NADPH oxidases in the cardiovascular system

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Chapter 10

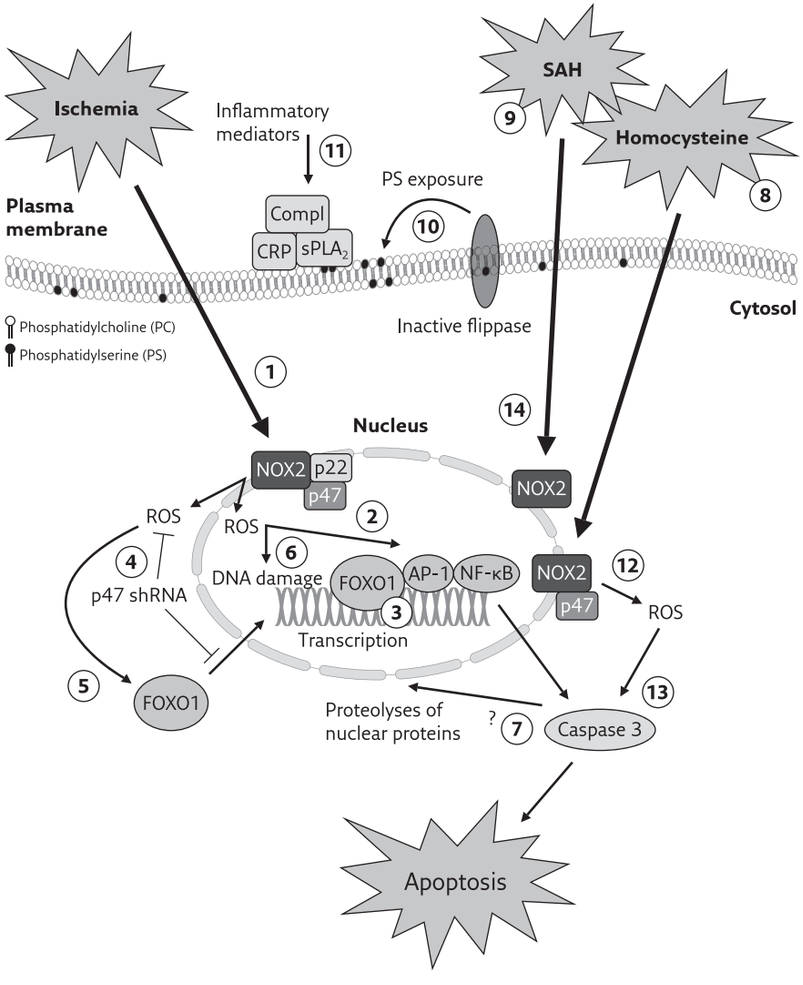
Discussion

Reactive oxygen species (ROS) play an important role in the pathophysiology of cardiovascular disease. They not only have a toxic effect in cardiovascular cells, but are also involved in cellular signal transduction via modification of signaling proteins, i.e. redox signaling. An important relative new source of ROS in cardiovascular cells is the family of NADPH oxidases (NOX).[1] Because ROS are diffusible and short-lived molecules, tight regulation of the activation and subcellular localization of NOX is essential for mediating redox signaling at the right place and time, facilitating distinct cellular functions.[2] In this thesis we studied the effect of different pathophysiological stimuli on the expression and subcellular localization of different NOX isoforms within different cardiovascular cells. The results of these studies are discussed here.

Ischemia-induced apoptosis in cardiomyocytes

(figure 1) NOX2 is one of the most abundantly described NOX isoforms within cardiomyocytes. We were the first to demonstrate that human cardiomyocytes express NOX2 and found an increase of cardiomyocyte NOX2 expression within the infarction area after acute myocardial infarction (AMI), suggesting a role(s) for this ROS-producing enzyme in the human infarcted heart.[3] Indeed, in ischemia, inhibition of NOX-derived ROS in H9c2 cells (cardiomyoblast cells), using apocynin and diphenylene iodonium (DPI), resulted in decreased caspase 3 activity, proving a role for NOX2 in the induction/regulation of ischemia-induced apoptosis in cardiomyocytes.[4] At the start of this PhD project, this mechanism of activation of NOX2 in cardiovascular cells was still less recognized.

