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The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology

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Bedard K, Krause, K-H. The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. *Physiol Rev* 87: 245–313, 2007; doi:10.1152/physrev.00044.2005.—For a long time, superoxide generation by an NADPH oxidase was considered as an oddity only found in professional phagocytes. Over the last years, six homologs of the cytochrome subunit of the phagocyte NADPH oxidase were found: NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2. Together with the phagocyte NADPH oxidase itself (NOX2/gp91$^{\text{phox}}$), the homologs are now referred to as the NOX family of NADPH oxidases. These enzymes share the capacity to transport electrons across the plasma membrane and to generate superoxide and other downstream reactive oxygen species (ROS). Activation mechanisms and tissue distribution of the different members of the family are markedly different. The physiological functions of NOX family enzymes include host defense, posttranlational processing of proteins, cellular signaling, regulation of gene expression, and cell differentiation. NOX enzymes also contribute to a wide range of pathological

processes. NOX deficiency may lead to immunosuppression, lack of otoconogenesis, or hypothyroidism. Increased NOX actvity also contributes to a large number or pathologies, in particular cardiovascular diseases and neurodegeneration. This review summarizes the current state of knowledge of the functions of NOX enzymes in physiology and pathology.

I. INTRODUCTION

The NOX family NADPH oxidases are proteins that transfer electrons across biological membranes. In general, the electron acceptor is oxygen and the product of the electron transfer reaction is superoxide. The biological function of NOX enzymes is therefore the generation of reactive oxygen species.

A. Reactive Oxygen Species

Reactive oxygen species (ROS) are oxygen-derived small molecules, including oxygen radicals [superoxide $(O_2^{\bullet-})$, hydroxyl $(^{\bullet}OH)$, peroxyl $(RO_2^{\bullet})$, and alkoxyl $(RO\cdot)$] and certain nonradicals that are either oxidizing agents and/or are easily converted into radicals, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen $(^1O_2)$, and hydrogen peroxide (H_2O_2). Nitrogen-containing oxidants, such as nitric oxide, are called reactive nitrogen species (RNS). ROS generation is generally a cascade of reactions that starts with the production of superoxide. Superoxide rapidly dismutates to hydrogen peroxide either spontaneously, particularly at low pH or catalyzed by superoxide dismutase. Other elements in the cascade of ROS generation include the reaction of superoxide with nitric oxide to form peroxynitrite, the peroxidase-catalyzed formation of hypochlorous acid from hydrogen peroxide, and the iron-catalyzed Fenton reaction leading to the generation of hydroxyl radical (468, 874).

ROS avidly interact with a large number of molecules including other small inorganic molecules as well as proteins, lipids, carbohydrates, and nucleic acids. Through such interactions, ROS may irreversibly destroy or alter the function of the target molecule. Consequently, ROS have been increasingly identified as major contributors to damage in biological organisms. In 1956, Harmann made his ground-breaking observations on the role of ROS in the aging process (350), and the concept of ROS as agents of cellular damage became widely accepted in theories of aging (73). Yet, at least one beneficial function of ROS production was also realized quite early, namely, the importance of ROS in host defense. This point became particularly clear when the link was made between deficiency in ROS generation and reduced killing ability in leukocytes. However, over the last decades, a second important concept of ROS has been evolving. In fact, ROS are involved not only in cellular damage and killing of pathogens, but also in a large number of reversible regulatory processes in virtually all cells and tissues. This review discusses both the physiological and pathophysiological role of ROS generated by the NADPH oxidase family of enzymes.

B. Physiological Sources of ROS

The physiological generation of ROS can occur as a byproduct of other biological reactions. ROS generation as a byproduct occurs with mitochondria, peroxisomes, cytochrome P-450, and other cellular elements (50, 307, 314, 356, 588, 636, 715, 791, 874). However, the phagocyte NADPH oxidase was the first identified example of a system that generates ROS not as a byproduct, but rather as the primary function of the enzyme system. The discovery of other members of the NOX family of NADPH oxidases demonstrated that enzymes with the primary function of ROS generation are not limited to phagocytes. In fact, the ROS-generating enzymes described in this review are found in virtually every tissue. This review focuses on novel homologs of the phagocyte NADPH oxidase. A complete coverage of the biochemistry and physiology of the phagocyte NADPH oxidase itself is beyond the scope of this review. However, we give an overview of the phagocyte NADPH oxidase to serve as a framework to understand the specific features of novel NOX isoforms (see sect. II*A1*).

C. ROS-Generating NADPH Oxidase: A Historical Overview

Although the NADPH oxidase was not yet identified, a respiratory burst by cells had already been described by the first half of the 20th century. These early observations were done in sea urchin eggs (938), phagocytes in 1933 (51), and spermatocytes in 1943 (565). In 1959, Sbarra and Karnovsky (787) demonstrated that the phagocyte respiratory burst was an energy-requiring process that depended on glucose metabolism. Shortly after, in 1961, Iyer et al. (419) showed that the phagocyte respiratory burst results in the generation of hydrogen peroxide. There was a major controversy over whether the main substrate for the enzyme system was NADPH or NADH. In 1964, Rossi and Zatti (755) correctly proposed that an NADPH oxidase was responsible for the respiratory burst. In 1970, Klebanoff (466) demonstrated a contribution of myeloperoxidase to the respiratory burst-dependent antimicrobial activity of phagocytes. In 1973, Babior et al. (43) reported that the initial product of the respiratory burst oxidase was superoxide and not hydrogen peroxide.

A second important line of study that led to the discovery of the phagocyte NADPH oxidase came from clinical research. In 1957, Berendes et al. (83) recognized a new and relatively rare syndrome in young boys who suffered from recurrent pyogenic infections that was accompanied with granulomatous reaction, lymphadenopathy, and hypergammaglobulinemia. The genetic disorder is now referred to as chronic granulomatous disease (CGD). Quie et al. (721) showed that CGD phagocytes have diminished bactericidal capacity, although many phagocyte functions, such as chemotaxis, phagocytosis, and degranulation, were found to be intact in CGD phagocytes. In 1967, it was recognized that the respiratory burst was absent in the phagocytes of CGD patients (47, 385, 721).

Further characterization of ROS generation by phagocytes revealed that this enzyme system *1*) produced superoxide and its downstream metabolite hydrogen peroxide; *2*) was insensitive to cyanide, distinguishing it from mitochondria and myeloperoxidase (MPO); *3*) was present in phagocytes from MPO-deficient patients, but absent in those of CGD patients; and *4*) was selective for NADPH over NADH by a factor of 100 (42).

The identification of proteins responsible for ROS production in phagocytes was the next challenge. A breakthrough occurred in 1978, when Segal, Jones, and colleagues (798, 799) identified cytochrome b_{558}, which was missing in the leukocytes of many CGD patients. In the late 1980s, the gene coding for the catalytic subunit of the phagocyte NADPH oxidase, commonly referred to as gp91^{phox}, was cloned by Royer-Pokora et al. (762) and Teahan et al. (865). In the novel NOX terminology, gp91^{phox} is called NOX2.

However, it was rapidly understood that NOX2 was not the only component of the phagocyte enzyme. In 1987, the transmembrane protein p22^{phox} was discovered as the membrane subunit associated with NOX2 (216, 693, 795). The development of a cell-free system allowed activation of the phagocyte NADPH oxidase using purified cytosol and membrane fractions (110, 373). This system provided the tools to discover the cytosolic subunits p47^{phox} and p67^{phox} (660, 923) and to define the roles of the small GTP-binding proteins Rac1 and Rac2 (4, 470). In 1993, Wientjes et al. (951) described a third cytosolic subunit, p40^{phox}.

In parallel with the progress toward understanding the phagocyte NADPH oxidase, a series of observations suggested that enzyme systems similar to the phagocyte NADPH oxidase exist in many other cell types, including fibroblasts (600), various tumor cells (855), and vascular smooth muscle (321). Fibroblasts from NOX2-deficient patients had normal ROS generation, suggesting that the phagocyte NADPH oxidase was not the source of ROS in fibroblasts (249). However, the molecular identity of the NADPH oxidase-like systems in nonphagocytic cells remained obscure. That was fundamentally changed by the availability of the human genome sequence. Two groups independently identified a first homolog of NOX2, which was initially referred to as mitogenic oxidase 1 (mox-1; Ref. 841) or NADPH oxidase homolog 1 (NOH-1; Ref. 55); this isoform has been named NOX1 in the novel terminology.

The identification of NOX1 was quickly followed by the cloning of NOX3 (143, 454), NOX4 (294, 813), and NOX5 (56, 143). In parallel with the identification of NOX1 to NOX5, two very large members of the NOX family were discovered, namely, DUOX1 and DUOX2, initially also referred to as thyroid oxidases (189, 228).

The identification of the new NOX/DUOX proteins was not, however, always followed by an immediate demonstration of their biochemical function. Indeed, the closest NOX2 homolog, NOX1, is usually inactive when transfected by itself. A search for homologs of the cytosolic subunits of the phagocyte NADPH oxidase (p47^{phox} and p67^{phox}) led to the cloning of a novel set of cytosolic subunits, NOXO1 and NOXA1 (53, 297, 857). Similarly, heterologous expression of DUOX enzymes is only successfully achieved since the identification of the DUOX maturation factors DUOXA1 and DUOXA2 (319).

Is the NOX family complete? Given the essentially complete databases of several mammalian genomes, it appears likely that most specific elements of the NOX system have now been identified: seven NOX isoforms, two organizer subunits (p47^{phox}, NOXO1), two activator subunits (p67^{phox}, NOXA1), and two DUOX-specific maturation factors (DUOXA1 and DUOXA2). There is, however, some space for continuing gene discovery: nonessential, modulatory subunits such as p40^{phox} might have gone unrecognized, and the possible existence of an unidentified p22^{phox} homolog has been suggested (844).

II. THE NOX FAMILY OF NADPH OXIDASE: INTRODUCING THE PLAYERS

A. The NOX Family Members

The description of NOX family members in this review focuses on mammalian NOX homologs and subunits (Table 1). Some features that are invariable for mammalian NOX enzymes (e.g., electron transfer to oxygen) might be different in nonmammalian organisms (e.g., ferric reductases in yeast).

All NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. In accordance with this preserved function, there are conserved structural properties of NOX enzymes that are common to all family members. Starting from the COOH terminus, these conserved structural features include *1*) an NADPH-bind-

TABLE 1. *NOX enzymes and subunits*

	Other Names	Chromosome Location	Gene Length	Amino Acids	Total SNP	cnSNP	cnSNP Amino Acid Change	cnSNP Nucleotide Substitution
NOX1	NOH-1, MOX1, GP91-2	Xq22	30374	564	79	1	His315Arg	A944G
NOX2	CYBB, gp91phox	Xp21.1	33451	570	69	3	Ser472Gly, Asn303His, Asp500Gly	A1414G, A907C, A1499G
NOX3	GP91-3	6q25.1-26	60534	568	228	1	Lys171Thr	A512C
NOX4	RENOX, KOX-1, KOX	11q14.2-q21	165139	578	858	1	Ile315Met	A945G
NOX5		15q22.31	42392	747	263	8	Gln134His, Leu362Phe Gly741Arg, Glu593Lys Gly191Glu, Phe133Leu Asn505Lys, His558Arg	G402T, C1084T G2221A, G1777A G572A, T399G T1515G, A1673G
DUOX1	Thox1, LNOX1, NOXEF1	15q21	35583	1,551	188	4	Arg1026Cys, Thr962Ile Phe1178Leu, His1216Arg	C3076T, C2885T T3532C, A3646G
DUOX2	Thox2, LNOX2, NOXEF2, p138lox	15q15.3-q21	20757	1,548	188	6	His683Arg, Glu143Asp Arg200Gly, Gln1009Arg Pro138Leu, Ser1067Leu	A2048G, A429C A598G, A3026G C413T, C3200T
p22phox	CYBA	16q24	9486	195	117	7	Gln158Arg, Thr75Ala Phe38Ser, Thr29Ala Val174Ala, His72Tyr Arg24Gly	A473G, A223G T113C, A85G T521C, C214T* A70G
p47phox	NOXO2, NCF1, NCF47K	7q11.23	15349	390	76	0		
NOXO1	p41NOX	16p13.3	2522	370	13	0		
p67phox	NOXA2, NCF2	1q25	34845	526	125	4	Met279Thr, Trp395Arg Arg181Lys, Gln389His	T836C, T1183C A542G, A1167C
NOXA1	p51NOX	9q34.3	11011	483	38	0		
p40phox	NCF4	22q1,3.1	17028	339	100	4	Thr29Ile, Ans118Ser Glu304Ala, Pro272Leu	C86T, A353G A911C, C815T

Alternative names, chromosomal localization, gene length, number of amino acids, total number of single-nucleotide polymorphisms (total SNP), number of coding nonsynonymous single-nucleotide polymorphisms (cnSNP), amino acid changes in cnSNPs, and nucleotide substitutions in cnSNPs are shown. The number of SNPs is based on presently available NCBI databank entries. The position of the nucleotide substitution is given relative to the start codon (207). * C214T in p22phox was originally called C242T (411), a name still widely used in the literature.

ing site at the very COOH terminus, *2*) a FAD-binding region in proximity of the most COOH-terminal transmembrane domain, *3*) six conserved transmembrane domains, and *4*) four highly conserved heme-binding histidines, two in the third and two in the fifth transmembrane domain (Fig. 1). Given the additional NH_2-terminal transmembrane domain, the histidines are in the fourth and sixth transmembrane domains in DUOX proteins. Additional features, such as EF hands, an additional NH_2-terminal transmembrane domain, and/or a peroxidase homology domain, are limited to some of the family members and are discussed in the respective sections.

Most data suggest that the new NOX family members are also selective for NADPH over NADH. However, some researchers feel that the issue is not entirely resolved and therefore apply the term *NAD(P)H oxidase* when referring to NOX family members. We discuss in the respective sections the present knowledge about NADPH versus NADH usage by the different NOX family members.

1. NOX2: prototype NOX

NOX2, also known as gp91phox, is the prototype NADPH oxidase. Its biochemical features have been ex-

tensively studied and abundantly reviewed in the recent past (44, 175, 652, 750, 917). Thus an extended coverage of NOX2 goes beyond the scope of this review. However, we summarize the most important features of NOX2, with a particular focus on the properties of NOX2 that allow a better understanding of other NOX isoforms.

Much of what is known about the topography and structure of the NOX isoforms is derived from studies on NOX2. Yet, even for NOX2, the suggested topographical features are deduced from indirect data and hence are putative assignments. Definitive assignments will have to await crystallographic studies, which unfortunately have not been achieved yet. Still, it is likely that the basic features, outlined in Figure 1, are correct. Hydropathy plots predict between four and six transmembrane domains for NOX2 (365, 800). Phage display library screening provide experimental data defining the extracellular domains (118, 406, 644). Antibody mapping studies demonstrate a cytoplasmic localization of the COOH terminus (119, 406, 757). Sequencing data and antibody mapping confirm a cytoplasmic NH_2 terminus (676, 865), over the alternative suggestion that the NH_2 terminus is proteolytically cleaved (143). Taken together, the available data suggest that NOX2 has six transmembrane domains
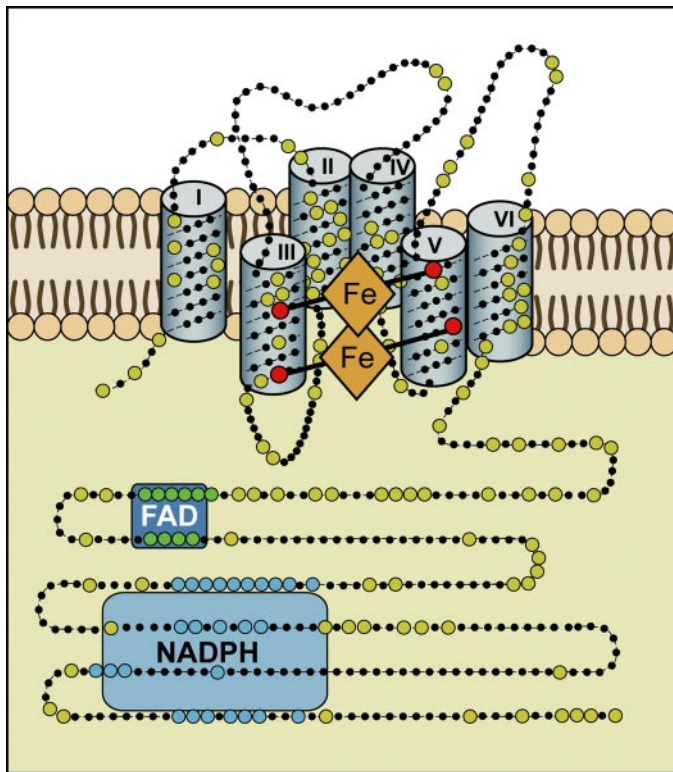
FIG. 1. Proposed structure of the core region of NADPH oxidase (NOX) enzymes. No crystal structure data of NOX enzymes are presently available. A consensus regarding basic features of the core region of NOX enzymes has emerged based on indirect data. All NOX family members share six highly conserved transmembrane domains. Transmembrane domains III and V each contain two histidines, spanning two asymmetrical hemes. The cytoplasmic COOH terminus contains conserved flavin adenine dinucleotide (FAD) and NADPH binding domains. NOX enzymes are thought to be single electron transporters, passing electrons from NADPH to FAD, to the first heme, to the second heme, and finally to oxygen. Enlarged circles represent amino acids that are conserved through human NOX1, NOX2, NOX3, and NOX4.

and that its COOH terminus and its NH_2 terminus are facing the cytoplasm.

Human NOX2 is a highly glycosylated protein that appears as a broad smear on SDS-PAGE reflecting the heterogeneity of glycosylation. The fully glycosylated form runs with an apparent molecular mass of $\sim$70–90 kDa. Removal of the carbohydrates by endoglycosidase F leaves a protein that runs at 55 kDa, demonstrating the extent of glycosylation (351). The carbohydrate chains are composed of N-acetylglucosamine and galactose and, to a lesser extent, frucose, mannose, and glucose (351). A mutagenesis approach demonstrates that the carbohydrates are bound to asparagine residues (^{132}Asn, ^{149}Asn, and ^{240}Asn) in the second and third predicted extracellular loops (929).

The activation of NOX2 occurs through a complex series of protein/protein interactions (Fig. 2; for more detailed recent reviews, see Refs. 328, 652, 844). NOX2 constitutively associates with p22^{phox}. Indeed, the NOX2

protein is unstable in the absence of p22^{phox}, and phagocytes from p22^{phox}-deficient patients have no detectable NOX2 protein (217, 692, 828). Activation of NOX2 requires translocation of cytosolic factors to the NOX2/p22^{phox} complex (Fig. 3). The present working model is as follows. Phosphorylation of p47^{phox} leads to a conformational change allowing its interaction with p22^{phox} (327, 843). It is thought that p47^{phox} organizes the translocation
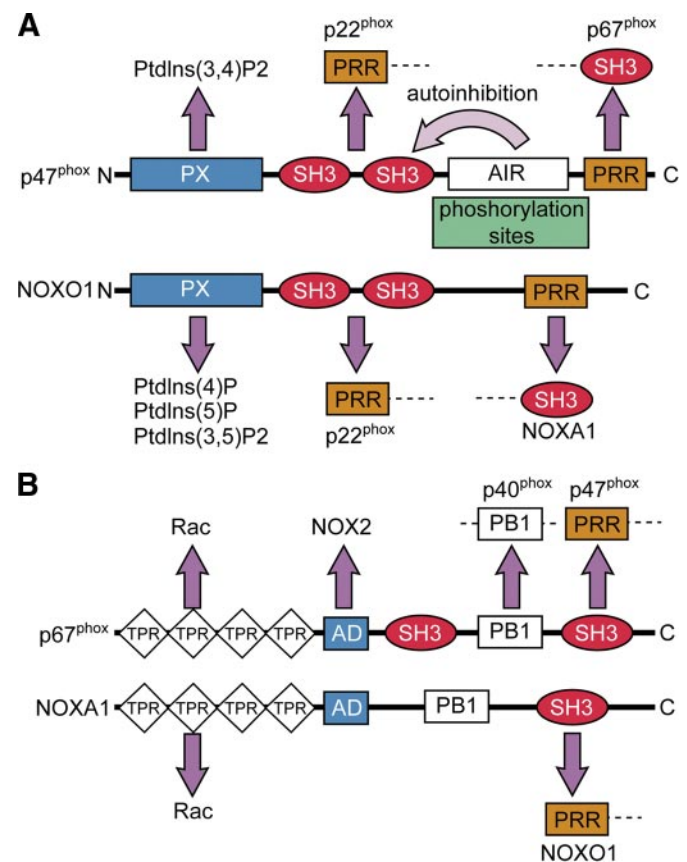

FIG. 2. Molecular interactions of cytoplasmic organizer and activator subunits. NOX1, NOX2, and NOX3 require cytoplasmic subunits for activation. In general, p47^{phox} is thought to be the organizer subunit of NOX2, and NOXO1 for NOX1 and NOX3. p67^{phox} is the activator subunit for NOX2, and NOXA1 is the activator subunit for NOX1. In the human system, NOX3 does not appear to require an activator subunit. A: the two organizer homologs, p47^{phox} and NOXO1, share a similar set of motifs. Both have NH_2-terminal "phox homology" (PX) domains that bind to phospholipids in the membrane, although they differ in phospholipid specificity. They also both interact through tandem Src homology 3 (SH3) domains with the proline-rich region (PRR) of p22^{phox}. Only p47^{phox}, but not NOXO1, has an autoinhibitory region (AIR) that is inactivated upon phosphorylation, allowing interaction of the tandem SH3 domain with p22^{phox}. Finally, both p47^{phox} and NOXO1 contain COOH-terminal PRRs, which allow interaction with p67^{phox} and NOXA1, respectively. Both p47^{phox} and NOXO1 might also directly interact with their respective NOX enzymes. B: the two activator homologs, p67^{phox} and NOXA1, also share a similar overall domain structure. Both have NH_2-terminal tetricopeptide repeat (TPR) domains that interact with Rac. Both have activation domains (AD); the functional role of this domain is thus far only documented for the p67^{phox}-NOX2 interaction. p67^{phox} and NOXA1 have COOH-terminal SH3 binding domains, which interact with p47^{phox} and NOXO1, respectively. The phox and Bem1 (PB1) domain of p67^{phox} interacts with p40^{phox}.
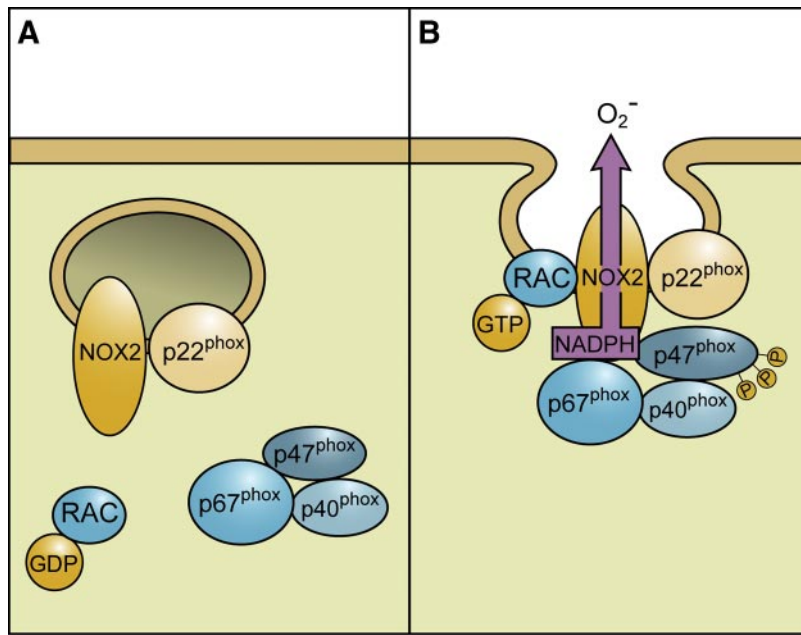
FIG. 3. Assembly of the phagocyte NADPH oxidase NOX2. The phagocyte NADPH oxidase was the first identified and is the best studied member of the NOX family. It is highly expressed in granulocytes and monocyte/macrophages and contributes to killing of microbes. In resting neutrophil granulocytes, NOX2 and $p22^{phox}$ are found primarily in the membrane of intracellular vesicles. They exist in close association, costabilizing one another. Upon activation, there is an exchange of GDP for GTP on Rac leading to its activation. Phosphorylation of the cytosolic $p47^{phox}$ subunit leads to conformational changes allowing interaction with $p22^{phox}$. The movement of $p47^{phox}$ brings with it the other cytoplasmic subunits, $p67^{phox}$ and $p40^{phox}$, to form the active NOX2 enzyme complex. Once activated, there is a fusion of NOX2-containing vesicles with the plasma membrane or the phagosomal membrane. The active enzyme complex transports electrons from cytoplasmic NADPH to extracellular or phagosomal oxygen to generate superoxide (O_2^-).

of other cytosolic factors, hence its designation as "organizer subunit." The localization of $p47^{phox}$ to the membrane brings the "activator subunit" $p67^{phox}$ into contact with NOX2 (342) and also brings the small subunit $p40^{phox}$ to the complex. Finally, the GTPase Rac interacts with NOX2 via a two-step mechanism involving an initial direct interaction with NOX2 (214), followed by a subsequent interaction with $p67^{phox}$ (476, 508). Once assembled, the complex is active and generates superoxide by transferring an electron from NADPH in the cytosol to oxygen on the luminal or extracellular space.

NOX2 can be regarded as a transmembrane redox chain that connects the electron donor (Fig. 1), NADPH on the cytosolic side of the membrane with the electron acceptor, oxygen on the outer side of the membrane. It transfers electrons through a series of steps involving a flavin adenine dinucleotide (FAD), binding to amino acids $_{337}$HPFTLSA and $_{355}$IRIVGD (917) and two assymetrical hemes found in transmembrane domains III and V, with the inner heme binding to histidines H101 and H209 and the outer heme binding to histidine H115 and H222 (262).

In the first step, electrons are transferred from NADPH to FAD, a process that is regulated by the activation domain of $p67^{phox}$ (658). NOX2 is selective for NADPH over NADH as a substrate, with K_m values of $40–45$ μM versus 2.5 mM, respectively (160). In the second step, a single electron is transferred from the reduced flavin $FADH_2$ to the iron center of the inner heme. Since the iron of the heme can only accept one electron, the inner heme must donate its electron to the outer heme before the second electron can be accepted from the now partially reduced flavin, FADH. The force for the transfer of the second electron, while smaller (31 vs. 79 mV), is

still energetically favorable. However, the transfer of the electron from the inner heme to the outer heme is actually against the electromotive force between these two groups. To create an energetically favorable state, oxygen must be bound to the outer heme to accept the electron (175, 223, 917).

