Biochemical Pharmacology 80 (2010) 778–785

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Commentary

Peptide-based inhibitors of the phagocyte NADPH oxidase

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ARTICLE INFO

Article history:
Received 30 March 2010
Accepted 17 May 2010

Keywords:
Phagocytes
NADPH oxidase
Inhibitors
gp91phox/NOX2
p22phox
p47phox

ABSTRACT

Phagocytes such as neutrophils, monocytes and macrophages play an essential role in host defenses against pathogens. To kill these pathogens, phagocytes produce and release large quantities of antimicrobial molecules such as reactive oxygen species (ROS), microbicidal peptides, and proteases. The enzyme responsible for ROS generation is called NADPH oxidase, or respiratory burst oxidase, and is composed of six proteins: gp91phox, p22phox, p47phox, p67phox, p40phox and Rac1/2. The vital importance of this enzyme in host defenses is illustrated by a genetic disorder called chronic granulomatous disease (CGD), in which the phagocyte NADPH oxidase is dysfunctional, leading to life-threatening recurrent bacterial and fungal infections. However, excessive NADPH oxidase activation and ROS over-production can damage surrounding tissues and participate in exaggerated inflammatory processes. As ROS production is believed to be involved in several inflammatory diseases, specific phagocyte NADPH oxidase inhibitors might have therapeutic value. In this commentary, we summarize the structure and activation of the phagocyte NADPH oxidase, and describe pharmacological inhibitors of this enzyme, with particular emphasis on peptide-based inhibitors derived from gp91phox, p22phox and p47phox.

1. Introduction

Reactive oxygen species (ROS) produced by phagocytes are one of the most powerful host defenses against bacteria, yeasts and fungi [1,2]. ROS produced by phagocytes include superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$) and hypochlorous acid (HOCl). These ROS are produced in large quantities when phagocytes are stimulated by pro-inflammatory agents or by particles such as bacteria. This process, known as the "oxidative burst" or "respiratory burst", is characterized by a rapid, cyanide-insensitive increase in oxygen uptake and glucose consumption [1,2]. Superoxide anion ($O_2^{\bullet-}$), the precursor of the other ROS, is first produced by an enzyme called NADPH oxidase, as follows [2,3]:

$$2O_2 + NADPH \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+$$

Once produced, $O_2^{\bullet-}$ is immediately transformed into H_2O_2 by spontaneous dismutation at acid pH in the phagosome, or through enzymatic dismutation by superoxide dismutase in the cytosol. Interaction between H_2O_2 and $O_2^{\bullet-}$ can, through the Haber–Weiss reaction in the presence of a transition metal (or the Fenton reaction in the presence of iron), give rise to the hydroxyl radical ($OH^\bullet$), one of the most powerful oxidants. H_2O_2 is also a substrate of myeloperoxidase, an enzyme stored in neutrophil azurophilic granules and released during neutrophil activation. Myeloperoxidase catalyses the transformation of H_2O_2, in the presence of a halogen (Cl^-, Br^-, I^-), into highly toxic molecules such as hypochloric acid ($HOCl^-$). Other reactions between OCl^- and H_2O_2 can lead to the formation of singlet oxygen ($^1O_2$). Most of the hypochlorous acid (OCl^-) thus generated is converted into toxic chloramines. MPO can also use H_2O_2 to oxidize tyrosines into tyrosyl radicals. HOCl, chloramines and tyrosyl radicals, which are toxic species that serve to kill bacteria and other pathogens. The crucial role of phagocyte NADPH oxidase in host defenses against microbial pathogens is illustrated by a human genetic disorder called chronic granulomatous disease, which is associated with life-threatening recurrent bacterial and fungal infections [4]. However, excessive ROS production can damage healthy bystander tissues. ROS hyper-production by neutrophils is believed to cause direct tissue insult in a broad range of inflammatory diseases, including rheumatoid arthritis, inflammatory bowel diseases, acute respiratory distress syndrome, sepsis, diabetic complications, cardiovascular disease, ischemic tissue injury and neurodegenerative diseases [5,6]. Pharmacological

Abbreviations: DPI, diphenylene iodonium; CGD, chronic granulomatous disease; fMLF, formyl-methionyl-leucyl-phenylalanine; MPO, myeloperoxidase; phox, phagocyte oxidase; ROS, reactive oxygen species.