We first analyzed ischemia-induced nuclear targeting and activation of NOX2 (1) (The numbers in the text refer to the bold numbers in the figures) and found colocalization of the NOX subunits $p22^{phox}$ and $p47^{phox}$ with the nuclear pore complex in the nuclear envelope (Chapter 2).[4] The activation of NOX2 at this subcellular location offered different mechanistic possibilities regarding the function of NOX2. Firstly, it could affect proteins translocating in or out of the nucleus, including transcription factors. There are namely a number of redox-sensitive transcription factors that are either activated or inactivated through redox modifications. Several redox-sensitive transcription factors, i.e. the activator protein 1 (AP-1), nuclear factor kappa B (NF-κB) and forkhead box 01 (FOXO1), have also been implicated in the regulation of cardiomyocyte apoptosis, partly dependent on their subcellular location (2).[5/6] For instance, nuclear translocation and activation of FOXO1 in response to ischemia/reperfusion has been suggested to have a pro-apoptotic role in mouse cardiomyocyte (3).[7] We found that $p47^{phox}$ down-regulation by small hairpin (sh)RNA significantly



1. Regulation of cardiomyocyte apoptosis by NOX2 | NOX2/p47^{phox} targeting to the (peri) nuclear region plays an important role in pro-apoptotic signaling in cardiomyocytes subjected to ischemia or high homocysteine concentrations.

inhibited nuclear ROS production and nuclear translocation of FOXO1 (4), resulting in reduced caspase 3 activity (Chapter 3). This indicates that p47^{phox}-mediated ROS production and nuclear FOXO1 are functionally related in ischemia-induced apoptosis of cardiomyocytes (5).

ROS production at the nuclear region might also induce DNA damage via the formation of peroxynitrite, as such facilitating apoptosis (6).[8] Furthermore, increased leakage of the nuclear pore complex at the nuclear envelope has also been linked to oxidative stress.[9] Even more, it was described that the permeability of the nuclear pore increases through caspase-mediated proteolysis of specific nuclear proteins (7).[10] Therefore, ROS-mediated oxidation of nuclear pore complexes might contribute to increased permeability of the nuclear pore at different stages of apoptosis.

Taken together, we have demonstrated that ischemia induces nuclear translocation of p22^{phox}, p47^{phox} and NOX2, resulting in local ROS production that facilitates apoptosis, at least in part by promoting FOXO1 translocation to the nucleus.

