Regulation of NADPH Oxidases: The Role of Rac Proteins

Peter L. Hordijk

Abstract—The role for reactive oxygen species (ROS) in cellular (patho)physiology, in particular in signal transduction, is increasingly recognized. The family of NADPH oxidases (NOXes) plays an important role in the production of ROS in response to receptor agonists such as growth factors or inflammatory cytokines that signal through the Rho-like small GTPases Rac1 or Rac2. The phagocyte oxidase (gp91phox/NOX2) is the best characterized family member, and its mode of activation is relatively well understood. Recent work has uncovered novel and increasingly complex modes of control of the NOX2-related proteins. Some of these, including NOX2, have been implicated in various aspects of cardiovascular disease, including vascular smooth muscle and endothelial cell hypertrophy and proliferation, inflammation, and atherosclerosis. This review focuses on the role of the Rac1 and Rac2 GTPases in the activation of the various NOX family members. (Circ Res. 2006;98:453-462.)

Key Words: NADPH oxidase □ Rac1 □ reactive oxygen species

The cellular responses to external stimuli involve the activation of multiple signaling pathways through processes such as receptor dimerization, protein phosphorylation and the generation of intracellular messenger molecules. The classical “second messengers” cAMP and intracellular Ca2+ have turned out to be among the first of an ever-growing list of intracellular signaling intermediates that includes protons, proteins, (phospho)lipids, and reactive oxygen species (ROS). ROS, a collection of oxygen-derived molecules such as oxygen radicals, hydrogen peroxide, peroxynitrite, and NO, are rapidly diffusing, short-lived messenger molecules, of which their role in signal transduction has only recently been fully recognized. There are various enzymes that can generate (intra)cellular ROS, including xanthine oxidase, cyclo-oxygenases, NO synthases, mitochondrial oxidases, and the NADPH oxidases (NOXes). Biochemically, ROS act by oxidative modification of nucleic acids, sugars, lipids, and proteins. This results in, for example, DNA damage but also in regulated signaling through the inactivation of specific enzymes such as protein tyrosine phosphatases, which leads to increased tyrosine kinase activity. To preserve the cellular redox balance, control cell signaling, and to prevent potential damage, ROS are removed or neutralized by, for example, glutathione and vitamins as well as by enzymes such as superoxide dismutases, catalase, and peroxidases.
Figure 1. Schematic summary of the available information on the control of NOX1 to NOX5 activity. Superoxide/ROS are produced in cells after stimulation by pathogens, receptor agonists, and shear stress. NOXes 1 through 4 but not NOX5 associate with p22phox. NOX2 (gp91phox) is simulated by phosphorylation-activated p47phox and by p67phox, in conjunction with activated Rac. The hematopoietic Rac GEFs Vav1 and P-Rex1 can activate NOX2. NOX1 is activated by the NOXO1 and NOXA1 proteins; NOXO1 is phosphorylation independent, but NOXA1 can associate with activated Rac1. The Rac GEF β-PIX binds and activates NOX1, although a role for NOXA1 remains to be demonstrated. NOX3 is activated by NOXO1 but is insensitive to additional stimulation by NOX1 or activated Rac. Regulation of NOX4 activity is controversial. There are no data on regulatory proteins for NOX4, as represented by the black box. NOX5 is activated by Ca2+ and does not require the regulatory subunits. Dashed lines indicate additional or potential ways of cross-talk or activation.

It has recently been shown that most, if not all, types of cells generate intracellular ROS and that the well-established phagocyte oxidase is in fact member of a family of various NOXes that show widespread, differential tissue expression. The generation of ROS through the activation of the phagocyte NOX (gp91phox/NOX2) is an extensively studied and well-coordinated process in which various means of regulation, such as phosphorylation, GTPase activation, and protein–protein interactions, act in sequence.5–6 The contribution of Rac proteins to the activation of the phagocyte oxidase has been studied extensively and has recently seen important progress in the analysis in mouse models (see below). Many studies have shown Rac-mediated production of ROS in nonphagocytic cells, and there are a few recent studies that indeed link Rac signaling to the activation of the “new” NOXes (Figure 1).

