

REVIEW

NADPH oxidase(s): new source(s) of reactive oxygen species in the vascular system?

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Reactive oxygen species play an important role in a variety of (patho)physiological vascular processes. Recent publications have produced evidence of a role for putative non-phagocyte NADP oxidase(s) in the vascular production of reactive oxygen species. In the present review, we discuss the detection of the different components of NADP oxidase(s) in the vascular system, together with the putative role of reactive oxygen species produced by vascular NADPH oxidase(s), in both in vitro and in vivo studies.

dase. Upon activation, the NADPH oxidase catalyses the one electron reduction of oxygen, using NADPH as the electron donor¹:



The superoxide anion radicals (O_2^-) generated in this way play a prominent role in oxygen dependent bacterial killing. O_2^- can be dismutated to hydrogen peroxide (H_2O_2), either spontaneously or by the antioxidant enzyme superoxide dismutase, and H_2O_2 may subsequently be converted into a variety of active oxygen species, such as singlet oxygen and hydroxyl radicals.² The NADPH oxidase of phagocytes is composed of at least five protein subunits. Gp91phox and p22phox are transmembrane proteins that need each other to form a stable flavocytochrome b_{558} .³ Three other components, p47phox, p67phox, and p40phox are localised in the cytosol⁴ of resting cells and, upon stimulation of the cells by various agents, these components translocate to the flavocytochrome to form the active enzymatic NADPH oxidase complex⁵ (fig 1). In addition to these subunits, the regulatory low molecular weight GTPases, Rac1 (in macrophages),⁶ Rac2 (in neutrophils),⁷ and Rap1A,⁸ participate in the assembly of the active complex.

The NADPH oxidase of phagocytic leucocytes is the source of the reactive oxygen species (ROS) that these cells need to kill invading pathogens. In recent years, numerous reports have produced evidence of a role for putative non-phagocyte NADPH oxidase(s) in the vascular production of ROS, because several of the components of the phagocyte NADPH oxidase have been detected in vascular tissue of several animal species, including humans. However, these studies suggest distinct differences in amino acid sequence (and antibody reactivity) between the central subunits of the phagocyte and non-phagocyte NADPH oxidase(s). ROS produced by these putative non-phagocyte NADPH oxidase(s) are involved in various (patho)physiological responses of vascular cells, such as mitosis, apoptosis, migration, hypertrophy, and modification of the extracellular matrix. Accordingly, ROS have been implicated in several major intracellular signalling pathways leading to changes in gene transcription and protein synthesis. Therefore, it is not surprising that ROS are thought to play an important role in disease processes, such as atherosclerosis and tumour progression, among others. In this review, we will discuss the evidence for a role of NADPH oxidase(s) in various (patho)physiological processes of the vascular system.

“Reactive oxygen species have been implicated in several major intracellular signalling pathways leading to changes in gene transcription and protein synthesis”

THE NADPH OXIDASE OF PHAGOCYtic LEUCOCYTES

NADPH oxidase has been studied extensively in phagocytes (neutrophilic and eosinophilic granulocytes, monocytes, and macrophages). Exposure of these cells to any of a large number of stimuli activates a respiratory burst, caused by an activation of the plasma membrane bound NADPH oxi-

NON-PHAGOCYTE, VASCULAR NADPH OXIDASE(S)

NADPH oxidase components have also been detected in non-phagocytic cells. However, the catalytic subunit of the non-phagocyte NADPH oxidase(s) appears to be genetically and structurally distinct from its phagocyte counterpart gp91phox. This has been demonstrated—for example, in patients with the X linked form of the immunodeficiency syndrome chronic granulomatous disease (CGD),⁹ which is characterised by the lack of or strongly reduced NADPH oxidase activity in phagocytes caused by an absent or defective gp91phox protein.^{10 11} However, fibroblasts of such patients contain a spectroscopically normal cytochrome b_{558} content compared with control fibroblasts and show a comparable O_2^- production.⁹