NOX2 was first described in neutrophils and macrophages and is often referred to as the phagocyte NADPH oxidase. NOX2 is still widely considered to have a very limited, essentially phagocyte-specific tissue expression (e.g., Ref. 844), yet when tissue distribution of total mRNA from various organs is investigated, NOX2 appears to be among the most widely distributed among the NOX isoforms (Table 2). It is described in a large number of tissues, including thymus, small intestine, colon, spleen, pancreas, ovary, placenta, prostate, and testis (143). Mostly this wide tissue distribution is due to the presence of phagocytes and/or blood contamination in the tissues from which total mRNA has been extracted. However, there is now also increasing evidence at both the message and the protein level for expression of NOX2 in nonphagocytic cells, including neurons (806), cardiomyocytes (372), skeletal muscle myocytes (426), hepatocytes (739), endothelial cells (313, 434, 538), and hematopoietic stem cells (704).

In phagocytes, NOX2 localizes to both intracellular and plasma membranes in close association with the membrane protein $p22^{phox}$ (97, 394). In resting neutrophils, most of the NOX2 localizes to intracellular compartments, in particular secondary (i.e., specific) granules (26, 97, 439) and tertiary (i.e., gelatinase-containing) granules (465). Upon phagocyte stimulation, there is a translocation of NOX2 to the surface as the granules fuse with the

phagosomal or the plasma membrane (97, 286, 304). This fusion is thought to be a key event for the microbicidal activity of NOX2. However, NOX2 can also be activated within the granules without a need for fusion with surface membranes (562, 898). The resulting intracellular ROS generation might be involved in signaling functions of NOX2 (439).

In cells other than phagocytes, the subcellular distribution varies depending on the specific cell type. In smooth muscle cells, NOX2 is found to colocalize with the perinuclear cytoskeleton (538). In hippocampal neurons, NOX2 is suggested to be localized in the membranes of synaptic sites (866), possibly playing a role in superoxide-dependent long-term potentiation and memory function.

The human and mouse NOX2 gene is located on the X chromosome. At the transcriptional level, a promoter region sufficient for NOX2 expression in monocytes lies in the 450-bp region before the transcriptional start site (818). However, additional regulatory regions located up to 60 kb upstream from the start site also influence expression, with sensitive regions at -13, -15, -28, and -29 kb (545). NOX2 gene expression is regulated by repressing and activating factors. Repressing factors include the CCAAT displacement protein (CDP) (563, 819), HOXA10, and Meis1 (76). Activating factors include PU.1, Elf-1, YY1, and interferon regulatory factors IRF1 and IRF2 (243, 422, 850, 924), NFκB (28), HOXA9 (76), and PB1 (76). The PU.1 transcription factor appears to have a particularly important role in the expression of NOX2, as PU.1-deficient mice fail to express NOX2 (850). Mutations in the NOX2 promoter region at positions -53, -52, or -50 interfere with PU.1 binding, and CGD patients with such mutations do not express NOX2 in neutrophils, monocytes, and B lymphocytes. However, the importance of the PU.1 promoter may be somewhat cell specific, as these patients do express NOX2 in eosinophils (850).

NOX2 gene expression is inducible. This has been demonstrated in phagocytes in response to interferon-γ (mRNA) (654), in myofibroblasts after carotid artery injury (mRNA) (856), and in cardiomyocytes after acute myocardial infarction (protein) (488). NOX2 expression is also increased in response to angiotensin II in adipose tissue (mRNA) (359), aorta (mRNA) (359), heart (mRNA) (359), resistance artery vascular smooth muscle cells (mRNA and protein) (881), and pancreatic islets (protein) (648). Note however that an increase in NOX2 levels is not invariably due to transcriptional activation. In the case of resistance arteries, the angiotensin II-induced NOX2 elevation may be due to increased de novo protein synthesis, regulated at a posttranscriptional level (881).

2. NOX1

NOX1 was the first homolog of NOX2 (then called gp91$^{\mathrm{phox}}$) to be described (55, 841). NOX1 and NOX2

genes appear to be the result of a relatively recent gene duplication, as the number and the length of the exons is virtually identical between the two genes (53). Similarly, at the protein level, there is a high degree of sequence identity ($\sim$60%) between NOX1 and NOX2 (55, 841). The human and mouse NOX1 gene is located on the X chromosome. An alternatively spliced form of NOX1 lacks exon 11 (55, 296, 353). It appears that this splice variant encodes a protein incapable of producing superoxide (296). The existence of a second very short isoform of NOX1 had been suggested; however, this turned out to be an artifact due to the formation of a stable loop in the NOX1 mRNA (see Refs. 55, 296, 353). In mouse, several other splice variants due to the use of alternative promoters have been described (29). Most studies suggest a molecular mass of NOX1 in the range of 55–60 kDa (24, 178, 425). If these values are correct, NOX1 is most likely not *N*-glycosylated, despite the presence of two NXT/S consensus glycosylation sites in the extracellular domains.

The message for NOX1 is most highly expressed in colon epithelium (53, 841, 854); however, it is also expressed in a variety of other cell types, including vascular smooth muscle cells (510, 841), endothelial cells (14, 473), uterus (55, 841), placenta (178), prostate (55, 841), osteoclasts (516), retinal pericytes (576), as well as in several cell lines, such as the colon tumor cell lines Caco-2 (159, 701), DLD-1 (701), and HT-29 (701) and the pulmonary epithelial cell line A549 (320). Expression of NOX1 in the gastric mucosa is species dependent. It was not found in human stomach (770) but is functionally expressed in guinea pig stomach pit cells and mucosal cells (444, 447, 868).

In addition to its constitutive expression in a variety of tissues, the NOX1 message is induced under many circumstances. In vascular smooth muscle, platelet-derived growth factor (PDGF), prostaglandin F$_{2\alpha}$, and angiotensin II induce NOX1 expression (441, 510, 841, 955). NOX1 is upregulated in restenosing carotid artery after injury (856) and in prostate in response to castration (859). Other conditions where NOX1 upregulation is observed include interferon-γ addition to Caco-2 colonocytes (295), Ras expression in rat kidney cells (614), BMP4 stimulation of endothelial cells (823), and *Helicobacter pylori* lipopolysaccharide stimulation of guinea pig gastric mucosal cells (443). Studies on the inducible expression of NOX1 in the vascular system suggest an involvement of epidermal growth factor (EGF) receptor transactivation and involvement of ATF-1, phosphatidylinositol 3-kinase, and protein kinase C (PKC)-δ (252). In renal mesangial cells, nitric oxide downregulates NOX1 (mRNA and protein) (707).

The 5'-region of the human NOX1 gene contains binding elements for signal transducers and activators of transcription (STATs), interferon regulatory factor (IRF), AP-1, NFκB, CREB, CBP/p300 elements (498), and GATA

TABLE 2. *Tissue distribution of NOX enzymes*

	High-Level Expression	Intermediate- to Low-Level Expression	Reference Nos.
NOX1	Colon	Smooth muscle, endothelium, uterus, placenta, prostate, osteoclasts, retinal pericytes	14, 53, 55, 178, 473, 510, 516, 576, 841, 854
NOX2	Phagocytes	B lymphocytes, neurons, cardiomyocytes, skeletal muscle, hepatocytes, endothelium, hematopoietic stem cells, smooth muscle	143, 313, 372, 426, 434, 538, 704, 739, 806, 844
NOX3	Inner ear	Fetal kidney, fetal spleen, skull bone, brain	54, 143, 454, 677
NOX4	Kidney, blood vessels	Osteoclasts, endothelium, smooth muscle, hematopoietic stem cells, fibroblasts, keratinocytes, melanoma cells, neurons	14, 105, 135, 170, 176, 211, 247, 294, 383, 392, 425, 510, 699, 704, 813, 836, 900, 901, 969, 973
NOX5	Lymphoid tissue, testis	Endothelium, smooth muscle, pancreas, placenta, ovary, uterus, stomach, various fetal tissues	56, 143, 770
DUOX1	Thyroid	Airway epithelia, tongue epithelium, cerebellum, testis	189, 229, 271, 299, 794, 931
DUOX2	Thyroid	Salivary and rectal glands, gastrointestinal epithelia, airway epithelia, uterus, gall bladder, pancreatic islets	189, 229, 230, 246, 271, 299, 794, 931

NOX enzymes are expressed in a small number of tissues at high levels (readily detected by Northern blotting) but show intermediate- to low-level expression in many other tissues.

factors (107). Constitutive expression of NOX1 in intestinal epithelial cells depends on the GATA-binding sites (107), while interferon-γ-enhanced expression is regulated by the binding of activated STAT1 dimers to the γ-activated sequence (GAS) element (498). Note that the GATA sites are within the 520-bp region upstream of the transcription initiation site, while the GAS elements is located at −3818 to −3810 bp (498). In the mouse NOX1 gene, additional promoters up to 110 kb upstream from the transcription initiation site give rise to NOX1 splice variants (29). Within the colon, there is a gradient of NOX1 expression with levels being low in the proximal and high in the distal colon (302, 854). However, at this point, it is not clear whether this gradient is constitutive or secondary to bacterial colonization.

Data on the subcellular localization of NOX1 are scarce, mainly because the generation of high quality antibodies against NOX1 (and other NOX isoforms) turned out to be a challenge and some of the antibodies used were not subjected to rigorous validation protocols. With this limitation in mind, there are several studies reporting a subcellular localization of NOX1; in keratinocytes, there was a weak cytoplasmic and a strong nuclear staining (134). One study in vascular smooth muscle suggests an ER pattern (425), while another describes punctate patches along cell surface membranes, possibly corresponding to a caveolar localization (378).

In studies using a cell-free system, NOX1 is selective for NADPH over NADH as a substrate (977).

After the initial discovery of NOX1, it was not immediately obvious whether NOX1 was indeed a superoxide generating enzyme. While one group reported a very low level of superoxide generation in NOX1-transfected cells

without the need of a stimulus (841), other groups did not observe such ROS generation by NOX1 alone (53, 295, 297). The discovery of colon homologs of the cytosolic subunits of the phagocyte NADPH oxidase resolved the issue (53, 146, 297, 857): superoxide generation by NOX1 depends on cytosolic subunits. The novel cytosolic subunits were named NOXO1 (NOX organizer 1 = p47phox homolog) and NOXA1 (NOX activator 1 = p67phox homolog). Details concerning these proteins are discussed below. The discovery of the subunit dependence of NOX1 introduced new complexities. First, in transfected cells, NOX1 is also able to use the p47phox and p67phox subunits, suggesting that cytosolic subunits are not specific for a given NOX protein (53). For example, it might be possible that p47phox acts as a subunit of NOX1 in the vascular system (see below). Second, while expression systems using the mouse proteins suggest a constitutive activity of the NOX1/NOXO1/NOXA1 system, studies using human proteins show only a weak constitutive activity, and full activation depends on activation through the PKC activator phorbol 12-myristate 13-acetate (PMA) (297, 857). There are indeed significant differences between the mouse and the human proteins, in particular, in the region of the phox homology domain which is distinct in human NOXO1. Yet, at this point, it is not clear whether the difference in PKC dependence is really due to a difference between the mouse and human NOX1/NOXO1/NOXA1 system or whether this reflects some experimental details, such as cell lines or the use of transient versus stable expression systems.

In addition to its dependence on cytosolic subunits, NOX1 requires the membrane subunit p22phox (24, 446). The p22phox dependence of NOX1 might be less stringent

than the one observed for NOX2 and NOX3 (844). There is now also ample evidence for an involvement of the small GTPase Rac in the regulation of NOX1 activity (144, 443, 615, 687, 844, 857, 892). Rac binds to the TPR domain of the activator subunit NOXA1 (144, 857, 892), but in analogy with NOX2, Rac activation of NOX1 might be a two-step process that also includes a direct binding to NOX1.

3. NOX3

NOX3 was described in 2000 based on its sequence similarity to other NOX isoforms (454), although the first studies on the function of the protein did not appear until 2004 (54, 677). NOX3 shares ~56% amino acid identity with NOX2. The gene for human NOX3 is located on chromosome 6. Sequence alignment and hydropathy plot analysis predict the overall structure of NOX3 to be highly similar to that of NOX1 and NOX2, in terms of transmembrane domains, the length of the extracellular loops, NADPH- and FAD-binding sites, and the localization of the heme-coordinating histidines (143, 454). To date, no splice variants of NOX3 have been reported.

Two different approaches led to the definition of NOX3 as an NADPH oxidase of the inner ear. A characterization of the "head tilt" mutant mouse through reverse genetics revealed underlying mutations in the NOX3 gene (677); as the head tilt mouse has vestibular defects, a functional role of NOX3 in the inner ear was established. Based on an EST clone derived from the inner ear, another study performed detailed analysis of NOX3 distribution by real time PCR and in situ hybridization and found very high NOX3 expression in the inner ear, including the cochlear and vestibular sensory epithelia and the spiral ganglion (54). Low levels of NOX3 can also be detected in other tissues, including fetal spleen (454), fetal kidney (54, 143), skull bone, and brain (54).

Nothing is currently published about the promoter region of NOX3; however, given the highly restricted tissue distribution, it seems likely that the expression is under the control of a distinct set of regulatory factors. Similarly, nothing is known about the subcellular localization of NOX3.

Our present knowledge on subunit dependence of NOX3 is as follows.

A) P22^{PHOX}. NOX3 is a p22^{phox}-dependent enzyme. NOX3 expression stabilizes the p22^{phox} protein (891) and leads to p22^{phox} translocation to the plasma membrane (892). In functional studies, p22^{phox} is required for NOX3 activation (446, 891), and truncated p22^{phox} inhibits ROS generation by NOX3 (446). Yet, there remain some doubts about the in vivo relevance of p22^{phox} for NOX3 function, as no vestibular dysfunction has been reported for p22^{phox}-deficient CGD patients.

B) ABSENCE OF CYTOSOLIC SUBUNITS. In the absence of cytosolic subunits, heterologously expressed NOX3 was found either to be inactive (147), weakly active (54, 892), or substantially active (891).

C) NOXO1. An enhanced activation of NOX3 in the presence of NOXO1 was found in all studies (54, 147, 891, 892). The strongest argument for a crucial role of NOXO1 in NOX3 activation however comes from in vivo studies demonstrating that inactivation of NOXO1 mimicks the phenotype of NOX3-deficient mice (463). Thus there can be little doubt that NOXO1 is in vivo an essential partner of NOX3.

D) NOXA1. The results on the requirement for NOXA1 are contradictory: while some studies found enhancement of NOX3 activity through NOXA1 (54, 892), others did not (147, 891). Thus the results of heterologous expression studies depend on the experimental conditions, and in vivo data with NOXA1-deficient animals will be necessary to clarify the issue.

E) P47^{PHOX} AND P67^{PHOX}. In heterologous expression studies, p47^{phox} and p67^{phox} are capable of activating NOX3 (54, 147, 891, 892); however, the physiological relevance of this finding is, at least with respect to p47^{phox}, questionable as the loss of NOXO1 function in mice suffices to mimick the NOX3 deletion phenotype (463) and no vestibular symptoms have been described in p47^{phox}-deficient mice or patients.

F) RAC. The Rac dependence of NOX3 is also still a matter of debate. Two studies suggest a Rac independence (144, 891), while the results of a third study suggest an effect (892). The differences in the findings may be due to a less strict requirement for Rac in NOX3 activation, or to the presence of endogenous Rac in the cells which found Rac independence.

Is the NOX3 system activation dependent or constitutively active? Present biochemical evidence would rather argue in favor of a constitutive activation: NOXO1, the key subunit for NOX3 activation, constitutively activates NOX3 in reconstituted systems (see references above). However, this is much less clear from a physiological point of view: why should there be a constitutively active ROS-generating system in the inner ear? Thus in vivo studies will be necessary to clarify the point.

4. NOX4

NOX4 was originally identified as an NADPH oxidase homolog highly expressed in the kidney (294, 813). While NOX1-NOX3 represent an evolutionarily closely related subgroup of NOX enzymes, NOX4 is more distant, sharing only ~39% identity to NOX2. The gene for human NOX4 is located on chromosome 11. The existence of four NOX4 splice variants has been suggested (315). NOX4 antibodies recognize two bands, one of 75–80 kDa and a second of 65 kDa from both endogenous NOX4-expressing cells (smooth muscle and endothelium) and NOX4-transfected Cos7 cells (378, 403, 813). The subcellular distribution of

the two bands was distinct (378). The fact that two molecular masses are detected and that NOX4 contains four putative *N*-glycosylation sites might suggest that NOX4 is glycosylated, although treatment with *N*-glycosidase F failed to reduce the protein to a single band (813).

In addition to its strong expression in the kidney, NOX4 mRNA is also found in osteoclasts (969, 973), endothelial cells (14, 392, 901), smooth muscle cells (247, 383, 425, 510, 699, 836), hematopoietic stem cells (704), fibroblasts (170, 176, 211), keratinocytes (135), melanoma cells (105), and neurons (900).

Induction of NOX4 mRNA expression is observed under the following conditions: in response to endoplasmic reticulum stress (699), shear stress (402), carotid artery injury (856), hypoxia and ischemia (842, 900), and transforming growth factor (TGF)-β1 and tumor necrosis factor (TNF)-α stimulation of smooth muscle (619, 836). Upregulation of NOX4 (mRNA and protein) has been reported in response to angiotensin II (377, 955, 964) (but one study found an angiotensin II-induced downregulation in NOX4 mRNA, Ref. 510). The angiotensin II-induced upregulation of NOX4 mRNA was prevented by pigment epithelium-derived factor (PEDF) (964). Downregulation of NOX4 mRNA and protein is observed in response to PPAR-γ ligands (403).

In vascular smooth muscle, NOX4 is described in proximity to focal adhesions (378). In transfected cells, NOX4 localization is mostly observed in the endoplasmic reticulum (ER), whether green fluorescent protein (GFP)-tagged NOX4 is used (901) or distribution is assessed by immunofluorescence (584). While a functional role for NOX4 in the ER is entirely possible, such a localization may also represent an accumulation at its site of synthesis. Puzzling observations come from vascular smooth muscle and endothelial cells, where NOX4 expression in the nucleus is suggested by several lines of arguments (immunofluorescence, electron microscopy, nuclear Western blots, and nuclear ROS generation) (378, 494). It is however difficult to understand how a protein that spans the membrane six times can be found in a presumably membrane-free space, such as the interior of the nucleus.

Our present knowledge on subunit dependence of NOX4 is as follows.

A) p22$^{\text{PHOX}}$. NOX4 is a p22$^{\text{phox}}$-dependent enzyme. NOX4 colocalizes and coimmunoprecipitates with p22$^{\text{phox}}$; NOX4 also stabilizes the p22$^{\text{phox}}$ protein (24). Importantly, functional studies also demonstrate a p22$^{\text{phox}}$ requirement for NOX4-dependent ROS generation (446, 584). p22$^{\text{phox}}$ mutants lacking the proline-rich COOH terminus are still fully active in supporting NOX4 activity, while such mutants are not sufficient for NOX1, -2, and -3 activation.

B) NOX4 DOES NOT REQUIRE CYTOSOLIC SUBUNITS. NOX4 does not require cytosolic subunits for its activity, and upon heterologous expression, it is active without the need for cell stimulation (294, 584, 813).

C) RAC. In heterologously NOX4-expressing cells, Rac is not required for activity (584). Yet, at least in some endogenously NOX4-expressing cells, a Rac requirement has been documented (311, 410). Whether such a Rac requirement reflects a direct Rac/NOX4 interaction or is rather indirect remains to be seen.

As discussed above, NOX4 might be a constitutively active enzyme. However, not all available data favor this concept. NOX4 activation is observed under the following experimental conditions: *1*) lipopolysaccharide (LPS)-stimulated HEK293 cells (686), *2*) insulin-stimulated adipocytes (569), *3*) angiotensin II- or high glucose-stimulated mesangial cells (311, 410), and *4*) PMA-stimulated vascular endothelial cells (494). Mechanisms of NOX4 activation might include a direct binding of TLR4 to NOX4 (686). The angiotensin II and the high glucose stimulation are attributed to a Rac1-dependent NOX4 activation (311, 410).

A peculiarity of NOX4 is the fact that upon its heterologous expression, hydrogen peroxide, rather than superoxide, is detected (584). This should not, however, be taken as proof of direct hydrogen peroxide generation by the enzyme. Indeed, the most likely explanation is that the localization of the enzyme within intracellular organelles results in the release of superoxide into the lumen of the organelles where it rapidly dismutates into hydrogen peroxide. It is then the nonpolar hydrogen peroxide that is able to diffuse through membranes and reach the extracellular space.

5. NOX5

NOX5 was discovered in 2001 by two groups. Cheng et al. (143) described it as a cDNA predicting a protein with 565 amino acids, while Banfi et al. (56) described it as cDNA predicting a protein of over 700 amino acids. The human NOX5 gene is located on chromosome 15. The NOX5 isoforms described by Banfi et al. (NOX5α, -β, -γ, and -δ) distinguish themselves from the NOX1–4 enzymes by the presence of a long intracellular NH$_2$ terminus containing a Ca^{2+}-binding EF hand domain (56, 58). The fifth isoform described by Cheng et al. (NOX5ϵ or NOX5-S) lacks the EF-hand region and therefore has an overall structure more similar to NOX1–4 (143). On immunoblots, NOX5 is described as an 85-kDa protein (103). This would be consistent with its predicted molecular mass and suggests that the protein is not glycosylated. As seen for NOX2, NADH cannot replace NADPH as a cytoplasmic electron donor for NOX5 (58).

NOX5 mRNA expression is described in testis, spleen, lymph nodes, vascular smooth muscle, bone marrow, pancreas, placenta, ovary, uterus, stomach, and in various fetal tissues (56, 143, 770). Within the testis, the NOX5 message is localized to pachytene spermatocytes.

Within the spleen, NOX5 shows a distinct localization within the mantle zone, which is rich in mature B cells, and in the periarterial lymphoid sheath area, which is enriched with T lymphocytes (56). Interestingly, NOX5 could not be detected within circulating lymphocytes (56). These data are based on mRNA expression; no data on the tissue distribution or subcellular distribution of the NOX5 protein are published. Presently there is also no information on the NOX5 promoters or on factors controlling gene expression of the EF-hand expressing NOX5 isoforms (α-δ). However, a recent study shed first light on mechanisms regulating expression of the NOX5ϵ isoform: acid induces NOX5ϵ expression in Barrett's esophageal adenocarcinoma cells through mechanisms involving the cAMP response element binding protein CREB (274).

Nothing is known about the activation of the EF hand-deficient NOX5ϵ; thus the activation mechanisms summarized below are based on studies using EF hand-containing NOX5 isoforms. NOX5 does not require p22^{phox} for activity, as demonstrated by siRNA suppression of p22^{phox} leading to a decrease in the activity of NOX1 to NOX4, but not of NOX5 (446). NOX5 does not require cytosolic organizer or activator subunits (56) and has been shown to function in a cell-free system without the requirements of any cytosolic proteins (58). As predicted by the presence of EF hands, activation of NOX5 is mediated by an increase in the cytoplasmic Ca^{2+} concentration (58). The Ca^{2+}-binding domain of NOX5 behaves as an independent folding unit and undergoes conformational changes in response to Ca^{2+} elevations (58). This is thought to activate the enyzme through an intramolecular protein-protein interaction between the Ca^{2+}-binding region and the catalytic COOH terminus of the enzyme (56, 58).

6. DUOX1 and DUOX2

For several novel NOX isoforms, the identification of the protein preceded the definition of its function. In the case of DUOX1 and DUOX2, the situation was reversed. It had been known for a long time that thyroid epithelial cells produce H$_2$O$_2$ at the apical plasma membrane in a Ca^{2+}- and NADPH-dependent manner (88). Researchers in the thyroid field were actively looking for an NADPH oxidase. It took 15 years from the discovery of this function to the identification of DUOX proteins (originally called thyroid oxidase). They were identified from thyroid gland by two groups using different methods: purification and partial sequencing of the DUOX2 enzyme followed by rapid amplification of cDNA ends polymerase chain reaction (RACE PCR) (228) and low-temperature hybridization of a thyroid cDNA phage library with a NOX2 probe (189, 228). The genes for both human DUOX isoforms are located on chromosome 15. The two DUOX genes are somewhat unusual in their arrangement. They are arranged in a head-to-head configuration, separated by a

relatively short (16 kb) region with the direction of transcription away from one another (675).

In addition to a NOX1–4 homology domain and an EF-hand region, DUOX proteins have a seventh transmembrane domain at the NH$_2$ terminus with an ecto-facing peroxidase like domain. Within the NOX backbone, DUOX isoforms share $\sim$50% identity with NOX2 (189). An NH$_2$-terminally truncated form of DUOX2 mRNA has been found in rat thyroid cell lines (625).

DUOX enzymes are glycosylated. Both DUOX1 and DUOX2 have two N-glycosylation states: the high mannose glycosylated form found in the ER, which runs by gel electrophoresis at 180 kDa, and a fully glycosylated form found at the plasma membrane that runs at 190 kDa (188, 624). Carbohydrate content analysis of plasma membrane DUOX revealed specific oligosaccharides indicative of Golgi apparatus processing (623). When totally deglycosylated, the molecular mass of both DUOX1 and DUOX2 drops to 160 kDa (188).

It is not clear whether the peroxidase homology domain of DUOX enzymes functions as a peroxidase. One study suggests that DUOX peroxidase homology domains, when expressed as recombinant proteins, have a peroxidase function (239). However, from a structural point of view, this is surprising. Indeed, the DUOX peroxidase homology domains lack many amino acid residues identified as essential for peroxidase function (168, 181, 653). The fact that a peroxidase is usually coexpressed in DUOX expressing systems, e.g., thyroid peroxidase in the thyroid gland and lactoperoxidase in salivary glands, also questions the peroxidase function of DUOX. This is particularly well documented for the thyroid, where thyroid peroxidase deficiency leads to severe hypothyroidism, due to a lack of peroxidase-dependent hormone synthesis (690). Still, the peroxidase homology region of DUOX2 seems to be of functional importance, as hypothyroidism in patients with mutations in the extracellular domain has been reported (918).

Based on its homology with NOX2 and the fact that heme enzymes are monoelectron transporters, DUOX enzymes should generate superoxide. However, a generation of hydrogen peroxide by thyrocytes has been detected in many studies. This led to a heated debate over the question of whether the thyroid oxidase directly generates hydrogen peroxide or whether the hydrogen peroxide generation occurs via a superoxide intermediate (231, 527, 645, 646). In a recent study, the immature, partially glycosylated form of DUOX2 generated superoxide, while the mature form generated hydrogen peroxide (27). The authors speculate that posttranslational modifications favor intramolecular dismutation of superoxide to hydrogen peroxide. Taken together, it is likely that the primary product of DUOX enzymes is superoxide and that a rapid dismutation precludes in many instances the detection of a superoxide intermediate. The substrate selec-

tivity for human DUOX has not been defined; however, the GXGXXPF sequence typical of NADPH over NADH substrate selectivity is conserved, and in the sea urchin DUOX homolog Udx1 has been shown to favor the substrate NADPH to NADH (957).

