HHS Public Access

Author manuscript

Asian J Androl. Author manuscript; available in PMC 2015 April 07.

Published in final edited form as:

Asian J Androl. 2008 January ; 10(1): 6–13. doi:10.1111/j.1745-7262.2008.00371.x.

NADPH oxidase: recent evidence for its role in erectile dysfunction

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Abstract

Important roles for reactive oxygen species (ROS) in physiology and pathophysiology have been increasingly recognized. Under normal conditions, ROS serve as signaling molecules in the regulation of cellular functions. However, enhanced ROS production as a result of the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase contributes significantly to the pathogeneses of vascular diseases. Although it has become evident that increased ROS is associated with erectile dysfunction (ED), the sources of ROS in the penis remain largely unknown. In recent years, emergent evidence suggests the possible role of NADPH oxidase in inducing ED. In this review, we examine the relationship between ROS and ED in different disease models and discuss the current evidence basis for NADPH oxidase-derived ROS in ED.

Keywords

reactive oxygen species; erectile function; superoxide; penis; nitric oxide

1 Introduction

Erectile dysfunction (ED) affects millions of men worldwide and reduces quality of life [1]. Although ED may result from psychological, neurological, and hormonal defects, vascular impairment accounts for a major portion of male ED [2, 3]. ED is often associated with chronic vascular diseases such as atherosclerosis, hypertension, and heart disease. Elucidation of erectile mechanisms has led to the discoveries of therapeutic targets, among which nitric oxide (NO) has been recognized as a critical molecule in erection physiology. Sexual stimuli induce the release of NO from penile nerve endings and endothelial cells, which in turn relaxes corpus cavernosal smooth muscle and increases blood flow to penis. Studies have shown that increased reactive oxygen species (ROS) reduce NO production or bioavailability, leading to impaired endothelial function and erectile function [4–7].

ROS include free radicals such as superoxide and hydroxyl radicals and non-radicals such as hydrogen peroxide. Abundant evidence has demonstrated the importance of ROS in physiologic functions and the pathogeneses of various diseases. ROS are produced either

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through non-enzymatic ways or through enzymatic systems such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, un-coupled endothelial NO synthase, cytochrome P450, and the mitochondria respiratory chain. Under normal conditions, cells are capable of self-protecting against the continuous formation of low levels of ROS via major intracellular antioxidant enzymes including superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase. For example, studies have shown that cavernous endothelial cells are able to detoxify ROS within a well-defined range using these enzymes [8]. These antioxidant enzymes are usually up-regulated as a compensatory mechanism when ROS production is increased [5, 9]. However, sustained high levels of ROS generation will diminish antioxidant enzyme activities and increase oxidative stress, leading to cell damage.

The effects of ROS on erectile function have been investigated with most of the emphasis placed on the activities of antioxidant enzymes. However, the sources of ROS remain largely unknown. It has become evident that NADPH oxidase is a major source for ROS formation in the vascular wall. In recent years, emergent evidence also suggests the possible role of NADPH oxidase in inducing ED. In the first part of this review, we will briefly examine the relationship between ROS and ED in different disease models. In the second part, we will focus on the basic molecular actions of NADPH oxidase and discuss the current knowledge of NADPH oxidase-derived ROS in ED.

2 ROS and ED

2.1 Hypertension

Most forms of hypertension are characterized by impaired endothelium-dependent vasodilation, which is partly due to an increase in ROS production. Results from clinical studies indicate the high prevalence of ED in hypertensive patients when compared to the general population [2, 10]. In experimental hypertensive animal models, ED has been demonstrated in both spontaneously hypertensive rats (SHR) and deoxycorticosterone acetate-salt hypertensive rats [11, 12]. The levels of thiobarbituric acid reactive substances (TBARS), an indicator for oxidative stress, were increased in SHR penes, leading to reduced NO-dependent relaxations of isolated cavernosal smooth muscle [6]. Diminished SOD activity was observed in SHR rat penes when compared to that of Wistar-Kyoto rats, which may be one of the causes for this increase in ROS production in SHR.