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Abbreviations: Ang, angiotensin; CAD, coronary artery disease; CGD, chronic granulomatous disease; DPI, diphenylene iodonium; ER, endoplasmic reticulum; HUVEC, human umbilical vein endothelial cell; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; RT-PCR, reverse transcriptase polymerase chain reaction; SMC, smooth muscle cell; TNF- α , tumour necrosis factor α ; VSMC, vascular smooth muscle cell

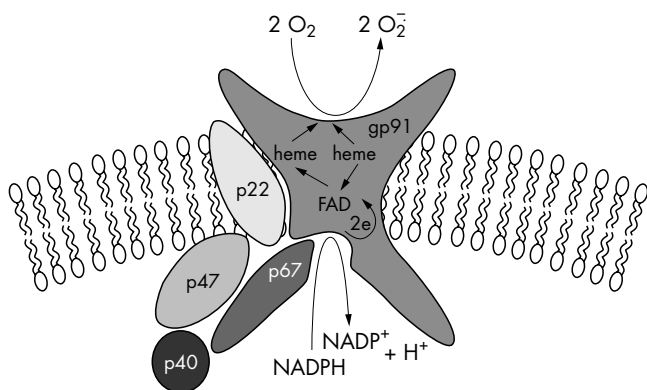


Figure 1 Composition of the NADPH oxidase in phagocytic cells.

In recent years, the expression of components of the NADPH oxidase in the vascular system has been studied by various methods (tables 1 and 2).

DETECTION OF P22PHOX IN BLOOD VESSELS

Endothelial cells

Animal

p22phox mRNA and protein have been detected in rat non-atherosclerotic coronary microvascular endothelial cells with the reverse transcriptase polymerase chain reaction (RT-PCR),^{12, 13} cDNA sequence analysis,¹² northern blotting,¹² and western blot analysis.^{12, 13}

In these cells, p22phox was also detected with confocal immunofluorescence microscopy in perinuclear regions, in the vicinity of the endoplasmic reticulum (ER) and towards the cell membrane.¹² Non-atherosclerotic porcine pulmonary artery endothelial cells have been shown to express p22phox by immunohistochemistry and RT-PCR.¹⁴

Human

In humans, p22phox expression has been demonstrated in atherosclerotic coronary artery endothelial cells by means of immunohistochemistry and western blotting.²⁶ p22phox expression was also detected by northern blotting,²³ RT-PCR,²³⁻²⁵ cDNA library screening,²⁴ and western blotting in the membrane fraction of the endothelium^{24, 25} in humans. The same cells have also been reported to express membrane associated flavocytochrome b₅₅₈.²⁵ In contrast, heme spectroscopy failed to detect the flavocytochrome b₅₅₈ in these cells.²³

Vascular smooth muscle cells (VSMCs)

Animal

p22phox expression has been demonstrated in non-atherosclerotic rat thoracic aorta VSMCs by RT-PCR,¹⁵ northern blot analysis,¹⁵⁻¹⁷ and sequencing of p22phox cDNA.¹⁵ Furthermore, p22phox expression was suggested by downregulation after transfection with antisense p22phox oligonucleotides in human umbilical vein endothelial cells (HUVECs).¹⁶ In addition, a cDNA for flavocytochrome b₅₅₈ (composed of p22phox and gp91phox) has been isolated from rat VSMCs; this molecule shows a substantial resemblance to both the mouse and the human amino acid sequence and nucleotide sequence, respectively.¹⁵ In contrast, in another study, the presence of p22phox could not be demonstrated in the non-atherosclerotic rat thoracic aortic medial layer by immunohistochemical staining, which exclusively showed adventitial expression of p22phox.¹⁸

Human

In humans, p22phox expression has been detected in atherosclerotic coronary artery VSMCs by means of immunohistochemistry, western blot analysis,²⁶ and RT-PCR, and with northern blotting in non-atherosclerotic aortic smooth muscle cells (SMCs).²⁷ In addition, the expression of p22phox was suggested from antisense p22phox transfection experiments in VSMCs²⁷ and in mesenteric artery SMCs.¹⁶ Western blot analysis pointed to the expression of p22phox at the plasma membrane of non-atherosclerotic aortic SMCs.²⁴

