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Hyperhomocysteinemia is Associated with Decreased Erythropoietin Expression in Rats

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Key Words

S-adenosylhomocysteine hydrolase • Erythropoietin • Hyperhomocysteinemia • CO exposure

Abstract

Background/Aims: Elevated plasma homocysteine (Hcy) levels have been identified as a pathogenic factor causing a variety of pathological changes in different cells and tissues. In vertebrates, Hcy is produced solely from S-adenosylhomocysteine (AdoHcy) through the catalysis of AdoHcy-hydrolase. The direction of AdoHcy-hydrolase activity is determined by its cytosolic substrate concentrations, thereby controlling intracellular AdoHcy levels. Most S-adenosylmethionine (AdoMet)-dependent methyltransferases are regulated *in vivo* by the ratio of AdoMet/AdoHcy, which is termed "methylation potential" (MP). To test whether high rates of erythropoietin (EPO) expression is reduced by a low MP in vivo we choosed the model of increased EPO production following carbon monoxide (CO) exposure in rats in which high transcriptional activity is responsible for renal EPO production. Results: To induce a sustained hyperhomocysteinemia in rats, we infused i.v. a low or high dose of Hcy resulting in Hcy plasma levels of 87.4±6.2 and 300.8±23.7 µmol/l, respectively. Renal tissue contents of AdoHcy, AdoMet, and adenosine (Ado) were measured after freeze clamp by means of HPLC. Within 4h of CO exposure EPO serum levels increased from 13.6±0.4 (control) to 2254.8±278.3 mIU/ml. Only high dose of Hcy reduces both, the MP from 40.8±2.0 to 8.2±1.0 in the kidney as well as EPO serum levels by 40% compared to control rats. Conclusion: Our data show that severe hyperhomocysteinemia (HHcy) affects the MP in the renal tissue and lowers EPO expression following CO induced intoxication. This result supports the concept that efficient EPO production requires an unimpaired MP.

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Introduction

Homocysteine (Hcy) is a precursor of S-adenosylmethionine (AdoMet), the primary methyl group donor for most biological methylations and is produced in vertebrates solely from S-adenosylhomocysteine

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(AdoHcy). Since AdoHcy is a potent inhibitor of most AdoMet-dependent methyltransferases [1, 2] a fast removal of AdoHcy is necessary to maintain AdoHcy tissue content at a low level [3]. Therefore the ratio of AdoMet/AdoHcy, defined as the methylation potential (MP), is considered to be a reliable indicator of the flow of methyl group transferred from AdoMet to the methyl acceptor within the cells [4]. The hydrolysis of AdoHcy to Hcy and adenosine (Ado) is a reversible reaction by the enzyme AdoHcy-hydrolase (EC 3.3.1.1). Therefore, the metabolisms of AdoMet, AdoHcy, Ado and Hcy are functionally linked and alteration in one pathway may affect the other (Fig.1). The direction of AdoHcy-hydrolase activity is determined by its cytosolic substrate concentrations. Moreover, AdoHcy-hydrolase controls the intracellular AdoHcy levels. Although the equilibrium of the reaction favors the synthesis of AdoHcy the hydrolysis of AdoHcy prevails under physiological conditions because both reaction products are rapidly removed [5, 6]: Ado by adenosine deaminase (EC 3.5.4.4) or adenosine kinase (EC 2.7.1.20) and Hcy by cystathionine ß-synthase or betaine-homocysteine S-methyltransferase (EC 2.1.1.5) or 5'-methyltetrahydrofolate-homocysteine S-methyl-transferase (EC 2.1.1.13) see also Fig. 1. Therefore, not only defects in the remethylation or transsulfuration pathways can cause hyperhomocysteinemia (HHcy) but also vitamin deficiencies [7] or impaired kidney function [8, 9]. Moreover, a moderately elevated plasma Hcy has been shown to be an independent atherothrombotic risk factor and is often found in patients with end-stage renal disease [10-12]. Chronic renal failure has been reported to be associated with elevated plasma Hcy, not because of impaired urinary excretion, but because of impaired metabolism of Hcy by the kidney [13, 14]. Recent studies support the hypothesis that elevated Hcy is rather a marker for renal impairment than an independent cardiovascular risk factor [14, 15]. A series of studies has shown that elevation of plasma Hcy correlates with increased AdoHcy levels and with a decreased MP [16]. The low MP is associated with a decreased global DNA methylation [17, 18] and these epigenetic changes can lead to inappropriate gene expression and promotion of disease [19, 20]. However, a case of AdoHcy-hydrolase deficiency was reported in which the patient's leukocyte DNA was hypermethylated relative to controls despite extremely high levels of plasma AdoHcy [21]. Correspondingly, we have shown that a reduced MP changes mainly mRNA methylation and gene expression levels whereas the global DNA methylation is not affected [22, 23]. Since increased AdoHcy levels lead to a reduced

