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REVIEW ARTICLE

Hyperhomocysteinaemia and vascular injury: advances in mechanisms and drug targets

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Homocysteine is a sulphur-containing non-proteinogenic amino acid. Hyperhomocysteinaemia (HHcy), the pathogenic elevation of plasma homocysteine as a result of an imbalance of its metabolism, is an independent risk factor for various vascular diseases, such as atherosclerosis, hypertension, vascular calcification and aneurysm. Treatments aimed at lowering plasma homocysteine *via* dietary supplementation with folic acids and vitamin B are more effective in preventing vascular disease where the population has a normally low folate consumption than in areas with higher dietary folate. To date, the mechanisms of HHcy-induced vascular injury are not fully understood. HHcy increases oxidative stress and its downstream signalling pathways, resulting in vascular inflammation. HHcy also causes vascular injury *via* endoplasmic reticulum stress. Moreover, HHcy up-regulates pathogenic genes and down-regulates protective genes *via* DNA demethylation and methylation respectively. Homocysteinylation of proteins induced by homocysteine also contributes to vascular injury by modulating intracellular redox state and altering protein function. Furthermore, HHcy-induced vascular injury leads to neuronal damage and disease. Also, an HHcy-activated sympathetic system and HHcy-injured adipose tissue also cause vascular injury, thus demonstrating the interactions between the organs injured by HHcy. Here, we have summarized the recent developments in the mechanisms of HHcy-induced vascular injury, which are further considered as potential therapeutic targets in this condition.

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Abbreviations

ADMA, asymmetric dimethylarginine; Ang II, angiotensin II; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DNMT, DNA methyltransferase; ECs, endothelial cells; FABP4, fatty acid-binding protein 4; GRP78, glucose-regulated protein 78; Hcy-TL, homocysteine thiolactone; HHcy, hyperhomocysteinaemia; HO-1, haem oxygenase-1; IGF2, insulin-like growth factor 2; MMP, matrix metalloprotease; MS, methionine synthase; MT, methyltransferase; MTHFR, methylene tetrahydrofolate reductase; NOXs, NADPH oxidases; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; sEH, soluble epoxide hydrolase; THF, tetrahydrofolate; VSMCs, vascular smooth muscles cells

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Introduction

Homocysteine is a non-essential, sulphur-containing, non-proteinogenic amino acid. It was first isolated from a urinary bladder stone in 1933 by Vincent du Vigneaud, who later received the Nobel Prize in Chemistry, in 1955. Under normal conditions, the concentration of homocysteine ranges from 5 to 10 μM in human plasma but does not exceed 15 μM (Seshadri *et al.*, 2002). Elevation of the plasma levels of homocysteine above 15 μM is defined as hyperhomocysteinaemia (HHcy) (Ji and Kaplowitz, 2004). Plasma homocysteine exists in three different forms defined as free homocysteine, protein-bound homocysteine and oxidized forms of homocysteine (Jakubowski, 2008). Among these forms, the majority of homocysteine exists in the protein-bound form and is *N*-linked and *S*-linked to γ-globulins or albumins, accounting for 80–90% of the total plasma homocysteine. Moreover, approximately 10–20% of total homocysteine is in the oxidized form, presented as homocysteine-**cysteine (Cys)** and homocysteine-homocysteine dimers in plasma. As a result, less than 1% of total plasma homocysteine exists as the free, reduced amino acid (Jakubowski, 2002).

In 1969, a Harvard pathologist, Kilmer McCully, reported that two children with homocystinuria, a genetic disorder characterized by the existence of homocysteine in the urine as a result of severe HHcy (>100 μM), displayed vascular pathologies (McCully, 1969). Among these children, one 2-month-old boy had an advanced stage of arteriosclerosis that closely resembled that observed in elderly individuals with advanced cardiovascular disease. The other child, an 8-year-old, died of a stroke related to arteriosclerosis in the carotid artery. These were the first suggestions that elevated plasma homocysteine was a potential cause of premature vascular disease. In 1976, a human population study, which included 25 patients with coronary artery disease and 22 control subjects with normal coronarography, first identified higher homocysteine levels as a result of abnormalities in methionine (Met) metabolism in patients (Wilcken and Wilcken, 1976). To date, a substantial amount of data from case–control cohort studies and meta-analyses support the association of HHcy with a range of vascular diseases, including hypertension, ischaemic stroke, aortic aneurysm and dissection, coronary heart disease, coronary artery calcification, vascular dementia and cervical artery dissection. as HHcy has also been identified as a risk factor for these diseases (Fallon and Ben-Shlomo, 2003; Ravaglia *et al.*, 2005; Takagi and Umemoto, 2005; Holmes *et al.*, 2011; Clarke *et al.*, 2012; Liu *et al.*, 2012, 2016; Luo *et al.*, 2014; Kim *et al.*, 2015; Wang *et al.*, 2015b).

Homocysteine metabolism

In this review, homocysteine metabolism includes both the production and the disposal of this amino acid and the normal physiological balance between these two pathways maintains plasma homocysteine at its physiological level (<15 μM). Thus, HHcy is the result of increased production and/or decreased disposal of homocysteine.

Homocysteine is produced in all human tissues, by a single pathway, through the transmethylation of the essential amino acid **Met**. The process of Met transmethylation involves three steps sequentially catalysed by **S-adenosyl-L-methionine** (SAM) synthase, methyltransferase (MT) and **S-adenosyl-L-homocysteine** (SAH) hydrolase. SAM synthetase catalyses the reaction of Met with ATP to form SAM. SAM is converted into SAH *via* an MT-catalysed methyl transfer reaction. Finally, SAH is rapidly metabolized by SAH hydrolase to **adenosine** and homocysteine (Skovierova *et al.*, 2016). Homocysteine production may be enhanced by increased intake of Met-rich protein, which results in HHcy. Mice fed a high Met diet provide a widely used animal model of HHcy (Yang *et al.*, 2015a) and, clinically, limiting Met intake improved HHcy in patients (Wang *et al.*, 2015b).

Compared with the production of homocysteine, its disposal involves many pathways. First, approximately 50% of homocysteine is re-methylated to form Met *via* two distinct mechanisms, folate/vitamin B12-dependent and folate/vitamin B12-independent re-methylation. Folate in the form of **N-5-methyl tetrahydrofolate** (THF), derived from *N*-5,10-methylene tetrahydrofolate reductase (MTHFR)-catalysed THF modification, donates a methyl group to homocysteine in the re-methylation catalysed by the vitamin B12-dependent enzyme methionine synthase (MS) (Castro *et al.*, 2006). In the other mechanism, betaine derived from choline by betaine–homocysteine *S*-methyltransferase also behaves as a methyl group donor and contributes to the folate/vitamin B12-independent re-methylation of homocysteine to form Met (Skovierova *et al.*, 2016). Second, homocysteine is resynthesized into SAH through the reversal of SAH hydrolase activity. High concentrations of SAH strongly inhibit the transmethylation of Met because SAH is a potent allosteric inhibitor of MT (Kerr, 1972). Third, homocysteine is metabolized to form Cys *via* trans-sulphuration, sequentially catalysed by the vitamin B6-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Skovierova *et al.*, 2016). These trans-sulphuration enzymes CBS and CSE, exhibit other enzymic activities that contribute to the biosynthesis of the gasotransmitter **H_2S**, from homocysteine and/or **Cys** (Weber *et al.*, 2016). Deficiencies in re-methylation and trans-sulphuration are the main causes of the accumulation of homocysteine (Figure 1). A dietary deficiency of folate and vitamin B12 or a dysfunctional mutation in MTHFR also inhibits homocysteine re-methylation, whereas insufficient intake of vitamin B6 or CBS deficiency impairs the trans-sulphuration of this amino acid (Chen *et al.*, 2001; Fohr *et al.*, 2002). The MTHFR and CBS genes have been reported to be the genetic determinants in severe HHcy and homocystinuria (Hannibal and Blom, 2017). CBS-deficient mice provide a model of HHcy that can be used for mechanistic studies (Zhang *et al.*, 2012b). The polymorphisms MTHFR C677T, MTHFR A1298C and MS A2756G, are the critical dominant negative mutations and they jointly add to the risk of folate deficiency, which results in HHcy in humans (Li *et al.*, 2015). The MTHFR C677T polymorphism has been identified as a risk locus in patients with coronary heart disease, coronary artery calcification, cervical artery dissection, aortic aneurysm and dissection (Fallon and Ben-Shlomo, 2003; Takagi and Umemoto, 2005; Holmes *et al.*, 2011; Clarke *et al.*, 2012; Liu *et al.*, 2012; Luo *et al.*, 2014; Kim *et al.*, 2015; Wang *et al.*, 2015b). To further assess the causality of this single-nucleotide polymorphism in HHcy-related vascular injury and disease, Mendelian randomization studies have

