INTRODUCTION

In 1962 Carson and Neill were the first to describe extremely high levels of homocysteine (Hcy) in the urine of mentally retarded children\(^1\). This homocystinuria was later discovered by Mudd et al. to be due to a genetic defect in the cystathionine \(\beta\) synthase (CBS) gene\(^2\). These patients were found to be suffering from premature atherosclerosis and thromboembolism. From these patients, approximately 25% died of cardiovascular events before the age of 30\(^3,4\). The vascular pathology in these patients was later described by McCully in 1969, including smooth muscle proliferation, progressive arterial atherosclerosis and haemostatic changes leading to thrombosis\(^5\). Subsequent research revealed that other genetic defects in enzymes involved in the methionine cycle can also cause elevated Hcy levels, for instance, in the methylenetetrahydrofolate reductase (MTHFR) gene and the methionine synthase (MS) gene\(^6,7\).

Since the discoveries of Carson, Neill, Mudd and McCully many patient trials and numerous in vitro studies have been performed to determine the potential of Hcy, including mild forms of hyperhomocysteinemia (HHC), as an independent risk factor for cardiovascular disease\(^8-13\).

Methionine and Homocysteine metabolism

Hcy is a thiol-containing amino acid derived from methionine metabolism (depicted in Fig. 1). Methionine is derived from dietary proteins and is normally converted to S-adenosylmethionine (SAM) which is the most important methyl donor for methylation of DNA, RNA and proteins\(^14\). Donation of the methyl group to methyltransferase gives rise to S-adenosylhomocysteine (SAH). This in turn is hydrolysed to adenosine and Hcy by SAH hydrolase (SAHH). Remethylation of Hcy to methionine using co-factors folate and vitamin B12 occurs when there is a demand for SAM\(^15\). If SAM levels are sufficient, transsulfuration occurs using vitamin B6 as a co-factor to form cysteine and glutathione\(^15,16\). Both pathways occur predominantly in the liver.

In plasma, Hcy is protein bound for 99%, mostly to albumin (70%)\(^17\). Hcy also has the capacity to form disulfide linkages with a second Hcy molecule (creating homocystine) or to a cysteine molecule. Only 1% of Hcy exists free in its reduced form\(^16\).

Normal Hcy plasma levels in humans are defined as lower than 15 \(\mu\)M. Moderate HHC indicates Hcy plasma levels of 15 to 30 \(\mu\)M. Intermediate HHC is defined as Hcy levels between 30-100 \(\mu\)M, and severe HHC is defined as Hcy plasma levels over 100 \(\mu\)M\(^16,18,19\). Patients suffering from homocystinuria who have a genetic defect in the CBS gene have extremely elevated plasma Hcy levels of up to 400 \(\mu\)M, since CBS is responsible for most of the clearance of Hcy. Increased levels of Hcy above 15 \(\mu\)M occur in 14% of Dutch males, and 9% of Dutch females in the age of 20 to 65 years\(^20\). After the age of 65 the percentages rise to approximately 25%. Also, 25% of patients suffering from cardiovascular disease in The Netherlands have increased levels of Hcy\(^20\).
Since vitamins B6, B12 and folate are required for the pathways that metabolise Hcy and shortages of these vitamins often underlie HHC in particular in developing countries, the general idea developed that supplementing HHC patients with vitamin B6, B12 and/or folic acid would reduce the risk for cardiovascular events. Over the last three decades large patient trials have been performed to determine whether lowering the Hcy levels in these patients with vitamin supplementation was effective\textsuperscript{21-23}; however the risk for cardiovascular events did not decrease significantly in these patients who were already suffering from cardiovascular problems. Therefore it is most important to further evaluate the mechanisms through which Hcy causes its detrimental cardiovascular effect in a way to develop effective therapies related to Hcy-induced cardiovascular disease.

AIM OF THE STUDY

While a detrimental effect of homocysteine on vascular cells is generally accepted, several lines of evidence also point to an effect of hyperhomocysteinemia on cardiomyocytes. The aim of the present study is to clarify but also extend our
understanding of the effect of Hcy on the viability of cardiomyocytes and vascular endothelial cells with specific emphasis on apoptosis.

To that end, we had several objectives. First we wanted to establish whether Hcy induces apoptosis in endothelial cells and cardiomyocytes. When this appeared to be the case, we set up experiments to decipher the pathways involved in the induction of apoptosis in cardiomyocytes with specific attention on changes in transbilayer phospholipids asymmetry of the plasma membrane, the role of RhoA/Rho kinase signalling, and the role of NADPH oxidases (NOX-2 and NOX4). Finally we evaluated the role of S-adenosylhomocysteine in apoptosis induction in cardiomyocytes and endothelial cells.

