Universidade de Lisboa
Faculdade de Farmácia

Crossroads of Homocysteine, Nitric Oxide and Asymmetric Dimethylarginine metabolisms

Involvement of S-adenosylhomocysteine and impaired cellular methylation

Mónica Eunice dos Santos Rocha

Doutoramento em Farmácia
Bioquímica

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Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para obtenção do grau de Doutor em Farmácia (Bioquímica)

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To my mother...
Para ti, mãe...

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Abbreviations

5,10-MTHF	5, 10-methylenetetrahydrofolate
5-MeTHF	5-methyltetrahydrofolate
ADMA	asymmetric dimethylarginine
AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
BH_4	tetrahydrobiopterin
BHMT	betaine-homocysteine methyltransferase
C	cytosine
CAT	cationic amino acid transporters
CBS	cystathionine β-synthase
CGL	cystathionine γ-lyase
DDAH	dimethylarginine dimethylamino hydrolase
DNMT	DNA methyltransferase
eNOS	endothelial nitric oxide synthase
FAD	flavine adenine dinucleotide
FGF2	fibroblast growth factor-2
GAMT	guanidinoacetate methyltransferase
GNMT	glycine N-methyltransferase
HAT	histone acetyltransferase
Hcy	homocysteine
HDAC	histone deacetylase
HHcy	hyperhomocysteinemia
HMT	histone methyltransferase
MAT	methionine adenosyltransferase
MBP	methyl-CpG binding proteins
mC	5-methylcytosine
miRNA	micro-RNA
MMA	monomethylarginine

MS	methionine synthase
MTHFR	5,10-methylenetetrahydrofolate reductase
ncRNA	noncoding RNA
NO	nitric oxide
PEMT	phosphatidylethanolamine methyltransferase
PLP	pyridoxal phosphate
PRMT	protein arginine methyltransferase
RNAi	RNA interference
ROS	reactive oxygen species
SDMA	symmetric dimethylarginine
sGC	soluble guanylate cyclase
SHMT	serine hydroxymethyltransferase
tHcy	total homocysteine
THF	tetrahydrofolate

Summary

Elevated homocysteine, or hyperhomocysteinemia, is an independent risk factor for vascular disease. However, the precise mechanisms underlying this association, although intensively studied, are still incompletely solved. The precursor of all homocysteine produced in the body is S-adenosylhomocysteine (AdoHcy), a strong methyltransferase inhibitor. When homocysteine accumulates, AdoHcy accumulates as well, potentially disturbing most of the transmethylation processes within the cell. Notably, the role of AdoHcy has gained increased attention, regarding the pathophysiology of hyperhomocysteinemia. In fact, in recent studies, not homocysteine, but rather AdoHcy emerged as a more insightful indicator of vascular disease and tissue damage. Thus, in the present work, we postulate that elevated homocysteine itself may not be the major causative factor for the development of vascular disease, and sought to investigate whether AdoHcy accumulation, by disturbing cellular transmethylation reactions, would influence vascular homeostasis. Specifically, we will focus on the endothelium production of nitric oxide (NO), a potent anti-atherogenic molecule, and on the metabolism of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of the endothelial nitric oxide synthase (eNOS).

Chapter 1 comprises a general introduction on homocysteine metabolism, including a review of the major concepts concerning regulation, determinants of hyperhomocysteinemia and proposed pathophysiological vascular mechanisms, with special interest on the NO/ADMA pathways. **Chapter 2** presents the aims and outline of this thesis.

We first investigated the impact of an impaired methylation environment due to AdoHcy accumulation on NO bioavailability. For that, we cultured human umbilical vein endothelial cells (HUVEC) with adenosine-2,3-dialdehyde (ADA), a well-known inhibitor of the enzyme that reversibly converts AdoHcy in homocysteine, to elevate the intracellular levels of AdoHcy (but not of homocysteine), thus establishing our work model characterized by cellular hypomethylation. We observed a decreased NO production due to lower protein levels and activity of the enzyme eNOS, but yet up-regulated levels of the correspondent mRNA. Taken together, these data suggest that eNOS expression is target of post-translational mechanisms possibly involving methylation steps, hence influenced by AdoHcy (**Chapter 3**).

Since the analysis of global DNA methylation was a central point in this work, we compared two most frequently used methods for measuring global DNA methylation, available in our laboratory: a traditional assay using methylation-sensitive enzyme digestion, and other involving DNA acid hydrolysis. Data confirmed that both methods are feasible tools for DNA methylation

studies, but the latter approach presented considerable advantages, not only by allowing an absolute quantification, but also because displayed lower intraday variability (**Chapter 4**).

The next objective was to evaluate the effect of AdoHcy and impaired cellular hypomethylation on ADMA metabolism, namely upon its metabolic degradation by the enzyme dimethylarginine dimethylaminehydrolase (DDAH) (**Chapter 5**). HUVEC were cultured with ADA or 5-aza-2-deoxycitidine (AZA), which targets DNA methyltransferases and specifically inhibits DNA methylation without accumulation of AdoHcy. Although both compounds induced global DNA hypomethylation, our data showed that only ADA (not AZA) increased DDAH activity, suggesting that AdoHcy stimulates the activity of DDAH by a mechanism independent of DNA methylation.

In **Chapter 6** we aimed to determine the extent to which protein arginine methylation status is affected by accumulation of AdoHcy. For that, protein-incorporated ADMA levels were quantified in our cell model. Interestingly, the data indicated that protein methylation is more sensitive to AdoHcy accumulation than DNA methylation, pinpointing a possible new player in homocysteine-related diseases. In fact, protein methylation is emerging as crucial to cellular homeostasis, and its dysregulation may lead to disease.

In **Chapter 7**, using a diet-induced hyperhomocysteinemia rat model, we investigated the *in vivo* effect of chronic hyperhomocysteinemia on tissue global DNA methylation. We verified that diets capable of inducing hyperhomocysteinemia also disturb cellular methylation potential in liver and heart, but only severely elevated AdoHcy levels affect global DNA methylation, and in a tissue-specific manner.

Increased levels of ADMA have been suggested to contribute for the homocysteine-induced vascular toxicity. CBS (cystathionine β-synthase) deficiency, or Classical Homocystinuria, is an inherited disorder of homocysteine metabolism that results in abnormally elevated levels of homocysteine in the blood, and serious complications in the cardiovascular system. In **Chapter 8** we present our study showing that severe hyperhomocysteinemia is not associated with increased ADMA plasma levels in CBS deficient patients, supporting the possibility that intracellular accumulation of AdoHcy, as result of high homocysteine levels, impairs protein methylation processes, thereby the synthesis of ADMA.

Lastly, **Chapter 9** presents a general discussion, major conclusions and future perspectives disclosed by this thesis.

In summary, the work here described provides evidence to what extent a disturbed cellular methylation due to elevated AdoHcy may contribute to the pathophysiology of homocysteine.

Sumário

A doença vascular é a principal causa de morbilidade e de mortalidade no mundo ocidental industrializado. Ao longo das últimas décadas, níveis elevados de homocisteína, ou hiper-homocisteinémia (HHcy), têm sido considerados um factor de risco independente para esta patologia. A diminuição da biodisponibilidade do óxido nítrico (NO), metabolito crucial para a homeostasia vascular, é uma manifestação-chave da disfunção endotelial que precede a aterosclerose, e um fenótipo consistentemente associado aos estados de HHcy. Contudo, os mecanismos pelos quais níveis aumentados de homocisteína induzem a disfunção endotelial, apesar de intensamente estudados, não se encontram completamente elucidados.

A S-adenosil-homocisteína (AdoHcy) é o precursor metabólico de toda a homocisteína produzida no organismo, e um inibidor da maioria das metiltransferases celulares. Por esta razão, tem sido sugerido um mecanismo indirecto para a toxicidade da homocisteína, secundário a uma acumulação de AdoHcy, e a uma possível alteração dos processos celulares de transmetilação. Com efeito, e independentemente da causa subjacente, a níveis elevados de homocisteína corresponderá uma acumulação de AdoHcy, que poderá perturbar os processos de metilação celular, nomeadamente do DNA e das proteínas, daí resultando consequências fisiológicas importantes. Deste modo, o papel da AdoHcy tem vindo a assumir uma relevância crescente na patofisiologia da HHcy. De facto, dados recentes, quer experimentais quer clínicos, sugerem que a AdoHcy possa vir a ser considerada como o biomarcador adicional à Hcy, ou mesmo de excelência, para a susceptibilidade à doença vascular.

Neste trabalho, postulamos então que o aumento dos níveis de homocisteína não são directamente o factor causador de doença vascular, mas sim a acumulação de AdoHcy secundária a esse aumento de homocisteína, a qual, por inibição de mecanismos de metilação celular, nomeadamente, da metilação do DNA e/ou das proteínas, poderá influenciar o metabolismo do NO e/ou do seu principal inibidor endógeno, a dimetilarginina assimétrica (ADMA), diminuindo assim a biodisponibilidade de NO e promovendo a disfunção endotelial.

Para testar a nossa hipótese de trabalho, usámos como modelo de estudo, numa primeira abordagem, culturas de células endoteliais humanas. Os dados obtidos através destas experiências *ex vivo*, foram posteriormente confirmados *in vivo,* nomeadamente em situações de HHcy quer endógena, em doentes com alterações hereditárias do metabolismo da homocisteína (défice em cistationina β-sintase, CBS), quer induzida, em animais sujeitos a dietas com teores manipulados em co-enzimas e co-factores intervenientes no metabolismo da homocisteína.

O **Primeiro Capítulo** da presente tese inclui uma revisão geral do metabolismo da homocisteína, incluindo os principais conceitos referentes à regulação, determinantes do estado de HHcy e mecanismos propostos como reponsáveis pela sua associação com a disfunção endotelial, nomeadamente o involvimento das vias metabólicas do NO e da ADMA. Finalmente, é apresentada uma revisão global dos principais mechanismos epigenéticos, e exemplos actualizados de estudos referentes ao papel da homocisteína na modulaçao da metilação do DNA e expressão génica. O **Capítulo 2** apresenta os principais objectivos de trabalho e o delineamento dos capítulos constituintes desta tese.

O primeiro objectivo do trabalho consistiu então na avaliação do efeito de um ambiente celular hipometilante sobre a biodisponibilidade do NO (**Capítulo 3**). Para tal, recorremos a células endoteliais isoladas da veia do cordão umbilical (HUVEC) para estabelecer o modelo de estudo. Assim, as culturas celulares foram tratadas com adenosina-2,3-dialdeído (ADA), um potente inibidor da AdoHcy hidrolase (enzima que converte a AdoHcy em homocisteína), que induziu efectivamente uma acumulação exclusiva de AdoHcy. Verificámos que a produção de NO diminuía, devido a uma diminuição do teor proteico da isoforma endotelial do NO sintase (eNOS) e da sua actividade enzimática, embora a transcrição do respectivo gene estivesse aumentada. Em conjunto, estes dados apontam para a existência de mecanismos pós-transcricionais, modulados pelo potencial de metilação celular alterado e induzido pela acumulação de AdoHcy, os quais conduzem a uma diminuição da produção de NO.

Sendo os processos de metilação celular, nomeadamente do DNA, um tema central deste trabalho, importava utilizar o método mais correcto para os avaliar. Assim, no **Capítulo 4** fomos comparar dois dos métodos mais utilizados para esta quantificação: um método tradicional, fundamentado no uso de enzimas de restrição sensíveis à metilação, e um método mais recente, baseado na hidrólise química do DNA seguida da detecção específica das citosinas metiladas. Os resultados revelaram que ambos os métodos possibilitam uma quantificação fiável do grau de metilação do DNA, mas que o último método, cujo processo de detecção usa a espectrometria de massa em tandem, apresenta sólidas vantagens pois, ao invés do método enzimático que oferece uma quantificação relativa, permite uma quantificação absoluta da metilação global do DNA e apresenta menores variações intra-dias. Por esta razão, o método LC-MS/MS foi o método eleito para os estudos posteriores.

A ADMA é um inibidor endógeno da síntese de NO endotelial, logo o seu metabolismo assume particular importância no contexto da homeostasia vascular. O objectivo seguinte, apresentado no **Capítulo 5**, pretendeu elucidar o efeito do ambiente hipometilante ao nível do metabolismo da ADMA, e especificamente sobre a enzima responsável pela sua hidrólise, a dimetilarginina dimetilamino-hidrolase (DDAH). As culturas de HUVEC foram tratadas em paralelo com ADA e com 5-aza-2-deoxicitidina (AZA), um inibidor específico das DNA metiltransferases e que não induz acumulação de AdoHcy. Embora os dois fármacos utilizados induzissem uma hipometilação do DNA, os resultados revelaram que apenas o tratamento com ADA aumentou a actividade da DDAH, reflectindo-se numa diminuição dos teores de ADMA extracelulares. Assim, os nossos dados sugerem que a acumulação intracelular de AdoHcy estimula a actividade da DDAH por um mecanismo independente da metilação do DNA.

No **Capítulo 6** fomos analisar os efeitos da acumulação intracelular de AdoHcy no estado de metilação das proteínas, o qual foi avaliado indirectamente pela quantificação dos resíduos metilados de arginina, nomeadamente ADMA e dimetilarginina simétrica (SDMA). Surpreendentemente, os resultados revelaram que a metilação das proteínas é mais susceptível de alteração devido à acumulação intracelular de AdoHcy do que a metilação do DNA. Estes dados são de grande interesse, pois sugerem que a metilação das proteínas poderá ser um novo interveniente na patofisiologia da HHcy. Com efeito, a metilação de proteínas é um regulador emergente da função proteica crescentemente implicado na fisiopatologia humana.

No **Capítulo 7** fomos analisar as consequências *in vivo* de uma situação de HHcy na metilação do DNA. Para tal, induzimos em ratos uma gama variável de níveis de homocisteína através de dietas ricas em metionina e/ou pobres em vitaminas do grupo B, e fomos analisar os seus reflexos ao nível dos padrões de metilação do DNA em diversos órgãos. Os resultados revelaram que dietas indutoras de HHcy afectam a capacidade de metilação celular, mas que o seu reflexo a nível do DNA está dependente dos níveis de AdoHcy atingidos e sobretudo do tecido em causa, nomeadamente da presença ou ausência de vias metabólicas específicas.

Os dados por nós obtidos nas anteriores experiências *ex vivo* contradizem diversas publicações que referem um aumento concomitante de homocisteína e de ADMA em doentes cardiovasculares. Assim, pretendemos investigar o que acontece *in vivo* em doentes com deficiência em CBS, nos quais se verifica uma situação de HHcy crónica associada a risco elevado de doença vascular (**Capítulo 8**). Os resultados revelaram uma ausência de correlação entre os níveis plasmáticos

de homocisteína e de ADMA, o que sugere que a acumulaçao destes dois metabolitos resulta de mecanismos patogénicos independentes. No entanto, verificou-se que nestes doentes os níveis de arginina e a razão arginina/ADMA (indicador da taxa de inibição da síntese de NO) se encontravam diminuídos. Estes dados corroboram os obtidos na experiência anterior efectuada em culturas celulares, e sugerem que a elevação de ADMA nos doentes vasculares é independente do aumento dos níveis de homocisteína, e estará provavelmente relacionada com outros factores, tais como disfunção renal.

Por último, no **Capítulo 9** é apresentada uma discussão dos resultados obtidos, incluindo a análise integrada de todo o trabalho e respectivas conclusões, bem como algumas perspectivas de trabalho futuro.

Em resumo, este trabalho contribuiu para a elucidação do envolvimento da acumulação de AdoHcy e consequente alteração do padrão de metilação celular no mecanismo patogénico subjacente à toxicidade vascular da homocisteína.

CHAPTER

General Introduction

1. Homocysteine Metabolism

Homocysteine is a sulphur amino acid produced during transmethylation of the essential amino acid methionine, and not used in protein synthesis (FIGURE 1). First, methionine is activated by the enzyme methionine adenosyltransferase (MAT, EC 2.5.1.6) and ATP to form S-adenosylmethionine (AdoMet). AdoMet is the primary methyl group donor for many vital biological processes, including methylation of DNA, RNA, proteins, lipids and neurotransmitters. Upon transmethylation, AdoMet is converted to S-adenosylhomocysteine (AdoHcy), which is further hydrolyzed by the enzyme AdoHcy hydrolase (EC 3.2.2.9) to homocysteine and adenosine. This reaction is reversible and favors AdoHcy synthesis. Because AdoHcy is a strong inhibitor of most AdoMet-dependent methyltransferases [1], an efficient removal of homocysteine and adenosine is necessary to prevent intracellular AdoHcy accumulation, which might otherwise lead to a disturbance of cellular methylation processes (SECTION 7). However, under physiological conditions, both homocysteine and adenosine are promptly metabolized, warranting a low intracellular concentration of AdoHcy.

Homocysteine is located at a metabolic branch point and can either be irreversibly degraded to cysteine via the transsulfuration pathway, or conserved by remethylation back to methionine (FIGURE 1).

The transsulfuration pathway, mainly limited to liver and kidneys, is initiated with the condensation of homocysteine and serine to form cystathionine, in a reaction catalyzed by the enzyme cystathionine β-synthase (CBS, EC 4.2.1.22), with pyridoxal phosphate (PLP, or vitamin B_6) as co-factor. Cystathionine is further metabolized to produce cysteine by another PLP-requiring enzyme, cystathionine γ-lyase (CGL, EC4.4.1.1; FIGURE 1). Besides protein synthesis, cysteine is used in the synthesis of glutathione, an important cellular antioxidant also involved in detoxification of many xenobiotics.

Homocysteine remethylation occurs by receiving the methyl group from 5-methyltetrahydrofolate (5-MeTHF), which links the folate cycle with the homocysteine metabolism (FIGURE 1). 5-MeTHF is the active folate derivative and the main circulating form of folate in plasma. It is produced from 5,10-methylenetetrahydrofolate (5,10-MTHF) by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), which uses FAD (flavine adenine dinucleotide; the active form of vitamin B_2) as co-factor. The methyl group from 5-MeTHF is transferred via vitamin

B_{12} to homocysteine, in a reaction catalyzed by methionine synthase (MS, EC 1.16.1.8), with production of tetrahydrofolate (THF). THF is then recycled to 5,10-MTHF in the presence of serine and vitamin B_6 by the enzyme serine hydroxymethyltransferase (SHMT, EC 2.1.2.1). The folate-dependent remethylation pathway is present in nearly all cells, except red blood cells. Alternatively, in liver and kidney, methyl groups can also be donated by betaine (also known as trimethylglycine, an intermediate of choline oxidation), in a reaction catalyzed by the enzyme betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5; FIGURE 1).

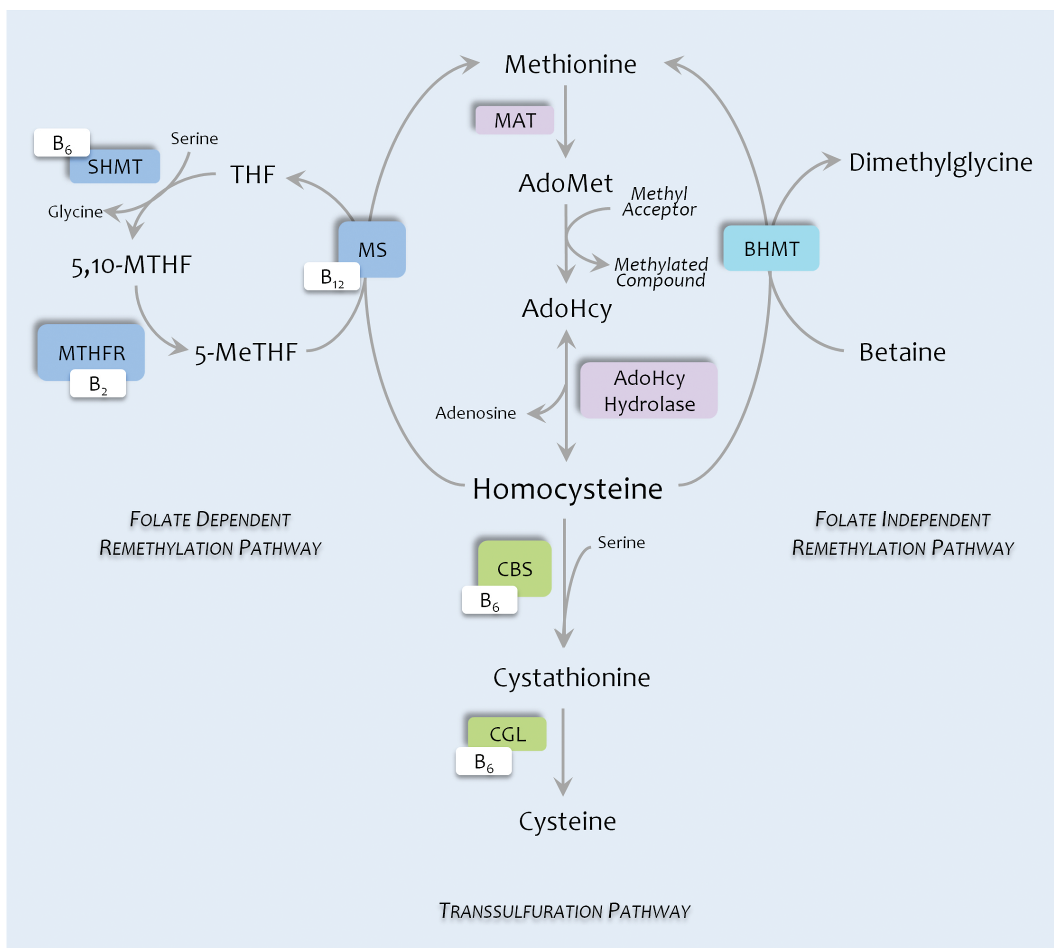

FIGURE 1 | SCHEMATIC REPRESENTATION OF HOMOCYSTEINE METABOLISM

(MAT) methionine adenosyltransferase, (AdoMet) S-adenosylmethionine, (AdoHcy) S-adenosylhomocysteine, (CBS) cystathionine β-synthase, (CGL) cystathionine γ-lyase, (5-MeTHF) 5-methyltetrahydrofolate, (5,10-MTHF) 5, 10-methylenetetrahydrofolate, (MTHFR) 5, 10-methylenetetrahydrofolate reductase, (MS) methionine synthase, (THF) tetrahydrofolate, (SHMT) serine hydroxymethyltransferase, (BHMT) betaine-homocysteine methyltransferase.

The intracellular concentration of homocysteine is under tight control. Once formed in the cell, homocysteine is quickly either metabolized to cysteine or remethylated to methionine. In addition, if one of these pathways is compromised, leading to higher intracellular production than elimination, the excess of homocysteine is rapidly exported to the blood. Hence, cellular export of homocysteine reflects the balance between homocysteine production and catabolism. Persisting high concentrations of homocysteine in the blood creates the condition called hyperhomocysteinemia.

In plasma, homocysteine is predominantly present as disulfide bound to proteins (mainly albumin). The remaining non-protein bound homocysteine exists mostly in the form of the disulfide homocystine and mixed disulfides, such as homocysteine-cysteine, and only a very small portion remains unbound, free homocysteine. Generally, total homocysteine (tHcy) is measured, which refers to the sum of all forms of homocysteine present in the plasma.

1.1 Regulation of Homocysteine metabolism

Liver and kidney, due to a unique set of enzymes, are major organs in maintaining homocysteine within optimal levels, whereas in most other tissues homocysteine metabolism appears to be fully reliant on the folate-dependent remethylation pathway or on cellular export. In fact, CBS activity is measurable in several tissues, but outside the liver and kidneys seems to be too low to significantly contribute to the regulation of homocysteine levels [2,3].

AdoMet plays a central role in the regulation of homocysteine metabolism, by coordinating the fate of homocysteine towards remethylation or transsulfuration pathways; it is an allosteric inhibitor of MTHFR and an activator of CBS activity [3]. When the levels of AdoMet are adequate to sustain methylation demand, the partitioning of homocysteine between both metabolic pathways is approximately equal [4]. In case of excess of methionine supply, an increase in tissue AdoMet levels occurs, and homocysteine degradation to cysteine is favored. Moreover, AdoMet also regulates homocysteine remethylation through inhibition of BHMT activity [3]. Conversely, if methionine levels are low, for example during fasting, low AdoMet levels will neither activate CBS nor inhibit MTHFR, resulting in conservation of homocysteine via remethylation back to methionine.

In liver, with regard to nutritional status, especially in response to high protein and methionine intake, AdoMet concentrations are subject of tight regulation. As already mentioned, AdoMet is produced from methionine and ATP by the enzyme MAT. In mammalian tissues, three isoforms of this enzyme, with distinct tissue distribution, kinetics and regulatory proprieties, have been identified (MAT I, II and III). In extra-hepatic tissues, the predominant MAT II has relatively low affinity for methionine and is inhibited by AdoMet. The liver-specific MATI and MAT III isoforms, with higher $K_{M,}$ respond to elevated methionine, resulting in high hepatic AdoMet levels; in addition, AdoMet is a positive effector of these isoforms [5].

In liver, the transmethylation flux, i.e. the rate of conversion of AdoMet into AdoHcy, is mainly determined by the activity of three AdoMet-dependent methyltransferases: guanidinoacetate methyltransferase (GAMT), responsible for the methylation of guanidinoacetate to form creatine; phosphatidylethanolamine methyltransferase (PEMT), which synthesizes phosphatidylcholine from phosphatidylethanolamine; and glycine N-methyltransferase (GNMT), which methylates glycine to form sarcosine. Both GAMT and PEMT consume large amounts of AdoMet and contribute significantly to homocysteine production and plasma levels [6,7].

Regarding the methylation demand imposed by physiologic substrates on Hcy metabolism, hepatic GNMT activity ensures the conservation of methyl groups, when their availability is compromised, or otherwise, their disposal when the supply is excessive. Compared to most other methyltransferases, GNMT is less sensitive to AdoHcy inhibition and is allosterically inhibited by 5-MeTHF [8]. During high methionine intake, elevated levels of AdoMet limit the synthesis of 5-MeTHF, thereby activating GNMT, and then sarcosine production. Conversely, a lack of methyl groups increases MTHFR activity and 5-MeTHF levels, which inhibit GNMT activity, thereby preserving methyl groups for other transmethylation reactions. Consequently, GNMT activity has been proposed as a key regulator of the cellular methylation potential, by optimizing the AdoMet/AdoHcy ratio [9,10].

As mentioned before, besides folate as primary substrate for homocysteine remethylation, other B vitamins, such as vitamin B_{12}, vitamin B_6 and riboflavin (vitamin B_2), are co-factors for the main homocysteine regulating enzymes (Figure 1). In fact, low B vitamin intake is a common non-genetic determinant of elevated homocysteine levels in the general population (Section 3). On the other hand, supplements containing folic acid (synthetic form of folate) either alone or in combination with riboflavin, vitamin B_6, and vitamin B_{12} are used as therapy to effectively reduce plasma tHcy concentrations.

2. Determinants of Homocysteine

The major determinants of homocysteine levels have been extensively reviewed by many authors [11-16], and include genetic, dietary and lifestyle factors, as summarized in Table 1.

In the fasting state, normal plasma levels of homocysteine are between 5 and 15 µmol/L. Values above this range define hyperhomocysteinemia, which can be classified into mild (15-30 µmol/L), intermediate (30-50 µmol/L) and severe (>50 µmol/L) hyperhomocysteinemia.

Table 1 | Determinants of Homocysteine
Genetic factors
Variants in genes
Gene-nutrient interaction
Male sex
Diet
Low intake of folate
Low intake of vitamin B_6 and B_{12}
High intake of methionine-containing proteins
Lifestyle
Smoking
Coffee
Alcoholism
Lack of exercise
Others
Certain medications
Renal dysfunction
Increasing age

Mild to intermediate hyperhomocysteinemia results from a combination of genetic, nutritional and lifestyle factors. The *MTHFR* 677C>T polymorphism, causing reduced enzyme activity and thermolability, is the strongest known genetic determinant of plasma homocysteine levels in the general population. When associated with low folate levels, this variant predisposes to approximately 20% increase in plasma tHcy concentrations [14]. Circulating levels of tHcy can be restored to normal values, by increasing folate or riboflavin status.
Severe hyperhomocysteinemia is caused by genetic defects in either homocysteine transsulfuration or remethylation pathways. Classic homocystinuria or CBS deficiency is the most common inborn error of homocysteine metabolism [13]. As mentioned before, CBS deficient patients have severe hyperhomocysteinemia and homocystinuria, and develop premature serious vascular complications [17]. Mutations in the *MTHFR* gene resulting in complete loss of enzyme activity have also been reported in association with extremely high homocysteine levels in plasma, homocystinuria and premature cardiovascular disease [18]. In addition, the combination of the MTHFR 677TT genotype with moderate folate deficiency may also result in severe hyperhomocysteinemia. Other genetic causes of severe hyperhomocysteinemia include disorders of the cobalamin metabolism, namely functional MS deficiencies due to defects on transport or synthesis of cobalamin [13,19].
Plasma homocysteine concentrations increase with age and males have higher levels than females [16]. In the last decades, it has become clear that a diet deficient in folate and vitamin B_{12} is an important and common non-genetic determinant of mild to moderate hyperhomocysteinemia [13,20]. Lifestyle factors such as smoking, lack of exercise, coffee consumption or alcoholism also increase plasma homocysteine concentrations [15]. Finally, normal kidney function plays a prominent role in maintaining optimal homocysteine levels, and impaired renal function is often associated with high levels of homocysteine [13].

3. Homocysteine and Vascular Disease

Disturbances in homocysteine metabolism have been associated with a host of pathologic conditions, including neurological disorders, congenital abnormalities, bone diseases, diabetes, hepatic and renal dysfunction, cancer and cardiovascular diseases.
Vascular disease is the leading cause of death in the Western industrialized world [21]. In addition to traditional risk factors, such as diabetes, hypertension, smoking and hypercholesterolemia, hyperhomocysteinemia is also associated with vascular disease.
In 1969, Kilmer McCully was the first to acknowledge the potentially harmful effects of homocysteine in the vascular wall [22]. In two young patients with markedly elevated homocysteine concentrations due to different genetic causes, McCully found similar vascular lesions. The observation that the only common metabolic abnormality between those two patients was the striking hyperhomocysteinemia formed the basis for the so-called *Homocysteine Theory*. According to this theory, homocysteine (or one of its derivatives) could be atherogenic and responsible for the development of premature vascular complications. In addition, McCully raised the important question whether mild to moderate elevations of homocysteine, common in the general population, would also increase the risk of vascular disease [23].
In 1976, Wilcken *et al* provided the first direct evidence for a relationship between abnormal homocysteine metabolism and coronary artery disease [24]. Since these observations, data from a large number of clinical and epidemiological studies have implied an important role for moderately elevated levels of plasma homocysteine as an independent risk factor for cardiovascular events and mortality [25-33]. In a large meta-analysis, Humphrey *et al* found that every raise of 5 µmol/L in plasma tHcy concentration increases the risk of coronary disease events by approximately 20%, independently of traditional risk factors [34]. However, it is important to note that these observational studies do not unequivocally establish the direction of the cause-effect association, i.e. is an elevated homocysteine concentration a cause or a consequence of the disease? In fact, one cannot exclude the possibility that increased plasma tHcy levels may be associated with some other factor, and merely represent an epiphenomenon of the disease. To address this important question, several randomized controlled trials were initiated, aiming to study the effect of homocysteine-lowering B-vitamin therapy on prevention of clinically defined vascular disease outcomes. The majority of these intervention trials and following meta-analysis published so far have shown no clear clinical benefit on vascular disease risk and

mortality, despite a substantial, quick and long lasting homocysteine-lowering effect [35-40]. These observations have raised doubt about the causality of plasma homocysteine in vascular disease pathology [41]. Is the *Homocysteine Theory* still valid? Or does homocysteine merely represent a surrogate for another related metabolite that is the true atherogenic culprit? Actually, the current view holds that the vascular toxicity of homocysteine cannot be rejected solely on the basis of the negative trial evidence. Most of the conducted intervention studies have been neglecting the impact of several confounding factors (summarized in Table 1) that may have led to bias and inappropriate interpretation of the data [42-44].