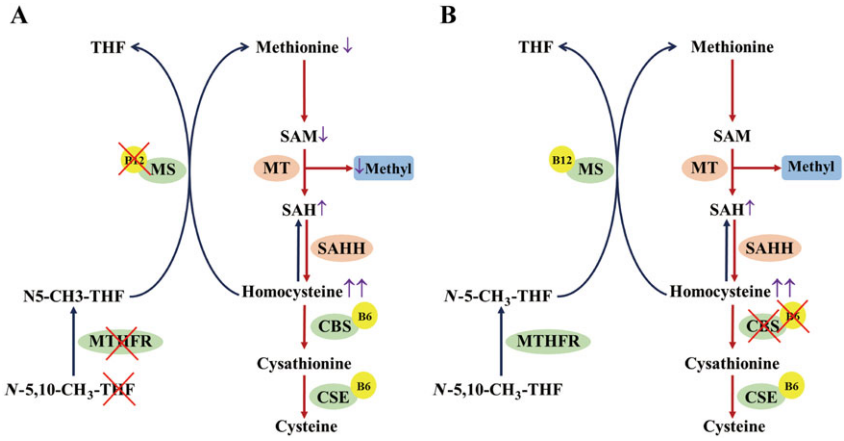

Figure 1

(A) Schematic overview of homocysteine metabolism changes in HHcy induced by re-methylation (i) or trans-sulphuration (ii) deficiency. Dietary deficiency of folate (THF) and vitamin B12 or a MTHFR dysfunctional mutation inhibits the re-methylation of homocysteine into Met and induces homocysteine accumulation. Low levels of Met decrease SAM production, while elevated homocysteine levels contribute to SAH production, catalysed by *S*-adenosylhomocysteine hydrolase (SAHH). Thus, the MT-mediated transformation of SAM into SAH markedly decreases, leading to the lack of methyl donors. (B) Insufficient intake of vitamin B6 or CBS deficiency impairs the trans-sulphuration of homocysteine and leads to the elevation of this amino acid. Increased levels of homocysteine induce enhanced SAH production, catalysed by SAHH. Because homocysteine re-methylation is only marginally affected, SAM and methyl donor production is not significantly influenced. B6/B12, vitamin B6/B12.

been carried out by several groups. The results showed that HHcy induced by MTHFR C667T did not have a causal role in high systolic BP, cardiovascular disease mortality, coronary heart disease or ischaemic stroke (Bentley *et al.*, 2010; Clarke *et al.*, 2012; Yang *et al.*, 2012; Borges *et al.*, 2016). However, there are important limitations in these Mendelian randomization analyses (Smulders and Blom, 2011). For instance, it could not be guaranteed that all potential confounders had been excluded; that an unknown indirect association may exist between MTHFR C677T and HHcy and, finally, that genetic heterogeneity, pleiotropy, population stratification and canalization may also be involved in the process of the study. Thus, the outcomes of the Mendelian randomization studies do not provide strong enough evidence to reject the pathogenic effects of homocysteine on vascular injury.

The mechanisms of vascular injury induced by HHcy

HHcy displays a close relationship with many vascular diseases, even though the underlying mechanisms are not fully understood. Vascular remodelling, the architectural alteration of the vascular wall, contributes substantially to the pathogenesis of vascular injury. The vascular wall is composed of the intima, media and adventitia, with endothelial cells (ECs) constituting the main cellular fraction of the vascular intima. These cells regulate the vascular tone and maintain the inflammatory balance. Endothelial dysfunction, defined as a disorder of endothelial-dependent dilation, as well as endothelial activation, which refers to the overexpression of adhesion molecules, are two major pathological changes taking place in ECs in vascular diseases. Vascular smooth muscles cells (VSMCs) located in the media exhibit an extensive plasticity in which fully differentiated VSMCs may de-differentiate from a contractile phenotype into other phenotypes in physiological processes, including vascular remodelling and angiogenesis, and in pathological processes, such as hypertension, atherosclerosis and vascular calcification. The de-differentiation of VSMCs, defined as the alteration in contractile genes and functions, contributes to vascular remodelling. The vascular adventitia mainly consists of adventitial fibroblasts. Over the last 10 years, an outside-in vascular injury model has emerged, which suggests an initiating role for the adventitial layer in vascular injury (Stenmark *et al.*, 2013; Majesky, 2015). Most vascular injuries involve vascular inflammation through the interaction between vascular resident cells and activated inflammatory cells. Circulating leukocytes, including monocytes/macrophages, lymphocytes and neutrophils, are all involved in the pathogenesis of vascular injuries. Several *in vitro* and *in vivo* studies, using homocysteine at pathogenic concentrations and HHcy animal models, respectively, have demonstrated that homocysteine induces pathogenic effects on both vascular resident cells and circulating leukocytes.

Oxidative stress in HHCy

Oxidative stress as a result of ROS accumulation is the major mechanism that mediates homocysteine-induced vascular injury (Tyagi *et al.*, 2005). In addition to the interaction of homocysteine with Cys to form a disulphide bond, homocysteine also directly inhibits the activity of antioxidants, thereby disrupting SOD, activating NADPH oxidases (NOXs) and subsequently producing superoxide anion, causing an accumulation of ROS. The generated ROS further activate the transcriptional activity of NF-κB, which results in the expression of pro-inflammatory genes and vascular inflammation (Skovierova *et al.*, 2016) (Figure 2).

ROS production. Homocysteine at a pathogenic concentration leads to significant ROS production in both vascular resident cells and circulating leukocytes

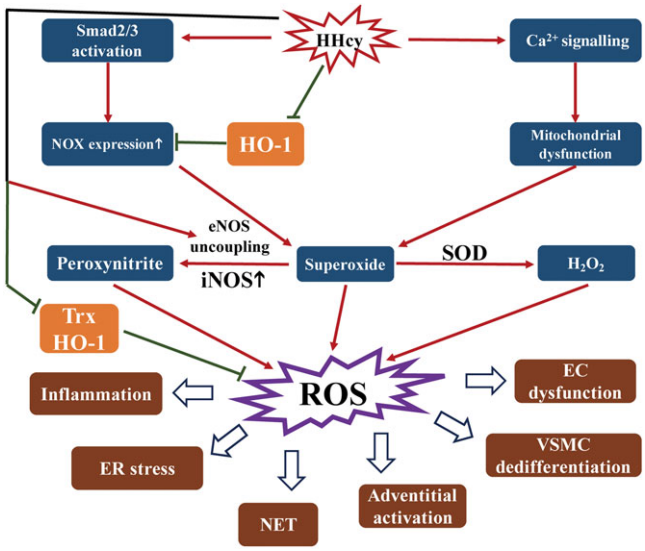

Figure 2

Schematic overview of HHcy-induced oxidative stress. HHcy up-regulates NOX expression by Smad2/3 activation, while HHcy induces mitochondrial dysfunction *via* activating calcium signalling. Both these effects of HHcy result in superoxide production. Superoxide is converted to H_2O_2 catalysed by SOD. Moreover, HHcy up-regulates iNOS and enhances eNOS uncoupling, which further induces peroxynitrite formed by NO and superoxide. Superoxide, H_2O_2 and peroxynitrite all are ROS. HHcy also inhibits the activity of antioxidants, such as thioredoxin (Trx) and HO-1, to up-regulate NOX expression and attenuate antioxidant-mediated elimination of ROS. NET, neutrophil extracellular trap.