Rac-Mediated Production of ROS

High levels of ROS are generated in neutrophils upon cell stimulation and phagocytosis, and these ROS aid in the killing of ingested pathogens. In most other types of cells, low levels of ROS are implicated in growth, differentiation, migration, and angiogenesis, as well as in inflammation, vascular hypertrophy, and atherosclerosis (see below).7–12 The stimuli that induce the production of intracellular ROS include growth factors, inflammatory stimuli, integrins and their ligands, and shear stress.9,13–17 Most of these intracellular ROS are supposedly derived from NOXes, based on sensitivity of the responses to the flavoprotein inhibitor diphenylene iodonium, to a NOX2-inhibitory peptide,18 to downregulation by antisense oligos19 or through the use of gene-deficient mice.20–22

The direct link between Rac activity and ROS production in nonphagocytic cells was shown for the first time in 1996.13 These authors showed that expression of an activated mutant of Rac1, V12Rac1, resulted in increased production of ROS in fibroblasts. This response was mimicked by V12Ras, and it was suggested that Rac acts downstream of Ras in initiating ROS production induced by EGF or by platelet-derived growth factor (PDGF), as well as by cytokines such as tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β).13 Rac-mediated production of ROS was also found in HeLa cells and implicated in IL-1β-mediated activation of nuclear factor κB (NF-κB),9 further strengthening the idea that Rac-mediated production of intracellular ROS is instrumental in signal transduction, downstream (of cytokine) receptors.

Several studies have subsequently implicated Rac-mediated production of ROS in a variety of cellular responses, in particular in endothelial cells. In bovine aortic endothelial cells, shear stress can activate protein tyrosine phosphorylation and extracellular-signal regulated kinase activation in a Rac- and ROS-dependent manner.17 These findings were based on expression of N17Rac1 and the use of antioxidants without demonstration of direct stimulation of either Rac1 or ROS production, albeit that Rac1 activation and ROS synthesis by shear in endothelial cells has been observed by others as well.23,24 In addition, depolarization of endothelial cells (seen in hypertensive vessels and implicated in platelet aggregation) results in ROS production in a phosphatidylinositol 3-kinase (PI3K) and Rac-dependent manner.25 ROS also negatively regulate endothelial cell–cell adhesion, which is controlled by junctional molecules such as vascular endothelial (VE)-cadherin.26–29 VE-cadherin associates to β- and γ-catenin, which link the transmembrane molecules to the actin cytoskeleton. These complexes are subject to regulation by (ROS-induced) tyrosine phosphorylation. In line with this, endothelial ROS production is stimulated by permeability-increasing agonists such as TNF-α. In addition, the immunoglobulin-like cell adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells initiates ROS production that mediate Rac1-induced permeability as well as leukocyte transendothelial migration.16,19,27 Various mechanisms of action of the VCAM-1–Rac-induced ROS have been proposed: Deem and Cook-Mills suggested that endothelial NOX2 releases these ROS extracellularly and stimulate matrix metalloprotease activity,19 whereas our laboratory provided evidence for a role of the redox-sensitive kinase Pyk2 in ROS-mediated loss of cell–cell contact.28 In conclusion, there is ample evidence for Rac-dependent production of ROS by NOXes in cellular signaling, in particular in vascular cells.

The Role of Rac in the Activation of NOXes NOX2

The NOX of human neutrophils (NOX2) was identified ∼20 years ago,29,30 and the control of its activation is intensely
This NOX comprises the flavocytochrome b<sub>558</sub>, which is a heme-binding heterodimer composed of a large (gp91<sup>phox</sup>) and a small (p22<sup>phox</sup>) subunit. The gp91<sup>phox</sup> subunit has 6 transmembrane regions in its N terminus, whereas its C-terminal portion contains the flavin-adenine dinucleotide (FAD) and NADPH binding domains that are essential for activity. Oxidase activation is controlled by the recruitment of regulatory proteins to the flavocytochrome, including the p40<sup>phox</sup>, p47<sup>phox</sup> and p67<sup>phox</sup>, of which p47<sup>phox</sup> and p67<sup>phox</sup> are essential for activity (Figure 1).<sup>35,36</sup>