Table 2 | Summary of possible limitations of homocysteine-lowering intervention studies
1. Short follow-up (in comparison with disease development)
2. Analysis based on secondary prevention
3. Patients with higher homocysteine levels (>20µmol/L) are underrepresented
4. Dual effects of B-vitamins supplementation, such as:
Enhanced inflammation and cell proliferation of existing atherosclerotic lesions
Folic acid (synthetic oxidized form) may cause folate-deprivation on target cells, due to its higher affinity for folate receptors
Folic acid affects natural killer cell function and impairs enzymes for which reduced folates are natural substrates
5. Bias by folic acid food fortification and/or multivitamins consumption
6. Bias from the concomitant use of statins and aspirin

First, although based on a large number of patients, most intervention trials had a follow-up duration of less than 5 years, which is very short with reference to the time frame of the development of atherosclerotic disease. This is supported by a meta-analysis on primary stroke published by Wang and colleagues, showing that folic acid supplementation had no effect in the short-term studies (<36 months), but a statistical significant risk reduction of 29% in the studies with at least 36 months of follow-up [45]. Second, the majority of the observational studies focused on homocysteine as a risk factor for primary events, whereas most intervention trials were designed to study recurrent events. It is reasonable to think that homocysteine-lowering

therapy might only be successful in the prevention of a primary vascular incident, and have no strong effect in improving secondary incidents.

In most cases, the intervention trials were performed in individuals with baseline levels of homocysteine roughly above the values found in the general population (≤15 µmol/L). Patients with higher levels of homocysteine were underrepresented in these studies, and might actually benefit from therapy [42].

A central explanation for the lack of positive effect of intervention trials addresses the possible dual effect of B-vitamins supplementation. One cannot exclude the possibility that homocysteine-lowering therapy with folic acid and B-vitamins may produce adverse vascular effects that mask the benefit of lowered homocysteine. For example, folates are well known as major intervenient on important biologic processes, such as the synthesis of nucleic acids, and therefore it is possible that this intervention may enhance cell proliferation and inflammation, accelerating the progression of the disease [42].

Lastly, a high percentage of men and women with increased risk for cardiovascular diseases, independent of their cholesterol levels, takes statins or aspirin (or both) to prevent primary or secondary events, which may very likely confound or obscure any possible effect of vitamin intake [44].

Another additional possibility is that, not homocysteine, but rather a related metabolite could be the trigger of some pathological changes associated with elevated homocysteine levels. Possibly explaining the failure of homocysteine-lowering vitamins to reduce vascular events, it was recently reported that supplementation with B-vitamins does not efficiently lower plasma AdoHcy levels [46].

Notably, one must not forget that the major evidence for increased plasma homocysteine as a risk factor for vascular disease comes from studies in CBS deficient patients. These patients have markedly elevated homocysteine concentrations in plasma and urine, and develop occlusive vascular disease in early adulthood or even in childhood [17]. When diagnosed, they undergo efficient homocysteine-lowering therapies including pyridoxine, folic acid, cobalamin, or betaine supplementation, which reduces the risk of vascular events by 90% [47].

In addition, accumulating data from *in vivo* and *in vitro* studies support the hypothesis that homocysteine plays a role in the pathogenesis of vascular disease, since it initiates various atherogenic mechanisms (Section 4).

4. Pathophysiology of Hyperhomocysteinemia

Although many studies have demonstrated significant biological effects and important molecular events triggered by homocysteine, the exact biochemical mechanism by which hyperhomocysteinemia contributes to vascular disease still remains poorly understood. Many potential mechanisms, involving significant functional and structural changes of the endothelium, have been suggested (TABLE 3) [48-53]. Endothelial dysfunction due to impaired bioavailability of NO is a consistent finding in hyperhomocysteinemia and will be further discussed (SECTION 5). Disturbed cellular methylation capacity, as consequence of increased AdoHcy levels, will be a focal point.

TABLE 3 | PATHOPHYSIOLOGICAL MECHANISMS INVOLVED IN HYPERHOMOCYSTEINEMIA
Endothelial dysfunction/injury
Impaired bioavailability of nitric oxide
Increased asymmetric dimethylarginine
Vascular inflammation
Proliferation of smooth muscle cells
Impaired activity of coagulant and fibrinolytic systems
Endoplasmatic reticulum stress and activation of unfolded protein response
Homocysteine thiolactone and protein homocysteinylation
Oxidative stress
Increased production of reactive oxygen and nitrogen species
Lipid peroxidation
Reduced expression of antioxidant enzymes
Activation of NAD(P)H oxidases
eNOS uncoupling
Impaired methylation
DNA methylation
Protein methylation

5. Hyperhomocysteinemia and Endothelial dysfunction

The endothelium has a vital function in regulating and maintaining the integrity and functionality of the vascular system. In response to physical and chemical stimuli (such as shear stress, acetylcholine, or thrombin), endothelial cells produce and release a variety of regulatory mediators important to preserve the balance of the vascular tone, coagulation, fibrinolysis and thrombosis, inflammation and vascular growth [54,55].

Endothelium-dependent relaxation is largely determined by the continuous production of nitric oxide (NO), an important vasodilator molecule with antithrombotic and antiatherogenic properties [56]. The synthesis of NO is catalyzed by endothelial NO synthase (eNOS, EC 1.14.13.39), by oxidizing its substrate L-arginine, in the presence of cofactors, such as NADPH, FAD, and tetrahydrobiopterin (BH_4). Besides NO, this reaction also yields L-citrulline. Within endothelial cells, eNOS targets to plasma membrane invaginations named caveolae, where is inhibited by binding to caveolin-1, the integral membrane protein of these microdomains. NO synthesis is triggered off by increased cytosolic calcium which complexes with calmodulin, and then binds and activates eNOS. NO rapidly diffuses from the endothelial cells into the layer of vascular smooth muscle cell (VSMC), where it activates soluble guanylate cyclase, converting GTP to the second messenger cGMP. The subsequent activation of a protein kinase then leads to inhibition of calcium influx into the VSMC, causing vascular relaxation (Figure 2).

Apart from mediating vasodilatation, NO is a highly reactive signaling molecule involved in the inhibition of platelet aggregation, leukocyte-endothelium adhesion, and vascular smooth muscle cell proliferation [56].

Endothelial dysfunction is the first step in the development of atherosclerosis. In both human and animal models of hyperhomocysteinemia, impairment of endothelial vasomotor function is a consistent finding [13,52,57-62]. However, the exact mechanism behind this association has not yet been established and may be multifactorial (Table 3).

Much of the endothelial dysfunction attributed to homocysteine is thought to involve oxidative stress [63]. In fact, increased levels of reactive oxygen species (ROS) in the vasculature contribute to vascular pathology by decreasing NO bioavailability. Due to the reactivity of the homocysteine SH group, oxidative stress was the first of now many mechanisms proposed to explain the association between hyperhomocysteinemia and vascular disease. Oxidation of homocysteine itself leads to increased generation of ROS, like superoxide anion, hydrogen peroxide and hydroxyl radical that

contribute to oxidative inactivation of endothelium-derived NO and endothelial dysfunction [63,64]. Homocysteine may also promote oxidative stress by directly impairing expression and activity of glutathione peroxidase-1, one of the most important antioxidant enzymes of the vascular endothelium [65]. In addition, hyperhomocysteinemia promotes NAD(P)H oxidases activation and eNOS uncoupling , both leading to increased production of ROS (TABLE 3) [52,66].

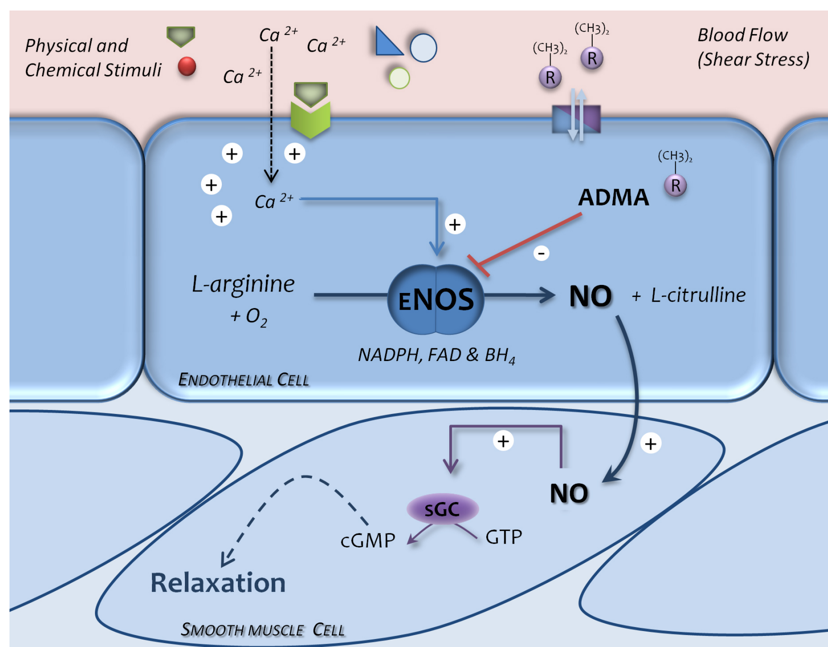

FIGURE 2 | SYNTHESIS OF NITRIC OXIDE

Increased influx of Ca^{2+} due to physical and chemical stimuli triggers nitric oxide (NO) production within the endothelial cell by the endothelial isoform of nitric oxide synthase (eNOS), in the presence of the co-factors NADPH, FAD and tetrahydrobiopterin (BH4). NO is a free radical gas that diffuses and activates its target soluble guanylate cyclase (sGC) in the smooth muscle cell, enhancing cyclic guanosine monophosphate (cGMp) production, which in turn mediates vasodilatation. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthesis.

Endogenous asymmetric dimethylarginine (ADMA) inhibits NO synthesis and plays an important role in vascular (dys)function (FIGURE 2) [67]. Many studies have clearly shown that ADMA influences both functional and structural properties of the vessel wall, and increased levels of ADMA are recognized as an emerging cardiovascular risk factor [68-70]. In addition to inhibiting NO synthesis, ADMA may also promote eNOS uncoupling, thereby directly contributing to increased oxidative stress [71].

The association between hyperhomocysteinemia and ADMA is discussed below and is a major focal point of this thesis. The metabolism of these two molecules is interconnected in several ways (FIGURE 4), supporting the hypothesis that ADMA, by impairing NO production, may mediate, at least in part, the endothelial dysfunction during hyperhomocysteinemia.

5.1 Homocysteine, ADMA and Vascular Disease

In 1992, Vallance and colleagues described ADMA as an endogenous competitive inhibitor of nitric oxide synthase (NOS) activity [67]. Since then, the role of this metabolite in the regulation of endothelial function has been extensively investigated, especially with regard to cardiovascular diseases.

The first indication suggesting that the apparent adverse vascular effects of homocysteine could be related to ADMA accumulation was reported by Böger *et al* [72]. In diet-induced hyperhomocysteinemic monkeys, they observed that experimental hyperhomocysteinemia could elevate ADMA concentrations and result in endothelial dysfunction [72]. Following studies confirmed that plasma levels of ADMA increase rapidly after oral methionine loading also in humans [73,74]. In a second study, Böger and colleagues showed that after methionine loading in healthy subjects, homocysteine and ADMA were positively correlated and associated with a decline in endothelium-dependent vasodilatation [73]. It should be noted, however, that other investigators have reported only a moderate increase [75] or no increase at all [76] of plasma levels of ADMA after methionine loading. The basal plasma levels of homocysteine and ADMA are positively correlated in humans with vascular disease [74,77-82], as well as in the general population [83], although in the majority of these studies the association was weak to moderate at most. It has been suggested that impaired kidney function, which is a common cause for elevation of both plasma homocysteine and ADMA levels, may be the underlying cause for this positive association between homocysteine and ADMA in vascular disease [84-86], a subject still under debate. Indications for a metabolic link connecting homocysteine and ADMA have also been provided by studies with cultured cells [87-89]. For example, incubation of human endothelial cells with methionine or homocysteine produced dose-dependent increases of the ADMA levels present in the culture medium [87,88].

It is generally accepted that lowering elevated levels of homocysteine is easily accomplished by treatment with B vitamins, of which folic acid is especially effective. The effect of therapy

with B vitamins on ADMA levels has been investigated in both human and animal models. Although one study found a parallel decrease of ADMA and homocysteine levels in subjects with hyperhomocysteinemia during folic acid treatment [81], many other studies are consistent in reporting that ADMA levels are not affected by B-vitamin therapy [72,73,78,90-92].
In summary, there is some evidence in support of ADMA as the missing link connecting homocysteine, endothelial dysfunction and vascular disease [69,93,94]. However, some authors hold that homocysteine and ADMA are independently related with the vascular complications observed in hyperhomocysteinemia [75,76,90,95,96], and suggest that other factors, such as impaired renal function, may explain some of the reported associations.

6. Metabolism of ADMA

ADMA is a naturally occurring amino acid released into the cytosol after the proteolysis of proteins containing methylated arginine residues (Figure 3) [97]. Protein arginine methylation is an important post-translational modification carried out by a group of enzymes described as protein arginine methyltransferases (PRMTs) [98]. These enzymes use AdoMet as a methyl donor and are classified into type I and type II, depending on their specific catalytic activity. Both types of PRMTs catalyze the transfer of a methyl group to a guanidino nitrogen atom of arginine and form monomethylarginine (MMA), whereas a second methyl group can be added to the same guanidino nitrogen atom by type I PRMTs to produce ADMA. Type II PRMTs methylate both guanidino nitrogen atoms of protein arginine residues resulting in formation of symmetric dimethylarginine (SDMA). Many substrates for PRMTs are proteins found in the nucleus, which interact with nucleic acids and are involved in the regulation of important cellular mechanisms, such as gene transcription, maturation and processing of RNA, but also translation and protein subcellular localization [98].
The synthesis of NO is directly inhibited by MMA and ADMA, but not by SDMA [99-102]. However, the physiological plasma concentrations of ADMA are approximately tenfold higher than MMA, thus ADMA is likely to be the principal endogenous inhibitor of NO production [102,103]. ADMA, MMA and SDMA export to and uptake from the circulation by neighboring cells or distant organs is mediated by cationic amino acid transporters (CAT). Importantly, all

the three free methylarginines are able to interfere with NO synthesis by competing with arginine for CAT-mediated cellular uptake, but the physiological consequence of this inhibition remains unclear [101,102].

Following its synthesis, free ADMA is either degraded in the cell of origin or exported to the circulation to be metabolized by liver and kidney. SDMA is mainly eliminated by the renal route; specific enzymatic clearance for SDMA is unknown.

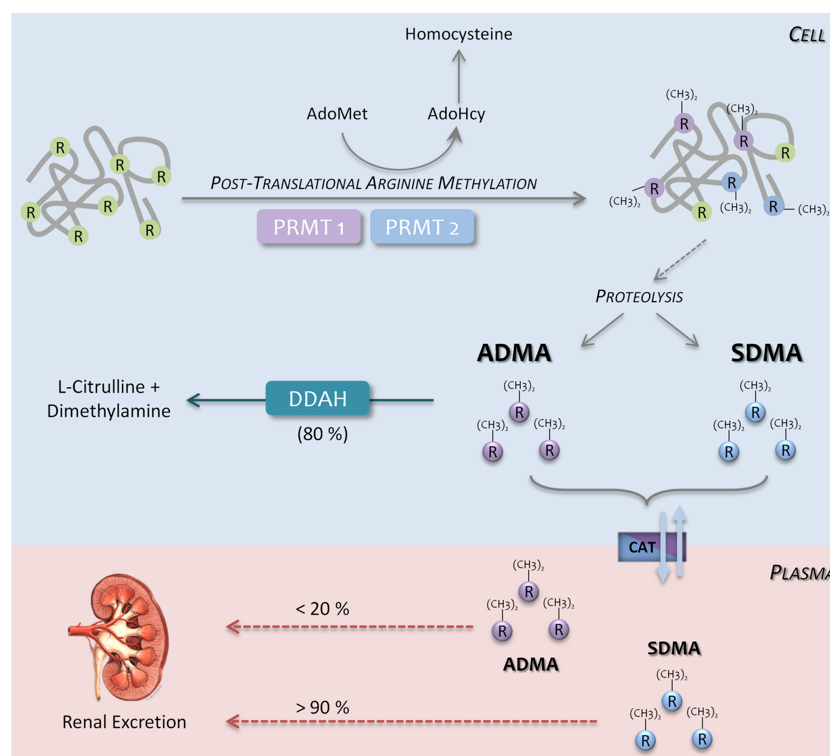

FIGURE 3 | SIMPLIFIED SCHEME OF ADMA AND SDMA METABOLISMS

Free asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are produced after proteolysis of proteins which arginine residues (R) have been methylated. Type I protein arginine methyltransferases (PRMT1) catalyze the formation of ADMA, whereas type II protein arginine methyltransferases (PRMT2) are responsible for SDMA synthesis. Both PRMTs use S-adenosylmethionine (AdoMet) as methyl group donor; consequently, S-adenosylhomocysteine (AdoHcy) and homocysteine are formed. Intracellular ADMA is degraded by dimethylarginine dimethylaminohydrolase (DDAH) into citrulline; only a small part of ADMA is eliminated in urine. SDMA is mainly eliminated by the renal route. Transport of ADMA and SDMA across the cell membrane is mediated by cationic amino acid transporters (CAT).

Although the kidney is involved in removing ADMA from the circulation, only a small part (less than 20%) is excreted in urine (Figure 3) [97]. The major specific metabolic pathway for ADMA degradation involves the activity of a cytosolic enzyme called dimethylarginine dimethylaminohydrolase (DDAH), which degrades ADMA into citrulline and dimethylamine (Figure 3) [104]. In humans, two isoforms of DDAH have been identified, DDAH1 and DDAH2, encoded by two different genes whose open reading frames show 63% homology [105,106]. Analysis of mRNA expression levels in a range of different human tissues showed a distinct tissue distribution of the two genes; DDAH1 is typically found in tissues expressing neuronal NOS, whereas DDAH2 predominates in tissues containing the endothelial isoform of NOS [106]. Nevertheless, co-expression of both proteins has been found within the cardiovascular system [105,107]. DDAH activity is found in many tissues, including pancreas, spleen and endothelium, but is particularly high in liver and kidney [97]. By taking up large amounts of ADMA from the systemic circulation, the renal and hepatic systems are key players in the regulation of ADMA levels [108,109]. DDAH expression and activity are impaired in pathological conditions associated with endothelial dysfunction, and represent potential therapeutically targets [105].

The exact contribution of both DDAH isoforms to the control of ADMA levels and regulation of NO production is yet to be elucidated. A detailed biochemical comparison of the kinetics of both DDAH1 and DDAH2 enzymes has been hampered by the inability to obtain an active purified recombinant DDAH2 isoform [110].

Initial studies with inhibitors of DDAH activity revealed that this enzyme is indeed a potential indirect regulator of NO synthesis, via ADMA metabolism [111]. DDAH gene silencing techniques, and later the development of DDAH transgenic mice, have been instrumental in further elucidating the role of DDAH in endothelial dysfunction and associated diseases [105]. However, which DDAH isoform plays the major role in ADMA degradation in endothelial cells is still unclear.

Assuming that total DDAH activity reflects the combined abundance of both isoforms, the most highly expressed enzyme would be mainly responsible for ADMA clearance. However, while there is ample evidence for a role of DDAH1 in ADMA clearance, the role of DDAH2 in ADMA metabolism has been questioned by several authors [110,112,113]. Using *in vivo* siRNA technology, Wang and colleagues observed that *DDAH1* gene silencing increased ADMA plasma levels by 50% without affecting NO-mediated vascular relaxation, whereas *DDAH2* gene silencing had no influence on ADMA plasma levels, but resulted in endothelial dysfunction [112]. The authors suggested DDAH1 as the primary enzyme responsible for ADMA clearance, whereas loss of

DDAH2 activity is associated with impaired endothelium-dependent relaxation, without leading to accumulation of ADMA in the circulation [112]. On the other hand, using DDAH1$^{(+/-)}$ mice, Leiper *et al* showed that loss of DDAH1 activity lead to accumulation of ADMA with reduction of NO production, which was accompanied by vascular pathophysiology, including endothelial dysfunction [113].

Of interest, a recent study introducing a global *DDAH1* gene-deficient mouse strain provided strong evidence for the importance of the DDAH1 isoform for *in vivo* ADMA metabolism [114]. The authors generated a viable DDAH1$^{(-/-)}$ mice with normal growth and development, demonstrating that DDAH1 is not required for embryonic development, as previously reported by others [113]. Importantly, ADMA degradation was impaired in all analyzed tissues (kidney, brain and lung) of the DDAH1$^{(-/-)}$ mice, even though DDAH2 expression was not altered. ADMA tissue and plasma levels were significantly increased in these mice, indicating that DDAH1 is critical in metabolizing NOS inhibitors and excluding DDAH2 from this role. In addition, NO production was also decreased, indicating that DDAH1 activity is important for the preservation of the normal properties of the cardiovascular system. This relevant study points towards an ADMA-independent regulation of endothelial function by DDAH2 [110,114].

Although it has been suggested that DDAH2 may not be involved in ADMA metabolism, this proposal is not supported by studies involving overexpression of DDAH genes, where enhanced expression of DDAH2 (or DDAH1) protected or improved the properties of the endothelium, by regulating vascular ADMA levels and NO bioavailability [115-118]. Furthermore, genetic variations in *DDAH1* and *DDAH2* genes have been strongly and additively associated with elevated ADMA serum concentrations in several pathological conditions involving endothelial dysfunction [119,120]. Overall, the data published so far indicate that both DDAH isoforms are important for the regulation of vascular function, but DDAH1 appears to have an effect dependent on ADMA levels, whereas the role of DDAH2 seems to be unrelated to ADMA concentrations.

6.1 Potential metabolic links between Homocysteine and ADMA

As previously stated, accumulation of ADMA during hyperhomocysteinemia has been observed and may, at least in part, explain the homocysteine-induced endothelial dysfunction due to impaired bioavailability of nitric oxide.

Homocysteine and ADMA metabolisms are interconnected in several ways (FIGURE 4) [102]. First, ADMA synthesis is carried out by PRMTs, which use AdoMet as methyl group donor. During this transmethylation reaction, AdoMet is converted into AdoHcy, which is subsequently hydrolyzed to homocysteine. Because ADMA contains two methyl groups, during its synthesis two equivalents of homocysteine are formed. On other hand, it is important to note that increased levels of homocysteine cause AdoHcy to accumulate, by shifting the equilibrium of AdoHcy hydrolase (SECTION 1). As strong inhibitor of the transmethylation process, AdoHcy may then possibly decrease the production of protein-bound ADMA. Second, homocysteine may elevate free ADMA levels by increasing proteolysis, either by destabilizing protein structure or by increasing oxidative stress [121]. Finally, it has been shown *in vitro* that homocysteine is able to inhibit DDAH activity, the major metabolic route of ADMA clearance [89,122].

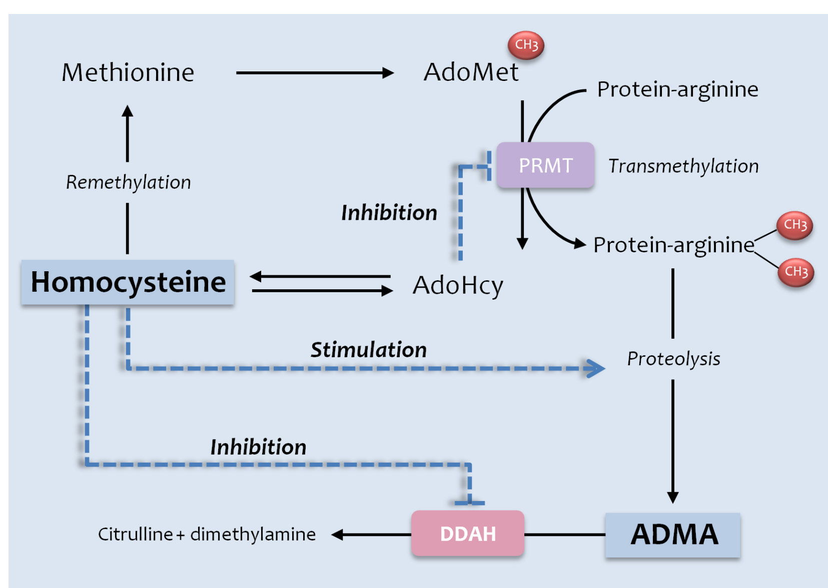

FIGURE 4 | POTENTIAL LINKS BETWEEN THE METABOLIC PATHWAYS OF HOMOCYSTEINE AND ADMA

Asymmetric dimethylarginine (ADMA) is generated by methylation of protein arginine residues, with concomitant formation of S-adenosylhomocysteine (AdoHcy) from the methyl group donor, S-adenosylmethionine (AdoMet). The transmethylation process, assisted by protein arginine methyltransferase (PRMT) activity, is inhibited by AdoHcy, possibly leading to decreased production of protein-bound ADMA. AdoHcy is subsequently hydrolyzed into homocysteine, which in turn stimulates protein degradation and inhibits dimethylarginine dimethylaminohydrolase (DDAH) activity, thus increasing ADMA levels. Adapted from [81].

The catalytic site of DDAH contains an essential reactive Cys residue [123], which is sensitive to oxidative or nitrosative modification leading to loss of enzymatic activity [109,124]. In a cell-free system, Stühlinger and colleagues demonstrated that homocysteine can directly bind to DDAH and reduce its activity [122]. Another possible mechanism for inhibition of DDAH activity in hyperhomocysteinemia is oxidative inactivation by homocysteine-induced reactive oxygen species. In cultures of both endothelial and neuronal cells, homocysteine decreased DDAH activity in a dose-dependent manner, but not in the presence of antioxidants [89,122]. In addition, homocysteine can react with NO to form S-nitroso-homocysteine, which has been reported to inactivate DDAH1 [125,126]. Despite *in vitro* data suggesting redox inactivation of DDAH by homocysteine, it is not yet clear whether this mechanism is operational *in vivo* as well. Dayal *et al* observed that homocysteine causes tissue-specific downregulation of DDAH expression in a CBS-deficient mouse model of hyperhomocysteinemia, suggesting that homocysteine may be able to regulate both activity and expression of DDAH enzymes [127].

7. Homocysteine and Impaired Methylation

As already detailed in Section 1, under physiological conditions, the intracellular levels of homocysteine seem to be precisely regulated and its accumulation prevented by rapid removal through metabolic pathways or cellular export. Intracellular homocysteine production exclusively results from AdoHcy hydrolysis, in a reversible reaction with equilibrium dynamics that strongly favors AdoHcy formation over homocysteine synthesis. By binding with higher affinity to the catalytic site of most AdoMet-dependent methyltransferases, AdoHcy acts as a powerful product inhibitor. When homocysteine accumulates, its precursor AdoHcy increases as well, thereby potentially disturbing methylation processes that are crucial for normal cell function and viability, such as methylation of DNA, RNA and proteins [128].

Next to the individual concentrations, the AdoMet/AdoHcy ratio is frequently used as an indicator of cellular methylation potential, with a decrease of this ratio predicting reduced cellular methylation capacity [129]. Gene expression profiles of both human and mouse tissues showed that AdoHcy is the only homocysteine metabolite significantly correlated with both homocysteine and transcript levels of enzymes involved in methylation processes, such as MAT, GNMT and MTHFR [130].

The metabolic link between homocysteine and the methylation cycle supports the concept of an alternative mechanism of homocysteine toxicity, secondary to AdoHcy accumulation, involving disturbed epigenetic mechanisms of gene regulation [128].

7.1 Epigenetic Mechanisms

Epigenetics refers to changes in the pattern of gene expression caused by mechanisms other than alterations in the primary DNA sequence [131]. The major epigenetic mechanisms are represented in FIGURE 5.

In eukaryotes, histones and other chromosomal proteins form a complex with DNA called chromatin. The primary unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped around a protein octamer of two copies each of the histone proteins H2A, H2B, H3 and H4. Chromatin is a dynamic structure, existing in many configurations between heterochromatin - highly compacted and silenced chromatin, and euchromatin - transcriptionally active open chromatin.

Epigenetic mechanisms ensure a stable transmission through multiple cell divisions of a transcriptionally activated state for some target genes or genome regions, or otherwise organize the chromatin to adopt a highly condensed inactive form. The most intensely studied epigenetic mechanisms of gene regulation include post-translational modifications of histones and DNA methylation. Recently, a new level of epigenetic regulation mediated by small noncoding RNAs (ncRNAs) has been described. A brief overview of the epigenetic mechanisms and their interplay in regulating gene expression is given below.

7.1.1 RNA-based mechanisms

RNA-based mechanisms of epigenetic control involve small ncRNAs species (with approximately 18 to 25 nucleotides), namely micro RNAs (miRNA) and small interfering RNAs (siRNA), which are well-known mediators of gene silencing, via RNA interference (RNAi) pathways [132,133]. RNAi is a mechanism of post-transcriptional regulation based on the degradation and/or translational repression of target mRNAs, leading to a reduction or loss of gene activity (FIGURE 5). miRNAs are derived from nuclear transcripts with characteristic stem–loop structures and exported to the cytoplasm. Alternatively, siRNAs result from long double-stranded RNA precursors, which can be either synthesized endogenously or introduced into the cell cytoplasm [132].

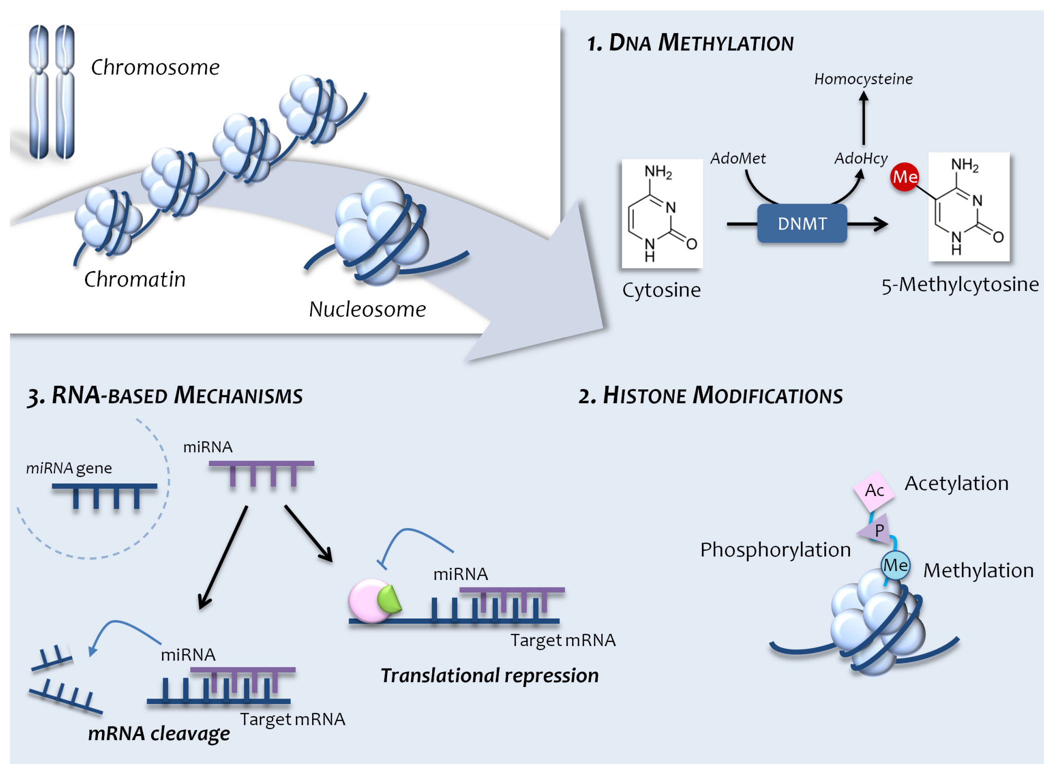

Figure 5 | Overview of epigenetic mechanisms of gene regulation.

Epigenetic modifications fall into three main categories: DNA methylation, histone modifications and RNA-associated gene silencing. These mechanisms are functionally interactive, forming a complex regulatory network that modulates genome function. 1. DNA methylation refers to the addition of a methyl group to the C5-position of a cytosine, usually in a context of CpG dinucleotides, to form 5-methylcytosine. This process is carried out by DNA methyltransferases (DNMT), using S-adenosylmethionine (AdoMet) as methyl group donor. 2. DNA strands wrapped around histone octamers form units termed nucleosomes, which organize into a dynamic structure called chromatin. Phosphorylation, acetylation and methylation are post-translational modifications of histone tails that affect the structure of the chromatin and influence gene expression. 3. RNA-based mechanisms involve small non-coding RNAs, such as micro-RNAs (miRNA), in a mechanism of post-transcriptional regulation based on the degradation and/or translational repression of target mRNAs, leading to a reduction or loss of gene activity.