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doi:10.1016/j.bcp.2010.05.020

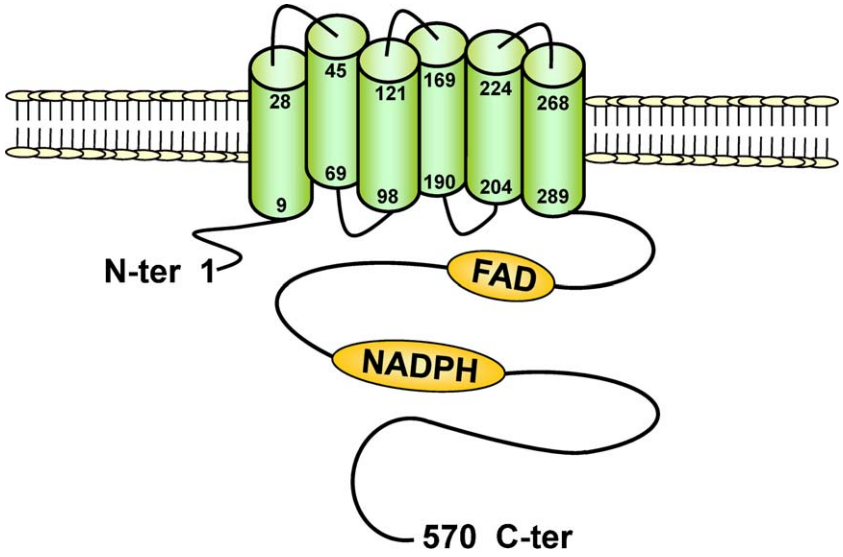

Fig. 1. Predicted structure of gp91phox/NOX2. The protein has a short N-terminal cytosolic sequence, 6 transmembrane helices, and one long C-terminal cytosolic tail containing the FAD binding site and one NADPH binding site.

NADPH oxidase inhibition might therefore be beneficial in patients with these disorders. Currently, there are no specific inhibitors of the phagocyte NADPH oxidase. Peptide-based inhibitors derived from specific subunits of the enzyme could provide a certain degree of specificity. In the first part of this manuscript, we review the principal features of NADPH oxidase in order to provide the information necessary to understand the action of the inhibitory peptides described in the second part.

2. Components of the phagocyte NADPH oxidase

The phagocyte NADPH oxidase is a multicomponent enzyme complex comprising six proteins, namely p22phox (phox: phagocyte oxidase), gp91phox, p47phox, p67phox, p40phox and the small G-protein Rac1 or Rac2. In resting cells the NADPH oxidase is inactive because its components are distributed between the cytosol (p47phox, p67phox, p40phox and Rac1/2) and the plasma membrane and membranes of specific granules (p22phox and gp91phox/NOX2, which form the flavocytochrome b_{558}). When cells are activated, the cytosolic components migrate to the membranes, where they associate with the membrane-bound components to assemble the catalytically active oxidase [7,8].

Flavocytochrome b_{558} is the central membrane-bound component of NADPH oxidase [9]. It is composed of a 1:1 complex between a glycosylated 91-kDa protein subunit (gp91phox) of 570 amino acids (Fig. 1), and a nonglycosylated 22-kDa subunit (p22phox) of 195 amino acids (Fig. 2). Flavocytochrome b_{558} contains one FAD and two hemes and forms the NADPH oxidase electron transfer chain [9]. It serves as the central docking station for the cytosolic components, via numerous interaction sites [8]. P22phox is phosphorylated on threonine residues by a phosphatidic acid-activated kinase and PKC [10]. Gp91phox phosphorylation in human neutrophils enhances its enzymatic activity and its binding to p47phox, p67phox and Rac1 [11].

P47phox is a cytosolic protein composed of 390 amino acids. Its COOH-terminal sequence is very basic and rich in serine and arginine [12,13]. The amino acid sequence of p47phox also contains two src-homology 3 (SH3) domains, one phox homology (PX) domain, a proline-rich region and one auto-inhibitory region (AIR) (Fig. 3). P47phox binds to the flavocytochrome b_{558} during

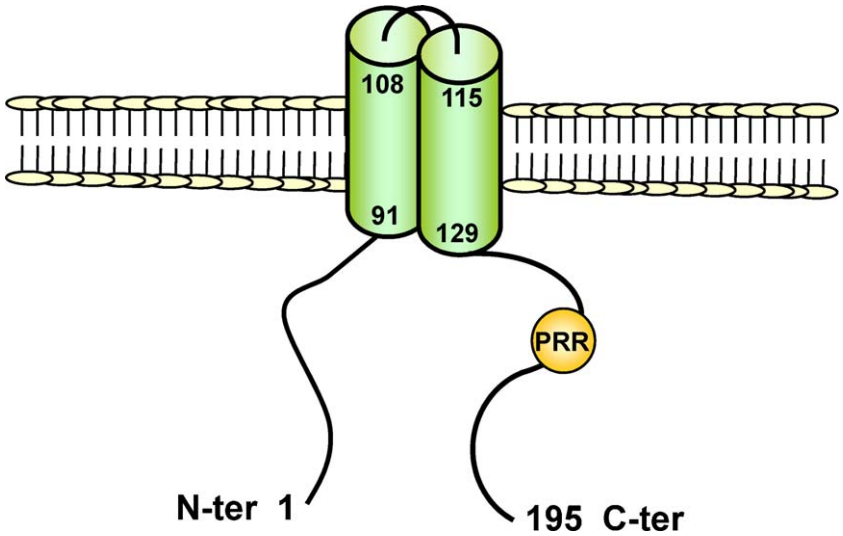

Fig. 2. Predicted structure of p22phox. The protein has an N-terminal cytosolic sequence, 2 transmembrane helices, and one long C-terminal cytosolic tail containing a proline-rich region (PRR) which binds to p47phox SH3 domains.