Homocysteine-induced apoptosis in cardiomyocytes

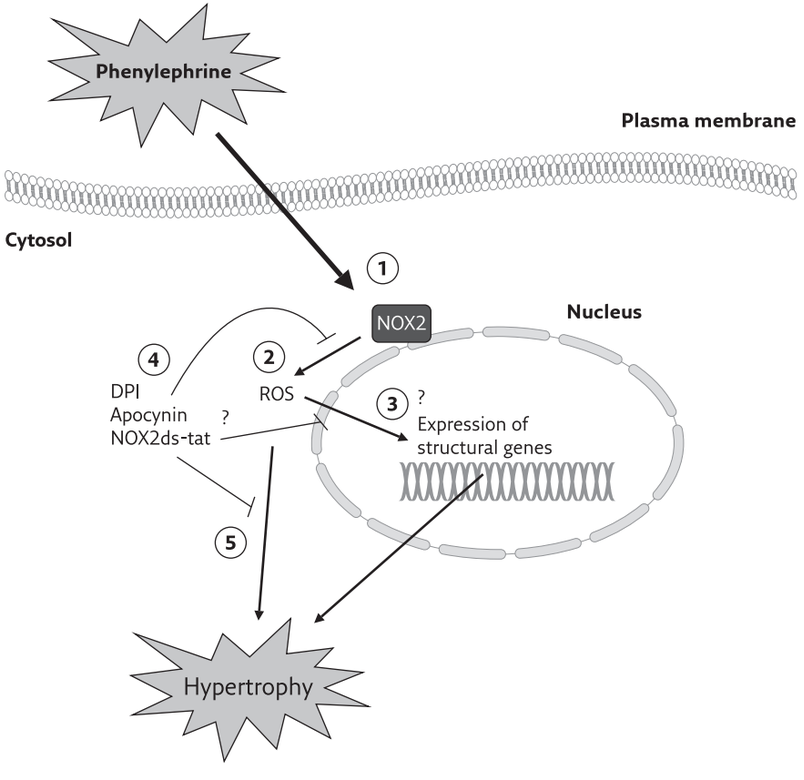
(figure 1) Homocysteine (Hcy) is a well-known vascular risk factor. However, we have shown previously that Hcy not only has a direct toxic effect on endothelial cells but also on cardiomyocytes. Hcy namely can induce cardiomyocyte apoptosis, in which nuclear NOX2 is playing an important role also.[11] Recent studies suggest that *S*-adenosylhomocysteine (SAH) is the main causative factor in Hcy-induced pathogenesis of vascular disease.[12/13] The role of SAH in Hcy-induced cardiomyocyte apoptosis however is unknown. Since we have found that p47^{phox} is essential for the translocation and activation of NOX2 in ischemia-induced apoptosis, we analyzed the role of p47^{phox} in NOX2-mediated apoptosis in cardiomyocytes related to Hcy (8) and have subsequently analyzed the role of SAH herein (9) (Chapter 4).

Firstly, we have found that Hcy not only induced apoptosis of H9c2 cells, but also resulted in phosphatidylserine (PS) exposure to the outer leaflet of the plasma membrane, via inactivation of flippase (10). Flippase is an ATP-dependent transmembrane protein that regulates transbilayer phospholipid asymmetry by translocating PS from the outer to the inner membrane layer.[14] Inactivation of flippase thus allows PS to be exposed in the outer membrane layer. This PS exposure represents a pro-inflammatory status of the plasma membrane and allows binding of acute phase proteins such as type IIA secretory phospholipase A_2 (sPLA$_2$-IIA) and C-reactive protein (CRP) that facilitate binding and activation of complement (Compl) (11), resulting in additional cell death.[15/16] Furthermore, we found that, similar to ischemia, Hcy induced (peri)nuclear NOX2/p47^{phox} expression, local ROS production (12), and subsequent caspase 3 activation (13), resulting in cardiomyocytes apoptosis.[17] Conversely,

although SAH did induce an increase in (peri)nuclear expression of NOX2, it was not activated as no local ROS production was found (14). Interestingly, SAH failed to induce (peri)nuclear $p47^{phox}$ expression. Since the lack of $p47^{phox}$ translocation by SAH in theory does prevent NOX2 activation, these results thus suggest that NOX2-related apoptosis induction of cardiomyocytes is not induced by increased SAH alone,[17] but instead needs Hcy.