Recently, it has been reported that small ncRNAs can induce epigenetic changes of gene expression [133]. For example, exogenously administered siRNAs result in transcriptional gene silencing, by inducing site-specific DNA methylation [134] and repressive post-translational histone modifications [135]. Moreover, important epigenetic regulators, such as DNA methyltransferases and histone deacetylases, are also targets for miRNA regulation [136]. This represents an exciting new mechanism by which miRNAs can control gene expression, in addition to the RNAi pathway. Furthermore, accumulating data indicate that the expression of miRNAs may be controlled by epigenetic mechanisms as well [137].

Small ncRNAs participate in a wide diversity of regulatory events associated with development and physiological pathways, and have been shown to be perturbed in cancer and other diseases, such as vascular complications [138]. Up to now, nearly a thousand of small ncRNAs have been identified in the human genome, and accumulating data suggest that a complete understanding of the interplay between miRNA and epigenetics is necessary to better understand the complexity of disease and further development of potential therapeutic agents.

7.1.2 Histone modifications

The amino terminal tails of histones are susceptible to a range of modifications, such as acetylation, phosphorylation and methylation (FIGURE 5). These post-translational modifications are carried out by different enzymes, whose activities are regulated by other specific modulators. Acetylation is one of the most important types of histone modifications [139,140]. This process is dynamic and results from the interplay between two key enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylated histones are products of HAT activity, which catalyzes the transfer of an acetyl group from acetyl-CoA to specific lysine residues. This reaction neutralizes the positive charges in the histones and reduces their attraction to the DNA backbone (which is negatively charged), resulting in an open configuration of the chromatin structure and gene activation. Conversely, HDAC induce gene repression by removing the acetyl groups, leading to a condensed inactive chromatin (FIGURE 6) [140].

Methylation occurs either on lysine or arginine residues of histone proteins, and is associated with both gene activation and silencing, depending on the specific methylated site [141,142]. Histone methyltransferases (HMTs) transfer a methyl group from AdoMet to the target residue, whereas histone demethylases eliminate those methyl groups (FIGURE 6) [143].

The resulting overall pattern of histone residue modifications in specific regions of the chromatin has been suggested to form a histone code, which dictates a particular biological outcome. Perturbation of the biological pathways involved in histone coding has great impact on the development and progression of disease [144].

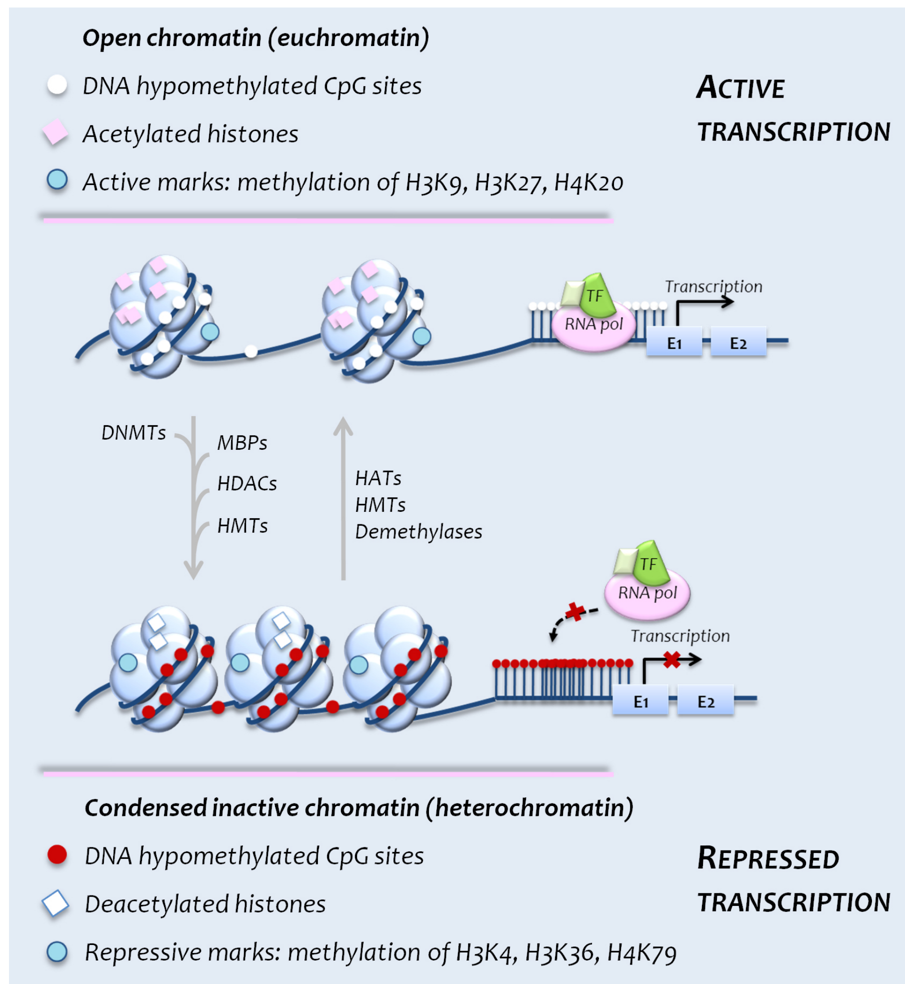

FIGURE 6 | EPIGENETIC MARKS OF GENE REGULATION.

Active genomic regions are characterized by an open expanded chromatin, associated with unmethylated CpG dinucleotides in the gene promoter, which facilitates the binding of transcription factors (TF) and RNA polymerase II (RNA pol) to target nucleotide sequences, initiating gene transcription. Conversely, methylated CpG islands results in the binding of methyl-CpG binding proteins (MBPs), which recruits a protein complex of histone deacetylases (HDACs) and histone methyltransferases (HMTs) that catalyze specific histone modifications, causing the chromatin to adopt a transcriptional inactive closed conformation. (DNMTs) DNA methyltransferases, (HATs) histone acetyltransferases.

7.1.3 DNA *methylation*

DNA methylation is an important epigenetic modification of mammalian genomes. In addition to its significant role in regulation of gene expression, DNA methylation is involved in many other important biological processes, such as X chromosome inactivation, defense against transposon proliferation, control of genomic imprinting, embryonic development and cellular lineage specification [128,145,146].

DNA methylation is the result of a chemical reaction carried out by enzymes called DNA-methyltransferases (DNMTs), which add a methyl group derived from AdoMet to the carbon number five of the cytosine (C) residue, resulting in the formation of 5-methylcytosine (mC) and AdoHcy (Figure 5) [147]. In mammals, three distinct DNMTs have crucial functional roles in the methylation of DNA: DNMT1, DNMT3a and DNMT3b. DNMT1 is the maintenance methyltransferase; it preferentially methylates hemimethylated double-stranded DNA, being responsible for the propagation of methylation patterns following DNA replication during mitotic division. In contrast, DNMT3a and DNMT3b are the *de novo* methyltransferases, important for the establishment of DNA methylation patterns in the early embryo. During development and cell differentiation processes, significant changes in DNA methylation patterns are observed, that correlate with expression/repression of explicit genes [148].

In differentiated cells, DNA methylation typically occurs in CpG dinucleotide rich-sequences, which are often concentrated in distinct areas of the genome, the so-called CpG islands [128,142,145]. Approximately 60% of human gene promoters are associated with CpG islands and usually remain free of methylation. Methylated CpG islands within regulatory regions commonly lead to loss of expression of the associated gene [142].

DNA methylation does not occur exclusively at CpG islands [149]. A recent work suggests that about half of the CpG islands is not associated with annotated promoters, but located within or between genes. Over 80% of CpG dinucleotides located outside CpG islands are commonly methylated [146,150]. In addition, it has been observed that most of the tissue-specific DNA methylation, in close association with transcriptional silencing, seems to occur not at CpG islands, but at CpG shores. CpG shores correspond to regions of relatively lower, but functional relevant CpG density located up to 2Kb upstream and downstream a CpG island [151]. Differentially methylated CpG shores are sufficient to distinguish between specific tissues and are conserved between human and mouse [152]. Furthermore, gene body methylation is a common observation in ubiquitously expressed genes and is positively correlated with gene expression [153]. Finally, a

significant fraction of heavily methylated DNA is found on repetitive elements, and is crucial to protect from transposon activity, gene disruption and chromosomal instability [153].
The mechanisms responsible for demethylation of DNA are currently being explored. Recently, it has been described that mC residues can be target of hydroxylation to form 5-hydroxy-mC, which may represent an intermediary in the process of DNA demethylation [154-156].
As mentioned above, methylation of CpG regulatory regions (*cis*-DNA binding elements surrounding gene promoters) may directly regulate gene expression by hindering the binding of the transcriptional machinery. DNA methylation and histone modifications are known to be functionally interactive (Figure 6). DNA methylation recruits a family of proteins, called methyl-CpG binding proteins (MBPs), that recognize methylated DNA sequences and can directly modulate gene expression by interfering with the binding of transcription factors (activators or repressors), or in turn, recruit enzymes that catalyze histone modifications, changing the structure of the chromatin to a transcriptional inactive conformation (Figure 6) [128,157]. For example, MeCP2 is a known MBP that acts as a gene repressor by recruiting HDACs, HMTs and the chromatin remodeling complex. H3K9 (histone 3, lysine 9) acetylation is associated with relaxed active chromatin. However, if a region of the chromatin is methylated at CpG level, MeCP2, upon recognition and binding to the methylated region, promotes deacetylation and following methylation of H3K9, which is associated with condensed inactive chromatin [158,159].

7.2 Homocysteine, DNA methylation and Vascular Disease

Available data indicate that DNA methylation plays a critical role in the development of atherosclerosis and vascular disease, in both humans and experimental models [160-162]. For example, hypomethylation of DNA has been found in advanced human atherosclerotic plaques [163], as well as in atheromas of apoE$^{-/-}$ mice, which exhibit decreased DNA methylation prior to any histological changes related to atherosclerosis [164].
As already referred to in Section 1, hyperhomocysteinemia may induce AdoHcy increase and disturb important transmethylation reactions, such as DNA methylation. In 2000, Yi and colleagues provided the first *in vivo* indication that high levels of homocysteine are associated with increase plasma and lymphocyte AdoHcy concentrations and loss of DNA methylation, in healthy young women [165].

Hyperhomocysteinemia, impaired DNA methylation and vascular disease have been found to be associated in several studies. Castro *et al* demonstrated that patients with advanced atherosclerosis have increased levels of plasma homocysteine and intracellular AdoHcy, which correlated with global DNA hypomethylation in lymphocytes [166]. Other studies in both humans [167-169] and animal models of hyperhomocysteinemia [170-173] confirmed this inverse correlation between homocysteine and the extent of DNA methylation. MTHFR- and CBS-deficient mice develop endothelial dysfunction in association with hyperhomocysteinemia, increased tissue AdoHcy levels and decreased AdoMet/AdoHcy ratio, which suggests that altered AdoMet-dependent methylation may contribute to vascular dysfunction in hyperhomocysteinemia [59,173]. The studies with genetically and/or diet-induced animal models of hyperhomocysteinemia have disclosed a tissue-specific sensitivity to AdoHcy accumulation and DNA methylation [129,172]. Using CBS-deficient mice in combination with control or methyl-deficient diet to modulate levels of AdoHcy and AdoMet, Caudill *et al* observed that increased AdoHcy levels, either alone or in association with a decrease in AdoMet concentrations, were most consistently associated with DNA hypomethylation, whereas AdoMet depletion only was not sufficient to affect DNA methylation in this model [129]. Moreover, differences in effects on global DNA methylation observed in the four analyzed tissues (liver, kidney, brain and testis) underlined the significance of tissue-specific metabolic pathways in establishing the sensitivity to AdoHcy accumulation [129]. The expression of *H19*, an imprinted gene, has been used to study the effect of impaired methylation due to hyperhomocysteinemia. Imprinting is a genetic mechanism by which genes are selectively expressed from maternal or paternal chromosomes. Under physiological conditions, *H19* is expressed from the maternal allele, whereas the paternal allele is repressed by methylation. Devlin *et al* reported tissue-specific changes in *H19* methylation and expression in mice with hyperhomocysteinemia [174]. The results showed a decreased *H19* methylation in liver, but increased gene methylation together with decreased expression in brain [174]. Ingrosso and colleagues were the first to demonstrate, in a human study, that male patients with hyperhomocysteinemia (and uraemia) have reduced leukocyte global DNA methylation associated with altered expression of *H19* and *SYLB*, supporting a causal link between increased Hcy levels and impaired gene expression, via DNA hypomethylation [175]. Moreover, *H19* and *SYLB* showed disturbed gene-specific methylation, which affected the allelic expression pattern. Interestingly, both global DNA methylation and allelic gene expression were restored after folate administration and subsequent normalization of plasma homocysteine levels.

The authors also suggested that different genes respond with different expression patterns to increased Hcy concentrations [175]. However, in patients with classical homocystinuria and hyperhomocysteinemia, global DNA methylation and *H19* methylation patterns were not altered [176].

The study of methylation patterns of disease-associated genes has contributed to a better understanding of the toxic effects of homocysteine in vascular complications [128]. For example, it has been shown that clinically relevant concentrations of homocysteine inhibit endothelial cell growth by transcriptional repression of the *cyclin A* gene, via a hypomethylation-related mechanism. The authors found that incubation of endothelial cells with homocysteine increased intracellular AdoHcy levels and demethylated a specific CpG site, located in a repressive cell-cycle dependent element of the *cyclin A* gene [177]. Of additional interest was the observation that homocysteine inhibited DNMT1 activity in this cell system, which provided evidence that homocysteine may directly interfere with specific DNA methylation reactions [177]. Another study showed that homocysteine may also impair endothelial cell growth by repressing fibroblast growth factor-2 (*FGF2*) gene expression [178]. However, in this case and as opposed to the *cyclin A* example, the authors found that the promoter region of *FGF2* was hypermethylated. The promoter region of the estrogen receptor α gene was also found to be hypermethylated in vascular patients and positively associated with increased homocysteine plasma levels and severity to atherosclerotic lesions [179]. Recently, it has been documented that homocysteine stimulates the expression of p66shc, a protein known to promote oxidative stress, resulting in decreased NO production and endothelial dysfunction. Importantly, the authors demonstrated that homocysteine induced hypomethylation of specific CpG sites in the *p66shc* promoter in endothelial cells, through a mechanism mediated by inhibition of DNMT3b activity [180].

The aforementioned studies, along with several other examples [181-185], clearly support the notion that homocysteine is able to regulate gene expression by inducing epigenetic modifications, such as DNA methylation, in a complex network of mechanisms that contribute to the development and progression of vascular disease.

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Chapter 2

Objectives and Outline of this Thesis

1. Objectives

Since first purposed in 1969 by McCully, the hypothesis that elevated homocysteine might cause vascular disease has been target of extensive investigation, and in fact many independent studies have established that hyperhomocysteinemia is a risk factor for cardiovascular diseases. Endothelial dysfunction due to impaired NO bioavailability precedes the development of atherosclerosis and is one of the most consistent findings during hyperhomocysteinemia, but the underlying mechanisms are still not fully understood and appear to be multifactorial. It has been shown that homocysteine induces vascular oxidative stress through imbalanced redox status and inhibition of critical antioxidant enzyme, resulting in increased oxidative inactivation of NO. Another potential mechanism involves the metabolism of ADMA, an endogenous inhibitor of NO synthesis, which along with homocysteine, contributes to the impaired bioavailability of NO observed in hyperhomocysteinemia. Over the last decade, attention has increased towards a mechanism based on homocysteine as a modulator of the methylation capacity of the cell. Increased levels of homocysteine result in the accumulation of its precursor AdoHcy, which is a strong inhibitor of methylation processes. Hypomethylation of both DNA and proteins have been reported in association with increased levels of homocysteine and AdoHcy. Accordingly, the involvement of epigenetic alterations in the homocysteine-induced vascular disease, as consequence of accumulation of AdoHcy, is a promising possibility.

The **Major Aim** of this work was to investigate whether AdoHcy accumulation and impaired methylation processes could be one of the mechanisms involved in the decrease of NO bioavailability observed in HHcy. Several studies were designed in order to address the following objectives:

(i) To investigate whether AdoHcy accumulation influences NO bioavailability in endothelial cells.
(ii) To explore whether AdoHcy accumulation, via epigenetic modifications, disturbs the expression and/or activity of gene products involved either in NO synthesis or in the metabolism of its endogenous inhibitor, ADMA.
(iii) To study the consequences of AdoHcy accumulation on global DNA and protein methylation.

2. Outline

A general introduction on homocysteine and ADMA metabolisms is shown in **Chapter 1**, providing background information about the major concepts relevant to the design of the experimental work ascribed to this thesis. **Chapter 2** includes the aims and outline of this thesis.

The hypothesis of AdoHcy as a causative factor in homocysteine-induced vascular dysfunction is discussed in **Chapter 3**, where the impact of an impaired methylation environment due to AdoHcy accumulation on NO bioavailability is investigated in human endothelial cells.

DNA methylation is one of the most prominent epigenetic modifications of mammalian genomes and an important player on the development of many pathologies, including atherosclerosis and vascular disease. In **Chapter 4**, we compare two most frequently used methods for measuring global DNA methylation and determine which method to use in the subsequent chapters.

Chapter 5 explores the ability of AdoHcy accumulation in disturbing epigenetic mechanisms, namely DNA methylation, and its consequences on the expression and activity of the genes involved in ADMA degradation.

Protein arginine methylation gives rise to ADMA, the major endogenous inhibitor of NO synthesis, and represents an important post-translational modification essential for protein function and cell homeostasis. In **Chapter 6,** we investigate the extent to which protein arginine methylation status is affected by accumulation of AdoHcy, in comparison with DNA methylation, in cultured human endothelial cells.

In **Chapter 7**, using a diet-induced hyperhomocysteinemia rat model we sought to investigate the *in vivo* effect of moderate to severe chronic hyperhomocysteinemia on tissue global DNA methylation status.

The study of possible *in vivo* associations between homocysteine and ADMA metabolisms are explored in **Chapter 8**, where plasma levels of homocysteine and ADMA were determined in CBS deficiency patients, in which intracellular AdoHcy accumulation results from endogenously altered homocysteine metabolism.

Finally, **Chapter 9** presents a general discussion, major conclusions and future perspectives disclosed by this thesis.

Chapter 3

Cellular hypomethylation is associated with impaired nitric oxide production by cultured human endothelial cells

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Abstract

Hyperhomocysteinemia (HHcy) is a risk factor for vascular disease, but the underlying mechanisms remain incompletely defined. Reduced bioavailability of nitric oxide (NO) is a principal manifestation of underlying endothelial dysfunction, which is an initial event in vascular disease. Inhibition of cellular methylation reactions by S-adenosylhomocysteine (AdoHcy), which accumulates during HHcy, has been suggested to contribute to vascular dysfunction. However, thus far, the effect of intracellular AdoHcy accumulation on NO bioavailability has not yet been fully substantiated by experimental evidence.

The present study was carried out to evaluate whether disturbances in cellular methylation status affect NO production by cultured human endothelial cells. Here, we show that a hypomethylating environment, induced by the accumulation of AdoHcy, impairs NO production. Consistent with this finding, we observed decreased eNOS expression and activity, but, by contrast, enhanced *NOS3* transcription.

Taken together, our data supports the existence of regulatory post-transcriptional mechanisms modulated by cellular methylation potential leading to impaired NO production by cultured human endothelial cells. As such, our conclusions may have implications for the HHcy-mediated reductions in NO bioavailability and endothelial dysfunction.

Keywords |

S-adenosylhomocysteine, Methylation, Endothelial function, Nitric oxide bioavailability, Endothelial nitric oxide synthase

Introduction

Hyperhomocysteinemia (HHcy) is a risk factor for vascular disease, but the underlying mechanisms remain incompletely defined [1].

Cellular methylation potential is determined by the concentrations of the homocysteine (Hcy) precursor, S-adenosylhomocysteine (AdoHcy). AdoHcy inhibits virtually all S-adenosylmethionine (AdoMet)-dependent-methyltransferases, which in turn, catalyze the transfer of a methyl group from AdoMet to a wide variety of target molecules (including DNA and proteins), forming AdoHcy and the methylated substrate [2]. AdoHcy is further converted into Hcy and adenosine by a reversible reaction catalyzed by AdoHcy hydrolase, which favours AdoHcy production. When Hcy accumulates, its precursor AdoHcy will accumulate as well. Elevated Hcy was shown to be directly associated with increased extracellular and intracellular AdoHcy concentrations *in vivo* [1]. Increasing evidence indicates that Hcy may be regarded as a cellular demethylating agent, at least at the DNA level. In fact, we and others have observed an association between high levels of Hcy and AdoHcy, which, in turn, is correlated with global DNA hypomethylation status [1]. In addition, several studies support the existence of epigenetic mechanisms in the context of Hcy-related endothelial dysfunction [2].

Vascular disease begins with endothelial dysfunction, a sensitive indicator of the atherosclerotic process, occurring even before the clinical manifestations of the pathology and predicting adverse clinical outcomes [3,4]. Reduced bioavailability of nitric oxide (NO) and consequent impairment of endothelium-dependent vasodilatation result in endothelial dysfunction. Nitric oxide is synthesized by endothelial cells, from the amino acid L-arginine, via the enzymatic action of endothelial nitric oxide synthase (eNOS), and is an important mediator of intracellular signalling and a potent anti-atherogenic molecule responsible for the maintenance of vascular homeostasis [5]. Endothelial NOS expression is subject to significant degrees of regulation from transcriptional to post-translational levels [3,4]. Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of eNOS, and is derived from the proteolysis of methylated arginine residues in proteins [6]. Methylation of arginine in proteins is carried out by type I protein arginine methyltransferases (PRMTs 1), transferring one methyl group from AdoMet to various proteins and forming, in addition, AdoHcy [7]. AdoHcy is further hydrolysed to Hcy. Because of the metabolic link between ADMA and Hcy, ADMA has been suggested as a potential mediator of endothelial dysfunction in HHcy [7-10]. Evidence from animal models and clinical studies have

suggested that accumulation of ADMA contributes to impaired NO generation and disease pathogenesis [11].
Several *in vivo* studies, either in animal models [12-14] or in humans with severe [15], mild [16,17], or transient [18,19] HHcy, showed that endothelial dysfunction is a key vascular phenotype occurring in the setting of HHcy; however, the mechanistic understanding of the association between HHcy and impaired NO bioavailability remains to be completely defined. The present study was designed to evaluate whether a hypomethylating environment, induced by accumulation of the Hcy precursor, AdoHcy, perturbs NO production by cultured human endothelial cells.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cords of healthy foetuses from uncomplicated pregnancies and vaginal deliveries from healthy mothers, as described [20], or commercially obtained from Lonza (Walkersville, MD, USA). The cord was collected in buffered solution [4 mmol/L KCl, 140 mmol/L NaCl, 11 mmol/L D-glucose (all from Merck, Darmstadt, Germany), 10 mmol/L HEPES (Gibco, Grand Island, NY, USA), and 1% antibiotic–antimycotic solution (Sigma, St. Louis, MO, USA)], and stored at 4°C. Within 4 days, cells were isolated by collagenase (Gibco, Grand Island, NY, USA) treatment, and cultured essentially as previously described [20]. Cells were grown in coated six-well plates (6 x 10 cm^2), 75 cm^2 flasks or 100 cm^2 disks, depending on subsequent analysis.
All experiments were performed at 80% confluence and between the fourth and sixth passage. After the removal of the culture medium, fresh medium was added, either without (control) or with increasing concentrations (5, 10, and 20 μmol/L) of an AdoHcy hydrolase inhibitor (adenosine-2,3-dialdehyde, ADA). After 24 h of incubation, lactate dehydrogenase (LDH) release was evaluated in the cell culture medium using the Cytotoxicity Detection Kit (Roche Diagnostic GmbH, Mannheim, Germany).

Metabolite analysis

Aliquots of the medium were collected from cells grown in six-well plates after 24h of incubation and stored at –20°C, for further Hcy and ADMA analysis. HUVEC were then extensively washed with ice-cold phosphate-buffered saline (PBS) and exposed to denaturation buffer for 15 min at 4°C. Denaturation buffer consisted of 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L DTT (all from Merck, Damstadt, Germany), 10 mmol/L HEPES (Gibco, Grand Island, NY, USA), and 1.0 mmol/L PMSF (Sigma, St Louis, MO, USA) (pH 7.9), containing 1% (V/V) Triton X-100 (from Merck, Darmstadt, Germany). The cell lysate was centrifuged at 1200 rpm for 10 min at 4°C. The supernatant (cytosol) was collected, and two aliquots were taken. One was promptly deproteinized with an equal volume of 10% perchloric acid and stored at –20°C for further AdoMet and AdoHcy evaluation, and the remainder was immediately frozen and kept at –20°C until protein determination.

Extracellular total Hcy levels, defined as the total concentration of Hcy after reductive cleavage of all disulfide bonds, was determined by HPLC analysis according to Araki and Sako [21], with minor modifications. Extracellular ADMA concentrations were measured by HPLC with fluorescence detection as described previously [22], using modified chromatographic separation conditions [23]. Intracellular AdoHcy and AdoMet concentrations were analysed by LC-MS/MS [24].

Nitric oxide levels

The NO production in response to intracellular AdoHcy accumulation was measured by the Griess reaction [25]. For this purpose, cells were grown in six-well plates. Culture medium was replaced 24 h before incubations, which were conducted in 0.8 mL of basal endothelial cell culture medium without phenol red. After 12 h and 24 h of incubation with ADA, aliquots of culture medium were collected for NO_x quantification. Incubations for 18 h with 1 mmol/L of L-N^G-nitroarginine (L-NNA, from Cayman Chemicals, Ann Arbor, MI, USA), an eNOS inhibitor, in the presence and absence of 20 μmol/L of ADA, were also performed. Freshly collected (200 μL) cell culture supernatants were mixed with 200 μL Griess reagent (1:1 mixture of 1% sulphanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 minutes at room temperature. Absorbance at 543 nm was recorded. Cells were lysed and protein concentrations were determined. Nitrite concentration was calculated using sodium nitrite standards and normalized by cellular protein concentration

Western Blotting

Western blot was performed for analysis of eNOS levels, using four independent cultures of HUVEC (30 cm^2 for each condition). Cells were washed 3 times with ice-cold PBS, directly lysed in cell lysis buffer containing protease inhibitors (Sigma, St Louis, MO, USA), collected with a cell scraper, and sonicated. After centrifugation, the obtained supernatant was used for total protein determination and Western blot analysis. Protein concentrations were quantified using the DC Protein Assay (Biorad, Hercules, CA, USA).

Protein samples (20-30 μg) were separated on 10 % SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond ECLTM, Amersham, GE Healthcare, Chalfont St. Giles, UK). The membranes were incubated with anti-eNOS (at a 1:500 dilution; Cell Signalling, Danvers, MA, USA) and anti-β-actin (at a 1:400 dilution; Sigma, St. Louis, MO, USA) antibodies. A secondary anti-rabbit IgG HRP secondary antibody (Cell Signalling, Danvers, MA, USA) at a 1:2000 dilution was used. Primary antibody incubation was performed overnight at 4°C, and secondary antibody incubation was performed for 1-1.5 hours at room temperature. An ECL Plus Western Blotting Detection System was used for protein detection (GE Healthcare, Chalfont St. Giles, UK), membranes were exposed to Amersham Hyperfilm HCl (GE Healthcare, Chalfont St. Giles, UK), and a VersaDoc scanning system (BioRad, Hercules, CA, USA) was used for densitometry analysis.

eNOS activity

eNOS activity was determined by measuring the efficacy of conversion of [^{3}H]-Arg to [^{3}H]-citrulline in protein extracts from cultured cells using a commercial kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's instructions. Four independent cultures were used. Briefly, cells were grown to confluence in 100 cm^2 disks, treated with 20 μmol/L of ADA for 24 hours, washed with PBS, harvested in PBS containing 1 mmol/L of EDTA, and centrifuged. The obtained pellet was lysed in 100 μL of lysis buffer, centrifuged, and 10 μL of the obtained supernatant were incubated with 40 μL of reaction buffer containing [^{3}H]-Arg for 60 minutes at 37°C. Reactions were terminated by addition of 400 μL of Stop Buffer from the kit. Cell lysates were applied to a cation exchange resin column from the kit. The citrulline was eluted, radioactivity determined, and normalized via protein concentration.

Reverse transcription and quantitative PCR

Total RNA was extracted from three independent cultures using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and was reverse transcribed (2 µg) into cDNA using oligo(dT) SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Specific primers for *NOS3* gene (NM_000603.3) F:5'-CGTCCCTGTGGAAAGACAAG-3' and R:5'-CACGATGGTGACTTTGGCTA-3' were designed with the Universal Probe Library Assay Design Center (Roche Applied Science). *EIF4A2* gene (NM_001967.3) served as an internal control with F:5'-GTGTGAACTGGACCCTGTTG-3' and R:5'-TATTTAACATTCAAACTTCATTAAGACATG-3' primers. Amplification reaction assays were performed in triplicate and contained 1×SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) and primers at optimal concentration. A hot start at 50°C for 2 min, was followed by an extension period at 95°C for 10 minutes and then for 40 cycles at 95°C for 15s and 60°C for 1 min, using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Fluorescence emission was detected for each PCR cycle and the C_T (threshold cycle) values were determined.

Statistics

Data are presented as means ± SD. Statistical evaluation of the data was performed by paired Student's *t* test for comparisons between two groups, and by one-way analysis of variance followed by Dunnett's test for comparisons between more than two groups. A value of $p < 0.05$ was regarded as significant.

Results

Intracellular AdoHcy and AdoMet levels

As shown in Figure 1A, ADA was effective in promoting a significant AdoHcy intracellular accumulation, confirming its inhibitory effect on AdoHcy hydrolase. AdoMet levels remained unaltered (189 ± 50, 216 ± 22, 169 ± 50 and 203 ± 30 pmol/mg protein for ADA 0, 5, 10 and 20 µmol/L, respectively, p not significant for trend). A significant ($p < 0.01$ *versus* Control) decrease

in intracellular AdoMet/AdoHcy ratio was observed (5.8 ± 1.6, 1.8 ± 0.2, 1.0 ± 0.3 and 0.8 ± 0.1 for ADA 0, 5, 10 and 20 µmol/L, respectively), which was exclusively defined by the increasing levels of AdoHcy.

Hcy and ADMA export, the effect of AdoHcy hydrolase inhibition

In the presence of increasing concentrations of ADA, there was a significant decrease in both Hcy and ADMA extracellular concentrations, showing that both export levels were reduced in an ADA dose-dependent manner (Figures 1B and 1C).