Both DUOX1 and DUOX2 are highly expressed in the thyroid (189, 228). In addition, DUOX1 has been described in airway epithelia (271, 299, 794) and in the prostate (931).

DUOX2 is found in the ducts of the salivary gland (299); in rectal mucosa (299); all along the gastrointestinal tract including duodenum, colon, and cecum (230, 246); in airway epithelia (271, 794); and in prostate (931).

Induction of DUOX enzymes has been described. DUOX1 is induced in response to interleukin (IL)-4 and IL-13 in respiratory tract epithelium (352). DUOX2 expression was induced in response to interferon-γ in respiratory tract epithelium (352), in response to insulin in thyroid cell lines (625), and during spontaneous differentiation of postconfluent Caco-2 cells (246). Some studies (624, 625), but not others (189), find effects of forskolin, an adenylate cyclase activator, on DUOX expression. The putative promoters of DUOX1 and DUOX2 do not resemble each other and differ from promoters of other known thyroid-specific genes. The DUOX1, but not the DUOX2, promoter is GC rich and has putative SP-1 binding sites (675).

In thyrocytes, DUOX enzymes localize to the apical membrane (189, 228), although it appears that substantial amounts are found intracellularly, presumably in the ER (188). In airway epithelia, DUOX enzymes also localize to the apical membrane, as revealed by staining with an antibody that recognizes both DUOX1 and DUOX2 (794).

When heterologously expressed, DUOX enzymes tend to be retained in the ER, and superoxide generation can be measured only in broken cell preparations (27). This observation led to the discovery of DUOX maturation factors, which are ER proteins termed DUOXA1 and DUOXA2 (319). DUOX maturation factors seem to be crucial in overcoming ER retention of DUOX enzymes. DUOX enzymes do not require activator or organizer subunits; however, the p22^{phox} requirement is still a matter of debate. DUOX enzymes coimmunoprecipitate with p22^{phox} (931), but there is no evidence for enhanced DUOX function upon coexpression of p22^{phox} (27, 188, 931).

Studies on the activation of heterologously expressed DUOX2 in membrane fractions indicated that the enzyme *1*) does not require cytosolic activator or organizer subunits and *2*) can be directly activated by Ca^{2+}, suggesting that its EF-hand Ca^{2+}-binding domains are functional (27). Studies using *Clostridium difficile* toxin B conclude that DUOX activation in thyrocytes does not require Rac (270). A recent study found interaction of EF-hand binding protein 1 (EFP1) with DUOX1 and DUOX2, and it was suggested that this protein might be involved in the assembly of a multiprotein complex allowing ROS generation by DUOX enzymes (931).

B. NOX Subunits and Regulatory Proteins

NOX2 requires the assembly of at least five additional components for its activation. Other NOX isoforms vary in their requirements for these proteins or their homologs (Fig. 4). The additional proteins involved in NOX activation include the membrane-bound p22^{phox}, which helps stabilize the NOX proteins and dock cytosolic factors and the cytosolic proteins p47^{phox}, p67^{phox}, the small GTPase Rac, and the modulatory p40^{phox}, which together lead to the activation of the NOX enzyme. Cell stimulation leads to the translocation of p47^{phox} to the membrane. Because p67^{phox} is bound to p47^{phox}, this process also translocates p67^{phox}. Thus the role of p47^{phox} is that of an organizer. At the membrane, p67^{phox} directly interacts with and activates NOX2. Thus the role of p67^{phox} is one of an activator (857). The GTP binding protein Rac is also recruited to the membrane upon cell stimulation and is required for activation of the complex (722). Finally, the most recently discovered subunit p40^{phox} (951) appears to be modulatory, rather than obligatory.

1. p22^{phox}

Early attempts to purify the NADPH-dependent cytochrome *b* oxidase from neutrophils led to size estimates that ranged from 11 to 127 kDa (564, 700). This discrepancy in size was partially explained by heterogeneous glycosylation; however, it soon became clear that the flavocytochrome b_{558} was actually a heterodimer consisting of NOX2 and p22^{phox} (691).

The gene for human p22^{phox} is located on chromosome 16. p22^{phox} is a membrane protein, which closely associates with NOX2 in a 1:1 ratio (394). The membrane topology of p22^{phox} is difficult to predict based on hydropathy plots, and models have been proposed with two (528, 864), three (328, 917), and four transmembrane domains (180). In the absence of crystallization data, there is no consensus on this matter. However, the weight of evidence favors a two transmembrane structure with both the NH$_2$ terminus and the COOH terminus facing the cytoplasm (117, 406, 864). p22^{phox} runs on Western blots with an apparent molecular mass of 22 kDa and is not glycosylated (691).

The mRNA for p22^{phox} is widely expressed in both fetal and adult tissues (143) and in cell lines (693). The expression of p22^{phox} increases in response to angiotensin II (622), streptozotocin-induced diabetes (250), and hypertension (982).

The p22^{phox} promoter contains consensus sequences for TATA and CCAC boxes and several SP-1 binding sites located close to the start codon which, based on deletion
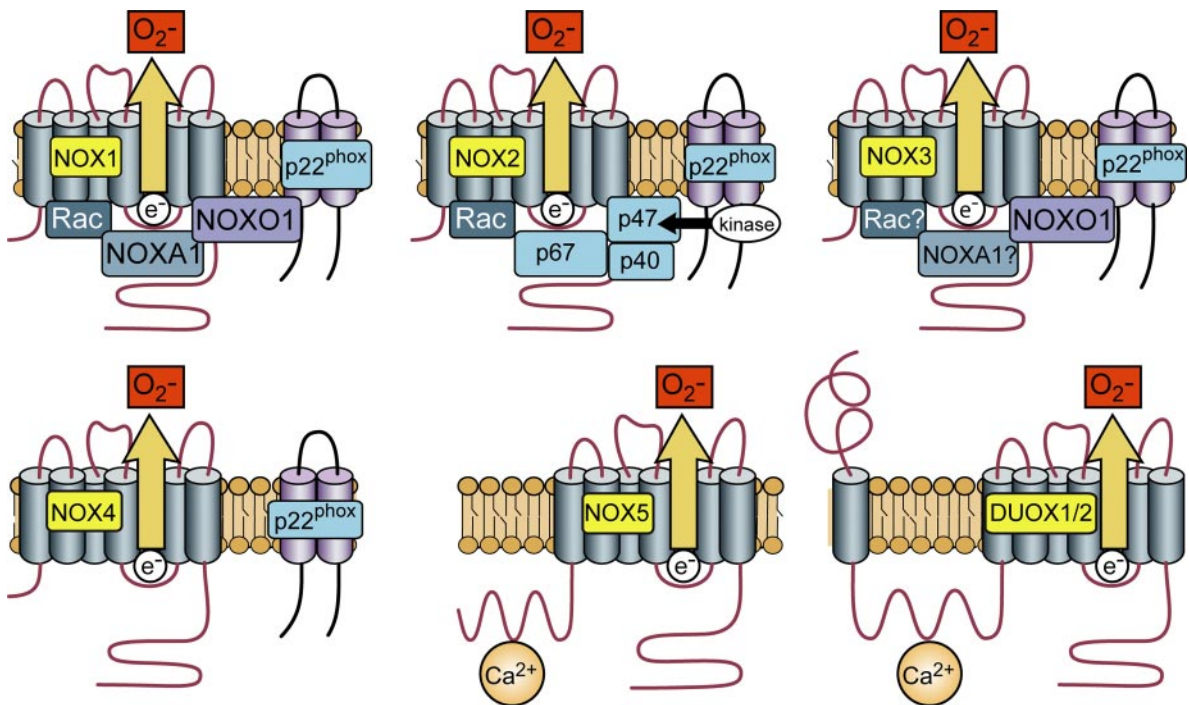
FIG. 4. Activation of NADPH oxidase isoforms. Despite their similar structure and enzymatic function, NOX family enzymes differ in their mechanism of activation. *A*: NOX1 activity requires p22^{phox}, NOXO1 (or possibly p47^{phox} in some cases) and NOXA1, and the small GTPase Rac. *B*: NOX2 requires p22^{phox}, p47^{phox}, p67^{phox}, and Rac; p47^{phox} phosphorylation is required for NOX2 activation. Although not absolutely required, p40^{phox} also associates with this complex and may contribute to activation. *C*: NOX3 requires p22^{phox} and NOXO1; the requirement for NOXA1 may be species dependent, and the requirement of Rac is still debated. *D*: NOX4 requires p22^{phox}, but in reconstitute systems it is constitutively active without the requirement for other subunits. However, in native NOX4-expressing cells, activation, possibly including Rac, has been described. *E* and *F*: NOX5, DUOX1, and DUOX2 are activated by Ca^{2+} and do not appear to require subunits.

mutants, appear to be important for the minimal promoter activity (632). The promoter region also contains consensus sequences for transcription factors including interferon (IFN)-γ, Elk1, GAGA, and NFκB (632).

There are several studies investigating the subcellular distribution of p22^{phox}. In phagocytes, p22^{phox} colocalizes with NOX2 in intracellular storage sites and translocates to the phagosome upon activation (427). p22^{phox} also colocalizes with NOX2 in coronary endothelial cells (68), with NOX1 in vascular smooth muscle (345), and with NOX1 and NOX4 in transfected cells (24, 584). Thus it appears that p22^{phox} localization is a function of the NOX isoform coexpressed with p22^{phox} in a given cell type. This is compatible with the concept that p22^{phox} forms heterodimers with various NOX enzymes.

p22^{phox} has two major functions: *1*) binding to NOX proteins, leading to protein stabilization, and *2*) binding to organizer subunits. p22^{phox} is thought to interact with NOX1 (511, 857), NOX2 (202, 693), NOX3 (446, 891), and NOX4 (24, 584). The underlying concept is that NOX proteins and the p22^{phox} protein are stable only as a heterodimer, while monomers are degraded by the proteosome (202). In line with this concept, NOX2-deficient CGD patients do not have detectable p22^{phox} protein within phagocytes, and p22^{phox}-deficient CGD patients do

not have detectable NOX2 protein (692). siRNA-mediated p22^{phox} downregulation leads to decreased function of NOX1, NOX2, NOX3, and NOX4 (446, 584), but not of NOX5 (103, 446). Interaction of tagged NOX1 and NOX4 with tagged p22^{phox} has also been demonstrated by fluorescent resonance energy transfer and by immunoprecipitation (24). Interestingly, DUOX enzymes also coimmunoprecipitate with p22^{phox} (931), but so far, no impact of p22^{phox} on DUOX function has been demonstrated.

The second function of p22^{phox}, namely, interaction with organizer subunits, is relevant only for NOX1, NOX2, and NOX3, but not for NOX4. The region of p22^{phox} responsible for this interaction is the COOH terminus, which contains proline-rich regions (205, 528), capable of interacting with SH3 domains of the organizer subunits p47^{phox} or NOXO1. Truncation of p22^{phox} or mutations within the COOH-terminal domain lead to a loss of activation of NOX1, NOX2, and NOX3 (446, 530). In agreement with the concept that NOX4 activation does not involve cytosolic organizer subunits, truncations or mutations of the p22^{phox} COOH terminus do not decrease NOX4 activity.

The importance of the p22^{phox} subunit for the phagocyte NADPH oxidase was revealed with the identification of CGD patients with mutations in p22^{phox} (217, 692).

Given the good in vitro data on the role of p22^{phox} for NOX1, NOX3, and NOX4, it is puzzling that the phenotype of these p22^{phox}-deficient CGD patients did not show obvious differences from CGD patients with other underlying mutations. This may in part be due to the small number of cases, the young age of the patients, and the limited scope of the clinical examination performed in these patients. Thus, at this point, it is unclear whether p22^{phox} is indispensable in vivo for the other NOX enzymes.

2. Organizer subunits: NOXO1, p47^{phox}

Two NOX organizer subunits are known: p47^{phox} (alias NOXO2) and NOXO1. p47^{phox} was discovered in the 1980s together with p67^{phox} (660, 923). The existence of a p47^{phox} homolog was first suggested by Geiszt and Leto at the first international meeting on NOX family NADPH oxidases (organizer: H. Schmidt) and subsequently confirmed by several groups (53, 147, 297, 857).

The genes for human p47^{phox} and NOXO1 are located on chromosomes 7 and 16, respectively. To our knowledge, no splice variants of p47^{phox} exist. In contrast, a total of four different splice variants of NOXO1 have been described (145).

The proteins NOXO1 and p47^{phox} share ~25% sequence identity to one another and share a high degree of similarity in their functional domains (Fig. 2A). NOXO1 and p47^{phox} each have phox (PX) domains that interact with membrane phospholipids. Both also have two SH3 domains that interact with the proline-rich regions in the COOH terminal of p22^{phox} (528, 857). p47^{phox} has an autoinhibitory region (AIR) that prevents this interaction until the protein is phophorylated and undergoes a conformational change. This AIR is absent in NOXO1. Finally, both NOXO1 and p47^{phox} also contain a COOH-terminal proline-rich region that can interact with SH3 domains in NOXA1 and p67^{phox}, respectively (531, 857). The molecular masses of p47^{phox} and NOXO1 are 47 and 41 kDa, respectively. Both are cytosolic proteins and not glycosylated.

p47^{phox} is highly expressed in myeloid cells (751, 923), although it has also been detected in other tissues, including the testis (145), inner ear (54, 145), neurons (866), hepatocytes (739), hepatic stellate cells (9), lung (23), glomerular mesangial cells (433), endothelial cells (434), and vascular smooth muscle (61). NOXO1 is highly expressed in the colon (53, 147, 297, 857), but also found in other tissues, including testis, small intestine, liver, kidney, pancreas, uterus, and inner ear (53, 145, 857).

The expression of p47^{phox} is induced under various circumstances, including retinoic acid-induced differentiation of monocytes and granulocytes (751), in the rat kidney in streptozotocin-induced diabetes (37), and in thrombin-stimulated smooth muscle cells (61). *Helicobac-*

ter pylori LPS activates transcription of NOXO1 in guinea pig gastric mucosa (443).

The minimal promoter activity for the p47^{phox} gene lies in the first 86 bp of the 5′-flanking region. The myeloid-specific transcription factor PU.1 is absolutely required for expression in phagocytes (543). However, p47^{phox} has been found in numerous other tissues, where distinct regulatory elements are likely to play a role. The NOXO1 promoter has so far not been studied.

The designation of p47^{phox} and NOXO1 as organizer subunits comes from observations in the phagocyte NADPH oxidase: the activator subunit p67^{phox}, the small p40^{phox} subunit, and the GTPase Rac all fail to translocate to the membrane in neutrophils from patients lacking p47^{phox} (235, 374). The organizer role of p47^{phox} and NOXO1 is also visible from the motifs found within the two proteins (Fig. 2A): an NH$_2$-terminal phox homology (PX) domain allows interaction with membrane phospholipids, centrally located tandem SH3 domains allow interaction with p22^{phox}, and a COOH-terminally located proline-rich repeat allows interaction with p67^{phox} or NOXA1. The major difference between p47^{phox} and NOXO1 is the presence of an autoinhibitory domain in p47^{phox}, which is absent in NOXO1. The autoinhibitory domain of p47^{phox} binds to the tandem SH3 domain within the same molecule, preventing association with p22^{phox}. Upon phosphorylation of the autoinhibitory domain, the tandem SH3 domain is exposed and allows p47^{phox} to translocate to the plasma membrane and bind p22^{phox}. The lack of an autoinhibitory domain in NOXO1 might suggest that it is constitutively active. Indeed, NOXO1 is found localized at the cell membrane with NOX1 and p22^{phox} in transfected HEK293 cells (146), while p47^{phox} translocates to the membrane only after its autoinhibition has been released by phosphorylation (163, 408, 866). However, constitutive activation of NOX1 by NOXO1/NOXA1 has been observed only in the mouse system (53, 54), while in the human system stimulation by the PKC activator PMA was necessary to get maximal activation (297, 857). Another difference between these two organizer homologs is the affinity of the PX domain for phosphatidylinositols. p47^{phox} binds to 3′-phosphorylated phosphatidylinositols, the products of phosphotidylinositol 3-kinase, while NOXO1 binds to monophosphorylated phosphatidylinositols, which are abundant in the membranes of nonactivated cells (146). The physiological consequence of this differential affinity for phospholipids remains to be determined.

The two organizer proteins NOXO1 and p47^{phox} can combine interchangeably with the two activator proteins, but the degree to which these complexes lead to activation of NOX1 and NOX2 varies markedly with the specific combination of subunit used (53, 54, 145, 857).

While there is no doubt that p47^{phox} and NOXO1 are crucial for the organization of active NOX1, -2, and -3 complexes, some evidence also points toward a more

direct role in NOX activation. A direct interaction of p47^{phox} and NOX2 has been suggested (203), and one study suggested that NOXO1 is sufficient to activate NOX3 without the need for activator subunits (147).

3. Activator subunits: p67^{phox} and NOXA1

The two activator subunits p67^{phox} (alias NOXA2) and NOXA1 were discovered in parallel with the respective organizer subunits. The human p67^{phox} and NOXA1 genes are found on chromosomes 1 and 9, respectively. To our knowledge, no splice variants of p67^{phox} and NOXA1 have been described.

Although p67^{phox} and NOXA1 share only ~28% amino acid identity, their overall domain structure is similar (Fig. 2B). Their molecular masses are 67 and 51 kDa, respectively. They are both cytoplasmic proteins and are not glycosylated. Both contain 1) an NH$_2$-terminal tetratricopeptide repeat (TPR), 2) a highly conserved activation domain (AD), 3) a less conserved "Phox and Bem 1" (PB1) domain, and 4) a COOH-terminal SH3 domain.

p67^{phox} is expressed in phagocytes (529), B lymphocytes (317), glomerular mesangial cells (433), endothelial cells (434), neurons (659), astrocytes (659), kidney (132), and hepatic stellate cells (9).

The expression of p67^{phox} is inducible in response to a variety of stimuli, including IFN-γ in myelomonocytic U937 cells (242) and promyelocytic HL60 cells (532), in response to zinc in neurons and astrocytes (659), and in response to angiotensin II in aortic adventitial fibroblasts (678) and whole aortas (156).

NOXA1 is expressed in the spleen, inner ear (54), stomach, colon, small intestine, uterus (54, 297), prostate, lung, thyroid, salivary glands (297), guinea pig gastric mucosal cells (443), basilar arterial epithelial cells (14), airway-like normal human bronchial epithelial cells (513), and vascular smooth muscle cells (25).

Like NOX2 and other genes preferentially expressed in myeloid tissues, p67^{phox} is under the control of the PU.1 transcription factor (290), and the induction of the p67^{phox} gene by interferon is regulated by the same set of factors as the NOX2 gene, namely, PU.1, IRF1, and ICSBP (242). The promoter region of p67^{phox} contains three PU.1 binding sites, and mutagenesis of any one of those sites reduced the transcriptional activity by ~50% (290, 542), demonstrating that all three sites are involved in the promoter function. Binding sites for AP-1 transcription factors, found in the first intron of p67^{phox}, are essential for p67^{phox} expression (290, 542). No information about the promoter or the transcriptional regulation of NOXA1 is currently available.

The general view of NOX2 activation events is that p47^{phox} phosphorylation leads to translocation of the p47^{phox}/p67^{phox} complex to the plasma membrane where p47^{phox} interacts with p22^{phox}, and p67^{phox} subsequently

acts as the NOX activator through a direct protein-protein interaction (163, 652). Evidence in line with the concept that the translocation of p67^{phox} requires the presence of p47^{phox} is found in p47^{phox}-deficient neutrophils, where p67^{phox} does not translocate to the membrane upon stimulation (235, 374, 894). However, the situation might be more complex, and there is evidence that p67^{phox} can also be phosphorylated (81, 184, 233, 265, 266, 317, 652, 987).

NOXO1 constitutively associates with the membrane (see above and Ref. 146). Thus it is likely that NOXA1 is constitutively associated with membranes as well, although this has not been experimentally proven to our knowledge. Similarly, it is presently unknown whether NOXA1 can be phosphorylated.

p67^{phox} and NOXA1 interact through their COOH-terminal SH3 domain with the proline-rich repeats of p47^{phox} and NOXO1 (192, 328, 857). p67^{phox} and, presumably, NOXA1 also interact directly with NOX proteins (NOX1 to NOX3) through their activation domain (53, 298, 342, 658, 857). Both p67^{phox} and NOXA1 possess NH$_2$-terminal tetratricopeptide repeats, which interacts with Rac (325, 476, 508, 844, 857). Thus the overall structure and protein-protein interactions of the two homologous subunits are highly similar. However, there are some notable differences. The PB1 domain of NOXA1 has important differences from that of p67^{phox} and fails to interact with p40^{phox} (857). NOXA1 also lacks the central SH3 domain found in p67^{phox} (53, 297, 857). The function of the central SH3 domain of p67^{phox} is not known.

4. p40^{phox}

p40^{phox} was detected by coimmunoprecipitation with p47^{phox} and p67^{phox} (951). The human p40^{phox} gene is located on chromosome 22. A splice variant of p40^{phox} has been described (357). The full-length p40^{phox} is a nonglycosylated cytosolic protein with an apparent molecular mass of 40 kDa. The structural domains of p40^{phox} include an SH3 domain, a PX domain, and a PB1 domain. p40^{phox} has been shown to interact with p47^{phox} and p67^{phox} with a 1:1:1 stoichiometry (507).

p40^{phox} protein is expressed in phagocytes (951), B lymphocytes (317), spermatozoa (816), hippocampus (866), and vascular smooth muscle (881).

p40^{phox} expression is induced by angiotensin II treatment in rostral ventrolateral medulla (285) and in vascular smooth muscle (881). INF-γ and TNF-α did not induce p40^{phox} in monocytes (234).

Deletion analysis of ~6 kb of the 5'-flanking sequence of the p40^{phox} gene demonstrated that the proximal 106 bp are responsible for most of the promoter activity (541). PU.1 binding at multiple sites is required for p40^{phox} gene transcription in myeloid cells (541).

p40^{phox} is absent from CGD patients who lack p67^{phox} (889), suggesting that the protein is stable only

upon binding to p67^{phox}. p40^{phox} is involved in the regulation of NOX2. One study suggests that p40^{phox} might inhibit NOX2 function (778); however, most evidence indicates that it enhances oxidase function (173, 493, 888). Unlike p47^{phox} and p67^{phox}, p40^{phox} is dispensable for NOX2 activity and no CGD patients with p40phox deficiency have been described (585). Presently available data also suggest that p40^{phox} is specific for NOX2. However, as NOX1 and NOX3 could potentially be activated by the p47^{phox}/p67^{phox} complex, a role of p40^{phox} in this scenario cannot be excluded.

5. Rac GTPases

There are three highly homologous Rac proteins in mammals: Rac1, ubiquitously distributed; Rac2, mostly expressed in myeloid cells; and Rac3, predominantly found in the central nervous system. Rac proteins are clearly involved in the regulation of the phagocyte NADPH oxidase NOX2. However, Rac proteins are not NOX subunits in the strict sense because they regulate other cellular functions, in particular the cytoskeleton. Thus we do not discuss Rac GTPases in detail in this review and refer readers to specific articles (92, 93, 197, 215, 776).

Of relevance for this review is our present knowledge on the importance of Rac proteins in the activation of different NOX isoforms. The involvement of Rac in NOX2 and NOX1 activation is established beyond doubt. The involvement of Rac in NOX3 activation is a matter of debate (615, 891). NOX4 activity appears to be Rac independent upon heterologous expression (584); however, some data from cells endogenously expressing NOX4 provide arguments compatible with a role of Rac in NOX4 activation (311, 410). Presently there are no data in favor of a role for Rac proteins in NOX5 activation. Finally, DUOX-dependent ROS generation in thyroid cells does not involve Rac1 activation (270).

While there is no doubt that Rac GTPases are important activators of NOX2 and NOX1 and possibly some other NOX isoforms, it should be kept in mind that they may also be involved in regulating ROS generation from other sources, particularly mitochondria (724, 948). Thus the numerous studies suggesting the involvement of a NOX NADPH oxidase in biological processes based on inhibition by dominant negative Rac should be taken with caution.

C. NOX Inhibitors

At present, no specific NOX inhibitors exist. Yet, many studies are based on the use of inhibitors (for a recent review, see Ref. 155). We therefore briefly describe some of the most commonly used compounds.

1. Diphenylene iodonium

The most commonly used NOX inhibitor is the iodonium-derivative diphenylene iodonium (DPI). The compound acts by abstracting an electron from an electron transporter and forming a radical, which then inhibits the respective electron transporter through a covalent binding step (661). In the case of NOX enzymes, it is not clear whether the iodonium radical formation occurs through interaction with the flavin group (661) or the heme group (224). As suggested by the mechanism of action, DPI is a nonspecific inhibitor of many different electron transporters. It inhibits not only all of the NOX isoforms, but also nitric oxide synthase (835), xanthine oxidase (225), mitochondrial complex I (544), and cytochrome P-450 reductase (713). To some extent, the lack of specificity of DPI can be addressed by using specific inhibitors of other sources of ROS.

2. Apocynin

The plant phenol 4-hydroxy-3-methoxyacetophenone, referred to as apocynin, was described around 1990 as a low-affinity inhibitor (IC$_{50}$ ~10 μM) of the phagocyte respiratory burst (817). Studies on the effect of the phagocyte NADPH oxidase suggested that apocynin 1) must be metabolized by peroxidases to generate the inhibitory compound and 2) acts by inhibiting the translocation of cytoplasmic subunits (833). According to statement by Lafeber et al. (502), the compound has been used in patients without signs of toxicity. While these initial studies on apocynin appear solid, a low-affinity prodrug is not an ideal drug candidate, nor is it a powerful tool for research. Nevertheless, apocynin is now used indiscriminately as a bona fide NOX inhibitor. Apocynin at extremely high concentrations has been used as a NOX4 inhibitor (e.g., Refs. 247, 758) and as a NOX5 inhibitor (274), although there is presently no evidence for a subunit dependence of these isoforms. Apocynin is also widely used in cell types where there is no evidence for the presence of a peroxidase. A final complicating factor in the use of apocynin comes from recent studies which suggest that apocynin may actually stimulate ROS production in nonphagocytic cells (747, 913).

3. AEBSF

4-(2-Aminoethyl)benzenesulfonylfluoride (AEBSF) is a serine protease inhibitor that inhibits NOX enzymes by interfering with the association of the cytoplasmic subunit p47^{phox} (213). It is not clear whether AEBSF really inhibits NOX enzymes or rather acts on signaling steps towards p47^{phox} activation. AEBSF is also likely to have NOX-unrelated effects due to serine protease inhibition (155).