(Zhang *et al.*, 2001; Zhang *et al.*, 2002). The mechanisms underlying the generation of ROS by homocysteine includes the up-regulation of NOX, mitochondrial dysfunction, abnormal NO generation and inhibition of antioxidants, as discussed below in more detail.

NADPH oxidase. NOXs are enzymes that mediate ROS production. Homocysteine increases NOX2 and NOX4 expression in ECs (Lai and Kan, 2015) and, in adventitial fibroblasts, up-regulates the expression of NOX4 *via* Smad2/3 activation, *in vitro* (Liu *et al.*, 2012). Furthermore, ROS production induced by homocysteine is blocked by NOX inhibitors in VSMCs, monocytes/macrophages, neutrophils and lymphocytes, which indicates that NOX also contributes to homocysteine-induced ROS production in these cells (Zeng *et al.*, 2003; Dai *et al.*, 2008; Zanin *et al.*, 2015).

Mitochondrial dysfunction. Mitochondria are an important source of ROS within most mammalian cells. Superoxide, the proximal mitochondrial ROS, further forms peroxynitrite by interacting with nitrate and is the substrate for SOD to form **H_2O_2**. In general, superoxide accumulates in mitochondria following the mitochondrial disorders that exhibit dysfunctions of oxidative phosphorylation and the respiratory chain (Murphy, 2009). In ECs, homocysteine at a pathological concentration leads to mitochondrial toxicity. First, homocysteine induces an elevated Ca^{2+} level that impairs mitochondrial function. Second, homocysteine enhances the mitochondrial dehydrogenase activity, which suggests increased oxidation in mitochondria, whereas the down-regulation by homocysteine of nitrate and SOD2 leads to a decreased consumption of superoxide. Third, homocysteine decreases mitochondria membrane potentials and ATP production, which suggests the dysfunction of the respiratory chain and accumulation of superoxide (Kamat *et al.*, 2015).

Abnormal NO generation. In general, endothelial NOS (**eNOS**) plays a vasoprotective role by producing NO in ECs. homocysteine inhibits eNOS-mediated NO production, as well as inducing eNOS uncoupling, both of which synergistically generate peroxynitrite production derived from superoxide and NO. Moreover, homocysteine up-regulates **iNOS** in ECs and VSMCs, which further increases peroxynitrite production (Welch *et al.*, 1998; Wang *et al.*, 2000).

Antioxidant levels. Inhibition or down-regulation of antioxidants is involved in the process of HHcy-related vascular injury and remodelling. Homocysteine markedly down-regulates the expression of the antioxidant proteins, thioredoxin and **haem oxygenase-1** (HO-1), which also contribute to oxidative stress (Sawle *et al.*, 2001; Dong *et al.*, 2005). Moreover, the antioxidant compound *N*-acetyl cysteine (NAC) and thioredoxin markedly inhibited the generation of ROS induced by homocysteine in monocytes/macrophages (Zeng *et al.*, 2003; Dai *et al.*, 2008; Zanin *et al.*, 2015). Piceatannol, a resveratrol analogue, protected ECs against homocysteine-induced apoptosis, oxidative stress and endoplasmic reticulum (ER) stress. Moreover, piceatannol markedly increases HO-1 expression *via* the activation of the transcription factor Nrf2. The inhibitory effects of piceatannol on apoptosis, ROS generation and ER stress were blocked by silencing HO-1 and were mimicked by treating the ECs with the HO-1 inducer, haemin. These results suggest the antioxidant enzyme HO-1 may protect ECs against apoptosis, oxidative stress and ER stress induced by homocysteine (Sawle *et al.*, 2001).

Effects of ROS generated by homocysteine. The major effect of ROS-induced oxidative stress is to induce inflammatory responses, mediated particularly by NF-κB, downstream of ROS. In addition to inflammation, homocysteine-induced oxidative stress contributes to dysfunction of EC, differentiation of VSMCs, adventitial activation and formation of the neutrophil extracellular trap.

Inflammation. Inflammation is the major pathogenic process involved in several vascular injuries and diseases, and homocysteine is a potent inducer of inflammatory interactions between vascular resident cells and leukocytes. NF-κB, **ERK1/2** and redox factor 1, as downstream targets of ROS, as well as **PKC** and **calmodulin** as signalling molecules upstream of ROS, are involved in the secretion of inflammatory cytokines, induced by homocysteine, from monocytes/macrophages in atherosclerosis (Zeng *et al.*, 2003; Dai *et al.*, 2006; Zanin *et al.*, 2015). In ECs,

homocysteine up-regulated **VCAM-1** and matrix metalloprotease (**MMP)-9** *via* NF-κB signalling, both *in vitro* and *in vivo* (Au-Yeung *et al*., 2004). Moreover, homocysteine induced production and secretion of the chemokine CCL2, IL-8 and **IL-1β** from monocytes/macrophages which was blocked by inhibitors of NOX or antioxidants, such as NAC and thioredoxin, indicating that oxidative stress was involved in this effect of homocysteine (Zeng *et al*., 2003; Dai *et al*., 2008; Zanin *et al*., 2015). Furthermore, homocysteine-induced oxidative stress potentiated the proliferation and inflammatory activation of **concanavalin A**-elicited splenic T-lymphocytes *in vitro*, which implied overactivation of T-lymphocytes (Zhang *et al*., 2002).

In addition to these *in vitro* studies, several *in vivo* studies have also demonstrated that HHcy activates inflammation partly *via* increased oxidative stress. CBS-deficient mice exhibit HHcy as a result of a disorder of homocysteine trans-sulphuration. *In vivo* studies have shown that CBS deficiency-induced HHcy increases inflammatory monocyte ($ly6C^{high}$) differentiation, a process dependent on superoxide anion production, and HHcy-activated monocytes produce TNF-α, **IL-6** and CCL2, which exacerbates atherosclerosis in mice (Zhang *et al*., 2009, 2012b). Interestingly, CBS-mediated trans-sulphuration of homocysteine to Cys also plays a critical role in the macrophage-involved host defence against invading pathogens. Inhibition of the trans-sulphuration pathway by **propargylglycine** impairs pathogen clearance by macrophages (Garg *et al*., 2006). Recently, pyruvate kinase 2-dependent metabolic reprogramming has been demonstrated to mediate proliferation and IgG secretion of B cells induced by homocysteine and HHcy-exacerbated atherosclerosis (Deng *et al*., 2017). In apolipoprotein E-deficient ($ApoE^{-/-}$) mice with HHcy, treatment with CTLA**4**-IgG attenuated the atherosclerotic lesions, by competing with CD28 to inhibit T-lymphocyte overactivation (Ma *et al*., 2013). Moreover, regulatory T-cells ameliorated HHcy-exacerbated atherosclerosis in $ApoE^{-/-}$ mice by inhibiting the inflammatory activation of T-lymphocytes (Feng *et al*., 2009). In contrast, homocysteine, *in vitro,* inhibited proliferation of concanavalin A-elicited blood T-lymphocytes *via* induction of DNA fragmentation, which is suggestive of immunosenescence (Picerno *et al*., 2007). Homocysteine also promoted LPS-induced B-lymphocyte proliferation and IgG secretion dependent on the PKC–ROS–p38MAPK–NF-κB pathways. Treatment with the **liver X receptor** agonist **T0901317** decreased formation of ROS, activation of NF-κB and secretion of IgG, induced by homocysteine, in B-lymphocytes *in vitro* (Zhang *et al*., 2001; Chang *et al*., 2007).