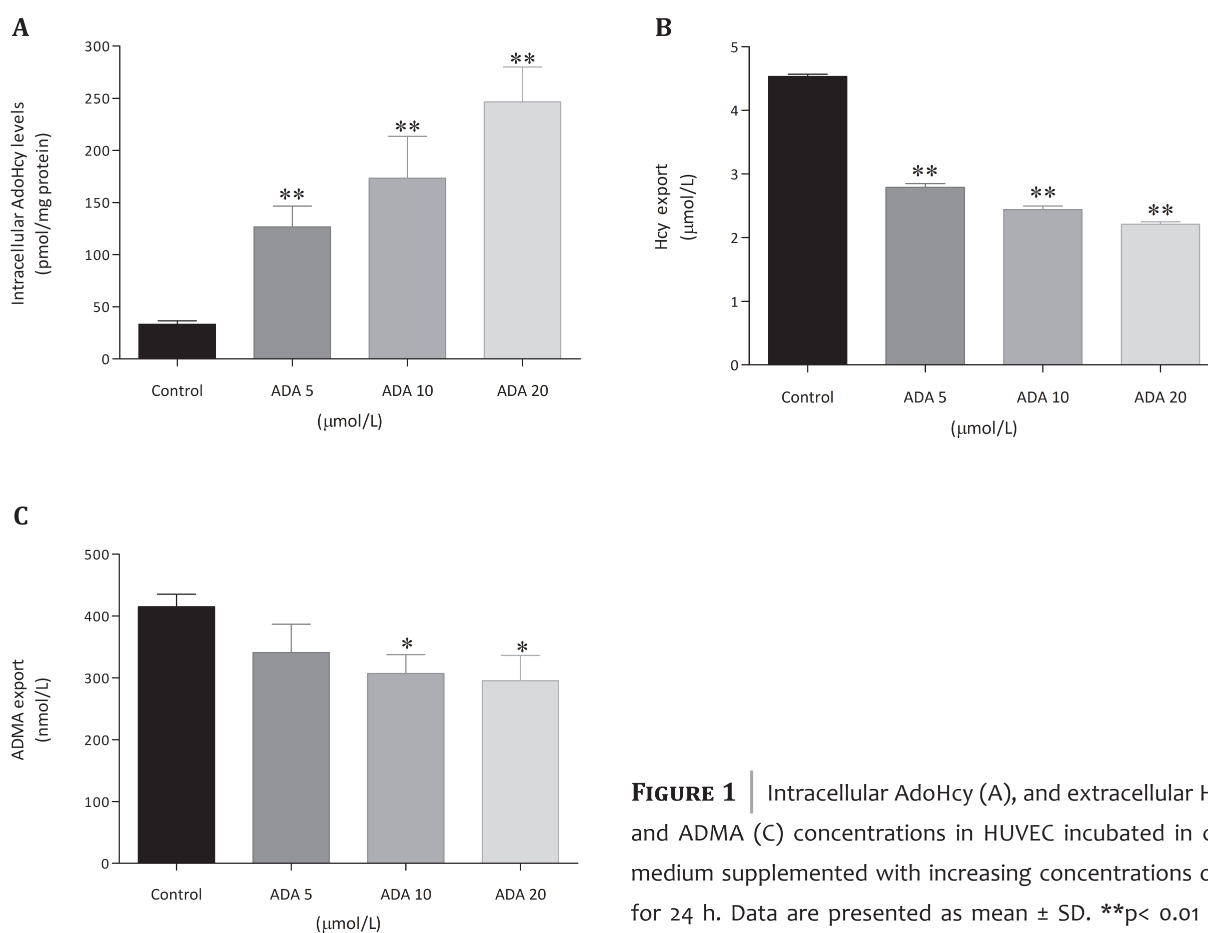

Figure 1 | Intracellular AdoHcy (A), and extracellular Hcy (B) and ADMA (C) concentrations in HUVEC incubated in culture medium supplemented with increasing concentrations of ADA for 24 h. Data are presented as mean ± SD. **p< 0.01 versus control, n≥ 3; *p< 0.05 *versus* Control, n= 6.

Lactate dehydrogenase (LDH)

Cell LDH release levels (mean ± SD, n=3), an indicator of cytotoxicity, after 24 h of incubation in the absence (control: 100 ± 8 %) or presence of the tested ADA concentrations. were similar [(ADA = 5 μmol/L: 111 ±16 %; ADA = 10 μmol/L: 110 ± 17 % and ADA = 20 μmol/L: 105 ± 12 %]. Therefore, a possible cytotoxic effect for the ADA was ruled out in the present study.

Nitric oxide production under intracellular AdoHcy accumulation

We measured nitrite, a metabolite of NO, as an index of endothelial NO production. We observed a decrease in extracellular nitrite concentrations, after 12 and 24 h of incubation, which reached statistical significance for the highest ADA concentration tested (20 μmol/L, Figure 2). The extracellular nitrite concentrations observed in the presence of ADA was abolished by co-incubation with L-NNA, an eNOS inhibitor (Figure 3).

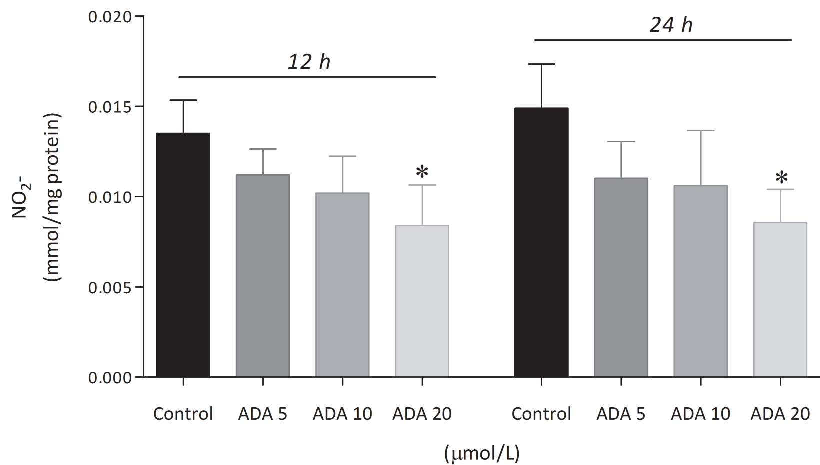

FIGURE 2 | Nitrite levels in HUVEC culture medium, measured by the Griess reaction. HUVEC were incubated in culture medium supplemented with increasing concentrations of ADA for 12 or 24h. Data are presented as mean ± SD, and are representative of 3 different cell lines. *$p < 0.05$ *versus* Control.

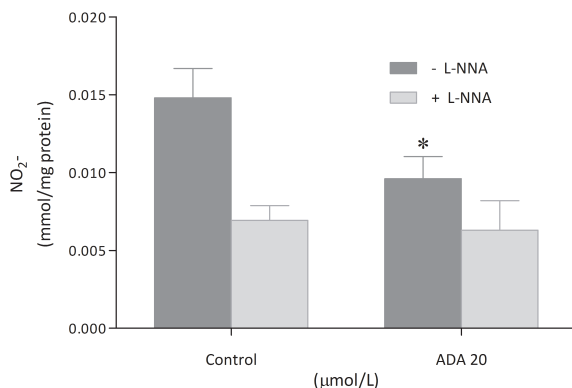

FIGURE 3 | Nitrite levels in HUVEC culture medium supplemented with and without 20 μmol/L of ADA, in the absence and presence of L-NNA (1 mmol/L), after 18 h of incubation. Data are presented as mean ± SD. *$p < 0.05$ *versus* Control, n= 3.

eNOS protein levels and enzyme activity

The expression of eNOS protein was studied using Western blot analysis. A typical band with a molecular mass of approximately 140 kDa was detected with an anti-eNOS antibody, with β-actin used as reference. Relative expression of eNOS was significantly reduced by intracellular AdoHcy accumulation (Figure 4A).

The functionality of the eNOS protein was assessed by monitoring the efficacy of L-arginine to L-citrulline conversion in cell lysates. Compared to controls, cells incubated with 20 µmol/L ADA for 24 h showed a significant decrease in eNOS activity ($p < 0.05$) (Figure 4B).

NOS3 mRNA levels

NOS3 mRNA levels were quantified by RT-qPCR. As opposed to the ADA-induced decrease of eNOS protein and activity, a significant increase of *NOS3* gene transcription was found directly related to the intracellular accumulation of AdoHcy (Figure 4C).

Discussion

The major finding of the present study is that intracellular accumulation of AdoHcy resulted in impaired NO production by cultured human endothelial cells. Furthermore, decreased eNOS protein expression and corresponding enzymatic activity were observed, but increased *NOS3* mRNA levels were found.

Hcy metabolism is biochemically linked to the principal epigenetic tag (5-methylcytosine) found in DNA [2]. Inhibition of cellular methylation reactions by AdoHcy, which accumulates in the setting of HHcy, has been suggested to contribute to vascular dysfunction. Accordingly, several studies support the existence of epigenetic mechanisms in the context of Hcy-related endothelial dysfunction [2]. However, thus far, the effect of intracellular AdoHcy accumulation on NO bioavailability has not yet been fully examined. The present study was designed to evaluate whether a hypomethylating environment, caused by intracellular AdoHcy accumulation, contributes to the impairment of endothelium-dependent NO production in cultured human endothelial cells.

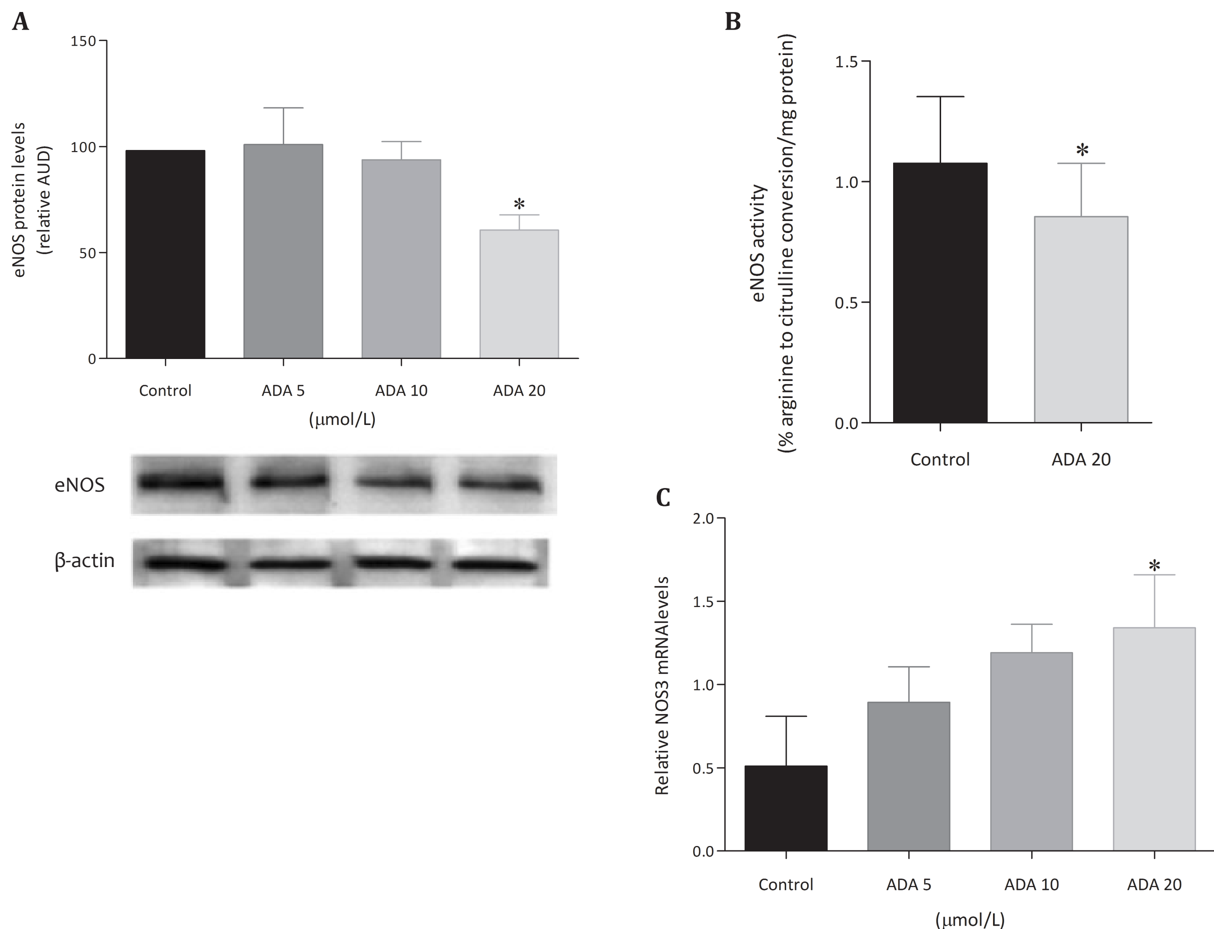

Figure 4 | eNOS expression and enzyme activity in HUVEC cultured with increasing concentrations of ADA for 24 h. (A) Relative eNOS protein levels were determined by Western blotting. β-actin was used as loading control. Densitometry was performed on 4 blots. A representative blot is shown. (B) eNOS activity was measured in HUVEC incubated with and without 20 μmol/L of ADA after 24 h by measuring the conversion of L-arginine to L-citrulline in cell lysates. Four different experiments were performed, each in duplicate. (C) Real-time quantitative RT-qPCR of relative NOS3 mRNA levels of three or more independently prepared cDNA pools, representing independent RNA isolations. Data are mean ± SD. *$p<0.05$ versus control.

Because AdoHcy hardly crosses the cell membrane [20,26], the intracellular accumulation of the Hcy precursor, AdoHcy, was achieved through the pharmacological inhibition of AdoHcy hydrolase by an adenosine analogue, ADA. As shown in Figure 1, increasing concentrations of ADA were effective in increasing intracellular AdoHcy levels. Supporting our previous observations, the levels of AdoHcy exclusively determined the decrease in AdoMet/AdoHcy ratio since AdoMet levels remained constant over the range of ADA concentrations used [20]. Hcy export from the HUVEC reflects an imbalance between its intracellular production and metabolism. Therefore, and as expected, a decrease in the concentration of extracellular Hcy was observed (Figure 1B), confirming the inhibitory effect of ADA upon AdoHcy hydrolase.

The rapid metabolism and short half-life of NO brings additional difficulties to its analytical assessment [25]. However, the measurement of nitrite, a metabolite of NO, is currently used as an index of endothelial NO production [25]. As shown in Figure 2, with increasing concentrations of ADA, a dose-dependent decrease in extracellular nitrite levels was observed in our work model. This effect was abolished in the presence of the eNOS inhibitor L-NNA (Figure 3). This finding confirmed that the decreased nitrite formation caused by intracellular AdoHcy accumulation was due to impaired NO synthesis by eNOS. Previous studies also found that NO release from cultured endothelial cells was impaired in the presence of elevated Hcy [27]. In addition, in HHcy mice with endothelial dysfunction, elevated AdoHcy and decreased AdoMet/AdoHcy ratio were observed [13].

ADMA is an endogenous and potent inhibitor of eNOS that was found to accumulate in different study models including in humans with HHcy and vascular disease [11]. Consequently, the accumulation of ADMA was suggested to contribute to reduced NO generation in Hcy-dependent endothelial dysfunction [11]. Our results suggest that the accumulation of the Hcy precursor, AdoHcy, is not responsible for the ADMA accumulation observed in the setting of HHcy. In fact, an AdoHcy intracellular accumulation (Figure 1A) was associated with decreased extracellular ADMA levels (Figure 1C) in our cellular model. In addition, a significant impaired NO production was observed (Figures 2 to 4). Therefore, our results suggest the existence of an ADMA-independent mechanism triggered by AdoHcy accumulation that may contribute to the HHcy-related impaired NO bioavailability. Interestingly, it has been recently suggested that the increase in ADMA levels, seen in humans with HHcy and vascular disease, is probably due to the decline of renal function and is not directly related to HHcy-dependent metabolic changes, nor contribute to endothelial dysfunction [28,29].

Nitric oxide is generated in endothelial cells from conversion of L-arginine to L-citrulline by the enzymatic action of eNOS, which in turn is encoded by the *NOS3* gene. To investigate whether the observed impairment in endothelial NO bioavailability was caused by decreased eNOS expression, we focused on its translational and transcriptional expression. As shown in Figure 4A, decreased eNOS protein levels were observed, suggesting eNOS down-regulation by intracellular AdoHcy accumulation. In agreement with these observations, an Hcy-induced decrease in eNOS protein levels was seen using human aortic endothelial cells, in a prior study [30]. We also examined the functionality of eNOS protein, monitoring the efficacy of L-arginine to L-citrulline conversion by cell lysates. Compared to controls, cells incubated with ADA for 24 h

showed a significant decrease in eNOS activity (Figure 4B), which is consistent with our findings of decreased nitrite levels (Figure 2) and decreased eNOS levels (Figure 4A). Previous reports concerning Hcy-induced disturbances in eNOS activity are controversial. In fact, no effect [31] or down-regulation [32] by Hcy upon eNOS activity had been reported previously in cultured endothelial cells.

Surprisingly, we found that relative *NOS3* mRNA levels were significantly increased by intracellular AdoHcy accumulation (Figure 4C). We, and others, have already shown that global DNA hypomethylation is induced by AdoHcy accumulation [1,20]. It is well known that DNA methylation is an epigenetic mechanism of gene regulation and that decreased methylation patterns are usually related to increased transcription of target genes by several mechanisms [2]. However, it has been previously shown in eNOS-expressing cells that the core promoter CpG dinucleotides are fully demethylated [33]. Accordingly, it was reported that treatment of human endothelial cells with a DNA methyltransferase inhibitor did not alter the levels of *NOS3* mRNA, as it did in eNOS non-expressing cells [33]. These observations led us to exclude DNA hypomethylation as causing the increase in *NOS3*m RNA levels observed in our model. However, DNA is not the only target for the AdoHcy-mediated inhibition of AdoMet-dependent methyltransferases, and multiple targets may be affected, including proteins implicated in eNOS expression in human endothelial cells. An example of this point is the forkhead box O1 (FoxO1) transcription factor, which acts as transcriptional repressor of *NOS3* expression. Recent data showed that protein arginine methyltransferase 1 (PRMT1) methylates FoxO1, which in turn hinders its phosphorylation and export to cytoplasm, thus augmenting the expression of FoxO1 target genes [34]. The same authors showed that, in a HUVEC model, the knockdown of PRMT1 induced *NOS3* expression [34]. Moreover, our data show that intracellular AdoHcy accumulation was associated with decreased extracellular ADMA concentrations, as well as increased relative *NOS3* mRNA levels. Recent observations by our group showed that protein-incorporated ADMA is strongly decreased by AdoHcy intracellular accumulation in HUVEC [35], indicating that PRMT1 is a target for AdoHcy-mediated inhibition and suggesting that FoxO1-methylation may be subsequently reduced by AdoHcy. Taken together, these findings suggest that PRMT1 was subjected to AdoHcy-mediated inhibition, causing FoxO1 hypomethylation and the observed AdoHcy-dependent enhanced *NOS3* transcription (Figure 4C).

Regulation of eNOS protein levels is a complex process that is mediated at several levels, including post-transcriptionally [36]. In fact, our contradictory observations concerning eNOS

transcriptional and translational levels disclose the existence of post-transcriptional events to be ascertained. Similar observations have been reported in different contexts. For example, in livers from patients with alcoholic hepatitis, *NOS3* mRNA was found to be increased, but eNOS activity decreased, despite no differences in eNOS protein expression compared with non-alcoholic livers [37]. In addition, exercise training of diabetic animals was shown to lead to an increase in left ventricular eNOS protein and a concomitant decrease in *NOS3* mRNA [38].

In conclusion, we observed that a hypomethylating environment, due to increased intracellular levels of AdoHcy, impairs NO production by cultured human endothelial cells. This finding may have implications in the HHcy-mediated reduction in NO bioavailability and endothelial dysfunction. Taken together, our results clearly suggest that post-transcriptional events may be crucial mechanisms in the regulation of NO synthesis, and their modulation by the cellular methylation potential demands further investigation.

Acknowledgements

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Chapter 4

Global DNA methylation: Comparison of enzymatic- and non-enzymatic -based methods

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Abstract

The most frequently used methods for measuring global DNA methylation are based on two different principles: the use of methylation-sensitive restriction endonucleases followed by analysis of the obtained fragments, or the hydrolysis of genomic DNA followed by specific detection and quantification of the 5-methylcytosine content.

We aimed to compare two different methods for evaluation of global DNA methylation: the cytosine extension assay after enzymatic digestion of DNA (Cyt-Ext) and a recently described method using liquid chromatography-electrospray ionization-tandem mass spectrometry after DNA hydrolysis (LC-MS/MS).

Both approaches were applied to evaluate global DNA methylation in leukocyte DNA from 96 healthy subjects. Calf thymus and pBR322 DNAs were used as hyper- and hypo-methylated references, respectively. Using the Cyt-Ext method, the DNA from healthy individuals displayed a radiolabel incorporation of 11312 ± 1600 Dpm/µg DNA, while LC-MS/MS method showed 4.55 ± 0.1 % of methylation. Results are shown as mean ± SD. The analysis of hypo- and hyper-methylated references showed that both methods are practical to discriminate different levels of methylation.

Cyt-Ext and LC-MS/MS are viable methods in evaluating global DNA methylation status. However, LC-MS/MS assay allows an absolute quantification and displayed a far superior intra-day precision, and therefore we consider it as a better approach for use in global DNA methylation studies.

Keywords |

Global DNA methylation, Cytosine extension assay, LC-MS/MS

Introduction

DNA methylation is one of the most prominent epigenetic modifications of mammalian genomes, whereby a methyl group derived from *S*-adenosyl-methionine is covalently linked to carbon number 5 of the cytosine (C) residue, resulting in the formation of 5-methylcytosine (mC) and S-adenosyl-L-homocysteine. This mechanism typically occurs in CpG dinucleotide rich-sequences, which are often concentrated in distinct areas of the genome called CpG islands. Most CpG islands are located in gene promoter regions and remain free of methylation [1,2]. An inverse relationship often exists between the extent of methylation at a regulatory CpG island and gene transcription, leading to the activation or inactivation of a functional gene. Besides its significant role in regulation of gene expression [3,4], cytosine DNA methylation is an epigenetic silencing mechanism involved in many other important biological processes, such as X chromosome inactivation, defence against transposon proliferation and control of genomic imprinting [5].

Disruption of proper DNA methylation levels has been associated with pathogenic mechanisms underlying embryonic malformation, mental retardation and, more recently, cardiovascular disease [6,7]. Similarity, some reports have shown an association between global hypomethylation of DNA and several types of cancers [8]. Accordingly, the evaluation of global DNA methylation status may be envisaged as a potential biomarker for several disease states.

DNA methylation has become the most widely studied epigenetic marker and therefore an increasing need has arisen for simple and reliable quantitative methods of analysis. In fact, over the last decade, the field of DNA methylation has grown dramatically and became one of the most dynamic and rapidly developed branches of molecular biology. As a result, many technologies have been developed, but accessible, accurate and reproducible quantification of DNA methylation remains a challenge.

The most frequently used analytical methods for measuring global DNA methylation are based on two different principles. One principle relies on the use of methylation-sensitive restriction endonucleases followed by analysis of the obtained fragments. Usually, genomic DNA is incubated with a pair of isoschizomer endonucleases which recognizes specific methylated or unmethylated DNA sequences [9,10]. The second principle involves the hydrolysis of total genomic DNA followed by detection and quantification of the mC content. The hydrolysis can be performed enzymatically [11,12], resulting in nucleotides or nucleosides, or by addition of an

acid [13,14], resulting in the free bases. Acid hydrolysis producing the free bases, C and mC, is more time-efficient and easier to perform than enzymatic hydrolysis, since the latter consists of multiple steps involving different enzymes.

To our knowledge, we are the first to perform a study to compare two methods for evaluation of global DNA methylation, one based on restriction endonucleases and the other on mass spectrometry. Namely, the so-called cytosine extension assay (Cyt-Ext), which was first described by Pogribny *et al* [9] in 1999, and still widely used, and the more recently described method by Kok *et al* [13], using acid hydrolysis and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS).

In the Cyt-Ext method, the analysis of global DNA methylation employs the methylation-sensitive restriction enzyme *Hpa*II (EC 3.1.21.4), which targets the sequence CCGG, thereby producing a single guanine overhang when the internal cytosine is unmethylated. Using an extension assay, a radiolabeled cytosine will link to that guanine and will be detected by scintillation counting. The radiolabel incorporation is proportional to the number of unmethylated sites. Therefore, an increase in radiolabel incorporation will reflect a hypomethylated status. The results are expressed relatively to the amount of DNA, thus implicating its precise quantification.

Global DNA methylation measured by LC-MS/MS according to Kok *et al* involves an initial hydrolysis of genomic DNA by formic acid resulting in free hydrophilic bases. Due to the polarity of C and mC, the selectivity of the chromatographic system is improved using nonafluoropentanoic acid (NFPA), an ion-pairing agent, which increases C and mC retention times allowing their proper separation by reverse-phase chromatography, and quantification using stable isotope dilution LC-MS/MS. The results are expressed in 5-methylcytosine/total C (mC/tC) ratio, hence avoiding the problems associated with precise DNA quantification. A lower percentage of mC/tC will logically reflect a hypomethylated status.

The two above-mentioned methods were compared regarding the precision and feasibility to detect significant changes in genome-wide methylation levels of hyper- and hypo-methylated DNA references and DNA extracted from leukocytes of healthy volunteers.

Materials and Methods

Subjects

Ninety six healthy subjects, with a mean age of 36 ± 11.3 years, were recruited from hospital staff by advertisement. The major clinical and biochemical characteristics of the study population are described in [13]. Briefly, the criteria for inclusion were: no history of metabolic, renal or vascular pathology, no supplementary intake of vitamins or use of any drug other than oral contraceptives within in the preceding year, and absence of pregnancy. A written informed consent was obtained from all participants and the Local Ethical Committee approved this study.

Standards and Calibration curve

Calibration curves were constructed using five standard solutions ranging from 0.5 to 6.5% of methylation prepared with DNA from *Escherichia coli* pBR322 DNA (no.D-9893, Sigma) known to have about 0.5% of methylation [15], and Calf thymus DNA (no.D-1751, type V, sodium salt, Sigma) with about 6.5% of methylation [13]. The intermediate points of the calibration curve were prepared by mixing the above mentioned DNAs in order to have a theoretical methylation of 2, 3.5 and 5 % of methylation. Each sample was analysed ten times by both methods.

DNA extraction

Venous blood was collected in EDTA-containing tubes (Becton-Dickinson, Plymouth, UK) and stored at –80°C. Specific for this study, DNA was isolated, stored at –20°C and analysed within 6 months. Prior to DNA extraction, 200 µL of the blood sample was treated with 4 µL of ribonuclease A solution (100 mg/mL, from bovine pancreas, Sigma). DNA was further isolated and purified using a QIAamp DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Extracted DNA was quantified by measuring the UV absorbance at 260 nm (NanoDrop® 1000, Thermo Scientific) and purity was confirmed by the ratio of absorbances at 260 and 280 nm, which was ≥ 1.8.

Global DNA methylation analysis by cytosine extension assay

The assessment of global DNA methylation levels was performed as previously described [9] with small modifications. Briefly, 1.0 µg of genomic DNA was digested overnight at 37°C with a 20-fold excess of the methylation-sensitive endonuclease *Hpa*II (New England Biolabs, MA, USA), according to manufacturer's protocol. A second DNA aliquot was incubated without restriction enzyme addition, serving as a background control. The extension reaction was then performed in a 25 µL volume reaction mixture, containing 0.5 µg of DNA, 1x PCR buffer II, 1.0 mM $MgCl_2$, 0.25 units of Amplitaq DNA polymerase (Applied Biosystems), 57.4 Ci/mmol [^{3}H] dCTP (Amersham Biosciences, Buckinghamshire, England) and incubated for 1h at 56°C. After incubation the samples were immediately placed on ice. Duplicate 10 µL aliquots from each extension reaction were applied onto Whatman DE-81 ion-exchange filters and, after drying for 4 h, were washed three times, during 10 minutes each, with Na-phosphate buffer (pH = 7.0), at room temperature. The filters were dried over-night and processed for scintillation counting. Background radiolabel incorporation was subtracted from enzyme-treated samples and the results were expressed as [^{3}H]dCTP incorporation/µg DNA.

Global DNA methylation analysis by LC-MS/MS

1. DNA hydrolysis

As described [13], a 10 µL aliquot containing approximately 1 µg of genomic DNA was transferred to a 300 µL microvial (VWR International, Amsterdam, The Netherlands). Then, 20 µL of aqueous internal standard solution containing 10.904 ng/µL [$^{13}C_2$, $^{15}N_3$]-cytosine (Isotec, St. Louis, MO, USA) and 0.445 ng/µL [$^2H_4$]-5-methylcytosine (CDN Isotopes, Quebec, Canada) were added and vacuum-dried for up to 48 h. To hydrolyze DNA to the free bases C and mC, the residue was dissolved in 20 µL of formic acid (98%). The microvials were sealed with a double-sided PTFE-faced silicone septum with an aluminium crimp cap (VWR International) and heated at 150°C for 3 h in an electric oven. After cool down to room temperature, the formic acid content was once more vacuum-dried overnight (17 h) and the residue was dissolved in 100 µL of a 5.0 mmol/L aqueous NFPA solution (Acros Organics, Morris Plains, NJ, USA). The samples were stored at –20°C until analysis.

2. LC-MS/MS analysis

All analyses were performed as previously described [13] and carried out on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems) equipped with a Perkin-Elmer Series 200 HPLC pump, a Perkin-Elmer Series 200 autosampler and a Harvard Apparatus Pump 11 infusion pump (Applied Biosystems). Briefly, liquid chromatography was performed on an Xterra MS C_{18} reversed-phase analytical HPLC column (50×4.6 mm, L×i.d., Waters Corporation) with a particle size of 3.5 µm. Mobile phase A consisted of an aqueous solution of 5.0 mmol/L NFPA and mobile phase B was a solution of 5.0 mmol/L NFPA in acetonitrile. The compounds were eluted with a linear gradient from 4% to 35% B in 4 min. Thereafter, the strongly retained compounds were removed by a gradient of 35% to 70% B over the next 2 min. Finally, the percentage of the mobile phase B was reduced to the initial conditions and the column was equilibrated for 6 min prior to the next injection. The injection volume used was 10 µL. The flow rate was 1.0 mL/min using a split ratio of 7:3. Ionization was performed using positive electrospray ionization. Collision-activated dissociation was started in the second quadrupole using nitrogen as the collision gas. Data acquisition and analysis were performed using Analyst for Windows NT software (Applied Biosystems). The results were expressed in the percentage of methylated to total cytosine (mC/tC).

Statistical analysis

Data are presented as mean ± SD. Statistical differences were calculated using Student *t* test for independent samples. Correlations were calculated using Pearson coefficient method.Statistical significance was set at $p < 0.05$.

Results

Here we present a comparison between the Cyt-Ext and LC-MS/MS methods, currently used to evaluate global DNA methylation. Calf thymus and pBR322 DNAs were analysed as hyper- and hypo-methylated references, respectively. The obtained results are summarized in Table 1, where intra-assay precision is expressed as coefficient of variation (CV). The values obtained by

LC-MS/MS method for DNA methylation of both DNA references favourably compare with those previously described [15,16]. Cyt-Ext assay does not permit an absolute quantification of global DNA methylation.

TABLE 1 - Global DNA methylation of pBR322 DNA (hypomethylated standard) and calf thymus DNA (hypermethylated standard) using cytosine extension assay (Cyt-Ext) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS) method. Results are shown as mean ± SD with the correspondent obtained coefficient of variation (CV).

	Cyt-Ext (Dpm/µg DNA)		**LC-MS/MS (% mC/tC)**	
DNA	*(Mean ± SD) ×10³*	*CV (%)*	*Mean ± SD*	*CV (%)*
pBR322 (n = 10)	19.4 ± 1.1	5.7	0.452 ± 0.02	4.4
Calf thymus (n = 10)	8.2 ± 0.7	8.8	6.402 ± 0.1	1.4

The five-points calibration curves were linear in the range of 0.5-6.5 % of methylation tested, with a mean correlation coefficient of −0.976 for the Cyt-Ext assay (Figure 1A), and 0.999 for LC-MS/MS method (Figure 1B). The same five-points were used for correlating the LC-MS/MS method with the Cyt-Ext assay and a significant negative correlation was found (Figure 2, Pearson coefficient, r= −0.972, p< 0.001).