A human X-linked genetic disorder, chronic granulomatous disease (CGD) has been invaluable in elucidating the relative contribution of these regulatory proteins and their specific domains involved in the activation of the phagocyte oxidase. CGD is characterized by severely impaired phagocyte function attributable to a defect in the production of superoxide by phagocytes. CGD results from each of more than 400 different mutations that have been identified in members of the oxidase complex, including gp91<sup>phox</sup>, p22<sup>phox</sup>, p67<sup>phox</sup>, p47<sup>phox</sup>, or Rac2.<sup>37–40</sup> This leads to a lack of proper complex assembly or to an inactive p91<sup>phox</sup> subunit.

Assembly of the neutrophil–NOX complex is initiated on phosphorylation of p47<sup>phox</sup>, releasing it from its autoinhibitory conformation. The p47<sup>phox</sup> N-terminal SH3 domain then binds the proline-rich region in p22<sup>phox</sup> and the PX domain of p47<sup>phox</sup> binds 3-phosphoinositides, the products of PI3K. Activation of the small GTPase Rac2 is associated with recruitment of p67<sup>phox</sup>, which associates with p47<sup>phox</sup> and with the cytochrome. The interaction of activated Rac2 with the neutrophil oxidase complex is mediated by the N terminus of the p67<sup>phox</sup> subunit.<sup>49</sup> Intriguingly, Rac-mediated translocation of p67<sup>phox</sup> to the cytochrome does not occur in p47<sup>phox</sup>-deficient cells, indicating Rac2 activation is not the sole event that drives p67<sup>phox</sup> translocation. Finally, direct binding of Rac2 to the flavocytochrome has been implicated in the initial steps of the electron transfer reaction: a Rac2 function, dependent on the insert domain (see below).<sup>50</sup>

**NOX1**

The NOX1 protein, initially identified by homology-based polymerase chain reaction as mitogenic oxidase 1, is predominantly expressed in colon epithelium as well as in vascular smooth muscle cells (VSMCs). NOX1 is efficiently activated by homologues of p47<sup>phox</sup> and p67<sup>phox</sup>, the NOXO1 (NOX-organizer 1) and NOXA1 proteins (NOX-activator 1), respectively (Figure 1). The NOXO1 protein does not contain an autoinhibitory region, which, in p47<sup>phox</sup>, is involved in the phosphorylation-induced activation and subsequent binding to the flavocytochrome b<sub>558</sub>. As a result, NOXO1 is considered “constitutively active,” and significant activation of NOX1 can already be observed in coexpression experiments in the absence of PMA.<sup>54,55</sup> NOXA1 and p67<sup>phox</sup> both have four so-called tetratricopeptide repeats in their N termini. These repeats mediate the binding to activated Rac2 and are required for NOX2 activation. In NOXA1, a R102E mutation that in p67<sup>phox</sup> impairs Rac2 binding, also blocks its interaction with activated Rac1 and Rac2. GDP-bound Rac1 or Rac2 does not bind NOXA1, nor does active or inactive Cdc42. These data suggest that Rac proteins activate NOX1 through NOXA1. However, whereas NOX2 requires additional activated Rac1 in cotransfection studies in HEK293 cells, NOX1, in the presence of the NOXO1 and NOXA1 proteins, apparently does not.<sup>55</sup>

In contrast, in gastric mucosal cells, lipopolysaccharide-induced superoxide production was blocked by the PI3K inhibitor LY 294002, which, in turn, was bypassed by activated Rac1, suggesting that Rac1 activates NOX1 in these cells.<sup>57</sup>