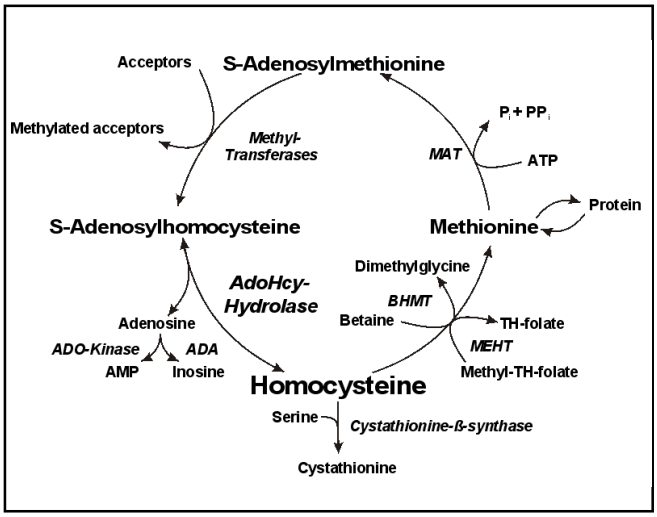


Fig. 1. Homocysteine (Hcy) metabolic pathway. Schematic overview. ADA, adenosine deaminase; BHMT, betaine-homocysteine methyltransferase; MAT, methionine adenosyltransferase; MEHT, methinone synthase.

erythropoietin (EPO) expression in HepG2 cells we analyzed in the present *in vivo* study the effect of Hcy on renal AdoHcy tissue content, on renal EPO mRNA, EPO plasma levels and AdoHcy-hydrolase expression after carbon monoxide (CO) exposure.

Materials and Methods

The following materials were purchased from the different sources indicated: EPO-ELISA (medac), anti-AdoHcy-hydrolase antibody [24], bovine serum albumin (BSA), Tris-(hydroxymethyl)-aminomethan (Tris), AdoMet, AdoHcy, Ado, DL-Hcy, methyladenosine, AMP, ADP, ATP, nucleoside phosphorylase (Sigma), adenosine deaminase, xanthine oxidase (Roche), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Serva), HPLC grade methanol and acetonitrile (Merck).

Animal preparation

The experiments were performed with male Sprague-Dawley rats in accordance to the German Law for Animal Protection. The experiments were approved by the Animal Care and Use Committee of the University of Tübingen. Male Sprague-Dawley rats (280-350 g) were obtained from Charles River, UK and fed with standard laboratory rat chow and tap water *ad libitum*. To induce CO intoxication, anesthetized rats with thiopental 90 mg/ kg i.p. (Trapanal® Byk Gulden, Germany) were provided with CO mixed with room air to generate the CO concentration of 1200 ppm via a mask. The actual CO concentration was monitored by a CO sensor (Testo, Reutlingen). This CO exposure results in a nearly maximal

Gene	Primer sequences (F, forward; R, reverse)	Product size (bp)
EPO	F: CAC GAA GCC ATG AAG ACA GA	100
	R: GGC TGT TGC CAG TGG TAT TT	
18S rRNA	F: CGG CTA CCA CAT CCA AGG AA	187
	R: GCT GGA ATT ACC GCG GCT	

Table 1. Nucleotide sequence of oligonucleotide primers used for amplification of erythropoietin (EPO) mRNA by real-time quantitative RT-PCR.