Adventitial fibroblasts

Animal

Adventitial localisation of fibroblastic p22phox expression seems to predominate, as demonstrated by immunohistochemical staining in non-atherosclerotic rat thoracic aorta adventitial fibroblasts¹⁸ and rabbit aorta adventitial fibroblasts.¹⁹

Human

Immunohistochemical staining and western blotting also showed primarily adventitial expression of p22phox in human coronary artery fibroblasts, which increased in atherosclerotic coronary artery lesions, although at the same time extending throughout the entire vessel wall.^{26, 28}

DETECTION OF GP91PHOX IN BLOOD VESSELS

Endothelial cells

Animal

RT-PCR and western blot analysis have demonstrated the expression of gp91phox in rat non-atherosclerotic coronary

Table 1 NADPH subunits detected by various molecular biological techniques in animal experiments

Subunit	Cell type	Western blotting	Northern blotting	RT-PCR	ICH	DNA sequencing	Anti-sense	Refs
p22phox	ec	r +	r +	r, p +	r, p +	r +		12-14
	vsmc		r +	r +	r +	r +	r +	15-18
gp91phox	fb				r -			18
	ec	r +		r, p +	r, rb +	r +		18, 19
	vsmc	c +	r -		r, p +			12-14
p47phox	fb				r, rb +			12
	ec	b +			p, b +			20
p67phox	vsmc				r, rb +			18, 19
	fb				p +			14, 21
p40	ec							18, 19
	vsmc		rb +	rb +	r, rb +			14
	fb							18, 19, 22

Cell type: ec, endothelial cell; fb, fibroblast; vsmc, vascular smooth muscle cell. Animal species: b, bovine; c, calf; m, mouse; p, porcine; r, rat; rb, rabbit; +, present; -, absent. ICH, immunohistochemistry; RT-PCR, reverse transcription polymerase chain reaction.

Table 2 NADPH subunits detected by various techniques in human experiments

Subunit	Cell type	Western blotting	Northern blotting	RT-PCR	ICH	DNA sequencing	Spectroscopy	Anti-sense	Refs
p22phox	huvec	+	+	+		+			23–25
	ca ec	+			+		–		23
	vsmc	+	+	+	+			+	26
	fb	+			+				16, 26, 27
gp91phox	huvec	+		+					23, 28
	huvec	+					–		23–25
	vsmc	–		–					23
p47phox	fb								24
	huvec	+		+	+				23, 25
	vsmc	+		+					29
p67phox	fb								
	huvec	+		+	+				23, 25
	vsmc	–		–					29
fb									

Cell type: ca ec, coronary artery endothelial cell; fb, fibroblast; huvec, human umbilical vein endothelial cell; vsmc, vascular smooth muscle cell. ICH, immunohistochemistry; RT-PCR, reverse transcription polymerase chain reaction.

microvascular endothelial cells.¹³ Another study reported the expression of gp91phox in non-atherosclerotic rat coronary endothelial cells by RT-PCR and consecutive cDNA sequence analysis, but failed to do so by northern blot analysis.¹² Interestingly, using confocal immunofluorescence microscopy the same study demonstrated gp91phox expression extending from perinuclear areas and in the vicinity of the ER towards the cell membrane.¹² In non-atherosclerotic porcine pulmonary artery endothelial cells gp91phox expression was detected by immunohistochemistry and RT-PCR.¹⁴

Human

RT-PCR^{23–25} and western blotting²⁵ have demonstrated gp91phox mRNA and protein expression in HUVEC lines.^{23–25} In contrast, the gp91phox component of the flavocytochrome b₅₅₈ could not be demonstrated by heme spectroscopy in HUVECs.²³

Vascular smooth muscle cells

Animal

Western blot analysis indicated gp91phox expression in non-atherosclerotic calf pulmonary artery VSMCs.²⁰

Human

Görlach *et al* were unable to detect gp91phox mRNA or protein expression by means of RT-PCR or western blotting in human non-atherosclerotic aortic SMCs.²⁴

Adventitial fibroblasts: animal

Adventitial fibroblasts in the non-atherosclerotic rat thoracic aorta¹⁸ and rabbit aorta¹⁹ were shown to express gp91phox as detected by immunohistochemical staining.