More direct evidence for a role of the PI3K–Rac pathway in NOX1 activation was provided by Park et al. This study showed that the Rac–guanine nucleotide exchange factor (GEF) β-PIX is required for and even potentiates EGF-induced ROS production in Caco-2 and HEK293T cells. Park et al showed that β-PIX or activated Rac1 binds the C-terminal domain of NOX1, and that, in particular, the membrane proximal portion acts as a dominant negative in that it blocks EGF-induced ROS production. The relevance for NOX1 was further demonstrated by depletion of NOX1 in Caco-2 cells, which prevented ROS production by EGF. Although NOXO1 and NOXA1 proteins further potentiated EGF-induced ROS in a β-PIX dependent fashion, it was not clarified whether for NOX1 activation, Rac1 interaction with NOXA1 is required or whether direct Rac1 binding to NOX1 is sufficient.<sup>58</sup>

**NOX3**

For NOX3, the situation is quite different. NOX3 is expressed in the vestibular and cochlear epithelia of the inner ear and is involved in the morphogenesis of otoconia and thus in control of the sense of balance.<sup>59,60</sup> and it is not clear which, if any, regulatory subunits are coexpressed with NOX3. Studies on its regulation have so far relied on cotransfection experiments, mainly in HEK293, COS, or Chinese hamster ovary (CHO) cells. For CHO cells, which lack p22<sup>phox</sup>, coexpression of p22<sup>phox</sup> is required to induce a basal level of NOX3 activity. Different from NOX1, NOX3 is already activated after coexpression with the NOXO1 protein, whereas coexpression of just NOXA1 does not activate NOX3 (Figure 2). In contrast, p67<sup>phox</sup> by itself does activate NOX3, and this is further potentiated by p47<sup>phox</sup>. Ueno et al showed that p67<sup>phox</sup> R102E, which does not bind Rac proteins, also stimulated NOX3. Although the role of p67<sup>phox</sup> or NOXA1 in NOX3 activation remains unclarified, activation of NOX3 apparently is Rac independent. In line with this notion, expression of N17Rac1 had little effect on NOX3 activity in CHO cells.<sup>61</sup>

**NOX4**

NOX4 was originally identified in the kidney and was named Renox. Expression of NOX4 by itself already results in ROS production in NIH3T3 cells, suggesting that NOX4 might be constitutively active. Later studies have shown that NOX4, similar to NOX1, NOX2, and NOX3, at least requires p22<sup>phox</sup> for its activity. Stable expression of NOX4 in HEK293 tk<sup>2</sup> cells, which have endogenous p22<sup>phox</sup>, results in significant ROS production, up to 100-fold over basal levels. NOX4 was not further activated by p47<sup>phox</sup>/p67<sup>phox</sup> or the NOXO1/NOXA1 proteins. Using the Cos–PHOX system, it was shown that whereas NOX2 in that system is inactivated after
a Rac1 knockdown, activation of NOX4 is undisturbed, supporting the notion that, like NOX3, NOX4 is Rac independent. Also, expression of Rac1N17, Rac1V12, or of a region of p21-activated kinase (PAK) that binds activated Rac1, did not affect NOX4 activity in the COS cell system. Thus, the lack of effect of coexpressed Rac1, p67phox, or NOXA1 indeed suggests that NOX4 is Rac1 independent.

In contrast to studies that claim NOX4 to be constitutively active, some recent reports suggest that NOX4 is, in fact, regulated. In mesangial cells, which express high levels of endogenous NOX4, antisense oligos to NOX4 reduced intracellular ROS production by arachidonic acid (AA), which is produced upon angiotensin II (Ang II) stimulation and leads to Rac1 activation. Similarly, Ang II/AA-induced and L61Rac1-induced activation of Akt was blocked by reduction of NOX4 expression. Although this study did not show direct activation of NOX4 by Rac1, it suggests that in mesangial cells, NOX4 activation is part of a Rac1-dependent signaling pathway. Along similar lines, Mahadev et al showed in 3T3L1 adipocytes, which also express high levels of NOX4, that insulin induces a 10-fold increase in intracellular ROS production, which was further increased by expression of NOX4. Importantly, NOX4 mutants that were mutated in their FAD- or NADPH-binding regions blocked insulin-induced ROS production for 80%, suggesting that these mutants act as dominant negatives, something one would not expect for an unregulated protein. Competition for p22phox does not explain this effect because expression of NOX4, which is p22phox dependent, increased ROS production, suggesting that p22phox was not limiting.