stimulation of EPO production by the kidney [25, 26]. Elevation of Hcy plasma levels was induced by Hcy infusion in isotonic saline (3 ml/h) via the right jugular vein. Low dose Hcy administration was started with a prime dose of 1.4 mg/100 g and a subsequent dose of 0.43 mg/h/100 g body weight. High dose Hcy administration was started with a prime dose of 5.5 mg/100 g and a subsequent dose of 1.3 mg/h/100 g body weight for 4 h. After 4 h of CO exposure the left kidney was exposed by flank incision and placed on a Lucite holder and immediately snap-frozen with clamps precooled to the temperature of liquid nitrogen. Blood samples were then collected and stored at –80°C until analysis of EPO and Hcy. For protein determination, measurement of AdoHcy-hydrolase activity, and Western blot analysis the right kidney was removed subsequently and stored at -80°C. Rats infused with saline only served as controls.

Tissue preparation and analysis

The frozen left kidneys were prepared as described previously [3]. In brief, the frozen kidneys were pulverized under liquid nitrogen and the tissue protein was precipitated with 0.6 N ice-cold perchloric acid. After centrifugation at 11,000 x *g* for 30 min at 4°C the supernatants were adjusted to a pH between 5.5 and 6.5 to measure Ado, AdoHcy and AdoMet or to a pH of 7.8 to measure the adenine nucleotides by means of HPLC as previously described [27]. Measurements of Hcy concentrations in plasma were performed by HPLC (Bio-Rad Laboratories) in the Department of Internal Medicine IV, University Hospital Tübingen.

Erythropoietin assay

Serum EPO concentrations were quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Medac, Wedel Germany). The EPO-ELISA is based on two mouse monoclonal anti-EPO antibodies. Recombinant EPO was used as calibrator (2.5 – 160 mIU/ml). Results are expressed in International Units (mIU/ml). Assays were performed in duplicate, as described by the manufacturer's protocol. Serum samples from rats exposed to CO were diluted 1:10.

Activity of renal AdoHcy-hydrolase

The enzymatic activity of AdoHcy-hydrolase was measured in total renal homogenate in the hydrolytic direction as described previously [6]. This spectrometrical method is based on the reduction of MTT to formazan at 578 nm with the following components: adenosine deaminase (1 U), nucleoside phosphorylase and xanthine oxidase (0.5 U each), gelatine (0.14 %) and MIT (1 mmol/l). The reaction was started with addition of AdoHcy (80 µmol/l).

SDS-Gelelectrophoresis and Western Blot

Hundred micrograms of protein from total kidney homogenate, determined by the Bradford method [28], were separated on 7-19 % sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and electrophoretically transferred to a Protran B 85 nitrocellulose membrane (Schleicher and Schuell, Duesseldorf, Germany) at 400 mA. The membranes were labeled with an AdoHcy-hydrolase-specific primary polyclonal antibody (1:250) raised in rabbits against the native bovine AdoHcy-hydrolase [24], followed by horseradish-peroxidase-linked secondary goat anti-rabbit IgG (DAKO, Denmark). The staining was performed using 500 µl *o*-toluidine [saturated in 7% (v/v) acetic acid], 100 µl 4-chloro-1-naphthol [2% (w/v) in diethylene glycol], and 500 µl H_2O_2 (3% v/v) in 50 ml PBS.

RNA isolation

RNA of non-hypoxic and hypoxic kidney tissue (4-7 animals/group) was isolated to analyze mRNA expression of EPO. Briefly, total RNA was extracted from an aliquot of pulverized frozen kidney with Tri-Reagent/Trizol (PeqLab, Erlangen, Germany) by the method of Chomczynski and Sacchi [29]. cDNA was synthesized with AMV reverse transcriptase (PeqLab, Erlangen, Germany) using random hexamers (Promega, Mannheim, Germany).

Real-time reverse transcription-polymerase chain reaction

Real-time-PCR analysis was performed on the Light Cycler instrument with FastStart DNA Master SYBR Green reagents (Roche, Mannheim, Germany) as described previously [22]. The relative amount of the specific mRNA of interest was normalized to 18S rRNA. Gene expression was calculated according to a mathematical model for relative quantification established by Pfaffl [30]. Primer sequences are shown in Table 1.