DETECTION OF P47PHOX IN BLOOD VESSELS

Endothelial cells

Animal

Immunohistochemistry^{14, 21} and western blotting²¹ have demonstrated p47phox expression in non-atherosclerotic porcine pulmonary artery endothelial cells¹⁴ and bovine pulmonary artery endothelial cells.²¹

Human

In HUVECs, p47phox mRNA expression was demonstrated by means of RT-PCR^{23, 25} and western blotting,²⁵ and p47phox was localised in the cytosol of these cells by means of immunohistochemistry.²³

Vascular smooth muscle cells: human

RT-PCR and western blotting demonstrated p47phox mRNA and protein expression in non-atherosclerotic human aortic SMCs.²⁹

Adventitial fibroblasts: animal

Immunohistochemical staining showed p47phox expression in non-atherosclerotic aortic adventitial fibroblasts of rats¹⁸ and rabbits.¹⁹

DETECTION OF P67PHOX IN BLOOD VESSELS

Endothelial cells

Animal

Porcine non-atherosclerotic pulmonary artery endothelial cells were shown by immunohistochemistry to express p67phox.¹⁴

Human

Immunohistochemistry,²³ RT-PCR,^{23, 25} and western blotting²⁵ have demonstrated p67phox mRNA and protein expression in HUVECs.^{23, 25}

Vascular smooth muscle cells: human

Neither RT-PCR nor western blot analysis could detect p67phox protein expression in non-atherosclerotic human aortic smooth muscle cells.²⁹

Adventitial fibroblasts: Animal

p67phox expression was detected by RT-PCR,²² northern blot analysis,²² and immunohistochemical staining in non-atherosclerotic rat^{18, 19} and rabbit aortic adventitial fibroblasts.²²

In summary, most, but not all of the phagocyte NADPH oxidase components have been found in the various cell types composing the atherosclerotic and non-atherosclerotic vasculature. However, when interpreting these sometimes conflicting reports, the lack of appropriate negative controls—material from patients with CGD or relevant knockout mice—has to be noted in most of these studies. Particularly for the central subunit of the NADPH oxidase, gp91phox, the possibility of crossreactivity of oligonucleotides or antibodies with one or more of the gp91phox homologues (see below) has to be taken into account. So far, to our knowledge, no extraphagocyte deficiencies of NADPH oxidase components in patients with CGD have been described.²⁴

GP91PHOX HOMOLOGUES

Over the past two years several novel gp91phox homologues have been described, which will be briefly mentioned in the

following section, with the nomenclature recently accepted by the HUGO Human Gene Nomenclature Committee (<http://www.gene.ucl.ac.uk/nomenclature/>). The names in parentheses indicate various names used in the original descriptions.

Nox2 (gp91phox)

The prototype (and for a long time the only) member of the new family of NADPH oxidases, gp91phox, is a 91 kDa glycosylated protein with six hydrophobic, probably membrane spanning, segments in its N-terminal half. Four histidine residues in this transmembrane cluster have been shown to participate in the ligation of two hemes. The cytosolic C-terminal half of the protein contains FAD and NADPH binding sites homologous to those found in some other flavoproteins; it also encompasses as yet poorly defined regions of interaction with the cytosolic oxidase components p47phox and p67phox.³

All the gp91phox homologues described so far have conserved the overall structure of six transmembrane segments with heme coordinating histidines, followed by a cytosolic part that contains highly conserved binding sites for FAD and NADPH. Although the homologues share other regions of high homology, nothing can yet be said about possible interactions with the other subunits of the phagocytic NADPH oxidase.