Phenylephrine-induced hypertrophy in cardiomyocytes

(figure 2) In the human infarcted heart we not only found increased NOX2 expression in jeopardized cardiomyocytes but also in morphological normal cardiomyocytes in the border area.[3] Since these cardiomyocytes can become hypertrophic in time, a role for NOX2 in hypertrophic signaling was hypothesized.
In isolated rat cardiomyocytes it was shown that phenylephrine (PE) could induce early changes in expression of structural genes that are associated with the induction of hypertrophy.[18/19] We therefore analyzed the role of NOX2 in PE-induced cardiomyocyte hypertrophy (Chapter 5). We found that PE stimulation induced a significant temporal increase in NOX2 expression between 1 and 4 hours after stimulation (1) with concomitant significant ROS production after 2 hours (2), resulting in hypertrophy after 24 and 48 hours of stimulation. An interesting finding is that early induction of NOX2-mediated ROS results in long-term hypertrophy. In isolated adult rat cardiomyocytes it already was shown that PE induced elevated levels of intracellular ROS after 5 minutes of stimulation, based on oxidation of dichlorofluroescein (DCF), that declined to basal levels after 60 minutes.[20] In isolated rat cardiomyocytes PE also induced early changes in gene expression (e.g. *c-Jun* and connective tissue growth factor (CTGF)) within 1 hour and subsequent changes in expression of structural genes associated with hypertrophy (increased over 4 to 24 hours).[18/19] However, a role for NOX/ROS-mediated in these early changes was not studied yet (3). We now found that inhibition of NOX2-mediated ROS (4), using apocynin, DPI or NOX2ds-tat (a specific inhibitor of NOX2)[21] during the first 4 hours after PE stimulation, significantly reduced hypertrophy (5). This thus proves that NOX2 not only plays a role in cell death of cardiomyocytes experiencing ischemia (figure 1), but also in PE-induced cardiomyocyte hypertrophy.



2. Regulation of cardiomyocyte hypertrophy by NOX2 | Early NOX2 activation at the nucleus plays a role in phenylephrine-induced hypertrophy of cardiomyocytes.

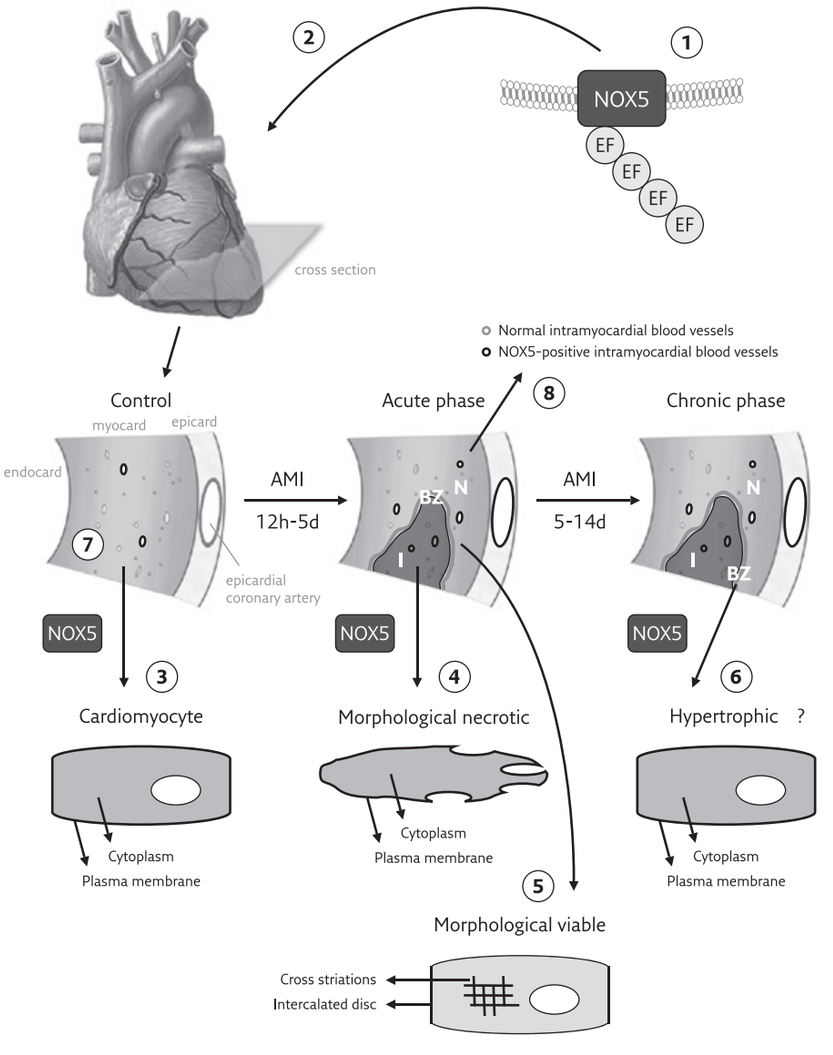
Ischemia-induced NOX5 expression in cardiomyocytes and intramyocardial blood vessels