Clearly, the mechanism of NOX4 regulation is not solved at this point. It is important to note that in all their experiments, Mahadev et al never found elevated NOX4-mediated ROS production in the absence of insulin, arguing against a constitutively active NOX4. Also, the studies by Gorin et al and Mahadev et al were based on cells that express endogenous NOX4, whereas the work by Geiszt et al and Martyn et al was based on overexpression of NOX4 in 3T3 or HEK293 cells. Whether these differences in experimental setup may relate to the findings on Rac-mediated regulation of NOX4 activity remains to be seen. In addition, several groups have noted clear cell type–specific differences in the mechanisms of NOX activation. Future studies on endogenously expressed NOXes, in combination with knockdown and genetic approaches, likely will provide the most informative results.

NOX5
For NOX5, little information is available. NOX5 is expressed in lymphoid cells and the testis as well as in prostate cancer cells. NOX5 contains four EF-hand domains in its N terminus, is FAD and NADPH dependent but p22phox independent, and is activated by calcium (Figure 1). The N terminus of NOX5 binds its C terminus, and the binding of calcium disrupts this intramolecular interaction, resulting in activation of the oxidase. There are currently no data on the role of the various regulatory proteins or Rac in the activation of NOX5.

Localization of NOX Proteins
In neutrophils, NOX2 resides in intracellular vesicles in resting cells. NOX2 can be recruited to the plasma membrane after cell stimulation and is found in the membrane of phagosomes after phagocytosis. The topology of NOX2 with six transmembrane domains predicts that the superoxide is released either into the extracellular milieu or into the phagosome. Based on sequence homology, this organization is likely similar for the other NOXes. In VSMCs and endothelial cells, NOX proteins have been detected at the plasma membrane and in the nucleus, the endoplasmic reticulum (ER), caveolae, and focal adhesions. NOX localization presently is a controversial issue because whereas Ambasta et al could not detect NOX1 in the plasma membrane of transfected HEK293 cells, Cheng and Lambeth found GFP-tagged as well as untagged versions of NOX1 and its regulator NOXO1 in the plasma membrane. NOX2 has been claimed in endothelial cells to reside in the plasma membrane, leading to extracellular release of ROS after cell stimulation. Others have detected NOX2 and its regulatory proteins as a preassembled complex in an intracellular, perinuclear compartment in resting endothelial cells. Thus, it may well be that, comparable to the situation in neutrophils, NOX proteins in nonhematopoietic cells reside in ER-associated vesicles that can fuse with the plasma membrane upon stimulation. This would also correlate with the membrane recruitment of activated Rac proteins.

Structural Requirements for Rac-Mediated Production of ROS
Several isoforms of Rac proteins exist: Rac1, Rac1b, Rac2, and Rac3. For the studies on oxidase activation, most information has been obtained on the Rac1 and Rac2 proteins. Rac1b is a Rac1 splice variant that is constitutively active and that has recently been linked to mitochondria-derived production of ROS in a model for epithelial–mesenchymal transition. Rac2 is expressed in hematopoietic cells only and is the most relevant Rac GTPase for activation of NOX2 in human neutrophils. In contrast, Rac1 is ubiquitously expressed and is likely the main Rac GTPase for NOX activation in nonhematopoietic cells. Expression studies have shown that Rac1 can also activate NOX2 in heterologous systems. However, Rac1 and Rac2 are not completely redundant. Various studies have shown that the intracellular targeting of Rac1 and Rac2 proteins, mediated by their hypervariable C termini, is not identical and that this, in part, determines differential effects on cell functions such as superoxide production and chemotaxis (discussed in more detail below).

