKC isoenzymes, including PKCα, PKCβI, PKCβII, PKCε, PKCδ, and PKCζ. The expression of each of these isoenzymes was induced upon monocyte activation.27 PKC activity also increased and translocated from the soluble to the particulate cell fraction. The activity of the cPKC group of PKC isoenzymes is regulated by calcium, and, based on our prior studies showing the critical role for calcium, this group was therefore singled out as a likely participant for regulating NADPH oxidase. The cPKC isoenzymes consist of PKCα, PKCβI, PKCβII, and PKCγ. PKC inhibitors and antisense ODN specific for a conserved mRNA sequence shared among the cPKC family members blocked NADPH oxidase activity.29 The cPKC isoenzymes expressed in monocytes are PKCα, PKCβI, and PKCβII. Additional studies using isoenzyme-specific antisense ODN revealed that PKCα was required for superoxide anion production and the related oxidation of LDL, whereas PKCβI and PKCβII were not involved.7

Relationship Between PKCα and cPLA2

cPLA2 activity is regulated not only by calcium but also by phosphorylation. Lin et al.40 have shown that activation of cPLA2 is associated with increased serine phosphorylation. Activation of monocyte PKC, as well as induction of calcium release from internal stores or calcium influx, all enhance PLA2 activity.31 Likewise, inhibitors of PKC have been shown to block agonist-induced cPLA2 activity.32,33 cPLA2 also contains a consensus site for phosphorylation by mitogen-activated protein (MAP) kinase; indeed, recent studies show that MAP kinase phosphorylation activates cPLA2 in vitro.34 Using PD098059, a selective inhibitor of the immediate upstream activator of MAP kinase, the dual-specificity kinase called MEK, we have recently shown that although monocyte/macrophage MAP kinase activation is substantially inhibited by this drug, cPLA2 activity remains unaffected. In contrast, PKCα was shown to be required for mediating the phosphorylation and activation of cPLA2. When monocytes were rendered deficient in PKCα expression, also using specific antisense ODN, the activity of NADPH oxidase could be restored by AA.7 This finding reveals that PKCα is solely required for mediating the phosphorylation and activation of cPLA2 and thereby regulates the production of superoxide anion (Q. Li et al, unpublished data, 2003) (Figure).

Although the aforementioned studies demonstrate that PKCα is the cPKC enzyme that is required for NADPH oxidase activity, these studies do not rule out the involvement of other PKC isoenzymes in regulating superoxide anion production by NADPH oxidase, eg, members of the aPKC and nPKC groups. In addition to the dedicated role of PKCε in regulating NADPH oxidase activity via cPLA2, we have recently discovered that another PKC isoform regulates NADPH oxidase activity.

Using a pharmacologic inhibitor and specific antisense ODN, our recent studies indicate that PKCδ expression and activity are also required for NADPH oxidase activity. Our results indicate that PKCδ is required for the phosphorylation of both p47phox and p67phox (E.A. Bey and M.K. Cathcart, unpublished data, 2003; X. Zhao and M.K. Cathcart, unpublished data, 2003). Translocation of both of these NADPH oxidase components is also regulated by PKCδ. It thus appears that PKCδ controls the phosphorylation of both p47phox and p67phox and regulates their translocation to the membrane fraction and controls the assembly of the active NADPH oxidase complex.

We are careful to interpret these data at face value. Although it is tempting to speculate that PKCδ directly phosphorylates p47phox and p67phox, our data do not prove that this is the case. Our studies indicate that PKCδ regulates phosphorylation of these components, but direct phosphorylation in vivo remains to be determined. To begin to assess this, we examined whether recombinant PKCδ could phosphorylate p47phox and p67phox in vitro and found that both NADPH oxidase components were directly phosphorylated (E.A. Bey and M.K. Cathcart, unpublished data, 2003; X. Zhao and M.K. Cathcart, unpublished data, 2003). We are now investigating the location of the phosphorylation sites on p47phox and p67phox obtained in vitro and are comparing them to those that are induced in intact cells on monocyte activation.

