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REVIEW

The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction

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ABSTRACT

The NADPH oxidase was originally identified as a key component of human innate host defence. In phagocytes, this enzyme complex is activated to produce superoxide anion and other secondarily derived ROS (reactive oxygen species), which promote killing of invading micro-organisms. However, it is now well-established that NADPH oxidase and related enzymes also participate in important cellular processes not directly related to host defence, including signal transduction, cell proliferation and apoptosis. These enzymes are present in essentially every organ system in the body and contribute to a multitude of physiological events. Although essential for human health, excess NADPH-oxidase-generated ROS can promote numerous pathological conditions. Herein, we summarize our current understanding of NADPH oxidases and provide an overview of how they contribute to specific human diseases.

INTRODUCTION

ROS (reactive oxygen species) represent a diverse family of molecules that play roles in a myriad of physiological and pathophysiological events. ROS are comprised of highly unstable and thus short-lived oxygen free radicals, including $O_2^{\bullet -}$ (superoxide anion), $^1O_2$ (singlet oxygen) and $OH^{\bullet}$ (hydroxyl radical), as well as more stable and freely diffusible radical and non-radical oxidants, such as $NO^{\bullet}$ (nitric oxide), H_2O_2 and O_3 (ozone). In addition, interaction between ROS can form secondary reactive oxidants, such as $ONOO^{\bullet -}$ (peroxynitrite), which is generated by diffusion-limited reaction between NO and $O_2^{\bullet -}$ [1]. Virtually all aerobic organisms generate ROS through cellular metabolism and utilize these molecules to maintain cellular homoeostasis; however, excess ROS induce oxidant stress, leading to pathological processes and damage to cellular components. Indeed, ROS-mediated damage has been linked to the aging process itself [2]. Although ROS are produced by a variety of

Key words: cellular homoeostasis, host defence, NADPH oxidase, pathogenesis, phagocyte, reactive oxygen species (ROS), superoxide anion.

Abbreviations: ARDS, acute respiratory distress syndrome; CAD, coronary artery disease; CGD, chronic granulomatous disease; CNS, central nervous system; DPI, diphenyleneiodonium; DSS, dextran sodium sulphate; DUOX, dual oxidase; GI, gastrointestinal; HIF-1α, hypoxia-inducible factor-1α; HOCl, hypochlorous acid; IBD, inflammatory bowel disease; IFN-γ, interferon-γ; Mox, mitogenic oxidase; MPO, myeloperoxidase; NEB, neuroepithelial bodies; NF-κB, nuclear factor κB; NOH, NADPH oxidase homologue; NOX, NADPH oxidase; NOXA, NOX activator; NOXO, NOX organizer; $O_2^{\bullet -}$, superoxide anion; $OH^{\bullet}$, hydroxyl radical; RA, rheumatoid arthritis; ROS, reactive oxygen species; SHR, spontaneously hypertensive rat; SOD, superoxide dismutase; TGF-β, transforming growth factor-β; TLR, Toll-like receptor; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor.

As per Human Gene Nomenclature Committee guidelines, all letters of human NOX genes and proteins are capitalized, whereas those of non-human species have only the first letter capitalized. All genes names are in italics.

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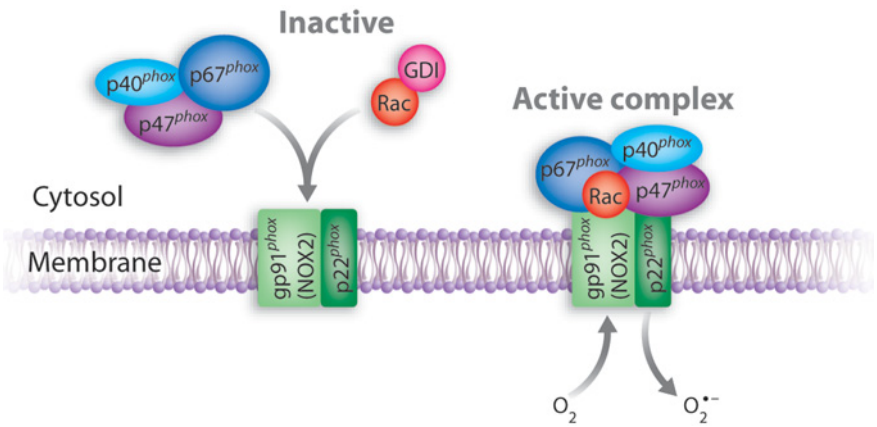

Figure 1 Model of NADPH oxidase activation

Cytosolic *phox* proteins and Rac translocate to the phagosomal or plasma membrane, where they assemble with flavocytochrome b_{558} to form the active $O_2^{\bullet -}$-generating complex. See text for further details.

intracellular mechanisms, one of the predominant cellular sources of these molecules is a family of multisubunit enzymes known as the NADPH oxidases. These enzymes catalyse the univalent reduction of molecular oxygen (O_2), thereby forming $O_2^{\bullet -}$ [3].

The classical NADPH oxidase was first described and characterized in phagocytes, such as neutrophils, and it was originally thought that the enzyme was restricted to leucocytes and used solely for host defence [4]. However, subsequent studies over the past two decades indicate that similar NADPH oxidases are present in a wide variety of non-phagocytic cells and tissues (reviewed in [5,6]). These enzymes are distinct from the phagocyte oxidases and appear to play essential roles in functions other than host defence; however, structural features of many non-phagocyte oxidase proteins seem similar or even identical with those of their phagocyte counterparts. Thus there has been a high level of interest in understanding the physiological functions of these enzymes and their potential roles in pathophysiological events.

In this review, we provide a brief overview of structural features of the phagocyte and non-phagocyte NADPH oxidases and then discuss what is currently known regarding the roles of these enzymes in human health and disease.

GENERAL FEATURES

Activation of the classical NADPH oxidase involves assembly of cytosolic and integral membrane proteins to form a multisubunit enzyme complex (reviewed in [3,7]) (Figure 1). Two essential membrane-associated proteins ($gp91^{phox}$ and $p22^{phox}$) form a non-covalent heterodimer, which was originally named cytochrome b_{558}, due to its α-band absorption maximum of 558 nm, but is now known as flavocytochrome b_{558} because of the association of an FAD moiety with $gp91^{phox}$ (reviewed in [7]). It appears that flavocytochrome b_{558} contains the entire electron transport apparatus of the phagocyte NADPH oxidase and thus may act as a physical conduit for electron transfer across the membrane (reviewed in [7]). In support of this concept, Koshkin and Pick [8] showed that flavocytochrome b_{558} alone generates $O_2^{\bullet -}$ if it is relipidated into a defined artificial phospholipid bilayer system.

Originally, it was thought the NADPH oxidase was specific to phagocytic cells; however, subsequent studies revealed the presence of analogous systems and possibly homologous proteins in non-phagocyte tissues [6] (Figure 2 and Table 1). These enzymes are functionally distinct from the phagocyte oxidase and respond to a variety of humoral mediators, such as growth factors, cytokines and hormones, as well as mechanical inputs, such as shear stress and cyclic stretch (reviewed in [9–11]). In an effort to characterize the nature of these oxidases, various tissues were probed for the presence of homologues of $gp91^{phox}$ because of its key importance in electron transport. The first of these homologues to be identified was designated as Mox1 (mitogenic oxidase 1) [12] or NOH-1 (NADPH oxidase homologue 1) [13] (Table 1). Because of the imminent problem with terminology, the nomenclature for the $gp91^{phox}$ homologues was changed such that Mox-1/NOH-1 was renamed NOX1 (NADPH oxidase 1), and $gp91^{phox}$ was renamed NOX2 [6]. NOX1 is apparently not expressed in phagocytes, but is expressed primarily in colon epithelium and at lower levels in prostate, uterus and vascular smooth muscle cells [12]. Shortly after the cloning of NOX1, two novel $gp91^{phox}$ homologues were identified in thyroid tissue and were designated as ThOX1 and ThOX2 (thyroid oxidase 1 and 2) [14,15]. Whereas the C-termini of these proteins are similar in structure to NOX2, they are three times longer because of the addition of