2.2 Diabetes

Diabetes mellitus is the most common risk factor for ED [13]. The risk of ED is three-fold greater for diabetic men and occurs at an earlier age than non-diabetic men. The link between ROS and diabetes-associated ED has been investigated. One possible source of ROS is from circulating monocytes. Morano *et al.* [14] reported that ROS generated by monocytes were significantly increased in patients with ED when compared to those of patients without ED. In streptozotocin (STZ)-induced type I diabetic rats, endothelial NO synthase and neuronal NO synthase-mediated relaxations of cavernosal muscle strips are reduced [4]. Treatment with the antioxidant, α-lipoic acid, partially restores the relaxations, suggesting increased ROS production in diabetic penes. Another group studied the effects of

ROS during the progression of diabetes. They found that increased ROS formation, indicated by increased TBARS levels and reduced glutathione levels, were associated with impaired ED in STZ-induced diabetic rats. More interestingly, oxidative stress is further increased in rats with long-term diabetes which is correlated with more severe ED when compared to rats with short-term diabetes [15]. Protein kinase C (PKC) is an important molecule that regulates a variety of cellular functions such as smooth muscle contraction [16]. Hyperglycemia increases ROS production and protein expression of PKC isoforms in cultured rat cavernosal smooth muscle cells [17]. The up-regulation of PKC is dependent on ROS because exposure of these cells with the antioxidant, vitamin E, prevents this increase. Taken together, these data suggest that ROS contribute significantly to diabetes-associated ED. This conclusion is further supported by the evidence that gene transfer of extracellular-SOD reduces superoxide production and restores erectile function in STZ-induced diabetic rats [18]. Data are lacking at present to suggest that ROS production contributes to ED in type 2 diabetes.

2.3 Hypercholesterolemia and hyperlipidemia

High levels of ROS were detected in cavernosal strips from hypercholesterolemic rabbits which is correlated with impaired endothelium-dependent relaxation but not endothelium-independent relaxation [5]. Increased ROS production may increase oxidized low density lipoprotein, leading to enhanced contractility of cavernosal strips [19]. Although it is not clear whether hypercholesterolemic rabbits have impaired erectile function *in vivo*, these data provide some evidence to support the clinical observations that hypercholesterolemia, hyperlipidemia and subsequently developed atherosclerosis are risk factors for ED [20].

2.4 Hyperhomocysteinemia

A high plasma concentration of homocysteine is an independent risk factor for the development of cardiovascular diseases such as atherosclerosis. Homocysteine-induced endothelial injury is associated with elevated ROS production [21–23]. The adverse effects of homocysteine were demonstrated in isolated rabbit cavernosal strips. Pre-incubation of cavernosal strips isolated from normal rabbits with homocysteine decreases endothelium-dependent relaxation responses, which are reversed by SOD or catalase [24]. Similarly, cavernosal strips isolated from hyperhomocysteinemic rabbits also display impaired endothelium-dependent relaxation along with increased ROS formation [25]. Copper exaggerated the ability of homocysteine to produce ROS through the Fenton reaction. Hyperhomocysteinemic rabbits having received penicillamine, a copper-chelator, have reduced superoxide generation in penes and better endothelium-dependent relaxation [25].

2.5 Aging

The prevalence of ED is significantly increased from 5% in men aged between 20 to 39 years to more than 70% in men aged 70 years and above [26]. Strong evidence has supported the concept that ROS are the key players of the aging process. Increased ROS levels were detected in aged Brown-Norway rat penes by lucigenin-enhanced chemiluminescence and dihydroethidium (DHE) [27]. Moreover, increased oxidative stress is implicated with a decreased ratio of the reduced form of glutathione versus oxidized

glutathione in aged penes [28]. It is associated with decreased SOD protein expression in penes from aged rats when compared to that of young rats [28]. *In vivo* adenoviral gene transfer of extracellular-SOD restores erectile responses to cavernous nerve and agonist-stimulation to the levels observed in young rats [27]. These data suggest that ROS play significant roles in the development of aging-associated ED.

2.6 Nerve injury

Nerve injury-related ED is common after radical prostatectomy. Using a unilateral cavernous nerve injury rat model, our group demonstrated that nerve injury induces oxidative stress in rat penile tissue indicated by increased nitrotyrosine staining [9]. The injury induces a rapid compensatory response in penile tissue by increasing glutathione peroxidase protein expression. However, this effect is not sustained in saline-treated nerve injured rats. Treatment with FK506, a nerve protecting agent, maintains the high levels of glutathione peroxidase expression and improves erectile function [9]. Consistently, studies from another group showed that increased oxidative stress and ED occurred within three weeks in the rats that had unilateral nerve dissection [29]. Resection of the cavernous nerve causes more severe injury, which leads to worse erectile function and greater ROS formation when compared to that of rats underwent nerve dissection [29].

