NADPH oxidases in the cardiovascular system

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Chapter 1

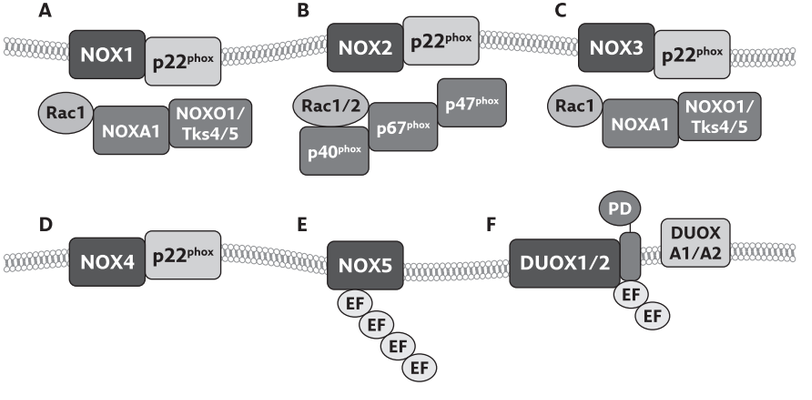
General introduction

Reactive oxygen species

Reactive oxygen species (ROS) are oxygen-containing molecules with one or more unpaired electrons that can be highly reactive with other molecules. They namely can induce cell damage by reacting with macromolecules such as DNA, lipids and proteins.[1] At the same time it is known that ROS at lower concentrations function as signaling molecules that, for instance, react with cysteine residues on certain proteins and thereby alter their functional state, the so called redox signaling.[2] As such, ROS are involved in the regulation of different processes like cell proliferation, migration, gene expression, cell growth and death. All these processes play an important role in the cardiovascular system also.
ROS can be produced by a variety of enzymes and non-enzymatic systems including the mitochondrial respiratory chain, xanthine oxidase, uncoupled nitric oxide synthase (NOS), cytochrome P_{450} and the family of NADPH oxidases (NOX).[3] The mechanism of activation of NOX is well characterized in neutrophilic granulocytes.[4] At the start of this PhD project in 2008 less was known about its role in cardiovascular cells as a relatively new sources of ROS.[5]

Different NADPH oxidase isoforms

Seven members of the NOX family (NOX1 trough NOX5, DUOX1 and DUOX2), each encoded by a separate gene and with distinct tissue distribution, have so far been described (figure 1).[5]



1. Members of the NADPH oxidase family | Overview of the interaction and regulation of the different NOX isoforms (A) NOX1, (B) NOX2, (C) NOX3, (D) NOX4, (E) NOX5 and (F) DUOX1/2.

The prototypic NOX2 ($gp91^{phox}$), a multi-subunit enzyme complex was first identified in neutrophilic granulocytes where it is involved in non-specific host defence against microbes during phagocytosis. NOX2 consists of the membrane-bound catalytic subunit $gp91^{phox}$ that forms a complex with the smaller molecular weight protein $p22^{phox}$ (figure 1B). The heterodimer forms a flavocytochrome that catalyses the transfer of electrons from NADPH to oxygen, thereby generating superoxide (O^{2-}). The binding of the regulatory cytosolic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small GTP-binding protein Rac, stimulates the catalytic activity of the heterodimer. $p47^{phox}$ forms a ternary complex with $p67^{phox}$ and $p40^{phox}$ within the cytosol and is responsible for chaperoning the entire complex to the membrane upon activation. Therefore, $p47^{phox}$ plays a critical role in activation of the complex. It contains an auto-inhibitory region (AIR) in the C-terminal end that, under resting conditions, prevents binding of $p47^{phox}$ to $p22^{phox}$.[6] However, in the presence of an appropriate stimulus, serine residues on $p47^{phox}$ become phosphorylated relieving the auto-inhibition, resulting in subsequent targeting to $p22^{phox}$ and the plasma membrane, respectively.[6] Rac is recruited to the membrane independently of the cytosolic ternary complex where GDP is replaced by GTP, allowing Rac to bind to $p67^{phox}$.

Other NOX isoforms variably require the binding of distinct regulatory subunits for their activity. Both NOX1[7] (figure 1A) and NOX3[8] (figure 1C) bind to $p22^{phox}$, and produce O^{2-} in the presence of NOXO1 (NOX organizer) and NOXA1 (NOX activator), which are homologues to $p47^{phox}$ and $p67^{phox}$, respectively. Novel NOX-related organizers (i.e. tyrosine kinase substrate (Tks4 and Tks5) were shown to selectively support NOX1 and NOX3 activity by interacting with NOXA1.[9]