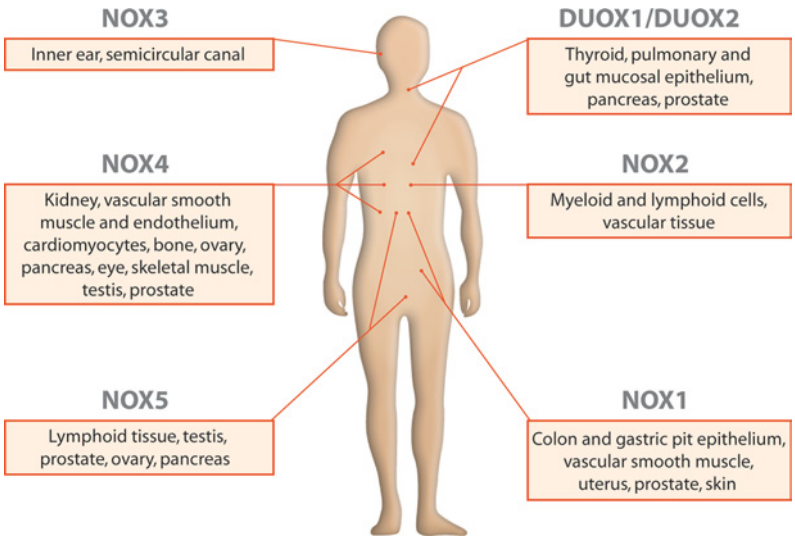

Figure 2 Locations of NOX proteins in the body

Tissues reported to express NOX homologues throughout the human body are indicated. See text for further details.

Table 1 Tissue expression of the NADPH oxidase proteins

Oxidase protein	Tissue expression
NOX1	Vascular smooth muscle, gastric pit and colon epithelium.
NOX2 (gp91^{phox})	Phagocytes, lymphocytes, vascular smooth muscle, fibroblasts, endothelium, skeletal muscle, neurons, lung, carotid body and kidney.
NOX3	Fetal tissue and inner ear.
NOX4	Fetal tissue, kidney, pancreas, placenta, ovary, testis, skeletal, carotid body, muscle, melanocytes, osteoclasts, eye, lung and kidney.
NOX5	Fetal tissue, lymphocytes, spleen, testis, ovary, placenta, pancreas, stomach, mammary glands and cerebrum.
DUOX1	Thyroid, salivary glands, colon, rectum, bronchi and cerebellum.
DUOX2	Thyroid, salivary glands, colon, rectum, bronchi, pancreas and prostate.
p22^{phox}	Phagocytes, lymphocytes, testis, placenta, ovary, kidney, liver, lung, spleen, pancreas, skeletal muscle, neurons, eye, vascular smooth muscle, fibroblasts, endothelium, lung, carotid body, kidney, melanocytes and osteoclasts.
p47^{phox}	Phagocytes, lymphocytes, testis, placenta, ovary, kidney, liver, lung, spleen, pancreas, skeletal muscle, neurons, eye, vascular smooth muscle, fibroblasts, endothelium, lung, carotid body and kidney.
p67^{phox}	Phagocytes, lymphocytes, testis, placenta, ovary, kidney, liver, lung, spleen, pancreas, skeletal muscle, neurons, eye, vascular smooth muscle, fibroblasts, endothelium, lung carotid body and kidney.
NOXO1 (p41^{nox})	Colon, liver, small intestine, gastric mucosal cells, cochlea, liver, pancreas, thymus and testis.
NOXA1 (p51^{nox})	Colon, uterus, salivary gland, small intestine, stomach, lung, thyroid, liver, kidney, pancreas, spleen, prostate, testis and ovary.

N-terminal extensions containing two proximal EF hand motifs that function in Ca^{2+} binding, an additional transmembrane helix and a distal peroxidase homology domain. Based on the presence of both NADPH oxidase and peroxidase homology domains, these proteins are now known as DUOX1 and DUOX2 (dual oxidase 1 and 2) [6]. Concurrent with the identification of the *DUOX* genes, a gp91^{phox} homologue was identified in the kidney and was originally designated as Renox [16], but was later renamed NOX4 by Cheng and co-workers [17], who also reported the cloning of *NOX4*, as well as two additional gp91^{phox} homologues, designated as *NOX3* and *NOX5*. NOX3 and NOX4 are homologous in size and domain structure to NOX2, but are quite distinct in their patterns of tissue expression. For example, NOX3 is expressed exclusively in the inner ear vestibular system [18], whereas NOX4 is expressed in a wide variety of tissues, including the renal cortex [16], vascular smooth muscle and endothelium, cardiomyocytes, bone, ovary, eye, skeletal muscle, testis and prostate. Unlike NOX1–4, NOX5 contains an N-terminal extension with three EF hand-like Ca^{2+}-binding motifs and can be activated in a Ca^{2+}-dependent manner [19]. NOX5 is expressed in spleen, lymph nodes and testis [19].

In phagocytes, p22^{phox} and NOX2 are essential subunits of flavocytochrome b_{558} and the absence of either protein results in a non-functional NADPH oxidase [20]. However, it was noted early on that p22^{phox} is expressed

in a variety of non-phagocytic cells, some expressing NOX2 and others, distinct NOX homologues [21]. Thus one of the key questions raised with the identification of NOX2 homologues was whether these proteins also required interaction with $p22^{phox}$. Indeed, recent studies addressing this issue have demonstrated a functional association between $p22^{phox}$ and NOX1 [22,23], NOX3 [24] and NOX4 [23,25]. Furthermore, Kawahara et al. [26] recently showed that mutations in the proline-rich region of $p22^{phox}$ result in dominant inhibition of ROS production by NOX1–4, but not by NOX5. Therefore these data indicate that $p22^{phox}$ association is essential for the function of many, but not all, NOX2 homologues.

Although flavocytochrome b_{558} contains all NADPH oxidase redox components, it does not function independently in the cell and requires several cofactor proteins for enzymatic activity, presumably to initiate and/or regulate electron transfer. In phagocytes, the cytosolic NADPH oxidase proteins include $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and a small GTPase (Rac2), which translocate to the phagosome or plasma membrane and associate with flavocytochrome b_{558} during activation (reviewed in [3]) (Figure 1). Based on the similarity between NOX2 ($gp91^{phox}$) and the other NOX proteins, it was hypothesized that these non-phagocyte oxidases require the participation of analogous cofactors for proper function. This idea was confirmed in studies showing that cells co-transfected with NOX1, $p47^{phox}$ and $p67^{phox}$ produce significant levels of ROS, whereas those expressing NOX1 alone generate very low levels of oxidants, indicating that these cytosolic *phox* proteins could functionally associate with NOX1 [27]. However, it was also proposed that other NOX-related cofactors must exist, since some NOX-expressing cells do not contain these *phox* proteins (reviewed in [28]). Indeed, this issue was resolved concurrently by several groups, who identified homologues of $p47^{phox}$ and $p67^{phox}$ in colon tissue [29–31]. These $p47^{phox}$ and $p67^{phox}$ homologues were subsequently designated as NOXO1 (NOX organizer 1) and NOXA1 (NOX activator 1), due to their respective roles in oxidase organization and activation [29–31]. NOXO1 is expressed primarily in colon and testis, but is also found at low levels in pancreas, liver, thymus and small intestine [29–31]. Although NOXO1 is structurally similar to $p47^{phox}$, it lacks the polybasic 'auto-inhibitory' domain that becomes phosphorylated during $p47^{phox}$ activation [29–31]. NOXO1 has been reported as a cofactor for NOX1 and NOX3 and may be constitutively bound to these proteins, which would be consistent with the constitutive activity observed for the NOX1- and NOX3-based enzymes [12,26]. Like NOXO1, NOXA1 is highly expressed in the colon [29–31], but also seems to be expressed in a wider range of tissues than NOXO1 [31]. NOXA1 is also structurally similar to $p67^{phox}$, although it is significantly shorter due to the absence of the first SH3 domain and a small region near the second SH3 domain [29–31]. It has been reported to be a cofactor for NOX1 and possibly NOX3, although there seems to be some dispute regarding this issue [24,32,33]. Recent studies by Ueno et al. [24] suggest that human NOX3 is constitutively active and this activity is significantly enhanced by NOXO1 and, to a lesser extent, by $p47^{phox}$. Interestingly, addition of NOXA1 or $p67^{phox}$ to the NOX3/NOXO1 system inhibits ROS production, while addition of these activators to the NOX3/$p47^{phox}$ system enhances activity [24]. Thus NOX3 activity seems dependent on the presence of a specific combination of organizers and activators. In contrast with NOX1–3, NOX4 seems to function independently of additional cytosolic oxidase cofactors or Rac2, indicating that it is unique among these homologues [23,25]. Currently, not much is known regarding the role of cofactors in regulating NOX5 or DUOX activity, and this will probably be an area of intense investigation in the near future.