In the following paragraph we shall briefly introduce these objectives as organised according to the subsequent chapters.

**NOX**

The NADPH-oxidase (NOX) complex normally is responsible for the generation of reactive oxygen species (ROS) during the respiratory burst in phagocytic cells, but it has become clear that NOX is also involved in signalling and more pathological processes in other cell types. Interestingly, it has already been shown that Hcy activates the NOX complex in neutrophils. ROS, specifically generated by individual NOX proteins, can also act as secondary messengers. For this selective inhibition of NOX proteins might be a novel approach to prevent and treat cardiovascular disease.

The NOX complex consists of several subunits. Gp91phox and p22phox form the membrane part of the complex and stabilize one another in a tightly associated heterodimer which is referred to as flavocytochrome b558. The other subunits are cytosolic, namely p67phox, p47phox and p40phox. In fact, the presence and composition of the NOX complex seems to be cell specific. Meanwhile, several NOX homologs have been indentified. The NOX complex subunits p22phox, gp91phox (NOX2) p67phox, p47phox have already been identified in cardiomyocytes, and were found to increase after induction of hypertrophy and heart failure in guinea pig, but also subsequent to myocardial infarction in humans. Unknown however was the relation between intracellular localization of NOX2 in cardiomyocytes and its putative function in this ischemic process. This we have studied in chapter 2.

**Homocysteine and Endothelial Dysfunction**

Since it became evident that HHC increased the risk of atherosclerosis the main focus for in-vitro studies has been endothelial cells (ECs), and to a lesser extent smooth muscle cells (SMCs). Growing evidence suggests that endothelial dysfunction plays a major role in the vascular injury seen in hyperhomocysteinemia. This has been observed in numerous animal models suggesting an involvement of increased oxidative stress with a depletion of bioactive nitric oxide (NO), resulting in impaired endothelial vasodilatation, and an impaired response to endothelium-derived hyperpolarizing factor. It has been shown in several in vitro and in vivo studies that Hcy produces superoxide which can react with NO to form
peroxynitrite, thereby depleting NO$^{44-50}$. Furthermore, Hcy also increases the NO synthase inhibitor ADMA which in turn additionally decreases the available nitric oxide$^{51}$. Finally Hcy decreases the amount of L-arginine which is necessary for the production of NO by endothelial nitric oxide synthase (eNOS)$^{52}$. A direct cytotoxic effect of Hcy on human ECs was found, via induction of necrosis and apoptosis$^{53}$. Several studies have examined the mechanisms of Hcy-induced apoptosis in endothelial cells and some suggested involvement of the JNK pathway. A study by Suhara et al. revealed that Hcy induced endothelial cell apoptosis via NF-κB mediated upregulation of Fas (CD95) expression, which leads to activation of the JNK pathway$^{54}$. Zhang et al. found that Hcy induced apoptosis in endothelial cells through activation of the unfolded protein response (UPR)$^{55}$ which in turn also caused activation of JNK$^{56}$. In a study on microvascular endothelial cells, Tyagi et al. demonstrated that Hcy resulted in ROS production in mitochondria coinciding with apoptosis$^{57}$. Dong et al. however, suggested that Hcy induced oxidative stress and apoptosis via the above mentioned NOX protein$^{58}$.

It has also been suggested that NOX proteins play a pathogenic role in the anatomic and functional changes of the arterial wall occurring in children with premature atherosclerosis$^{59}$. Petry et al. demonstrated that NOX2 and NOX4, but not NOX1, contributed to ROS production by and proliferation of endothelial cells, even under basal conditions$^{60}$. The importance of p47$^{61}$phox as a crucial subunit in the active endothelial NOX complex has also been shown using stimuli such as high glucose$^{61}$, angiotensin$^{62}$, phorbol ester or TNF-α$^{63}$.

Since all these studies point to several mechanisms of action for Hcy induced endothelial dysfunction with a significant role for NOX-mediated ROS production, we wanted to further elucidate the mechanism of Hcy-induced EC apoptosis, which was described in chapter 3.