(figure 3) NOX5 is the latest identified member of the NOX family. In contrast to most other NOX isoforms, NOX5 does not require cytoplasmic subunits for its activation. Instead it is regulated via calcium, which induces a conformational change of the NOX5 N-terminus, resulting in enzyme activation (1).[22] Although expression of NOX5 has been shown in human atherosclerotic diseased epicardial coronary arteries,[23] knowledge regarding expression of the NOX5 isoform in the heart was lacking (2). We found NOX5 expression in normal hearts in a limited number of scattered cardiomyocytes, staining the cytoplasm and plasma membrane (3). Its expression significantly increased in the heart after AMI (Chapter 6). In the acute inflammatory phase (between 12 hours to 5 days post-AMI) NOX5 expression was found in the cytoplasm and at the plasma membrane of necrotic cardiomyocytes (4) but also in morphologically viable cardiomyocytes. In these viable cardiomyocytes also staining of cross striations and intercalated discs was found (5). In the chronic inflammatory phase (5 to 14 days post-AMI) we also found NOX5-positive cardiomyocytes adjacent to the infarcted area. In this border zone cardiomyocytes NOX5 staining was again only found in the cytoplasm and plasma membrane (6). Since these cardiomyocytes can become hypertrophic in time,[24] similar to NOX2, a role for NOX5 in cardiomyocyte hypertrophy can also be postulated.

Next to cardiomyocytes, NOX5 expression was also found in intramyocardial blood vessels. In control hearts, we namely found NOX5 in a limited number of intramyocardial blood vessels (7), while AMI significantly increased NOX5 positive blood vessels, not only in het infarct area but also beyond that (8).

Structural and functional aberrations have been shown to occur in intramyocardial blood vessels. We have found, for instance, thickening of the basement membrane (BM), accumulation of advanced glycation end products (AGES) and expression of E-selectin in intramyocardial blood vessels in AMI patients but also in a AMI rat model,[25/26] suggestive of a pro-inflammatory status of these blood vessels, subsequent to but also preceding AMI. Whether NOX5 is playing a role herein is still unknown. We also found NOX5 expression in blood vessels of granulation tissue in chronic phase infarctions. This could point to a role of NOX5 in the revascularization process of ischemic myocardial tissue also. Indeed, overexpression of NOX5 was found to induce proliferation and the formation of capillary-like structures of human endothelial cells,[27/28] while knock-down of NOX5 with small interference (si)RNA attenuated these effects, indicative for a role of NOX5 in the process of angiogenesis, also post-AMI.

Taken together, we demonstrated for the first time NOX5 in the human heart and its increased presence after AMI. The different cellular and subcellular locations of NOX5 suggest multiple roles for NOX5 in the infarcted heart.