4. Neopterin

Neopterin is a pteridine that is generated by macrophages as a catabolic product of GTP, and serum levels are elevated during some cancers and during human immunodeficiency virus (HIV). Neopterin inhibits the phagocyte NAPDH oxidase with an IC_{50} in the low micromolar range (478). However, neopterin is not specific (inhibition of xanthine oxidase, Ref. 942) and may even increase the generation of singlet oxygen, nitric oxide, and the hydroxyl radical (732).

5. gp91ds-tat

The peptide inhibitor gp91ds-tat was designed specifically to inhibit NOX2 by mimicking a sequence of NOX2 that is thought to be important for the interaction with p47[phox]. The peptide is a low-efficacy inhibitor, inhibiting neutrophil ROS generation by ~25% at 50 μM (743). Also, as the region targeted by the peptide is homologous in other NOX isoforms, the peptide may lack specificity. Indeed, gp91ds-tat was most potent in inhibiting superoxide generation in the vascular system (743), where other NOX isoforms, such as NOX1 and NOX4, play a key role.

In summary, there are currently no potent and specific NOX inhibitors, and studies that base their conclusions solely on the use of the above-described compounds should be taken with some caution.

D. Polymorphisms in NOX Enzymes and Subunits

Polymorphisms are variants of the genetic code with a frequency of at least 1% in the general population. In this section, we discuss our present knowledge of polymorphisms for each of the NOX family members. The mutations in NOX2 and its subunits that lead to CGD are rare disease-causing mutations and not polymorphisms and are therefore not included.

An overall analysis of single nucleotide polymorphisms (SNPs) in various NOX enzymes and NOX subunits is shown in Table 1. This analysis is based on current data base entries, not all of which have been confirmed, thus the precise numbers should be taken with caution. Nevertheless, an interesting picture is beginning to emerge. The number of presently known SNPs per gene ranges between 13 for NOXO1 and 858 for NOX4. When normalized by gene size, the differences are much smaller, ranging from 2.1 SNPs per 1,000 bp for NOX2 to 12.3 SNPs per 1,000 bp for p22[phox]. When looking at coding nonsynonymous SNPs, the differences become more marked: the number of coding nonsynonymous SNPs per 1,000 amino acids is 35.9 for p22[phox], compared with 1.7 for NOX4 and 0 for NOXO1, NOXA1, and p47[phox]. Thus there are differences in the degree of amino acid conservation among different NOX isoforms and sub-

units. The degree of conservation of most NOX isoforms and subunits is high, suggesting an essential role of these enzymes. The relatively low conservation of NOX5 together with its loss in rodents suggest that mammalian organisms depend less on this isoform. The relatively low conservation of p22[phox] is puzzling, given the fact that this subunit interacts with four NOX isoforms. Polymorphisms in p22[phox] affect NOX2 activity, and it is tempting to speculate that p22[phox] variability is a cause of biological variations in ROS generation in the human population.

1. NOX1–5 and DUOX

In humans, NOX1, NOX3, and NOX4 each have one coding nonsynonymous SNP described. NOX2 has three coding nonsynonymous SNP, and additionally, in the region upstream of the NOX2 gene, there is also a highly polymorphic region of dinucleotide TA repeats. Similar tandem repeats have been identified in the 5'-region of the Duchenne's muscular dystrophy gene (259) and the hemophilia A locus (503). It has been suggested that the number of these dinucleotide repeats regulates NOX2 expression and activity (893).

The Lys171Thr polymorphism in NOX3 has been validated by frequency and genotype data and occurs with greater frequency in the East Asian population (~40%) than in individuals of European or African descent (<3%).

NOX4 is a large gene, and consequently, the total number of SNPs is high. Yet present databank entries suggest only one coding nonsynonymous SNP within the human gene, namely, an Ile315Met polymorphism.

For mammals, NOX5 is not an essential gene, as the gene is lacking in rodents (see above). In line with this observation, NOX5 in humans seems to be more permissive to genetic variations than other NOX family members. Currently eight entries with coding nonsynonymous SNPs are found in the database. Some of them are frequent and of potential significance, including the Glu191Gly polymorphism (~40% allelic frequency), the Phe133Leu polymorphism (14% allelic frequency with marked racial variations), and the His558Arg polymorphism (11% allelic frequency).

Four nonsynonymous coding SNPs have been reported for DUOX1 and six nonsynonymous coding SNPs have been reported for DUOX2.

2. Subunits and regulatory proteins

Potentially relevant SNPs in the p22[phox] gene include an Ala174Val polymorphism (33% allelic frequency) and a His72Tyr polymorphism (29% allelic frequency) (217, 821). The latter polymorphism is caused by a C to T substitution and is often also referred to as the C242T polymorphism (693). Most studies suggest a decreased ROS generation in phagocytes (631, 962) or vascular cells (336) from individuals with the His72Tyr polymorphism,

yet one study in granulocytes describes the opposite (812).

Studies on the effect of the His72Tyr polymorphism on cardiovascular disease yielded contradictory results.

1) An enhanced risk in His72Tyr carriers was described for cerebrovascular disease (416), progression of coronary heart disease (122), and hypertension in a high arsenic exposure group (389).

2) A decreased risk in His72Tyr carriers was described for blood levels of oxidized high-density lipoprotein (HDL) (647), development of diabetic nephropathy (590), progression of asymptomatic atherosclerosis in type 2 diabetes (360), coronary artery disease (411, 811), and hypertension (631).

3) Equal risk in His72Tyr carriers was described for cardiovascular disease or risk factors (123, 287, 484, 533, 586, 812, 827). One study also reports equal risk for cardiovascular risk factors, but increased salt sensitivity and decreased levels of nitric oxide metabolites in His72Tyr carriers (129).

Two $p22^{phox}$ polymorphisms outside of the coding regions have received attention: the A640G polymorphism located in the 3'-untranslated region of $p22^{phox}$ gene (187, 725) and the -G930A polymorphism located in the promoter (632). The A640G substitution has been linked to a protective effect in cardiovascular disease (287) and an improved response to exercise through an enhanced reduction of oxidative stress markers (688). A640G is also linked to a protective effect against drug-induced agranulocytosis (634). The -G930A gene is associated with higher promoter activity (632). The promoter variant -G930A is associated with hypertension (479, 981) and an increased level of oxidative stress (774, 981). Note that, at least in the caucasian population, the His72Tyr polymorphism is not in linkage disequilibrium with the -G930A polymorphism (631); thus it is difficult to unequivocally attribute an observed phenotype to one of the two polymorphisms.

In summary, research on the clinical impact of $p22^{phox}$ polymorphisms is at an early stage. A synopsis of results obtained with the most studied polymorphism, His72Tyr, has not yielded a clear trend, suggesting that it is not a major risk factor, at least for cardiovascular diseases. The results concerning the A640G and the -G930A polymorphism are less contradictory, but the number of studies is small.

There are currently four SNP entries for $p67^{phox}$ in the databank. Three other polymorphisms, not included in databank entries, Val166lle, Pro329Ser, and His389Gln, were identified by systematic sequencing of $p67^{phox}$ from healthy individuals (33). In a reconstituted Cos7 cell system, these variants show a functional activity similar to wild-type $p67^{phox}$ (33). Thus, presently, there is no indication that $p67^{phox}$ polymorphisms are of functional relevance.

There are four database entries for $p40^{phox}$ SNPs. No databank entries and no published evidence exist for polymorphisms of $p47^{phox}$, NOXO1, or NOXA1.

III. PHYSIOLOGICAL FUNCTION OF ROS AND NOX FAMILY NADPH OXIDASES

Details regarding the proposed physiological and pathophysiological function of NOX NADPH oxidases will be given in the sections on the respective organs and systems. In this section, we give an overview of how NOX-derived ROS are thought to exert their action (Fig. 5).

A. Host Defense and Inflammation

Host defense is a key function of NOX2, as evidenced by the severe infections observed in CGD patients. Host defense might also be an important role of other NOX family members. However, while a direct ROS-dependent killing was initially thought to completely explain the host defense function of NOX enzymes, it has become clear that the situation is more complicated and that several mechanisms cooperate to achieve successful oxygen-dependent killing. It is also important to remember that phagocytes have a number of oxygen-independent killing mechanisms, in particular, the release of microbicidal proteins from the granules (reviewed in Ref. 256). Indeed, studies with neutrophils from CGD patients demonstrate that NOX2 is not required for the killing of many types of bacteria (956). As a general rule, however, catalase-positive organisms (e.g., *Staphylococcus aureus*) can only be killed by NOX2-expressing neutrophils.

1. ROS-dependent killing

When the phagocyte respiratory burst was discovered, a direct killing of microorganisms by ROS was proposed. However, the type of ROS that actually kill bacteria is still debated.

A) SUPEROXIDE. Although superoxide is the species produced by NOX enzymes, it is not clear whether superoxide itself is directly involved in killing of microorganisms. It has been argued that superoxide is a rather "sluggish" ROS, not reactive enough to be an important player on its own (736). However, others have suggested that in nonpolar environments the reactivity of superoxide is enhanced and at low pH, the highly reactive protonated form of superoxide, $HO_2\cdot$, is abundant (467). Thus under some physiologically relevant conditions, such as the low pH in the phagosome and the nonpolar environment close to cell membranes, superoxide itself could potentially be a direct player in killing.

B) HYDROGEN PEROXIDE AND PEROXIDASE. Once generated, superoxide dismutates into hydrogen peroxide, either
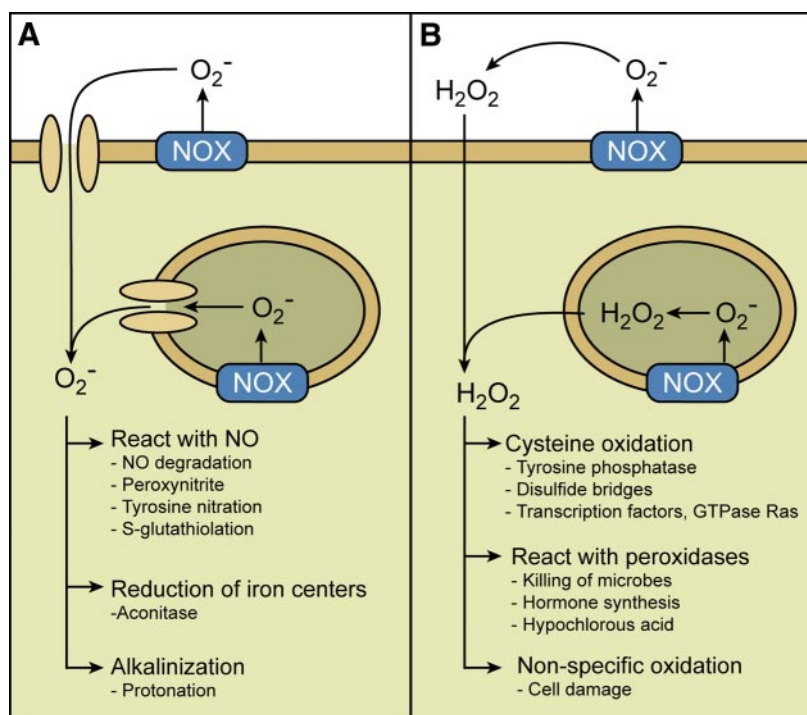
FIG. 5. ROS generation by NOX enzymes: topology and downstream effects. The immediate product of NOX enzymes is superoxide (O_2^-); however, due to spontaneous and enzymatic dismutation, hydrogen peroxide (H_2O_2) is also rapidly generated. The orientation of NOX enzymes is such that the NADPH binding NH_2 terminus is in the cytoplasm, and superoxide generation occurs either in the extracellular or an intraorganellar space. *A*: the negatively charged O_2^- does not permeate the lipid bilayer of biological membranes; however, at least in some cases seems to be able to pass through the pore of anion channels. Biological effects of NOX-derived O_2^- include *1*) reaction with nitric oxide (NO) leading to NO degradation (i.e., decreased bioavailability of a crucial signaling molecule), peroxynitrite formation, protein tyrosine nitration, and the addition of GSH to thiols; *2*) reduction of iron centers within enzymes (e.g., aconitase); and *3*) alkalinization of intracellular organelles. *B*: H_2O_2 is a well-established signaling molecule, which readily permeates biological membranes. Biological effects of NOX-derived H_2O_2 include the following: *1*) oxidation of low pK_a cysteine residues, which leads to inactivation of protein tyrosine phosphatases, disulfide bridge formation (e.g., inactivation in the protein serine-threonine phosphatase calcineurin), and activation of several transcription factors (e.g., NFκB and AP-1) and GTPases (e.g., Ras); *2*) reaction with peroxidases, important for the host defense and iodination of thyroid hormones; and *3*) toxicity through nonspecific oxidation of cellular components, in particular at high concentrations.

spontaneously, particularly at low pH, or facilitated by superoxide dismutase. Therefore, bacterial killing by hydrogen peroxide derived from superoxide has been suggested as a mechanism for NOX activity. A role in bacterial killing has also been suggested for DUOX2-derived hydrogen peroxide in combination with lactoperoxidase in the digestive tract (246, 298, 299). The combined effect of hydrogen peroxide and myeloperoxidase has been extensively studied, and it is clear that this system is powerful in killing bacteria and in inactivating bacterial pathogenicity factors. Killing by this system involves different types of peroxidation reactions, which have been discussed previously (467, 653). Within the phagosome, HOCl appears to be produced in sufficient concentrations to kill bacteria (428). However, while myeloperoxidase-deficient individuals are at an increased risk of infection (497), most are healthy, unlike the much more severe immunosuppression found in CGD patients deficient in NOX2. Thus, while myeloperoxidase undoubtedly provides an important amplification of NOX2-dependent killing mechanisms, it is not the only, and perhaps not even the major, mechanism.

C) RNS. Superoxide avidly reacts with nitric oxide to form the highly reactive intermediate peroxynitrite, which in turn can be metabolized to several other highly RNS. The relative importance of RNS in killing by phagocytes is unknown, but it is possibly more important in rodents than in humans (467).

D) OTHER ROS. Nonenzymatic conversion of superoxide and hydrogen peroxide can also lead to the formation of highly reactive free radicals. Catalyzed by iron, hydrogen peroxide can produce the hydroxyl radical in a reaction known as the Fenton reaction (245). While this reaction occurs readily in vitro, the extent to which it occurs in vivo is less clear. Indeed, evidence suggests that at least in neutrophils, the lack of an iron catalyst limits the amount of hydroxyl radical formed by Fenton-like reactions (108). Yet, the hydroxyl radical may still be formed in these cells through an iron-independent reaction between superoxide and hypochlorous acid catalyzed by myeloperoxidase (728). Hydrogen peroxide can also react with superoxide to produce the hydroxyl radical and singlet oxygen (566), or with hypochlorous acid to produce singlet oxygen and harmful chloramines (245). Indeed, bactericidal activity of phagocytes from CGD patients is reconstituted by the addition of a glucose oxidase system capable of generating peroxide and hypochlorous acid (300).

2. Inactivation of microbial virulence factors

Rather than killing of microorganisms directly, NOX enzymes may also act through inactivation of bacterial virulence factors. There is ample evidence for inactivation of virulence factors by the hydrogen peroxide myeloperoxidase system (158, 161). However, more recent studies suggest that such an inactivation may also occur through redox-sensitive elements within a given virulence factor. Pheromones, involved in bacterial quorum sensing, can be inactivated through NOX-derived ROS via oxidation of a COOH-terminal methionine (756).

3. Regulation of pH and ion concentration in the phagosome

The weight of evidence supports the idea that ROS generation by NOX2 is an important mechanism for killing of microbes and inactivation of microbial virulence factors (see above). However, recently an alternative concept has been proposed. This concept suggests that a direct effect of ROS is not crucial and that NOX2-dependent changes of phagosomal pH and ion concentrations are responsible for killing.

A) PHAGOSOMAL PH. NOX2 activation leads to a rise in phagosomal pH, and CGD neutrophils have therefore more acidic phagosomes (797). The NOX2 elevation of the phagosomal pH is probably due to the fact that superoxide is a weak base and that protons are consumed when superoxide dismutates into hydrogen peroxide (785, 797). NOX2-dependent phagosome alkalinization might allow for optimal function of neutral proteases in neutrophils (796). In dendritic cells, prolonged NOX2-dependent phagosomal alkalinization plays an important role in the cross-presentation of antigens (785).

B) PHAGOSOMAL ION CONCENTRATION. As an electron transporter, activation of NOX enzymes leads to a charge build-up that requires compensation (367, 792). It appears that most of the charge compensation occurs through H^+ channels (260). However, there has also been a suggestion that K^+ fluxes contribute to this charge compensation (16, 735, 796). Such K^+ fluxes could contribute to bacterial killing through changes in phagosomal osmolarity and unbinding of cationic proteases from their proteoglycan matrix (735).

In summary, most likely the contribution of NOX2 to microbial killing lies in both direct ROS effects and indirect effects through modulation of pH and ion homeostasis. Indeed, studies on the relative contribution of direct ROS killing of bacteria versus K^+ flux-dependent killing suggest that at low NOX2 activity K^+ flux are important, while at high NOX2 activity direct ROS-dependent killing is predominant (723).

4. NOX enzymes and proton channels

Electron transport by NOX enzymes leads to a charge build up, which requires compensation. While there is some evidence for involvement of K^+ channels and Cl^- channels in this process (see section above), most evidence points towards a key role of proton channels (194, 206). In general, the magnitude of the NADPH oxidase activity in cells is closely correlated with the magnitude of the proton channel activity (198, 367), and inhibition of H^+ channel activity inhibits ROS generation by NOX enzymes (366). Thus there is no doubt that there is a close relationship between NOX enzymes and proton channels; however, there are two different molecular interpretations: 1) NOX enzymes possess a proton channel domain,

suggested to reside within the histidine-rich third transmembrane domain (55, 57, 365, 368, 592, 639), and 2) NOX enzymes are closely associated and interact with physically distinct proton channels (195, 196). Candidates for such a distinct proton channels are the recently described voltage sensor-domain proteins (729, 777).

5. Anti-inflammatory activity

Since ROS are usually associated with inflammation, an anti-inflammatory activity of NOX enzymes seems counterintuitive. However, over the recent years there has been an impressive number of publications pointing in this direction. It may seem surprising that most data on an anti-inflammatory activity of NOX enzymes come from studies using mice deficient in the phagocyte NADPH oxidase (NOX2, p47^{phox}), which typically is considered a prototype proinflammatory enzyme. But one should also remember that the name *chronic granulomatous disease* (see sect. IC) refers to the hyperinflammation observed in the absence of NOX2. Indeed, chronic granulomas in CGD patients are thought to be sterile complications (124), and in CGD mice, a sterile hyperinflammation can be caused by injection of sterilized *Aspergillus fumigatus* extracts (633). This hyperinflammatory response might be due to the following: 1) a decreased capacity to degrade phagocytosed material in NOX2-deficient cells leading to the accumulation of debris (603, 789); 2) lack of ROS-dependent signaling in NOX2-deficient phagocytes, e.g., ROS-dependent apoptosis of inflammatory cells (111), hydrogen peroxide-induced inhibition of IL-8 production (524), and ROS-dependent attenuation of Ca^{2+} signaling (292, 293), may be impaired in CGD, contributing to enhanced inflammation; and 3) an impairment of oxidative inactivation of proinflammatory mediators may lead to a prolongation of the inflammatory response (354).

Hyperinflammation in phagocyte NADPH oxidase-deficient mice is not limited to hyperinflammation in response to inactivated *A. fumigatus*. It is also observed in mouse models of *Helicobacter* gastritis (90, 450), arthritis (399, 666), demyelinating disease (399), and sunburn (481). In experimental lung influenza infection, NOX2-deficient mice show heightened inflammatory infiltrates and accelerated viral clearance (820). Even more strikingly, genetic determination of the disease locus in arthritis-prone mice and rats (398) identifies p47^{phox} mutations as the underlying defect.

These observations in animal experiments might relate to clinical observations in CGD patients, where in addition to infections, patients with CGD suffer from a variety of inflammatory conditions (272, 956). These include inflammatory bowel disease, discoid and systemic lupus erythematosus, chorioretinitis, Behçet syndrome, and obstructive lesions of the esophagus, gastrointestinal tract, and urinary tract. In some instances, these inflam-

matory disorders are the first clinical manifestation of CGD. There is also a high incidence of lupus erythematosus in family members of CGD patients (956).

In summary, there is mounting evidence that NOX enzymes have a role in limiting the inflammatory response. This anti-inflammatory activity of NOX enzmyes represents an interesting and surprising function. The underlying molecular mechanisms await further studies.

B. Cellular Signaling

1. Inhibition of phosphatases

The best understood, and possibly most important, pathway by which ROS achieve regulation of cell function occurs through redox-sensitive cysteine residues. This has been most convincingly demonstrated for protein tyrosine phosphatases (PTP). PTPs control the phosphorylation state of numerous signal-transducing proteins and are therefore involved in the regulation of cell proliferation, differentiation, survival, metabolism, and motility (401). The catalytic region of PTPs includes cysteines (60, 771), which are susceptible to oxidative inactivation (208). Thus ROS decrease phosphatase activity that enhances protein tyrosine phosphorylation and thereby influences signal transduction (517). Consistent with this biochemical mechanism, NOX-derived ROS have been shown to regulate protein tyrosine phosphorylation in several different cell types (305, 499, 961, 967).

2. Activation of kinases

Treatment of cells with hydrogen peroxide leads to phosphorylation and activation of p38 mitogen-activated protein (MAP) kinase (219). There is abundant evidence for activation of elements of the MAP kinases system by NADPH oxidases (219, 279, 323, 344, 599, 614, 880, 885) (see also examples given in sect. IV). The precise redox-sensitive step(s) involved in kinase activation in response to NOX-dependent ROS production is presently unknown. It may be due to activation of signaling pathways upstream of ERK1/2 kinase (323), or it may rather be an indirect effect due to the inhibition of phosphatase activity by ROS (885).

3. Regulation of ion channels

ROS can regulate intracellular (375) and plasma membrane (862) ion channels. Such a regulation of ion channels may occur either directly or through ROS-sensitive signaling systems. NOX isoforms have been increasingly implicated in this function, but it should be kept in mind that other sources of ROS may also be important (387). NOX-derived ROS has been suggested in the regulation of K^+ channels (519, 845), plasma membrane Ca^{2+} channels (L-type, Refs. 933, 993), and intracellular Ca^{2+}

channels (ryanodine receptor, Ref. 976). In neurons for example, angiotensin II stimulation leads to an increase in Ca^{2+} currents and a decrease in K^+ currents, both of which are likely to be mediated by a NOX family NADPH oxidase (845, 933, 993). Unfortunately, no data from NOX-deficient animals exist, and the evidence therefore remains circumstantial. For other examples of NOX regulation of ion channels, see sections on Ca^{2+} signaling (sect. III*B4*) and oxygen sensing (sect. III*F*).

4. Ca^{2+} signaling

As described above, NOX enzymes may be activated through Ca^{2+} signaling. However, the reverse situation also occurs, namely, regulation of Ca^{2+} signaling through NOX enzymes.

The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is an important intracellular messenger system. In resting cells, this concentration is kept very low (100 nM) by Ca^{2+} pumps. In response to a variety of stimuli, $[Ca^{2+}]_c$ rises to micromolar levels, which leads to the activation of Ca^{2+}-dependent cellular processes. The rise in $[Ca^{2+}]_c$ occurs through Ca^{2+} influx across the plasma membrane and/or through Ca^{2+} release from intracellular stores. At least three types of proteins relevant for intracellular Ca^{2+} homeostasis are regulated through NOX enzymes: plasma membrane Ca^{2+} channels, intracellular Ca^{2+} release channels, and Ca^{2+} pumps. NOX enzymes may regulate the activity of such channels through two major mechanisms: ROS-dependent posttranslational modifications (e.g., cysteine oxidation, *S*-glutathiolation) and electron transport-dependent cell depolarization.

A) PLASMA MEMBRANE CALCIUM CHANNELS. NOX-derived ROS increase neuronal Ca^{2+} influx by increasing the opening of voltage-dependent L-type Ca^{2+} channels (933, 994). A ROS-dependent regulation has also been demonstrated for heterologously expressed neuronal P/Q-type voltage-dependent Ca^{2+} channels (534). Similarly, in plants, NOX-derived ROS activate Ca^{2+} channels, thereby regulating root hair outgrowth (268). The mechanisms of NOX enzyme regulation of voltage-dependent Ca^{2+} channels are not entirely understood. Oxidation of amino acid residues within the Ca^{2+} channel complex has been suggested (534); however, cellular depolarization through NOX-dependent electron transport should also be considered.

A second mechanism of Ca^{2+} influx, namely, store-operated Ca^{2+} influx, is also affected by NOX enzymes, however, in the opposite way. Store-operated Ca^{2+} channels are activated by the emptying of intracellular Ca^{2+} stores and not by plasma membrane depolarization. However, Ca^{2+} fluxes through this channel are modulated by an electrical driving force. Thus depolarization due to NOX-dependent electron transport inhibits Ca^{2+} influx through store-operated Ca^{2+} channels (293). In line with

this concept, there is an increased Ca^{2+} influx in phagocytes from CGD patients (293).

B) CALCIUM RELEASE FROM INTRACELLULAR STORES. ROS are also able to induce a rise in $[Ca^{2+}]_c$ through Ca^{2+} release from intracellular stores (316, 937). This is best documented for Ca^{2+} release channels of the ryanodine receptor family, which possess reactive cysteine residues, highly sensitive to oxidation by ROS (547). Activation of these Ca^{2+} release channels has not only been demonstrated for exogenous addition of H_2O_2 (257, 853) and superoxide (448), but also in response to NOX-dependent ROS generation (148, 376, 976). Recent studies suggest that NOX-derived ROS might not necessarily induce global cellular Ca^{2+} elevations, but might also act through rapid, localized intracellular Ca^{2+} transient, referred to as "Ca^{2+} sparks" (148). Ca^{2+} sparks occur as a result of the activation of a small cluster of ryanodine receptors. ROS also act on another type of Ca^{2+} release channel, namely, the inositol trisphosphate (IP_3) receptor family (390, 391).

C) CALCIUM PUMPS. ROS modulate the activity of Ca^{2+}-ATPase pumps (10, 316, 734) in a bimodal fashion. The mechanism of ROS-dependent Ca^{2+} pump activation involves an increasingly recognized mechanisms of ROS-dependent posttranslational processing, namely, *S*-glutathiolation (10). *S*-glutathiolation is a posttranslational modification of protein cysteines mediated by the interaction of peroxynitrite (derived from nitric oxide and superoxide) and glutathione, which ultimately leads to the formation of a reversible disulfide bond between the protein and glutathiolation (87). This *S*-glutathiolation Ca^{2+} pump activation occurs at low ROS concentrations. A stronger oxidative stress leads to an irreversible oxidation of thiols and thereby to enzyme inhibition (316).