EC dysfunction. Dysfunction of ECs is usually assessed as impaired NO production by eNOS. High levels of ROS inhibit the enzymic activity of **dimethylarginine dimethylaminohydrolase**, which results in an accumulation of **asymmetric dimethylarginine** (ADMA). As a competitor of the normal eNOS substrate **L-arginine**, ADMA can bind to eNOS and inhibit its activity (Dayal and Lentz, 2005). Homocysteine-induced ROS also decrease the activity of the **L-arginine transporter CAT-1** in ECs (Jin *et al*., 2007). A lack of L-arginine causes eNOS uncoupling (Forstermann and Munzel, 2006), which further impairs NO production and enhances ROS production.

VSMC de-differentiation. VSMC de-differentiation is mainly regulated by homocysteine-generated oxidative stress. ROS production, induced by this amino acid, up-regulated CCL2 and **MMP-2** *via* the NF-κB pathway in VSMCs, and led to VSMC de-differentiation into an inflammatory and secretory phenotype with a high proliferation rate (Wang *et al*., 2000; Ke *et al*., 2010). In addition to oxidative stress, homocysteine increased VSMC proliferation *via* a ROS-independent pathway (Taha *et al*., 1999). Moreover, this amino acid increased **caspase-3**-mediated VSMC apoptosis induced by other pathogenic stimulators, such as beta amyloid (Mok *et al*., 2002).

Adventitial activation. The adventitia plays a deleterious role *via* the production of NOX-derived ROS and recruitment of inflammatory cells involved in vascular injury and disease (Meijles and Pagano, 2016). HHcy induced by drinking water supplemented with homocysteine, enhanced aortic adventitial inflammation in $ApoE^{-/-}$ mice, caused by infused angiotensin II (Ang II), as shown by increased macrophage infiltration, IL-6 and CCL2 production, and MMP proteolysis activity, preferentially in the tunica adventitia. Correspondingly, the incidence of **Ang II**-induced abdominal aortic aneurysm (AAA) and aortic dissection in $ApoE^{-/-}$ mice was increased by such supplementation with homocysteine. Mechanistically, administration of a NOX4 inhibitor or siRNA abolished the homocysteine-induced adventitial activation (Liu *et al*., 2012). Moreover, NOX4 deficiency inhibited AAA formation-induced by Ang II in mice with hyperphenylalaninaemia and uncoupled eNOS (Siu *et al*., 2016). Thus, NOX4 mediates the adverse effects of homocysteine in adventitial inflammation and AAA formation (Liu *et al*., 2012). Furthermore, HHcy in rats, induced by a high Met diet, potentiated balloon injury-induced adventitial hyperplasia and collagen I deposition. Mechanistically, homocysteine inhibits MMP-2 activity, which thus decreases collagen I degradation, and favours its deposition in adventitial fibroblasts *in vitro* (Guo *et al*., 2008). Furthermore, HHcy-aggravated adventitial hyperplasia and collagen I deposition were reversed by the angiotensin AT_1 receptor antagonist, **valsartan**, *in vivo*, implying the involvement of these receptors in the pathogenic effects of homocysteine on adventitial remodelling (Yao and Sun, 2014).

Neutrophil extracellular trap. Neutrophil recruitment in the vascular wall is considered to be the early stage of inflammation related to atherosclerosis and aneurysm. Recently, a human study demonstrated that elevated homocysteine levels in type 2 diabetes induce the formation of neutrophil extracellular traps, a network of fibres composed of neutrophil DNA and proteins which bind and kill pathogens, without phagocytosis. Such traps are considered to represent an inflammatory activation of neutrophils. Mechanistically, homocysteine induces superoxide anion generated by NOX, which subsequently leads to the activation of ERK1/2 and **Akt** signalling related

to the formation of neutrophil extracellular traps (Joshi *et al.*, 2016). Furthermore, homocysteine promotes the chemotaxis and migration of human peripheral neutrophils *via* NOX-derived ROS production and subsequent activation of ERK1/2 (Alvarez-Maqueda *et al.*, 2004).

Potential cell surface receptors for homocysteine. Both groups of glutamate receptors, **AMPA** as well as **NMDA receptors**, have been identified as potential cell surface receptors for homocysteine. NMDA receptors are expressed in ECs, VSMCs, monocytes/macrophages, lymphocytes (Boldyrev *et al.*, 2012) and neutrophils (Bryushkova *et al.*, 2011; Boldyrev *et al.*, 2012) (Figure 3). An NMDA receptor antagonist blocked oxidative stress induced by homocysteine in various types of cells (Boldyrev *et al.*, 2013). Inhibition of NMDA signalling by a receptor antagonist or the relevant siRNA blocked homocysteine-induced proliferation and mitochondrial toxicity in ECs (Chen *et al.*, 2005; Kamat *et al.*, 2015). Moreover, homocysteine decreases claudin-5 expression in brain microvascular ECs *via* nuclear translocation of **β-catenin**, which further results in the disruption of the cell junction in the EC layer *in vitro* and the increased permeability of the blood–brain barrier in CBS-deficient mice *in vivo*. This homocysteine-induced EC injury was rescued by an NMDA receptor antagonist (Qureshi *et al.*, 2005). In murine macrophages, homocysteine enhanced **COX-2** expression by ROS generated *via* NMDA receptor-mediated calcium signalling pathways, as shown by the inhibition of these homocysteine-induced effects by the NMDA receptor inhibitor MK-801 or NMDA receptor siRNA (Lee *et al.*, 2013). In lymphocytes, homocysteine-induced ROS production and inflammatory cytokine release *in vitro* were attenuated by MK-801, implying that NMDA receptors may also mediate the effect of homocysteine in these cells (Boldyrev, 2005; Vladychenskaya *et al.*, 2011). However, most of these experiments have been performed *in vitro* and *in vivo* studies are required for confirmation. The possibility that other cell surface receptors are involved in the effects of homocysteine has still to be investigated. As homocysteine is not readily radiolabelled, because of the lack of a phenolic hydroxyl group and its ability to be easily oxidized (Tsen *et al.*, 2003; Yang *et al.*, 2015b), no direct evidence of the interaction of homocysteine with possible receptors, for example, ligand–receptor binding assays, is available to date.

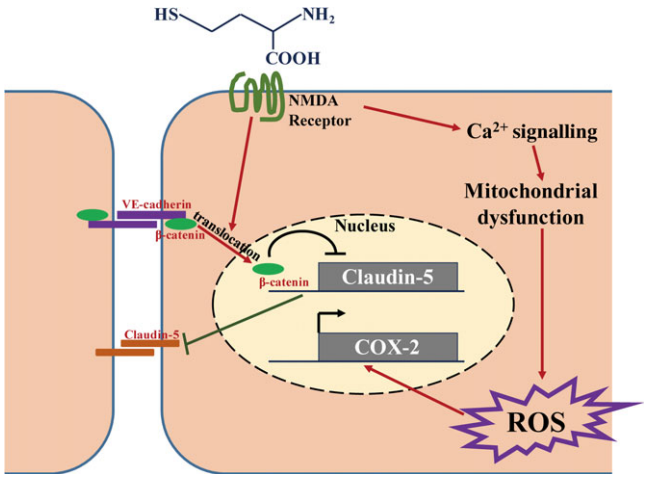

Figure 3

NMDA receptors may act as the cell surface receptor for homocysteine. NMDA receptor mediates homocysteine-induced ROS production by activating calcium signalling and impairing mitochondrial function. ROS accumulation further up-regulates COX-2 gene transcription. Homocysteine also leads to the nuclear translocation of β-catenin through NMDA receptors. The nuclear translocation of β-catenin results in disruption of vascular endothelial (VE)-cadherin–β-catenin interaction and inhibition of claudin-5 transcription. Thus, the tight junction of cells is damaged as the homophilic interactions of VE-cadherin and claudin-5 are reduced.