Experimental condition	(n)	Ado	AdoHcy	AdoMet	MP
normoxia	(8)	5.4±0.3	0.56±0.06	27.7±0.8	53.8±6
normoxia + low dose Hcy	(6)	4.3±0.6	0.91±0.08*	26.5±1.6	29.1±3*
normoxia + high dose Hcy	(5)	6.0±1.4	1.85±0.2*	28.8±1.5	16.8±2*
CO exposure	(7)	7.7±0.8*	0.76±0.06	31.0±0.8	40.8±2
CO exposure + low dose Hcy	(5)	4.2±1.2	3.03±0.9‡	32.3±3.1	12.3±3‡
CO exposure + high dose Hcy	(9)	5.6±0.6	4.60±0.7‡	30.2±2.5‡	8.2±1‡

Table 2. The data show tissue content of Ado, AdoHcy, and AdoMet in the rat kidneys after CO exposure. Data are means ± SEM for n determinations and are expressed in nmol/g wet weight. *p<0.05 vs. normoxia, ‡p<0.05 vs. CO exposure. MP, AdoMet/AdoHcy.

Statistical analysis

The statistical significance of the different experimental groups were assessed by ANOVA and the unpaired two-sided *t* test with α–adjustment according to Bonferroni-Holm. All values are presented as mean ± SEM. *P* values <0.05 were considered to be statistically significant.

Results

Determination of EPO and Hcy

As a proof of principle and to demonstrate that there is no difference in EPO secretion between awake and anesthetized rats we have carried out dose response curves to determine EPO serum concentrations under different CO concentrations (Fig. 2). Within 4 h of CO exposure (1200 ppm), EPO serum levels were strongly elevated from 13.6±0.4 (control) to 2254.8±278.03 mIU/ml.

Systemic infusion of low dose Hcy elevated plasma Hcy from 7.7±0.6 (n=8) to 87.4±6.2 µmol/l (n=5), whereas administration of high dose Hcy induces a HHcy with plasma levels of 300±23.7 µmol/l (n=9). These levels correspond to a severe HHcy in patients with hereditary homocysteineuria [31].

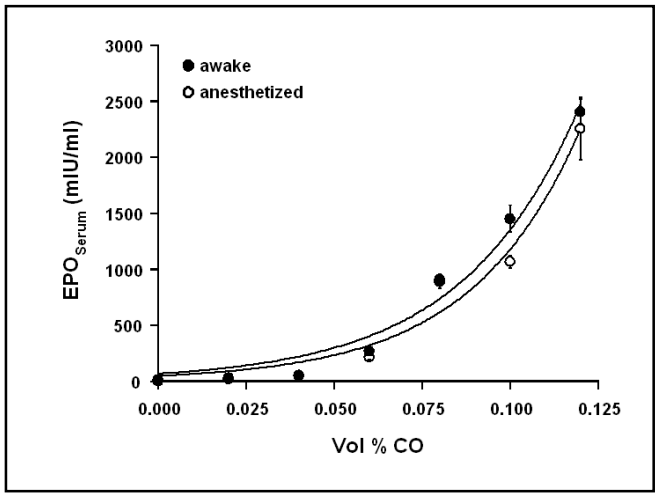


Fig. 2. Erythropoietin (EPO)/carbon monoxide (CO) dose-response curve in awake (●) and anesthetized (○) rats. For CO exposure, awake rats were placed into a cage with an air flow between 6-10 l/min. CO was mixed with room air to generate CO concentrations between 0.00 – 0.12 vol% CO. For anesthetized rats an aerating cap was developed which closed tightly around the neck of the rat. The cap was connected by a tube to a CO/room air reservoir resulting in CO concentrations from 0.00 to 0.12 vol%. Serum EPO concentrations were determined at the end of 4 h CO exposure. Values are means ± SEM for n=5-10.

Effect of CO exposure and HHcy on methylation potential and energy charge (EC)

To determine whether CO exposure (1200 ppm) and acute HHcy leads to changes in the AdoMet/AdoHcy ratio (MP) of the kidney, tissue levels of AdoHcy, AdoMet, and Ado were measured. Renal tissue content of AdoHcy was unchanged under CO exposure. However, MP is lowered from 53.8±6 (control) to 40.8±2. Furthermore, CO exposure increases the Ado tissue content (Table 2). Infusion of Hcy (low and high dose) results in accumulation of AdoHcy tissue levels under both, normoxic and hypoxic conditions. The resulting MP is reduced by 46% - 80%, respectively (Table 2).