C. Gene Expression

There is abundant evidence for the regulation of gene expression by ROS. NOX-dependent ROS generation has been shown to induce, for example, the expression of TNF-α (720), TGF-β1 (338), angiotensin II (338), monocyte chemoattractant protein-1 (338), and plasminogen activator inhibitor-1 (338).

Most studies investigating the mechanisms of mRNA upregulation in response to ROS have concluded that transcriptional upregulation is the underlying cause. This upregulation can occur through redox-sensitive second messenger systems (e.g., MAP kinase activation, Ref. 303) or through transcription factors, including NFκB, AP-1, and p53, which contain redox-sensitive, low-pK_a cysteine residues in their DNA binding domain (846). Indeed, NOX-derived ROS have been shown to effect gene expression through NFκB (96, 162, 582, 686) and AP-1 (1, 916). Alternatively, NOX-derived ROS may also alter gene expression through the alternation of mRNA stability (153, 989).

D. Cellular Death and Cellular Senescence

A large number of studies describe cell death in response to NOX activation (Table 3). ROS can trigger apoptosis either indirectly, through damage to DNA, lipids, and proteins or more directly by ROS-mediated activation of signaling molecules. Such proapoptotic signaling of ROS may occur through activation of MAP kinases, such SAPK/JNK, ERK1/2, and p38 (413). MAP kinase activation occurs in many instances through ROS-dependent inhibition of tyrosine phosphatase (437). At higher ROS concentrations, hydrogen peroxide can inhibit caspases and thereby lead to a switch from apoptosis to necrosis (340, 341).

In some circumstances however, NOX-derived ROS have a prosurvival effect (Table 3). NOX-derived ROS may act as antiapoptotic signals through activation of the NFκB (209) or the Akt/ASK1 pathway (618). It has also been suggested that superoxide is a natural inhibitor of Fas-mediated cell death (164).

Thus NOX activation is most commonly associated with cell death; however, under certain circumstances it may be antiapoptotic. Possible reasons for such apparently contradictory functions include *1*) the magnitude and duration of the ROS signal, *2*) the subcellular localization of the respective NOX isoform, *3*) the set of redox-sensitive signaling targets (e.g., transcription factors, kinases, phosphatases, caspases) expressed in a given cell type (504, 683), and *4*) the metabolism of superoxide (possibly antiapoptotic) versus hydrogen peroxide (proapoptotic) (512, 702).

E. Regulation of Cell Growth

Similar to what is described above for cell death and survival, there are arguments that NOX-derived ROS may lead to either cellular senescence or to enhanced cell growth.

1. Cellular senescence

ROS are thought to be a key mechanism in the aging process (73, 350), and there is abundant evidence for an acceleration of cellular senescence through oxidative stress (169). In the light of these observations, it is not surprising that several studies report NOX induction of cellular senescence and cell cycle arrest (346, 407). Indeed, the first description of NOX4 reported a rapid senescence of NOX4 overexpressing fibroblasts (294).

2. Cellular growth

Yet, despite the well-established role of ROS in cellular senescence, there is also evidence that under many circumstances ROS can accelerate cell growth (116). A

TABLE 3. *NOX enzymes as pro- or antiapoptotic*

Evidence of NOX	Stimulus/Model	Cell Type/Tissue	Reference Nos.
	NOX-induced cell death		
Inhibitors (DPI; AEBSF)	Brain-derived neurotrophic factor or serum deprivation	Neurons	456
Inhibitor (DPI) KO mice (NOX2)	Serum deprivation	Neurons	860
Antisense (p47^{phox})	CD95 ligand	Rat hepatocyte	739
KO mice (p47^{phox})	IFN-γ immune complex arthritis	Chondrocytes	906
Inhibitor (AEBSF)	IL-1β	Chondrocytes	975
Inhibitor (DPI)	Congugated linoleic acid	Jurkat T lymphocytes	85
Inhibitor (DPI)	Long-term insulin exposure	Brown adipose tissue	710
Antisense (p47^{phox}; p22^{phox})	Cerulein	Pancreatic acinar cells	980
siRNA (NOX1)	Branching morphogenesis	Sinusoidal endothelial cells	473
Inhibitor (DPI)	Hyperoxia	Lung epithelium	986
	Paracrine signal from activated myofibroblasts	Epithelial cells	
Inhibitors (DPI; apocynin)	Ischemia	Cardiac myocytes	601
Inhibitor (DPI) siRNA (Rac1)	Tumor promoter TPA	Keratinocytes	988
Inhibitor (DPI)	UVA	Keratinocytes	899
Inhibitor (apocynin)	Angiotensin II	Myocyte	719
Inhibitor (apocynin)	Hyperglycemia	Podocyte	849
Inhibitor (DPI)	TGF-β	Hepatocytes	127
Inhibitors (DPI, apocynin, neopterin)	Glutamate	Neuron	657
Inhibitors (DPI, apocynin, neopterin)	Caffeic acid, ferulic acid	HepG2 hepatoma	520
Inhibitor (DPI)	UVA	Keratinocytes	363
Dominant negative (p47^{phox}, p67^{phox}, Rac)	Palmate	Retinal pericytes	121
Inhibitors (DPI, apocynin, neopterin)	Hydrophobic bile salts	Hepatocyte	740
	NOX-induced cell survival		
Inhibitor (DPI)	Growth factor stimulation	Pancreatic cell lines	910
Inhibitor (DPI) antisense (NOX4)	Fibronectin and laminin	Pancreatic cell lines	238
Inhibitor (DPI)	EGF stimulation	A431 carcinoma cells	628
Inhibitors (DPI; neopterin)	Fas-mediated cell death	Melanoma cell line	164
Inhibitor (DPI)	Spontaneous apoptosis	Guinea pig gastric mucosal cells	868
Inhibitor (DPI) siRNA (NOX4)	Spontaneous apoptosis	PANC-1 pancreatic cancer cells	618
Gene expression (NOX1, NF-κB)	Human colon cancer samples	Colon	278
Inhibitor (DPI)	H_2O_2	Astrocytes	551
XCGD (NOX2)	Phagocytosis	PMN	474

The role of NOX enzymes can be regarded as either pro- or antiapoptotic and may depend on the stimulus, the model, or the method of study.

first hint came from a study showing that many rapidly growing tumor cells release large amounts of ROS (855). Subsequently, many studies have documented a role of ROS as second messengers in cell proliferation (9, 413, 414, 512, 552, 575, 609, 697, 783, 848, 990); in most of these studies there was at least circumstantial evidence that the source of mitogenic ROS was an NADPH oxidase.

There is little doubt that ROS are able to induce cell proliferation, but are NOX enzymes a source of this ROS? No proliferation defects were found in patients or mice deficient in the phagocyte NADPH oxidase NOX2. Thus, when new NOX enzymes were detected, the question of whether they are involved in the regulation of cell proliferation resurfaced. Initial studies showed a role for NOX1

in cell proliferation, and the enzyme was therefore even referred to as "mitogenic oxidase 1" (841). It was suggested that hydrogen peroxide mediates the cell growth and transformation caused by the Nox1 (35). Later, however, the authors of these studies detected the presence of V12 RAS in their cell lines, suggested that the observed transformation was probably due to RAS, and cautioned against the use of these cells (504). Indeed, NOX expression in other fibroblasts failed to produce transformation (504). Nevertheless, there is now a significant number of studies suggesting an involvement of NOX enzymes in cell proliferation. In vitro studies based on either antisense or siRNA suppression suggest a role of NOX4 and NOX1 in smooth muscle cell proliferation (602, 696, 836), a role of NOX5 in proliferation of esophageal adenocarcinoma cells (274), and a role for p22^{phox} in proliferation of endothelial cells (67). Note, however, that angiotensin II-induced aortic smooth muscle proliferation was conserved in NOX1-deficient mice (291).

Thus a critical review of the literature concerning the relationship between NOX enzymes and cell proliferation suggests that *1*) there is abundant evidence for a regulation of cell proliferation in vitro through reactive oxygen species, *2*) in vitro knock-down experiments argue in favor of NOX enzymes being involved in the regulation of cell proliferation, and *3*) there is so far no convincing data from knockout mice suggesting that NOX enzymes play a crucial role for cell proliferation in vivo.

F. Oxygen Sensing

Probably all of our cells are capable of sensing the ambient oxygen concentration and responding to hypoxia. However, some organs are specialized in oxygen sensing, particularly the kidney cortex, the carotid bodies, and the pulmonary neuroepithelial bodies. At least two cellular events allow cells to detect hypoxia (Fig. 6): stabilization of the transcription factor HIF (396) and activation of redox-sensitive K^+ channels (519, 713). In the case of HIF, under normoxic conditions, HIF prolyl hydroxylases mediate HIF hydroxylation at specific prolines and thereby promote its rapid degradation (8, 113). Under hypoxic conditions, this process is inhibited leading to stabilization of the HIF protein. While the hydroxylase is undoubtedly a directly oxygen-dependent enzyme, there is good evidence that increased ROS generation under hypoxic conditions can also contribute to HIF stabilization. The ROS effects may be mediated through oxidation of divalent iron, which is an obligatory cofactor for the hydroxylase. In the case of K^+ channels, normoxia is thought to maintain normal activity, while hypoxia inactivates K^+ channels and thereby leads to cellular depolarization. A well-documented mechanism of K^+ channel inactivation during hypoxia involves decreased
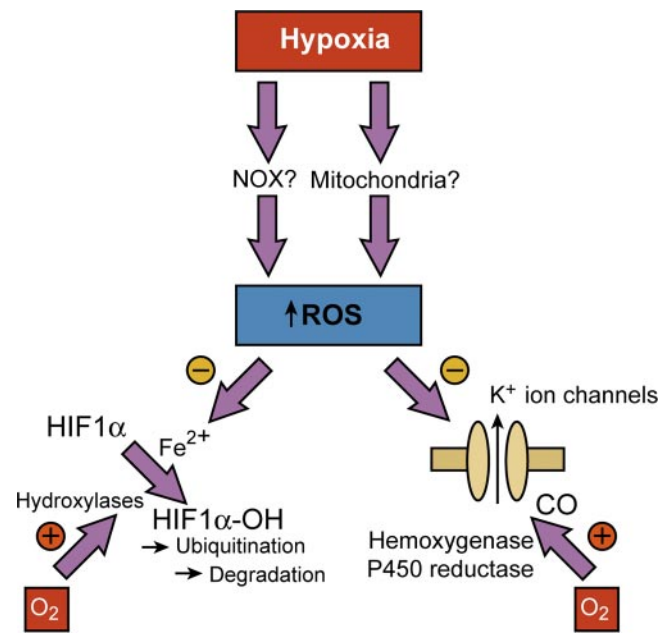

FIG. 6. Reactive oxygen species (ROS), NOX enzymes, and oxygen sensing: revised model based on recent findings. At least two cellular events allow cells to detect hypoxia: stabilization of the transcription factor HIF and activation of redox-sensitive K^+ channels. Under normoxic conditions, HIF is hydroxylated, which leads to its rapid degradation. Under hypoxic conditions, this process is inhibited leading to stabilization of the HIF protein. While the HIF hydroxylase is undoubtedly a directly oxygen-dependent enzyme, there is also good evidence that increased ROS generation under hypoxic conditions contributes to HIF stabilization, possibly mediated through oxidation of the hydroxylase cofactor Fe^{2+}. Under normoxic conditions redox-sensitive K^+ channels are active, maintaining the cellular resting membrane potential. Hypoxia inactivation of K^+ channels leads to cellular depolarization. Pathways leading to K^+ channel inactivation include hemoxygenase-dependent CO generation, but possibly also ROS. Traditionally, NOX enzymes were thought to be involved in oxygen sensing through a decreased ROS generation in hypoxic tissues. However, many recent results led to a revised model where hypoxia increases ROS generation. The source of the hypoxia-induced ROS might be mitochondria and/or NOX enzymes. The physiological effects of ROS, namely, inhibition of K^+ channels and stabilization of HIF, are best compatible with this revised model; however, the reasons why hypoxic cells generate more ROS are still poorly understood.

carbon monoxide (CO) generation (388). Under normoxic conditions, CO is generated by hemoxygenase through an oxygen- and *P*-450 reductase-dependent breakdown of heme. However, the hemoxygenase pathway of K^+ channel inhibition is not exclusive, and there are good arguments that ROS-dependent channel inhibition is also involved.

Traditionally NOX enzymes were thought to be involved in oxygen sensing through a decreased ROS generation in hypoxic tissues. ROS generation by NOX enzymes depends on the concentration of its electron acceptor, i.e., oxygen. Indeed, when NOX2-dependent respiratory burst is measured at oxygen concentrations below 1%, there is a steep drop in ROS generation by NOX2 (281). A reduction in ROS generation during hypoxia is also observed in isolated perfused lungs (31, 681).

Thus, although it is hard to extrapolate from these measurements to the in vivo situation, it is conceivable that under hypoxic conditions, ROS generation by NOX enzymes drops. However, from a mechanistic point of view, this traditional hypothesis is difficult to reconcile with observations that *1*) HIF is activated by ROS (136), *2*) increased ROS levels mimic the inhibition of redox-sensitive K^+ channels by hypoxia (912), and *3*) NOX4 overexpression enhances hypoxic inhibition of the TASK-1 K^+ channel (519).

A revised model where hypoxia increases ROS generation is depicted in Figure 6. Such a hypoxia-induced ROS generation, while counterintuitive, has now been amply documented (337, 362, 455, 581, 941). The source of the hypoxia-induced ROS might be mitochondria and/or NOX enzymes. There are solid arguments for both suggestions. In favor of a role of mitochondria, suppression of the mitochondrial cytochrome-*c* oxidase suppresses hypoxia-induced ROS generation in various cell lines (115, 337, 579). In favor of a role of NOX enzymes, studies using primary mouse carotid body chemoreceptor cells demonstrate that moderate hypoxia leads to increased ROS generation that is absent in cells derived from $p47^{phox}$-deficient mice (362). The mechanisms accounting for hypoxia activation of NOX-dependent ROS generation are not understood.

1. Kidney

In the kidney, hypoxia-dependent posttranslational stabilization of the transcription factor $HIF1\alpha$ leads to transcriptional activation of the erythropoietin gene, a key mechanism for the regulation of erythropoiesis. There are no mechanistic experiments demonstrating a role of NOX4 in oxygen sensing by the kidney, but such a role is conceivable based on the NOX4 localization in the kidney cortex (294, 813).

2. Carotid body

Carotid bodies are sensory organs composed of a small cluster of cells located near the bifurcation of the carotid artery. Carotid bodies detect changes in arterial oxygen saturation and respond to hypoxia by inducing tachycardia and increased ventilation. The carotid body is composed primarily of two cell types: the glomus cells, which act as the primary oxygen-sensing cells, and the sustentacular cells, which surround the innervated glomus cells (713). A decrease in oxygen is rapidly sensed by the glomus cells, resulting in Ca^{2+}-dependent neurotransmission and ultimately in increased respiratory and cardiac function.

Molecules and mechanisms implied in oxygen sensing in the carotid body include hemoxygenase (571), nitric oxide synthetase (712), the mitochondrial respiratory chain (45), a direct ion channel modulation by oxygen (559, 695), and NOX enzymes.

Early theories suggested a role for NOX2, which can be detected in carotid bodies (7, 174, 490); however, the presence of NOX2 in carotid bodies probably reflects the presence of macrophages (236). Several studies suggest normal oxygen sensing in NOX2-deficient mice (32, 361, 760), and there are no reports of any oxygen-sensing deficits in patients with CGD. However, while NOX2 deficiency does not alter oxygen sensing, there are two studies suggesting an altered oxygen-sensing in $p47^{phox}$-deficient mice (362, 775). The apparent discrepancy between NOX2-deficient mice and $p47^{phox}$-deficient mice hints at a role of a $p47^{phox}$-dependent NOX isoform other than NOX2.

3. Pulmonary oxygen sensing

In pulmonary neuroepithelial bodies, oxygen sensing involves redox regulation of K^+ currents. In this system, there is relatively strong evidence for an involvement of NOX2 in oxygen sensing: in NOX2-deficient mice, there is a decreased response to hypoxia in newborn animals (449) and in cells from neuroepithelial bodies (275). This NOX2 dependence of oxygen sensing appears to be specific for the pulmonary neuroepithelial bodies, as oxygen sensing was not impaired in pulmonary artery smooth muscle from NOX2-deficient mice (32).

4. Others

No role for NOX2 in oxygen sensing was observed in Epstein-Barr virus (EBV)-transformed B lymphocytes (946) or in cardiac fibroblasts (761). Endogenously expressed NOX in HEK293 is suggested to cooperate with the TASK-1 K^+ channel in oxygen sensing. This effect can be abolished by the expression of NOX4 siRNA and enhanced by overexpression of NOX4 but not NOX2 (519).

G. Biosynthesis and Protein Cross-Linking

Peroxidation reactions are important in physiology. One of the best-documented roles for NOX enzymes is the iodination of thyroid hormones, a reaction catalyzed by the thyroid peroxidase using DUOX-derived hydrogen peroxide (189, 228, 630). Another example is the H_2O_2 and peroxidase-dependent cross-linking of dityrosine residues in the extracellular matrix, which has been shown to be DUOX-dependent in cutaneous tissue of *Caenorhabditis elegans* (239). Dityrosine cross-linking is also important for the hardening of the fertilization envelope in sea urchin eggs, where it is mediated by the DUOX homolog Udx1 (957). Whether

NOX-dependent dityrosine cross-linking is also important in mammalian tissues is unknown.

H. Regulation of Cellular Redox Potential

Disulfide bond formation is determined by the redox potential. Thus NOX-dependent ROS generation might possibly have a broad influence on protein disulfide bridges in the entire cell or in restricted cellular compartments. Such a role of NOX enzymes has so far received little attention. However, the possible localization of NOX4 in the endoplasmic reticulum (425, 584, 901), the mediation of ER stress through NOX4 (699), and the association between NOX enzymes and protein disulfide isomerase (425) point towards such a role of NOX enzymes.

I. Reduction of Metal Ions

Most functions of NOX family members are linked to the reduction of molecular oxygen to superoxide. However, at least in yeast, the FRE homologs within the NOX family reduce metal ions, particularly trivalent iron (182, 183). Similar to yeast, mammals can absorb divalent but not trivalent iron. Thus a functional ferric reductase activity located within the small intestine has been known and studied for a long time. The fact that NOX1 is localized in the intestine led to initial speculations that it might be the long sought-after mammalian ferric reductase. However, NOX1 localizes mainly to the colon (55, 841), while ferric reductase activity localizes mainly to the small intestine, thus making this possibility rather unlikely. Also, a duodenal cytochrome b, Dcytb, which bears no structural resemblance to NOX enzymes, has been suggested to function as mammalian ferric reductase (596) (although this notion has been recently challenged, Ref. 333).

Iron reduction and absorption is not only relevant in the small intestine. Iron starvation of phagocytosed microorganisms is thought to be a host defense mechanism. Removal of iron from the phagosome is thought to occur in a similar manner to iron absorption in the colon, in two steps: *1*) reduction of trivalent iron through an oxidoreductase and *2*) translocation of divalent iron across the phagosome membrane by a divalent metal transport (Nramp1, Ref. 306). It has been suggested that NOX2-derived superoxide could indeed act to reduce iron (254); however, presently available data suggest that iron uptake in myeloid cells is NOX2 independent (204).

J. Regulation of Matrix Metalloproteinases

There is ample evidence that NOX-derived ROS are involved in the regulation of expression and/or activation of matrix metalloproteinases (130, 199, 329, 412, 540, 561, 763, 831, 906, 978). However, while most studies report a stimulatory role of NOX enzymes on matrix metalloproteinase expression, recent in vivo data from NOX2-deficient mice appear to suggest that the absence of NOX2 might also lead to elevated matrix metalloproteinase levels (440, 578, 902).

K. Angiogenesis

Various pathological conditions associated with neovascularization are associated with ROS generation (525, 815). ROS may directly activate hypoxia-inducible factor (Fig. 6) and thereby increase the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) (495). Angiogenesis-inducing ROS were found to be generated by NOX2 in endothelial cells (141, 896) and in a model of hindlimb ischemia (879), but by NOX1 in tumor models of angiogenesis (30). NOX-derived ROS have also been implicated in angiogenesis during differentiation of embryonic stem cells (782).

L. Cross-Talk With the Nitric Oxide System

Even before nitric oxide was identified as the endothelium-derived relaxation factor, it was recognized that the generation of ROS could lead to a "breakdown" of this factor (330). Indeed, superoxide readily reacts with nitric oxide to produce peroxynitrite, thereby decreasing nitric oxide levels (506, 509). However, it appears that other mechanisms, including uncoupling of the nitric oxide synthase and inhibition of dimethylarginine dimethylaminohydrolase, may also contribute to the antagonistic effect of ROS on the nitric oxide pathway (506).

Nitric oxide is an important mediator in a wide range of physiological and pathophysiological processes and is the subject of numerous articles (recent reviews include Refs. 89, 112, 114). The biological impact of nitric oxide modulation by NOX-derived superoxide is widespread and complex. A few examples are outlined here.

Nitric oxide and superoxide have opposing effects on vascular tone, and they react with one another negating their individual effects (638). NADPH-dependent ROS generation contributes to the development of nitrate tolerance by depleting nitric oxide and by promoting the uncoupling of nitric oxide synthase (637). LPS stimulation of microglia leads to generation of superoxide by NOX2 and nitric oxide by inducible nitric oxide synthase; these react to form peroxynitrite and lead to cell death in neighboring oligodendrocytes (535). Bradykinin reduces myocardial oxygen consumption through nitric oxide. NOX2-derived superoxide antagonizes this nitric oxide effect (460). Nitric oxide inhibits T-cell proliferation, and

this inhibition has been shown to be reversed by NOX-derived superoxide production (904).

A very recent study also suggests a reverse cross-talk through downregulation of NOX1 by nitric oxide (707).

In summary, NOX enzymes exert a broad range of actions beyond the direct killing of microorganisms. Most NOX activities are mediated through ROS; however, in some circumstances the electrical driving forces generated by these enzymes may mediate physiological functions in its own right (640, 792). One of the key activities of NOX-derived ROS is posttranslational modification of proteins (in particular phosphatases, transcription factors, and other signaling molecules). As opposed to earlier concepts, ROS interaction with proteins does not invariably lead to irreversible oxidative damage. In many instances, NOX-derived ROS rather interact as reversible signaling molecules. Other important aspects of the activity of NOX-derived ROS include the interaction with other macromolecules (lipids, carbohydrates, nucleic acids) or with small molecules (in particular nitric oxide), and also the superoxide-driven alkalinization. Many of the effects described here influence one another. For example, elevation of $[Ca^{2+}]_c$ can influence gene expression, cell death, and other cellular signaling pathways, and the putative role of NOX-derived ROS in oxygen sensing is mediated through posttransciptional modifications of transcription factors or ion channels. Thus, although there are still many unresolved issues, the molecular basis of physiological NOX functions is now becoming increasingly clear.

IV. NADPH OXIDASES IN SPECIFIC ORGAN SYSTEMS: PHYSIOLOGY AND PATHOPHYSIOLOGY

There are reports about ROS and ROS enzymes in virtually every tissue and organ system. For the purpose of this review, we selected organ systems that currently appear most pertinent. The sections are organized as follows: *1)* ROS generation in the specific organ or tissue, *2)* expression of NOX isoforms, *3)* physiological role of NOX, and *4)* implication in disease states.

A. Adipose Tissue

ROS generation in adipocytes occurs in response to insulin (486, 487, 569, 570) but has also been described to occur spontaneously and to be enhanced in obese animals (280). ROS generation by adipocytes has characteristics typical of NADPH oxidases. The message for both NOX4 and NOX2 isoforms has been identified in adipose tissues (280, 569). NOX2 and NOX4 mRNA appear to be upregulated in obese rats (280). Data from 3T3-L1 cells, a fibroblast cell line that can be differentiated into an adipocyte phenotype, are less clear. In one study, only NOX4 mRNA was found (569). Another study reported upregulation of

NOX2 mRNA upon exposure to hydrogen peroxide (280). Interestingly, most studies measuring ROS generation by adipocytes (primary cells and cell lines) detect release of hydrogen peroxide rather than superoxide, which potentially argues in favor of NOX4 (see section on NOX4).

ROS are suggested to enhance adipocyte differentiation (485). In differentiated adipocytes, release of ROS through NOX in response to insulin stimulation activates the distal insulin signaling cascade, including mobilization of glucose transporters to the surface of adipocytes (570). However, prolonged exposure to ROS can decrease glucose transporter expression and interfere with glucose uptake (764). The NOX-enhanced generation of ROS observed in obese rats promotes generation of factors involved in obesity-associated metabolic syndrome, such as plasminogen activator inhibitor 1 (PAI-1) and TNF-α, and decreases generation of the insulin-sensitizing, antiatherogenic factor adiponectin (280). NADPH oxidase-dependent ROS have an inhibitory effect on PTPs (149, 961). In obese patients, protein-tyrosine phosphatase activity was increased, and insulin-stimulated glucose uptake was decreased (961). In brown adipose tissue, chronic exposure to insulin induces apoptosis, by a mechanism involving NOX-derived ROS (710).

Thus ROS production in adipose tissue appears to be a physiologically relevant cellular signaling mechanism in the insulin response, exerting a protective antihyperglycemic action through enhanced adipocyte differentiation and glucose uptake by differentiated adipocytes. However, if there is a sustained surplus of glucose in the metabolic balance of the organism, NOX activity will contribute to the development of obesity.

B. Biology of Reproduction

1. Testis, spermatocytes, and fertilization

Probably the earliest descriptions of a respiratory burst came from studies on fertilization (565, 938). Since then, the generation of ROS by sperm has been demonstrated in a large number of species tested (17, 49, 267, 572). The source of ROS generated by sperm was suggested to be an NADPH oxidase within spermatozoa (19, 21, 52, 662, 663, 915). It was shown that Ca^{2+} ionophores induce ROS generation in spermatozoa (191) and that the enzyme responsible for superoxide generation was different from the one found in phagocytes (190). However, some studies suggested that spermatozoa themselves do not possess NADPH oxidase activity (34, 267, 746) and that sperm ROS generation might be explained by leukocyte contamination within sperm preparations (22, 451, 950) or by mitochondrial ROS generation (267).

The understanding of NOX isoforms in sperm advanced with the identification of NOX5, a Ca^{2+}-activated NOX isoform, which is highly expressed in human testis

(56). Yet, the conclusion that NOX5 is the spermatozoa NADPH oxidase is premature for the following reasons. First, in situ hybridization showed NOX5 mRNA only in very early stages of spermatogenesis, in particular pachytene spermatocytes; the NOX5 protein might be expressed in mature spermatozoa, but this has not been demonstrated. Second, although NOX5 is found in many mammalian species, it is not found in rodents, while rodent spermatozoa have also been reported to generate ROS. This apparent discrepancy might be explained by the possible expression of NOX2 and its subunits in mouse spermatozoa (816).