**Homocysteine and the Heart**

The primary focus in Hcy research has been predominantly on the effect of Hcy on the vasculature. A relation between increased Hcy and myocardial infarction has also been proposed. This is mostly correlated to the pro-atherogenic effect of Hcy on the myocardial vessels and not directly related to a possible detrimental effect on cardiomyocytes themselves$^{64-69}$. The Framingham Heart Study, however, revealed a positive correlation between HHC and new-onset heart failure not related to ischemia. These authors suggested that elevated Hcy levels induced left ventricular hypertrophy$^{70}$. An explanation for this hypertrophy was provided by other in vivo studies that showed that this hypertrophy can be caused directly by inducing cardiac interstitial$^{71}$ as well as replacement fibrosis$^{72}$, the latter of which is indicative for cardiomyocyte loss. Increased levels of Hcy as a risk factor for heart failure have been described by several other studies as well$^{73-77}$. In chapter 4 we have further elucidated the effect of increased Hcy levels on cardiomyocytes to determine whether Hcy indeed has a direct toxic effect on these particular cells themselves.
Homocysteine and Inflammation

A pro-inflammatory effect of Hcy itself has also been shown previously\textsuperscript{78,79}. Increased Hcy stimulates proinflammatory pathways in vascular cells, resulting in leukocyte recruitment to the vessel wall\textsuperscript{80,81}. This is mediated by the expression of adhesion molecules on endothelial cells\textsuperscript{82-84}, circulating monocytes\textsuperscript{85} and neutrophils\textsuperscript{27,86}. Hcy also stimulates the infiltration of leukocytes into the arterial wall mediated by increased secretion of chemokines\textsuperscript{87}, and induces the differentiation of monocytes into cholesterol-scavenging macrophages\textsuperscript{88}.

Next to the endothelium, cardiomyocytes also can undergo pro-inflammatory changes. The plasma membrane of an eukaryotic cell normally has an asymmetrical distribution of the phospholipids and plays an important role as a recognition site for inflammation. The outer leaflet mostly contains the hydrophobic phosphatidylcholine (PC) whereas the inner leaflet predominantly consists of the anionic phosphatidylinerine (PS) and phosphatidyethanolamine (PE). This membrane asymmetry is regulated by several membrane proteins. The ATP-dependent flippase has a preference for PS and translocates it back to the inner leaflet, whereas the ATP-independent scramblase translocates PC to the inner leaflet\textsuperscript{89-92}. Exposure of PS to the outer leaflet of the plasma membrane (so-called membrane flip-flop) is not only found in apoptosis, but also in reversible changed cells, including cardiomyocytes\textsuperscript{93}. It was shown previously that this PS exposure in reversibly changed cells forms a recognition site for inflammation which induces additional damage of cardiomyocytes after myocardial infarction\textsuperscript{94,95}. The amount of studies analysing the mechanisms of this flip-flop is limited. It has been shown that RhoA, a low molecular weight GTP-binding protein which is a member of the Ras superfamily\textsuperscript{96}, participates in PS exposure in megakaryocytes cells\textsuperscript{97}. This was also found by Grounds et al. who found that inhibition of RhoA activity by the RhoA specific inhibitor C3 exoenzyme resulted in a loss of normal cardiomyocyte morphology by inducing annexin V positivity, as a marker for PS exposure\textsuperscript{98}. In chapter 5 we have examined the mechanism of membrane flip-flop in cardiomyocytes as a pro-inflammatory condition in general and studied the role of flippase, RhoA and its downstream effector Rho-kinase\textsuperscript{99} in this respect. In addition we have examined in chapter 6 the mechanism of this PS exposure induced by Hcy in cardiomyocytes.

Homocysteine and Hypomethylation

An increase in Hcy also results in an increase in S-Adenosylhomocysteine (SAH). SAH is known as a potent inhibitor of methylation of DNA, RNA and proteins since it can also bind to methyltransferases but cannot donate a methyl group necessary for methylation to occur\textsuperscript{100-102}. Therefore there is a ongoing debate whether increased Hcy or the related increased SAH is the causative factor in cardiovascular disease. Increased SAH and therefore decreased methylation potential could lead to altered gene expression\textsuperscript{103} and altered cell differentiation\textsuperscript{104}, as reviewed by James et al.\textsuperscript{105}. According to these authors, hypomethylation caused by increased SAH is the main causative factor in HHC induced cardiovascular pathogenesis\textsuperscript{105}.
In addition, previous findings have shown, in patients with vascular disease, that increased plasma Hcy and increased SAH coincident with a decreased SAM/SAH ratio correlated positively with atherosclerotic vascular disease. Finally, Castro et al. have demonstrated in endothelial cells that increased SAH induced hypomethylation of DNA, resulting in malfunctioning gene expression, ultimately promoting endothelial dysfunction. In chapters 7 and 8 we finally have analysed, in endothelial cells and cardiomyocytes, differential effects of Hcy and SAH in the process of apoptosis induction.

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