Influence of HHcy on EPO and AdoHcy-hydrolase expression

Although HHcy reduces the MP under normoxic conditions, EPO serum levels remain unchanged. However, after 4 h of CO exposure only high doses of Hcy lowers EPO serum levels significantly (Fig. 3). As shown in Fig. 3 also EPO mRNA levels are decreased

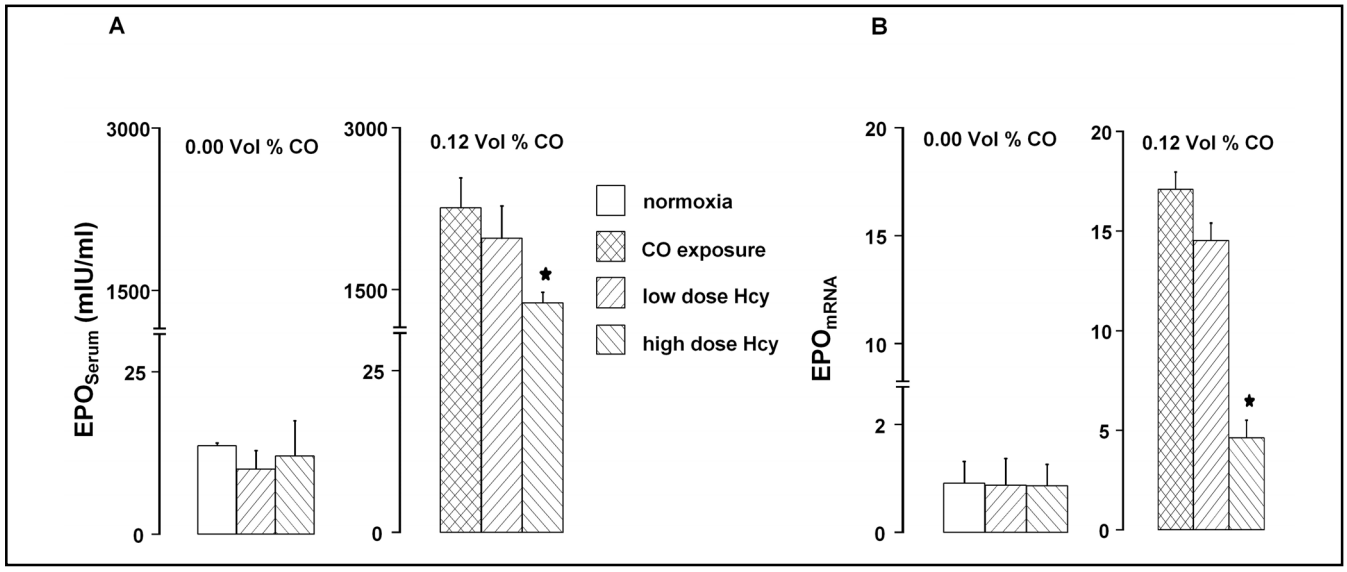
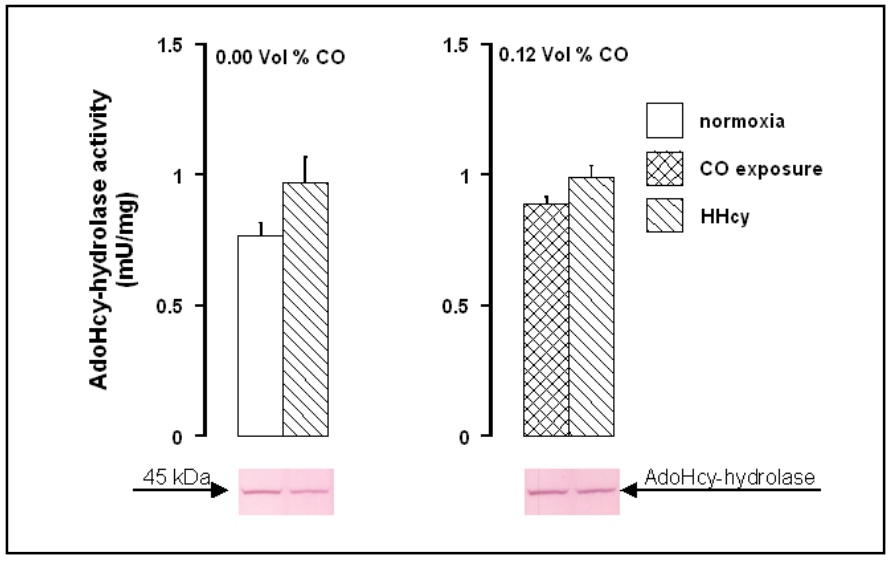