NOX4 also binds to $p22^{phox}$ but differs from the other NOX isoforms as it does not depend on at least the currently identified activator and organizer subunits which appear to be critical for activity of NOX1, NOX2 and NOX3 (figure 1D).[10] NOX4 has been described as a constitutively active NOX isoform, primarily generating hydrogen peroxide (H_2O_2),[10/11] whose activity is regulated through changes in expression levels.[12/13] NOX5 (figure 1E) is the latest discovered member of the NOX family. Meanwhile four different splice variants (e.g. NOX5α, NOX5β, NOX5γ and NOX5δ) have been identified.[14/15] In general, NOX5α and NOX5β are the most abundant splice variants expressed in cells. In endothelial and vascular smooth muscle cells (SMCs), all four splice variants are expressed together in varying proportions.[14/15] In contrast to the other NOX isoforms, NOX5 does not occur in rodents.[16/17] Like NOX4, NOX5 does not require any other NOX subunits for its activation,[18] nor does it need $p22^{phox}$ for membrane stabilization. NOX5 however does have a polybasic region that localizes the enzyme to the plasma membrane. Furthermore, NOX5 has a N-terminal calmodulin-like domain with four binding sites for calcium (EF hands), needed for calcium-dependent activation, and a C-terminal domain with binding sites for FAD and NADPH, necessary for O^{2-} production.[18/19]

Finally, the dual oxidases, DUOX1 and DUOX2, not only contain a NADPH oxidase domain but also a domain that is homologous to heme-containing peroxidases such as myeloperoxidase and lactoperoxidase (figure 1F).[20] DUOX1 and DUOX2 require activation subunits (DUOXA1 and DUOXA2, respectively) for proper translocation and H_2O_2 production, generated by the peroxidase domain (PD).[21] DUOX-mediated H_2O_2 has been found to play a role mainly in thyroid hormone formation, but has also been described in cardiomyocytes.[22]

NOX signaling in cardiomyocytes: schemia and apoptosis

At the start of this PhD thesis project, the NOX isoforms NOX1, NOX2, NOX4 and NOX5 were described in cells of the cardiovascular system, namely endothelial cells (NOX4 and NOX5),[14/23] SMCs (NOX1 and NOX5)[15/24] and cardiomyocytes (NOX2 and NOX4)[25-27] to regulate diverse functions varying from differentiation, proliferation, apoptosis, senescence up to inflammation. All these processes play a role in cardiovascular pathology.

Heymes *et al.*[28] reported in 2003 for the first time in total tissue homogenates of human hearts an increased NADPH oxidase activity in end-stage failing versus non-failing myocardium. Until then, the occurrence and/or expression pattern of different NOX isoforms in cardiomyocytes was not yet known. In 2003, our group demonstrated for the first time increased expression of NOX2 within human cardio-myocytes in patients who died subsequent to acute myocardial infarction (AMI).[25] We subsequently demonstrated *in-vitro* (in H9c2 cells: cardiomyoblast cells) that ischemia induced a nuclear expression of NOX2 coinciding with ROS production[26] Inhibition of NOX-derived ROS then resulted in decreased apoptosis. However, insight in the activation process of nuclear NOX2 was lacking.

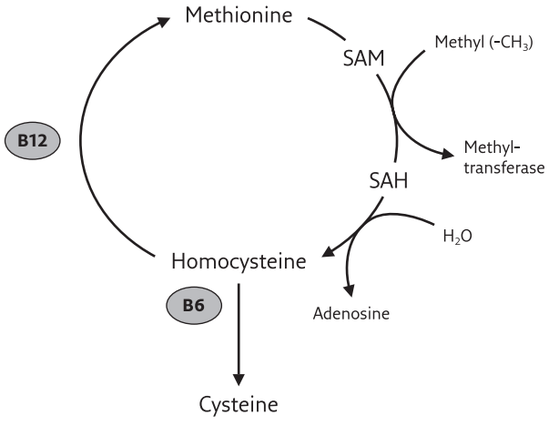
Since tight regulation of the activation and localization of NOX is essential for mediating redox signaling at the right place and time, we studied in *Chapter 2* targeting of NOX2 together with the subunits $p22^{phox}$ and $p47^{phox}$ to nuclear regions in ischemic cardiomyocytes. Next to this, it was also unknown how nuclear NOX2-derived ROS mediated apoptosis in ischemic cardiomyocytes. We hypothesized that upon induction of apoptotic signaling, NOX2 would translocate to the nucleus, resulting in local ROS production that then would modify redox-sensitive transcription factors that play a role in apoptosis signaling. Different redox-sensitive transcription factors, namely nuclear factor kappa B (NF-κB)[29/30] and forkhead box O1 (FOXO1),[31/32] indeed have been implicated in pro-apoptotic signaling in cardiomyocytes. It already was shown that NF-κB translocation, induced by high glucose levels, resulted in cardiomyocyte apoptosis that did depend on NOX-ROS formation.[33] Albeit the role of FOXO1 translocation herein was not studied yet.

A major aspect of FOXO1 activation is the regulation of its subcellular localization, having an active state after translocation to the nucleus. In *Chapter 3* we studied the role of NOX2/p47^{phox}-dependent ROS production herein.