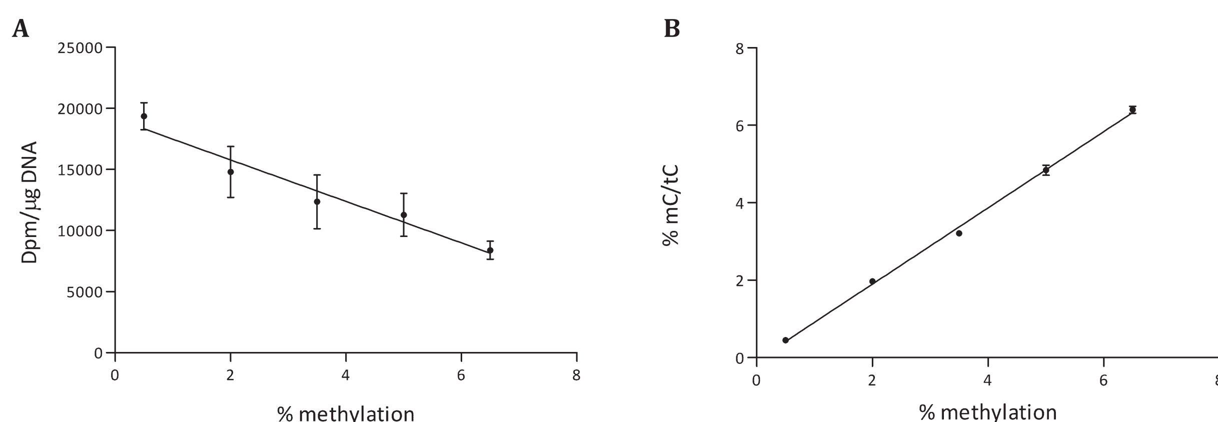

FIGURE 1 | Linearity analysis. Global DNA methylation obtained by (A) Cyt-Ext assay and (B) LC-MS/MS method of calibrating standards in the range of 0.5-6.5 % of methylated DNA. The mixtures were prepared with pBR322 DNA and calf thymus DNA, used as hypo- and hyper- methylated standards, respectively. Both methods showed linearity in the range tested (Pearson coefficient, r= −0.976 for Cyt-Ext assay and r= 0.999 for LC-MS/MS method; n=10).

The two methods were further applied to assess global methylation of genomic DNA extracted from peripheral blood leukocytes of 96 healthy volunteers. Using the Cyt-Ext method, the DNA from healthy individuals displayed a radiolabel incorporation of 11312 ± 1600 Dpm/μg DNA (mean ± SD). The same DNA showed a 4.55 ± 0.1 % (mean ± SD) of global DNA methylation, when analysed by LC-MS/MS method. As shown in Figure 3, the methods do not correlate among the studied population (Pearson coefficient, $r = -0.295$, $p < 0.003$; $n = 96$).
Both methods were able to discriminate the control population from hyper- and hypomethylated reference DNAs ($p < 0.05$ and $p < 0.001$, Student t test *versus* healthy volunteers for Cyt-Ext assay and LC-MS/MS method, respectively).

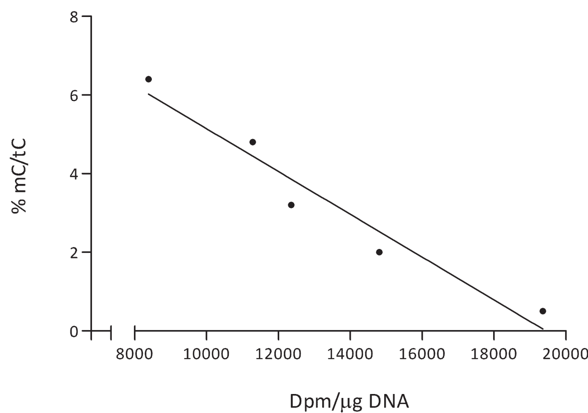

Figure 2 | Correlation between the global DNA methylation obtained by Cyt-Ext assay and LC-MS/MS method of standard mixtures prepared with pBR322 DNA (hypo-methylated standard) and calf thymus DNA (hyper-methylated standard). Each sample was analysed 10 times (Pearson coefficient, $r = -0.972$, $p < 0.002$).

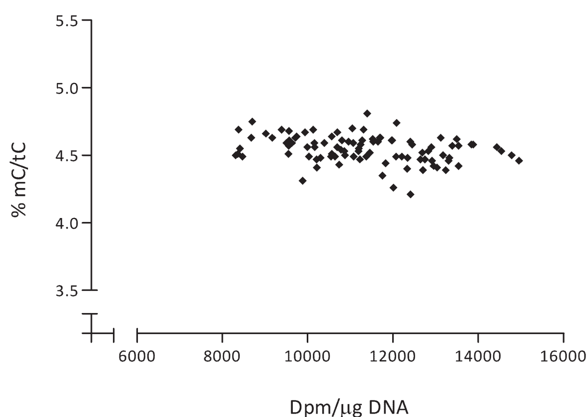

Figure 3 | Plot showing global DNA methylation obtained by Cyt-Ext assay and LC-MS/MS method of DNA from 96 healthy volunteers, where only a slight correlation was observed (Pearson coefficient, $r = -0.295$, $p < 0.003$; $n = 96$).

Discussion

The present study showed that both Cyt-Ext and LC-MS/MS methods can be used to evaluate global DNA methylation status. Only the LC-MS/MS assay provides an absolute quantification of global DNA methylation. In addition, the LC-MS/MS method has lower coefficient of variation values and permits a higher throughput.

Over the last decade, the rapidly expanding interest in the involvement of DNA methylation in developmental mechanisms and human diseases has highlighted the need for an accurate, quantitative and high-throughput assay for global DNA methylation determination. Several techniques to study genome-wide methylation rely primarily on the inability of methylation sensitive restriction enzymes to cleave sequences containing one or more methylated cytosines, with further analysis of the obtained fragments. With the advantage provided by mass differentiation of mass spectrometric detectors the hydrolysis of genomic DNA followed by detection and quantification of the mC content leads to another tool for evaluating global DNA methylation.

The current study addresses a comparison between two previously published methods [9,13] for assessing genome-wide methylation and their feasibility to disclose significant alterations in global methylation status of genomic DNA. With this purpose, both Cyt-Ext, an enzymatic-based assay, and LC-MS/MS, a non-enzymatic-based method, were used to evaluate DNA methylation in either hypo- or hyper-methylated references, as well as in leukocytes from healthy subjects.

The Cyt-Ext is a laborious method and demands a correct quantification of DNA, because the results are expressed as Dpm per amount of DNA. The LC-MS/MS method uses hydrolysis with formic acid, allowing a sample throughput of approximately 200 samples per week. The amount of DNA sample is half that used for Cyt-Ext assay and, most importantly, does not require the exact DNA quantification.

Precision of the methods was evaluated using hypo and hyper-methylated references namely, *Escherichia coli* pBR322 DNA and calf thymus DNA, with about 0.5 % and 6.5 % of methylation, respectively. The results showed that, from an analytical point of view, the LC-MS/MS method displays better intra-day precision (Table 1).

The LC-MS/MS method allows an absolute quantification of global DNA methylation, whereas Cyt-Ext assay only permits a comparative analysis of methylation status between test samples treated with the same enzyme [17,18]. The values disclosed by LC-MS/MS method for DNA

methylation of both hypo- (0.452 ± 0.02) and hyper-methylated (6.4 ± 0.1) controls compare very favourably with the expected ones (90 and 98%, respectively).
We further determined the global methylation of DNA extracted from leukocytes of 96 healthy volunteers with Cyt-Ext and LC-MS/MS methods. Figure 3 makes clear that the Cyt-Ext assay displayed a much higher dispersion of values. This variation is due to the sum of the analytical and biological variations. As shown in Table 1, the analytical variation of the Cyt-Ext assay is considerable, with CV's between 5 and 10%. However, addition influence of biological variability cannot be excluded out, and may reflect a polymorphic nature of the *Hpa*II recognition sequence, whose CpG sites are the only ones analysed by the Cyt-Ext method [17]. On the contrary, the analysis with the LC-MS/MS method showed a narrow distribution of DNA methylation values, indicating a quite low inter-individual variability (Figure 3). Since this is a quantitative analysis of DNA methylation, the results obtained with LC-MS/MS method suggest a very tight regulation of leukocyte global DNA methylation. The levels of DNA methylation achieved with LC-MS/MS compare favourably with values previously described [13].
As aforementioned, LC-MS/MS is the most informative methodology, since it assesses the methylation status of all the cytosine residues in the genome, whereas Cyt-Ext method yields information exclusively on the methylation status of the cytosine residues within the recognition sequence of the selected restriction enzyme.
Interestingly, the results obtained for hypo- and hyper-methylated DNA references displayed a significant negative correlation (Figure 2). This shows that even though different techniques of analysis of DNA methylation, they are related to each other. However, within the healthy volunteers group, no correlation was observed due to the very low variation found in human using the LC-MS/MS method (Figure 3).
Several methods have been published as further developments of the Cyt-Ext principle that circumvent the laborious part of this assay and its need of exact quantification of DNA input [19,20]. Studies were performed to improve the sensitivity and the loss of require hazardous radioactive disposal, making it safer and less expensive. Karimi *et al* [20] developed a novel method based on combined DNA cleavage by methylation sensitive restriction enzymes and polymerase extension assay by Pyrosequencing™ platform. The method is mentioned as LUminometric Methylation Assay (LUMA) and uses a luminometric technology to quantify methylation sensitive restriction digestions. It would be interesting in future studies to compare the improvements of the above mentioned method with the LC-MS/MS method referred on this article.

In conclusion, LC-MS/MS method offers a sensitive and accurate tool for quantification of global DNA methylation but requires special equipment, not available in all laboratories. Cyt-Ext method involves the use of radioactive chemicals and it is laborious, thus limiting its applicability to large-scale clinical trials. Despite the difference in the way of determining global DNA methylation, both methods can efficiently be applied to evaluate global methylation levels. However, only the LC-MS/MS method permits an absolute quantification of global DNA methylation. In addition, the LC-MS/MS method displayed a superior intra-day precision, and therefore we consider it as a better approach for use in global DNA methylation studies.

Acknowledgements

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Chapter 5

S-adenosylhomocysteine increases DDAH activity in human cultured endothelial cells, via a non-DNA dependent methylathion pathway

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Submitted

Abstract

Hyperhomocysteinemia, an independent risk factor for cardiovascular diseases, correlates with reduced nitric oxide (NO) bioavailability. Increased levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial NO synthesis may underlie this association. The key enzyme for ADMA degradation is dimethylarginine dimethylaminohydrolase (DDAH), which has two isoforms. Since homocysteine is directly involved in the methylation cycle, it has been suggested as an epigenetic effector. Under hyperhomocysteinemia, the accumulation of S-adenosylhomocysteine (AdoHcy) impairs methylation processes, and potentially contributes for the homocysteine-induced vascular toxicity.

This study was designed to dissect the effect of intracellular AdoHcy accumulation on DDAH expression and activity in cultured human endothelial cells. AdoHcy accumulation was induced using adenosine-2,3-dialdehyde (ADA), whereas DNA methylation was specifically inhibited by 5-aza-2-deoxycytidine (AZA).

ADA treatment increased AdoHcy levels, resulting in global DNA hypomethylation; *DDAH1* (but not *DDAH2*) mRNA levels were up-regulated, but no significant changes in protein expression were detected. ADA treatment increased DDAH activity, which was inversely associated with ADMA export. AZA induced global DNA hypomethylation, without affecting DDAH activity or ADMA export. Moreover, neither ADA nor AZA treatments altered the methylation pattern of *DDAH1* and *DDAH2* genes. In conclusion, our data suggest that AdoHcy accumulation stimulates DDAH activity by a non-DNA dependent methylation mechanism.

Keywords |

S-Adenosylhomocysteine, ADMA, DDAH, DNA methylation

Introduction

Over the past decades evidence has accumulated that hyperhomocysteinemia (HHcy) is an independent risk factor for cardiovascular diseases [1]. One of the most consistent findings in both humans and animal models of HHcy is a reduced bioavailability of nitric oxide (NO), leading to endothelial dysfunction. However, the underlying mechanisms are likely diverse and still not fully understood [1,2]. In the last decade, the involvement of epigenetic mechanisms in homocysteine (Hcy) vascular toxicity, as a result of an impaired cellular methylation potential, became an emergent possibility [3].
S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) are key-metabolites of the methylation cycle and precursors of Hcy. AdoMet is used by the majority of cellular methyltransferases, and after the transfer of the methyl group, AdoHcy is formed, which is subsequently hydrolyzed into Hcy and adenosine by the enzyme AdoHcy hydrolase. Due to its strong inhibitory effect on AdoMet-dependent methyltransferases, AdoHcy accumulation during HHcy potentially impairs crucial methylation processes. The ratio AdoMet/AdoHcy is often used as index of methylation potential and a decreased ratio typically indicates impaired methylation of important macromolecules, such as DNA, RNA and proteins [4]. In fact, HHcy has been widely reported in association with intracellular elevation of AdoHcy levels and global DNA hypomethylation (reviewed in [3]). Disruption of proper global DNA methylation has been associated with pathogenic mechanisms underlying the promotion and development of disease, including atherosclerotic lesions [5]. Prior investigations, including our own, have been supporting the hypothesis of an indirect mechanism of Hcy toxicity, secondary to AdoHcy accumulation, involving DNA hypomethylation and impaired expression of target genes [6-9].
Increased levels of Hcy have been associated with increased levels of asymmetric dimethylarginine (ADMA), an efficient endogenous inhibitor of NO synthesis [10], suggesting that ADMA may contribute, at least in part, to the Hcy-induced endothelial dysfunction due to impaired NO production [11], a subject still under debate. ADMA and symmetric dimethylarginine (SDMA) are released into the cytosol after proteolysis of proteins containing methylated arginine residues. Protein arginine *N*-methyltransferases (PRMTs) use AdoMet as a methyl donor and have been classified into two types, based on their specific catalytic activity. ADMA in proteins results from the action of type I PRMTs, whereas symmetric SDMA is produced by type II PRMTs . Unlike ADMA, SDMA does not inhibit NO synthases (NOS) [12]. Both ADMA and SDMA are cleared by

renal excretion. However, the major metabolic pathway specific for ADMA clearance involves the cytosolic enzyme dimethylarginine dimethylaminohydrolase (DDAH), which degrades ADMA into citrulline and dimethylamine [12,13]. In humans, two isoforms of DDAH have been identified, DDAH1 and DDAH2 [14], with distinct tissue distribution. Co-expression of both DDAH isoforms has been observed in cultured human endothelial cells [14,15]. The involvement of both DDAH1 and DDAH2 in the regulation of vascular function is supported by many studies, but which of these isoforms is mainly responsible for regulation of basal ADMA levels is still unclear [16]. A recent study using DDAH1$^{(-/-)}$ mice provided strong evidence that DDAH1 is largely responsible for the degradation of ADMA [17]. DDAH1 deficiency resulted in total loss of DDAH enzyme activity, accompanied with increased ADMA levels and impaired NO production [17]. In contrast, it has been shown that DDAH2 overexpression increased DDAH activity, resulting in decreased ADMA levels and improved vascular NO production [18,19]. The expression and activity of DDAH proteins is regulated at both transcriptional and translational levels [20-22]. For example, DNA methylation and histone acetylation have been shown to influence mouse *DDAH2* gene expression in trophoblast cell lineage [22], but the mechanisms of epigenetic regulation of the *DDAH1* gene are still unknown.

In the present study we investigate whether intracellular AdoHcy accumulation itself affects the expression of *DDAH* genes at the mRNA and protein levels, thereby altering enzyme activity (Figure 1), in cultured human endothelial cells. Our previous studies, using the same cell model, showed that intracellular AdoHcy accumulation resulted in a decrease of global DNA methylation [23], together with decreased ADMA export [24]. Here, we tested the hypothesis that the observed reduction of ADMA levels in our cell model is caused by enhanced degradation, due to epigenetic modifications of *DDAH* genes induced by AdoHcy accumulation.

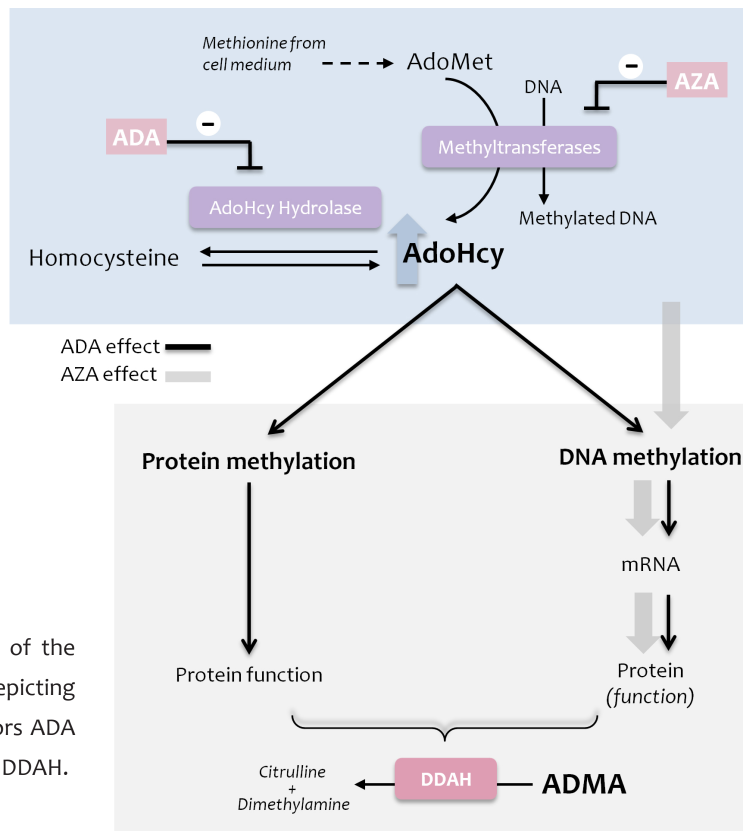

FIGURE 1 | Schematic representation of the HUVEC model and working hypothesis, depicting possible effects of the use of the inhibitors ADA and AZA on the expression and activity of DDAH.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords and cultured as previously described [23]. Cells were seeded on gelatin-coated dishes and grown under a 5% CO_2, 95% air atmosphere, in M199 medium supplemented with 10 % (v/v) heat-inactivated human serum, 10% (v/v) heat-inactivated newborn calf serum, 2 mmol/L L-glutamine, 5 U/mL heparin, 37.5 µg/mL endothelial cell growth factor (ECGF; crude extract isolated from bovine hypothalamus), 100 U/mL penicillin and 100 mg/mL streptomycin. For all experiments, 70-80% confluent passage two or three HUVEC cultures were used.

To induce intracellular AdoHcy accumulation and consequently cellular hypomethylation, HUVEC were cultured for 24h with medium supplemented with increasing concentrations (0, 2.5, 5 and 10 µmol/L) of adenosine-2, 3-dialdehyde (ADA; Sigma-Aldrich, St Louis, MO, USA), an

inhibitor of AdoHcy hydrolase. As positive control for DNA hypomethylation, cells were exposed to 5 µmol/L of 5-aza-2-deoxycytidine (AZA; Sigma-Aldrich, St Louis, MO, USA), a demethylating agent that binds to and inactivates DNA methyltransferases (DNMTs) (Figure 1).

After 24h, medium was collected for measurements of Hcy, ADMA and SDMA, and cells were harvested and processed for determination of intracellular levels of AdoMet and AdoHcy, and of total protein concentration using the BCA protein assay kit (Pierce, Rockford, USA).

Global DNA methylation

DNA was isolated and purified using QIAamp DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Global DNA methylation was measured using LC-MS/MS, as previously described [24,25]. Briefly, 1 µg of genomic DNA was hydrolyzed with formic acid, cytosine and 5-methylcytosine were separated using gradient-elution reversed phase chromatography, detected by LC-ESI-MS/MS and quantified using stable isotope dilution. Data is expressed as 5-methylcytosine to total cytosine ratio.

Metabolite assays

Total Hcy levels in fresh medium and after incubation with or without ADA and AZA were determined by high-performance liquid chromatography (HPLC), according to Akari and Sako [26]. Concentrations of ADMA and SDMA present in conditioned medium were measured by HPLC with fluorescence detection as previously described by Teerlink *et al* [27], using modified chromatographic conditions [28]. For AdoMet and AdoHcy quantification, cell lysates were promptly deproteinized with 10% perchloric acid and analyzed by stable-isotope dilution LC-MS/MS, as described [29].

Quantification of DDAH1 and DDAH2 mRNA levels

Total RNA was isolated from cultured cells using RNeasy kit (Qiagen, Hilden, Germany), and treated with DNAse I (Roche Applied Science, Mannheim, Germany) to remove contaminating genomic DNA, according to manufacturer's protocol. cDNA was synthesized from 1.5 µg of total RNA using random primers and Superscript II Reverse Transcriptase (Invitrogen, CA, USA). Quantitative real-time PCR was carried out in a total volume of 25 µL containing 5 µL of cDNA, 300

nmol/L of forward and reverse primers, and 1x SybrGreen PCR Mastermix (Applied Biosystems, CA, USA). Primer sets were designed using the Universal Probe Library (Roche Applied Science), according to the GenBank sequences (Table S1; human *DDAH1* mRNA NM_012137.3, human *DDAH2* mRNA NM_013974.1, and human β-*2-microglobuline* (B2M) mRNA NM_004048.2). PCR conditions were as follows: cycling was preceded by a hot start at 50°C for 2 min and denaturation at 95°C for 10 min, followed by 40 cycles of 15s at 95°C and 1 min at 60°C. After amplification, a melting curve was acquired by heating for 15s at 95°C, cooling to 60°C/30s and heating again to 95°C for 15s. Samples were run in triplicate on the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and fluorescence was measured during each annealing step. Expression levels of the genes of interest (GOI) were normalized to the reference gene B2M by comparative quantification, using the $\Delta\Delta C_T$ method [30]. For each sample, ΔC_T was calculated ($C_{T, GOI}$ - $C_{T, B2M}$). The mean relative expression was calculated as fold change $2^{-\Delta\Delta CT}$, ($\Delta\Delta C_T = \Delta C_T - \Delta C_{T, Control}$).

Western blot analysis

HUVEC cultured in 10 cm^2 were washed twice with cold PBS and lysed on ice with 100 µL of 20 mmol/L Tris-HCl buffer pH 8.0, containing 150 mmol/L NaCl, 90 mmol/L KCl, 2 mmol/L EDTA/NaOH, 5% Igepal, 0.5% Triton X-100 and supplemented with Mini EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany). After centrifugation, protein samples (15-30 µg) were separated on 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Hybond, Amersham, GE Healthcare). The blots were incubated for 2h with blocking buffer, containing Tris-buffered saline (TBS), 5% nonfat dry milk and 0.1% Tween-20, and probed overnight at 4°C with rabbit polyclonal antibody against DDAH1 or DDAH2 (both at a dilution of 1:500; Abcam, Cambridge, UK). After incubation with appropriate HRP-conjugated secondary antibody (at a dilution of 1:1000; Cell Signaling, MA, USA,), the bands were detected by enhanced chemiluminescence (ECL detection kit GE Healthcare, Amersham). The amount of protein loading was normalized by probing the blots with antibody against β-actin (at a dilution of 1:20000; Sigma-Aldrich, St Louis, MO, USA).

DDAH activity

The activity of DDAH in HUVEC was assessed by directly measuring the formation of L-citrulline from exogenous ADMA. HUVEC (60 cm^2) were trypsinized, pelleted and ressuspended in 500 µL of 100 mmol/L Na_2HPO_4 pH 6.5, supplemented with protease inhibitor cocktail tablets (Roche Applied Science). The cells were then sonicated three times for 3s at 10s interval, and centrifuged at 3405 *g* for 5 min at 4°C to remove cell debris. On ice, supernatants were divided in two aliquots of 200 µL and ADMA was added to both tubes at a final concentration of 4 mmol/L. To inactivate DDAH activity and determine baseline L-citrulline levels, 4% (dry) sulfosalicylic acid was immediately added to one tube. The other tube was incubated at 37°C for 6h, before the reaction was stopped. The L-citrulline concentration at baseline and after incubation was quantified by HPLC, as previously described [31]. DDAH activity was calculated from the increase in L-citrulline concentration during incubation. Data was normalized to protein content (BCA protein assay kit; Pierce, Rockford, USA) and the activity of DDAH measured in the control cells was defined as 100%. The DDAH activity evaluated under different cell culture conditions was expressed as a percentage relative to control. DDAH activity is not the only source of citrulline in the cell, for instance citrulline is also produced during NO synthesis. However, during control incubations in the absence of exogenous ADMA, no change in citrulline concentration was observed, demonstrating that other metabolic pathways did not significantly contribute to citrulline production (data not shown). Furthermore, enzyme inhibition by accumulation of the product, as well as possible consumption of citrulline by other metabolic pathways was also assayed; no significant changes were observed when compared with the control (data not shown).

Gene specific methylation analysis

The quantitative methylation analysis of the promoter regions of *DDAH1* and *DDAH2* genes was performed using the Sequenom MassARRAY® compact system. This system uses mass spectrometry (MS) for the detection and quantitative analysis of DNA methylation at CpG dinucleotides, using homogeneous MassCLEAVE™ (hMC), base specific cleavage and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [32]. DNA from HUVEC was isolated and purified as described above and forwarded to EpiTYPER™ assay. Briefly, this method involves the bisulfite treatment of genomic DNA, followed by PCR amplification. Each reverse

primer has a T7-promotor tag for in vitro transcription (5′-cagtaatacgactcactatagggagaaggct-3′), and the forward primer is tagged with a 10mer to balance the PCR (5′aggaagagag-3′). Assays were designed for the target regions using the online Sequenom Epydesigner software available online (http://www.sequenom.com) and the selected primers pairs are shown in Table S2. After Shrimp Alkaline Phosphatase treatment, which dephosphorylates unincorporated dNTPs, rendering them unavailable to future polymerase catalyzed reactions, PCR products are used as template for *in vitro* transcription and RNase A cleavage. Finally, the samples are conditioned, processed and analyzed in the MALDI-TOF mass spectrometer. EpiTyper software generates quantitative results for each CpG site or an aggregate of multiple CpG sites, referred as CpG units present on the target regions.

Statistical analysis

All experiments were performed using cells from several donors. Data are given as mean ± standard error of the mean (SEM). Tests of significance were conducted by paired Student's *t* test for comparison between two groups. Spearman test was used for correlation analysis. Statistical significance was accepted at $p < 0.05$.

Results

Manipulating cellular methylation potential

To induce intracellular accumulation of AdoHcy and thereby disturb cell methylation processes, we used ADA, an efficient inhibitor of AdoHcy hydrolase.

The presence of ADA significantly induced a dose-dependent cytosolic accumulation of AdoHcy, the endogenous inhibitor of AdoMet-dependent methyltransferases, while the levels of AdoMet did not differ significantly from control (Table 1). Therefore, the presence of ADA resulted in a significantly decreased AdoMet/AdoHcy ratio, exclusively defined by the increased levels of AdoHcy (Table 1). As a direct consequence of the blockage of AdoHcy hydrolase and reflecting impaired Hcy production, ADA significantly reduced Hcy export to cell culture media (Table 1).

The presence of ADA resulted in a significant decrease of ADMA export to the cell media, while SDMA levels did not change, within the 24h of incubation (Table 1).

A disturbed cellular methylation potential upon ADA incubation was confirmed by a decrease of about 10% of global DNA methylation (Table 1). However, only in the presence of the highest dose of ADA (10 µmol/L) the inhibition reach statistical significance. As positive control for DNA hypomethylation, cells were incubated with AZA, a specific inhibitor of DNMTs, which resulted in a significant decrease of 35% of global DNA methylation (Table 1). However, the levels of Hcy, AdoMet, AdoHcy and ADMA were not affected by AZA supplementation (Table 1).

Table 1 - Analysis of metabolites in HUVEC after incubation with ADA (µmol/L) or AZA (µmol/L) for 24h. Quantification of Hcy, ADMA and SDMA in the cell media (values are mean ± SEM and represent 6 to 20 independent experiments). Intracellular determination of AdoHcy, AdoMet and AdoMet/AdoHcy ratio (values are expressed as percentage of control ± SEM and represent 6 to 20 independent experiments). nd – not determined; * p< 0.01; ** p< 0.001 compared to control.

	Hcy (*µmol/L*)	**ADMA** (*nmol/L*)	**SDMA** (*nmol/L*)	**AdoMet** %	**AdoHcy** %	**AdoMet/ AdoHcy** %	**Global DNA Methylation** %
Control	**4.4 ± 0.1**	**411 ± 42**	**150 ± 3**	**100**	**100**	**100**	**100**
ADA 2.5	3.3 ± 0.1**	nd	nd	120 ± 19	335 ± 55*	40 ± 5**	100 ± 2
ADA 5	2.9 ± 0.1**	371 ± 36**	138 ± 3	122 ± 21	488 ± 75*	27 ± 3**	96 ± 3
ADA 10	2.6 ± 0.1**	365 ± 17**	145 ± 4	104 ± 20	707 ± 82**	17 ± 2**	88 ± 3*
AZA 5	4.4 ± 0.1**	417 ± 26	156 ± 4	98 ± 4	98 ± 2	100 ± 4	65 ± 4**

DDAH activity

To investigate whether the decrease of ADMA export under intracellular AdoHcy accumulation was due to enhanced ADMA catabolism, the activity of DDAH was determined in HUVEC lysates. As shown in Figure 2, DDAH activity was increased in the presence of ADA, but statistical significance was reached only for the highest dose (10 µmol/L), where an increase of 15 % relative to control was observed.

In line with the observation that ADMA levels remain unaltered compared to the control, AZA treatment did not change DDAH activity. This observation indicates that possible modifications on global DNA methylation status do not affect DDAH activity in HUVEC (Figure 2).
Correlation analysis revealed a significant negative association between DDAH activity in HUVEC and the levels of ADMA present in the culture media after 24h of incubation with or without ADA and AZA ($r = -0.40$, $p<0.01$; Figure 3).

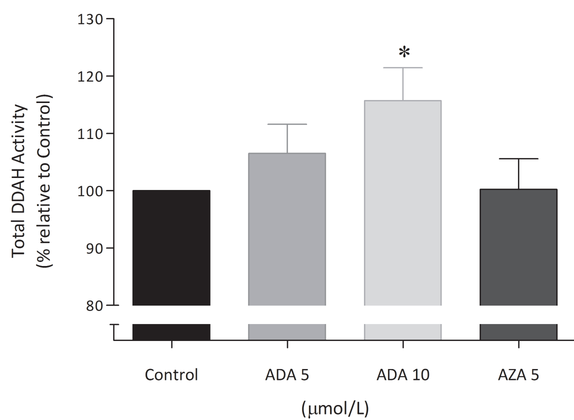

FIGURE 2 | Effect of ADA and AZA treatments on total DDAH activity in HUVEC. The data are expressed as percentage relative to non-treated HUVEC (control). The error bars represent the SEM (6 to 14 independent experiments); * $p< 0.05$ versus Control.

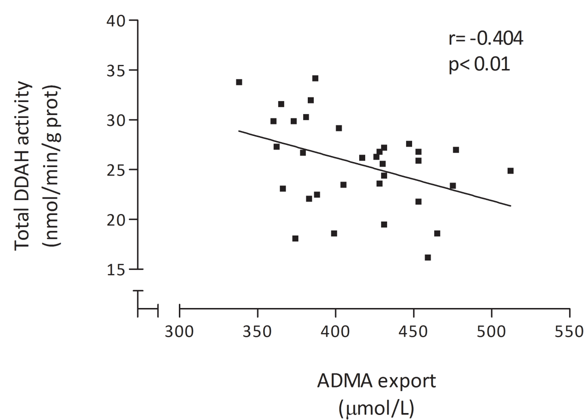

FIGURE 3 | Correlation between total DDAH activity and ADMA export to cell media. HUVEC were incubated with or without ADA and AZA for 24h ($n=33$, $r= -0.404$, $p< 0.01$; Spearman Test).

mRNA levels of *DDAH1* and *DDAH2* genes

The expression of both *DDAH1* and *DDAH2* transcripts was detected in HUVEC under normal conditions and evaluated after 24h of incubation with ADA or AZA. Compared to control, ADA treatment resulted in a significant increase of *DDAH1* gene expression, while *DDAH2* mRNA levels remained unaltered. In contrast, AZA had no effect on the mRNA levels of either *DDAH1* or *DDAH2* (Figure 4).

In the studied conditions, *DDAH1* (but not *DDAH2*) mRNA levels and ADMA export displayed a significant negative correlation (r = –0.57, p<0.01; Figure 5).