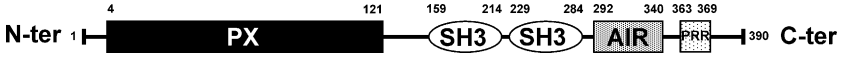

Fig. 3. Predicted structure of p47phox. The protein has a phox homology (PX) domain, two src homology (SH3) domains, one auto-inhibitory region (AIR) and a proline-rich region (PRR).

NADPH oxidase activation. P47phox is the subunit responsible for transporting the cytosolic complex (p47phox-p67phox-p40phox) from the cytosol to the membrane during oxidase activation, and for organizing the different NADPH oxidase subunits. In intact cells, p47phox is extensively phosphorylated [13]. P67phox is composed of 526 amino acids, with two SH3 domains, four tetratricopeptide-rich regions and a proline-rich region [7,12]. P67phox interacts with Rac1/2 and flavocytochrome b_{558} and can regulate its catalytic activity via a sequence called the activation domain [14]. P67phox is phosphorylated on serine and threonine residues by PKC-dependent and PKC-independent pathways [15]. P40phox is a 339-amino acid protein which was initially identified through its binding to p67phox. It contains one SH3 domain and one PX domain. P40phox is phosphorylated on serine 315 and threonine 154 by a PKC-dependent mechanism [16]. Rac2 is the most abundant rac protein in human neutrophils, but rac1 (92% homologous with Rac2) is also present [12].

3. Activation of the phagocyte NADPH oxidase

3.1. Activation in intact neutrophils

During neutrophil activation, 10–20% of cytosolic NADPH oxidase components migrate to the plasma membrane, where they bind to flavocytochrome b_{558} [7–9]. In intact cells, NADPH oxidase activation is accompanied by phosphorylation of p47phox, p67phox, p40phox, p22phox and gp91phox, along with several protein–protein interactions [10–14]. NADPH oxidase activation in phagocytes can be induced by a large number of particulate and soluble agents such as opsonized bacteria, opsonized zymosan, latex particles, formylated peptides such as formyl-Met-Leu-Phe (fMLF), and also by pharmacological compounds such as calcium ionophores and PKC activators such as phorbol esters [17]. Other agents, such as pro-inflammatory cytokines (TNFα, GM-CSF, IL-8, etc.) do not activate NADPH oxidase in neutrophils but instead induce a state of hyper-responsiveness to subsequent stimulation of NADPH oxidase and ROS production by agents such as fMLF, a process known as "priming" [17]. This phenomenon of "priming" or oxidative burst initiation is also observed *in vivo*, where it may play an important role in physiological regulation of bactericidal activity and also in host tissue injury in some inflammatory diseases.

During neutrophil stimulation, p47phox is phosphorylated on multiple sites in its carboxy-terminal portion, including serines 303–379, which play a central role in NADPH oxidase activation and regulation [13]. In human neutrophils, among the various protein kinases involved in the regulation of NADPH oxidase activity, the PKC family appears to play a major role in the activation [13,17]. Pro-inflammatory cytokines such as GM-CSF and TNFα induce partial phosphorylation of p47phox on Ser345 [13,18]. Phosphorylation of p47phox on Ser345 represents a critical mechanism in the priming of ROS production by neutrophils at inflammatory sites. Thus, this site may be considered as a "priming site". P47phox phosphorylation could potentially induce conformational changes that initiate assembly of the active enzyme via interaction of the SH3 domain with the proline-rich region of p22phox [12,17]. In resting conditions the two SH3 domains of p47phox interact intramolecularly with the auto-inhibitory region in the nonphosphorylated protein [7,8]. This interaction switches to the p22phox-polyproline sequence when the enzyme is activated by phosphorylation [8].

3.2. NADPH oxidase activation in cell-free systems

NADPH oxidase can be activated in a cell-free system by mixing cytosol and plasma membranes isolated from resting neutrophils or macrophages in the presence of Mg^{2+}, GTP and an anionic amphiphile such as arachidonic acid or sodium dodecyl sulfate, which are believed to mimic phosphorylation by providing negative charges [19]. Active NADPH oxidase can also be reconstituted from recombinant or highly purified cytochrome b558, p47phox, p67phox and Rac proteins [20]. P40phox is not required for NADPH oxidase activation in a cell-free system, although it enhances NADPH oxidase activation in intact cells. This cell-free system can also be activated by protein kinase C instead of anionic amphiphile agents [21] and by physiological concentrations of phosphatidic acid [22]. This system has been used to examine the effect of various peptide inhibitors on NADPH oxidase activation.