Nox1 (Mox-1, NOH-1)

Mox-1 mRNA is expressed in colon, prostate, uterus, and vascular smooth muscle cells, but was not found in peripheral blood leucocytes.³⁰ Interestingly, and in contrast to neutrophils, ROS produced by mox-1 seem to be generated intracellularly. Furthermore, overexpression of mox-1 resulted in increased cell growth, suggesting a possible role for mox-1 in growth regulation.³⁰

Nox3 (gp91-3)

To date, nothing is known about this homologue except for a seemingly exclusive expression in fetal kidney tissue (and the HepG2 cell line), as determined by RT-PCR.³¹

Nox4 (renox, Kox-1)

Another gp91phox homologue, Nox4, has been detected by *in situ* RNA hybridisation and by immunohistochemistry in the renal cortex, predominantly in proximal convoluted tubule epithelial cells, which is also the putative site of erythropoietin production.³² In addition, human embryonic kidney 293 cells appear to express Nox4 mRNA and protein as detected by RT-PCR and western blot analysis.³³ Based on the expression pattern it has been suggested that renox participates in the oxygen sensing mechanism that regulates the production of erythropoietin. Transfection of renox cDNA into NIH 3T3 fibroblasts resulted in enhanced superoxide production compared with control fibroblasts. However, in contrast to the results seen with mox-1, these modified fibroblasts displayed substantially diminished cell growth.^{32 33}

Nox5

Recently, the sequence of this homologue, reportedly expressed in renal epithelial cells, was deposited in GenBank (AK026011 and AF317889).

Duox1 and Duox2 (ThOX1 and ThOX2/p138^{Tox})

Discovered by low stringency screening of thyroid cell cDNA libraries with a gp91phox probe, ThOX1 and ThOX2 represent a separate subgroup in the family of gp91phox homologues.³⁴ Also called Duox1 and Duox2 (for dual oxidases 1 and 2), they are characterised by an N-terminal extension that contains a peroxidase homology domain, a conserved calcium binding motif, and an additional transmembrane segment. Expressed specifically in the thyroid gland, at the apical membrane of thyrocytes, the two oxidases are thought to be involved in the biosynthesis of thyroid hormone.³⁴

In summary, the discovery of the above described non-phagocyte gp91phox homologues represents the first confirmation of earlier research in different tissues that had postulated a role for such homologues in a variety of signalling pathways. Future developments, which may include the description of additional, tissue specific oxidases, will need to elucidate the regulation and the role of these enzymes that are thought to be central to a new, redox dependent signalling mechanism.

ROLES OF ROS PRODUCED BY VASCULAR NADPH OXIDASE(S)

The search outlined above for NADPH oxidase components in vascular tissues and for gp91phox homologues has been stimulated by the realisation that ROS are involved in several important processes of vascular functioning. Atherosclerosis, in particular, seems to be at the crossroads of a variety of ROS mediated pathophysiological signalling routes, such as SMC proliferation, the induction of early growth response gene transcription, the induction of adhesion molecule expression, and the consecutive activation of inflammatory response mechanisms. Although much of the evidence presented so far has been circumstantial, the discovery of key players, such as the novel gp91phox homologues, should accelerate the elucidation of these essential signalling networks. In the following section we will present an overview of the current knowledge concerning the role of NADPH oxidase generated ROS in the (patho)physiology of the vasculature. Several components of the NADPH oxidase(s) appear to be associated with important determinants of vascular disease progression, such as mechanical stress, inflammatory mediators, hypertension, adhesion molecules, and thrombosis. The involvement of these components in vascular disease, as studied in animal and human *in vitro* and *in vivo* experiments, will be reviewed separately.

Vascular NADPH oxidase produced ROS: animal *in vitro* studies

Phorbol-12-myristate-13-acetate (PMA)

PMA, which is known to activate the NADPH oxidase in phagocytes,³⁵ and NADPH administration resulted in increased ROS production in intact rat aortic segments, whereas only NADPH administration was able to enhance ROS production in mechanically denuded rat aortic endothelium.²⁴ In porcine pulmonary artery endothelial cells, lucigenin chemiluminescence demonstrated increased ROS production upon PMA treatment, which was inhibited by the non-specific NADPH oxidase inhibitor diphenylene iodonium (DPI).¹⁴ In accordance with this observation, chemiluminescence showed that aortic endothelial segments of gp91phox deficient mice exhibited an attenuated PMA induced NADPH dependent ROS production, whereas wild-type mice showed normal PMA induced increases in superoxide generation.²⁴