PHAGOCYTE NADPH OXIDASE

The primary function of the phagocyte NADPH oxidase is production of $O_2^{\bullet -}$ and its secondary metabolites for use in defence against invading micro-organisms [34]. Phagocyte ROS production is stimulated by a variety of soluble and particulate stimuli, such as cytokines, chemokines, microbes and microbial products, viruses and other foreign antigens [7]. Phagocytosis of micro-organisms is a major impetus for activation of the NADPH oxidase, and in neutrophils components of the oxidase assemble at the extraluminal surface of phagosomes which become enriched with flavocytochrome b_{558} through granule fusion [35]. Production of ROS during phagocytosis is dictated in part by the type of receptors triggered, and phagocytic cells utilize pattern recognition receptors to recognize molecules produced by bacteria and fungi [36]. Some pattern recognition receptors, such as TLRs (Toll-like receptors), play a prominent role in the recognition of microbes or microbial products, but do not activate the NADPH oxidase directly [37]. Rather, these receptors prime leucocytes for enhanced responses to subsequent stimuli, such as phagocytosis, which in turn fully activate cells to produce microbicidal ROS [38]. Interestingly, Laroux et al. [39] recently found that phagocytes derived from mice deficient in the TLR adaptor protein MyD88 are impaired in their ability to kill bacteria and showed that this defect results from diminished p38 MAPK (mitogen-activated protein kinase) activation and subsequent phosphorylation of $p47^{phox}$. Thus these data suggest additional links between TLR pathways and the phagocyte NADPH oxidase.

The efficiency of phagocytosis and subsequent production of ROS is enhanced if microbes are opsonized with host serum proteins, such as antibody and complement, and these proteins are recognized by multiple

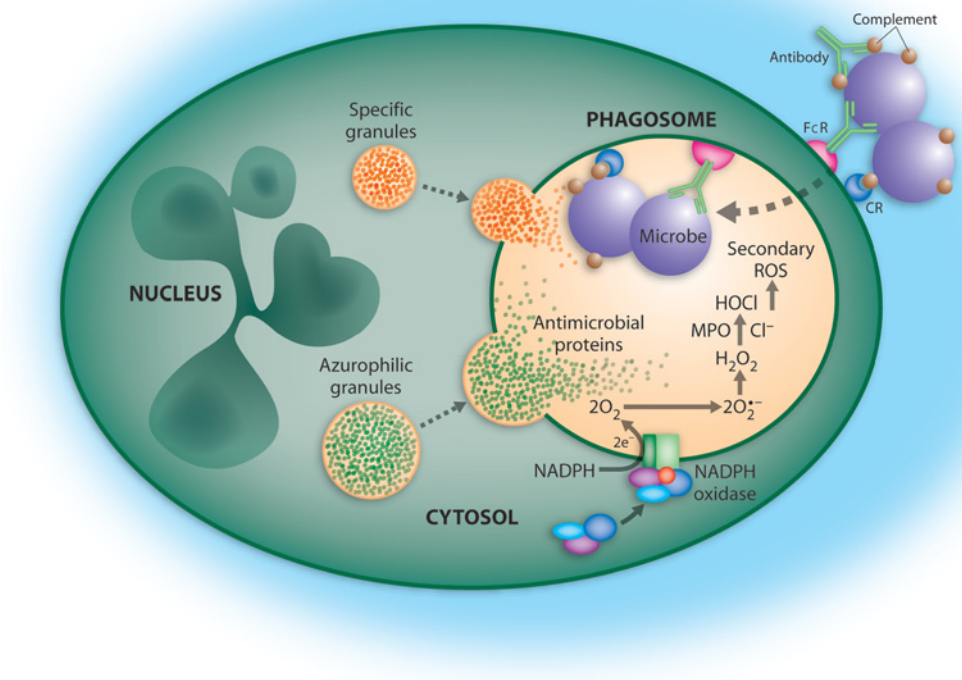

Figure 3 Activation of NADPH oxidase and microbicidal systems during phagocytosis

Complement and antibody receptors (CRs and FcRs) promote the uptake of micro-organisms by neutrophils, which in turn triggers degranulation and production of ROS. See text for details.

receptors on phagocytes, which either alone or in combination promote ingestion of micro-organisms and NADPH oxidase activation [40] (Figure 3). However, micro-organisms can also be ingested readily in the absence of host opsonins and such uptake also triggers production of ROS. For example, recent studies demonstrate that phagocytosis mediated directly by CD44 or dectin-1 elicits ROS production [41,42].

The essential importance of the phagocyte NADPH oxidase to host immunity is clearly demonstrated by a rare genetic disorder known as CGD (chronic granulomatous disease), which occurs with an incidence of 1 in 200 000–250 000 (reviewed in [43]). CGD is caused by defects in gp91^{phox}, p22^{phox}, p47^{phox} or p67^{phox} and results in an inactive NADPH oxidase (Table 2) [44]. As a result, patients with CGD experience severe recurrent bacterial and fungal infections and often develop granulomas, which may form initially by accumulation of phagocytes containing ingested bacteria. The accumulation of phagocytes occurs because they are unable to kill ingested pathogens and because apoptosis/turnover is abnormal, both of which are due to the defect in NADPH oxidase function [43,45]. Infections are caused mostly by catalase-positive organisms, such as *Aspergillus* spp. or *Staphylococcus* spp., and are often life-threatening [43]. The overall mortality in individuals with CGD was reported recently as 17.6 % [44], which is a dramatic improvement from an earlier study indicating the mortality rate was 48.9 % [46]. New advances in therapeutic interventions for CGD, such as treatment with IFN-γ (interferon-γ) or itraconazole, have probably contributed to the improved prognosis [47]. Although neutrophils from CGD patients are unable to generate ROS, they are still able to kill a number of pathogens, presumably through the action of antimicrobial components of neutrophil granules, such as α-defensins [48]. In addition, recent work by Kobayashi et al. [45] indicates that neutrophils from individuals with CGD have increased levels of

Table 2 Phagocyte NADPH oxidase defects

Data are from a registry of 368 patients with CGD [44].

Protein	Gene	Chromosome	Frequency
gp91^{phox} (NOX2)	*CYBB*	Xp21.1	76 %
p22^{phox}	*CYBA*	16p24	3 %
p47^{phox}	*NCF1*	7q11.23	18 %
p67^{phox}	*NCF2*	1q25	4 %
Rac2	*RAC2*	22q13.1	Unknown

transcripts encoding proteins that participate in host defence. Certainly, further studies are needed to elucidate the array of compensatory microbicidal mechanisms in phagocytes from patients with CGD.

Although $O_2^{\bullet-}$ and H_2O_2 alone have limited microbicidal capacity, they are essential to phagocyte bactericidal activity, presumably as substrates for the formation of secondarily derived ROS [49]. At about the time a correlation was made between defects in phagocyte $O_2^{\bullet-}$ production and CGD, Klebanoff and co-workers described the contribution of $O_2^{\bullet-}$ and H_2O_2 to MPO (myeloperoxidase)-mediated bactericidal activity, and subsequently demonstrated that microbicidal activity of NADPH-oxidase-derived ROS is augmented significantly by MPO (recently reviewed in [50]). MPO, an abundant haem-containing protein that resides in neutrophil primary (azurophilic) granules, is targeted to phagosomes and catalyses a reaction with chloride and H_2O_2 to produce HOCl (hypochlorous acid), a potent microbicidal agent [50]. *In vitro*, HOCl is a far more potent microbicide than H_2O_2, and mice deficient in MPO are much more susceptible to some micro-organisms compared with wild-type animals (e.g., see [51,52]). Conversely, humans with hereditary MPO deficiency, which is relatively common (incidence of 1 in 2000–4000), typically do not have an increased susceptibility to infection [53]. Thus it has been proposed that $O_2^{\bullet-}$ and H_2O_2 or secondarily derived ROS, such as $OH^{\bullet}$, may be sufficient to compensate for the lack of the MPO–halide system under certain conditions [50].