4. Homologues of phagocyte NADPH oxidases (NOXs)

Almost 30 years after the discovery of gp91phox, several homologues of this catalytic subunit have been described in human tissues such as lung, kidney and colon, and in various cell types (epithelial cells, endothelial cells, vascular smooth muscle cells) [6,23]. These homologues have been cloned and grouped together under the acronym NOX, for NADPH oxidase (NOX1–NOX5), or Duox, for dual oxidase (Duox1 and Duox2). NOX2 (gp91phox) is not restricted to phagocytes, as it is also expressed, albeit at lower levels, in endothelial cells, B and T lymphocytes, neurons, and other cell types [23]. The main difference between phagocytic gp91phox/NOX2 and its homologues resides in the fact that ROS are produced in much smaller amounts by the new homologues. The other difference lies in their regulation, including the nature of the cytosolic subunits and their requirement. These new findings suggest that ROS might be involved in several cellular functions such as local tissue-specific bactericidal activity (in colon or lung) and intracellular signaling. The NOX1 and NOX3 homologues are regulated by two NOX regulatory proteins called NOX organizer 1 (NOXO1) and NOX activator 1 (NOXA1) [24,25]. These are the respective homologues of p47phox and p67phox. Like p47phox, NOXO1 'organizes' the assembly of the fully active NOX1 enzyme complex. Indeed, it recruits NOXA1 to the membrane-bound NOX1 catalytic subunit through its interaction with p22phox and with membrane phosphoinositides [26,27]. The small G-protein Rac1 is also clearly involved in NOX1 activation, through its interaction with NOX activator 1.

5. Phagocyte NADPH oxidase inhibition

5.1. Chemical (nonpeptide) inhibitors

Many chemicals are known to inhibit ROS production by the phagocyte NADPH oxidase. Twenty years ago, Cross [28] listed numerous molecules with this effect, although few of them directly interact with or inhibit NADPH oxidase [29]. One of the first inhibitors of NADPH oxidase to be identified, and which is still widely used, is diphenylene iodonium (DPI), which inhibits electron transport by gp91phox/NOX2 [30]. This molecule is a flavoprotein inhibitor and is therefore nonspecific for NOX2. It inhibits not only other NOXs and DUOXs but also NO synthase and

probably other flavoproteins [31]. However, in animal models, DPI inhibits ROS production and has an anti-arthritic effect [32]. It has been shown to reduce ethanol-induced liver inflammation in mice [33], and to inhibit pancreatic inflammation and fibrosis [34].

Apocynin (4-hydroxy-3-methoxyacetophenone-substituted), a natural molecule structurally related to vanillin, inhibits the NADPH oxidase [35,36]. It was reported to target p47phox, and its effect requires a peroxidase such as MPO [37]. It might be more specific for NOX2 but can also inhibit NOX1 *via* NOXO1 in the presence of peroxidase. Apocynin also scavenges ROS, making it nonspecific for NOX2 [36,38]. *In vivo*, apocynin was shown to attenuate collagen-induced arthritis in rats, to lower IL-6 levels, and to reduce joint swelling [39] and zymosan-induced arthritis and inflammation in mice [40]. Apocynin also has other beneficial effects *in vivo* [41,36].

Other molecules such as phenylarsine oxide (PAO), a sulfhydryl reagent for vicinal or proximal thiol groups, and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), inhibit NADPH oxidase by preventing assembly of the complex [42,43], but are also known for their inhibitory effects on other enzymes. PAO is a phosphotyrosine phosphatase inhibitor and AEBSF is a serine protease inhibitor. In rats, PAO inhibits ROS production and also attenuates LPS- and carrageenan-induced inflammation [44]. Many other small molecules have been shown to inhibit the phagocyte NADPH oxidase and other NOXs [45], but there are currently no specific chemical inhibitors for the phagocyte NADPH oxidase. Peptide inhibitors could be more specific than chemical inhibitors because they are derived from specific subunit sequences. In the following part of the article we describe peptide inhibitors derived from each subunit described in the literature and discuss their specificity.