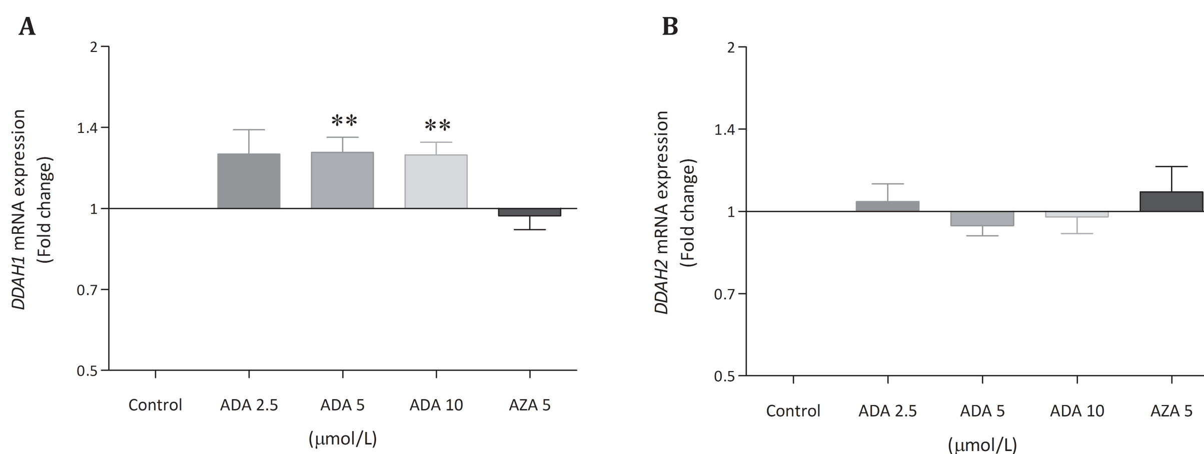

Figure 4 | Real-time RT-PCR quantification of mRNA levels of (A) *DDAH1* and (B) *DDAH2* in HUVEC after incubation with ADA or AZA for 24h. Data represent fold change relative to control, and values were normalized to β2M (n= 6 to 21 independent experiments). ** p< 0.01 compared to Control.

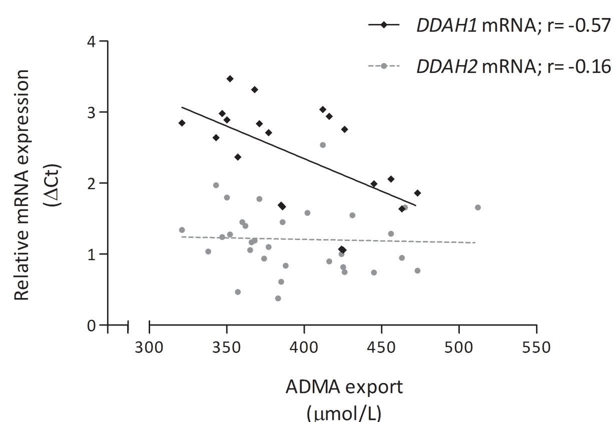

Figure 5 | Correlation of ADMA export and DDAH genes mRNA expression in HUVEC. HUVEC were incubated with or without ADA and AZA for 24h. A significant negative correlation is observed with DDAH1 (but not with DDAH2) mRNA levels (n=20, r= –0.57; p< 0.01; Spearman Test).

DDAH1 and DDAH2 protein expression

Under control conditions, HUVEC express both isoforms of DDAH protein. Upon treatment with ADA or AZA, and in disagreement with the observed increase in *DDAH1* transcript levels, protein analysis by Western blot did not reveal any substantial induction of DDAH1 expression (Figure S1). The expression of the DDAH2 isoform remained unaltered as well.

Gene specific methylation analysis

The methylation pattern of *DDAH1* and *DDAH2* genes was evaluated in cultured HUVEC under normal conditions and after treatment with ADA or AZA. The target regions encompassed the proximal promoter, the first exon and a significant portion of intron 1 (for sequence numbering, nucleotide +1 was assigned to the adenosine of the initiation translation codon ATG). *DDAH1* gene was analyzed between nucleotides -445 and +967, a region encompassing 138 CpG sites, of which 58 are in the promoter and the remaining in the coding region. *DDAH2* gene was analyzed between nucleotides -655 and +1870, a sequence displaying 155 CpG sites, 83 in the promoter and 72 in the coding region. The results revealed that both gene regions were fully demethylated under normal conditions, which agrees with their transcriptional activity. As expected, neither ADA nor AZA treatment altered the methylation status of both genes.

Discussion

The present study demonstrates that increased intracellular AdoHcy levels result in an increase of total DDAH activity in human endothelial cells, via a non-DNA methylation dependent mechanism. In addition, up-regulated *DDAH1* mRNA levels (but not *DDAH2*) were observed, which correlated with the decrease of ADMA export found in our cell model. Importantly, when DNA methylation was specifically inhibited by AZA treatment, we found no changes of DDAH activity or expression of *DDAH* genes, indicating that the increment of both *DDAH1* mRNA expression and total DDAH activity was not due to disturbed DNA methylation.

HHcy has been extensively associated with cardiovascular diseases, but the underlying mechanisms have not yet been fully resolved. ADMA is a potent endogenous NOS inhibitor, and

its accumulation may play an important role as mediator of endothelial dysfunction in HHcy. ADMA is partially eliminated by the kidney, but the primary metabolic route of its clearance from the body is its degradation by DDAH into dimethylamine and L-citrulline [13]. As important regulator of ADMA metabolism and NO production, DDAH has been gaining scientific interest within the study of vascular function.

The biochemical link between Hcy and DNA methylation supports the existence of epigenetic mechanisms in the background of Hcy-related endothelial dysfunction and vascular disease [1,3]. In fact, the ability of Hcy to modify epigenetic tags has been demonstrated. HHcy is accompanied by intracellular accumulation of AdoHcy in association with global DNA hypomethylation and impaired gene expression [3].

The present study was designed to dissect the effect of intracellular accumulation of AdoHcy on DDAH expression and activity in cultured human endothelial cells. The analysis of ADMA and SDMA in cell media, after 24h of incubation, showed that intracellular accumulation of AdoHcy decreased ADMA export, whereas SDMA levels remained constant (Table 1). We hypothesized that epigenetic changes, as a result of a reduced cellular methylation capacity due to AdoHcy accumulation, may have influenced the expression and/or activity of DDAH, the ADMA degrading enzyme (Figure 1).

In HUVEC, we found that AdoHcy accumulation increased *DDAH1* mRNA expression, but had no effect on *DDAH2* levels (Figure 4), showing that distinct regulatory mechanisms are involved in the expression of these two genes. In support, other authors have demonstrated that activators of *DDAH1* gene expression are not necessarily enhancers of the *DDAH2* variant [20,21,35]. Moreover, we observed that the increase of *DDAH1* mRNA levels was correlated with reduced extracellular levels of ADMA, in ADA-treated endothelial cells (Figure 5). In fact, DDAH1 was recently shown to be the major contributor to vascular DDAH activity and ADMA levels [17]. Importantly, we found that the significant reduction of DNA methylation by the specific inhibitor of DNA methyltransferases (AZA) had no effect on the mRNA levels of both DDAH genes (Figure 4). This observation suggets that both *DDAH1* and *DDAH2* gene expression is regulated at the transcriptional level by mechanisms independent of DNA methylation. Effectively, though HUVEC displayed a decrease on global DNA methylation status when treated with ADA or AZA, the specific methylation patterns of *DDAH1* and *DDAH2* genes showed no alterations.

Here we show that in cultured human endothelial cells, intracellular AdoHcy accumulation increased total DDAH activity, which in turn was negatively correlated with ADMA export (Figure s 2 and 3). Noteworthy, when DNA methylation was specifically inhibited, neither DDAH activity nor ADMA export was affected (Figure 2 and Table 1).

The findings that in endothelial cells 1) AdoHcy accumulation up-regulated *DDAH1* mRNA levels and increased DDAH activity associated with reduction of ADMA export, and that 2) specific inhibition of DNA methylation had none of the former effects, suggests that other cellular methylation reactions different than DNA methylation are pivotal regulatory mechanisms of DDAH expression and activity, and are primarily affected by AdoHcy.

DNA is not the only target for the AdoHcy-mediated inhibition of AdoMet-dependent methyltransferases, and multiple targets may be affected, including proteins implicated in DDAH expression in human endothelial cells. In fact, the identification of transcription factors that undergo important methylation processes which determine localization, activity or binding to other modulators is rapidly increasing [36]. Our recent observation that non-histone protein methylation is more prone to be affected by AdoHcy accumulation than DNA methylation in cultured human endothelial cells is in support of this mechanism [37].

Although DDAH activity was found increased, we were unable to observe a significant ADA-mediated increment of DDAH protein expression by Western Blot analysis (Figure S1). This discrepancy between DDAH protein levels and enzyme activity has also been documented in other studies [19,38,39], supporting the concept that DDAH enzymes are target of post-translational regulation. Studies addressing post-translational modifications of DDAH proteins resulting in increased enzyme activity have not yet been reported. It remains to be established whether the effect of ADA on DDAH activity is related to methylation of DDAH itself or that AdoHcy accumulation affected the methylation of important regulatory proteins of DDAH synthesis and protein function.

In conclusion, our data revealed, for the first time, that AdoHcy accumulation up-regulates DDAH activity, via a non-DNA methylation dependent mechanism. Epigenetic mechanisms other than methylation of DNA may be involved. For a better understanding of the pathophysiological effects of a disturbed cellular methylation capacity on ADMA metabolism, further studies are mandatory to identify key modulators of DDAH activity that can be target of AdoHcy inhibition.

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Supplementary material

Table S1 - Primer sets for quantitative real-time PCR.

Gene	Forward primer	Reverse primer	Amplicon size *(bp)*
DDAH1	TGCTTCTTTCATCATGTCAACC	CTTCCGGACTGCGTCTTC	114
DDAH2	TCGGATTTCTTAGTTTTCTTGTTTC	GGCTTGAAGACGGGGACT	120
β2M	GGCTATCCAGCGTACTCCAAA	CGGCAGGCATACTCATCTTTTT	225

Table S2 - Primer sets for quantitative DNA methylation analysis of DDAH1 and DDAH2 genes.

Gene		Forward primer	Reverse primer	Amplicon size *(bp)*	CpG sites
DDAH1					
	Amplicon #1	AAAAAATTCATTATTCACACCTCCC	AATTTGAGAAGTTTTAGGTTTGTGG	349	25
	Amplicon #2	CTCCCTAAAAAACACTCAAAATTACA	GTAGTAAGTTGGGGTTGTAGGTGGT	481	45
	Amplicon #3	ACAACTCCACCACCTACAACC	GAGTTGGGTTTTTTTTGTATTATATTT	331	43
	Amplicon #4	AAATATAATACAAAAAAAACCCAACTC	GGTTTAAATTGTTTAGGTAAAGATTAGGA	386	43
DDAH2					
	Amplicon #1	TAGAGTTGGTGGAGAAGGGAGTTTT	TCTTAAACTCAAAACAAAAACCAAA	420	29
	Amplicon #2	TGAGGAAATGAGAGGTAGAGAGTAGTT	ATCCTACCCATCCTTAAAATCACAA	458	17
	Amplicon #3	TAAAGTTTTGTAAGATTTGGGGTTT	ACTACTCTCTACCTCTCATTTCCTCAA	375	23
	Amplicon #4	GTTGTTAGAATTGTTATTTGAGGAGT	TCCCTATACCAAAACCTATACCTCC	377	24
	Amplicon #5	TTAAAGAATTTGATTTTTGAGGGTG	CAATTCTAACAACTATAACCCCAATC	445	15
	Amplicon #6	TAAGTAGGGGTAATAGGTGTTTTGG	CAAATCTTCCTCCTACCATCTCTAAA	176	4
	Amplicon #7	TGTTTGTATTAGATAGAGAGTATGGGGA	CCCCTCTTAACAACCACTAAAATAC	484	26
	Amplicon #8	GTTAGATTTGGGTGGGTTTGG	TCCCCATACTCTCTATCTAATACAAACA	434	31

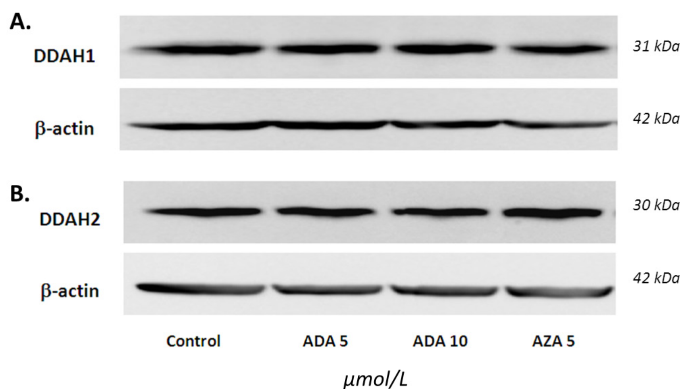

Figure S1 | Representative Western blot of whole-cell lysates of HUVEC after incubation of 24 hours with or without ADA or AZA, probed for (A) DDAH1 and (B) DDAH2, normalized to loading control, β-actin.

Chapter 6

Protein Arginine Methylation is more prone to inhibition by S-adenosylhomocysteine than DNA methylation in Vascular Endothelial cells

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Submitted

Abstract

Methyltransferases use S-adenosylmethionine (AdoMet) as methyl group donor, forming S-adenosylhomocysteine (AdoHcy) and methylated substrates, including DNA and proteins. AdoHcy inhibits most methyltransferases. Accumulation of intracellular AdoHcy secondary to Hcy elevation elicits global DNA hypomethylation.

We aimed at determining the extent at which protein arginine methylation status is affected by accumulation of intracellular AdoHcy. AdoHcy accumulation in human umbilical vein endothelial cells was induced by inhibition of AdoHcy hydrolase by adenosine-2,3-dialdehyde (ADA). As a measure of protein arginine methylation status, the levels of asymmetric and symmetric dimethylated arginine residues (ADMA and SDMA, respectively) in cell protein hydrolysates were measured by HPLC. A 20% decrease was observed at a 2.5-fold increase of intracellular AdoHcy. Western blotting revealed that the translational levels of the main enzymes catalyzing protein arginine methylation, protein arginine methyl transferases (PRMTs) 1 and 5, were not affected by AdoHcy accumulation. Global DNA methylation status was evaluated by measuring 5-methylcytosine and total cytosine concentrations in DNA hydrolysates by LC-MS/MS. DNA methylation decreased by 10% ($p < 0.05$) only when intracellular AdoHcy concentration accumulated to 6-fold of its basal value.

In conclusion, our results indicate that protein arginine methylation is more sensitive to AdoHcy accumulation than DNA methylation, pinpointing a possible new player in methylation-related pathology.

Keywords |

S-Adenosylhomocysteine, protein methylation, DNA methylation, homocysteine-related diseases

Introduction

Cellular methylation is a crucial event in regulating gene expression and protein function. DNA methylation is an important epigenetic mechanism of gene regulation that, in differentiated cells, occurs almost exclusively by methylation of cytosine at CpG dinucleotides, forming 5-methylcytosine. DNA methylation is catalyzed by DNA methyltransferases (DNMTs). Numerous studies have addressed DNA methylation in relation to disease [1,2].

Protein arginine methylation is a widespread post-translational modification that increases the structural diversity of proteins and modulates their function in the living cell. It is catalyzed by protein arginine methyltransferases (PRMTs), which are divided into two major classes depending on the type of methylarginine they generate [3]. Both type I and type II enzymes monomethylate the guanidinium nitrogen of arginine residues in proteins. The generation of asymmetric dimethylarginine (ADMA) is catalyzed by type I enzymes, whereas type II enzymes catalyze the formation of symmetric dimethylarginine (SDMA). PRMTs target a large number of distinct proteins involved in transcriptional regulation, signal transduction, RNA metabolism and DNA repair [4].

Methyl transfer is a single enzymatic process catalyzed by a methyltransferase. Both DNA and protein methyltransferases use S-adenosylmethionine (AdoMet) as methyl group donor [3]. Upon transfer of the methyl group, AdoMet is converted to S-adenosylhomocysteine (AdoHcy). AdoHcy is a competitive inhibitor of most AdoMet-dependent methyltransferases because it binds to their active sites with a higher affinity than AdoMet [5]. For this reason, the ratio AdoMet/AdoHcy is regarded as an indicator of cellular methylation capacity, and a decrease in this ratio may predict a reduced cellular methylation status. AdoHcy is hydrolyzed to homocysteine (Hcy) and adenosine by AdoHcy hydrolase. This reaction is reversible, favoring AdoHcy synthesis rather than its hydrolysis. Under normal physiological conditions, Hcy is promptly metabolized by the transsulfuration and remethylation pathways and the catabolic direction of the reaction is favored, warranting a low intracellular concentration of AdoHcy [6].

Elevation of plasma levels of homocysteine (Hcy), or hyperhomocysteinemia (HHcy), has been extensively associated with cardiovascular disease [7]. Intracellular accumulation of AdoHcy and cellular hypomethylation may underlie this relationship. Noteworthy, AdoHcy has been claimed as a more sensitive indicator of vascular disease than Hcy [8]. Yi and coworkers [9] provided evidence that moderate elevations in plasma Hcy correlate significantly with elevations in intracellular

AdoHcy levels and DNA hypomethylation in lymphocytes. This observation has been reinforced by other studies [10-13], highlighting the impact of one-carbon metabolism defects on global DNA methylation. Furthermore, DNA hypomethylation may be partially responsible for vascular complications associated with HHcy [14]. Thus, elevated Hcy may be regarded as a global DNA hypomethylation effector via AdoHcy accumulation.
Although there is a large body of literature relating AdoMet/AdoHcy ratio to the methylation status of the cell, the extent at which protein arginine methylation is affected by AdoHcy accumulation is not known. The present study was designed to determine the effect of intracellular AdoHcy accumulation on global protein arginine methylation status, in comparison with the known AdoHcy-mediated hypomethylating effect on DNA in cultured human endothelial cells.

Materials and Methods

Materials

Hepes, 5-aza-2-deoxycytidine (AZA) and adenosine-2,3-dialdehyde (ADA) were obtained from Sigma-Aldrich (St Louis, MO, USA). L-glutamine was purchased from Biochrom-AG (Berlin, Germany). Newborn calf bovine serum and endothelial cell growth factor were from Roche (Mannheim, Germany) and collagenase, M199 basal culture medium (with Earle's balanced salt solution and Hepes) and Hank's balanced salt solution were from Gibco (New York, NY, USA).

Cell culture

Umbilical cords were obtained from the Department of Obstetrics of the Amstelland Hospital in Amstelveen, The Netherlands. The investigation conforms to the principles outlined in the Declaration of Helsinki. Endothelial cells (human umbilical vein endothelial cells, HUVEC) were isolated from human umbilical veins and cultured essentially as previously described [15]. Briefly, freshly obtained human umbilical veins were collected in a buffer solution (pH 7.3) composed of KCl 4 mmol/L, NaCl 140 mmol/L, D-glucose 12 mmol/L, Hepes 11 mmol/L, 100 U/mL penicillin and 100 mg/mL streptomycin. Within 4 to 6 days, cells were isolated by collagenase treatment and ressuspended in complete M199 medium (cM199) consisting of M199 supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin (all Lonza, Verviers,

Belgium), 10% (v/v) heat-inactivated human serum (Sanquin CLB, Amsterdam, The Netherlands), 10% (v/v) heat-inactivated new-born bovine calf serum (Gibco, Grand Island, NY, USA), 5 U/mL heparin (Leo Pharmaceutical products, Weesp, The Netherlands) and 50 µg/mL crude endothelial cell growth factor prepared from bovine brains [16]. Cells were grown at 37°C in an atmosphere of 5% CO_2/95% air.

Cells from individual donors were grown until near confluence and passed as necessary. Before each experiment, culture medium was removed and cells were washed twice with Hank's Balanced Salt Solution. Incubation with ADA (2.5, 5 and 10 µmol/L), a specific inhibitor of AdoHcy hydrolase, was used to stimulate accumulation of intracellular AdoHcy. AZA (5 µmol/L) was employed to inhibit DNA methylation. Cells were grown during 24 h in cM199 medium with or without ADA or AZA. The cytotoxicity of ADA and AZA was evaluated by measuring the release of lactate dehydrogenase (LDH) into the culture medium using the Roche Cytotoxicity Detection Kit (Mannheim, Germany), in accordance with the instructions provided by the manufacturer.

Aliquots of the incubation medium were collected and stored at -20°C. Whole cell lysates were prepared by incubation in ice-cold lysis buffer (Cell Signaling Technology, Frankfurt am Main, Germany) with 1 mmol/L PMSF for 15 min and then centrifuged to remove cellular debris. Total protein was measured by the Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard. Lysates were stored at -80°C until further use.

Determination of Hcy and AdoMet and AdoHcy levels

Total Hcy levels in culture medium before and after 24 h of incubation in unsupplemented medium or in AZA or ADA supplemented medium were measured by the Abbott IMx fluorescence polarization immunoassay (Abbott Park, IL, USA) according to the manufacturer's instructions. For intracellular AdoMet and AdoHcy quantification, whole cell lysates were deproteinized with equal volumes of 10% perchloric acid, centrifuged at 4°C, 16000 × *g*, for 2 min, and the obtained supernatant was analyzed by stable-isotope dilution liquid chromatography-tandem mass spectrometry, as previously described in detail [17].

Evaluation of global DNA methylation status

DNA was obtained using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and quantified by measuring the absorbance at 260 nm (NanoDrop® 1000, Thermo Scientific). DNA purity was confirmed by the ratio of absorbance at 260 nm and

280 nm, which was always greater or equal to 1.8. DNA was hydrolyzed and cytosine (C) and 5-methylcytosine (mC) were analysed by LC-MS/MS, as previously described [18,19]. In short, 1 µg of genomic DNA was hydrolyzed using 1 mol/L formic acid. C and mC were separated using gradient elution reversed phase chromatography with a mobile phase containing 5 mmol/L nonafluoropentanoic acid as ion-paring reagent. Ionization was performed using positive electrospray ionization. C and mC were quantified using stable isotope dilution and the results were expressed as the percentage of methylated to total cytosine (mC/tC).

Protein hydrolysis

Cellular proteins were precipitated by mixing whole cell lysates with an equal volume of 20% (w/v) trichloroacetic acid. After centrifugation at 4°C, 16000 × *g*, for 10 min, the supernatants were removed and the protein pellets were washed twice with ice-cold acetone and allowed to dry at room temperature. Protein hydrolysis was carried out at 110 °C with 6 mol/L HCl for 16 to 24 hours. The hydrolysates were dried under a gentle stream of nitrogen gas and stored at -20°C until further analysis of ADMA and SDMA.

Determination of free and protein-incorporated ADMA and SDMA

ADMA and SDMA levels were measured in the incubation medium and in whole cell protein hydrolysates by high-performance liquid chromatography with fluorescence detection, as previously described [20,21]. Arginine (Arg) in protein hydrolysates was quantified using the same procedure. Briefly, after sample cleanup by cationic solid-phase extraction (MCX 1cc cartridges, Waters Oasis, Milford, MA, USA), the analytes were derivatized with *ortho*-phthaldialdehyde reagent containing 3-mercaptopropionic acid. Chromatographic separation of the fluorescent derivatives was performed on a Chromolith Performance RP-18e column (100 × 4.6 mm) protected by a matching guard cartridge (10 × 4.6 mm) from Merck (Darmstadt, Germany). For quantification of ADMA and SDMA levels in the incubation medium, monomethylarginine was used as internal standard. Homoarginine was used as internal standard for the analysis of Arg, ADMA and SDMA in cell protein hydrolysates, due to the presence of monomethylarginine in these samples. The results were normalized to total Arg content of the corresponding hydrolysate.

Real-time reverse transcription-PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and reverse transcribed (2 μg) into cDNA using oligo(dT) SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Specific primers were designed with the Universal Probe Library Assay Design Center (Roche Applied Science, Mannheim, Germany). The sequences of the primers used were: *PRMT1*, 5'-ccaacgccaagaacaacc-3' and 5'-tcagcgcatccggtagtc-3'; *PRMT5*, 5'-tgaattgtcgcctgagtgc-3' and 5'-gggatgctcacaccatcat-3'; and *EIF4A2*, 5'- gtgtgaactggaccctgttg-3' and 5'-tatttaacattcaaacttcattaagacatg-3'. *EIF4A2* served as reference gene. Amplification reaction assays were performed in triplicate and contained 1 × SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) and primers at optimal concentration. Real-time PCR was performed using 40 melting and annealing/extension cycles of 15 seconds at 95 °C and 1 minute at 60 °C , preceded by a 2 minute step at 50 °C and a 10 minute activation step at 95 °C, using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Fluorescence emission was detected for each PCR cycle and the Ct (threshold cycle) values were determined. Normalized fold expression was calculated as difference of transcription in cells supplemented with ADA compared to controls, using the ΔΔCt method.

Western blotting

Western blotting was performed for analysis of PRMT1 and PRMT5 levels, using three independent cultures. 30 cm^2 of 80% confluent HUVEC was used for each sample. Cells were washed 3 times with ice-cold PBS, lysed with cell lysis buffer containing protease inhibitors (Sigma, St Louis, MO, USA), collected with a cell scraper, and sonicated. After centrifugation, the obtained supernatant was used for total protein determination and Western blot analysis.
Protein samples (30-40 μg) were separated on 10 % SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond ECLTM, Amersham, GE Healthcare, Chalfont St. Giles, UK). The membranes were incubated with anti-PRMT1 (at a 1:500 dilution; Abcam, Cambridge, UK) or anti-PRMT5 (at a 1:500 dilution; Millipore, Billerica, MA, USA) and anti-β-actin (at a 1:600 dilution; Sigma, St. Louis, MO, USA) antibodies. A secondary anti-rabbit IgG HRP (Cell Signaling, Danvers, MA, USA) or anti-mouse IgG HRP (JIR, Suffolk, UK) antibody at a 1:2,000 dilution was used. Primary antibody incubation was performed overnight at 4°C, and secondary antibody incubation was performed for 1–1.5 hours at room temperature. An ECL Plus Western

Blotting Detection System (GE Healthcare, Chalfont St. Giles, UK) was used for protein detection, membranes were exposed to Amersham Hyperfilm HCl (GE Healthcare, Chalfont St. Giles, UK), and a VersaDoc scanning system (BioRad, Hercules, CA, USA) was used for densitometry analysis.

Statistical analysis

All experiments were performed with cells from individual donors (n ranged from 3 to 22). Results are expressed as percentage (mean ± SEM) relative to cells incubated in control cM199 medium, except for free ADMA and SDMA concentrations in the incubation medium. Statistical significance was tested using Student's paired *t* test and was accepted at $p < 0.05$, *versus* Control.

Results

Effect of ADA on intracellular AdoHcy and AdoMet levels and Hcy production

To attain intracellular accumulation of AdoHcy and thereby disturb global cellular methylation processes, we used ADA, an efficient inhibitor of AdoHcy hydrolase. As previously reported [22], ADA elicited AdoHcy accumulation in a dose-dependent manner (Figure 1A), whereas AdoMet levels did not change (data not shown). Furthermore, Hcy concentration in the incubation medium decreased in the presence of ADA (Figure 1B). Hcy level after 24 hours of incubation with the highest dose of ADA (10 µmol/L) did not differ from Hcy level present in fresh cM199 (3 µmol/L), suggesting a total inhibition of AdoHcy hydrolase for this condition. Incubation with AZA, a specific inhibitor of DNA methylation, did not affect Hcy export or intracellular AdoMet and AdoHcy levels.

Global DNA methylation analysis

DNA methylation status, defined by the ratio of 5-methylcytosine to total cytosine in DNA hydrolysates, decreased by 10% under the highest dose of ADA (10 µmol/L), whereas no effect was observed with lower (2.5 and 5 µmol/L) concentrations of the inhibitor (Figure 2). Incubation with 5 µmol/L AZA, a specific inhibitor of DNA methyltransferases (DNMTs), resulted in a 35% decrease of the 5-methylcytosine to cytosine ratio.

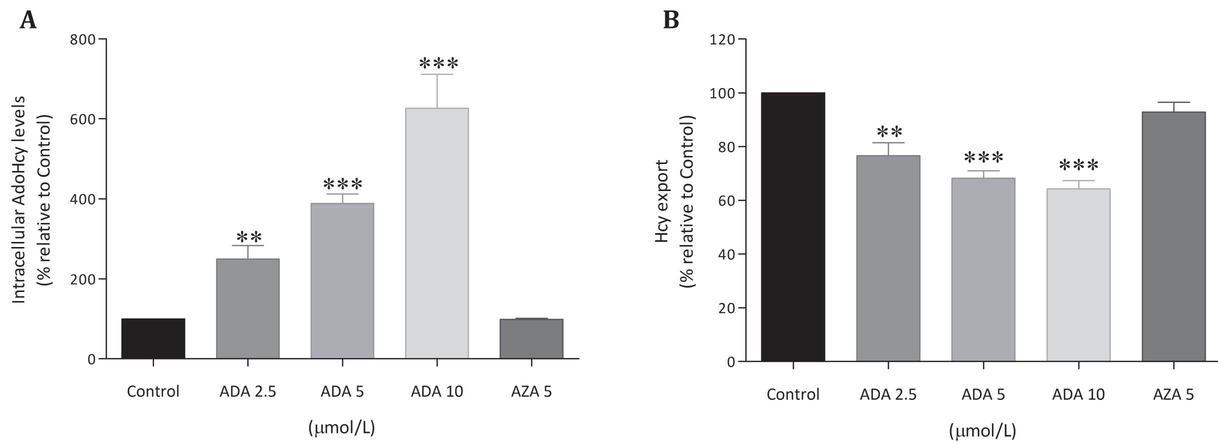

Figure 1 | Incubation of HUVEC with ADA, an inhibitor of AdoHcy hydrolase, induces intracellular AdoHcy accumulation and lowers Hcy production. Graphics represent the intracellular concentration of AdoHcy (A) and the concentration of Hcy in the incubation medium (B), after 24 hours of incubation in the absence and presence of ADA or AZA (see text for details), analysed as percentage of control. Data are mean ± SEM and represent 6 to 13 independent experiments performed with HUVEC obtained from individual donors. **p< 0.01 and ***p< 0.001, *versus* Control.

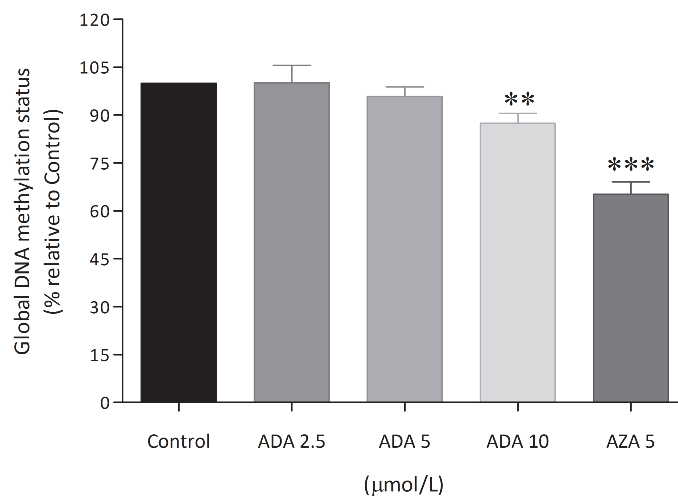

Figure 2 | Effect of ADA and AZA treatment on genomic DNA methylation status in HUVEC, evaluated as the ratio 5-methylcytosine / total cytosine (mC/tC). Data are mean ± SEM and represent 6 to 16 independent experiments performed with HUVEC obtained from different donors.
**P< 0.01 and ***P< 0.001, *versus* Control.

Global protein arginine methylation analysis

The effect of intracellular AdoHcy accumulation on protein arginine methylation status was evaluated by measuring ADMA and SDMA levels in the incubation medium and in cell protein hydrolysates. Basal ADMA and SDMA levels in cM199 medium were 219 nmol/L and 115 nmol/L, respectively. After 24 hours of incubation, these concentrations rose to 412 nmol/L and 146 nmol/L, respectively. Incubation with ADA elicited a significant decrease in extracellular ADMA concentration, whereas that of SDMA did not change significantly (Table 1).
In cell protein hydrolysates, ADMA level was about 1% of total Arg content, whereas that of SDMA was 0.1%. Both protein-incorporated ADMA and SDMA decreased significantly at all ADA concentrations, whereas those were not affect by AZA supplementation (Figure 3).

Table 1 - Quantification of both free ADMA and SDMA levels exported by HUVEC after 24h of incubation with or without ADA (μmol/L). Data are mean ± SEM and represent 7 independent experiments of individual donors. ** p< 0.01 *versus* Control.