Superoxide Anion, NADPH Oxidase, and Atherosclerosis

One way that superoxide anion is presumed to participate in atherogenesis is through the formation of oxidized lipids, particularly oxidized LDL. The altered bioactivity of oxidized compared with unoxidized LDL was first reported by Chisolm and colleagues.35,36 Shortly thereafter, our laboratory showed that monocytes/macrophages, the major inflammatory cell component of atherosclerotic lesions, could cause LDL oxidation.37 Thus, the hypothesis was formed that macrophage oxidation of LDL contributes to the pathogenesis of atherosclerosis.

Among the proatherogenic properties of oxidized LDL is the characteristic unregulated uptake of this modified lipoprotein by macrophages that thereby contributes to the formation of foam cells. Numerous other proatherogenic biologic activities have been attributed to oxidized LDL.38

As mentioned, our laboratory was the first to demonstrate that activated monocytes could oxidize LDL.37,39 This was found to be a unique activity of leukocytes, because they are the only lesion cells that oxidize LDL in the absence of added free metal ions.40 Macrophage-mediated LDL oxidation was shown to be entirely dependent on the production of superoxide anion by the NADPH oxidase enzyme complex.40,41
In atherosclerotic lesions, oxidized lipids are believed to be formed not only by free radical oxidation but also by a variety of enzymatic pathways, including cyclooxygenases and lipoxygenases. We found that oxidized lipid products of 15-lipoxygenase are present in human atherosclerotic lesions, and others have documented that 15-lipoxygenase is expressed in lesion macrophages.\textsuperscript{4,24,43} Myeloperoxidase and ceruloplasmin have also been implicated in mediating lipid and LDL oxidation in atherosclerotic lesions.\textsuperscript{40} It is important to note that superoxide anion is an important mediator of LDL oxidation both as a reactive oxygen radical and as a required substrate or cofactor in the oxidation reactions catalyzed by these enzymes, particularly myeloperoxidase, ceruloplasmin, and lipoxygenases.

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 p47phox or gp91phox.\textsuperscript{4,44,45} 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 this study, lesions were dramatically decreased in the p47phox-null, NADPH oxidase–deficient animals.\textsuperscript{4} The interpretation of these observations was that aortic sinus lesions may be more advanced and differences between the NADPH oxidase-deficient and control animals might be difficult to detect at this stage of lesion development. This interpretation remains to be confirmed.

In addition to the NADPH oxidases of monocytes and neutrophils, several other cell types have been reported to have lower-activity NAD(P)H 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 as outlined above; however, the relative contributions of NADPH oxidases from different cell sources remains to be determined.

In the murine studies described above, none of the genetic knockouts were cell- or tissue-specific. It is therefore not possible to determine which cell type, among those with the capacity to produce superoxide anion by this or a similar enzyme complex, was responsible for the observed protection against atherosclerosis. It is important to pursue these studies to discriminate between monocyte/macrophage NADPH oxidase and that of smooth muscle cells or endothelial cells in this disease process. It is of utmost importance to evaluate total aortic lesions in the gp91phox-null animals, because gp91phox is important for phagocyte NADPH oxidase but not for the NAD(P)H oxidase complex of smooth muscle cells. Additional confirmation with bone marrow transplants from p47phox-null mice into atherosclerosis-prone animals would also clarify the source of the disease-promoting NADPH oxidase activity observed in the studies by Barry-Lane et al.\textsuperscript{4}

In summary, it is likely that 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. Additional experiments are needed to determine the importance of monocyte/macrophage-derived superoxide anion in the pathogenesis of atherosclerosis and to identify the critical regulatory pathways that could serve as targets for therapeutic intervention.

The goal in designing therapeutic agents to regulate monocyte/macrophage NADPH oxidase is to preserve the function of the enzyme complex so that it can function properly in host defense while regulating excessive and chronic superoxide anion production that seems to contribute to inflammatory injury. Identification of the molecular pathways, such as those summarized in the Figure, that regulate NADPH oxidase assembly and activity will help to ascertain the optimal pathways for controlling oxidase activation.

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