ER stress in HHcy

The ER is the site in which newly synthesized proteins, undergoing post-translational modifications, fold into the correct conformation. Only correctly folded proteins are exported to the Golgi apparatus, whereas misfolded proteins are retained in the ER for refolding or ER-associated degradation. When unfolded and aggregated proteins accumulate in the ER they cause "ER stress" (Binet and Sapieha, 2015) and ER stress is known to be involved in the pathogenesis of vascular diseases. Homocysteine is known to elicit ER stress (Figure 4), as demonstrated by its up-regulation of the expression of ER stress response genes, including those for glucose-regulated protein 78 (GRP78), activating transcription factor-6 (ATF6), **protein kinase RNA-like endoplasmic reticulum kinase (PERK)**, ATF4, IRE1α and XBP1 spliced form (Outinen *et al.*, 1999; Hossain *et al.*, 2003). ER stress elicited by homocysteine mediates EC apoptosis and death and further accelerates the development of atherosclerosis in mice (Hossain

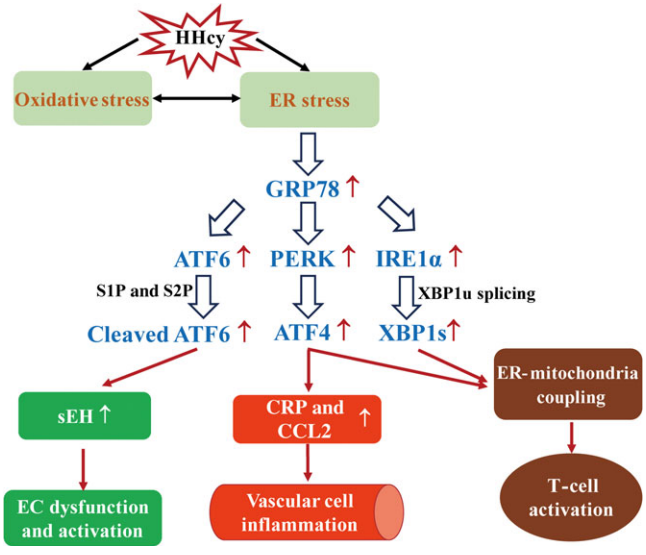

Figure 4

Schematic overview of HHcy-induced ER stress. HHcy-induced ER stress is mediated by oxidative stress. Conversely, HHcy can directly contribute to ER stress, which further leads to oxidative stress. Cleaved ATF6, ATF4 and X-box binding protein 1, spliced form (XBP1s) transcriptionally regulates their downstream genes separately. S1P, site 1 protease; S2P, site 2 protease; XBP1u, X-box binding protein 1, unspliced form.

et al., 2003; Kim *et al*., 2007). Furthermore, ER stress elevates both intracellular and plasma membrane cholesterol, which is mediated by the inhibition of **neutral sphingomyelinase 2** activity in ECs. Vasorelaxation is mediated by the **K_{Ca}3.1 (IK_{Ca})** and SK_{Ca} ion channels, and NO production derived from ECs. Homocysteine-induced ER stress impairs NO production, IK_{Ca} and SK_{Ca} channels, as well as NOX-generated ROS (Chaube *et al*., 2012; Wang *et al*., 2015a). Another target of homocysteine is soluble epoxide hydrolase (sEH), an enzyme that hydrolyses epoxyeicosatrienoic acids and attenuates their vasorelaxing and protective effects in ECs. Treatment of cultured human ECs with homocysteine dose- and time-dependently up-regulated sEH mRNA and protein, which was associated with up-regulation of adhesion molecules and activation of ATF6. Mechanistically, ATF6 binds to the promoter of sEH and up-regulates its expression. Consequently, siRNA knockdown of ATF6α blocks and ATF6 overexpression mimicks the effect of homocysteine on sEH up-regulation, indicating that ATF6 activation is involved in homocysteine-induced sEH expression and endothelial activation (Zhang *et al*., 2012c). Similarly, in VSMCs, homocysteine causes ER stress, as indicated by an up-regulation of GRP78 and PERK activation, and further enhances the secretion of the inflammatory cytokines CRP and CCL2 (Wang *et al*. 2000; Pang *et al*., 2014). In lymphocytes, homocysteine enhanced ER stress, as indicated by PERK activation and IRE1α-spliced XBP1, and inhibition of ER stress with 4-phenylbutyric acid blocked T-cell activation induced by homocysteine. Mechanistically, homocysteine increased ER–mitochondria coupling, and uncoupling ER from mitochondria with the microtubule inhibitor nocodazole attenuated homocysteine-stimulated mitochondrial reprogramming, IFN-γ secretion and proliferation in T-cells. These results suggest that juxtaposition of the ER and mitochondria is required for homocysteine-promoted mitochondrial function and T-cell activation *in vitro* (Feng *et al*., 2016). Furthermore, as previously described, the inhibition of oxidative stress or ROS production relieves ER stress, which suggests homocysteine-induced ROS production mediates ER stress (Kil *et al*., 2017). Interestingly, homocysteine thiolactone (Hcy-TL) directly up-regulates GRP78 expression and induces ER stress independent of *N*-homocysteinylation and subsequently leads to oxidative stress and endothelial inflammation (Wu *et al*., 2015). Overall, homocysteine-induced ER stress and oxidative stress constitute a mutually reinforcing system.

Epigenetic modification in HHcy

Homocysteine also exerts its pathogenic effects *via* increasing methylation (Kamat *et al*., 2016). SAM and SAH derived from the homocysteine metabolic pathway regulate DNA methylation. SAM serves as a methyl donor *via* transmethylation into SAH. HHcy-related MS or MTHFR deficiency inhibits the transformation of homocysteine into Met and further decreases SAM production, whereas the accumulation of homocysteine leads to the formation of SAH from homocysteine, thus substantially elevating the level of SAH, which further inhibits the transmethylation of SAM (Kamat *et al*., 2016). Taken together, the SAM/SAH ratio, reduced by the elevation of SAH and decrease of SAM, corresponds with the levels of global hypomethylation in cells. In apparent contradiction, HHcy was associated with a reduced SAM/SAH ratio but not global hypomethylation in CBS-deficient mice (Lee *et al*., 2017). One possible explanation is that the level of SAM did not decrease but rather increased as a result of the functional MS and MTHFR. Moreover, homocysteine also directly regulates the MT ,e.g. DNA methyltransferases (DNMTs), activity, which causes DNA, RNA and histone methylation (Castro *et al*., 2005; Yu *et al*., 2009).

SAM/SAH ratio. The decrease of the SAM/SAH ratio is related to reduced DNA methylation under conditions of HHcy. HHcy-induced SAH elevation and SAM reduction contribute to the genome-wide hypomethylation in homocysteine-treated VSMCs (Yideng *et al*., 2007) and in rats with HHcy induced by a high Met diet, there was DNA hypomethylation in aortic tissue (Jiang *et al*., 2007a), as demonstrated by a high-throughput quantitative methylation assay. Furthermore, an HHcy-attenuated SAM/SAH ratio leads to demethylation of DNA for human telomerase reverse transcriptase and p21 and subsequent up-regulation of the expression of these two genes, which contributes to the shortening of the telomere length and cell senescence in ECs both *in vitro* and *in vivo* (Wang *et al*., 1997; Zhang *et al*., 2015). In VSMCs, expression of PDGF, an inducer of VSMC proliferation, is up-regulated as a result of DNA demethylation induced by the HHcy-reduced SAM/SAH ratio (Han *et al*., 2014). Moreover, SAM and SAH exhibit directly opposite effects on VSMC proliferation and migration and neointimal formation *in vivo*. SAM protects against neointimal formation *via* the inhibition of ER stress and inflammation of VSMCs in obese diabetic rats (Lim *et al*., 2011), whereas SAH induces the proliferation and migration of VSMCs and enhances atherosclerotic lesions through an oxidative stress-ERK1/2 pathway in $ApoE^{-/-}$ mice (Luo *et al*., 2012).