	ADMA (*nmol/L*)	**SDMA** (*nmol/L*)
Control	**412 ± 9**	**146 ± 7**
ADA 2.5	391 ± 11**	142 ± 6
ADA 5	381 ± 9**	137 ± 8
ADA 10	376 ± 14**	135 ± 8

Effect of AdoHcy accumulation on PRMTs levels

Both mRNA and protein levels of PRMT1 and PRMT5 were quantified in HUVEC incubated with or without increasing concentrations of ADA. There was a significant increase in the expression of *PRMT1* and *PRMT5* genes (Figure 4A). However, the corresponding protein levels, evaluated by Western blotting analysis, did not change (Figure 4B).

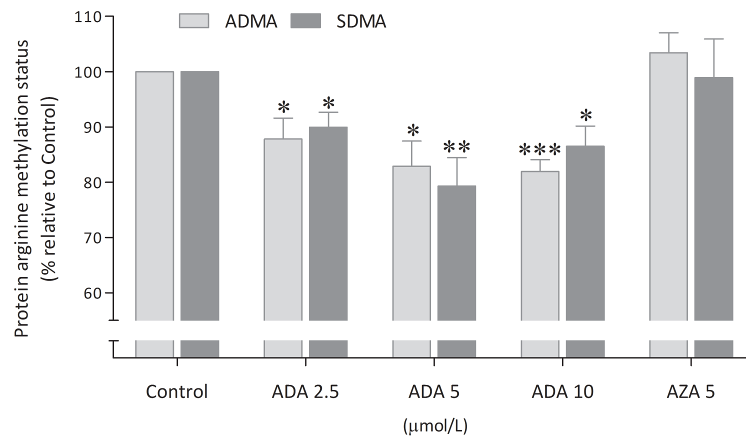

Figure 3 | Effect of ADA and AZA treatment on genomic protein arginine methylation status in HUVEC, evaluated as the ratios of dimethylated arginine residues / total arginine in protein hydrolysates after 24 hours of incubation in the absence and presence of ADA or AZA (see text for details). Data are mean ± SEM and represent 5 to 7 independent experiments performed with HUVEC obtained from individual donors.
*p< 0.05, **p< 0.01 and ***p< 0.001, versus control.

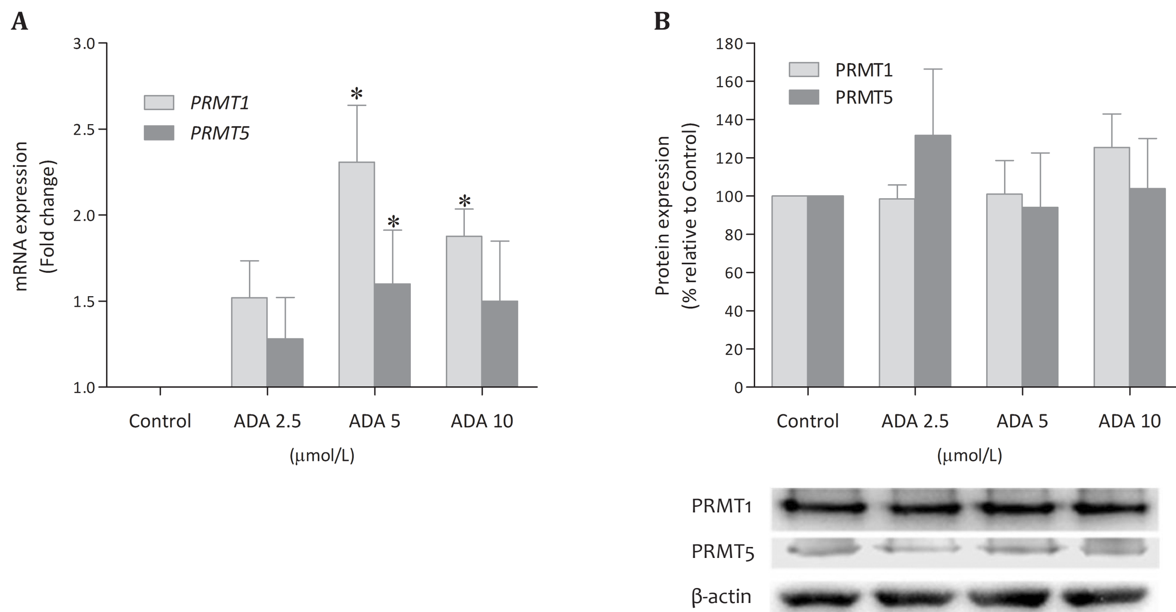

Figure 4 | Relative PRMT1 and PRMT5 expression in HUVEC, after 24 hours of incubation in the absence and presence of ADA. (A) Real-time RT-PCR analysis was performed in triplicate for quantification of mRNA levels of *PRMT1* and *PRMT5* relative to control, using *EIF4A2* as reference gene. (B) Protein levels of PRMT1 and PRMT5 determined by Western blotting, using β-actin as internal control. A representative blot is shown. Densitometry was performed on 3 blots obtained from independent experiments. Data are mean ± SEM. *p< 0.05, *versus* Control.

Discussion

The present study provides evidence that accumulation of intracellular AdoHcy decreases global protein arginine methylation in cultured human endothelial cells. Importantly, we show that protein arginine methylation is more sensitive to AdoHcy accumulation than DNA methylation. Several epidemiological surveys as well as observations from *in vitro* and *in vivo* studies, provided evidence that AdoHcy elevation parallels HHcy [9,10,13,23,24]. Since AdoHcy is a strong methyltransferase inhibitor, a reduced cellular methylation capacity due to accumulation of AdoHcy may account for the detrimental effects associated with HHcy. In this study we aimed at investigating the direct effect of AdoHcy on protein arginine and DNA methylation status.
Incubation of cells with ADA, a specific inhibitor of AdoHcy hydrolase, was effective in promoting build-up of intracellular AdoHcy (Figure 1), as expected from our previously published results [22]. DNA methylation status decreased (ca. 10%), at the highest dose of ADA (Figure 2). This effect was mild in comparison with the strong decrease (ca. 35%) in DNA methylation status elicited by AZA, a specific inhibitor of DNMTs.
On the other hand, free ADMA in the culture medium decreased after 24 hours of incubation of ADA, at all doses (Table 1). This observation led us to explore the effect of AdoHcy accumulation on protein arginine methylation status. In fact, cell free system studies have shown that PRMT1, the main enzyme responsible for ADMA synthesis, is strongly inhibited by AdoHcy [25,26]. The levels of free dimethylated arginines in the medium do not depend exclusively on the extent of protein arginine methylation. Important events such as the turnover rate of methylated proteins, cellular export and metabolization of ADMA by dimethylarginine dimethylamino hydrolase (DDAH) must be considered [27]. In order to exclude these variables, we developed a method to quantify the levels of protein-incorporated dimethylated arginines (ADMA and SDMA). In proteins, SDMA levels were 10 times lower than those of ADMA. This result, in line with previous studies [28,29], shows that asymmetric dimethylation is more extensive than symmetric dimethylation. As shown in Figure 3, accumulation of intracellular AdoHcy lowered protein-incorporated ADMA and SDMA levels in HUVEC, indicating that both asymmetrical and symmetrical dimethylation were affected.
To investigate whether the decrease in protein arginine methylation status under AdoHcy accumulation was due to decreased levels of the enzymes responsible for this post-translational modification, we measured the transcriptional and translational levels of PRMT1 and PRMT5,

which are the major type I and type II PRMTs, respectively, in human endothelial cells [30]. Figure 4A shows that the levels of the transcripts were rather higher upon accumulation of intracellular AdoHcy, possible reflecting a compensatory mechanism secondary to PRMTs inhibition. However, at the translational levels (Figure 4B), expression of PRMT1 and PRMT5 was not affected. We conclude that protein arginine hypomethylation was not due to reduced PRMTs levels.

In our experimental set-up, AdoHcy accumulation was accompanied by both DNA and protein hypomethylation. However, the effect on DNA methylation was only observed at the highest dose of the inhibitor (which corresponded to a 6-fold increase of AdoHcy), whereas protein arginine hypomethylation was already observed at the lowest dose of ADA (corresponding to a 2.5-fold increase of AdoHcy). This shows that proteins are more prone to be hypomethylated by intracellular AdoHcy accumulation than genomic DNA. In fact, results from kinetic studies regarding competitive inhibition of several methyltransferases by AdoHcy support that AdoHcy is a stronger inhibitor of PRMT1 than of DNMT1 [31-33]. Moreover, the fact that proteins are subject to turnover, while the fraction of DNA that is susceptible to AdoHcy inhibition arises mostly from newly synthesized molecules during cell division, may contribute to this difference.

In recent years, DNA methylation status has been envisaged as a potential biomarker for several pathologies and thoroughly assayed in epidemiological studies. Many *in vivo* and *in vitro* studies have attempted to link DNA methylation status and human disease [1,2]. Our results indicate that methylation of protein arginine residues is affected by intracellular accumulation of AdoHcy in a higher extension than DNA methylation. Future research is warranted to disclose the functional consequences of protein methylation disturbance in the context of Hcy-related diseases.

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Chapter 7

Diets inducing Hyperhomocysteinemia affect global DNA methylation in a tissue-specific manner

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Submitted

Abstract

The pathophysiology of homocysteine (Hcy) may be attributed to accumulation of its precursor S-adenosylhomocysteine (AdoHcy), which as a strong demethylating agent, may impair vital cellular methylation processes, including DNA methylation.

Here, we investigated the *in vivo* effect of diet-induced hyperhomocysteinemia (HHcy) on tissue global DNA methylation. Wistar rats (n= 8) were subject to one of the following diets: standard chow, high methionine (HM), low B-vitamins (LV) or a combined diet (HMLV). Plasma total Hcy levels were determined by HPLC. S-adenosylmethionine (AdoMet) and AdoHcy concentrations, and global DNA methylation levels in liver and heart were measured by LC-MS/MS methods.

All three experimental diets induced significant elevated levels of plasma Hcy, comparing to the control group. In liver of rats fed the HM diet, AdoHcy concentrations did not change, whereas the groups fed the LV and HMLV diets had higher levels than controls, yet only the HMLV group displayed hepatic global DNA hypomethylation. In heart, global DNA methylation was not affect by any of the studied conditions, despite the elevated AdoHcy content and lower AdoMet/AdoHcy ratio observed in the HMLV diet.

Our studies showed that elevated plasma Hcy is associated with a reduced methyl donor balance in liver and heart, but not, in general, with changes in tissue global DNA methylation levels, which seem to be affected in a tissue-specific manner, likely determined by a certain threshold of AdoHcy accumulation.

Keywords |

Diet-induced hyperhomocysteinemia, S-adenosylhomocysteine, Tissue global DNA methylation

Introduction

Hyperhomocysteinemia (HHcy) has been associated with several pathologic conditions, including cardiovascular disease. However, the mechanisms by which elevated levels of homocysteine (Hcy) predispose to vascular pathology are largely unknown [1]. The involvement of disturbed epigenetic mechanisms in Hcy toxicity is gaining increasing attention [2]. In HHcy, elevation of S-adenosylhomocysteine (AdoHcy), a potent inhibitor of most methylation reactions, is observed in association with global DNA hypomethylation and altered gene expression [3-7]. Methylation plays a vital role in numerous cellular processes, and abnormal patterns of methylation have been implicated in several human diseases, including vascular disease [2,8]. Accordingly, it has been proposed that Hcy vascular toxicity may in part be mediated by AdoHcy accumulation and impaired biological methylation processes, such as DNA methylation [2,9,10] .

There are evident metabolic links supporting Hcy as a possible modulator of the methylation cycle. During Hcy synthesis, methionine is activated by methionine adenosyltransferase (MAT) to form S-Adenosylmethionine (AdoMet), which is the donor for methylation of numerous molecules, including DNA and proteins. As a result of these reactions, AdoMet is converted to AdoHcy. AdoHcy is a competitive inhibitor of most AdoMet-dependent methyltransferases, because it binds to their active sites with a higher affinity than AdoMet [11]. For this reason, the ratio AdoMet/AdoHcy is often regarded as an indicator of the cellular methylation status, and a decrease in this ratio may predict a reduced cellular methylation capacity. AdoHcy is subsequently hydrolyzed to Hcy and adenosine, in a reversible reaction that strongly favors AdoHcy synthesis. Consequently, increased levels of Hcy result in intracellular AdoHcy accumulation which potentially promote a global cellular hypomethylation status [10].

In order to maintain physiological AdoHcy levels, the intracellular concentration of Hcy is under tight control. However, Hcy metabolism appears to be coordinated by multiple enzymes in a tissue-specific manner and if Hcy production exceeds the metabolic capacity of the cell, the overload of Hcy is exported into the blood. In most tissues, Hcy metabolism seems to be fully reliant on the folate-dependent remethylation pathway, where B-vitamins play as substrates and co-factors [12]. In liver, in particular, but also in kidney, Hcy can be remethylated back to methionine via betaine-homocysteine methyltransferase (BHMT), or enter the transsulfuration pathway to be irreversibly degraded to cysteine. In fact, liver and kidney are considered major organs in maintaining Hcy at proper levels [13] and, due to its enhanced metabolic capacity, may

be less prone to be affected by AdoHcy accumulation and changes on methylation potential of the cell.

Despite being target of research, the influence of moderate HHcy on tissue levels of AdoHcy and on the tissue-specific response to an altered methylation potential, regarding global DNA methylation, remains unclear. Based on the observation that Hcy, AdoMet and AdoHcy levels are significantly different across different tissues, it has been proposed that circulating Hcy does not equilibrate Hcy content in all tissues and that cellular methylation may be differently regulated at tissue level [14]. Recently, Wilson et al focused on tissue metabolic contributions to plasma Hcy levels and concluded that a considerable proportion of intracellular Hcy is imported from plasma into tissues such kidney, skin and heart [15]. To fully understand the underlying mechanism of Hcy vascular toxicity it is necessary to considerer the metabolism of all organs involved and their specific response to elevated levels of Hcy and impaired methylation capacity. To address these questions, murine models have proved to be particularly useful [16].

The present study aimed to investigate the influence of diets inducing HHcy on tissue-specific global DNA methylation. Using a quantitative LC-MS/MS method, we determined the percentage of global DNA methylation in liver and heart of rats subjected to different diet-induced levels of plasma Hcy. We observed that the cellular methylation status of liver and heart is disturbed under HHcy, but the effect on global DNA methylation appears to depend on metabolic characteristics of each tissue.

Materials and Methods

Animals and experimental diets

These studies were performed in 32 female Wistar rats, aged 4 weeks and with a body weight of approximately 250 g (Iffa Credo, Brussels, Belgium), in accordance with NIH and national guidelines for animal protection. Animals were randomly assigned to receive one of the four diets: a high methionine and low B-vitamins diet (HMLV; n=8); a low B-vitamins diet (LV; n=8); a diet high in methionine with adequate high levels of B-vitamins (HM; n=8); or a standard rodent chow (Control; n=8). The animals were maintained on these diets for 8 weeks. The exact composition of the experimental diets is shown on Table 1. The animals were

sacrificed and samples of liver and heart were collected, cut in pieces and immediately frozen in liquid nitrogen [17]. Frozen tissues were stored for 10 years at -80°C. Tissue homogenate was prepared using 10 volumes (10 mL/g wet weight of tissue) of 0.1 M HCl under ice with an OMNI-2000 homogenizer (Omni 2000, OMNI International, Waterbury, CT). Immediately after homogenization, part of the homogenate was processed for AdoMet and AdoHcy determination as described below. Protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), using bovine serum albumin as standard.

Table 1 - Composition of the diets: Control, High methionine (HM), Low B-vitamins (LV) and High methionine and low B-vitamins (HMLV). Data are expressed as gram per kilogram; (–) indicates residual vitamin levels.

Diet	Methionine	Folic acid	Vitamin B12	Pyridoxine	Choline
Control	3.8	0.002	0.00003	0.01	2.5
HM	7.7	0.002	0.00003	0.01	2.5
LV	3.8	-	-	-	2.5
HMLV	7.7	-	-	-	2.5

Biochemical analysis

Plasma total Hcy levels were determined by HPLC with fluorescence detection, as previously described in detail [18]. For quantification of AdoMet and AdoHcy, tissue homogenate was 10-fold diluted to a final volume of 250 µL with ice-cold 0.1 N HCl, and promptly deproteinized by addition of 156 µL of 10 % perchloric acid. After centrifugation at 4 °C for 10 min at 2000 g, the supernatant was subjected to a weak anion-exchange solid-phase extraction procedure and analyzed by stable-isotope dilution LC-MS/MS spectrometry, according to Struys *et al* [19].

Global DNA methylation

Genomic DNA was isolated and purified from tissue samples using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Global DNA methylation was measured using LC-MS/MS, as previously described [20,21]. Briefly, 1 µg of genomic DNA was hydrolyzed with formic acid; cytosine and 5-methylcytosine were separated using gradient-elution reversed phase chromatography, detected by LC-MS/MS and quantified using stable isotope dilution. Results are expressed as the percentage of methylated cytosine to total cytosine (mC/tC).

Statistical analysis

Data are given as mean ± standard error of the mean (SEM) or expressed in percentage relative to control ± SEM, when applicable. Tests of significance were conducted by Mann-Whitney U-test for comparison between two groups. Spearman test was used for correlation analysis. Statistical significance was accepted at $p < 0.05$.

Results

Plasma levels of Hcy

All three experimental diets efficiently induced significant elevated levels of plasma Hcy, when compared to the control group (Figure 1). Plasma levels were 4.8 ± 0.3 µmol/L in the control group. HMLV diet increased Hcy to 83.8 ± 12.3 µmol/L, the highest plasma Hcy concentrations achieved. Animals receiving a HM and LV diets had lower Hcy levels (22.7 ± 2.3 µmol/L and 34.8 ± 5.2 µmol/L, respectively), but still significantly elevated when compared to those displayed by rats fed with standard rodent chow.

Liver global DNA methylation, AdoMet and AdoHcy levels

The liver of rats fed with normal chow displayed 569.6 ± 19.9 nmol/g protein of AdoMet and 153.9 ± 3.6 nmol/g protein of AdoHcy. The level of global DNA methylation found in liver under normal diet was 1.55 ± 0.14 % of mC/tC.

In liver tissue, the methionine enriched diet (HM) did not affect the levels of AdoMet and AdoHcy, though global DNA methylation level showed a trend to decline (Figure 2). Comparing with the control, the low B-vitamins diet (LV) decreased hepatic AdoMet levels, which were accompanied by an increase of AdoHcy levels, resulting in a decreased of the AdoMet/AdoHcy ratio. However, global DNA methylation remained stable (Figure 2).

Finally, the diet enriched in methionine and deprived of B-vitamins (HMLV) did not affect AdoMet levels, but drastically increased AdoHcy levels, resulting in a lower AdoMet/AdoHcy ratio, which corresponded to a statistically significant impairment of global DNA methylation status in liver tissue (Figure 2).

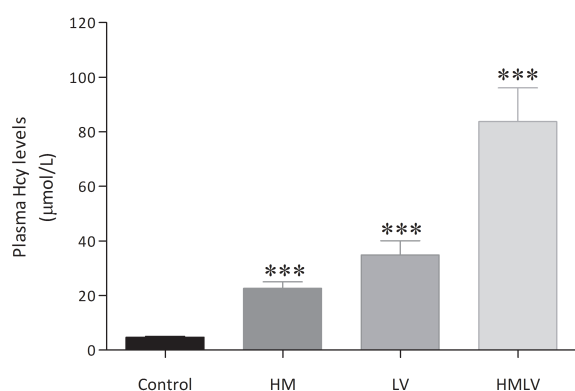


Figure 1 | Plasma Hcy concentrations in rats fed standard chow (Control), high methionine (HM), low B-vitamins (LV) or high methionine and low B-vitamins (HMLV) diets. Values are mean ± SEM (n= 8). *** p< 0.001 *versus* Control.

Heart global DNA methylation, AdoMet and AdoHcy levels

The levels of AdoMet and AdoHcy in heart tissue of rats fed with normal chow were 303.5 ± 15.4 nmol/g protein and 9.7 ± 1.3 nmol/g protein, respectively. Under normal diet, the level of global DNA methylation found in heart was 2.87 ± 0.18 % of mC/tC.

In heart tissue, both HM and LV diets lowered AdoMet levels, while AdoHcy levels tend to increase, resulting in a non-significant decreased AdoMet/AdoHcy ratio. Global DNA methylation status was not affected, comparing to the control group (Figure 2).

In the HMLV group, we observed no changes on heart AdoMet levels and a clear increase of AdoHcy, thus lowering AdoMet/AdoHcy ratio. However, global DNA methylation status showed no alteration (Figure 2).

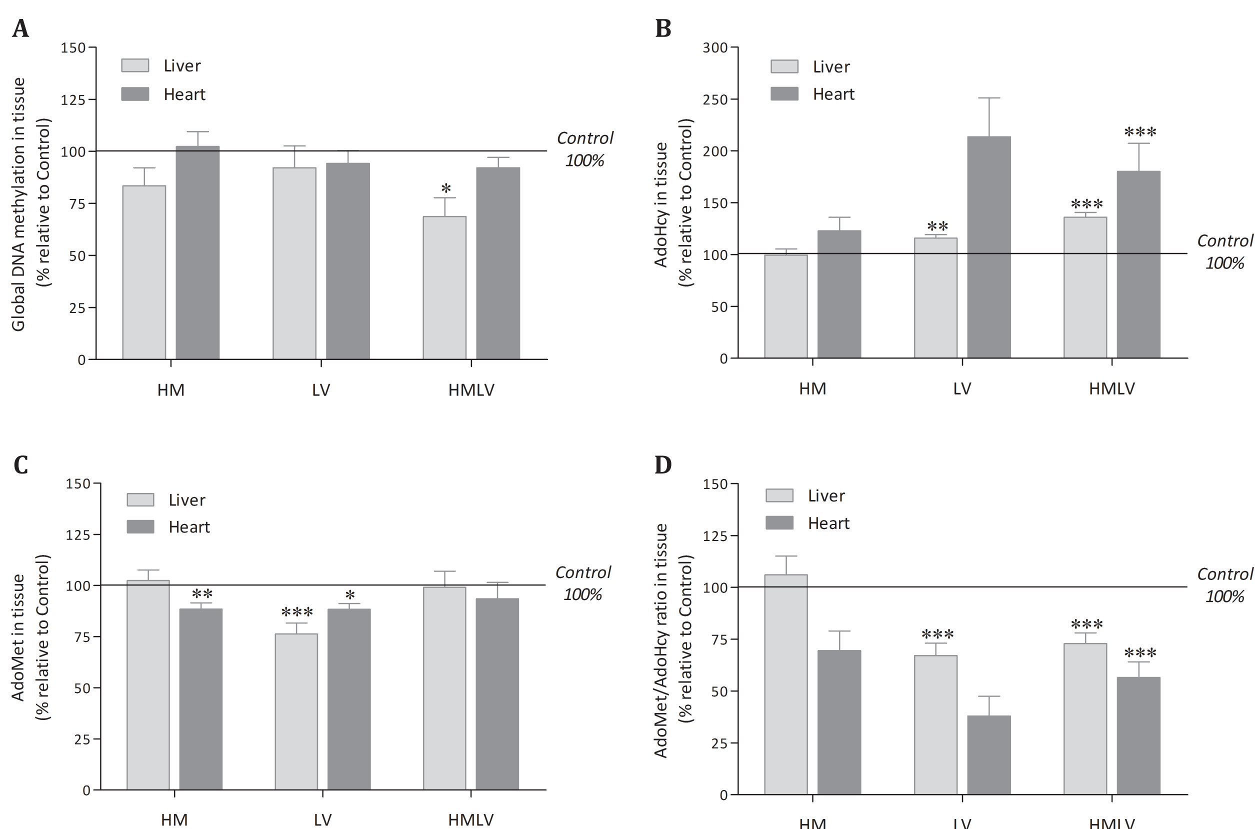


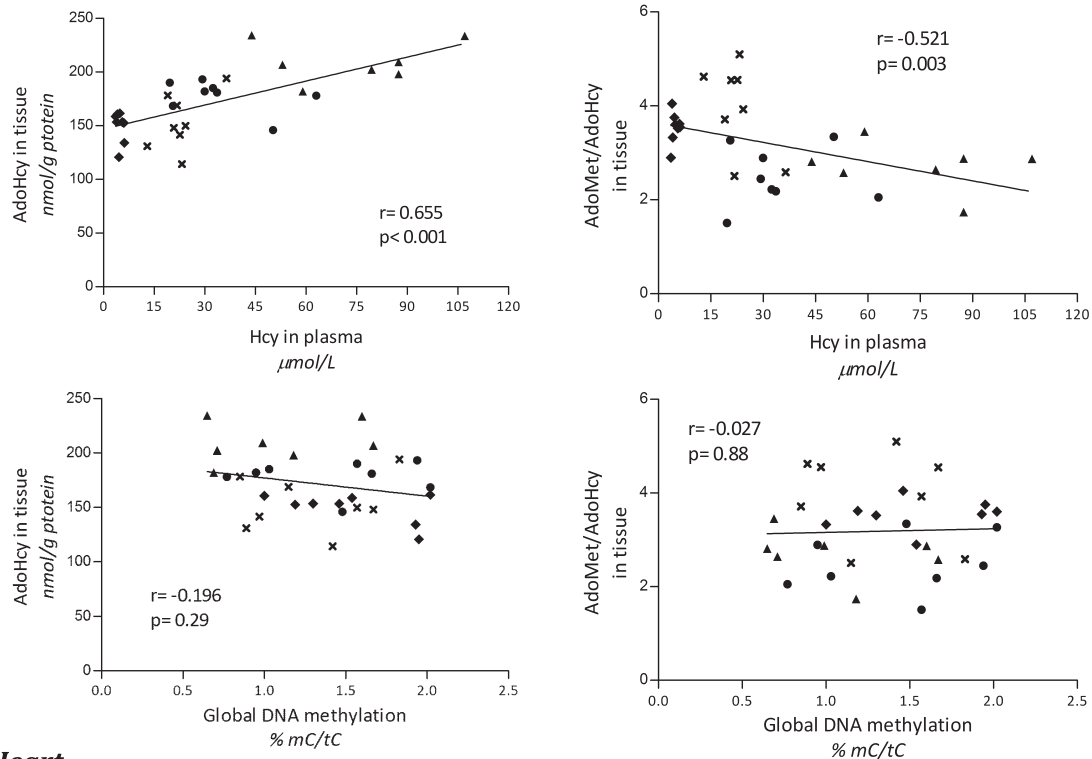
FIGURE 2 | (A) Global DNA methylation, (B) S-adenosylhomocysteine (AdoHcy), (C) S-Adenosylmethionine (AdoMet) and (D) AdoMet/AdoHcy ratios measured in liver and heart tissues from diet-induced hyperhomocysteinemic. Values are mean ± SEM (n= 8), expressed in percentage relative to Control diet. * p< 0.05, **p< 0.01, and *** p< 0.001 *versus* Control.

Correlation Analysis

As shown in Figure 3, regression analysis indicated strong positive correlations between plasma Hcy and AdoHcy levels in liver (r= 0.66, p< 0.001) and in heart (r= 0.47, p= 0.01). Inverse correlations were also observed between plasma Hcy and AdoMet/AdoHcy ratio in liver (r= -0.52, p= 0.003) and in heart (r= -0.70, p< 0.001).

Within the studied tissues, global DNA methylation was poorly or not associated with any of the parameters under analysis. In fact, we found no association with plasma Hcy levels either in liver (r= -0.31, p= 0.08) or in heart (r = -0.04, p = 0.80) (data not shown). Also, no correlation was observed between global DNA methylation and AdoHcy levels and AdoMet/AdoHcy ratio, either in liver (r= -0.20, p= 0.29 and r= -0.03, p= 0.88, respectively) or in heart (r= -0.39, p= 0.06 and r= 0.19, p= 0.36, respectively).

Liver



Heart

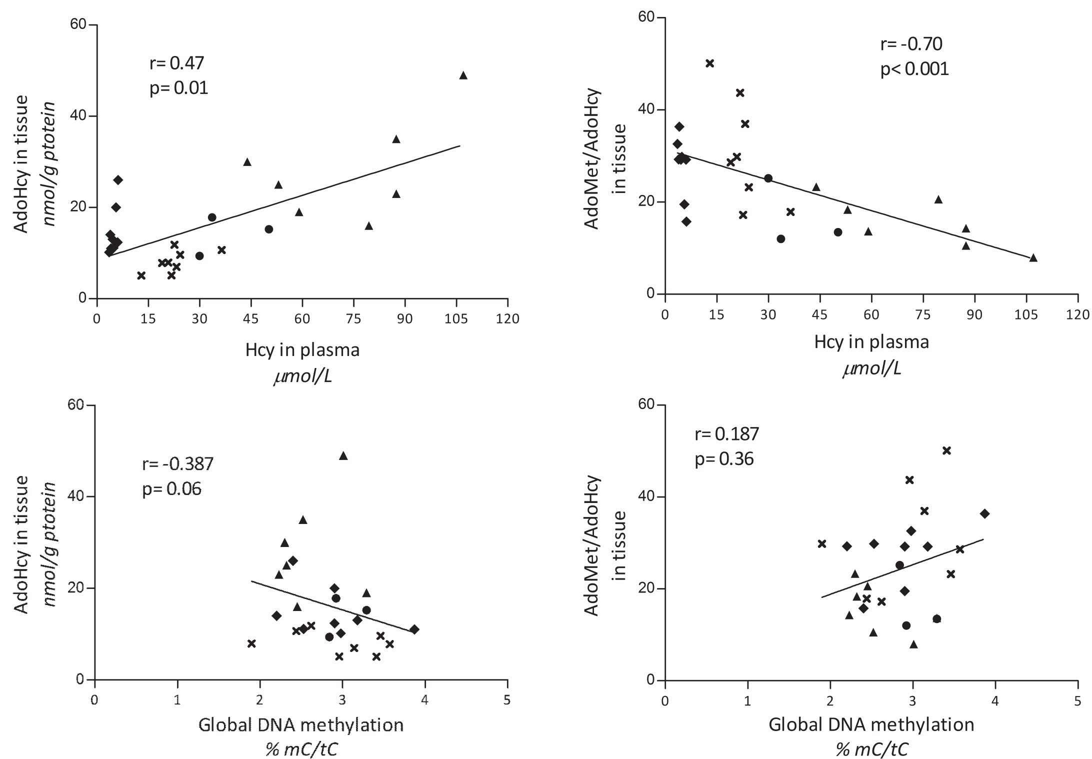


Figure 3 | Correlations of plasma levels of Hcy and tissue global DNA methylation as a function of intracellular levels of AdoHcy and AdoMet/AdoHcy ratios in liver and heart tissues of rats fed with different diets: Control (◆), high methionine (HM; •), low B-vitamins (LV; ✖) or high methionine and low B-vitamins (HMLV; ▲). Values are mean ± SEM (n= 8), expressed in percentage relative to Control diet. * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$ *versus* Control. Spearman rank correlation coefficient was calculated and statistical significance was accepted at $p< 0.05$.

Discussion

The major goal of the present study was to investigate the *in vivo* effect of moderate to severe HHcy on tissue global DNA methylation. Our results indicate that diets capable of inducing HHcy also disturb cellular methylation potential, but influence global DNA methylation in a tissue specific manner, which may be associated with the differences in tissue metabolic pathways that control the levels of AdoMet and AdoHcy. In addition, a contribution due to the biologic characteristics of each tissue cannot be ruled out.

Several *in vivo* and *in vitro* studies suggested that hypomethylation due to AdoHcy accumulation represents a potential mechanism involved in Hcy-mediated vascular toxicity [2,10]. In fact, vascular disease patients may have increased levels of both plasma Hcy and intracellular leukocytes levels of AdoHcy, in concert with decreased DNA methylation status [5]. Methylation is critical in maintaining cellular homeostasis. It targets a wide number of molecules, influencing not only DNA expression and stability, but also protein structure and function, among other purposes. Nevertheless, the measurement of genome-wide DNA methylation has been used to estimate the global cellular methylation status by many authors,

Here, we determine the levels of genome-wide DNA methylation of liver and heart of rats exposed to control diet and to different diets inducing HHcy. Dietary manipulations that result in HHcy may well elicit changes in the content of AdoMet and AdoHcy in a tissue-specific manner [22]. Therefore, the tissue levels of AdoMet and AdoHcy were quantified in the analyzed tissues. Thus far, little or no information was available regarding the influence of diets that induce elevated Hcy levels on global DNA methylation of heart tissue, nor on any other measure of methylation balance.