NOX-derived ROS might be important for maturation of spermatocyte maturation or the function of mature spermatocytes. During spermatogenesis, up to 75% of developing spermatocytes are eliminated through apoptosis (65). In analogy with apoptosis in other cell types (in particular neurons and hepatocytes), NOX enzymes might be involved in the mechanisms of apoptosis. Another interesting hypothesis is a potential role for NOX enzymes in cell proliferation and differentiation during spermatogenesis; however, this possibility has not been studied. More data are available on the function of ROS in regulation of activation and function of mature spermatocytes (18, 572). It has been suggested that NOX-derived ROS regulated acrosome formation and capacitation, the final steps in sperm maturation (20, 49, 190, 191, 364, 662, 663).

Finally, NOX enzymes are involved in the respiratory burst that occurs during fertilization (364, 957); in the case of sea urchin eggs, the burst is mediated through the DUOX homolog Udx1. This respiratory burst is thought to prevent the entry of supernumerary sperms through the stabilization of the fertilization envelope. The biochemical mechanism of this stabilization involves dityrosine cross-linking of proteins within the fertilization envelope. Two important points should, however, be considered in this context.

1) Most of the studies on this topic were performed in nonmammalian systems. While mammalian strategies to block polyspermy are often similar to those applied by nonmammals (288), the ROS-dependent stabilization of the fertilization envelope through dityrosine cross-link has to our knowledge not been reported in mammals.

2) The NOX-dependent ROS generation during fertilization is generally attributed to the eggs; whether there is a role for sperm-dependent ROS generation is not clear.

Excessive ROS generation might also be implicated in sperm pathology. Male infertility has been linked to excessive ROS generation (18). In male-factor infertility, oxidative stress is thought to affect the fluidity of the sperm plasma membrane, and ROS-induced DNA damage may accelerate the process of germ cell apoptosis, leading to the decline in sperm counts (13). In line with the janus-faced function of ROS in spermatocyte physiology and pathophysiology, there are reports that antioxidants may prevent oxidative damage to sperm (612) but may be detrimental to sperm development at high levels (867).

In summary, there is now little doubt about the importance of ROS in sperm development and function, and the implication of a spermatocyte NOX enzyme (NOX5, possibly another NOX family member in rodents) is likely.

2. Prostate

Little is known about ROS generation by cells of the prostate, although it is clear that NOX enzymes are expressed. NOX1 and NOX2 were found in total mRNA from prostate (55, 353, 454, 841). NOX5 was described in prostate cancer cell lines and in prostatic adenocarcinomas (103). In response to castration, upregulation of NOX1, NOX2, and NOX4 has been described in the rat prostate (859), indicating a hormonal control of NOX expression.

The physiological function of ROS generation in prostate cells is little understood, but may be related to cell growth (103). The possible role of ROS in prostate cancer development has received particular attention, because the consumption of the tomato-derived antioxidant lycopene is associated with a decreased prostate cancer risk (282). Both NOX1 and NOX5 have been associated with prostate cancer (103, 546). As in other types of cancer, ROS might be involved in carcinogenesis through ROS-dependent DNA damage or through ROS-dependent regulation of cell growth. Interestingly, NOX1-mediated ROS generation decreases P-glycoprotein levels in prostate cancer cell lines (940), raising the question of whether NOX-derived ROS generation might prevent multidrug resistance.

3. Ovary

Within the ovary, generation of ROS has mainly been described for the corpus luteum at regression and in response to prostaglandin $F_{2\alpha}$ (38, 75, 748, 786). The expression of NOX2, NOX4, and NOX5 has been reported in ovaries (143). NOX2 expression might be due to leukocytes, and indeed, it has been suggested that neutrophil NOX2 participates in corpus luteum ROS generation (610).

ROS may lead to cell cycle arrest and apoptosis in vitro, a mechanism that can be limiting for in vitro fertilization (140). However, in vivo, a role for NOX-derived ROS in meiotic maturation of oocytes has been suggested, as maturation was prevented by apocynin and diphenyleneiodonium (226). Angiogenesis in the context of follicular development might also involve NOX-derived ROS (12).

4. Uterus, placenta, and preeclampsia

A) UTERUS. ROS generation by the endometrial epithelium was observed already more than 20 years ago, and an

NADPH oxidase was suggested to be involved (415). ROS levels appear to be increased in the late phase of the menstrual cycle (840). As assessed in total mRNA from uterus, NOX1 (55, 841) and NOX5 (56, 143) are expressed. Proposed NOX functions in the uterus include NFκB activation leading to prostaglandin $F_{2\alpha}$ production (839) and regulation of angiogenesis during the menstrual cycle (12).

B) PLACENTA. Human placental trophoblasts have been suggested to generate ROS, through an NADPH oxidase (589). Studies on solubilized placental NADPH oxidase suggested it to be constitutively active and distinct from the phagocyte NADPH oxidase (577). Within total mRNA preparations of placenta, NOX2, NOX4, and NOX5 have been reported (143, 454). As assessed by Northern blots and RT-PCR, NOX1 mRNA was absent from the placenta (454, 841), although immunoreactivity with NOX1 antibodies has been reported (178). NOX2 expression might be due, at least in part, to the presence of placental macrophages, the so-called Hofbauer cells (641). The physiological role of ROS generation in the placental trophoblast has been suggested to include host defense and degradation of noxious substances (589) or signaling and oxygen sensing (577).

C) PREECLAMPSIA. Evidence continues to accumulate that oxidative stress contributes to the pathogenesis of preeclampsia (pregnancy-induced hypertension) (185). Increased ROS generation by neutrophils has been discussed; however, results are contradictory (172, 887). Studies in EBV-transformed B lymphocytes possibly point towards a genetically determined enhanced NOX2 activity in these cells (518). Microparticles are small membranous vesicles that are released from various cell types. These microparticles have been suggested to be involved in mechanisms of preeclampsia. Microparticles seem to contain a ROS-generating NADPH oxidase (109). It appears that the number of microparticles per se is not different in preeclampsia but that their cellular origin and/or their functional state might be different (308, 309, 908, 909). Specifically, it has been suggested that endothelium-derived (308, 309) and/or T cell- and granulocyte-derived (908) microparticles are increased in preeclampsia. Microparticles from women with preeclampsia, but not from controls, led to endothelial dysfunction through ROS-dependent mechanisms (109, 909).

In a different line of thought, it has been suggested that agonistic antibodies against the angiotensin II receptor AT1 in preeclamptic patients stimulates NADPH oxidases in vascular smooth muscle and in the trophoblast (193).

In summary, NOX enzymes are expressed in the uterus and the placenta, but little is known about their physiological function in this context. There is, however, increasing evidence that NOX enzymes are involved in the pathogenesis of preeclampsia.

C. Cardiovascular System

The involvement of NOX enzymes in cardiovascular physiology and pathophysiology has attracted enormous attention, with over 500 articles published on this topic. Several specialized reviews have been published recently (80, 102, 320, 636, 883).

Note also that statins, widely used drugs to treat cardiovascular diseases, have an indirect NOX inhibitory action through inhibition of Rac isoprenylation (94). There is evidence for the involvement of NOX enzymes in cardiac pathophysiology, and it has been suggested that the beneficial effects of statins in cardiac disease may, at least in part, be attributable to NOX inhibition (613, 642). A protective effect of statins in a stroke model has also been attributed to NOX inhibition (386).

1. Vascular system

ROS generation in the vascular system has been suspected for a long time. Initially, the source of ROS was thought to be mitochondria and thus an unavoidable side effect of aerobic respiration. Subsequent data, however, suggested an active enzymatic ROS generation, with NOX2, at that time the only known NOX family member, and xanthine oxidase as the main suspects (409). Direct measurements of NADPH/NADH oxidase activity in blood vessels or in blood vessel-derived cells were performed in the mid 1990s (321, 620, 621, 680), concluding that such enzymes are a major source of ROS in the vascular system. These measurements also quickly established that there were clear differences between the properties of the phagocyte NADPH oxidase and the enzymatic activity observed in vascular preparations, which led to the widespread use of the term *vascular NADPH oxidase*. However, there is in fact no vascular specific NOX isoform, but rather a complex expression of different NOX isoforms in different cells and regions of the vascular system (Fig. 7).

The message for NOX1, NOX2, NOX4, and NOX5 has been described in arteries (320). In endothelial cells, NOX4 appears to be the most abundant isoform (15, 377, 901), although NOX1 (mRNA and protein) (14, 377, 823), NOX2 (mRNA and protein) (279, 377, 901), and NOX5 (mRNA) (56) have also been described. In vascular smooth muscle, the message for NOX4 is also abundantly expressed (320, 510). The protein expression and activity of NOX4 in cerebral arteries appears to be much higher than in systemic arteries (608). While both NOX1 and NOX2 have also been described in vascular smooth muscle, there appears to be a distinct anatomical distribution: the NOX1 message is mainly expressed in large conduit vessels (510), while NOX2 mRNA is more strongly expressed in resistance vessels (320, 881). There is also a report of DUOX1 mRNA expression in aortic smooth muscle (435). In adventitial fibroblasts, expression of

NOX2 at the protein level is described (133, 744), as well as expression of NOX4 at the mRNA level (133). Little is known about ROS generation and NOX expression in veins, but one study suggests a predominant role of NOX2 in venous ROS generation (335).

The function of NOX-derived ROS in the vascular system is complex and depends not only on the NOX isoform but also on the cell type. Based on studies in vascular smooth muscle, it has been suggested that NADPH oxidase activity largely depends on NOX4 expression under resting conditions (247). Several studies suggest that NOX1 is upregulated at the mRNA and protein level upon angiotensin II stimulation (510, 955), while the data on NOX4 expression and angiotensin II stimulation are contradictory (247, 510, 955). ROS-dependent increase in blood pressure is thought to be a key function of NOX in the vasculature (884, 982). Strong data in favor of a role of NOX-derived ROS as a hypertensive signaling element come from studies demonstrating a decreased systolic blood pressure response to angiotensin II (505) and to BMP4 (611) in $p47^{phox}$-deficient mice. A decrease in basal blood pressure (291) and blood pressure response to angiotensin II (291, 591) is also found in NOX1-deficient mice. Studies on blood pressure in NOX2-deficient mice are less conclusive: angiotensin II-induced hypertension is moderately decreased (935) or not changed (882). Evidence for a role of $p47^{phox}$ in hypertensive signaling comes also from studies in patients with Williams-Beuren syndrome, where $p47^{phox}$ hemizygosity decreases risk of hypertension (201). Thus, taken together, these studies suggest a predominant role for NOX1, possibly functioning together with $p47^{phox}$ as an organizer subunit, in ROS-dependent blood pressure elevations.

In the vasculature, NOX-derived ROS are thought to be involved in nitric oxide inactivation (330, 509, 717), growth and cell division (413, 841), kinase activation (125, 895), activation of matrix metalloproteinases (613), activation of transcription factors and gene expression (730), extracellular matrix regulation (920), endothelial cell proliferation and migration (3), and neointimal formation (142).

The role of NOX family enzymes in vascular pathophysiology has received wide attention. The NOX-derived ROS have been implicated in a variety of vascular diseases, including hypertension (277, 627, 703, 744, 884, 982), aortic media hypertrophy (441, 548, 897), atherosclerosis (41, 137, 324, 355, 825, 832), and vascular diabetic complications (945). NOX-derived ROS produced in response to angiotensin II have been implicated in the vascular complications that are associated with insulin resistance (863). Recently, work in NOX2-deficient mice suggests that NOX2-derived ROS are involved in the pathological interaction between blood cells and vessel walls that occurs in sickle cell disease (958).

FIG. 7. NOX enzymes in the vascular wall. The scheme depicts NOX enzymes in the vascular wall and the text to the right their putative functions. All three layers of the vascular wall [intima (i.e., endothelial cells), media (i.e., smooth muscle cells), and adventitia (i.e., fibroblasts and macrophages)] express NOX family members. NOX4 is the predominant isoform in endothelial cells, NOX1 and NOX4 in smooth muscle cells, NOX4 in fibroblasts, and NOX2 in macrophages. NOX-derived superoxide (O_2^-) avidly reacts with nitric oxide (NO). The effects of NOX in the vascular system might therefore be at least in part due to depletion of the vasorelaxant NO. NOX-derived ROS also affect the extracellular matrix (ECM), influence gene expression, and might be involved in cell proliferation and differentiation.

2. Heart

ROS generation in cardiac tissues is low under basal conditions (232). However, it increases in response to activation by various stimuli (232, 671, 763, 847, 963). Several cell types within the heart contribute to this ROS generation, including cardiomyocytes, endothelial cells, and inflammatory cells (488, 601, 763, 847, 963). The source of ROS produced by cardiomyocytes was initially suggested to be mitochondria (232); however, there is increasing evidence for expression of NOX enzymes in the heart (see Table 4). Cardiac NOX2 is upregulated in response to activation of the aldosterone/angiotensin II pathway (671, 847).

Globally, NOX function in cardiomyocytes can be divided in a developmental function (716, 783, 784, 790) and a function in adult cardiomyocytes. During development, NOX4 is the predominant isoform and drives cardiac differentiation through activation of p38 MAP kinase (536). In the adult heart, NOX2 is the predominant isoform, and it is involved in the regulation of redox-sensitive signaling cascades, such as modulation of kinases and phosphatases (763, 769, 947), gene expression, protease activation (429), and superoxide-dependent nitric oxide inactivation (460).

A) MYOCARDIAL INFARCTION AND MYOCARDIAL REPERFUSION INJURY. The relative importance of NOX enzymes in tissue damage during myocardial infarction remains controversial. Circumstantial evidence in favor of such a role includes the following: *1*) ischemia-induced NOX2 expression leads to apoptosis in cardiomyocytes in vitro (601); *2*) NOX2 levels are increased in cardiomyocytes from patients with myocardial infarction (488); *3*) neutropenia, blockade of neutrophil adhesion receptors, or addition of the ethyl-gallimide (an antioxidant and/or NADPH oxidase inhibitor) leads to decreased infarct size in dog models of myocardial infarction (227, 430); and *4*) coronary arteries from patients with coronary heart disease show increased expression of NOX2 and its subunits, as well as increased ROS generation (334). In mouse models of myocardial infarction, there is no decrease in infarct size in NOX2- and p47^{phox}-deficient animals (273, 382).

Thus either NOX2 does not play a major role in tissue damage during myocardial infarction or compensatory upregulation of other NOX enzymes (273) masks a role of NOX2. The potential role of other NOX enzymes, such as NOX1 or NOX4, has not yet been investigated. In summary, despite appealing theoretical arguments for a role of NOX enzymes in myocardial infarction and ischemia/reperfusion injury, there are presently no strong data supporting this concept.

B) ISCHEMIC PRECONDITIONING. Ischemic preconditioning is an interesting concept that might have clinical applications in surgery. The basic observation is the following: preexposure of the heart to a mild stress [e.g., short ischemia (738) or angiotensin II pretreatment (553)] reduces damage from subsequent ischemic insults. Both ischemic and angiotensin II-dependent preconditioning depend at least partially on NOX2: angiotensin II-dependent preconditioning is attenuated by NOX inhibitors (458), while ischemic preconditioning is completely abolished in NOX2-deficient mice (78). Thus low-level ROS generation by NOX enzymes appears to upregulate protective factors within the heart.

C) CARDIAC HYPERTROPHY, FIBROSIS, AND HEART FAILURE. There is also evidence for the involvement of NOX enzymes in cardiac hypertrophy, fibrosis, and progression towards heart failure (79, 372, 429, 537, 643, 822). The role of various NOX isoforms in cardiac hypertrophy depends on the stimulus. Angiotensin II-dependent cardiac hypertrophy depends on Rac1 (780) and NOX2 (120), through mechanisms including ROS-dependent Akt activation (380). In contrast, pressure overload-induced myocardial hypertrophy does not require NOX2, but might possibly involve NOX4 (120, 593).

D) ATRIAL FIBRILLATION. NOX2-dependent ROS generation is increased in atrial myocytes from patients with a history of atrial fibrillation (457).

3. Shock and related pathologies

ROS are generally thought to play an important role in the pathophysiology of septic shock and organ failure (772). In some models, such as liver injury, there is indeed

TABLE 4. *Expression of NOX enzymes*

	NOX1	NOX2	NOX4
Heart	∅ (55, 143)	√√ (79, 143, 372, 454, 963)	√ (120, 143, 813)
Cardiomyocytes	∅ (457)	√√ (488, 537, 601, 963)	√ (120)
	√ (626)		∅ (457)
			√ (626)
Developing cardiomyocytes	∅ (536)	∅ (536)	√√ (536)
	√ (790)		
Endothelium		∅ (537)	
Fibroblast	∅ (176)	∅ (176)	√ (176)
Phagocytes	∅	√√√ (560)	∅

√, Expression of NOX enzyme; ∅, no expression of NOX enzyme. Reference numbers are given in parentheses.

some indication for a pathogenetic role of NOX-derived ROS. ROS derived from NOX2 in neutrophils are thought to be involved in the destruction of hepatocytes (332) and in intestinal tissue damage (258). A possible role for NOX in shock-associated organ failure is suggested by studies where the amount of tissue injury is reduced by NOX inhibitors such as DPI (2, 332) or apocynin (2, 82, 557). Also, platelet-derived exosomes from septic patients lead to apoptosis of endothelial cells in an ex vivo assay (424). Only limited data from knockout mice are available. p47^{phox}-deficient mice are protected from liver failure after hemorrhagic shock (523). In contrast, there is no survival advantage in NOX2-deficient mice after endotoxic shock (655).

In summary, it is likely that NOX enzymes are important in shock-related pathologies; however, more in vivo data with different NOX-deficient mice will be needed.

D. Central Nervous System

The presence of ROS in the central and peripheral nervous systems has received considerable attention over the past several decades. The nervous system accounts for over 20% of the oxygen consumed by the body, and as a result produces large quantities of ROS. Additionally, the nervous system is particularly sensitive to oxidative stress because of enrichment of polyunsaturated fatty acids in many of the membranes. The overwhelming majority of publications on the role of ROS in the central nervous system (CNS) have focused on the pathological effects of mitchondrial ROS production. However, there are also other sources of ROS in the nervous system, in particular NOX family NADPH oxidases and, as in other tissues, ROS also function as signaling molecules (438, 875, 900, 993). In total brain mRNA, predominantly NOX2 is detected, and only trace amounts of NOX4 and NOX5 were found in one study (143), while another study reported NOX2 only in the fetal, but not in the adult brain (454). NOX4 expression in the brain has been detected by RT-PCR, immunohistochemistry, and in situ hybridization, where it localizes in neurons (900).

The principal cellular constituents of the CNS are three cell types of neural descent, neurons, astrocytes, and oligodendrocytes, as well a one cell type of myeloid descent, namely, microglia.

1. Microglia

Microglia are macrophage-like cells and consequently produce large amounts of ROS through the phagocyte NADPH oxidase (514, 919). In accordance with microglia being a professional phagocyte, a key function of ROS in microglia is thought to be its participation in the host defense and the removal of debris from the CNS.

However, there is increasing evidence for a role of ROS in cell-to-cell communication in the CNS (see below).

2. Oligodendrocytes

Oligodendrocytes are responsible for myelination in the CNS. No ROS generation or expression of NOX enzymes in oligodendrocytes has been reported to our knowledge, although these cells are responsive to ROS generated in neighboring cells (40).

3. Astrocytes

Astrocytes are glial cells with a complex function within the CNS: they play an important role in providing substrates and regulatory molecules for neurons and also participate in the inflammatory response. Astrocytes produce ROS through NADPH oxidases (5, 698); activation of astrocyte ROS generation occurs in response to PKC activation and to Ca^{2+} ionophores (6). One study suggests the expression of the message for NOX4, but not NOX2 in human astrocytes (143). However, two more recent studies report NOX2 expression at both the mRNA and protein level and a decrease in ROS generation in astrocytes from NOX2-deficient mice (6, 698). Thus NOX2 is most likely the predominant NOX isoform expressed in astrocytes.

NOX-derived ROS are involved in astrocyte intracellular signaling (698); they might also be involved in the regulation of cell survival; however, data in that respect are contradictory (151, 551). Increased NOX2 expression is found in reactive astrocytes hinting towards the possibility that astrocyte NOX2 contributes to oxidative damage in neuroinflammation (6).

4. Neurons

In neurons, the expression of ROS-generating NADPH oxidases was considered unlikely for a long time because of their high suseptibility to oxidative damage. However, this paradigm has changed. Neurons express NOX2 (mRNA and protein, Refs. 860, 866), NOX4 (mRNA and protein, Ref. 900), and NOX1 (mRNA, Ref. 404). NOX4 was upregulated during stroke (900), and NOX1 was upregulated in response to nerve growth factor (NGF) (404).

Globally, two functions of NADPH oxidases in neurons have been proposed: alteration of cell fate and modulation of neuronal activity. Induction of neuronal apoptosis in response to serum deprivation or by brain-derived neurotrophic factor is mediated by NOX2 (456, 860). In PC12 cells, NOX1 negatively regulates NGF-induced neurite outgrowth (404). However, NOX enzymes may also modulate neuronal activity. Angiotensin II-stimulated ROS generation (992, 993) is thought to mediate neuronal chronotropic actions (845). There is also increasing evidence for a role of ROS in cognitive functions, and mice overexpressing superoxide dismutase (SOD) have im-
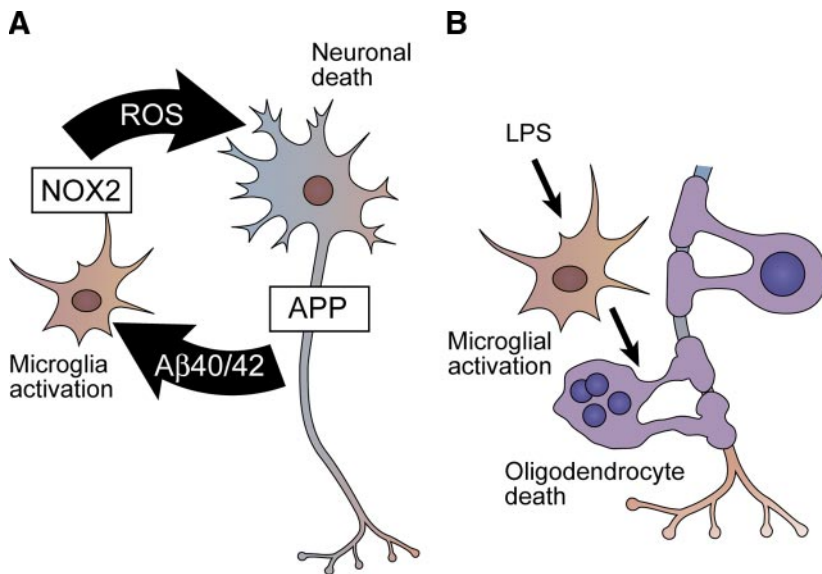
FIG. 8. Role of NOX enzymes in central nervous system (CNS) pathologies. Microglia are phagocytes of the CNS that express high levels of the phagocyte NADPH oxidase NOX2. ROS generation by microglia has been associated with the death of neighboring cells in the CNS. *A*: in the case of Alzheimer's disease, the release of the amyloid precursor protein (APP) fragments amyloid β 40 and 42 (Aβ40/42) activates neighboring microglia cells. Once activated, NOX2 within the microglial cells generates ROS leading to neuronal cell death. *B*: oligodendrocyte death is an early event in the progression of demyelinating diseases. In the case of periventricular leukomalacia, microglia activation, e.g., through maternal or fetal infection, may lead to oligodendrocyte death. More specifically, lipopolysaccharide (LPS) leads to activation of microglial inducible NO synthase and microglial NOX2, which generate peroxynitrate through the reaction of nitric oxide with superoxide. Peroxynitrate is a highly reactive oxygen species that is able to kill oligodendrocytes. Similar mechanisms might be involved in other demyelinating diseases such as multiple sclerosis.

paired memory (875, 876). More specifically, NOX enzymes, particularly NOX2, might be involved in long-term potentiation and learning (438, 469). Indeed, all elements of the phagocyte NADPH oxidase are found in hippocampal neurons (866), and an NADPH oxidase, presumably NOX2, is involved in NMDA receptor signaling (462). Most importantly, learning and memory are impaired in NOX2- and p47^{phox}-deficient mice (461), as well as in CGD patients (682). Note, however, that the degree of learning and memory impairment is mild, suggesting either that NOX2 plays only a modulatory role or that various NOX isoforms have a redundant function.

An interesting aspect of NOX enzymes in the CNS is their role in intercellular signaling. For example, ROS produced in hippocampal neurons during long-term potentiation diffuse into neighboring oligodendrocytes where they stimulate kinases (40); the resulting enhanced phosphorylation of myelin basic protein is thought to enhance myelinization (39).

5. NOX enzymes in pathologies of the CNS

ROS generation by NOX enzymes has been implicated in a variety of diseases of the CNS.

A) ISCHEMIC STROKE. Stroke size was markedly reduced in NOX2-deficient mice (927), while increased NOX2 expression in diabetic rats was associated with an aggravated ischemic brain injury (496). In a gerbil model of global cerebral ischemia-reperfusion injury, apocynin strongly diminishes damage to the hippocampus (936).

B) ALZHEIMER'S DISEASE, PARKINSON'S DISEASE, AND HIV DEMENTIA. There is also increasing evidence for a role of microglial NOX2 in inflammatory neurodegeneration, including Alzheimer's disease and Parkinson's disease (152, 983). In the case of Alzheimer's disease (Fig. 8*A*), several studies show a role of microglia in amyloid precursor protein (APP)-dependent neurodegeneration (152, 689, 810). APP fragments released from neurons activate NOX2 in neighboring microglia cells (718) through a Vav-dependent mechanism (953). The consequent ROS generation by microglial NOX2 leads to death of neighboring neurons (718). Several studies suggest similar mechanisms in Parkinson's disease (91, 283, 284); however, the microglia-activating ligands are less well defined. A strong argument for a role of NOX2 in Parkinson's disease also comes from experiments in NOX2-deficient mice, which are protected in a MPTP model of Parkinson's disease (985). Dementia is a common problem in advanced HIV disease, and microglia activation is thought to be a key element in the development of the disease (128); activation of NOX2 by the HIV-1 Nef suggests an involvement of NOX2 (919).