Regulation of DNA methylation. DNA methylation is one of several epigenetic mechanisms that cells employ to control gene expression. DNA methylation occurs at the cytosine bases of eukaryotic DNA, which are converted to 5-methylcytosine by DNMT enzymes. The altered cytosine residues are typically adjacent to a guanine nucleotide as CpG islands, which results in two methylated cytosine residues placed diagonally to each other on opposing DNA strands. In the human genome, 75% of all promoters are within CpG islands. Thus, the methylated cytosine typically leads to the decrease of interaction between promoters and transcriptional regulators (including transcriptional factors, suppressors and repressors). This decreased interaction results in the repression of transcription, whereas the blockage of suppressors and repressors that interacts with methylated promoter elements alternatively up-regulates gene expression (Robertson, 2005; Edwards *et al*., 2017). In addition, DNA methylation brings about the deacetylation of histone H4 and methylation of Lys^9 of histone H3 (H3-Lys^9) and prevents the methylation of Lys^4 of histone H3 (H3-Lys^4), thus generating a chromatin structure identical to that of methylated sequences in the genome (Hashimshony *et al*., 2003). HHcy has been reported to contribute to vascular injury and remodelling by directly

modulating DNMT activity and the corresponding gene expression.

The amino acid, homocysteine, down-regulates **DNMT1** activity and further induces DNA demethylation. Thus, down-regulation of DNMT1 by homocysteine enhanced PDGF and sEH expression by increasing the binding of ATF6 and SP-1 to the demethylated promoter of these genes in ECs. The increased expression of PDGF and sEH subsequently results in the degradation of EC-derived vasodilator epoxyeicosatrienoic acids and VSMC proliferation (Zhang *et al.*, 2012c,b). Both *in vitro* and *in vivo* studies have further demonstrated that DNMT1-mediated demethylation of the gene for **fatty acid-binding protein 4 (FABP4)**, induced by homocysteine at a pathogenic concentration, or by HHcy, induced by a high Met diet, up-regulate FABP4 expression, which leads to intracellular cholesterol accumulation in THP-1 monocyte-derived macrophages (Jiang *et al.*, 2016) and accelerates atherosclerosis in ApoE$^{-/-}$ mice (Yang *et al.*, 2015a) respectively. However, the inhibition of DNMT1 by homocysteine attenuates the expression of cyclin A as a result of the enhanced transcriptional suppressors bound to the demethylated promoter element and subsequently mediates homocysteine-inhibited EC growth (Jamaluddin *et al.*, 2007).

In contrast, homocysteine has been reported to increase DNMT activity and induce DNA methylation. Interestingly, this amino acid decreases the expression of the protective genes DDAH2 and p66shc through DNA methylation dependent on DNMT1 or DNMT3b activity. The down-regulation of DDAH2 and p66shc mediates homocysteine-induced ROS production, dysfunctional NO production and apoptosis in ECs (Zhang *et al.*, 2007; Kim *et al.*, 2011; Jia *et al.*, 2013). Moreover, homocysteine down-regulates the expression of FGF2 *via* DNA methylation and further inhibits EC growth and survival *via* the G_i protein pathway (Chang *et al.*, 2008). Similarly, as observed in homocysteine-treated VSMCs, the up-regulated **DNMT3a** (Zhang *et al.*, 2016) and DNMT3b increase p53 DNA methylation and decrease its gene expression, which further contributes to VSMC proliferation (Cao *et al.*, 2016). Furthermore, in an **insulin-like growth factor 2 (IGF2)**/H19 imprinting system of VSMCs (Sasaki *et al.*, 2000), homocysteine exhibits an inductive effect of methylation on the H19 enhancer element, which results in the down-regulation of H19 and up-regulation of IGF2 involved in VSMC proliferation (Li *et al.*, 2009). In monocyte/macrophages, *in vitro* studies have indicated that homocysteine up-regulates DNMT expression and modulates gene expression. First, the DNA methylation of the atheroprotective genes for **PPARα**, **PPARγ** and ApoE is significantly induced by homocysteine, which leads to the down-regulation of the expression of these genes and the promotion of atherogenesis (Yi-Deng *et al.*, 2007; Yideng *et al.*, 2008). Second, the expression of iNOS is down-regulated by homocysteine *via* DNA methylation, which is blocked by **PPARα/γ** ligands (Jiang *et al.*, 2007b). As the cholesterol efflux pathway protects against monocyte/macrophage-derived foam cell formation, homocysteine also enhances the DNA methylation of **ABCA1** transporter and **acetyl-CoA acetyltransferase 1**, two key proteins mediating cholesterol efflux and further markedly down-regulates their expression in THP-1 monocytes, which accelerates foam cell formation (Liang *et al.*, 2013).

Regulation of RNA methyltransferase. RNA methylation, usually present at the cytosine and adenine bases of eukaryotic DNA (5-methylcytosine and 6-methyladenine, respectively), has been observed in numerous types of RNA molecules, including mRNAs, tRNAs and non-coding RNAs; however, the function of RNA methylation remains unclear. A tRNA MT NSun2 activated by homocysteine increases ICAM-1 expression *via* mRNA methylation in ECs, and HHcy-induced ICAM-1 expression in ECs is abolished by NSun2 deficiency *in vivo* (Luo *et al.*, 2016). This study indicates that mRNA methylation is also involved in the pathogenic effect of homocysteine in EC injuries.

Regulation of histone methyltransferase. Histone methylation mainly occurs on the side chains of lysines or arginines. These residues are able to be methylated multiple times and repress or activate gene transcription. The histone lysine MTs, including **G9a** and **Suv39h1**, catalyse H3-Lys^9 methylation in mammalian cells. A recent study demonstrated that unstable plaque formation and the number of apoptotic cells in the lesion are significantly increased in ApoE$^{-/-}$ mice with HHcy, induced by a high-Met diet, accompanied by a decreased expression of H3-Lys^9 dimethylation. HHcy increases the apoptosis of macrophages and inhibits H3-Lys^9 dimethylation, as well as the expression of histone MT G9a *in vitro*. The inhibition of histone methylation by **BIX01294** enhances macrophage apoptosis and foam cell formation *in vitro*. In conclusion, HHcy-induced macrophage apoptosis mediates the progression of atherosclerosis, and histone methylation attributed to HHcy may be involved in this process (Cong *et al.*, 2017).

Collectively, homocysteine has been reported to regulate gene expression *via* DNA, RNA and histone methylation in ECs, VSMCs and monocytes/macrophages (Figure 5). In

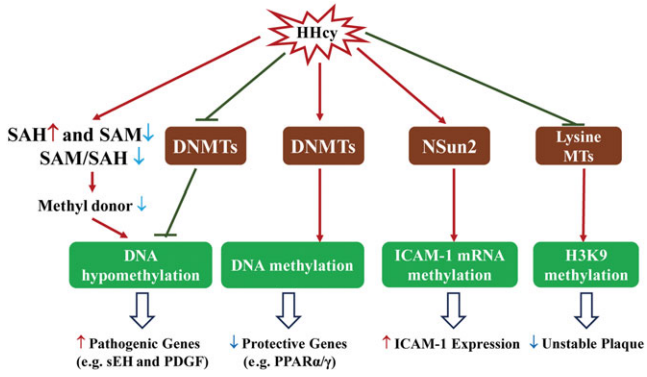

Figure 5

Schematic overview of HHcy-induced epigenetic methylation. HHcy decreases SAM/SAH ratio and inhibits expression of DNMTs, thus leading to DNA hypomethylation and up-regulation of pathogenic gene expression. In contrast, HHcy enhances DNMT expression and DNA methylation to down-regulate expression of protective genes. Furthermore, HHcy up-regulates tRNA MT NSun2 and promotes ICAM-1 mRNA methylation, which further increases its gene expression. Histone H3-Lys^9 (H3K9) methylation protects against unstable atherosclerosis plaque. HHcy inhibits lysine MTs and H3K9 methylation, further inducing unstable plaque.

general, a decreased reduced SAM/SAH ratio in HHcy leads to genome-wide hypomethylation *in vivo*. In contrast, the intake of a folate-deficient or vitamin B6-deficient high-fat diet markedly elevated plasma homocysteine levels and aggravated atherosclerotic plaques in ApoE$^{-/-}$ mice compared with a regular high-fat diet, with no effect on 5-methyldeoxycytidine levels in vascular tissues (McNeil *et al.*, 2011). Moreover, there are other discrepancies associated with DNMTs regulated by homocysteine at pathological concentration, between different *in vitro* studies. An explanation of the differing effects of HHcy on regulation of DNMTs expression is still to be provided. Because the conditions in cell cultures *in vitro* are not usually identical to those *in vivo*, additional studies in HHcy animal models *in vivo* are required. Furthermore, in view of the complexity of epigenetic modulation, other hidden mechanisms, particularly the mechanism by which homocysteine regulates DNMTs, should be investigated in the future.