As previously reported [23], a diet rich in methionine (HM), deficient in B-vitamins (LV) or a combined diet (HMLV) efficiently induced different levels of HHcy. In the present study, the combined diet resulted in more than 15-fold increase of plasma Hcy, comparing with the control group (Figure 1). We observed that severely elevated levels of plasma Hcy are associated with AdoHcy accumulation and decreased methylation capacity in both liver and heart tissues. However, while liver displayed global DNA hypomethylation, this parameter was not affected in heart (Figure 2). One possible explanation relies on that the levels of AdoHcy in heart, even under the most markedly HHcy conditions (HMLV group), were more than 10-fold lower than its levels in the liver (where hypomethylated DNA was observed), and thus, may well be bellow the

K*i* value of the enzyme responsible for maintaining the DNA methylation patterns. In support, Choumenkovitch and colleagues found increased AdoHcy levels, decreased methylation capacity and global DNA hypomethylation in liver of CBS-deficient mice [24]. In contrast, Devlin et al reported that liver global DNA methylation was not altered upon a diet-induced HHcy in heterozygous *MTHFR* mice [23]. Nevertheless, the circulating levels of Hcy in the study by Devlin and colleagues might have been too low to trigger AdoHcy accumulation and liver DNA hypomethylation. In fact, the authors observed a 3-fold increase in plasma Hcy levels, which is comparable to the one observed by us in the HM group, where, actually, we observed both unaltered hepatic AdoHcy and DNA methylation status (Figure 2). This observation suggests that the methylation of DNA in liver is well regulated and that only severe elevations of circulating Hcy, and as such of intracellular AdoHcy, will disturb this process.

Next to the ubiquitous expressed methionine adenosyltransferase II (MAT II), the liver expresses high levels of MAT I/III which have higher catalytic capacity for methionine. Hence, the liver is unique in responding to high concentrations of methionine. In this study, the hepatic levels of AdoMet were not increased by a diet enriched in methionine alone or in combination with low B vitamins (Figure 2). This observation might be related to the fact that the liver also expresses large amounts of glycine N-methyltransferase (GNMT) which, by methylating glycine to form sarcosine, ensures the disposal of methyl groups when the supply is excessive. Unlike most methyltransferases, GNMT is not sensitive to AdoHcy inhibition [25].

Claudill et al reported that a decrease of AdoMet alone may not be sufficient to affect DNA methylation status of liver, brain, kidney and testes, whereas an increase of AdoHcy, either alone or accompanied by lowered AdoMet levels, is frequently consistent with tissue DNA hypomethylation [22]. In line with this observation, we found that in rat liver global DNA hypomethylation was likely mediated by an increase of AdoHcy, since AdoMet levels remained unaltered, comparing to the control group.

Of interest, in the control group, the intracellular levels of AdoHcy and the percentage of methylated DNA were notably different between liver and heart. In fact, rapidly proliferating cells usually have lower total genomic DNA methylation, while differentiated cells have rather stable methylation patterns [26]. One may note that the turnover of liver cells is higher than of heart cells [27]. If cells do not divide, less effect of AdoHcy accumulation on global methylation status may be expected, since the DNA methylation patterns have already been established. In contrast, in proliferating tissues, during DNA replication and cell division, the maintenance of DNA methylation patterns may be target of AdoHcy inhibition.

Increasing evidence indicates that Hcy, via AdoHcy accumulation, may be regarded as a cellular demethylating agent, at least at the DNA level [2]. In support, we observed that increased levels of plasma Hcy were correlated with intracellular AdoHcy accumulation and decreased methylation capacity in both liver and heart tissues (Figure 1). However, in our animal model, global DNA hypomethylation in liver was found poorly or even not at all correlated with plasma Hcy, AdoHcy levels or AdoMet/AdoHcy ratios (Figure 3). This observation is in part intriguing given the close metabolic relationship between methylation and one carbon metabolites. The lack of correlation may be attributed to measurement inaccuracy, statistical power or animal variability. Moreover, one should consider the possibility that genomic DNA methylation may not reflect the methylation status of the cell, but being rather a suboptimal parameter of methylation. Our group recently reported that methylation of proteins is more prone to be affected by intracellular accumulation of AdoHcy than global DNA methylation [28], which may open a new perspective on cellular markers of global methylation status. Interestingly and in line with our observation, cardiac peroxisome proliferator-activated receptor-γ co-activator-1 (PGC-1 α), a master regulator of energetic metabolism in heart, was recently reported to be hypomethylated under a decreased AdoMet/AdoHcy ratio [29].

In conclusion, our results indicate that moderate to severe chronically diet-induced HHcy in rats impairs tissue cellular methylation potential, via accumulation of AdoHcy and consequent reduction of AdoMet/AdoHcy ratio, but affects global DNA methylation in a tissue-specific manner. We observed global DNA hypomethylation in liver, but not in heart, which may be due to the large differences in the tissue levels of AdoHcy, in particularly. Further studies are warranted to disclose the functional consequences of these observations, and their relevance to the understanding of the vascular pathophysiology of altered Hcy metabolism.

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Chapter 8

Asymmetric dimethylarginine in adults with Cysthathionine β-synthase deficiency

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Abstract

In hyperhomocysteinemia (HHcy), a independent risk factor for cardiovascular diseases, endothelial dysfunction due to reduced bioavailability of nitric oxide is a consistent finding. However, the underlying mechanisms remain unknown.

Increased levels of the nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA) have been associated with HHcy, and may contribute, at least in part, for the homocysteine-induced endothelial dysfunction, but whether cystathionine β-synthase (CBS) deficiency is associated with increased ADMA has hardly been investigated.

To address this question, we measured total homocysteine (tHcy), ADMA and symmetric dimethylarginine (SDMA) in plasma of 22 adult CBS deficient patients, using established HPLC techniques. Results showed that in CBS deficient patients with elevated levels of tHcy (median (total range): 33 (14-237) µmol/L), both ADMA and SDMA levels were normal. Moreover, tHcy and ADMA concentrations were not correlated (r_s= 0.017, p= 0.94). Our results favor the hypothesis that the negative vascular effects of HHcy have an ADMA-independent etiology.

Keywords |

Hyperhomocysteinemia, CBS deficiency, ADMA

Short Communication

Hyperhomocysteinemia (HHcy) is a recognized risk factor for cardiovascular diseases. Endothelial dysfunction due to impaired bioavailability of nitric oxide (NO) is a consistent finding in HHcy, but the underlying mechanisms are yet unknown [1]. Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of the enzyme NO synthase, which converts L-arginine (Arg) into NO, and thereby represents an important regulator of vascular function. Increased levels of homocysteine (Hcy) have been associated with increased ADMA levels, suggesting that ADMA may mediate, at least in part, Hcy-induced endothelial dysfunction due to reduced NO production [2].

Cystathionine β-synthase (CBS) deficiency is a rare inborn error of metabolism that results in marked accumulation of homocysteine (Hcy). Patients present severely elevated levels of Hcy in plasma (HHcy) and urine (homocystinuria), as well as premature vascular complications. Hcy-lowering therapy significantly reduces the risk of vascular events in these patients, but HHcy and endothelial dysfunction persist, despite treatment. Therefore, CBS deficient patients can be regarded as *in vivo* models to study the interplay between HHcy and vascular disease.

Hcy may affect ADMA metabolism in several ways (reviewed in [3]). ADMA is released into the cytosol after proteolysis of proteins containing methylated Arg residues. Protein Arg methylation is facilitated by a group of enzymes called protein-Arg-methyltransferases, which use S-adenosylmethionine (AdoMet) as methyl group donor. During these transmethylation reactions, AdoMet is converted into S-adenosylhomocysteine (AdoHcy), which is subsequently hydrolyzed into Hcy and adenosine. Because ADMA contains two methyl groups, two equivalents of Hcy are formed during its synthesis. Hcy may also enhance protein degradation, thereby increasing the release of free ADMA from methylated proteins. Another possible mechanism for elevation of ADMA in HHcy may be decreased activity of the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which represents the major metabolic route of ADMA clearance. It has been demonstrated in a cell-free system and in cultured cells, that Hcy inhibits DDAH activity in a dose-dependent manner, causing ADMA to accumulate. However, this inhibitory effect of Hcy on DDAH activity has not yet been observed *in vivo*. Dayal *et al* observed that Hcy causes tissue-specific downregulation of DDAH expression in a CBS deficient mouse model of HHcy, suggesting that Hcy may be able to regulate both activity and expression of DDAH enzymes [4].

The mechanisms described above, that all result in increased ADMA levels in the presence of HHcy, may be opposed by Hcy-induced protein hypomethylation. The rate of the protein Arg methyltransferase reaction, and thus ADMA synthesis, is in part regulated by intracellular concentrations of AdoMet and AdoHcy [4]. The hydrolysis of AdoHcy into Hcy is a reversible reaction that favors AdoHcy synthesis. Therefore, in situations where Hcy accumulates, AdoHcy levels increase as well. Because AdoHcy is a potent inhibitor of transmethylation reactions, its accumulation may result in protein hypomethylation. In fact, we have recently shown that global protein Arg methylation status is decreased by intracellular accumulation of AdoHcy in human endothelial cells [5].
Several studies have shown positive associations between plasma concentrations of Hcy and ADMA (reviewed in [2,3]), but, to date, only two groups of investigators have determined ADMA concentrations in patients with severe HHcy due to CBS deficiency [6,7]. In a first study, Wilcken and colleagues observed that ADMA levels in plasma of 23 treated CBS deficient patients were neither increased nor correlated with Hcy concentrations [6]. Later, Kanzelmeyer *et al* suggested that plasma ADMA levels in children with CBS deficiency were significantly higher than in healthy controls, but a possible association between ADMA and Hcy was not disclosed/reported [7]. These contradictory observations led us to investigate the relationship between Hcy and ADMA plasma levels in our patients with HHcy due to CBS deficiency.

We studied 22 adult CBS deficient patients diagnosed on the basis of clinical presentation, severely elevated Hcy and methionine levels, and impaired CBS activity in cultured fibroblasts. All patients received Hcy-lowering therapy, consisting of daily intake of pyridoxine (250-750 mg), folic acid (5 mg), vitamin B12 (10 µg) and/or betaine (6 g). Plasma levels of total Hcy (tHcy), Arg, ADMA and symmetric dimethylarginine (SDMA) were measured by high performance liquid chromatography (HPLC) methods with fluorescence detection, as previously described [8,9].

Plasma concentrations of the metabolites measured and the respective reference values are shown in Table 1. As expected, tHcy levels of most CBS deficient patients were severely elevated (median (total range): 33 (14-237) µmol/L) as compared to reference values, whereas ADMA and SDMA levels were within the normal range. Surprisingly, plasma levels of Arg were slightly decreased and negatively associated with tHcy concentrations (Figure 1A, r_s= -0.456, p= 0.03, Spearman rank correlation). Furthermore, the Arg/ADMA ratio, commonly used as index of NOS inhibition [10], was rather low and inversely correlated with tHcy

(Figure 1B, r_s= -0.523, p= 0.01), indicating that, even after treatment, CBS deficient patients are prone to endothelial dysfunction due to impaired NO production, as previously reported [6]. However, importantly, no correlation between tHcy and ADMA plasma levels was observed (Figure 1C, r_s= 0.017, p= 0.94).

We show that, in adult humans, severe HHcy due to CBS deficiency is not associated with increased ADMA levels, indicating that neither increased ADMA synthesis nor impaired ADMA catabolism are triggered by Hcy accumulation. This suggests that ADMA and Hcy-related vascular toxicities may have a different etiology.

Table 1 - Variables measured in CBS deficient patients under homocysteine-lowering treatment. Data are median (total range) for tHcy, and mean ± SD for ADMA, SDMA and Arg. Reference values for arginine and its methylated forms taken from [9].

	Patients *(n = 22)*	**Reference values**
tHcy *(µmol/L)*	33 (14-237)	5 - 15[a]
ADMA *(µmol/L)*	0.42 ± 0.07	0.42 ± 0.06
SDMA *(µmol/L)*	0.48 ± 0.08	0.47 ± 0.08
Arg *(µmol/L)*	82.8 ± 27.9	94.2 ± 25.8
Arg/ADMA	195 ± 49	223 ± 49

[a] Normal range for tHcy

Our results are in line with a study by Dayal *el al*., who observed normal plasma levels of ADMA in CBS deficient mice compared to wild-type controls, despite the evident increase in tHcy [4]. Our data also corroborate the results by Wilcken *et al* who reported that plasma ADMA levels were significantly increased only in CBS patients with an impaired glomerular filtration rate, and not in those with normal renal function [6]. Of interest, none of our CBS deficient patients had a history of renal disease, and plasma levels of SDMA, a marker of renal function [11], were within the normal range. In fact, since renal failure contributes to increased plasma levels of both tHcy and ADMA, it has been suggested that impaired kidney function may be the underlying cause for a positive association between Hcy and ADMA in vascular disease [12], a subject still under debate.

Interestingly, Kanzelmeyer *et al* recently reported that plasma levels of ADMA were higher in CBS deficient children than in age-matched controls [7]. It should be noted that plasma levels of ADMA decrease from infancy to adulthood [13], which may explain the disparate impact of CBS deficiency on ADMA levels in children as compared to adults. Possibly, in children, high levels of ADMA claim almost the full capacity of the DDAH-mediated degradation pathway, thereby increasing its vulnerability to inhibition by Hcy.

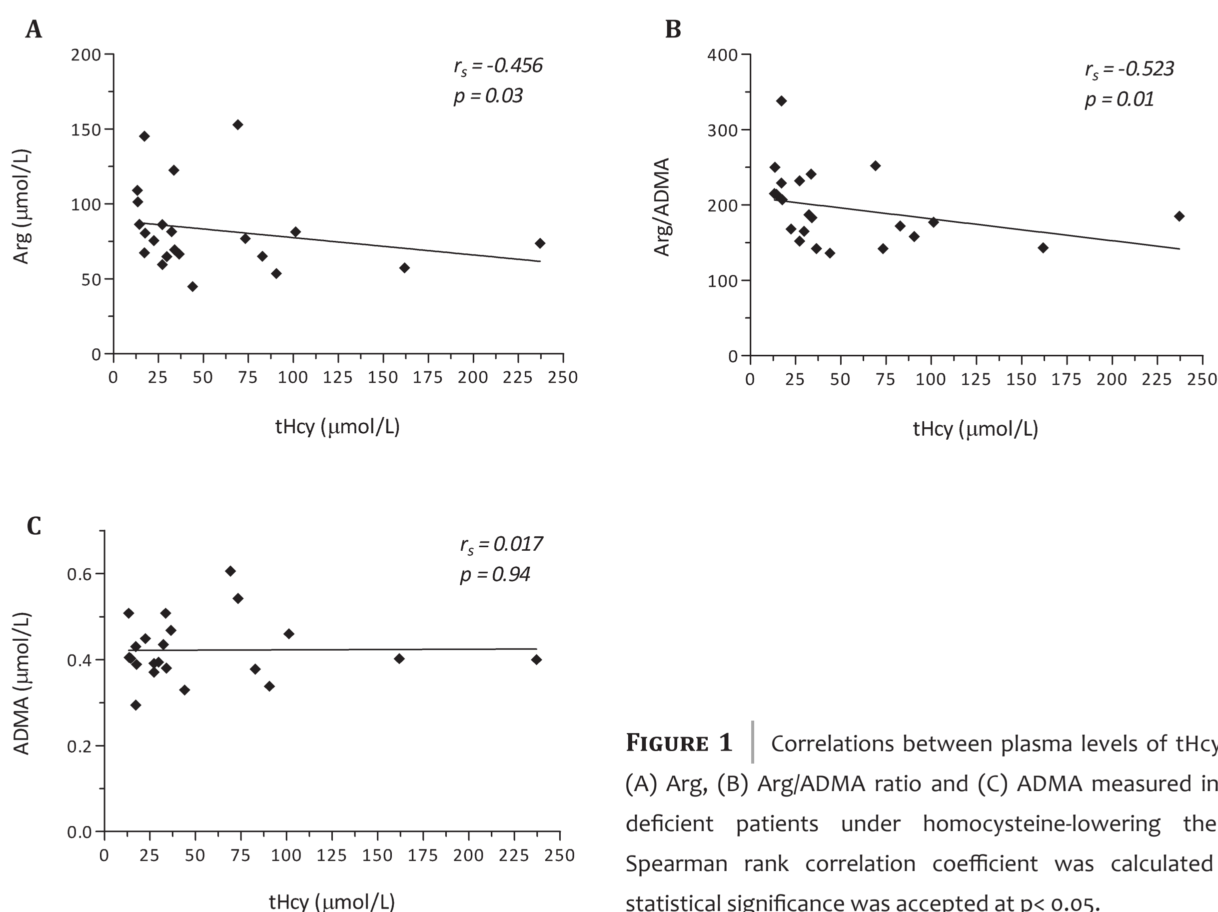

Figure 1 | Correlations between plasma levels of tHcy and (A) Arg, (B) Arg/ADMA ratio and (C) ADMA measured in CBS deficient patients under homocysteine-lowering therapy. Spearman rank correlation coefficient was calculated and statistical significance was accepted at $p < 0.05$.

Our study has some limitations. ADMA levels and the association between Hcy and ADMA may have been influenced by the fact that all patients included in this study were under B vitamin treatment, aimed to reduce circulating Hcy levels. However, although one study found that ADMA concentrations decreased in parallel with Hcy concentrations during folic acid treatment [14], many other studies are consistent in reporting that ADMA levels are not affected by B-vitamin therapy [15].

As outlined above, high Hcy may lower ADMA synthesis due to reduced methylation capacity, resulting from increased AdoHcy levels and subsequent reduction of the AdoMet/AdoHcy ratio. The levels of AdoMet and AdoHcy from the patients included in this study have been previously reported as elevated when compared with the control group, but no significant changes of the AdoMet/AdoHcy ratio were observed [16].

Our study shows that, in adults, a defect in the transsulfuration pathway due to CBS deficiency is not associated with elevated ADMA levels. Likewise, the transient HHcy after methionine loading was not associated with increased ADMA levels in healthy subjects and hypertensives [17,18]. However, we cannot exclude that the impact on ADMA is different in HHcy due to other causes, such as impairment of the remethylation pathway or renal dysfunction.

To understand the true association between Hcy and ADMA metabolism, intracellular levels of ADMA, which may be up to 10-fold higher than in plasma, are likely more relevant and should be considered for determination. For example, in freshly isolated brain tissue, Cardounel and colleagues observed that the intracellular levels of ADMA decreased more than 50% in the presence AdoHcy [19].

In conclusion, Hcy may influence cellular ADMA metabolism in several ways, making it difficult to disentangle its effect on specific steps. The mechanisms involved affect both ADMA synthesis and clearance and may lead to positive as well as negative associations between plasma levels of Hcy and ADMA. The overall net effect probably depends on Hcy levels and may be different in mild versus severe forms of HHcy. In this study we show that severe HHcy in adult patients with CBS deficiency is not associated with increased plasma levels of ADMA.

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Chapter 9

General discussion, Concluding remarks and Future perspectives

1. General Discussion

Over the last decades, many studies have established that elevated homocysteine is an independent risk factor for vascular disease. The decrease of NO bioavailability is a key manifestation of endothelial dysfunction that contributes to the pathogenesis of atherosclerosis, and one of the most consistent findings during hyperhomocysteinemia. However, the precise mechanisms by which hyperhomocysteinemia induces endothelial dysfunction are incompletely defined and may be multifactorial (CHAPTER 1, TABLE 2).

AdoHcy is the metabolic precursor of all homocysteine produced in the body. Several *in vivo* and *in vitro* studies have shown that hyperhomocysteinemia is related to disturbed transmethylation, suggesting an indirect mechanism for homocysteine toxicity, secondary to accumulation of AdoHcy (CHAPTER 1, SECTION 7.2). Irrespectively of the underlying cause, when homocysteine accumulates, its precursor AdoHcy may increase as well, thereby disturbing cellular methylation processes.

The role of AdoHcy on the pathophysiology of hyperhomocysteinemia has now gained increased attention. In fact, recent experimental and clinical evidences indicate that the accumulation of AdoHcy, rather than increased homocysteine levels, may be a better biomarker of vascular disease and tissue damage [1-5].

In the present work, we postulate that elevated homocysteine itself may not be the major causative factor for the development of vascular disease, and sought to investigate whether AdoHcy accumulation impacts the nitric oxide bioavailability pathway by disturbing cellular methylation capacity and key epigenetic mechanisms.

Endothelial cell model of AdoHcy accumulation and impaired methylation

In the present work we cultured human endothelial cells and used a strong inhibitor of AdoHcy hydrolase, ADA, to define an *in vitro* model of intracellular AdoHcy accumulation and impaired methylation potential. The supplementation of the culture medium with ADA not only increases AdoHcy levels, but also results in decreased homocysteine production within the cell. Therefore, in this cell system one can assume that any effects of ADA treatment are a consequence of AdoHcy, and not due to homocysteine accumulation.

As indicative of impaired cellular methylation potential, we observed a decrease of AdoMet/AdoHcy ratio, which was exclusively defined by the elevated levels of AdoHcy induced by ADA treatment, since the content of AdoMet remained unchanged. The ability of AdoHcy in disturbing transmethylation processes was further confirmed through the analysis of the methylation status of the genomic DNA, and protein arginine residues (CHAPTER 6).

The expanding interest of studying DNA methylation in human diseases has triggered the development of many methodologies, aiming to improve sensitivity, accuracy and quantification of a high throughput analysis. However, it is currently not known which method can be seen as the *gold standard*. In CHAPTER 3, we compared the widely used cytosine extension assay [6] with our recently published LC-MS/MS method [7], in order to determine which method to use in the following chapters. Even though this study showed that both methods can be used to evaluate global DNA methylation status, only the LC-MS/MS assay permits an absolute quantification and higher throughput analysis. In addition, this method displayed a superior intra-day precision and therefore we considered it a better approach for use in global DNA methylation studies.

Although many studies relate AdoMet/AdoHcy ratio to the methylation status of the cell, the extent at which protein methylation is affected by AdoHcy accumulation was yet unknown. Notably, a large number of distinct proteins involved in crucial cellular events, such as transcriptional regulation, signal transduction, RNA metabolism and DNA repair, are target of post-translational arginine methylation. In CHAPTER 6, we describe a simple method to quantify the levels of the major forms of protein-incorporated dimethylated arginines, ADMA and SDMA. Importantly, our data indicated, for the first time, that protein arginine methylation is more sensitive to AdoHcy accumulation than global DNA methylation. Supporting the role of AdoHcy as a hypomethylating agent, our *in vitro* model showed a significant decrease on both global DNA methylation and protein arginine methylation (CHAPTER 6).

Intracellular AdoHcy accumulation and endothelial nitric oxide bioavailability

Many of the plausible mechanisms that have been proposed to explain the impairment of NO bioavailability seen in hyperhomocysteinemia appear to be directly triggered by homocysteine itself. The studies performed in CHAPTERS 3 AND 5 of this thesis addressed the solely effect of elevated AdoHcy levels and impaired methylation processes on NO pathway.

In our *in vitro* model we demonstrated that intracellular accumulation of AdoHcy decreases NO synthesis by endothelial cells, independently of the homocysteine levels, by decreasing the expression and activity of eNOS (Chapter 3). Moreover, we noticed an impaired post-transcriptional regulation of *NOS3* gene expression, likely due to hypomethylation of associated regulatory proteins that undergo important methylation events.

ADMA is an endogenous inhibitor of all NO synthase isoforms and modulates the biological effects of NO, especially in the cardiovascular system. In Chapter 5 we investigate *in vitro* whether AdoHcy accumulation influence the expression and/or activity of DDAH, the major metabolic route of ADMA degradation, by disturbing epigenetic mechanisms, namely DNA methylation. Interestingly, our study showed that elevated levels of AdoHcy modulate the expression and activity of DDAH, and also suggested that other cellular methylation reactions, different than DNA methylation, seem to be involved. In effect, our observation that non-histone protein methylation is more vulnerable to AdoHcy accumulation than global DNA methylation is in support of this possibility (Chapter 6).

The role of AdoHcy in the pathophysiology of hyperhomocysteinemia has often been suggested, but hardly investigated. Our *in vitro* findings showed that AdoHcy, independently of homocysteine, induces changes on the NO/ADMA pathway in endothelial cells, and likely contributes for the impaired NO bioavailability observed during hyperhomocysteinemia. Recently, Sipkens and colleagues demonstrated that AdoHcy accumulation alone induces ROS increased formation and cell apoptosis [8]. In apoE-deficient mice, Lou and colleagues induced elevated plasma levels of AdoHcy by dietary administration of ADA and further supported that high AdoHcy, rather that high homocysteine, promotes the development of atherosclerosis [9].

Tissue global DNA methylation in diet-induced hyperhomocysteinemia

In Chapter 7, we extent our *in vitro* analyses to examine *in vivo*, in diet-induced hyperhomocysteinemic rats, the influence of elevated plasma homocysteine levels on global DNA methylation of different tissues. Our results indicated that homocysteine, via AdoHcy accumulation, impairs the cellular methylation potential of liver and heart, but the effect on global DNA methylation seems to be due to differences in the tissue contents of AdoMet and AdoHcy, associated to tissue-specific metabolic pathways. Moreover, and in line with our experiments in cultured endothelial cells, only severe elevated levels of AdoHcy were associated with tissue DNA hypomethylation.

ADMA and elevated homocysteine

Because of the metabolic links between ADMA and homocysteine, ADMA could be a potential mediator of endothelial dysfunction in hyperhomocysteinemia. For example, it has been suggested that the elevation of ADMA in hyperhomocysteinemia might be mediated by an inhibitory effect of homocysteine on the expression or activity of DDAH. In contrast, high homocysteine levels may also lower ADMA synthesis due to reduced methylation capacity and inhibition of PRMT activity, resulting from increased AdoHcy levels (Chapter 1, Figure 4). Some authors, however, hold that hyperhomocysteinemia is indirectly related to ADMA levels [10-12], and other studies suggest that hyperhomocysteinemia can produce endothelial dysfunction without elevating ADMA levels [13-16].

The mechanisms of how ADMA levels may become elevated in hyperhomocysteinemia remain to be elucidated. Our *in vitro* work suggests that elevation of ADMA observed in hyperhomocysteinemia is not due to AdoHcy accumulation: 1) we demonstrate by experiments with ADA that intracellular AdoHcy accumulation decreases both export of free ADMA and protein-incorporated ADMA levels in human endothelial cells (Chapter 6) and 2) showed that AdoHcy-induced hypomethylation is associated with increased DDAH activity (Chapter 5).

In Chapter 8, we investigate the possible *in vivo* associations between homocysteine and ADMA metabolisms in patients with genetic CBS deficiency, in which intracellular AdoHcy accumulation results from endogenously altered homocysteine metabolism. In our group of CBS-patients, plasma levels of homocysteine were severely elevated, but ADMA levels were within the normal range, suggesting that neither increased synthesis nor impaired ADMA degradation are triggered by homocysteine accumulation. It remains to be elucidated whether ADMA may accumulate inside the cell and inhibit endothelial function even in the absence of elevation of plasma ADMA.

Our study showed that severe hyperhomocysteinemia was not associated with increased ADMA plasma levels in CBS deficient patients, which suggests that high homocysteine, likely via intracellular accumulation of AdoHcy, impairs protein methylation processes, thereby the synthesis of ADMA. Moreover, it raises doubts about the possible *in vivo* inhibitory effect of homocysteine on DDAH activity.

The results disclosed by the present work indicate that ADMA may not be involved in homocysteine-induced endothelial dysfunction.

2. Concluding Remarks and Future Perspectives

The role of a disturbed transmethylation in the pathophysiology of hyperhomocysteinemia is a current subject of interest. Many authors have shown that elevated homocysteine is associated with global DNA hypomethylation, yet the interpretation of data must be done carefully. Global DNA methylation is not distributed uniformly across the genome, and may mainly reflect the methylation status of non-coding DNA regions, which are normally found methylated. Thus, global DNA methylation may poorly correspond to methylation status of specific genomic regions. Studies of key gene specific methylation, and associated consequences on gene expression, would provide more information on the functional effects of unbalanced methylation. For example, the CBS gene is known to have CpG islands in its promoter region, possible targets of epigenetic regulation. Studies of CBS promoter methylation in cellular or animal models of hyperhomocysteinemia or elevated AdoHcy and its possible impact on gene expression are warranted. The recent development of microarrays-based approaches for studying gene-specific methylation, which allow the identification of the methylation status of CpG sites of one gene or multiple genes for a given sample, brought powerful tools for the understanding of gene transcription regulation events in hyperhomocysteinemia [17].

Although different classes of molecules are subject to this structural modification, attention has been drawn essentially on DNA methylation, which is consensually recognized as being implicated in a variety of diseases. Surprisingly, the effect of AdoHcy accumulation on global protein methylation was until now unknown. Protein methylation is a common post-translational modification that takes place on specific amino acid residues, including arginine. Here we show that protein arginine methylation is more vulnerable to AdoHcy accumulation than DNA methylation in cultured human endothelial cells.

Methylation in biological systems modulates many crucial cellular functions. AdoHcy is a strong product inhibitor, and to date more than 50 AdoMet-dependent methyltransferases, acting upon a broad spectrum of cellular compounds including DNA, RNA and proteins, have been identified. An elevation of intracellular levels of AdoHcy targets a key house-hold metabolism and modulates the methyl donor balance within the cell. AdoHcy accumulation likely impairs the cellular homeostasis in a complex network of tissue-specific mechanisms, and thus may contribute to the establishment of vascular pathologies.

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Voltaire

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“Gratitude is not only the greatest of virtues, but the parent of all the others.”
Cicero

List of Publications

1. Castro R, Barroso M, Rocha MS, Esse R, Ramos R, Ravasco P, Rivera I and Tavares de Almeida I. **The TCN2 776CNG polymorphism correlates with vitamin B(12) cellular delivery in healthy adult populations.** *Clin Biochem 2010;43 (7-8):645-9*

2. Rocha MS, Castro R, Rivera I, Kok R, Smulders Y, Jakobs C, Tavares de Almeida I and Blom HS. **Global DNA Methylation: Comparison of enzymatic- and non-enzymatic -based methods.** *Clin Chem Lab Med 2010; 48(12):1793-8*

3. Barroso M, Rocha MS, Esse R, Goncalves Jr I, Gomes AQ, Teerlink T, Jakobs C, Blom HJ, Loscalzo J, Rivera I, Tavares de Almeida I and Castro R. **Cellular hypomethylation is associated with impaired nitric oxide production by cultured human endothelial cells.** *Amino Acids 2012; 42(5):1903-11*

4. Rocha MS, Teerlink T, Janssen M, Kluijtmans L, Smulders Y, Jakobs C, Tavares de Almeida I, Rivera I, Castro R and Blom HJ. **Asymmetric dimethylarginine in adults with cystathionine β-synthase deficiency.** *Atherosclerosis 2012; (222): 509-11*

5. Rocha MS, Teerlink T, Koolwijjk P, Smulders Y, Jakobs C, Tavares de Almeida I, Rivera I, Castro R and Blom HJ. **S-Adenosylhomocysteine increases DDAH activity in cultured endothelial cells, via a non-DNA dependent mechanism.** *Submitted*

6. Esse R, Rocha MS, Barroso M, Florindo C, Teerlink T, Kok R, Smulders S, Rivera I, Leandro P, Koolwijk P, Castro R, Blom HJ and Tavares de Almeida I. **Protein arginine methylation is more prone to inhibition by S-adenosylhomocysteine than DNA methylation in vascular endothelial cells.** *Submitted*

7. Rocha MS, Vis K, Esse R, Imbard A, de Vriese AS, Kok RM, Smulders Y, Tavares de Almeida I, Rivera I, Castro R and Blom HJ. **Diets inducing hyperhomocysteinemia affect global DNA methylation in a tissue-specific manner.** *Submitted*