5.2. Peptide inhibitors

5.2.1. Gp91phox-derived inhibitory peptides

Peptides derived from gp91phox/NOX2 have been used to inhibit NADPH oxidase. The first such peptides were the NOX2 carboxy-terminal peptides RGVHFIF (amino acids 559–565) and SNSESGPRGVHFIFNKEN (amino acids 552–569), which inhibit phagocyte NADPH oxidase activation in cell-free systems (50% inhibitory concentration (IC_{50}) = 32 μM) and, at higher concentrations, in electropermeabilized neutrophils [46–48]. Leusen et al. [49] showed that a point mutation of gp91phox (D500G) in X-linked chronic granulomatous disease patients resulted in an inactive NADPH oxidase and failure of p47phox/p67phox to translocate to membranes, and thereby identified a gp91phox/p47phox interaction domain encompassing D500 in gp91phox. The peptide sequence FAVHHDEEKDVITG, mimicking the 491–504 amino acids domain of gp91phox, inhibited superoxide generation in a cell-free system (IC_{50} = 10 μM) and also prevented membrane translocation of p47phox and p67phox. Using a random-sequence peptide phage display library technique, DeLeo et al. identified several potential sites of interaction between p47phox and cytochrome b558 [50]. A peptide mimicking one gp91phox/p47phox interaction sequence, corresponding to amino acids 77–93 (FLRGSSACCSTRVRRQL), had a potent inhibitory effect on human NADPH oxidase (IC_{50} = 1 μM). Another peptide, corresponding to amino acids 86–102, had a potent inhibitory effect, with an EC_{50} of 2 μM [51]. Later, Rey et al. identified the corresponding sequence in mouse gp91phox and called it "gp91phox ds", for gp91phox docking sequence [52]. They added a translocating TAT peptide (gp91ds-tat: RKKRRQRRR-CSTRIRRQL) to make it cell-permeable (see next section), and showed that it inhibited ROS production in neutrophils and mouse aorta, and also limited the increase in blood pressure elicited by an infusion of angiotensin II in mice [52]. Furthermore, gp91ds-tat inhibited ROS production in aged rats [41], lowered proatherogenic molecule levels in rats [53], and attenuated neurovascular dysfunction in aged mice [54]. It is noteworthy that this gp91phox/NOX2 sequence is conserved in NOX1 and NOX4, meaning that it is not specific for the phagocyte NADPH oxidase/NOX2 but could act as a NOX1, NOX2 and NOX4 inhibitor. However, this peptide inhibits NOX2 in cells, such as phagocytes, that express only this enzyme.

Park et al. showed that peptides corresponding to gp91phox amino acid residues 27–46, 87–100, 282–296, 304–321, 434–455 and 559–565 inhibited superoxide generation in a cell-free system with respective IC_{50} values of 34, 40, 30, 35, 25 and 53 μM [55]. A peptide corresponding to the predicted NADPH binding site in gp91phox also inhibited superoxide production in a cell-free system. The minimum sequence essential for this inhibitory effect consisted of amino acids 420–425 (KSVWYK) and has an IC_{50} of 30 μM [56]. Interestingly, unlike other peptides that inhibit NADPH oxidase, this peptide was effective even when added after system activation. Kao et al. identified amino acids 419–430 (ILKSVWYKYCNN) as a Rac interaction site within NOX2, and generated a cell-permeant peptide by adding a TAT sequence [57]. When used at 20 μM, this peptide inhibited ROS production in neutrophils stimulated by fMLF or PMA. This NOX2-Rac binding domain is conserved in NOX1 and NOX3 but not in NOX4, NOX5 or DUOXs, making it selective for NOX1, NOX2 and NOX3. Fig. 4 represents the amino acid sequence of gp91phox/NOX2 and indicates the locations of the major inhibitory peptides. The regions covered by these peptides have more than 60% homology with NOX1, NOX2, NOX4 and NOX5, meaning that these peptides might also affect these homologues. The reason for this lack of specificity is that these peptides were identified before the discovery of NOX homologues. However, there are still several other NOX2 specific sequences which could be tested in order to identify specific inhibitory peptides.

5.2.2. P22phox-derived inhibitory peptides

Nakanishi et al. first showed that the p22phox peptide AGGPPGGPQVNPIPVTDEVV (amino acids 175–194) inhibited superoxide production in a cell-free system, with an IC_{50} of 36 μM, and that it bound to p47phox [47]. A second peptide, corresponding to p22phox residues 82–95 (PFTRNYYVRAVLHL), was then found to inhibit superoxide production with an IC_{50} of 13 μM in a cell-free system consisting of solubilized neutrophil membranes and cytosol [58]. Using a "peptide-walking" technique, Pick's group [59], identified peptides located in p22phox domains corresponding to amino acids 9–23, 31–45, 47–61, 85–99 and 113–127, which inhibited NADPH oxidase activation in a cell-free system composed of macrophage membranes and recombinant proteins, with respective IC_{50} values of 5.7, 3.0, 4.6, 2.0 and 3.0 μM. As p22phox is a subunit common to all NOXs, p22phox-derived peptides are unlikely to specifically inhibit a particular type of NOX. Fig. 5 represents the amino acid sequence of p22phox and the locations of the major inhibitory peptides.

5.2.3. P47phox-derived inhibitory peptides

P47phox sequences have also been used to inhibit NADPH oxidase activation in a cell-free system or in electropermeabilized neutrophils. The p47phox peptide AYRRNSVRFL (amino acids 323–332) inhibits p47phox phosphorylation, p47phox translocation and NADPH oxidase activation in a cell-free system (IC_{50} = 54 μM) [60,61]. This peptide may compete either for p47phox phosphorylation, by acting as a pseudosubstrate for protein kinases, or for p47phox interaction with flavocytochrome b_{558} or p67phox [61]. A synthetic peptide comprising the amino acid sequence 323–332, corresponding to residues 314–331 (RSRKRLSQDAYRRNSVRF), also inhibits p47phox phosphorylation, p47phox translocation and NADPH oxidase activation in human neutrophils [62]. DeLeo et al.