3 NADPH oxidase

3.1 NADPH oxidase subunits

NADPH oxidase, a multicomponent enzyme, catalyzes the production of superoxide using an electron derived from NADPH. Superoxide is converted to hydrogen peroxide by SOD which subsequently leads to the formation of other ROS such as hydroxyl radicals.

NADPH oxidase was first identified in phagocytes. It consists of the catalytic subunit $gp91^{phox}$ (phox, phagocyte oxidase) and regulatory subunits $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ (Figure 1) [30, 31]. $gp91^{phox}$ contains a flavin adenine dinucleotide (FAD) and two heme groups and provides binding sites for NADPH and oxygen molecule. It exists together with $p22^{phox}$ as a stabilized membrane-bound complex called cytochrome b_{558} [32]. $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are located in the cytosol in the resting state. In addition, a small GTPase protein, Rac, participates in the assembly of the NADPH oxidase complex and is required for NADPH oxidase activation, Once stimulated, the cytosolic subunits migrate to the membrane, where they assemble with $gp91^{phox}$ and $p22^{phox}$ [33]. The active oxidase generates superoxide by transferring the electron from NADPH to oxygen.

3.2 Activation of NADPH oxidase

NADPH oxidase is activated by endothelin-1, angiotensin II (Ang II), thrombin, platelet-derived growth factor, tumor necrosis factor-α (TNF-α) and cytokines through receptor-mediated mechanisms [34–37]. Mechanical stress and phorbol-12-myristate-13-acetate (PMA) can also directly activate NADPH oxidase [38–40]. Phosphorylation of $p47^{phox}$ on several serine residues is a crucial step to initiate the translocation of cytosolic components to the membrane and activate NADPH oxidase [41], although some data suggest that only Ser379 is essential for oxidase activity and membrane association [42]. The phosphorylation

causes a conformational change of $p47^{phox}$ and enables the two SH3 (Src homology 3) domains of $p47^{phox}$ to bind to the prolinerich region on the carboxyl terminus of $p22^{phox}$ [43]. In addition, $p47^{phox}$ has a Phox-homology (PX) domain which binds to phosphoinositides on the membrane, contributing to membrane anchoring of $p47^{phox}$. $p47^{phox}$ facilitates the recruitment of $p67^{phox}$ to the membrane through tail to tail interactions between its proline-rich region and the SH3 domain of $p67^{phox}$. Therefore, $p47^{phox}$ functions as an organizer. $p67^{phox}$ is considered an activator because its main function is to transfer the electron from NADPH to FAD [44, 45]. This is a rate-limiting step in superoxide generation. The function of $p40^{phox}$ is less known and controversial. Some studies suggest that $p40^{phox}$ is not required for high level superoxide production or even down-regulates NADPH oxidase activity [46, 47]. In contrast, other reports indicate that $p40^{phox}$ enhances superoxide production through improving the efficiency of $p67^{phox}$ in the activation of NADPH oxidase [48, 49].

Activation of NADPH oxidase also requires a small GTP-binding protein-Rac. Rac is a member of the Rho family, and three isoforms of Rac have been identified: Rac1, Rac2 and Rac3. Rac1 and Rac3 are universally expressed except that Rac3 is not expressed in neutrophils. Rac2 is only expressed in hematopoietic cells. Rac functions as a molecular switch cycling between the GDP-bound inactive form and the GTP-bound active form. This process is tightly regulated by three proteins: GDP-dissociation inhibitor (GDI), Rho guanine exchange factor (RhoGEF), and GTPase activating protein (GAP). GDP-bound Rac is stabilized by GDI in the cytosol of the resting cells. During activation, RhoGEF facilitates the exchange of GTP for GDP, leading to the migration of Rac to the membrane along with the core cytosolic NADPH oxidase subunits. GAP inactivates Rac by increasing the intrinsic GTP hydrolytic activity of Rac. Studies have shown that $p67^{phox}$ has a tetratricopeptide repeat domain serving as a docking site specific for Rac [50].

