SUMMARY

Numerous studies have indicated that increased levels of homocysteine (Hcy), also known as hyperhomocysteinaemia (HHC), form a risk factor for the development of several diseases. HHC has been related to neurological disorders such as Parkinson’s disease and Alzheimer’s, but also to cancer, preeclampsia and cardiovascular disease. In this thesis we have studied the pathophysiological role of Hcy in endothelial cells and cardiomyocytes, with an emphasis on the role of NOX proteins herein.

NOX

The NADPH-oxidase (NOX) complex can generate reactive oxygen species (ROS), which was first described in phagocytic cells. Meanwhile it has become clear that NOX is involved in signalling and pathological processes in other cell types also, although less is known about the mechanism of activation of NOX proteins in these non-phagocytic cells. In chapter 2 we have shown that in an ischaemic environment the NOX2 subunit of the NOX complex translocates to the nucleus of cardiomyocytes, coinciding with local generation of ROS, which results in apoptosis. We subsequently have studied the effect of Hcy on NOX proteins in endothelial cells and cardiomyocytes.

Hcy and Endothelial Dysfunction

Growing evidence suggests that endothelial dysfunction, including cell death of endothelial cells, plays a major role in the vascular injury seen in HHC, in which oxidative stress is playing an important role. In chapter 3 we have described that incubation of human umbilical vein endothelial cells (HUVECs) with Hcy not only resulted in apoptosis of endothelial cells but interestingly also in transition of phosphatidylserine (PS) to the outer leaflet of the plasma membrane, also known as plasma membrane flip-flop, representing a pro-inflammatory state of the cell. Using digital imaging microscopy we found that Hcy increased expression and translocation of NOX2, NOX4 and p47phox to the nucleus, coinciding with nuclear and cytoplasmic ROS production. Inhibition of mitochondrial ROS and NOX-related ROS then indeed prevented apoptosis.

An increase in Hcy also results in an increase in S-adenosylhomocysteine (SAH), a very potent inhibitor of methylation of DNA, RNA and proteins. There is an ongoing debate whether Hcy itself, or increased SAH is the main causative factor in HHC-induced cardiovascular disease. We therefore compared the effect of increased Hcy and/or increased SAH on cell viability of HUVECs as described in chapter 8. We found that both Hcy, resulting in increased SAH, and also increased SAH alone induced plasma membrane flip-flop and endothelial apoptosis, coinciding with (peri)nuclear NOX2/NOX4/p47phox-expression and ROS production. From this we can conclude that increased levels of SAH, independent of Hcy, can be responsible for HHC-induced endothelial damage. Therefore, SAH reduction theoretically is a possible target to prevent endothelial dysfunction.
Homocysteine and the Heart

The role of Hcy in the development of cardiovascular disease mainly has been related to its effect on the vascular wall. However, in several studies, including the large scale Framingham Heart Study, a correlation was found between elevated Hcy and heart failure. It even was postulated that Hcy could have a direct effect on the heart by damaging cardiomyocytes resulting in cardiac fibrosis, although this has not yet been proven. Therefore we have studied whether Hcy had a direct effect on cardiomyocytes. In chapter 4 we indeed describe concentration-dependent effects of Hcy on cardiomyocytes. Low concentrations of Hcy resulted in activation of mitochondria, PS exposure to the outer leaflet of the plasma membrane and translocation of NOX2 to the nucleus, albeit without ROS production or apoptosis induction. The highest concentration of Hcy we tested however did induce apoptosis of cardiomyocytes, coinciding with (peri)nuclear NOX2/p47<sup>pox</sup>-mediated ROS production, and thus activation of the NOX complex. With this high concentration we also observed a thread-to-grain transition of the mitochondrial reticulum with a decrease of ΔΨ<sub>μ</sub>, coinciding with a decrease of ATP, indicative for an effect on mitochondria also.

Since we found induction of a pro-inflammatory status of cardiomyocytes by Hcy, visualized by PS exposure to the outer leaflet of the plasma membrane, we further examined this mechanism of PS exposure. In chapter 5 we describe that next to inhibition of flippase, also inhibition of RhoA and its downstream effector Rho-associated kinase, all resulted in the loss of the transbilayer phospholipid asymmetry, inducing PS exposure to the outer leaflet of the plasma membrane. We subsequently analyzed this in more detail; in chapter 6 we describe that Hcy caused PS exposure in the outer leaflet of the plasma membrane in cardiomyocytes via inactivation of flippase and Rho-kinase.

Similar to the endothelial cells, we compared the effect of Hcy and SAH on cardiomyocytes, as described in chapter 7. We then found that the jeopardizing effect of Hcy in cardiomyocytes was not induced by SAH in the absence of Hcy. SAH alone namely failed to induce p47<sup>pox</sup> expression in the (peri)nucleus, which is necessary to induce subsequent ROS production and apoptosis, while Hcy did induce this p47<sup>pox</sup> expression in the (peri)nucleus of cardiomyocytes.

Conclusion

NOX proteins play an important role in the induction of apoptosis by Hcy, offering a putative new therapeutic target to reduce cell death of both cardiomyocytes and endothelial cells, and as such preventing endothelial dysfunction and heart failure in HHC patients. In addition we have shown that increased Hcy induced PS exposure to the outer leaflet of the plasma membrane in both cardiomyocytes and endothelial cells, inducing a pro-inflammatory state of these cells. Finally we have found that SAH is cytotoxic in endothelial cells, whereas in cardiomyocytes, Hcy is the main culprit, indicative for different cytotoxic pathways in endothelial cells and cardiomyocytes.