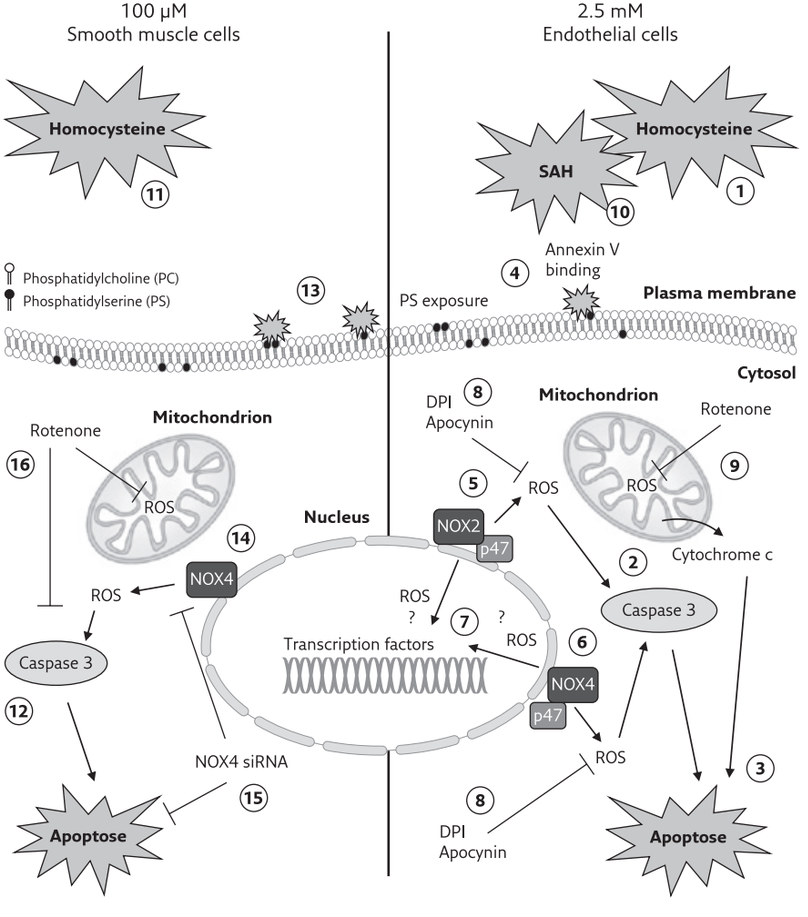
3. NOX5 in the heart | Overview of the (sub)cellular localizations of NOX5 in the human heart after AMI.

Homocysteine-induced apoptosis in endothelial cells

(figure 4) As discussed above, Hcy has been shown to play a role in vascular disease. Hcy was shown to not only cause endothelial cell injury and dysfunction, but also a pro-inflammatory state in these cells.[29] Hcy-induced apoptosis of the vasculature was found to relate to NOX activity.[30-32] However, the particular NOX isoforms involved herein were not specified. As we have shown that Hcy-induced translocation of NOX2/p47^{phox} to (peri)nuclear regions is crucial for the induction of apoptosis in cardiomyocytes (figure 1),[11/17] we wondered whether this nuclear NOX-mediated apoptosis pathway also plays a role in Hcy induced apoptosis of endothelial cells. We indeed found that pathophysiological concentrations of Hcy (2.5 mM) (figure 4, right part) (1) resulted in activation of caspase 3 and cytochrome c release in the cytoplasm of endothelial cells (2), indicative of apoptosis (3) (Chapter 7). Next to this we found that Hcy induced PS exposure in the outer plasma membrane layer of endothelial cells, as measured via binding of fluorescent labeled Annexin V (4). This PS exposure can occur in apoptotic cells, but has also been found in cells that are non-apoptotic, representing a reversible, but pro-inflammatory status of the cell.[16/33] Since NOX2 and NOX4 are the most abundantly expressed NOX isoforms in endothelial cells,[34-36] and previous studies on endothelial cells have shown that p47^{phox} is a crucial subunit in the active endothelial NOX complex by other stimuli,[37-40] we studied their expression pattern. We then found that 2.5 mM Hcy induced (peri)nuclear NOX2 expression (5), coinciding (peri)nuclear p47^{phox} and local ROS production. Furthermore, although evidence shows that NOX4 activity, unlike NOX2, does not depend on p47^{phox},[41/42] we also found NOX4 coinciding p47^{phox} and local ROS in the peri-nuclear region (6). Whether these NOX2/NOX4-mediated (peri)nuclear ROS can activate or modulate transcription factors is still unknown (7). We also found diffuse ROS generation in the cell after incubation with Hcy. Both apocynin en DPI partly reduced Hcy-induced caspase 3 activity (8). Inhibition of mitochondrial ROS production using rotenone also partly inhibited Hcy-induced apoptosis, indicative for a role of mitochondrial ROS in the Hcy-induced effects in endothelial cells also (9). Interestingly, in contrast to our findings in cardiomyocytes (figure 1), we found in endothelial cells that increased intracellular SAH (10) induced both PS exposure and apoptosis. This then coincided with (peri)nuclear localization of NOX2 and NOX4 and concomitant local ROS production, comparable with (2.5 mM) Hcy incubation (Chapter 8). It has to be noticed that the *in-vitro* concentration of 2.5 mM Hcy we have used is relatively high compared to its pathophysiological concentrations *in-vivo* (up to 400 µM).[43] However, we have found that Hcy is rapidly degraded *in-vitro.* As such, the short-term exposure of 6 hours to higher Hcy concentrations we used, might reflect a life-long exposure to moderately elevated levels of Hcy that occur in