Although there is abundant data implicating ROS in microbial killing, recent studies by Segal and co-workers have questioned the actual role of ROS as direct microbicidal agents (reviewed in [54]). As an alternative, this group proposed that ROS have limited direct microbicidal activity, and the primary role of $O_2^{\bullet-}$ is to alkalinize the phagocytic vacuole by consuming protons. Inasmuch as the phagocyte NADPH oxidase is estimated to produce $O_2^{\bullet-}$ at an extraordinary rate of 20 mM/s [55], a significant charge differential is generated across the phagosomal membrane. To compensate, Reeves et al. [56] proposed that K^+ ions are transported into the phagosome through large conductance Ca^{2+}-activated K^+ channels, and it is this combination of alkaline pH and K^+ accumulation that leads to granule protease release and activation. These activated enzymes, and not ROS, are then suggested to be the agents primarily responsible for the destruction of bacteria. Although these findings do suggest a unique mechanism for neutrophil antimicrobial activity, they still do not adequately explain away the large amount of evidence implicating direct function of ROS in microbial killing, and it is likely that both mechanisms contribute to microbial killing [57]. One possibility to consider is that the effectiveness of these respective killing mechanisms could also be dependent on the specific pathogen encountered.

CARDIOVASCULAR NADPH OXIDASE

Cardiovascular NADPH oxidases have been shown to play important roles in physiological processes, such as blood pressure regulation, as well as pathophysiological events, including hypertension and atherosclerosis (recently reviewed in [10,58]). The functions of NADPH-oxidase-generated ROS in the cardiovascular system seem to be quite complex, involving a range of signalling and regulatory mechanisms. In the vascular system, ROS production has been observed in adventitial fibroblasts, endothelial cells and smooth muscle cells and participates in regulating $NO^{\bullet}$ bioavailability and mitogenic signalling [10,58].

Maintenance of blood pressure is essential to health, and deviations from normal blood pressure levels can be life-threatening. One of the key factors regulating blood pressure is endothelial-derived relaxation factor (also known as $NO^{\bullet}$), and the role of this molecule in vascular homoeostasis has been characterized extensively [59]. Furthermore, reduced levels of endothelial $NO^{\bullet}$ causes endothelial dysfunction, which contributes to hypertension and other vascular diseases [59,60]. Although a number of mechanisms have been suggested to explain decreased $NO^{\bullet}$ in endothelial dysfunction, there is now a large body of data indicating that $NO^{\bullet}$ bioavailability is reduced primarily by reaction with vascular ROS [59,60]. Indeed, the reaction of $NO^{\bullet}$ and $O_2^{\bullet-}$ is one of the fastest known biochemical reactions, indicating that $NO^{\bullet}$ would be consumed anytime it encounters $O_2^{\bullet-}$ [1]. In support of this concept, Wang et al. [61] demonstrated that adventitial $O_2^{\bullet-}$ inactivated endothelial $NO^{\bullet}$ in isolated aorta and proposed that this process forms a barrier to limit $NO^{\bullet}$ availability. Based on these observations and later work by a number of laboratories, it is currently thought that vascular tone is modulated by the balance between $NO^{\bullet}$ and $O_2^{\bullet-}$ and that excessive levels of ROS upset this balance, leading to decreased $NO^{\bullet}$, vasoconstriction and hypertension [58,60,62]. Consistent with that notion, NADPH oxidase activity is increased in animal models of hypertension, such as renovascular hypertension and angiotensin-II-induced hypertension, whereas these hypertensive responses are inhibited in Nox2- and $p47^{phox}$-deficient mice respectively [62]. Likewise, RNA silencing of $p22^{phox}$ *in vivo* demonstrates a requirement for NADPH oxidase activity in development of angiotensin-II-induced hypertension [63]. Interestingly, studies of polymorphisms in *CYBA*, the gene encoding $p22^{phox}$, have shown that individuals with a C242T mutation, which results in an amino acid substitution ($His^{72} \rightarrow Tyr$), have reduced phagocyte NADPH oxidase activity and generally reduced vascular dysfunction (reviewed in [64]). Furthermore, a range of SOD (superoxide dismutase) mimetics and antioxidants, including specific inhibitors of NADPH oxidase assembly, significantly reduce hypertension (reviewed in [60]).

Note, however, that ROS-mediated effects in the kidney and brain also contribute to hypertension and other abnormalities in vascular homoeostasis (see further details below). Additionally, ROS-independent pathways must also be considered in the aetiology of hypertension [11].

In landmark studies investigating the source of vascular ROS, Pagano et al. [65] showed that a constitutively-active phagocyte-like (Nox2-based) NADPH oxidase is present in aortic adventitial fibroblasts and activity of this oxidase is enhanced by angiotensin II. Subsequent work has shown NOX2 is also expressed in endothelial cells of large vessels [66] and in smooth muscle cells of smaller resistance arteries [67]. Furthermore, endothelial cell Nox2-based oxidase has been implicated in responses to various cytokines and growth factors, such as TNF-α (tumour necrosis factor-α), angiotensin II and VEGF (vascular endothelial growth factor) [11]. Additional NOX homologues have been identified in vascular tissue, including NOX1, which is expressed in smooth muscle cells of large vessels [68], and NOX4, which is constitutively expressed in large vessel smooth muscle cells and endothelium [69,70]. Interestingly, NOX4 is expressed at much higher levels than NOX2 in vascular endothelial cells, suggesting that NOX4 may play a physiological role in this cell [71]. In support of this conclusion, Ago et al. [72] showed that down-regulation of NOX4 reduced $O_2^{\bullet -}$ production by endothelial cells. Multiple NOX homologues can also be present in the same vascular cell type. For example, Hilenski et al. [69] reported that NOX1 and NOX4 are localized distinctly in vascular smooth muscle cells, with NOX1 primarily in surface domains and NOX4 concentrated in focal adhesions. Thus this type of NADPH oxidase compartmentalization may facilitate the focusing of distinct signalling events within or between vascular cells. In addition, recent studies suggest that the relative levels or activities of different NOX homologues in a given cell may change, depending on physiological events. For instance, diabetes appears to enhance Nox1 expression in rat aorta, whereas Nox4 expression remains unchanged, suggesting that increased Nox1 activity may contribute to endothelial dysfunction associated with diabetes [73]. In addition to hypertension, vascular NADPH oxidases play important roles in a number of other vascular diseases [74]. Analysis of coronary arteries indicated that $O_2^{\bullet -}$ is distributed homogeneously throughout normal vessels, whereas intense ROS production is found in the plaque shoulder of atherosclerotic arteries, suggesting that increased oxidative stress contributes to coronary atherosclerosis [75]. Furthermore, examination of the plaque shoulder demonstrated the presence of abundant NOX2 and NOX4, which are expressed in plaque-associated macrophages and vascular smooth muscle cells, but revealed only low levels of NOX1 [75]. Interestingly, NOX4 expression does not appear to change during atherosclerosis, whereas NOX2 up-regulation correlates with disease pathogenesis [75]. In vessels from patients with CAD (coronary artery disease), expression of NOX2 and NOX4 is enhanced, which is consistent with a role of these oxidases in CAD [76]. During restenosis of carotid artery after balloon injury, Nox1, Nox2 and Nox4 are up-regulated sequentially at 3, 7–15 and >15 days after injury respectively [77].

Although the effect of ROS on $NO^{\bullet}$ bioavailability has been an area of intense investigation, a number of studies suggest that ROS also have direct effects on vascular tissues, independent of $NO^{\bullet}$. For example, ROS induce proliferation of vascular smooth muscle cells, which also contributes to the pathogenesis of cardiovascular disease (e.g., see [78,79]). Indeed, vascular NOX2-dependent oxidase activity is implicated in angiotensin-II-induced vascular hypertrophy [80], cardiac hypertrophy [81], aortic stenosis [82], angioplasty-induced neointimal hyperplasia [83], ischaemia-induced angiogenesis [84] and aldosterone-induced inflammation of the heart [85]. ROS are thought to serve as intracellular messengers, mediating vascular cell responses to various hormones and growth factors by regulating redox-sensitive intracellular targets, such as protein kinases, protein tyrosine phosphatases and transcription factors (reviewed in [10]). In addition, recent studies suggest a positive-feedback mechanism in endothelial cells whereby $p22^{phox}$ expression is up-regulated by NADPH-oxidase-generated ROS, leading to enhanced and sustained ROS production [86]. Similarly, H_2O_2 generated by dismutation of $O_2^{\bullet -}$ stimulates production of secondary ROS, resulting in a self-propagating phenomenon to prolong ROS-dependent pathological signalling in vascular cells [87]. Interestingly, interaction of MPO with vascular ROS generates HOCl, which is proposed to amplify H_2O_2-induced vascular injury and exacerbate inflammatory vascular diseases [88].