In contrast, porcine coronary SMCs were shown to be inert to PMA stimulation, because superoxide values remained indifferent, in agreement with the lack of NADPH oxidase components in VSMCs.²⁴

Mechanical stress

Flow adapted and shear stress exposed bovine pulmonary artery endothelial cells exhibited an increased ROS production compared with continuously perfused cells. This increase was inhibited by DPI, indicating that an NADPH oxidase may be activated by the shear stress.³⁶

Inflammatory mediators

Cytokines have been shown to upregulate ROS production in vascular tissue. In particular, tumour necrosis factor α (TNF- α) has been suggested to stimulate NADPH oxidase dependent superoxide production, because p22phox becomes

upregulated upon TNF- α administration in cultured rat hypertensive aortic VSMCs.³⁷ Interestingly, transfection with antisense p22phox nucleotides in rat aorta VSMCs abolished TNF- α induced superoxide production, implicating a p22phox based non-phagocyte NADPH oxidase as the source of ROS formation.³⁷ Lipopolysaccharide (LPS) stimulates ROS production in rat aortic rings.³⁸ This production could be inhibited by the non-specific NADPH oxidase inhibitor DPI.³⁸ Furthermore, LPS induced an increase in the expression of p67phox and of an approximately 75 kDa protein in rat aortic rings, as detected by western blotting with antibodies directed against the human p67phox and gp91phox proteins, respectively. In contrast, northern blotting showed increased p22phox mRNA upon LPS administration.³⁸

Angiotensin II

Angiotensin II (Ang II) plays an important role in the development of hypertension. It has been shown that upon Ang II administration superoxide values rise in rat aortic SMCs^{39,40} and throughout the rabbit aortic vascular wall (especially in endothelium and adventitia),⁴¹ and that this rise of superoxide values can be inhibited by DPI. It has been suggested that adventitial superoxide can inactivate endothelium derived nitric oxide, which enables the adventitia to play a role in the regulation of smooth muscle tone and in hypertension.^{19,28,42} Results of several groups indicate that this Ang II induced superoxide production is mediated by an NADPH oxidase,^{19,43} among other mechanisms by upregulation of the mRNA expression of the NADPH oxidase component p67phox.²⁹ In addition, transfection with antisense p22phox nucleotides in rat aortic SMCs inhibited Ang II induced superoxide generation.¹⁷

Ischaemia

Ischaemia of bovine pulmonary artery endothelial cells led to the generation of ROS and NF- κ B and AP-1 induced DNA synthesis, as detected by means of electrophoretic mobility shift assays. Both effects were inhibited by the addition of DPI.³⁶

In summary, most of the studies mentioned above have used pharmacological inhibitors of ROS production to show NADPH oxidase involvement. However, these compounds are not specific for NADPH oxidases,⁴⁴ so definitive proof of NADPH oxidase mediated ROS production is still lacking. Nonetheless, transfection studies with antisense oligonucleotides specific for p22phox have shown a reduced superoxide production, implicating p22phox and, by extrapolation, an NADPH oxidase in ROS mediated processes in the vasculature.¹⁷

Vascular NADPH oxidase produced ROS in human in vitro studies

PMA

Apocynin, DPI, and other non-specific inhibitors attenuated ROS production in HUVEC cultures pretreated with PMA.⁴⁴

Mechanical stress

Short term stretch increased superoxide generation in a DPI inhibitable manner in human aortic endothelial cells⁴⁵ and in human coronary artery SMCs.⁴⁶

Inflammatory mediators

In HUVECs, TNF- α stimulated ROS production,⁴⁷ and DPI was shown to inhibit this reaction.⁴⁸ The pro-atherosclerotic agent endothelin 1 upregulated gp91phox mRNA expression and superoxide production in HUVECs.⁴⁹

Platelet derived growth factor⁵⁰ increased ROS production and, consecutively, upregulated various intracellular second messengers^{47,48} and intercellular adhesion molecules, such as vascular cell adhesion molecule 1, in human endothelial

cells.^{47,48} This upregulation was inhibited by DPI and apocynin.^{47,48,50} In HUVECs, LPS and PMA induced E-selectin mRNA expression, as estimated by northern blot analysis, was inhibited by apocynin treatment, again suggesting a role of an NADPH oxidase in this process.⁵¹

