The importance of the contribution from Li et al. presented in this issue lies as much in the questions it raises as in those it answers. In setting out to prove that p47\textsuperscript{phox}, a regulatory subunit of the phagocyte NADPH oxidase, is equally vital to the superoxide-generating enzyme of vascular endothelial cells, which they successfully did, they also turned up a number of unexpected observations. The latter results, whose interpretation will necessarily remain speculative for the time being, may become fertile ground for future investigations, not only of the endothelium, but also of other tissues that express variants of this enzyme complex first characterized in phagocytes.

In the neutrophil, the bactericidal oxidative burst results from massive superoxide anion production by an NADPH oxidase, a complex of 5 major subunits, the membrane bound gp91\textsuperscript{phox} and p22\textsuperscript{phox}, and the regulatory cytosolic components p47\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac.\textsuperscript{2} The importance of p47phox is well documented because its phosphorylation appears to be the limiting step required for assembly of the active enzyme complex.\textsuperscript{2} Furthermore, mutations in p47\textsuperscript{phox} are a cause of chronic granulomatous disease, an immune deficiency resulting from impaired phagocytic activity.

It is now clear that many other tissues, including all layers of the vascular wall, also produce superoxide and its derivatives.\textsuperscript{3} These reactive oxygen species are no longer regarded simply as undesirable by-products of aerobic metabolism but also as essential second messengers because they control the function of redox-sensitive signaling proteins. They also appear to be key factors in the development of numerous pathologies, such as cancer and vascular disease.\textsuperscript{3,4} NAD(P)H oxidases have been identified as major enzymatic sources of superoxide in nonphagocytic tissues; although similar to the neutrophil oxidase, they differ from it in several respects, such as lower activity, distinct substrate specificity, and slower time course of activation. Because of these differences, recent work has aimed at establishing the molecular composition and mechanism of activation of nonphagocytic oxidases.

In the vascular endothelium, the 5 major subunits of the neutrophil enzyme appear to be expressed as mRNA\textsuperscript{5–12} and protein.\textsuperscript{5–11,13,14} The involvement of some subunits has been demonstrated functionally, for example, with the use of antisense p22\textsuperscript{phox} oligonucleotides,\textsuperscript{15} dominant-negative Rac1,\textsuperscript{16} and gp91\textsuperscript{phox} knockout mice.\textsuperscript{9} However, demonstration of a functional role for p47\textsuperscript{phox} in the endothelium has been lacking thus far, in contrast to other nonphagocytic tissues, such as vascular smooth muscle, in which p47\textsuperscript{phox} is expressed\textsuperscript{17–20} and functional, as shown by the use of inhibitors and knockout mice.\textsuperscript{18,20–23}

To establish the function of p47\textsuperscript{phox} in the endothelium, Li et al.\textsuperscript{1} performed elegant experiments by comparing superoxide production in coronary microvascular cells from wild-type and p47\textsuperscript{phox} knockout mice. They showed that PMA- and TNF\textsubscript{α}-induced superoxide production was abolished in the absence of p47\textsuperscript{phox}. Not only were similar results found in cells transfected with antisense p47\textsuperscript{phox} cDNA, but more dramatically, transfection of knockout cells with intact p47\textsuperscript{phox} restored oxidase activity, thus proving that this subunit is a functional part of the endothelial NADPH oxidase. Consistent results were obtained when superoxide production was measured using 3 different methods. These are the first data to directly demonstrate a functional role for p47\textsuperscript{phox} in endothelial NAD(P)H oxidase activity.

An unexpected observation from this study is that PMA and TNF\textsubscript{α} depressed oxidase activity in cells from knockout animals (Table and Figures 4 through 7),\textsuperscript{1} although this inhibition was not always significant (Figure 3).\textsuperscript{1} This effect, which is independent of p47\textsuperscript{phox}, may be mediated by phosphorylation of other enzyme subunits or proteins involved in signaling pathways. Thus, in wild-type cells, agonists must have 2 opposite effects: on the one hand, they greatly stimulate enzyme activity, presumably by phosphorylation of p47\textsuperscript{phox}; and on the other hand, they also reduce oxidase activation, possibly as part of a feedback mechanism.

Equally surprising is the fact that p47\textsuperscript{phox} is not required for basal superoxide production. On the contrary, in the absence of agonist, p47\textsuperscript{phox} actually inhibits enzyme activity. This negative effect might be understood, as suggested by the authors, if one recalls that the neutrophil enzyme can function in vitro without p47\textsuperscript{phox}, provided that p67\textsuperscript{phox} and Rac are sufficiently abundant.\textsuperscript{24,25} Thus, binding of unphosphorylated p47\textsuperscript{phox} to p67\textsuperscript{phox} may prevent this latter subunit from activating the enzyme. In contrast, as expected from the current model of activation of the phagocyte enzyme, in the presence of PMA or TNF\textsubscript{α}, p47\textsuperscript{phox} markedly increases oxidase activity. This effect, likely mediated by p47\textsuperscript{phox} phosphorylation, is all the more remarkable considering that taken in isolation, both p47\textsuperscript{phox} and agonists inhibit enzyme activity, as described earlier.
It is clear from Li’s study that endothelial cells, unlike phagocytes, generate small quantities of superoxide in the absence of stimulation, presumably for signaling purposes. This constitutive enzymatic activity is similar to that observed in other nonphagocytic tissues, such as smooth muscle, but was unexpected because the NADPH oxidase of endothelial cells is thought to have the same molecular composition as the phagocyte enzyme, which is inactive in the absence of stimulation. One explanation for this activity in wild-type endothelial cells may be that the regulatory subunits, which are cytosolic in the neutrophil, are partly preassembled with gp91phox and p22phox in nonphagocytic tissue. Indeed, in contrast to the neutrophil, oxidase activity is present in the particulate fraction of smooth muscle and endothelial cells, and in a recent report, p47phox was found in the particulate fraction rather than the cytosol of endothelial cells. However, the persistence of activity in cells from p47phox−/− knockout mice suggests that this subunit may not be absolutely required to activate the nonphagocytic oxidase, presumably because some p67phox and Rac remain bound to gp91phox. Alternatively, p47phox−independent activity could result from the presence of a gp91phox homologue in endothelial cells. Although nox1 was not found in the endothelium, preliminary results from our laboratory indicate that nox4 is expressed. From Li’s observations in cells from knockout mice, one would predict that nox4 may not associate with p47phox and may be inhibited by PMA and TNFα. Further studies using specific inhibitors of both enzymes will be required to test these hypotheses.

The report from Li et al thus conclusively demonstrates that p47phox is required for activation of the endothelial NADPH oxidase by agonists. This important result suggests that an enzyme with a molecular composition identical to that of the neutrophil is functional in the endothelium. Furthermore, unexpected findings from this study will provide a basis for further research. For example, the mechanism of the inhibitory effect of agonists and p47phox on the enzyme will have to be elucidated. Finally, the existence of a p47phox−independent oxidase activity remains to be investigated. It will be of great interest to determine if these new findings also apply to other nonphagocytic tissues.

References


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Out Phoxing the Endothelium: What's Left Without p47?
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