Based on the importance of NADPH oxidase activity in regulation of the vasculature, one would expect that individuals with CGD should have a number of cardiovascular abnormalities, especially in regulating blood pressure. However, a review of the literature describing CGD patients does not suggest problems with blood pressure regulation, regardless of the defect. Although it is possible that this information was simply not included in these publications, it is more likely that the absence of vascular abnormalities in these patients is due the compensatory effects of other NOX homologues. Indeed, it was found that the pressor response induced by angiotensin II is decreased or absent in Nox1-deficient mice [89,90]. NOX4 would be another potential compensatory oxidase, as it is abundant in the vasculature and apparently functions in the absence of the regulatory cytosolic factors missing in autosomal forms of CGD. Clearly, further work is needed to define the relative contributions of different NOX proteins in vascular physiology and their ability to functionally compensate for each other.

RENAL NADPH OXIDASE

A number of studies suggest NADPH-oxidase-generated ROS play important roles in renal function [91]. For example, ROS have been implicated in regulation of Na^+ transport, renovascular tone, tubuloglomerular feedback and renal oxygenation (reviewed in [92,93]). $O_2^{\bullet-}$ increases NaCl absorption in the thick ascending limb of Henle's loop in the absence of $NO^{\bullet}$, suggesting a direct effect on Na^+ transport, and Juncos et al. [94] recently demonstrated that enhanced salt absorption results from ROS modulation of Na^+/H^+ exchangers. Welch et al. [95] demonstrated that enhanced tubuloglomerular feedback responses observed in SHRs (spontaneously hypertensive rats) result from inactivation of $NO^{\bullet}$ by increased $O_2^{\bullet-}$ production in the JGA (juxtaglomerular apparatus). Furthermore, ROS generated in the renal outer medulla can exert a tonic regulatory action on renal medullary blood flow [96]. In response to hypoxia, NADPH-oxidase-derived ROS are thought to facilitate renal medullary function by regulating expression of HIF-1α (hypoxia-inducible factor-1α) [93].

The expression of several NADPH oxidase proteins has been demonstrated in various renal tissues. NOX2, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ are expressed in human podocytes, and $p67^{phox}$ is up-regulated by ATP treatment [97]. Nox1, Nox2, Nox4, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ are present in adult rat kidney, with many of the subunits localized to the nephron, and Nox protein expression is up-regulated in the SHR kidney, suggesting a role in renovascular hypertension [98]. Indeed, analysis of renal function in Nox2-deficient mice confirmed a role for this oxidase in maintenance of renal vascular tone [99]. NOX4 (also known as Renox) was originally discovered in kidney and is expressed at high levels in the renal cortex [16,100]. NOX4 is also expressed in mesangial cells, where it mediates angiotensin-II-induced activation of Akt/PKB (protein kinase B) [101]. Gorin et al. [102] reported that Nox4 was the major source of ROS in the kidney during early stages of diabetes and showed Nox4-derived ROS mediate renal hypertrophy. In addition, recent studies [103] suggest that up-regulation of mesangial cell ROS by high glucose is dependent on PKC (protein kinase C) activity and contributes to diabetic glomerulopathy.

Although ROS play essential roles in normal renal function, these molecules have also been implicated in pathological conditions related to abnormal kidney function. For example, NADPH oxidase activity is enhanced in diabetic nephropathy, presumably contributing to glomerular cell apoptosis, endothelial dysfunction, phagocyte adherence and impaired coagulation in the kidney [104]. Indeed, the NADPH oxidase inhibitor apocynin blocks proteinuria and glomerulopathy in diabetic rats [105], and the antioxidant α-lipoic acid improves albuminuria and pathology in diabetes by reducing NADPH oxidase subunit expression [106]. Additionally, Kondo et al. [107] reported that the antioxidant probucol, when added to an angiotensin II receptor blockade, fully arrested proteinuria and disease progression in mesangioproliferative glomerulonephritis. Recent studies by Yang et al. [108] showed that antioxidant responses induced by stimulation of D_5 dopamine receptor results from inhibition of NADPH oxidase protein expression (Nox2, $p47^{phox}$ and Nox4) and ROS production in kidney and brain. Thus the ability of D_5 receptor stimulation to decrease ROS production may explain, in part, the antihypertensive action of D_5 receptor activation.

PULMONARY NADPH OXIDASE

In the pulmonary system, ROS generated by NADPH oxidase play distinct physiological roles in airway and vasculature remodelling, as well as in O_2 sensing [109]. However, excessive phagocyte-derived ROS also plays a key role in lung injury during asthma and inflammatory events, such as ARDS (acute respiratory distress syndrome) [110]. In airway smooth muscle cells and pulmonary artery smooth muscle cells, NADPH-oxidase-generated ROS serve as important signalling molecules, and Brar et al. [111] showed that a $p22^{phox}$-dependent NADPH oxidase regulates proliferation of airway smooth muscle cells via activation of NF-κB (nuclear factor κB). Recently, Sturrock et al. [112] demonstrated that TGF-β (transforming growth factor-β) induces NOX4-dependent proliferation in human pulmonary artery smooth muscle cells, suggesting that NOX4 may be the predominant NOX protein in this system. However, it also appears that expression of NADPH oxidase components may be dependent on phenotype and anatomical location, and different homologues may be expressed during various physiological and pathological events. For example, a constitutively active Nox2-based oxidase is present in ventilatory skeletal muscle cells and is up-regulated during sepsis [113]. Furthermore, Nox2 stimulates muscle differentiation through activation of NF-κB and iNOS (inducible nitric oxide synthase) [114].

NADPH oxidases also play an important role in O_2 sensing throughout the body, and NOX-based enzymes have been reported to participate in O_2 sensing in pulmonary chemoreceptors [NEB (neuroepithelial bodies)], carotid body, EPO (erythropoietin)-producing cells in the kidney and other organ systems (reviewed in [115]). Originally, it was thought that a NOX2-based enzyme was involved in O_2 sensing, based on the presence of NOX2, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ in carotid body [116] and airway chemoreceptor cells [117]. In support of this concept, O_2 sensing and regulation of K^+ currents in pulmonary NEB requires a functional Nox2-based oxidase [118], and respiratory control is defective in neonatal CGD mice [119]. However, this issue has been controversial, as other groups reported that NADPH

oxidase inhibition does not block hypoxia sensing in carotid body chemoreceptor cells [120], normal hypoxia-induced gene expression occurs in NOX2-deficient CGD cells [121] and O_2 sensing is normal in pulmonary artery smooth muscle cells and carotid body isolated from Nox2-deficient mice [122,123]. Recently, Lee et al. [124] shed some light on this issue when they found that NOX4 could act as an O_2 sensor, leading to regulation of TASK-1 activity in HEK-293 (human embryonic kidney) cells. Thus it is possible that NOX4 may be the relevant NOX protein involved in O_2 sensing or it may be that various NOX enzymes are redundant in this tissue, substituting for each other when necessary. Additional, undefined O_2 sensors may also co-operate with the NOX-based systems.

As described above, regulation of endogenous NADPH oxidases in airway and pulmonary artery smooth muscle cells has important implications for the pathology of asthma and pulmonary vascular diseases. However, exogenous ROS from environmental sources or generated by inflammatory phagocytes are also involved in actual injury to the pulmonary system (reviewed in [125]). Activation of circulating neutrophils and transmigration into the alveoli is associated with development of acute lung injury, and phagocyte-derived ROS have been implicated in the pathogenesis of asthma, allergic rhinitis and ARDS [125]. For instance, Gao et al. [126] demonstrated that phagocyte ROS contribute to lung microvascular injury, and similar damage is absent in lung microvessels from Nox2- and $p47^{phox}$-deficient mice. Alcohol abuse increases the incidence of ARDS, and Polikandriotis et al. [127] recently reported that chronic ethanol ingestion increases ROS production and Nox2 oxidase expression in the lung. Since $NO^{\bullet}$ is important in airway smooth muscle tone and $NO^{\bullet}$ deficiency contributes to allergen-induced airway hyper-reactivity [128], it was thought that this effect resulted from the removal of $NO^{\bullet}$ by enhanced ROS production, as is the case for vascular tissues (see above). However, studies addressing this issue demonstrated that, while $O_2^{\bullet-}$ is responsible for decreasing $NO^{\bullet}$ bioavailability in normal tracheal tissues, $NO^{\bullet}$ deficiency in hyper-reactive trachea is due to down-regulation of constitutive NOS activity, rather than reaction with $O_2^{\bullet-}$ [129]. Thus the specific roles played by ROS in pulmonary tissues may also depend on the presence or absence of underlying pathological conditions.