Homocysteinylation in HHcy

Homocysteinylation mainly targets proteins and is classified as *S*-homocysteinylation and *N*-homocysteinylation (Figure 6). *S*-Homocysteinylation occurs when homocysteine binds a protein through its free thiol group to another free thiol group of a Cys residue in the protein molecule to form a disulphide bond. *N*-Homocysteinylation is mainly performed by the corresponding thiolactone, Hcy-TL, which is generated by an error-editing function of aminoacyl-tRNA synthetases with homocysteine and ATP. *N*-Homocysteinylation comprises the interaction of amino groups from Hcy-TL and a Lys of a target protein, which further produces a new free thiol group derived from Hcy-TL. Thus, homocysteinylation may not only affect protein function *via* amino acid modification but also affects the thiol-dependent redox status (Jakubowski, 1999). Protein homocysteinylation is enhanced in diabetes and atherosclerosis patients with HHcy (Jakubowski *et al.*, 2000; Jakubowski, 2001). Homocysteinylated LDL isolated from circulating blood exerts cytotoxic effects on ECs *in vitro* (Ferretti *et al.*, 2004, 2006; Nanetti *et al.*, 2012), whereas fibronectin homocysteinylated by homocysteine attenuates its interaction with fibrin, which indicates a potential delay in haemostasis (Majors *et al.*, 2002). Metallothionein homocysteinylated by homocysteine results in an antioxidative activity disorder and favours ROS accumulation in ECs (Barbato *et al.*, 2007), whereas homocysteinylated eNOS reduces the activity of NOS (Zhang *et al.*, 2000). In contrast, homocysteinylated **ACE** exhibits an increased ability to produce Ang II and consequently activate the Ang II–NOX–ROS pathway in ECs (Zhang *et al.*, 2007). However, the function of a substantial number of proteins homocysteinylated by homocysteine or its thiolactone in the vascular system has not been fully investigated.

The interaction between vascular injury and injury to other systems, induced by HHcy

As the vascular system is distributed in all organs throughout the entire body, HHcy-induced vascular injury may influence other organs. HHcy increases the permeability of the blood–brain barrier by disrupting the cell junction of the brain microvascular ECs (Qureshi *et al.*, 2005). A substantial amount of homocysteine subsequently accumulates in the CSF. Homocysteine may act as an agonist of the NMDA receptor, which further causes neural damage *via* calcium ion efflux or free radial generation (Williams and Schalinske, 2010). HHcy has been associated with pathogenic alterations in mental health, such as cognitive impairment, dementia, depression, Alzheimer's and Parkinson's diseases (Williams and Schalinske, 2010). Moreover, homocysteine-induced vascular injury contributes to renal vascular dysfunction and results in chronic renal disease (Skovierova *et al.*, 2016).

HHcy-induced injury of other systems secondarily contributes to vascular injury. Thus, homocysteine is excitatory on hypothalamic neurons, controlling cardiac functions *via* the sympathetic system. Consequently, homocysteine can cause hypertension *via* the neuronal system in addition to its effects on NO production (Ganguly and Alam, 2015). Resistin, an adipokine produced by perivascular adipose tissue, contributes to intimal hyperplasia and atherosclerosis (Nosalski and Guzik, 2017). Resistin induces the proliferation

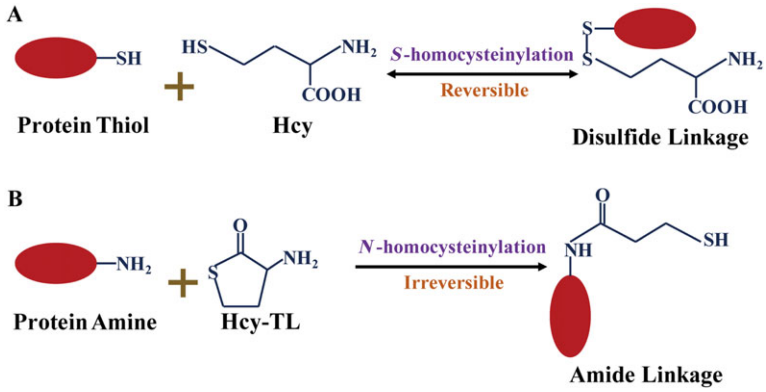

Figure 6
Chemical reactions of *S*-homocysteinylation (A) and *N*-homocysteinylation (B).

and migration of VSMCs *via* the ERK1/2 or PI3K-Akt (Calabro *et al.*, 2004) and integrin α5β1-FAK/paxillin-Rac1 (Jiang *et al.*, 2009) or PKC (Raghuraman *et al.*, 2016) signalling pathways respectively. Moreover, resistin leads to endothelial dysfunction as shown by the up-regulation of VCAM-1 and **ICAM-1** expression, as well as the increased secretion of endothelin-1 and CCL2 by ECs (Verma *et al.*, 2003; Jamaluddin *et al.*, 2012). Homocysteine promotes the production and secretion of resistin by adipocytes *via* the ROS–PKC–NF-κB pathways, and the expression of resistin is enhanced in epididymal adipose tissue from C57BL mice with HHcy, compared with normal mice (Li *et al.*, 2008). Taken together, these data indicate that homocysteine may indirectly induce EC and VSMC injury *via* the paracrine release of adipokines from perivascular adipose tissue.

Drug targets for HHcy-induced vascular injury

The development of drugs for the treatment of homocysteine-induced vascular injury should mainly target on the pathways of homocysteine metabolism and the underlying mechanisms of pathogenic effects of HHcy. Treatments aimed at lowering homocysteine levels have been clinically applied, while many natural chemicals and already available drugs have been shown to exert therapeutic effects in HHcy-induced vascular injury by inhibiting oxidative or ER stress (Figure 7).