3.3 Homologues of NADPH oxidase

Recently, $gp91^{phox}$ and its novel homologues were found in various types of cells other than phagocytes. These homologues are termed NOX (NADPH oxidase). NOX1, $gp91^{phox}$ (or NOX2), NOX3, NOX4 and NOX5 are expressed in smooth muscle cells, endothelial cells, fibroblasts and cancer cells [51, 52]. NOX1 through NOX4 have very similar structures. However, studies have shown that NOX4 activation requires only the presence of $p22^{phox}$ and not any cytosolic subunits [53]. Because NOX5 has an extra unique domain containing four binding sites for calcium, the regulation of NOX5 is dependent on intracellular calcium concentration and does not require any other subunits [54, 55].

In addition, homologues of $p47^{phox}$, NOX organizer 1 (NOXO1) and $p67^{phox}$, NOX activator 1 (NOXA1) were discovered in colon epithelial cells [56, 57]. Co-expression of NOXO1 and NOXA1 greatly increase the ability of NOX1 to generate superoxide.

3.4 Localization of NADPH oxidase

3.4.1 Endothelial cells—NOX2 and its regulators $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1 were all found in endothelial cells isolated from animals as well as human coronary artery and umbilical vein [58, 59]. In addition, NOX1, NOX3, and NOX4 were demonstrated in

mouse lung endothelial cells [60, 61]. Recently, NOX5 variants NOX5β, NOX5δ and NOX5S were identified in human microvascular endothelial cells [62].

3.4.2 Smooth muscle cells—In addition to NOX2, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, Rac1, and novel NOX family members were demonstrated in human and animal smooth muscle cells [63]. mRNA and protein expressions of NOX1 and NOX4 were detected in mouse lung smooth muscle cells, rat aortic smooth muscle cells and human aortic smooth muscle cells [64–66]. It is not known whether NOX3 and NOX5 are expressed in smooth muscle cells.

3.4.3 Fibroblast—NOX2, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ were detected in rat and rabbit aortic adventitial fibroblasts [67, 68]. NOX4 and NOX5 were predominantly expressed in human cardiac fibroblasts while NOX1 and NOX2 were barely detectable [69].

4 Physiologic role of NADPH oxidase

Phagocytes generate high levels of ROS to kill bacteria as a self-defense mechanism through activation of NADPH oxidase. In non-phagocytic cells, a small amount of NADPH oxidase-derived ROS is generated at physiologic levels, functioning as second messengers in response to cellular stimuli. ROS are involved in the regulation of cell growth and proliferation, cell migration, angiogenesis and apoptosis through activation of various signaling pathways. The actions of ROS on smooth muscle cell growth and proliferation in response to growth factors and hormones are mainly mediated by mitogen-activated protein kinases (MAPK) such as ERK1/2, p38MAPK, c-Jun N-terminal kinase [70, 71]. Protein kinase B (Akt) is another downstream molecular target of ROS. Hingtgen *et al.* [72] reported that ROS generated by NADPH oxidase activates Akt, which play a key role in Ang II-induced cardiomyocyte hypertrophy. NADPH oxidase also participates in cytoskeletal reorganization through interaction with the orphan adaptor TRAF4 and focal contact scaffold Hic-5 in lamellipodia at the leading edge of the cells, which is essential for cell migration [73, 74]. During the process of angiogenesis, NADPH-derived ROS not only up-regulate vascular endothelial growth factor (VEGF) expression, but also mediate VEGF induced phosphatidylinositol 3-kinase (PI3K)/ERK1/2 signaling cascade via VEGF receptor-2 [61, 75, 76]. In addition, NADPH oxidase participates in the control of cell cycle arrest and apoptosis via activation of cell cycle inhibitors $p21^{cip1}$ and p53 [77].

5 Pathophysiologic role of NADPH oxidase in ED

Lower levels of ROS are important in the regulation of physiologic function. However, excessive production of ROS may overwhelm the cellular antioxidant defense mechanisms, leading to pathologic changes observed in vascular diseases. ROS cause vascular damages by promoting smooth muscle cell growth and proliferation, increasing extracellular matrix protein deposition, activating matrix metalloproteinases, inducing endothelial dysfunction, and altering vascular tone. It has been well established that enhanced NADPH oxidase activity contributes to the development of atherosclerosis, hypertension, diabetes and hypercholesterolemia. The role of NADPH oxidase in ED is vastly understudied compared to other vascular diseases.