The structure–function analysis of Rac2 in NOX2 activation has initially been studied with the semirecombinant cell-free system. In this assay, recombinant Rac proteins, or mutants thereof, are added, together with recombinant p47phox or p67phox, to isolated neutrophil membranes that contain the NOX2 and p22phox subunits. An anionic amphiphile is also added, which induces a conformational change in p47phox to initiate activation of the oxidase. Superoxide production can subsequently be detected by a variety of methods. Later studies have used a reconstitution model in...
Figure 2. Overview of regions and residues in Rac that were found to be involved in NOX2 activation. Rac is represented by the black bar, and the switch I and switch II insert region and the C-terminal domain are indicated in white. Individual residues and domains that are required for activation of the NADPH oxidase are indicated in the top of the figure. The unique D57 mutation (#) was identified in a CGD patient. The bottom part of the figure indicates the amino acid differences between the Rac2 and Rac1 proteins. The hypervariable C termini are shown for comparison, with the divergent residues indicated by an asterisk. The curved line indicates the lipid anchor attached to the C-terminal cysteine residue.

Rac proteins are relatively small, 21-kDa proteins, a large series of residues and regions throughout the GTPase, have been implicated in the activation of NOX2 (Figure 2). Rac proteins, like other small GTPases, contain an effector region in the N terminus (approximately residues 20 to 40), which mediates interactions with effector proteins including p67phox.85–87 This part of the protein overlaps with the so-called switch I region (Figure 2; amino acids 32 to 40) that undergoes a strong conformational change on Rac activation.86 This was proposed to be the oxidase activation domain itself, perhaps analogous to Rac1 binding to the C-terminal domain of NOX1.85 This notion is further supported by studies showing that Rac2 binds directly to the cytochrome, through its insert region, before interacting with p67phox. The Rac2–cytochrome complex was proposed to have increased affinity for p67phox.86 Extensive mutational analysis of Rac and peptide-walking experiments have implicated amino acids 103 to 107, 163 to 169, and 183 to 188 in Rac1-mediated oxidase activation (Figure 2).85,86 Together with the switch I and II regions and the insert region, these domains are likely exposed on the outside of the GTPase.85 In particular, mutation of His103 was found to result in a dramatically reduced activation of the oxidase.83

Finally, there have been extensive studies on the C-terminal domain of Rac proteins in the activation of the oxidase. This domain is hypervariable and is the most divergent region among the usually highly homologous Rho-like GTPases (Figure 2). For example, 6 of the 15 amino acid differences between Rac1 and Rac2 are in the C-terminal region (Figure 2). The C terminus of Rho-like GTPases mediates membrane association, in part through the lipid anchor that is posttranslationally added to the C-terminal cysteine residue of the so-called CAAX box.86 However, for oxidase activation by Rac1 in the cell-free assay, the lipid anchor was found to be dispensable.86 The polybasic region in the Rac1 C-terminus has also been implicated in membrane association because of its net positive charge. However, these C termini additionally mediate specific protein–protein interactions, relevant for proper intracellular targeting of Rho-like GTPases.86,89,90

The isolated Rac1 C terminus was found to efficiently interfere with oxidase activation in the cell-free assay.97 Deletion of the entire C terminus of Rac1 or Rac2 or mutation of Lys183 or Lys186 blocks their capacity to activate the oxidase.97 Using a variety of peptides, derived from Rac1, RhoA, or RhoC, Joseph et al showed that the inhibitory effect of the Rac1 C-terminal peptide was most likely because of its basic nature.100 Although these data would suggest a nonspecific role for the C termini of Rac1 or Rac2, more recent studies show that an additional function of the C-terminal...
domain is to ensure proper post-translation prenylation of the Rac2 protein.101