ROS have also been implicated in the progression of the demyelinating disease (Fig. 8*B*). ROS produced by microglial NOX2 are required for the phagocytosis of myelin, yet these ROS can also cause damage to the myelin sheath (903). Periventricular leukomalacia is a focal necrosis of white matter in the brain that underlies most cases of cerebral palsy. In periventricular leukomalacia, the combination of NOX2-derived superoxide and inducible nitric oxide synthase-derived nitric oxide leads to the formation of peroxynitrate and thereby to the killing of oligodendrites (535). The data on the role of the phagocyte NADPH oxidase system in autoimmune encephalomyelitis yielded complex results: depending on the length of the antigen that was injected, p47^{phox}-deficient or p47^{phox} mutant mice developed either a less severe or a more severe disease (399, 904). Also, genetic intercross experiments in susceptible and resistant rats

identify a low ROS generating variant of p47^{phox} as an aggravating factor for autoimmune encephalitis (71). Thus the role of NOX autoimmune encephalitis lies in the boundary between the situation in neurodegenerative disease, where ROS are disease-causing factors and autoimmune disease where ROS appear protective.

E. Endocrinology

There is very strong evidence for an involvement of NOX family NADPH oxidase in thyroid function. There is also increasing evidence for their role in dysfunction of the endocrine pancreas and in the development of diabetes. In other endocrine organs, there is little evidence for a role of NOX family enzymes, albeit some immunolocalization data exist (491, 492).

1. Thyroid

H_2O_2 generation by thyrocytes was described by the early 1980s (88, 921), and an NADPH oxidase activity was quickly suggested to be the underlying mechanism (605). The discovery of the DUOX enzymes (see above) provided the molecular basis for the thyrocyte ROS generation.

The physiological role of DUOX in the thyroid is well studied and well understood. Synthesis of thyroid hormones requires hydrogen peroxide for oxidation and incorporation of iodine into thyroglobulin. DUOX provides the hydrogen peroxide for this reaction. Despite the presence of a peroxidase homology domain in DUOX enzymes, the peroxidase reaction requires a separate enzyme, namely, the thyroid peroxidase. The biochemical demonstration of DUOX function is strongly supported by recent data on patients with mutations in DUOX genes (629, 690). Biallelic inactivating mutations in the DUOX2 gene result in complete disruption of thyroid-hormone synthesis and are associated with severe and permanent congenital hypothyroidism, while monoallelic mutations are associated with milder, transient hypothyroidism (630). DUOX2 sequence variants associated with mild hypothyroidism have also been described (918). An unresolved issue is the question why DUOX1 cannot substitute for DUOX2 in thyroid hormone synthesis. DUOX1 is clearly expressed in the thyroid and is from a structural point of view very similar to DUOX2. Yet, DUOX2 mutations lead to a severe phenotype, while, to our knowledge, no DUOX1 mutations leading to hypothyroidism have been described.

2. Endocrine pancreas

At least under pathological conditions such as hyperglycemia, oxidative stress can be detected in pancreatic islet β-cells (405, 960). In contrast, islet cells appear to have a very low antioxidant capacity (318). Chronic long-term exposure to ROS has been associated with impairment of β-cell function and with complications of diabetes (305). Different sources for ROS generation by islets have been suggested; these include mitochondria and, more recently, NADPH oxidase isoforms (665, 886). Both NOX2 (protein) (648) and NOX4 (mRNA) (250) are expressed in β-cells. In both studies, an increased expression of these NOX isoforms in animal models of diabetes has been proposed (250, 648). Thus a role of ROS in the development of β-cell failure in diabetes has been widely discussed. In line with this concept are observations that glucose toxicity can be prevented by antioxidants (861).

F. Gastrointestinal System and Liver

1. Stomach and Helicobacter

Gastric epithelium is able to generate ROS. The traditionally held view is that sources of radicals in the gastrointestinal tract include mucosal xanthine oxidase and NADPH oxidase found in the resident leukocytes of the lamina propria (670). However, more recent results suggest that NOX family NADPH oxidases might also be expressed in gastric epithelial cells. This question has been addressed predominantly in guinea pig stomach, where ROS generation by gastric mucosa has been convincingly documented (869). On the basis of several lines of evidence, NOX-type NADPH oxidases were identified as the source of this ROS generation. The responsible enzyme was initially suggested to be NOX2 based on activity and expression at the protein level (870); however, more recent results based on mRNA suggest that it is rather NOX1 (868). *Helicobacter pylori* lipopolysaccharide activates NOX1 and increases expression levels of NOX1 and its cytosolic subunit NOXO1 (443, 447, 870). However, the expression of NOX1 in the stomach might be species specific. In the human stomach, message for NOX2 and NOX5, but not NOX1, is found (770). In the dog stomach, Northern blot analysis suggests the expression of DUOX2 (189). In the mouse system, the expression profile of NOX enzymes in the stomach has, to our knowledge, not been studied. The understanding of the role played by NOX enzymes in the stomach is at a preliminary stage. In the guinea pig, NOX1 is suggested to be involved in the regulation of growth and apoptosis of gastric mucosal cells (868). In mice, the role of NOX2 in response to anti-inflammatory agents and infection has been studied. NOX2-deficient mice are less susceptible to nonsteroidal anti-inflammatory drug (NSAID) injury than wild-type mice (72), raising the possibility that NOX2 is involved in gastric injury by NSAID. Two studies have investigated gastric *Helicobacter pylori* infection and found markedly increased inflammation in NOX2-deficient mice (90, 450). Importantly, this was not due to a decreased host defense function, as bacterial load was either comparable (450) or

even decreased (90). Thus the mouse model suggests that *Helicobacter* infection is another example for the hyperinflammatory response associated with NOX2 deficiency (see sect. IIIA5). Increased gastric inflammation has also been described in patients with CGD (62, 580).

2. Colon

For a long time, it was thought that oxidative stress in the colon was essentially derived from NOX2 due to leukocyte invasion. However, colon epithelial cells themselves are capable of generating superoxide (445, 668, 669, 701). After the discovery of NOX homologs, it rapidly became clear that, in addition to a low-level expression of NOX2 (143, 454), NOX1 is highly expressed in the colon (55, 454, 841). NOX1 expression follows a gradient with low levels in the proximal colon, and high levels in the distal colon (64, 854). Within the colon wall, in situ hybridization suggests either even distribution between apical surface and crypts (854), or a greater localization to the lower part of the crypts (295), while protein expression appears to be highest on the mucosal surface (445). Thus reminiscent to the situation of NOX2 in phagocytes, it appears that the NOX1 gene is turned on in immature precursor cells; however, the mature protein is found predominantly in well-differentiated cells. DUOX2 is also expressed in the distal gastrointestinal tract, in particular, cecum, sigmoidal colon, and rectal glands (230, 246, 299). The more precise anatomical localization is suggested to be highly differentiated enterocytes within the apical membrane of the brush border in one study (246), while another study suggests the lower half of the rectal glands (299). The function of NOX1 and DUOX2 in the distal gastrointestinal tract is not understood. Basically, there are two major working hypotheses: a role in host defense and a role in the regulation of cell proliferation. Given the high degree of similarity between the NOX1 and NOX2 systems, the host defense hypothesis is appealing; however, no convincing evidence has been produced so far. The evidence in favor of the host-defense hypothesis includes the observation that NOX1 expression is enhanced by IFN-γ (295, 498), is activated by LPS (447) and flagellins (445), and is more highly expressed in the distal colon, similar to the pattern of bacterial colonization (854). The alternative working hypothesis is that NOX1 acts as a mitogenic oxidase in the colon. Given the high turnover of colonocytes, high-level expression of a mitogenic enzyme makes sense. However, as with the proposed host defense function, there is limited direct experimental evidence concerning the role of NOX1 in colonocyte proliferation. One study reports that NOX1 levels are increased upon differentiation and growth arrest in colonocytes (295), while another study suggests that highest NOX1 levels are found in the subconfluent state (701). If NOX1 is to act as a mitogenic oxidase, one would predict that it should be found in the colonic crypts. However, while this might be the case for NOX1 mRNA (295, 854), it appears that the protein is predominantly located at the luminal surface (445). Note also that NOX1 knockout mice are healthy and have normal weight, arguing against a severe cell growth defect in the colon epithelium (291). The possible function of DUOX2 in the distal gastrointestinal tract includes a role in host defense, possibly in collaboration with lactoperoxidase (246, 295).

ROS generation has been implicated in the progression from inflammatory bowel disease to cancer (171, 417). NOX1 might contribute to development of colon cancer through at least two mechanisms: ROS-dependent DNA damage and ROS-dependent enhancement of cell proliferation. However, little experimental data concerning this question are presently available. With the use of monoclonal antibodies against NOX1, a positive correlation between the level of NOX1 expression and the degree of differentiation in adenocarcinomas was observed (278). However, studies using in situ hybridization and mRNA array analysis observed no difference between normal and tumor colon samples (295, 854), and antisense downregulation of NOX1 did not decrease proliferation of Caco-2 colonocytes (295).

Oxidative stress is thought to be involved in the pathogenesis of inflammatory bowel disease (595). Thus the discovery of NOX1, highly expressed in the colon, raised the question to which extent an overactivity of this enzyme could be involved in the pathogenesis of inflammatory bowel disease. However, to date, this question has received little attention. One surprising piece of evidence came from in situ hybridization studies of bowel biopsies from patients with Crohn's disease or ulcerative colitis, which demonstrate expression of NOX1 in lesional lymphocytes (854). The role of NOX1 in lymphocytes and the possible link with the pathophysiology remains unknown. There is another possible link between inflammatory bowel disease and NOX family NADPH oxidases. CGD patients develop an inflammatory bowel disease, suggesting that NOX2-derived ROS might play a role in the defense against colon inflammation (395, 580, 788) (see also sect. IIIA5).

3. Liver

A) HEPATOCYTES. Hepatocytes generate ROS in response to a wide variety of endogenous and exogenous stimuli, including CD95 (Fas) ligand (CD95L) (741, 742), TGF-β (371), and alcohol (48). Hepatocytes contain a number of systems that might be involved in the generation of ROS. In addition to NOX family NADPH oxidases, mitochondria, cytochrome *P*-450 enzymes, ER oxidoreductases, and cytosolic peroxisomal and xanthine oxidases may also generate substantial levels of ROS in

hepatocytes. Already in 1981, an NADPH oxidase-like activity had been described in membrane preparations from hepatocytes; as opposed to membranes from phagocytes, the hepatic system appeared to be able to use either NADH or NADPH (727). Histochemical localization placed the NADPH oxidase-like activity within the bile-canalicular plasma membranes (617). ROS generation by hepatocytes in response to capsaicin (521), tamoxifen (522), CD95 (Fas) ligand (CD95L) (739), and TGF-β (371) is thought to be NOX mediated. In total liver mRNA, relatively large amounts of NOX2 (143, 454) and trace amounts of NOX4 (143) have been described. Little information is currently available regarding the NOX isoforms present specifically in hepatocytes. PCR analysis suggested that rat hepatocytes expressed mRNAs of NOX1, NOX2, NOX4, DUOX1, and DUOX2 (739). However, these results were obtained by PCR exclusively, and the large set of NOX isoforms identified in hepatic stellate cells (739) is not in line with other studies (63).

The main function attributed to NOX-derived ROS in hepatocytes is apoptosis (521, 739). Several arguments point towards a role of NOX-derived ROS in CD95L-dependent hepatocyte apoptosis: 1) p47^{phox} is phosphorylated in response to CD95L, 2) NADPH oxidase inhibitors prevent ROS generation and reduce apoptosis, and 3) antisense RNA against p47^{phox} prevents CD95L-mediated ROS generation and reduces apoptosis (739). Hepatocyte ROS generation in response to TGF-β is also thought to lead to apoptosis through NOX activation (371). Thus it appears that a p47^{phox}-containing NADPH oxidase (possibly NOX1 or NOX2) mediates hepatocyte apoptosis.

There is, however, a difference between the results obtained in vitro in isolated hepatocytes and those obtained from in vivo studies. In one study, treatment of rats with a peroxisome proliferator compound led to an enhanced hepatocyte proliferation through NOX-dependent hepatic ROS generation (767). The different outcome of hepatocyte exposure to ROS (proliferation vs. cell death) might be explained by the different source and/or quantity of ROS. While TGF-β and CD95L activate an NADPH oxidase within hepatocytes, the peroxisome proliferator compound is thought to generate ROS in Kupffer cells. Thus ROS generated by hepatocytes themselves might lead to apoptosis, while exposure to ROS, generated by neighboring cells, might rather cause hepatocyte proliferation. There may also be species-specific factors involved, as the phosphorylation events in response to peroxisome proliferator agents that regulate kinase and phosphatase activity do not occur in human hepatic-derived cells (694).

In addition to hepatocytes, the liver consists of many nonparenchymal cells types, including endothelial cells, pit cells, Kupffer cells, and hepatic stellate cells (also known as Ito cells, hepatic pericytes, perisinusoidal cells, and lipocytes).

B) HEPATIC STELLATE CELLS. The many names of hepatic stellate cells (HSC) reflect their many proposed functions, which include regulation of microvascular blood flow as well as storage of fat and vitamin A. In response to hepatocyte injury, HSCs transform from star-shaped vitamin A-rich to myoblast-like, vitamin A-deficient cells and are involved in both the secretion and degradation of extracellular matrix (805, 837).

HSCs generate ROS in response to various stimuli (9, 63, 793, 838), particularly TGF-β (177). HSCs express p22^{phox} (46), but which NOX isoform is expressed is less clear. Both NOX1 (mRNA) and NOX2 (mRNA and protein) are found in HSC primary culture and cell lines (9, 63). However, in freshly isolated human HSCs, the situation is different: neither isoform is found in healthy controls, while NOX1, but not NOX2, is found in samples from patients with hepatic fibrosis (63). Interestingly, p47^{phox}-deficient mice are protected from hepatic fibrosis in a bile duct ligation model of hepatic injury, suggesting that a p47^{phox}-containing NOX complex is involved (63). Taken together, these data are compatible with p47^{phox} acting as the organizer subunit for NOX1 in HSCs, although this model presently remains speculative. Proposed functions of NOX-derived ROS in HSCs include the induction of cell proliferation (9, 793, 838) and the production of collagen (177, 838). Consequently, a role for NOX enzymes in hepatic fibrosis, characterized by HSC proliferation and accumulation of extracellular matrix proteins, has been suggested (9, 63).

C) KUPFFER CELLS. Kupffer cells are specialized tissue macrophages within the liver. ROS generation in Kupffer cells occurs in response to stimuli such as LPS, peroxisome proliferator compounds (767), as well as to the yeast cell wall extract zymosan (86). ROS generation by Kupffer cells also occurs under pathological conditions such as hypoxia-reoxygenation (768) and in response to ethanol (358).

Being tissue macrophages, Kupffer cells express the phagocyte NADPH oxidase NOX2 and its subunits (379, 767). LPS-stimulated ROS generation is absent in p47^{phox}-deficient Kupffer cells (767), demonstrating the importance of a p47^{phox}-containing NOX enzyme, presumably NOX2, in this cell type. NOX2-derived ROS in Kupffer cells most likely have several functions. They have been suggested to play an important role in clearing pathogens derived from the gut (500). They are also involved in the regulation of gene expression, particularly the expression of the CD95 ligand (890) and NFκB-dependent production of TNF-α (753). Finally, they have been suggested to be essential mediators in antigen presentation by Kupffer cells (568).

D) LIVER CIRRHOSIS AND ALCOHOLIC LIVER DISEASE. The involvement of oxidative stress in alcohol-induced liver disease was suggested 40 years ago (212) and has been largely confirmed since (36). Exposure to alcohol leads to

increased hepatic generation of ROS (101, 482). ROS may activate signaling cascades leading towards hepatic fibrosis (see sect. iv*F3*b) and may have a direct toxicity on hepatocytes, particulary through the formation of peroxynitrite and α-hydroxyethyl (α-OH-ethyl) radical (36, 597). However, while a role of ROS in alcohol toxicity is widely accepted, there is disagreement concerning whether the predominant source is a cytochrome *P*-450 (CYP2E1) (475) or an NADPH oxidase (358, 482). Inhibition of alcohol-induced ROS generation by DPI has been suggested as an argument for a role of an NADPH oxidase (358); however, DPI also inhibits cytochrome *P*-450 enzymes (991). Mice deficient in NOX or NOX subunits appear better suited to study the question. In favor of a role of NOX family NADPH oxidases, p47^{phox}-deficient mice show reduced alcohol-induced hepatic CD14 upregulation (949), and reduced hepatic pathology and ROS generation, as detected by spin trap (482). Conversely, in another study, alcohol induction of oxidized purines and DNA repair genes is absent in CYP2E1-deficient mice, but preserved in the p47^{phox}-deficient mice (101). Thus, in mouse models, NOX-derived ROS contribute to alcoholic liver damage, but their relative importance compared with cytochrome *P*-450-derived ROS remains to be elucidated.

E) HEPATIC CANCER. Hepatic carcinogenesis is thought to involve ROS-induced DNA damage and/or mitogenic signaling. ROS play a role in development of hepatic cancer in a variety of rodent models including mice overexpressing c-*myc* and TGF-α (251) and exposure to carcinogens such as peroxisome proliferator compounds (733) and diethylnitrosamine (871). NOX2 is thought to be important in this context, and indeed, the ROS response of Kupffer cells to a peroxisome proliferator is abolished in p47^{phox}-deficient cells (766, 767). p47^{phox}-deficient mice have diminished genotoxic and cytotoxic effects of diethylnitrosamine (DEN) in the liver (871). However, one publication using p47^{phox}-deficient mice suggests that the activation of NADPH oxidase in Kupffer cells is not involved in peroxisome proliferator-mediated DNA damage (765). Increased NADPH oxidase activity has also been implicated in the progression of nonalcoholic fatty liver disease (126).

F) LIVER ISCHEMIA AND REPERFUSION INJURY. Ischemia reperfusion injury in many organs is characterized by a hypoxic insult to tissue during the ischemic phase, followed by a massive ROS generation during the reperfusion phase. In many cases it is thought that the insult through the ROS generation is as important as the insult through hypoxia. First arguments that NOX enzymes might be important in liver ischemia/reperfusion injury came from studies showing that hepatic expression of a dominant negative Rac1 suppressed ischemia/reperfusion-induced production of ROS (724) and resulted in a reduction of liver necrosis (673). Several other studies

confirmed such an effect of Rac inhibition (349, 672, 674). However, while Rac GTPases are indeed important activators of NOX1 and NOX2 (see above), they may also be involved in regulation of ROS generation from other sources, in particular mitochondria (724, 948). Yet, several more recent studies support a role of NOX2 in liver ischemia/reperfusion injury. ROS generation by Kupffer cells (presumably NOX2) plays an important role in liver reperfusion injury after cold preservation (809). Importantly, NOX2-deficient mice show reduced hepatic reperfusion injury (348), although this effect might be relevant only after prolonged periods of ischemia and reperfusion (379).

G. Kidney and Urinary Tract

1. ROS and NOX in kidney physiology

Formation of ROS is observed in many cell types of the kidney, in particular the cortical area (331). ROS generation increases in response to certain physiological stimuli, including angiotensin II (312, 346, 347, 556, 558, 616), chemokine receptors (397), and aldosterone (616).

At least three different NOX isoforms are expressed in the kidney cortex: NOX4 (132, 143, 294, 616, 813), NOX2 (11, 132, 143, 454, 911), and NOX1 (132, 454, 616). Although no strict comparisons have been performed, based on mRNA levels it appears that NOX4 is most abundant. The predominant NOX4 localization within the kidney cortex is renal tubular cells (294, 813), but at lower levels it is also found in other cell types, including glomerular mesangial cells (311).

The proposed function of NOX-derived ROS in the kidney can be classified into three major categories, namely, *1*) regulation of renal blood flow, *2*) alteration of cell fate, and *3*) regulation of gene expression.

A) RENAL BLOOD FLOW. ROS are involved in the regulation of renal blood flow. A key mechanism is the reaction of superoxide with NO, which limits its relaxing effect on afferent arterioles (558, 952). On the basis of studies using NOX2-deficient mice and the inhibitor apocynin, NOX enzymes are a likely source of the ROS involved in the regulation of renal blood flow (347, 558).

B) CELL FATE. NOX-derived ROS can alter renal cell fate in at least three ways: *1*) enhancement of epithelial-mesenchymal transition (745, 877), presumably through MAP kinase activation (745); *2*) induction of mesangial cell apoptosis (556); and *3*) promotion of cellular hypertrophy through activation of ERK1/ERK2 (312) and through p27Kip1-dependent cell cycle arrest (346).

C) GENE EXPRESSION. NOX-dependent oxidative activation of transcription factors [NF-κB (220), c-*jun* (179)] leads to enhanced expression of renal target genes [phospholipase A$_2$ (220), MCP-1 and CSF-1 (781), COX-2 (261,

423)]. Note that mitochondrial ROS might also contribute to COX-2 expression (974).

2. ROS and NOX in kidney pathophysiology

In general, enhanced generation of ROS by NOX enzymes is thought to participate in nephrotoxic pathways.

A) DIABETIC NEPHROPATHY. A role for NOX-derived ROS in diabetic nephropathy is now widely accepted (343, 515, 539, 779). There are also first indications that (nonspecific) NADPH oxidase inhibitors may prevent renal damage in diabetic animals (37). However, there is still a debate regarding the NOX isoforms involved in mediating ROS-dependent tissue damage in diabetic nephropathy.

Upregulation of NOX2 or subunits thereof have been reported in several studies (37, 264, 310, 779), and antisense RNA against p47^{phox} prevents high glucose-induced ROS generation in mesangial cells (393). Increased expression of NOX4 mRNA and protein in diabetic nephropathy has also been reported (250, 310), and treatment of diabetic animals with NOX4 antisense RNA decreases kidney pathology (310). It is tempting to speculate that NOX2 and NOX4 act synergistically in generating ROS-dependent damage in diabetic nephropathy, but more studies will be necessary to clarify this point.

3. High salt and hypertension

High-salt diet induces p47^{phox} and NOX2 mRNA expression in the renal cortex (276, 464), and increased expression of p47^{phox} and NOX2 mRNA and protein was found in the kidney of spontaneously hypertensive rats (11, 132, 984). A moderately decreased blood pressure in NOX2-deficient animals has also been reported (935), but it is not clear whether vascular NOX2 or renal NOX2 is most relevant for this decrease in blood pressure.

4. Others

Chronic renal failure is associated with elevated NOX2 protein expression (911), and there is also evidence that ROS generation is involved in the pathogenesis of acute renal failure (651). Aminoglycosides are thought to induce nephrotoxicity at least in part through ROS. They have been suggested to stimulate mitochondrial ROS generation in the renal cortex (928), although more recent results suggest that a NOX enzyme participates in aminoglycoside-dependent mesangial cell contraction and proliferation (583).

H. Lung and Airways

Total lung or airway mRNA contains solid amounts of NOX2 (143, 454) and DUOX1 (299) as well as low amounts of NOX4 (143). The bulk of the NOX2 is likely due to alveolar macrophages, typical phagocytes which are crucial for host defense, but which also participate in a variety of inflammatory lung diseases. The low-level expression of NOX4 and the lack of detection of other NOX enzymes, however, does not exclude an important role of these isoforms in a particular pulmonary cell type.

1. Airway epithelium

Airway epithelia are able to generate ROS (139, 271). NOX isoforms expressed in airway epithelia are DUOX1 (299, 352, 513, 794), DUOX2 (271, 352, 794), and possibly NOX2 (513). Duox1 expression is increased by treatment with Th2 cytokines IL-4 and IL-13, whereas Duox2 expression is induced after treatment with the Th1 cytokine IFN-γ (352). Proposed functions for NOX enzymes in airway epithelia include host defense (299), acid production (794), response to mechanical stress (139), regulation of gene expression (513, 684), activation of TNF-α-converting enzyme leading to mucin expression (808), and induction of cell death (986).

2. Alveolar cells

Type II pneumocytes release ROS (459, 706). This ROS generation has been attributed in part to mitochondria (459, 706), and at least in part to an NADPH oxidase-like enzyme (706, 905). However, so far no NOX isoforms have been reported. Proposed functions of ROS generation in alveolar type II cells include host defense (905), induction of IL-8 expression (200), and oxidative protein inactivation (930).

3. Lung vasculature and pulmonary hypertension

NADPH oxidase-dependent ROS generation has been described in pulmonary endothelial cells (263, 609, 685) and smooth muscle cells (104). It has been suggested that NOX4 is the predominant NOX isoform in pulmonary smooth muscle (383). Proposed function of NOX-derived ROS include NFκB activation (104), MAP kinase activation (685), and cell proliferation (104, 609). It appears that in the pulmonary vasculature, NOX enzymes play a particularly important role in the response to changes in the oxygen concentration. Paradoxically, an increase in ROS generation is observed in response to both hyperoxia (154) and hypoxia (263, 581, 943, 944). Hypoxia-induced NOX activation is thought to lead to ROS-dependent vasoconstriction. Such a model would suggest that NOX activation plays an important role in pulmonary hypertension (432). Indeed, there are many experimental data that hint towards such a role of NOX, including an NADPH oxidase activation in response to stimuli thought to be involved in the development of pulmonary hypertension (218, 326, 587), an enhancement of pulmonary vasoconstriction through exogenously added superoxide (549),

and an increase in NADPH oxidase expression or activity in hypoxic pulmonary hypertension (106, 326, 550).

4. Pulmonary fibroblasts and pulmonary fibrosis

In pulmonary fibroblasts, ROS generation with pattern typical for NOX enzymes is observed in response to TGF-β1 (873), irradiation with alpha particles (649), and rhinovirus infection (211).

p47^{phox}, p67^{phox}, p22^{phox}, and NOX4, but not NOX2, are expressed in lung fibroblasts (211), and p67^{phox} upregulation occurs in response to rhinovirus infection (211). Oxidative stress in response to rhinovirus infection is reduced upon treatment with p47^{phox} antisense RNA (211). Proposed consequences of NOX in pulmonary fibroblasts include IL-8 upregulation (211) and induction of epithelial cell death through a paracrine mechanism (926).

Pulmonary fibrosis is characterized by lung inflammation and excessive generation of ROS (939). Antioxidant treatment protects against bleomycin-induced lung damage in rats (807), and mice deficient in extracellular SOD develop an exaggerated fibrosis in response to bleomycin (255). Most importantly, the fibrotic response to bleomycin is markedly attenuated in p47^{phox}-deficient mice (578). Thus a p47^{phox}-containing NADPH oxidase appears to be involved in the development of pulmonary fibrosis; however, whether this is simply due to the release of ROS by the macrophage NADPH oxidase (NOX2) or whether it also involves p47^{phox}-dependent ROS generation in fibroblasts remains to be seen.