CNS (CENTRAL NERVOUS SYSTEM) NADPH OXIDASE

Elevated oxidative stress is characteristic of normal brain aging, as well as disease states, such as Alzheimer's disease, Parkinsons's disease, ischaemic injury and stroke (reviewed in [130,131]). Although the roles of NADPH oxidase in CNS function and disease pathogenesis are incompletely defined, it is clear that these enzymes are key participants in CNS processes. For example, microglia express Nox2 and $p22^{phox}$, but generate lower levels of $O_2^{\bullet-}$ than phagocytes [132], and recent studies suggest this oxidase participates in the regulation of microglial proliferation [133]. Tammariello et al. [134] demonstrated that Nox2, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ are present in sympathetic neurons and showed that activity of this oxidase contributes to neuronal apoptosis during NGF (nerve growth factor) deprivation. Likewise, Abramov et al. [135] reported that astrocytes express a Nox2-based oxidase, which is important in astrocyte response to inflammatory agents. Nox2-dependent NADPH oxidase activity also contributes to angiotensin II signalling in the nucleus tractus solitarius [136] and hypothalamic cardiovascular regulatory nuclei [137], suggesting that ROS are also important signaling molecules in central regulatory networks.

Based on the importance of NOX proteins to vascular homoeostasis (discussed above), it is not surprising that ROS also play important roles in regulating cerebral vascular tone (reviewed in [138]). For example, chronic exposure to nicotine increases $p47^{phox}$ expression in the parietal cortex, enhances ROS production and impairs NOS-dependent vasodilation of pial arterioles, suggesting that Nox-generated ROS are reducing $NO^{\bullet}$ bioavailability in these cerebral vessels [139]. Likewise, cerebral artery Nox4 expression and activity are increased during chronic hypertension, suggesting a role for this Nox protein in the regulation of cerebral vasodilation [140].

ROS associated with inflammatory and ischaemic CNS injury are produced predominantly by recruited phagocytes, and antibody treatment to prevent leucocyte recruitment to the CNS reduces experimental cerebral injury [141]. In support of this idea, neutrophil depletion prior to hypoxic/ischaemic brain injury is neuroprotective [142]. During ischaemic injury, ROS-induced lipid peroxidation plays an important role in tissue pathology, and antioxidants represent an important therapeutic approach to prevention of this process [143]. Indeed, brain-penetrating antioxidants have even better efficacy as neuroprotective agents in CNS ischaemic injury [144]. Phagocyte ROS production is enhanced in patients with Parkinson's disease [145], and Wu et al. [146] found that a Nox2-based oxidase is important in inflammation-induced toxicity to dopaminergic neurons. Additionally, Qin et al. [147] reported that ROS generated by the microglial Nox2-based NADPH oxidase contribute to dopamine neuron toxicity and also amplify pro-inflammatory gene expression in microglia. Interestingly, Martignoni et al. [148] proposed that L-dopa, the standard treatment for Parkinson's disease, may promote ROS formation in patients, possibly accounting for some of the cytotoxicity of this drug. Alzheimer's disease is also an important CNS disease associated with ROS stress [149], and it has

been proposed that NOX proteins are involved in the protein and lipid oxidation associated with this disease [150]. Indeed, amyloid β peptide has been reported to activate astrocyte NADPH oxidase (Nox2), generating oxidative stress that leads to neuronal death.

GI (GASTROINTESTINAL) NADPH OXIDASE

ROS generation by resident and recruited phagocytes represents an essential component of GI defence against a myriad of pathogens [151]. However, recent studies indicate other NOX-based enzymes may also be essential to healthy gut immunity. For example, NOX1 is abundantly expressed in colon and gastric pit epithelial cells [12,13,152] and is induced during epithelial cell differentiation, with the highest levels of NOX1 present on crypt cells [27]. NOXO1 and NOXA1 are also present in colon epithelial cells, which is consistent with their role in regulating NOX1 activity [29–31]. NOX1 appears to be important in normal gut function. For example, H_2O_2 derived from Nox1-generated $O_2^{\bullet-}$ in colon mucosa promotes serotonin biosynthesis, which is important in regulating secretion and motility [153]. Although the NOX1-based oxidase appears to generate constitutive low levels of $O_2^{\bullet-}$ in colon epithelial cells, there is recent evidence to suggest that this oxidase may also contribute to gut immunity. For instance, treatment of guinea pig gastric mucosal cells with *Helicobacter pylori* LPS (lipopolysaccharide) activates Rac1 and transcription of Nox1 and Noxo1 [154], which appears to be mediated by TLR5 [155]. In addition, Kuwano et al. [156] recently reported that IFN-γ activates NOX1 transcription and up-regulates $O_2^{\bullet-}$ production by colon epithelial cells, supporting further a role for NOX1 in mucosal host defence. In addition to Nox1, Duox2 has been suggested to play a role in gut immunity [157]. In *Drosophila*, *Duox2* expression is found throughout the digestive tract [158], and silencing *Duox2* in adult flies markedly increases mortality, even after minor infection [158]. Importantly, analysis of human GI tissues shows DUOX2 expression in barrier epithelial cells of the colon and rectum, as well as in rectal glands [159]. These data suggest a potential role for DUOX2-generated ROS in mucosal barrier defence.

A significant amount of research suggests that ROS potentiate GI tissue damage and pathology, resulting in IBDs (inflammatory bowel diseases), such as Crohn's disease and ulcerative colitis [160]. Abnormalities in vascular structure and function, in addition to inflammatory lesions, in the gut may be initiated and exacerbated by members of the NADPH oxidase family, including NOX1, NOX2, and NOX5. Indomethacin and several other NSAIDs (non-steroidal anti-inflammatory drugs) are known to induce injury to the gut mucosa, and this damage appears to be mediated, in part, by phagocyte-generated ROS (e.g., [161]). In human stomach biopsies, *NOX2* and *NOX5* transcripts are expressed, and while *NOX2* message increases as a function of age and inflammation, *NOX5* transcript levels fail to correlate with age-dependent inflammatory disease [162]. In normal human colon, *NOX1* and *NOX5* are present in colonic lymphocytes, whereas lymphocytes in lesions from patients with Crohn's disease or ulcerative colitis express *NOX1* but not *NOX5* [162]. Interestingly, phagocytes from patients with Crohn's disease exhibit impaired inflammatory responses [163] and generate lower levels of ROS than those from healthy individuals [164], a phenomenon linked to diminished flavocytochrome b_{558} expression [165]. Similarly, *NOX2* message is down-regulated or completely absent in macrophages from normal mucosal tissue as compared with cells from IBD mucosa [166]. Thus it is clear that factors in addition to phagocyte-generated ROS are involved in the inflammatory activity of Crohn's disease. Consistent with this idea, studies on IBD showed that the anti-inflammatory cytokine TGF-β is essential for down-regulating excessive inflammation in human disease and in mouse models [167]. In mice, NF-κB activation plays a key role in the induction of DSS (dextran sodium sulphate)-dependent colitis, and suppression of NF-κB activity protects mice from DSS-induced colitis [168]. Although the DSS model generally indicates a role for NADPH-oxidase-mediated tissue damage in the induction of IBDs, the data are mixed. For example, studies in Nox2-deficient mice indicate that NADPH oxidase contributes to DSS-induced colitis and vascular dysregulation, and transgenic expression of SOD restores the healthy gut [169]. However, a separate study in Nox2-deficient mice showed significantly increased gastritis [170]. Additionally, gut inflammation due to DSS treatment does not differ between $p47^{phox}$-deficient and wild-type mice, and SOD transgenic mice exhibit more severe colitis than wild-type mice [171]. A consideration clearly absent from these models is the potential involvement of other NOX homologues. Thus a more comprehensive understanding of NOX enzymes in gut inflammation will need to be considered before conclusions are drawn from current IBD models.