Rac1 and Rac2 proteins are 92% homologous and obviously show great overlap in their biological effects. However, mice that were made deficient in Rac2 show defects in neutrophil function that could not be rescued by the remaining fraction of Rac1.20 These defects include chemotactant-stimulated actin polymerization, chemotaxis, and NOX activity.20,102 These defects were also not rescued by fMLP-induced Rac1 activation in these cells,103 and it was concluded that the specific defects that were observed in neutrophils from Rac2−/− mice were the result of isoform-specific signaling, rather than of the reduction in overall Rac protein levels.105 This is also in line with previous findings that Rac2 binds ∼6-fold better to p67phox compared with Rac1 and is more efficient in activating the oxidase.85,91,104 In human neutrophils, Rac2 is the major isoform expressed and it is concluded that the specific defects that were observed in neutrophils from Rac2−/− mice were the result of isoform-specific signaling, rather than of the reduction in overall Rac protein levels.105 This is also in line with previous findings that Rac2 binds ∼6-fold better to p67phox compared with Rac1 and is more efficient in activating the oxidase.85,91,104 In human neutrophils, Rac2 is the major isoform expressed and thus the key GTPase for oxidase activation by chemoattractants and the Fcγ receptor.103,105 But in human monocytes, Rac1 is the predominant isoform, and in these cells, Rac1 was shown to be the most important GTPase for oxidase activation.106 Clearly, expression levels, together with specificity of signaling, control the relative importance of the Rac isoforms in NOX2 activation.

Recent studies in mouse models have provided further insight in the control of differential signaling by the Rac1 and Rac2 GTPases. In Rac2−/− progenitor cell-derived neutrophils, reintroduction of Rac2 but not of Rac1 restores the fMLP-induced production of superoxide.84,105,107 Similarly, the defect in fMLP-induced chemotaxis was restored by Rac2 but not by Rac1, in line with the notion that Rac1 in neutrophils84,102,107 like in macrophages,108 is not essential for migration. In contrast, deletion of Rac1 in murine neutrophils impairs actin assembly and chemotaxis but leaves PMA- or fMLP-stimulated oxidase activity normal.109 Using chimeric Rac proteins, Filippi et al showed that the Rac2 C terminus in the background of Rac1 is sufficient for restoration of superoxide production but not of fMLP-induced chemotaxis.84 Detailed mutational analysis revealed that a negatively charged residue at position 150 (aspartic acid in Rac2) is required for cell migration. Rac1 has a glycine at this position, and mutating this to an aspartic acid, in conjunction with a Rac2 C terminus, allowed the Rac1 chimera to fully restore migration as well as actin polymerization in Rac2−/− neutrophils. In addition, the C terminus and residue 150 were shown to be critical determinants for the intracellular localization of Rac2.84

Finally, the complex activation of the neutrophil NOX is complicated even more by additional modes of regulation. Diebold et al recently found that Cdc42, which, by itself, is not able to activate the oxidase, acts as a negative regulator in the cell-free assays as well as in the Cos–Phox system because it can bind, just like Rac1 and Rac2, to the flavocytochrome b558 via its insert region.110 In line with this, a Cdc42-binding domain of Wiskott-Aldrich syndrome protein directly, as was proposed for the RacGEF PIX, binding to NOX1.58

Rac and ROS in Cardiovascular Disease

There is a vast body of literature that links vascular ROS production to cardiovascular disease.53,113 ROS production can be detected in VSMCs as well as in endothelial cells, and ROS may also be derived from infiltrating leukocytes. Important stimuli for ROS production in the vasculature are cytokines (eg, TNF-α), growth factors (eg, PDGF), and G-protein–coupled receptor agonists (eg, Ang II). Vascular ROS production as well as Rac1 activation have been associated with hypertrophy and smooth muscle cell prolif-
eration, endothelial dysfunction as well as endothelial cell migration and proliferation, hypertension inflammation, and atherosclerosis (Figure 3).7,53,113,114

Vascular hypertrophy has been ascribed to the effects of various receptor agonists, including the extensively studied Ang II. Ang II induces ROS production in VSMCs in a Rac1-dependent fashion.115 Recent studies showed that this Ang II–induced ROS production also requires the membrane adapter caveolin, which is involved in Rac1 activation,116,117 and the lipid kinase PI3K-γ.115,118 Ischemia-reperfusion injury also is accompanied by production of high levels of ROS. Here, Rac1 has clearly been implicated through expression of dominant-negative N17Rac1. Kim et al showed that in VSMCs, cell death induced by hypoxia/reoxygenation is prevented by N17Rac1.119 Similarly, Harada et al, showed that N17Rac1 expression in the liver protects from ischemia-reperfusion–induced production of ROS and activation of NF-κB.120