Although increased ROS production is linked to ED in different disease models, the sources of ROS have never been investigated *in vivo*. Some initial *in vitro* experiments provide indirect evidence for the possible involvement of NADPH oxidase in the development of ED. Greater superoxide production is detected in penile tissue isolated from hypercholesterolemic rabbits than that in control rabbit penes [78]. NADPH oxidase inhibitors apocynin and diphenyleneiodonium chloride (DPI) significantly reduce not only the basal production of superoxide in control rat penes but also that in hypercholesterolemic penes. Inhibitors of xanthine oxidase, NO synthase and the mitochondrial electron transport chain have no effect on superoxide formation. Apocynin and DPI also partially restore the impaired endothelium-dependent smooth muscle relaxation. These data suggest that NADPH oxidase-derived ROS are produced at low levels in normal penes and enhanced NADPH oxidase activity is responsible for ED in hypercholesterolemic rabbits. The phosphodiesterase 5 (PDE-5) inhibitor NCX 911 recovers cavernosal relaxation partly through inhibition of NADPH oxidase activity.

Another study demonstrated that the NADPH oxidase subunit $p47^{phox}$ is expressed in rabbit cavernosal smooth muscle cells [79]. Incubation with U46619, a thromboxane mimetic, induces ROS production along with increased $p47^{phox}$ protein expression. The PDE-5 inhibitor sildenafil reduces ROS in smooth cells by reducing NADPH oxidase subunit $p47^{phox}$ expression. Teixeira *et al.* [80] recently examined the role of NADPH oxidase in mouse penes. NADPH oxidase-dependent superoxide generation is significantly increased after incubating mouse cavernosal tissue with U46619 for 1 h or 8 h. This effect is reversed by the soluble guanylyl cyclase activator, BAY 41-2272, through decreasing protein expression of $p22^{phox}$ and $gp91^{phox}$. On the other hand, NADPH oxidase-derived ROS may regulate PDE-5 protein expression. TNF-α and nicotine stimulate ROS production and increase PDE5 protein expression in rabbit cavernosal smooth cells [81]. Apocynin, SOD, catalase as well as sildenafil reduce PDE-5 expression through inhibition of ROS formation [81]. These data suggest that NO signaling inhibits NADPH oxidase activity. Conversely, ROS generated from NADPH oxidase negatively regulates the NO pathway.

Although these *in vitro* studies suggest that NADPH oxidase activity is increased in disease states or can be stimulated by agonists, it remains unclear whether and to what extent NADPH oxidase functions *in vivo* in the penis or whether it plays an important role in the pathogenesis of ED. We recently performed a series of experiments designed to investigate the role of NADPH oxidase in the development of ED in hypertension [82]. Consistent with other studies showing that hypertensive rats exhibit ED [11, 12], in our Ang II-infused hypertensive rats (4 weeks infusion of subpressor doses of Ang II), erectile function was significantly impaired. ROS generation was significantly increased in penes isolated from Ang II-infused hypertensive rats accompanied by increased NADPH oxidase subunit $p47^{phox}$ protein expression. Chronic treatment with apocynin reduced NADPH oxidase protein expression and ROS levels in Ang II-infused hypertensive rat penes. Correspondingly, preserved erectile function in hypertensive rats by apocynin treatment provides further evidence that elevated NADPH oxidase activity is an important mechanism for hypertension-associated ED. Furthermore, we investigated the downstream targets of ROS. We found that increased ROS formation in Ang II-infused hypertensive rat penes led

to up-regulation of RhoA protein expression, a key component in the RhoA/Rho-kinase pathway of contractile mechanism in the penis. ROS also decreased endothelial NO synthase expression in hypertensive rat penes. Apocynin treatment reversed these changes to the levels similar to that of control penes (unpublished data, Jin *et al.*). This is the first study to our knowledge that demonstrated a relationship between NADPH oxidase-derived ROS and ED at an in vivo level.

6 Conclusion

Accumulating evidence has demonstrated the importance of NADPH oxidase-derived ROS in both physiologic and pathophysiologic processes. There is a dynamic balance between antioxidant systems and ROS generating systems within cells. Cells may be protected against the unfavorable effects caused by moderate increases in ROS production through compensatory mechanisms of up-regulated antioxidant enzyme activity. However, excessive production of ROS in response to pathogenic stimuli destroys these antioxidant mechanisms and causes cell damage. In recent years, the link between elevated ROS levels and ED has been established and therapeutic strategies have centered on restoring the antioxidant ability of cells in the penis. However, another important aspect is blockage of sources of ROS formation since ROS forms are extremely active and interchangeable. It may not be completely effective to simply scavenge one of the ROS. Therefore, it is crucial to investigate the mechanisms of ROS generation. Ours and other studies have demonstrated that NADPH oxidase represents a major source for ROS in the development of ED. Yet, further characterization of NADPH oxidase and molecular targets of ROS in penile tissue are essential to obtain a better understanding of the pathogenesis of ED and develop pharmacological and molecular interventions.