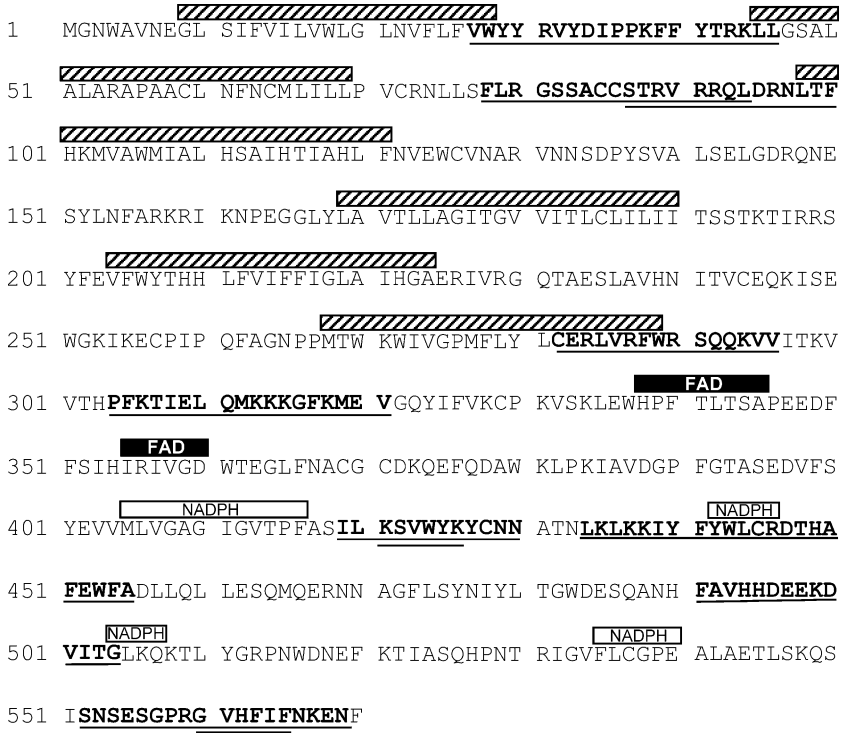

Fig. 4. Amino acid sequence of human gp91phox/NOX2 and sites of the major inhibitory peptides. Heavily hashed boxes represent predicted transmembrane alpha helices. Also indicated are putative FAD and NADPH binding sites. Bold and underlined sequences represent inhibitory peptides.

identified sites of p47phox interaction with flavocytochrome b_{558} and used the corresponding peptides to inhibit NADPH oxidase [61]. In addition to peptide 323–332, they found that peptides corresponding to amino acids 315–332 and 334–347 inhibited superoxide production in a cell-free system and in electropermeabilized neutrophils, with respective IC_{50} values of 15 and 18 μM. Morozov et al. used the peptide-walking technique to map p47phox functional domains involved in NADPH oxidase activation [63]. They showed that peptides corresponding to amino acids 5–19 (IC_{50} = 10.6 μM), 17–31 (IC_{50} = 8.8 μM), 105–119 (IC_{50} = 10.3 μM), 149–163 (IC_{50} = 10.9 μM), 193–207 (IC_{50} = 8.5 μM), 305–319 (IC_{50} = 11.5 μM) and 325–339 (IC_{50} = 3.4 μM) inhibited NADPH oxidase activation in a cell-free system. The most effective was the peptide corresponding to amino acids 325–339, which overlaps with the peptide described above by other investigators [60–62]. Three other inhibitory peptides are located in the PX domain (amino acids 5–19, 17–31 and 105–119), two in the SH3 domain (amino acids 149–163 and 193–207), and two in the carboxy-terminal phosphorylated domain (amino acids 305–319 and 325–339). P47phox is heavily phosphorylated in neutrophils, and this phosphorylation plays a major role in NADPH oxidase activation. Two peptides corresponding to p47phox phosphorylated sites (amino acids 301–320 and 314–335) inhibit NADPH oxidase activation (IC_{50} = 7 and 6.7 μM respectively) by inhibiting p47phox interaction with p22phox and its membrane translocation [64]. Among the p47phox phosphorylated sites, Ser345 plays a key role in NADPH oxidase hyperactivation or priming by pro-inflammatory cytokines such as TNFα and GM-CSF, and also in inflammatory diseases such as rheumatoid arthritis [18] and intestinal inflammation [65]. Interestingly, a TAT-peptide sequence containing Ser345 (amino acids 334–347) was shown to inhibit NADPH oxidase hyperactivation in human neutrophils induced by TNFα and GM-CSF, and hyperactivation of PMN from synovial fluid of RA patients, while preserving the physiological ability of bacterial N-formyl peptide to activate neutrophils [18]. Fig. 6 represents the sequence of p47phox and the sites of the major inhibitory peptides. Since the PX and SH3 domains are conserved between p47phox and NOXO1, peptides located in these domains are unlikely to be specific for NOX2. However, the AIR domain of p47phox (amino acids 286–340) is missing in NOXO1, and peptides derived from this sequence could therefore represent good candidates for developing very specific inhibitors of the phagocyte NADPH oxidase, the activity of which requires the organizer subunit p47phox.