Several NOX isoforms have been implicated recently in Rac1-dependent ROS production. VSMCs express NOX1, NOX2, and NOX4, but NOX1 appears to be the most relevant oxidase.52 NOX1 and NOX4 are expressed at higher levels compared with NOX2, and superoxide-producing agonists such as PDGF or Ang II downregulated NOX4 expression and upregulated NOX1.52 In line with the requirement for Rac1 and ROS production in endothelial cell migration and proliferation (Figure 3),10,121–123 neovascularization in an ischemic hindlimb model, which is associated with increased expression of NOX2 and increased production of ROS, was found to be impaired after treatment with antioxidants or in NOX2-deficient animals.22,124 Use of a peptide (gp91ds-tat) that interferes with the p47phox–NOX2 interaction has provided further in vivo evidence for a role of NOX2 in Ang II–induced hypertension and smooth muscle cell hypertrophy.18,125 Similarly, in NOX2-deficient mice, induction of renovascular hypertension resulted in less endothelial dysfunction and reduced hypertension compared with the responses in wild-type animals.21

The Rac1–ROS signaling pathway clearly is a crucial mediator of cardiovascular disease (Figure 3). Based on current literature, the most relevant NOX isoforms in vascular cells appear to be NOX1 and NOX2, which can both be regulated through Rac1. NOX1 is, in endothelial cells as well as in smooth muscle cells, expressed at higher levels compared with NOX2.52,74 However, NOX2 appears to be important as well, based on studies with the NOX-inhibitory peptide, the NOX2-deficient animals, and the observations of its upregulation after, for example, ischemia and in restenosis.22,126 The role for NOX4, although highly expressed in, for example, endothelial cells and in VSMCs, is presently unknown. Increased as well as decreased expression of NOX4 has been described in various models for vascular disease as well as in human atherosclerosis52,126–129; NOX4-specific inhibitors or NOX4-deficient mice will likely provide more detailed insight into its role in cardiovascular disease.

Conclusions

The mechanism of activation of the neutrophil NOX2 has been the subject of intensive research for ~30 years. Still, new aspects are being uncovered, and the details of its activation remain topic of discussion. Elegant mouse models now allow the testing of specific mutants in a physiologically relevant background. These approaches have provided important insights in the activation processes for NOX2. In the meantime, related NOX enzymes and novel regulators have been discovered, of which the activation or assembly may prove equally complex as for the neutrophil NOX complex. Based on currently available evidence, NOX1 is most likely activated through Rac1 in combination with NOXA1 and NOXO1. NOX3 appears p67phox/NOXA1, and thus Rac1 independent, and also NOX5 seems to be independent of Rac1. The regulation of NOX4 remains to be investigated in detail, also because the suggestion that NOX4 is a constitutively active oxidase is hard to reconcile with its relatively high expression levels, for example, in endothelial cells. Although many aspects of NOX regulation are still unknown, the available data do suggest that the different NOXes are regulated through distinct mechanisms or regulators. This suggests that in cells, where several NOXes are coexpressed, as in VSMCs and in endothelial cells, these may be activated individually, depending on the stimulus, their localization, or the condition of the cells. There is increasing evidence for an important role of Rac-mediated activation of NOXes in cardiovascular disease, which makes these signaling complexes an important therapeutic target. The challenge lies in the use of appropriate model systems that allow generation of consistent data. The use of appropriate mouse models or knockdown approaches targeting endogenous NOXes or their regulators will likely provide important progress in our understanding of the activation of this multimolecular protein complex.

Acknowledgments

D. Roos is gratefully acknowledged for critical reading. The author is a fellow of the Landsteiner Foundation for Blood Transfusion Research.

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Regulation of NADPH Oxidases: The Role of Rac Proteins
Peter L. Hordijk

Circ Res. 2006;98:453-462
doi: 10.1161/01.RES.0000204727.46710.5e
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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