Bradykinin was shown to induce the generation of ROS, phosphorylation of cytosolic mitogen activated protein kinases p42/p44, and c-fos mRNA expression in aortic VSMCs in a DPI and *N*-acetylcysteine inhibitable manner.⁵²

Angiotensin II

Administering Ang II to human vascular endothelial cells resulted in a rise in superoxide concentration as detected by lucigenin enhanced chemiluminescence.⁵³

Coagulation factors

The administration of thrombin, which is known to induce VSMC proliferation, and is therefore probably involved in atherogenesis, to human aortic SMCs, resulted in p47phox mRNA and protein upregulation and increased DPI inhibitable ROS production.²⁹ In HUVECs, thrombin induced apocynin inhibitable ROS production.⁵⁴ Exposure of aortic SMCs and mesenteric SMCs to products released from activated human platelets resulted in enhanced p22phox expression and intracellular ROS production, in addition to upregulation of tissue factor mRNA expression in a DPI inhibitable manner.¹⁶ Tissue factor is a major activator of the coagulation cascade leading to the generation of thrombin.

In summary, there is an increasing amount of evidence, circumstantial at the moment, for an important role of NADPH oxidase(s) in the human vasculature.

Vascular NADPH oxidase produced ROS: animal in vivo studies

Although in vivo experiments have been performed on a very limited scale so far, this experimental approach is of vital importance in investigating the influence of NADPH oxidase dependent ROS production on vascular disease. RT-PCR indicated increased p22phox mRNA expression in aortic medial VSMCs,⁵⁵ in addition to adventitial smooth muscle tissue⁵⁶ of hypertensive rats. In wild-type and gp91phox knockout mice, no differences in plasma lipid values and atherosclerotic lesion size were found after a high fat diet of 20 weeks. The authors concluded that the phagocyte NADPH oxidase does not contribute greatly to the aetiology of atherosclerosis.⁵⁷ Immunohistochemistry in male Sprague Dawley rats demonstrated increased p47phox expression throughout the rat aortic vascular wall following catheter induced injury, whereas uninjured arteries showed only adventitial and low level medial VSMC p47phox expression.²⁹ However, in p47phox knockout mice, no differences were found in atherosclerotic lesion size and basal blood pressure compared with a p47phox positive control group, although vascular superoxide generation was decreased by 50% in aortic rings from the knockout mice.⁵⁸

Clearly, this experimental approach is just beginning to unravel the functional contribution of gp91phox and its homologues to vascular pathophysiology.

Vascular NADPH oxidase produced ROS: human in vivo studies

Studies on the role of ROS produced by the vascular NADPH oxidase(s) in humans are restricted to mutational analysis or polymorphism studies; that is, the analysis of the effects of mutations or polymorphisms in one of the oxidase components on the phenotype of the affected individuals. So far, only patients with CGD (individuals with defects in one of the phagocyte NADPH oxidase components) have been the subject of mutational analysis. No vascular deficiencies have been described in these patients.

In recent years, several studies have investigated the possible effects of polymorphisms of p22phox on the vasculature. Four different polymorphisms have so far been reported,⁵⁹ and two of these, C242T and A640G, have been analysed as to their role in atherogenesis.

“Studies on the role of reactive oxygen species produced by the vascular NADPH oxidase(s) in humans are restricted to mutational analysis or polymorphism studies”