patients. Furthermore, we found that 2.5 mM D,L-Hcy represents only 1.2 mM of L-Hcy (only the L-form of Hcy has a biological effect, not the D-form),[29] indicating that we in fact studied up to a 3-fold higher concentration than occurs pathophysiologically.

Thus, similar to cardiomyocytes, NOX activation is involved in Hcy-induced apoptosis in endothelial cells. However, in contrast to cardiomyocytes, increased levels of SAH were sufficient to induce toxic effects in endothelial cells, suggesting partially different pathways in the induction of damage in these cells.



4. NOX signaling in homocysteine-induced vascular dysfunction | Overview of the effects of homocysteine (2.5 mM) on endothelial cell viability (right part) and of homocysteine (100 µM) on smooth muscle cell viability (left part), and the role of different NOX isoforms hereon.

Homocysteine-induced apoptosis in smooth muscle cells

(figure 4) In vascular smooth muscle cells (SMCs) contradicting effects of Hcy have been described in different studies, varying from the induction of migration and proliferation of vascular SMCs up to induction of apoptosis in SMCs.[44/45] In line with the latter findings, we found in human femoral arterial biopsies that severely increased blood levels of Hcy (54±12 μM) coincided with significantly decreased numbers of medial SMCs, indicative of cell death.[46] We have elaborated this finding *in-vitro*. We then found that incubation of SMCs with 100 μM Hcy (figure 4, left part) (11) resulted in activation of caspase 3 (12) and PS exposure in the plasma membrane outer layer (13) (Chapter 9). This Hcy-induced apoptosis coincided with increased expression and translocation of NOX4 to the (peri)nuclear region and local ROS production (14), whereas the expression levels and subcellular localization of NOX1 and NOX2 were not affected. NOX4 knock-down, using siRNA, totally reduced Hcy-induced caspase 3 activity, as well as (peri)nuclear NOX4 expression and ROS production, indicative for an important role of NOX4 in Hcy-induced apoptosis of arterial SMCs (15). We also found a partial contribution of ROS produced by mitochondria in Hcy-induced apoptosis as the mitochondrial ROS inhibitor rotenone partially inhibited caspase 3 activity (16).

Thus, Hcy-induced apoptosis in SMCs is mediated by (peri)nuclear NOX4 activity. Furthermore our studies indicate that SMCs are more sensitive for Hcy compared with endothelial cells and cardiomyocytes.

Conclusion

Oxidative stress plays an important pathological role in cardiovascular cells. As such there is great interest in targeting ROS as a new therapeutical approach in human cardiovascular disease. This thesis describes further mechanistic insight as to how a relatively new source of ROS, namely NOX isoforms, are involved in cell fate decisions in cardiovascular cells and how their subcellular localization is particularly important for their functioning. We have shown that the expression, activation and (sub)cellular localization of the different NOX isoforms in response to different pathological stimuli can differ between cardiovascular cell type, suggesting different regulation of these NOX isoforms within different cells. More important, we found that different NOX isoforms, or even one NOX isoform, in response to different stimuli, can have opposing functional effects, also within one cell type.

Although multiple compounds have been registered that inhibit different NOX isoforms (recently reviewed in),[47-53] our results indicate that further research is needed before these can be applied successfully as a therapeutic for cardiovascular disease.

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