5. Asthma and chronic obstructive pulmonary disease

ROS-mediated damage is thought to be important in the pathogenesis of asthma and chronic obstructive pulmonary disease (726), and NADPH oxidases are thought to be a relevant source of ROS in this context (383). NOX-derived ROS in the lung may be generated by inflammatory cells, including macrophages, neutrophils, and eosinophils (501), but also within airway smooth muscle (383). Antioxidant treatment and overexpression of extracellular SOD appears to decrease airway hyperresponsiveness in animal models (369 and references therein). To our knowledge, the involvement of NOX enzymes in asthma has not been directly tested in knockout models. Interestingly, Picrorhiza, a traditional medicine used as a treatment for asthma (221), contains the NOX inhibitor apocynin as one of its active ingredients. A new element has been provided by the observation that ROS generated by pollen NADPH oxidase may also participate in airway inflammation (95, 749).

However, the role of NOX derived in lung is not soley destructive. NOX2-deficient mice develop spontaneous emphysematous destruction of their peripheral airspaces, presumably through an increased matrix metalloprotein-

ase activity (440). This may be another example for the hyperinflammatory response observed in NOX2-deficient mice (see sect. III*A5*).

I. Musculoskeletal System

1. Bone

A) OSTEOCLASTS. Osteoclasts are macrophage-like cells that play an important role in bone turnover (100). They resorb bone material, a function that is important for bone health, as it is the basis of bone regeneration. An increased osteoclast activity, however, leads to osteopenia and/or osteoporosis, while a decreased osteoclast activity may lead to osteopetrosis.

Osteoclasts generate ROS through NADPH oxidase-dependent mechanisms (289, 452, 971). Stimuli activating ROS generation in osteoclasts or osteoclast precursors included RANKL and IFN-γ (516, 829, 970). Given their macrophage nature, it is not suprising that osteoclasts express high levels of NOX2 at the protein and mRNA levels (516, 971). However, it was suprising that spontaneous ROS generation is conserved in osteoclasts from NOX2 knockout mice (969). This observation could be explained at least in part by the expression of NOX4 mRNA and protein in mature osteoclasts (969, 973) and NOX1 in osteoclast precursors (516).

Presently available data strongly suggest that NOX enzymes play a role in osteoclast differentiation and in the function of mature osteoclasts.

I) Osteoclast differentiation. Abundant evidence points towards a role of ROS in osteoclast differentiation (289, 516, 830). More specifically, NOX1 appears to be required for the differentiation of precursor into mature osteoclast in response to the receptor activator of the NFκB ligand RANKL (516).

II) Activity of mature osteoclasts. Antisense experiments indicate that both NOX4 and NOX2 participate in bone resorption by mature osteoclasts (969). However, there is an apparently normal bone resorption in CGD patients and in NOX2-deficient mice; it has been suggested that this might be due to compensatory NOX4 upregulation (969). A proposed role of NOX enzymes in the activity of mature osteoclast is also supported by observations that NOX4 is expressed at a higher level in osteoclasts than that in precursor cells (973), and stimulation of osteoclast activity through IFN-γ involves NOX2 upregulation (970). NOX enzymes might enhance the osteoclast activity through ROS-dependent signaling, e.g., p38 MAP kinase activation (477). This is supported by the observation that addition of exogenous hydrogen peroxide stimulates osteoclastic bone resorption and cell motility (66). There are, however, also good arguments that NOX enzymes might be part of the bone resorption machinery. Indeed, a key step in bone resorption is acidifi-

cation of the extracellular space through proton secretion by osteoclasts (759). Thus the proton secretion observed concomitant with NADPH oxidase activation (98) might be of functional importance in bone resorption. In this scenario, the biological purpose of the electron transport by NOX enzymes would be the generation of proton-motive forces (792), rather than ROS generation. However, a direct role of ROS through fragmentation of matrix proteins has also been discussed (453).

Given the abundant evidence for a role of ROS in osteoclast differentiation and function, it is tempting to speculate that increased NOX activity in osteoclasts might be involved in the mechanism of osteoporosis. However, to our knowledge, such a link has not yet been documented. There is however a potential link to a disease called osteopetrosis, which is associated with decreased osteoclast activity and increased bone mass. Neutrophils of patients with osteopetrosis show a decreased respiratory burst (70). In immortalized B lymphocytes from osteopetrosis patients, decreased ROS generation and decreased p47^{phox} levels are detected (972). IFN-γ treatment, which increases osteoclastic bone resorption in vivo in patients with malignant osteopetrosis, enhances in vitro osteoclast generation from peripheral blood leukocytes from osteopetrotic patients and normalizes their superoxide generation (567).

In summary, there are now convincing in vitro data that NOX enzymes play an important role in osteoclasts: NOX1 plays a role in RANKL-induced osteoblast differentiation and NOX2/NOX4 in bone resorption by osteoblasts. The in vivo relevance and the implication of NOX in diseases associated with abnormal osteoclast activity (osteopetrosis and osteoporosis) remain to be established.

B) OSTEOBLASTS. Little is known about ROS generation and NADPH oxidases in osteoblasts, although a recent publication suggests that NOX-derived superoxide activation is involved in osteoblast response to shock waves (932).

C) CARTILAGE. Chondrocytes are able to generate superoxide (381, 555). The presence of p22^{phox} and p47^{phox} is reported in chondrocytes, although no NOX2 is detected (381, 635). In the chondrocyte-like ATDC5 cell line, however, NOX2 is detected and its expression is enhanced by IL-1β (975). Possible functions of NOX-derived ROS in chondrocytes include the mediation of cytokine- and growth factor-induced gene expression (554, 555) and the induction of apoptosis (975).

There is a long-standing concept that NOX-derived ROS are involved in the pathogenesis of arthritis (244, 253, 339). And, indeed, the nonspecific NOX inhibitor DPI decreases disease severity in a mouse model of potassium peroxochromate arthritis (606). However, recent genetic data challenge this concept (see sect. IIIA5). Many studies hint towards the opposite possibility, namely, an in-creased severity of arthritis in animals with either loss of function mutations (398, 399, 666) or deletions (902) in components of the phagocyte NADPH oxidase (NOX2, p47^{phox}). However, the effect of NOX-derived ROS might depend on the arthritis model: in IFN-γ-enhanced immune complex arthritis, p47^{phox}-deficient animals showed a less severe joint destruction and decreased chondrocyte death (906).

2. Skeletal muscle

Skeletal muscle generates ROS; the release is low under resting conditions and increases in response to activity (99, 737). While the contribution of mitochondria to such a ROS generation is undisputed, a potential role of NADPH oxidase activity is suggested by some (77), but not by others (995).

Total mRNA from skeletal muscle contains NOX4 (143, 813) and trace amounts of NOX2 (143). One study in the ventilatory muscles suggests expression of the phagocyte NADPH oxidase complex (p22^{phox}, NOX2, p47^{phox}, and p67^{phox}) (426). Recent studies suggest that NOX-derived ROS may lead to Ca^{2+} release from the sarcoplasmic reticulum, a key signaling step in contraction (376).

J. Platelets and Leukocytes

In total mRNA from spleen, NOX2 and NOX5 are abundantly expressed (56, 143). Total mRNA from peripheral blood leukocytes contains only NOX2 (143), while mRNA from bone marrow contains NOX2 (914) and NOX5 (53).

1. Hematopoietic stem cells

In hematopoietic stem cells, ~30% of oxygen consumption cannot be explained by mitochondria but has characteristics of an NADPH oxidase activity (704). The presence of NOX enzymes in hematopoietic stem cells was more directly evidenced through detection of b-type cytochrome, NOX4 and NOX2 mRNA, as well as NOX2 immunoreactivity (704). However, the detected ROS generation was apparently spontaneous and not enhanced by PMA, arguing in favor of NOX4 as the relevant source of ROS. NOX-derived ROS have been suggested to act as intracellular messengers modulating cell growth and development in hematopoietic stem cells (704).

2. Granulocytes

Neutrophil granulocytes are the prototype of a NOX2-expressing phagocyte. Upon stimulation, neutrophils generate a large respiratory burst. The expression and role of NOX2 in neutrophils has been abundantly investigated, and a detailed description is beyond the scope of this review (for recent review articles, see Refs. 44, 157, 245, 652, 752, 796). NOX2-dependent respiratory

burst is also found at high levels in eosinophil granulocytes (792, 965). There are also some limited indications about a NOX-type enzyme in basophils and mast cells (852). To our knowledge, no expression of NOX isoforms other than NOX2 has been reported in granulocytes. The function of NOX2 in neutrophils is mainly host defense. The situation might be similar for eosinophils, which are involved in the host defense against metazoan parasites, while in basophils and mast cells, a regulation of cellular responses by NOX-derived ROS has been suggested (852). Increased generation of ROS by NOX2 from granulocytes has been implicated in a large number of inflammatory disease (for review, see Refs. 44, 157, 245, 652, 752, 796). From a clinical perspective, the most widely studied NOX2-related neutrophil disease is CGD, a syndrome that can be caused by genetic deficiency of NOX2, p47^{phox}, p67^{phox}, or p22^{phox}. The relatively rare disease is characterized by an enhanced sensitivity to bacterial and fungal infections (384, 801). Many aspects of the disease phenotype could be reproduced in NOX2- and p47^{phox}-deficient mice (421, 709). CGD and its mouse models are also characterized by increased inflammation in response to noninfectious material (see sect. IIIA5).

3. Macrophages

Macrophages are tissue-localized phagocytes derived from circulating monocytes. Many tissues have particular types of macrophages with specific features and function, including microglia in the CNS, osteoclasts in the bone, and Kupffer cells in the liver. Particularities of tissue-specific macrophages are discussed in the respective sections. The topic of the NOX expression in macrophages (see, for example, Ref. 269) cannot be covered in detail in this review. We will only briefly summarize some relevant aspects with respect to the NOX family.

It appears that all types of macrophages express the phagocyte NADPH oxidase NOX2 and its subunits p47^{phox}, p67^{phox}, p40^{phox}, and p22^{phox}. However, as opposed to granulocytes, there are indications that macrophages may also express other types of NOX. Osteoclasts express NOX4 (969, 973). Kupffer cells have even been suggested to express several NOX isoforms in addition to NOX2 (739).

Obviously one of the key functions of NOX2 in macrophages is killing of phagocytosed microorganisms, as described before for neutrophil granulocytes. However, the situation in macrophages is more complex than in granulocytes. In addition to killing, NOX enzymes in macrophages and macrophage-related cells are likely to be of importance in activation of kinases and transcription factors, gene expression (in particular proinflammatory genes), regulation of proliferation, and apoptosis (59, 96, 269, 753, 754).

4. Dendritic cells

Dendritic cells are antigen-presenting cells of myeloid origin. Dendritic cells express NOX2 and its subunits, even though the phagocyte respiratory burst declines during differentiation of monocytes towards dendritic cells (979). Preincubation with proinflammatory ligands or pathogen-derived molecules leads to an activation of the phagocyte NADPH oxidase NOX2 in dendritic cells (248, 925). Whether NOX2 in dendritic cells plays a role in bacterial killing is debated (248, 785, 925). However, NOX2 plays an important role in the presentation of antigens, more specifically in the so-called cross-presentation. For cross-presentation, proteins must be partially degraded in the phagosome, followed by *1*) translocation to the cytosol, *2*) further degradation, *3*) transport into the ER, *4*) loading to major histocompatibility complex class I (MHC I), and finally *5*) presentation to CD8$^+$ T cells. NOX2 is involved in the initial degradation of proteins in the phagosome. It regulates this process through alkalinization of the phagosome, which limits the activity of lysosomal proteases. Dendritic cells lacking NOX2 show enhanced phagosomal acidification and therefore overshooting antigen degradation, which destroys potential peptides for T-cell recognition (785).

Thus NOX2 is at the center of the function of dendritic cells, namely, the regulation of antigen presentation.

5. B lymphocytes

B lymphocytes, including EBV-transformed B cells, B cell lines, tonsillar B lymphocytes, and peripheral B cells, generate ROS (431, 471, 472, 573, 574, 814, 922). At least for EBV-transformed B cells and lymphomas, it is convincingly documented that the ROS generation occurs through the phagocyte NADPH oxidase NOX2 (431, 472, 573, 814, 922). B cells within lymphoid tissues are also likely to express NOX5 (56). The relative contribution of NOX2 and NOX5 to B-cell ROS generation and whether additional NOX isoforms are also of relevance is presently unknown. The discovery of NOX2-dependent ROS generation by EBV-transformed B cells is of particular importance for several reasons. First, it is a lymphocyte system where phagocyte contamination as the source of NADPH oxidase can be excluded. Second, it allows the establishment of cell lines from patients. These can subsequently be used as model systems to study genetic diseases, including CGD (711), but also other diseases where NOX2-dependent oxidative stress might play a role (656). Third, it allows experiments on NOX2 function requiring prolonged cell culture and genetic manipulations, which is impossible with neutrophils (222). And fourth, it provides a model for studying gene transfer for correction of CGD (138, 167, 878). Possible functions of NOX enzymes in B

lymphocytes include proliferation (400) and regulation of tyrosine phosphorylation (851).

6. T lymphocytes

ROS generation by T cells has been observed in response to mitogens, cognate antigens, superantigens, and antibodies to the TCR complex (954). Thymocytes and peripheral T cells generate ROS through an NADPH oxidase-like system (241, 420, 480, 667, 705, 959). One study using a mixed culture of primary lymph node cells concluded that NOX2 is not a relevant source of ROS generation by T cells (907). A more recent study with purified T cells, however, suggests that ROS generation in response to T-cell receptor activation is at least partially dependent on p47[phox] and NOX2 (420). Possible functions of NOX-derived ROS in T cells include the regulation of expression of cytokines (420) and receptors (436), the regulation of tyrosine phosphorylation (851), regulation of cell adhesion through oxidative inhibition of the phosphotyrosine phosphatase SHP-2 (499), and mediation of conjugated linoleic acid-induced apoptosis (85).

7. Platelets

In response to a variety of stimuli, platelets generate ROS with mechanisms characteristic of NADPH oxidases (418, 489, 526, 804). This ROS generation does not appear to be due to granulocyte contamination in platelet preparations (489). ROS generation through mechanisms possibly involving an NADPH oxidase has also been reported in megakaryocyte cell lines (714, 804).

NOX2 appears to be the isoform expressed in platelets based on Western blots, where immunoreactivity toward p47[phox], p67[phox], and p22[phox] (489, 804) as well as NOX2 (708) are found. This is compatible with functional data from antibody-induced lysis of platelets, which is reduced in NOX2-deficient mice, strongly suggesting the presence of NOX2 (650).

Generally, ROS have been suggested to act as second messengers in platelet activation (418). Specific proposed functions of NOX-derived ROS in platelets include regulation of platelet aggregation (166, 773), adhesion (74), and recruitment (489).

In terms of pathophysiology, NOX2 has been suggested to be involved in antibody-induced lysis of platelets and thus in the mechanism of autoimmune thrombocytopenia (650). In patients with heart failure, a TNF-α-induced increase in platelet ROS generation has been suggested to contribute to the deterioration of cardiovascular function (186). Similarly, hypertensive patients have an increased platelet NADPH oxidase activity (301). Platelet NOX has also been implicated in the development of nitrate tolerance through the inactivation of nitric oxide by superoxide (598).

K. Sensory Organs

1. Eye

A) LENS. Recent results suggest that lens epithelia are able to generate superoxide and that they express an NADPH oxidase-like enzyme (731). A role for NOX-derived ROS in mediating the effects of growth factors on lens growth and development has been proposed (731). In terms of pathophysiology, ROS have been suggested to be involved in cataract formation through oxidation of lens proteins, in particular crystallins, and lens phospholipids (824); however, the role for NOX has not yet been investigated.

B) RETINA. At least two cell types within the retina have been suggested to express NADPH oxidases: retinal pigment epithelium and retinal pericytes. Retinal pigment epithelium is involved in phagocyte removal of debris within the retina. Photoreceptors are exposed to intense levels of light that lead to accumulation of photodamaged, toxic proteins and lipids. These are removed through shedding of photoreceptor outer segments, followed by their phagocytosis through retinal pigment epithelium (834). This phagocytosis is accompanied by an intracellular respiratory burst, presumably through an NADPH oxidase (604). A specific NOX isoform in retinal pigment epithelium has not been identified. However, chorioretinal lesions, characterized by accumulation of pigment, have been observed in patients with CGD, raising the possibility that NOX2 is involved in the degradation of material of photodamaged material through retinal pigment epithelium.

Pericytes are smooth muscle-like cells surrounding the retinal capillaries, presumably involved in the regulation of capillary blood flow, limiting endothelial cell proliferation, and maintaining vascular stability. An NADPH oxidase-like enzyme was suggested to be expressed in retinal pericytes (121, 576); in one study NOX1 is identified as the expressed isoform (576). A role of NOX-derived ROS in pericyte apoptosis, thought to be involved in diabetic retinopathy, has been suggested (121, 576).

2. Inner ear

ROS generation by cells of the inner ear has been documented in response to ototoxic agents and to noise (165, 483, 966). NOX3 mRNA is highly expressed in the inner ear (54, 677), in both the cochlear and the vestibular system system (54). More specifically, NOX3 expression is found in spiral ganglion neurons and the organ of Corti within the cochlear system, and the sensory epithelial cell layer of the saccule within the vestibular system (54). NOX3 expression is not observed in nonsensory layers, such as stria vascularis. It is interesting that the cochlear and vestibular sensory epithelia develop from a common ectodermal thickening at the head region, called placode,

which also gives rise to the neurons that will form the spiral ganglion, suggesting that NOX3-expressing cells have a common developmental origin (54). The precise cellular localization of NOX3 (sensory vs. supporting cells) and its subcellular localization (vesicles vs. plasma membrane) is unknown.

NOX3 plays a key role in the generation of otoconia. Otoconia are small crystals found in the vestibular apparatus which contribute to the detection of linear acceleration and gravity. They are made up of a combination of organic material, primarily the protein otocin 90 (OC90), and of the inorganic salt calcium carbonate. The importance of NOX3 for otoconia formation is clearly demonstrated in mice with loss of function mutations in NOX3. These mice are unable to form otoconia and therefore suffer from a disturbed equilibrium leading to the so-called "head-tilt" phenotype (677). A strain of mice with a loss of function mutation in the NOX3 subunit NOXO1 have a very similar phenotype ("head-slant") (463). The role of NOX3 in otoconia formation is not understood, and indeed, the present knowledge of otoconia formation itself is incomplete. The predominant model (Fig. 9) suggests the following elements: Ca^{2+}-enriched vesicles (also referred to as "globular substance") are released from supporting cells of the sensory epithelium into a gelatinous layer within the endolymph. Nonsensory epithelium secretes the organic component OC90 (872). Within the endolymph, the OC90 undergoes a conformational change and interacts with the Ca^{2+}-containing vesicles. Finally, the vesicles accumulate carbonate in the bicarbonate-rich endolymph, and crystallization occurs leading to the formation of the otoconia.

The role of NOX3 in the biosynthesis of otoconia is not clear. Basically, the following options have been suggested: 1) NOX3, expressed within the sensory epithelia, may influence the Ca^{2+} loading of the vesicles (463); 2) NOX3-derived ROS may induce a conformational change in the OC90 protein (677); and 3) NOX3-derived ROS, in combination with lactoperoxidase, might induce lipid peroxidation, changing the membrane properties of the Ca^{2+}-containing vesicles and thereby allowing an interaction with OC90 (463).

The role of NOX3 in the cochlear system is unknown. Indeed, the head-tilt mice appear to have normal hearing (677). However, as these studies were performed in C57BL/6 mice, which are characterized by a severe, early hearing loss (607, 826), further studies on this topic are necessary.

There is strong evidence that ROS play a role in the pathophysiology of age-, noise-, and drug-associated hearing loss (165, 483, 594, 966). Thus the ROS-generating enzyme NOX3 might be involved in hearing loss. To date, however, only limited experimental data are available. Two points would suggest a role of NOX3 in hearing loss. First, the anatomic NOX3 localization in the inner ear (54) corresponds to the anatomic sites of oxidative damage in hearing loss, as described previously (664, 858). Second, the ototoxic drug cisplatin activates NOX3 in vitro (54).

L. Skin

1. Keratinocytes, fibroblasts, and melanocytes

ROS generation in skin cells has been widely observed. Keratinocytes generate ROS in response to ultra-
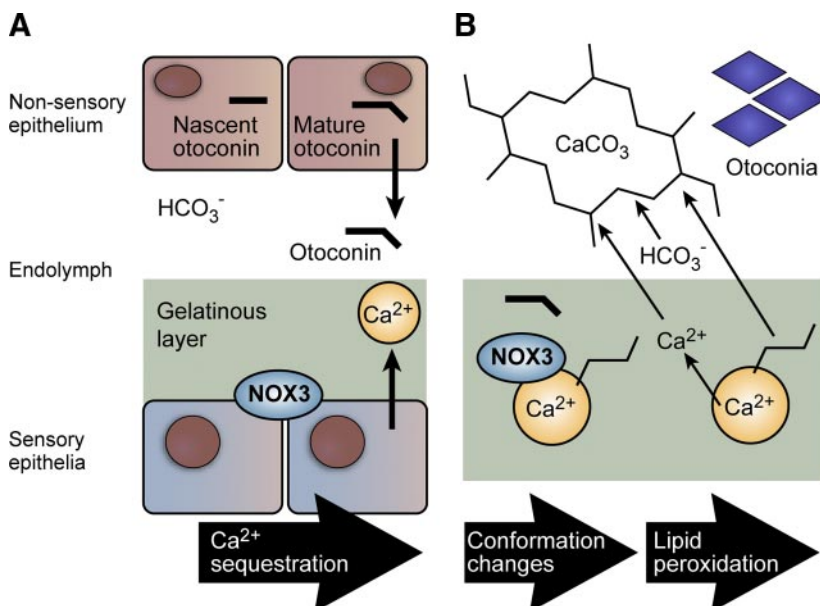

FIG. 9. NOX enzyme function in otoconia formation. Mice with NOX3 mutations are unable to form otoconia. NOX3 is found in sensory epithelia of the vestibular system. However, its precise cellular localization (sensory vs. supporting cells) and its subcellular localization (vesicles vs. plasma membrane) are unknown. The black arrows indicate the potential sites of NOX3 action in otoconia formation. Otoconia are formed from inorganic material, calcium carbonate, and organic material, principally otoconin. A: otoconin is secreted from NOX3-negative nonsensory epithelia. Supporting cells of the sensory epithelia are thought to release Ca^{2+}-enriched vesicles into the Ca^{2+}-poor HCO_3^--rich endolymph. NOX3 may influence the sequestration of Ca^{2+} into vesicles or the Ca^{2+} release into the endolymph. B: a more detailed view of putative events during otoconogenesis. Models of otoconia formation propose that otoconin interacts with Ca^{2+}-enriched vesicles, leading to a localized Ca^{2+} release. NOX3 (possibly localized on vesicle membranes) might participate in this process either through initiating a conformational change in otoconin (disulfide bridges, cysteine oxidation) or through a peroxidation of the vesicle membrane rendering it sensitive to otoconin. The interaction of Ca^{2+}, HCO_3^-, and otoconin initiates crystallization and formation of otoconia.

violet light (69, 934) and to phorbol esters (831). Interestingly, these ROS responses can be blocked by NADPH oxidase inhibitors, raising the possibility that ROS generation even in response to ultraviolet light is not simply a physicochemical process, but might involve NOX enzymes.

At the mRNA level, NOX1, NOX2, and NOX4 are detected (135). Putative functions of NOX-derived ROS in keratinocytes include VEGF expression (803), MAP kinase activation (134, 150), and anchorage-independent growth (134). In line with a possible involvement of NOX enzymes in enhanced cell growth, Ras-transformed keratinocytes produce increased amounts of ROS, possibly through NOX-dependent pathways (968); the connection between Ras and NOX has also recently been suggested in other cell types (614). ROS generation in fibroblasts has been documented for a long time and is suggested to occur through an NADPH oxidase (600, 873). Fibroblasts generate ROS in response to platelet-derived factors (84), Ca^{2+} ionophores or IL-1 (600), very-long-chain fatty acids (210), TGF-β1 (873), or insulin (131). Many studies point towards expression of NOX4 in fibroblast (170, 211, 320), and the release of hydrogen peroxide, rather than superoxide (873), is reminiscent of the recently described hydrogen peroxide release by NOX4-transfected HEK293 cells (584). The concept of NOX4 as the main NOX in fibroblasts is also compatible with previous observations of normal ROS generation in fibroblasts from CGD patients, suggesting that the enzyme system in fibroblasts is distinct from NOX2 (249). However, in some types of fibroblasts, in particular adventitial fibroblasts, NOX2 is expressed and of functional importance (133, 320, 679). In a model of rhinovirus infection of skin fibroblasts, p47^{phox} is crucial for ROS generation and IL-8 expression, while NOX2 does not appear to be involved (442). It remains to be seen whether this is due to a subunit-dependent NOX isoform other than NOX2 (i.e., NOX1 or NOX3), or whether this hints towards a hitherto unknown role of p47^{phox}.

Little is known about ROS generation in normal epithelial melanocytes, although based on RT-PCR results, they appear to express p22^{phox} and NOX4. In melanoma cells, however, NOX2 are expressed in addition to NOX4. On the basis of antisense experiments, NOX4 is suggested to promote cell growth in melanoma cells (105).

2. ROS and wound healing

There are arguments for redox control of wound repair, which are not simply due to the antimicrobial effects of NADPH oxidases (802). Impaired wound healing is observed in CGD patients (237), suggesting that NOX2-derived ROS are important for wound healing. However, other NOX isoforms are also thought to play a role in regulating the redox environment in wounds, including NOX1 and NOX4 (802). Of particular interest in that respect is the NADPH oxidase-dependent secretion of tissue factor by vascular smooth muscle cells (370). NADPH oxidase control of wound repair is an emerging field, and while high-level ROS generation is involved in direct bacterial killing, low-level ROS generation likely contributes to its regulatory mechanism.

V. CONCLUSIONS

NADPH oxidase research has gone through an amazing transformation. From a focused topic with a small group of specialists working on the respiratory burst as a peculiar property of phagocytes, it has turned into a broad field that involves virtually every domain of biomedical sciences. The last 10 years were characterized by gene discovery and attempts to understand the underlying biochemistry. Although this phase might not be completely finalized, major progress has been made. The study of the role of NOX family NADPH oxidases in physiology and pathophysiology should be considered as a work in progress. There are now a large number of publications on these aspects; however, many key questions still await an answer. The most solidly documented physiological functions of NOX include the host defense (NOX2), involvement in biosynthesis (DUOX2), and regulation of blood pressure (NOX1). However, good evidence for NOX function in many other domains is accumulating, and hopefully there will be an increasing availability of knockout models and high-quality antibodies that should help to rapidly sort out several of the unresolved issues. Also, the domain of human NOX mutations and polymorphisms and their relationship to health and disease is likely to acquire a major importance over the coming decade. Finally, given their likely involvement in various disease processes, NOX enzymes are likely to become prime drug targets.

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