5.2.4. Rac1-derived inhibitory peptides

The Rac1 peptide, corresponding to aa 178–188, inhibited NADPH oxidase activity in a cell-free system, with an IC_{50} of about 10 μM [66,67]. Interestingly, the corresponding peptide sequence of Rac2 (aa 178–188) had no effect. The Rac1 sequence could thus

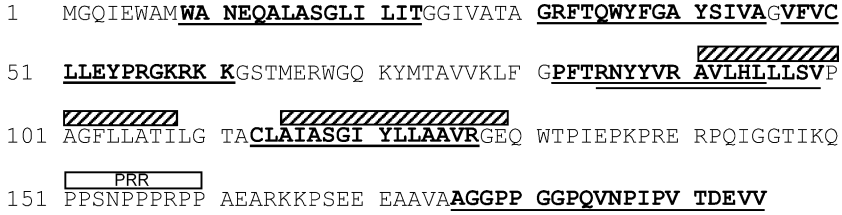

Fig. 5. Amino acid sequence of p22phox and sites of the major inhibitory peptides. Heavily hashed boxes represent predicted transmembrane alpha helices. Also indicated is the proline-rich region (PRR). Bold and underlined sequences represent inhibitory peptides.

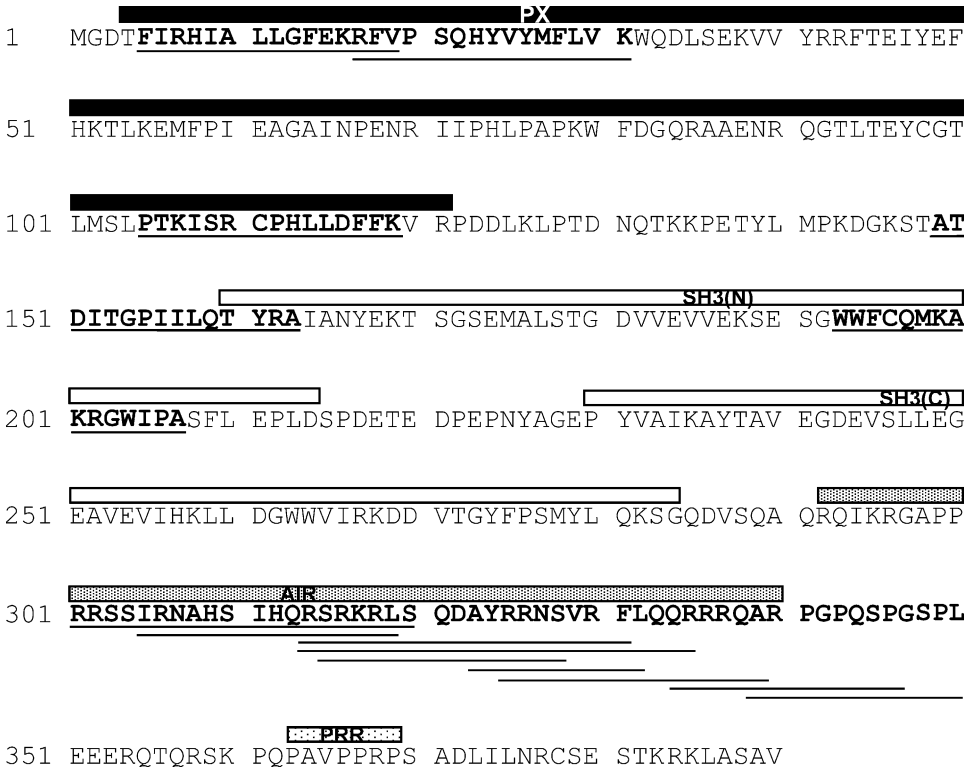

Fig. 6. Amino acid sequence of p47phox and sites of the major inhibitory peptides. Black boxes represent the phox homology domain (PX), white boxes the src homology domain 3 (SH3) domains, hashed boxes the auto-inhibitory region (AIR) and the proline-rich region (PRR). Bold and underlined sequences represent inhibitory peptides.

be used to study the specific role of Rac1. Mapping of Rac1 functional domains by peptide walking has identified several inhibitory peptides (residues 73–81, 103–107, 123–133, 163–169 and 183–188) [68]. Like p22phox, Rac1 is a subunit common to all NOXs, making it unlikely that Rac1-derived peptides would have a specific effect on a particular NOX.