NOX signaling in cardiomyocytes: homocysteine and apoptosis

Our research group has also shown that, next to ischemia, high concentrations of homocysteine (Hcy) also induce cardiomyocyte apoptosis, in which (peri)nuclear NOX2 was suggested to play an important role.[34] Increased plasma levels of Hcy, leading to hyperhomocysteinemia, has become apparent since 1969 to form a risk factor in the development of cardiovascular disease.[35] Normally, Hcy is converted to methionine by addition of a methyl group (figure 2). Methionine then is converted into *S*-adenosylmethionine (SAM), which is the main source of methyl for methylation of DNA, RNA and proteins. Once the methylgroup is transferred to methyltransferases, *S*-adenosylhomocysteine (SAH) remains. SAH is subsequently hydrolyzed to Hcy and adenosine. Due to either genetic defects or deficiencies in co-factors such as vitamin B6 and B12, accumulation of Hcy occurs.[36/37] However, whether Hcy is the causative factor of cell damage or the increased SAH remained unclear.

As depicted above, we already had shown in cardiomyocytes that Hcy did induce apoptosis in a concentration-dependent manner, in which (peri)nuclear NOX2 played a role.[34] Since we hypothesized that p47^{phox} is essential for translocation and activation of NOX2 (Chapter 2), we subsequently analyzed in *Chapter 4* the role of the subunit p47^{phox} in NOX2-mediated apoptosis of cardiomyocytes under conditions of high concentrations Hcy or intracellular SAH.



2. Homocysteine pathway | Homocysteine as part of the methionine cycle. *S*-adenosylhomocysteine (SAH) is formed during *S*-adenosylmethionine (SAM)-dependent methylation reactions, and the hydrolysis of SAH results in homocysteine. Homocysteine can be remethylated to form methionine.

NOX signaling in cardiomyocytes: hypertrophy

Redox-sensitive signaling pathways are not only playing an important role in mediating apoptotic signaling, but remarkably also in cardiac hypertrophy.[38-42] Cardiac hypertrophy is an adaptive response of the heart to stress or disease,[43] for instance due to hypertension[44] or AMI.[25] In the above mentioned AMI study we not only found NOX2 expression in jeopardized cardiomyocytes in the necrotic areas but also in viable cardiomyocytes in its border areas.[25] As part of these cardiomyocytes become hypertrophic in time, a role for ROS produced via NOX2 in the process of hypertrophy was postulated.[25/45]

Byrne *et al.*[46] indeed demonstrated in 2003 that angiotensin II-induced cardiac hypertrophy coincided with an increased left ventricular NADPH oxidase activity. Hypertrophy then was reduced in NOX2 knock-out mice compared to wild-type controls, indicative for an important role of NOX2 in angiotensin II induced cardiac hypertrophy.[46] Interestingly, it was shown that both angiotensin II and phenylephrine (PE) are coupled to similar downstream signaling pathways and hypertrophic responses in cardiomyocytes, albeit PE was a more effective inducer of hypertrophy.[47/48] We wondered whether NOX proteins might play a decisive role herein. This we have studied in *Chapter 5*.

NOX signaling in cardiomyocytes: NOX5

The latest identified member of the NOX family is NOX5. In contrast to most other NOX isoforms, NOX5 does not appear to require cytoplasmic subunits for its activation,[49] but instead is regulated through calcium, which induces a conformational change of the NOX5 N-terminus, leading to enzyme activation.[18] NOX5 was first described to be functional active in endothelial cells in 2007.[14] In this study, NOX5 was demonstrated to promote endothelial ROS production, its proliferation, and the formation of capillary-like structures. As such, this could point to a role of NOX5 in the regenerative phase post-AMI. NOX5 indeed was shown in the endothelium and SMCs of epicardial coronary arteries obtained from explanted hearts of patients with coronary artery disease and AMI.[50] However, detailed information of NOX5 within the heart was lacking. For this we have analyzed in *Chapter 6* the expression of NOX5 in the heart of patients who died as a result of AMI.

NOX signaling in endothelial cells and smooth muscle cells: homocysteine and apoptosis

Hyperhomocysteinemia not only plays a role in cardiomyocyte pathology but was first described in vascular disease.[35] Several patient and animal studies namely have shown a correlation between hyperhomocysteinemia, and atherosclerosis or vascular dysfunction.[51-56] Growing evidence suggests that endothelial dysfunction plays a major role in vascular injury seen in hyperhomocysteinemia. *In-vitro* studies have shown that elevated levels of Hcy induce oxidative stress resulting in apoptosis of endothelial cells, wherein NADPH oxidase-mediated ROS play an important role.[57-62] However, in these studies the NOX isoform involved was not further specified. In *Chapter 7* we have now studied the involvement of the different NOX isoforms in pro-apoptotic signaling of endothelial cells under high concentrations Hcy. Additionally, in *Chapter 8* we have compared the role of Hcy and SAH herein.
Albeit a direct pathological effect of hyperhomocysteinemia on endothelial cells was shown, its effect on SMCs is contradicting, varying from induction of proliferation up to induction of cell death.[36/63/64] Also herein ROS do play an important role.[36] Even more, in isolated rat aortas it was shown that increased levels of Hcy (induced by high-methionine diet) not only did induce increased ROS levels but also NADPH oxidase activity, indicative for a role of NOX in SMC also.[65] The role herein of the different NOX isoforms was however unknown. This is the subject that we studied in *Chapter 9.*

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