Acknowledgement

We acknowledge the support from the National Institute of Health (DK073531 to Liming Jin and DK67223 to Arthur L. Burnett) and American Heart Association (0530007N to Liming Jin).

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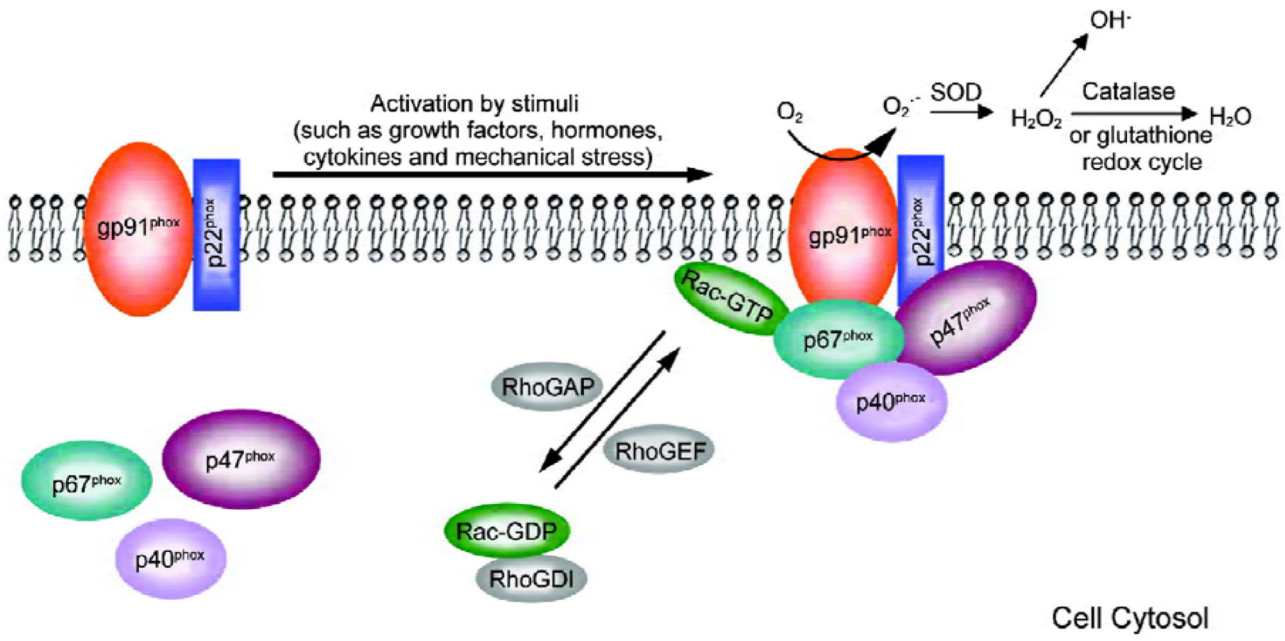

Figure 1.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation. In the resting state, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ are located in the cytosol while $p22^{phox}$ and $gp91^{phox}$ form a cytochrome b_{558} complex on the membrane. Rac-GDP is stabilized by GDI in the cytosol. Following stimulation, the cytosolic subunits migrate to the membrane, where they assemble with $gp91^{phox}$ and $p22^{phox}$. After RhoGEF facilitates the exchange of GTP for GDP, Rac-GTP binds to $p67^{phox}$, helping to activate the NADPH oxidase and generate superoxide ($O_2\cdot^-$). $O_2\cdot^-$ is catalyzed to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), and H_2O_2 subsequently either is converted to H_2O by catalase or becomes hydroxyl radicals. In addition, H_2O_2 is degraded via glutathione redox cycle catalyzed by glutathione peroxidase and glutathione reductase. RhoGDI, Rho GDP-dissociation inhibitor; RhoGEF, Rho guanine exchange factor; RhoGAP, Rho GTPase activating protein.