HEPATIC NADPH OXIDASE

The liver plays an important role in innate immunity, specifically through the action of Kupffer cells and liver-specific natural killer cells (reviewed in [172]). Kupffer cells are the resident macrophage population of the liver and utilize a Nox2-based NADPH oxidase to generate ROS in defence against pathogens [173]. However, excessive ROS generation by Kupffer cells has been shown to play a role in hepatotoxicity, and studies in $p47^{phox}$-deficient mice suggest that Kupffer cell oxidant production contributes to ethanol-induced liver disease [174] and hepatocarcinogenesis [175]. Hepatic stellate

cells also generate ROS via a Nox2-containing NADPH oxidase, and these oxidants are reported to mediate angiotensin II signalling during liver fibrosis [176]. Finally, NADPH oxidase components are expressed in hepatocytes, and Reinehr et al. [177] found message for *Nox1*, *Nox2*, *Nox4*, *Duox1* and *Duox2*, as well as Nox2 protein expression, in rat hepatocytes. In addition, this group reported that hydrophobic bile salts activated hepatocyte NADPH oxidase, leading to apoptosis in these cells [178]. Thus it is evident that there is a fine line dividing the physiological and pathological roles of hepatic NADPH-oxidase-generated ROS, and severe inflammatory hepatotoxicity can result if the balance is shifted toward ROS excess (e.g., alcohol-induced injury).

NADPH OXIDASE AND ARTHRITIS

Although the neutrophil-oxidant-producing system is designed to restrict oxidant generation to the smallest possible region where the pathogen is located, some ROS inevitably leak into surrounding areas where they have the capacity to inflict tissue damage. Indeed, ROS have been implicated as the damaging agent in arthritic diseases, including RA (rheumatoid arthritis) [179,180] and acute inflammatory arthritis [181]. Neutrophils represent the majority of the cells found in joint synovial fluid of RA patients [182], and many of these cells are activated by soluble factors, such as immune complexes [179], resulting in higher levels of ROS in the synovial fluid of arthritics compared with non-arthritic patients [183]. Furthermore, phagocytes from patients with arthritis have a significantly increased ability to produce ROS [184], and this phenomenon is attributed in part to priming by TNF-α [185]. In support of these observations, pharmacological agents that destroy or inhibit the production of ROS, such as apocynin [186,187], methotrexate [188] and DPI (diphenyleneiodonium) [189], can suppress the development of inflammation and symptoms associated with arthritis. In contrast, Crandall et al. [190] recently reported that the pathogenesis of Lyme arthritis is independent of NADPH oxidase activity. Thus the role of NADPH oxidase in arthritis appears to be complex and may depend on the type of disease. Notably, other work suggests that the role of NADPH oxidase may extend even beyond the simple contribution of ROS to tissue destruction. For example, joint inflammation and bone erosion are worse in $p47^{phox}$- and $gp91^{phox}$-deficient CGD mice with experimentally induced arthritis, compared with wild-type mice, and the authors concluded that NADPH-oxidase-derived ROS might actually play a role in limiting the disease process under certain conditions [191]. In support of this idea, Olofsson et al. [192] reported that a naturally occurring structural polymorphism of the gene encoding $p47^{phox}$ (*NCF1*) regulates the severity of arthritis and proposed that NADPH-oxidase-derived ROS reduced arthritis by regulating arthritogenic T-cells, rather than causing tissue damage [192,193]. Although this finding seems counterintuitive, ROS are known to induce T-cell apoptosis [194], and this may limit expansion of T-cell-dependent autoimmune responses directed to self-antigens [193]. Thus it may be that, under healthy conditions, ROS work to prevent arthritis and other autoimmune conditions, whereas, once these conditions develop, oxidants can contribute directly to pathogenesis.

NADPH OXIDASE AND CANCER

ROS have long been known to contribute to the pathogenesis of cancer, although the precise mechanisms involved are still being defined (recently reviewed in [195]). It is clear, however, that inflammation associated with chronic infection or inflammatory syndromes, such as ulcerative colitis, can increase the risk of malignancy [196,197]. Moreover, recent work has increasingly pointed to a critical role for NADPH oxidases in the induction and progression of malignant cells [198]. Note, however, that ROS can also be harmful to tumour cells, via their ability to induce cellular senescence and apoptosis. For example, Huang et al. [199] showed that inhibiting SOD, which leads to increased cellular ROS, results in selective killing of cancer cells and suggested this might be a good approach for treating cancer.

If NADPH oxidase is essential to tumorigenesis, one might expect reduced incidence of malignancy in CGD patients; however, malignancies still frequently occur in individuals with CGD [200], suggesting a potential role of other NOX homologues in this process. NOX family members appear to play functional roles in many stages of cancer. For example, accumulation of genetic abnormalities leads to malignant transformation, and ROS have been tied to genomic instability through chemically induced changes in DNA, changes in nucleic acid structure and conformation and inhibition of repair enzymes [201]. Furthermore, oxidative damage to DNA results in single- and double-strand breaks, frame-shift mutations and chromosomal abnormalities [202]. Thus the same ROS generated by phagocytes in defence of the host can also contribute to the development of malignancies. Indeed, epidemiological studies indicate that increased leucocyte count is a risk factor for cancer [203], and a number of studies have shown that ROS produced by human neutrophils can directly damage DNA and can transform bystander cells [204,205].

Although mild oxidant stress can induce apoptosis, prolonged exposure of some cell types to ROS can prevent caspase activation and, thus, the initiation of apoptosis [206]. Chronic exposure to ROS may also alter normal cellular signalling events. For instance, ROS have

been shown to activate transcription factors NF-κB and AP-1 [207], and NF-κB has been found to be constitutively activated in a variety of malignancies [198,208]. NF-κB contributes to abnormal cell growth via enhanced expression of anti-apoptotic factors, such as Bcl-2, and proliferative factors, including cyclin D1 [198]. The role of ROS in constitutive activation of NF-κB is supported by studies showing that treatment with antioxidants and ROS scavengers decreases constitutive NF-κB activation in malignant cells [209]. Additionally, NOX-based enzymes have been implicated as important sources of ROS involved in constitutive NF-κB activation in various malignant cells [198]. Brar et al. [111] showed that treatment of melanoma cells with antisense oligonucleotides to knock down $p22^{phox}$ inhibits cell proliferation, confirming the role of NADPH oxidase in maintaining these cancer cells. Likewise, targeted inhibition of NOX4 results in induction of apoptosis in pancreatic cancer cells which were previously resistant to apoptosis [210]. These cells also require growth factors, such as IGF-1 (insulin-like growth factor-1) and FGF-2 (fibroblast growth factor-2), which induce ROS production via NOX4 to facilitate evasion of apoptosis [211]. Overexpression of NOX1 causes transformation of a variety of cell types, including fibroblasts [12], and recent studies suggest that NOX1 expression is associated with redox-mediated cellular activation, which may be an obligatory step for advanced stages of cell transformation, eventually leading to tumorigenic conversion [212]. In addition, analysis of colon tumour biopsy revealed cells constitutively expressing high levels of NOX1 [213]. Note, however, that the role of NOX1 as a mitogenic factor has been an issue of debate because the original work on NOX1 was performed in NIH3T3 cells containing constitutively active Ras [6]. In addition, subsequent studies from Geiszt et al. [27] indicated that NOX1 is predominantly expressed in differentiated colon epithelial cells and fails to induce proliferation of colon carcinoma cells. Thus it is possible that the mitogenic effect noted previously represents a combination of mutated Ras and ROS generation in NOX1-expressing cells. In support of this idea, substantial evidence indicates that ROS play a role in Ras-mediated transformation, as mutated Ras is known to be present in a significant fraction of malignant cells [214], and recent studies demonstrate that ROS actually promote Ras guanine nucleotide exchange by reacting with redox-sensitive residues in Ras to promote guanine nucleotide dissociation [215]. Current research suggests that additional NOX family members might be essential to cell transformation and mitogenic growth. For example, overexpression of NOX4 enhances proliferation, and inhibition of NOX4 blocks proliferation of melanoma cells [209]. In addition, NOX5 was found to be responsible for the generation of ROS required for prostate cancer cell growth [216] and for maintenance of malignant B-cells in hairy cell leukaemia [217].