The C242T polymorphism results in a His72 → Tyr substitution in the p22phox protein. Cahilly *et al* found that the homozygous T genotype and the TC genotype of the C242T polymorphism of p22phox are associated with a greater loss in mean minimum lumen diameter, increased progression of coronary artery disease (CAD), and less regression of the disease under treatment as assessed by coronary angiography than the homozygous C genotype.⁶⁰ Cai *et al* showed in a white Australian population that the TC and TT genotypes of the C242T polymorphism were more abundant in young male patients with CAD (≤ 45 years old), whereas an association of the C242T polymorphism with CAD could not be established in the total patient population.⁶¹ In contrast to these results, Inoue *et al* showed that in a Japanese population, the TC + TT genotypes of the C242T polymorphism were found significantly more frequent in control subjects than in patients with CAD,⁶² and a study of an American population of 252 patients undergoing angiography for the diagnosis of CAD found no significant difference in the prevalence of the C242T allele between controls and patients with CAD.⁶³ A combined biracial study of Asian Indians and Chinese from Singapore did not reveal an increased allelic prevalence in either the control group or the patients with CAD.⁶⁴ However, a study of 2205 male white Europeans, although not detecting an association of the C242T polymorphism with CAD, found a clear association of the A640G polymorphism with CAD, the AA genotype being found preferentially in patients with CAD.⁶⁵ This is again in contrast to the findings of Inoue *et al* in a Japanese population, which showed an equal allele distribution of the A640G polymorphism among controls and patients with CAD.⁶² In another study, patients with cerebrovascular disease were shown to express the TC and TT genotypes of the C242T polymorphism more frequently than controls, suggesting that p22phox polymorphism possibly constitutes a risk factor for cerebrovascular disease.⁶⁶

DISCUSSION

Although there can be no doubt about the important contribution of ROS to a variety of (patho)physiological vascular processes such as atherosclerosis, the source, regulation, and precise functional relevance of vascular ROS production still remain to be clearly defined. Many findings point in the direction of one or more NADPH oxidase(s) central to ROS producing system(s), but despite the recent emergence of non-phagocytic gp91phox homologues definitive proof of this assumption is still lacking. In vitro inhibition studies of ROS production have established the role of ROS in the upregulation of adhesion molecules,^{67,68} activation of transcription factors,^{69,70} hypertrophy,^{40,71} cell proliferation,¹⁷ migration,¹ and ultimately atherosclerosis.^{28,42} However, the reliance of these experiments on pharmacological non-specific inhibitors and scavengers, such as DPI and N-acetylcysteine, has limited the conclusions to be drawn from this research.

A very suggestive, yet circumstantial, piece of evidence implicating an NADPH oxidase system comparable to that found in phagocytes in the generation of ROS in the vasculature is the demonstration of almost all phagocyte NADPH oxidase components throughout all layers of the vascular wall. It

Take home messages

- Almost all phagocyte NADPH oxidase components are found throughout all layers of the vascular wall
- In non-phagocytes, reactive oxygen species have a role in a variety of essential intracellular and intercellular signal transduction pathways and are active at much lower physiological concentrations
- The source, regulation, and precise functional relevance of vascular reactive oxygen species still remain to be clarified

will be very interesting to learn which gp91phox homologues serve as catalytic centres in these systems and the exact functional relevance of the presence or absence of the other oxidase subunits in the different vascular tissues. Indeed, in phagocytic cells the cytosolic components function in the regulation of a very intense, burst-like generation of ROS corresponding to the need for killing pathogens. In contrast, in non-phagocytic cells, ROS have a role in a variety of essential intracellular and, possibly, intercellular signal transduction pathways, such as cell differentiation and proliferation, apoptosis, and oxygen sensing, and are therefore active at much lower physiological concentrations. The amounts of superoxide produced by the Nox1 based oxidase system, for instance, are much lower than those produced by phagocytic oxidase.³⁰ How then, is the regulatory machinery of the oxidase(s) adapted to this difference in function and, probably, subcellular localisation? Which are the target molecules modified by the generated ROS and what are the consequences of the dysregulation of these systems under pathological conditions? The elucidation of these and other questions will benefit enormously from the rapidly progressing characterisation at the molecular level of the enzyme systems involved.

“In non-phagocytic cells, reactive oxygen species have a role in a variety of essential intracellular and, possibly, intercellular signal transduction pathways and are therefore active at much lower physiological concentrations”

This characterisation will also permit the establishment of mouse models for the further elucidation of the role ROS based signalling plays in vascular health and disease and the genotypic and phenotypic characterisation of affected human individuals, thereby opening new diagnostic and, possibly, therapeutic options for such vascular disease states as hypertension and atherosclerosis.

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