5.2.5. P67phox- and p40phox-derived inhibitory peptides

To our knowledge, no peptide sequences derived from p67phox or p40phox have yet been shown to inhibit the phagocyte NADPH oxidase. One possible reason is that p67phox was long considered to be an unstable protein with only a minor function in NADPH oxidase activation. However, in 1998, Han et al. showed that p67phox has an activation domain (amino acids 199–210) [14], and Diekman et al. showed that it binds Rac during activation by its N-terminal region (amino acids 1–199) [69]. These two p67phox sequences might be used to develop new competitive inhibitory peptides. The reason for the lack of p40phox-derived peptides may be that p40phox is not required for NADPH oxidase activation in the cell-free system, making it more difficult to assess the effect of inhibitory peptides in intact cells.

5.2.6. Inhibitory peptides not derived from NADPH oxidase components

A proline- and arginine- (PR) rich antibacterial peptide designated PR-39 (because it contains 19 prolines and 10 arginines) inhibits NADPH oxidase activity (IC_{50} = 1 μM) by targeting the interaction of the p47phox SH3 domain with p22phox [70]. This peptide has protective effects in experimental myocardial ischemia-reperfusion [71]. Because SH3 domain/polyproline region interaction is involved in several protein/protein interactions, this peptide might inhibit processes other than NADPH oxidase activation.

A cell-permeable dominant negative peptide (DN-Ets-1-TAT) derived from Ets-1, a transcriptional regulator of p47phox, was found to lower angiotensin II-induced p47phox expression and ROS production *in vitro*, and also to attenuate medial hypertrophy of the thoracic aorta in mice [72]. This strategy targets p47phox expression and not NADPH oxidase activation. Inhibition of NADPH oxidase expression is not a suitable strategy for neutrophils, because p47phox is already expressed in mature neutrophils; however, this peptide might alter p47phox expression in monocytes, macrophages or other cells which continuously express p47phox.

Qin et al. showed that a tripeptide (GGF) contained in the dynorphin opioid peptide inhibited microglial NADPH oxidase at fentomolar concentrations and had neuroprotective and anti-inflammatory effects [73]. The mechanism of action of this peptide is not known.

6. Peptide delivery into living cells

The main obstacle to the use of peptides to inhibit NADPH oxidase in intact living cells is their inability to cross the plasma membrane lipid bilayer. The use of cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) is an attractive approach to this problem [74]. The most widely used CPPs are derived from the human immunodeficiency virus (HIV1) transactivator protein TAT, the *Drosophila* transcription factor antennapedia protein, and the herpes simplex virus (HSV) type 1 protein VP22 [75]. Although the mechanism of peptide transduction is controversial, these peptides can deliver biologically active proteins, DNA, RNA, liposomes and nanoparticles across plasma membranes [74,75]. Several NADPH oxidase peptide inhibitors have already been coupled to TAT peptide and have been shown to inhibit ROS production *in vitro* and *in vivo* [18,52–54,57,72].

7. Conclusion and future perspectives

NADPH oxidases/NOXs have essential roles in many physiological processes, including innate immunity, cell proliferation, gene

expression, blood pressure regulation, antigen presentation and intracellular signaling. However, excessive ROS production by NADPH oxidases, and especially by the phagocyte NADPH oxidase NOX2, has been implicated in many inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, acute respiratory distress syndrome, sepsis, diabetic complications, cardiovascular diseases, ischemic tissue injury and neurodegenerative diseases, in which ROS are believed to cause direct tissue insult. Thus, pharmacological targeting of the phagocyte NADPH oxidase/NOX2 might be beneficial in patients with these disorders. No specific chemical inhibitors for NOX2 have so far been identified, and peptide-based inhibitors appear to be the most promising candidates, provided the target sequence is specific for a particular phagocyte NADPH oxidase component. The best targets for NADPH oxidase inhibitors are gp91phox/NOX2, p47phox and p67phox, because their expression is more specific for phagocytes. This tends to rule out the use of peptides derived from p22phox and Rac1/2, which are components common to all NOXs.

The main problem with inhibitory peptides used to be their cell delivery, but this can now be achieved by coupling to cell-permeable peptides such as TAT or antennapedia peptides. Several cell-permeant peptide antagonists of NOX2 have already been shown to inhibit ROS production *in vitro* and *in vivo*, and have also served to identify new biological functions of the NADPH oxidase system. The other problem with peptides is their stability in live organisms. Interestingly, subcutaneous or intravenous infusion of NOX2-inhibitory peptides has been shown to attenuate vascular disorders in experimental animals. Oral administration is unlikely to provide efficient peptide delivery because of the hostile gastrointestinal environment. Finally, candidate inhibitors should be specific for the phagocyte NADPH oxidase in order to avoid cross-inhibition. The ideal NADPH oxidase inhibitor would prevent hyper-activity while preserving the physiological functions of this key element in host defenses, as described above for the peptide derived from Ser345-p47phox sequence. Drugs that prevent NADPH oxidase priming and ROS over-production would be particularly valuable.

Competing interest

The authors have no competing interests to declare.

Acknowledgements

This work was supported by grants from Agence Nationale de la Recherche (ANR), Arthritis Fondation Courtin, INSERM and CNRS.

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