During final stages of tumour formation, excessive cellular proliferation creates an environment lacking vascularization, and cells must adapt to hypoxic conditions and induce vascularization of the tumour mass through angiogenesis [218]. Of note, recent studies indicate that up-regulation of NOX1 during hypoxia leads to HIF-1α activation in human pulmonary epithelial cells [219]. Studies addressing hypoxia adaptation in tumour cells suggest that they utilize this type of hypoxia-sensitive transcriptional regulation and that ROS can stimulate angiogenesis via HIF-1α activation [220]. For instance, ROS-dependent induction of VEGF facilitates rapid tumour growth and invasion in nude mice, and overexpression of Nox1 not only increases VEGF expression, but also increases VEGF receptor expression and MMP (matrix metalloproteinase) activity, thereby contributing to tumour angiogenesis [221]. Further investigation of the role of ROS in VEGF signalling and angiogenesis-associated responses shows that VEGF stimulates $O_2^{\bullet -}$ production in a Rac1- and NOX2-dependent manner, as well as endothelial cell proliferation and migration [222]. Moreover, sponge implant models in Nox2-deficient CGD mice confirm that a Nox2-containing NADPH oxidase plays an important role in VEGF signalling and angiogenesis.

The essential role of the NADPH oxidase in cancer pathology suggests that inhibition of NADPH oxidase might represent a relevant approach for treating cancer, and antioxidants have been the focus of many anticancer therapies. Indeed, some anticarcinogenic agents inhibit ROS production and inflammation [195], and it has been proposed that high doses of antioxidant vitamins may also improve efficacy of standard cancer therapy [223]. In support of this approach, treatment of NOX1-expressing tumour cells with antioxidants or ROS scavengers inhibits cell growth [224]. Similarly, vitamin E treatment inhibits NADPH-oxidase-dependent transformation in a murine liver cancer model [225]. In the pursuit of antioxidant treatments for human cancer, SOD mimetics have been considered for colon and liver malignancies [226]. In addition, various plant extracts have shown promise as anticancer agents. For example, Lee [227] recently reported that phenolic acids induce NADPH-oxidase-dependent apoptosis in HepG2 hepatoma cells.

NADPH OXIDASE AND RESOLUTION OF INFLAMMATION

Apoptosis is widely accepted as essential for many physiological processes, including neutrophil homoeostasis [228]. Inasmuch as neutrophils are the most abundant white cell in humans and contain or generate numerous cytotoxic molecules, including ROS, there is significant potential for host injury and trauma associated with infection and inflammatory diseases. The

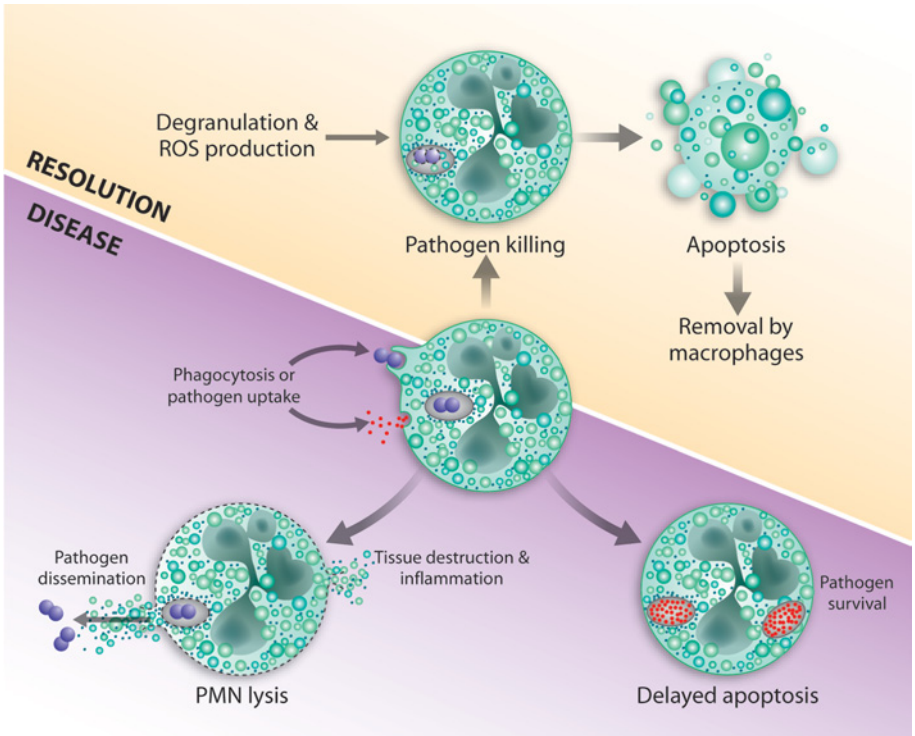

Figure 4 **A paradigm illustrating the role of ROS in the resolution of infection**

See text for details.

importance of neutrophil apoptosis in the resolution of infections is underscored by diseases in which tissue destruction and inflammation are associated with neutrophil activation or lysis (see above and reviewed in [229]).

ROS are implicated in spontaneous neutrophil apoptosis and, thus, putative mechanisms involve NADPH oxidase (e.g., see [230,231]). Furthermore, numerous studies indicate that ROS are critical for bacteria-induced neutrophil apoptosis and/or phagocytosis-induced cell death. For example, Watson et al. [232] showed that phagocytosis of *Escherichia coli* induces neutrophil apoptosis, a phenomenon dependent on production of ROS, and Zhang et al. [233] demonstrated that ROS promote cleavage of caspases 3 and 8 following CR3 (complement receptor 3)-mediated phagocytosis. Additionally, DPI blocks phagocytosis-induced cell death, supporting the critical role of ROS in this process [233].

In addition to suffering recurrent infections, individuals with CGD develop granulomas that inhibit function of large vital organs, such as the GI tract, liver and lung [43]. The formation of granulomas is linked to altered neutrophil apoptosis, and studies of neutrophils from patients with CGD provide compelling evidence for the involvement of ROS in neutrophil apoptosis [45,234,235]. Coxon et al. [234] were the first to use neutrophils from CGD patients to show that phagocytosis-induced cell death requires NADPH oxidase, and more recent studies by Kobayashi et al. [45] demonstrate that ROS directly or indirectly modulate expression of the pro-apoptosis molecule BAX, which increases concurrently with phagocytosis-induced cell death. Hampton and co-workers [235] demonstrated that ROS are critical for clearance of neutrophils from inflammatory sites, thereby providing further support for the notion that ROS are essential for the resolution of neutrophil-mediated inflammation.

Recent advances in genomics and genome-scale techniques provide new approaches for elucidating host–pathogen interactions, and Kobayashi et al. [45,236] have used these methods to define processes in neutrophils during interaction with bacterial pathogens. These studies showed that phagocytosis of bacteria, activation of NADPH oxidase, killing of micro-organisms and induction of apoptosis were accompanied by global changes in neutrophil gene expression common to a range of bacterial pathogens. Based on this work and numerous other studies (reviewed in [237]), a paradigm for the resolution of bacterial infections is emerging (Figure 4). On one hand, phagocytosis triggers production of ROS and killing of bacteria, which results in the induction of neutrophil apoptosis and subsequent removal by macrophages. Alternatively, bacterial pathogens can modulate neutrophil apoptosis and, thereby, survive and cause disease (Figure 4). Thus, for intracellular pathogens, such as *Anaplasma phagocytophilum*, failure to trigger production of neutrophil ROS

and/or the ability of these bacteria to inhibit NADPH oxidase appear to be key to pathogenesis [238,239].

CONCLUDING REMARKS

Discovery of phagocyte NADPH oxidase proteins in the mid-to-late 1980s triggered a major effort to characterize the biochemical and functional aspects of the enzyme. What ensued were 10–15 years of intense research by a number of laboratories worldwide that ultimately led to a detailed understanding of how ROS are generated by phagocytic cells. Since that time, focus has shifted to better understand the role NADPH oxidases in a broader sense, and it is now widely accepted that these enzymes are not simply agents of microbial death and destruction; they also play key roles in many organ systems and cell types, thereby contributing to normal physiological processes and, when in excess, to the pathogenesis of various human diseases. With complete sequencing of the human genome and advances in research techniques, such as gene silencing, proteomics, microarray approaches, bioinformatics and systems biology, we are poised to fully understand the role of these enzymes in human health and disease.

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