Homocysteine-lowering therapy

As low levels of folic acids and vitamin B in the human body cause HHcy, dietary supplementation with folic acids and vitamin B may favour the lowering of homocysteine in the plasma and improve HHcy-related vascular diseases. Folic acid supplementation has been associated with a reduction in the progression of carotid atherosclerosis (Vermeulen *et al.*, 2000). Further clinical trials have also shown that lowering homocysteine with folic acid and vitamin B reduces the risk of stroke (Saposnik *et al.*, 2009; Liu *et al.*, 2015). However, several clinical trials did not show beneficial effects of lowering plasma homocysteine through folate and vitamin B supplementation on the risk of major cardiovascular events in patients with vascular disease (Lonn *et al.*, 2006; Ebbing *et al.*, 2008) and kidney disease (Bostom *et al.*, 2011; Jardine *et al.*, 2012). These trials with negative results were performed in a population with normally high folate consumption (the USA, Australia and New Zealand). High folate consumption may induce a state of HHcy with a higher basal level of plasma folate. As a result, the further administration of folic acids and vitamin B results in a less effective lowering of plasma homocysteine. Strikingly, it has been suggested that clinical trials of homocysteine-lowering interventions through folic acids and vitamin B supplementation in vascular disease prevention should be conducted in regions with low folate consumption rather than in areas where foods are more commonly fortified with folate (Holmes *et al.*, 2011). Besides folic acids and vitamin B supplementation, a recent clinical trial has indicated an association

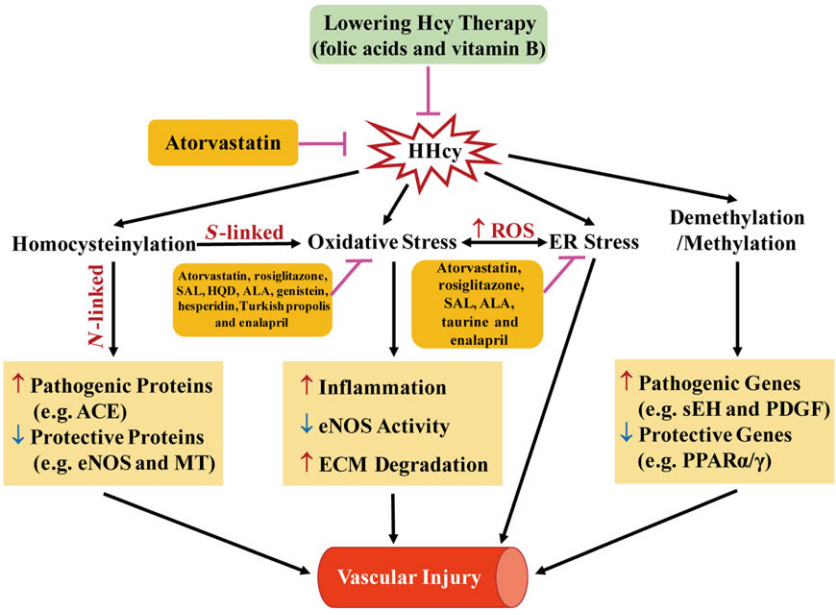

Figure 7

Mechanisms and drug targets of HHcy-induced vascular injury. HHcy induces vascular injury by promoting oxidative stress, ER stress and protein homocysteinylation as well as regulating methylation. Among them, oxidative stress and ER stress aggravate mutually *via* ROS as a mediator. For homocysteinylation, the *S*-linked reaction induces oxidative stress, whereas the *N*-linked reaction regulates the property and activity of target proteins. Lowering homocysteine levels and inhibition of oxidative/ER stress are the potential therapeutic targets for drugs or natural chemicals. ALA, α-lipoic acid; ECM, extracellular matrix; HQD, Huang Qi decoction; MT, metallothionein; SAL, salidroside.

between atorvastatin and lowering homocysteine in renal transplantation patients (Monfared *et al.*, 2016), implying that atorvastatin may be used as a homocysteine-lowering therapy, especially in the areas with dietary folate fortification.

Oxidative stress

Because oxidative stress is the major mechanism mediating homocysteine-induced vascular injury, several natural chemicals or known drugs are protective against homocysteine-induced injury, through their antioxidant activity. **Atorvastatin** is widely used to lower serum cholesterol levels and has been found to protect against HHcy-induced oxidative stress in ECs both *in vitro* and *in vivo* (Bao *et al.*, 2010; Jia *et al.*, 2012, 2016). The PPAR agonist **rosiglitazone**, developed for type II diabetes, also ameliorates EC dysfunction *via* suppressing HHcy-induced oxidative stress in rats (Yang *et al.*, 2015c). Salidroside, an active component of *Rhodiola rosea*, attenuated homocysteine-induced EC dysfunction by reducing oxidative stress (Leung *et al.*, 2013). **Genistein**, a soy isoflavone, blocks homocysteine-induced pathogenic alterations in human ECs *via* down-regulating antioxidative pathways (Han *et al.*, 2015). Hesperidin, a citrus flavonoid, protects against HHcy induced by a high Met diet, by blocking oxidative stress, EC dysfunction and neurotoxicity in Wistar rats (Hemanth Kumar *et al.*, 2017). Huang Qi decoction (HQD) is a traditional Chinese medical formula, and its components exert antioxidant effects. HQD alleviates EC dysfunction initiated by HHcy through antioxidant mechanisms (Chu *et al.*, 2016). **α-Lipoic acid**, a disulphide-containing compound, can scavenge ROS, inhibit the formation of free radicals and chelate metal ions, to maintain cellular homeostasis. Thus, α-lipoic acid suppressed homocysteine-induced oxidation and reduced EC apoptosis and inflammation (Hu *et al.*, 2016). Turkish propolis is a natural product, made by honey bees from various plant oils, pollens, resins and wax materials. Turkish propolis protects human ECs *in vitro* from HHcy-induced apoptosis by decreasing HHcy-induced ROS overproduction and lipid peroxidation levels (Darendelioglu *et al.*, 2016). **Enalapril**, a widely used ACE inhibitor, effectively inhibits aortic ROS production induced by a high Met diet in rats (Zhou *et al.*, 2015).

ER stress

As ER stress also plays a critical role in HHcy-induced vascular injury, it could be another therapeutic target for HHcy-related vascular diseases. Atorvastatin attenuates atherosclerotic plaque destabilization by inhibiting endothelial ER stress in HHcy mice (Jia *et al.*, 2016) and the activation of **AMP-activated protein kinase** may be involved in this protective effect (Jia *et al.*, 2012). **Taurine** prevents the decrease in expression and secretion of extracellular SOD induced by homocysteine in VSMCs by decreasing ER stress (Nonaka *et al.*, 2001). Salidroside protects against HHcy-induced injury in HUVEC *via* the regulation of ER stress (Zhu *et al.*, 2017). Moreover, α-lipoic acid and enalapril protect ECs and improve hypertension through inhibiting ER stress as well (Zhou *et al.*, 2015; Hu *et al.*, 2016).

Conclusion and perspective

HHcy exerts its pathogenic effects on vascular injury *via* various pathways, including oxidative stress, ER stress, epigenetic modulation and protein homocysteinylation. To date, the therapy for HHcy mainly focuses on lowering plasma homocysteine *via* dietary supplementation with folic acids and vitamin B. However, many *in vitro* and *in vivo* studies have demonstrated that many natural chemicals or established drugs are protective against homocysteine-induced vascular injury. Further animal studies and clinical trials will be required to confirm these potential therapeutic applications.

Nevertheless, several problems and controversies remain unsolved. First, many mechanisms have only been demonstrated *in vitro*, such as the agonist-like effect of homocysteine on NMDA receptors. Clearly, the role of NMDA receptors in HHcy-induced vascular injury should be further explored *in vivo*. Moreover, the concentration of homocysteine applied in cell culture experiments varies considerably between different studies, even up to millimolar concentrations, which are rarely observed in HHcy patients. Some effects such as the regulation of DNMTs by homocysteine, appear to provide opposing results, related to the different times and concentrations used. Thus, many of the findings from *in vitro* experiments should be further validated in *in vivo* studies. Second, many new studies have demonstrated that the effects of HHcy derive from the targeting of multiple systems, rather than a single system. For example, HHcy-induced vascular injury further induces the injury of other organs, whereas vascular injury could also be secondarily caused by HHcy-induced injury of other systems. This observation indicates that HHcy is more likely to be a disease that involves the dysfunction of several organs. Advanced studies should be broadened to focus on the interaction of multiple organs or systems in the entire body. Third, homocysteine-lowering therapy by the intake of folic acids and vitamin B shows little or no curative effect in patients who are normally exposed to diets with folate fortification. Thus, the application of this therapy may be more preferable for patients with low daily folate intake (as in Asia), which would indicate a region-dependent use. For patients accustomed exposed to dietary folate fortification, the development of another therapy to lower homocysteine levels may be beneficial for their HHcy-induced vascular injury.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015,a,b,c,d,e,f,g).

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Conflict of interest

The authors declare no conflicts of interest.

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