Fig. 3. Influence of HHcy and CO exposure on EPO expression. Rats were exposed to 0.12 vol% CO. EPO and Hcy serum concentrations were determined at the end of 4 h CO exposure. Values are means ± SEM for n=8-9.

Fig. 4. Influence of HHcy and CO exposure on AdoHcy-hydrolase activity. AdoHcy-hydrolase activity was measured in the hydrolytic direction as described under Materials and Methods. Values are means ± SEM for n=8-9.



(-70%) during high doses of Hcy. A moderate, but not significant reduction of EPO plasma levels by 15% is found during the infusion of low dose Hcy.

Interestingly, AdoHcy-hydrolase activity is neither changed by CO exposure nor by Hcy infusion (Fig. 4).

Discussion

AdoHcy is the sole precursor of Hcy production via the reversible AdoHcy-hydrolase mediated reaction, which has a thermodynamic equilibrium towards AdoHcy synthesis rather than hydrolysis [5]. In turn, AdoHcy, as the metabolite of AdoMet-dependent transmethylation reactions functions as a product inhibitor of AdoMet-dependent methyltransferases, including methylation of mRNA. The present study was designed to evaluate the effect of intracellular AdoHcy accumulation on EPO and AdoHcy-hydrolase expression in the rat kidney *in vivo*. EPO secretion is a good model for increased transmethylation activity in the rat kidney, since EPO is not stored in vesicles but is produced on demand following CO exposure, resulting in extensive EPO mRNA. Therefore, it can be expected that hyperhomocysteinemia (HHcy) induced an accumulation of AdoHcy resulting in a decreased MP which may lead to an impaired EPO production following CO exposure.

In the first series of experiments we established a dose-response curve of CO mediated increases of EPO plasma concentration in *anesthetized* rats confirming data from the literature obtained in *conscious* animals [32]. The infusion of Hcy induced a moderate (low dose) and a severe (high dose) HHcy resulting in a 2-3 fold increase of AdoHcy tissue content. Since AdoMet was unchanged the MP was reduced by 40-70 % following 4 hours Hcy infusion. Unstimulated EPO plasma levels were unchanged by Hcy infusion. When CO (1200 ppm) was added to the inhaled room air we found a slight increase in AdoHcy and a small reduction of the MP. In contrast, during CO exposure HHcy (low and high dose) lead to a 5-8-fold increase in AdoHcy, suggesting that under these *in vivo* conditions high Hcy concentrations favor the synthesis of AdoHcy by AdoHcy-hydrolase confirming earlier studies in which adenosine and Hcy administered directly to the kidney [3]. Interestingly, elevated renal Ado tissue content during CO exposure without Hcy infusion was reduced from 7.7 to 5.6 nmol/g wet weight during high dose of Hcy, respectively (Table 2) which is compatible with the assumption that a fraction of the intracellular Ado pool enters the AdoHcy pool due to high Hcy concentration. We can exclude that changes in AdoHcy-hydrolase activity may have contributed to elevated AdoHcy levels since enzymatic activity of renal AdoHcy-hydrolase was unchanged (Fig. 4).

Concomitant with the elevated renal AdoHcy and unchanged AdoMet levels the MP is markedly reduced. Thus, the capacity of AdoMet dependent methylation reactions is most likely impaired [1]. Our results show that only under conditions with HHcy and CO exposure EPO secretion by the kidneys is significantly reduced (Fig.3). The plasma EPO levels at the end of 4 hours CO exposure reflect the sum of secreted EPO minus degraded EPO since the half life of EPO is in the order of ~ 60 min [33]. Therefore, our plasma EPO levels are only indirectly related to the actual rate of renal EPO release. Correspondingly the mRNA of EPO is much more reduced by HHcy than EPO plasma levels. Nevertheless, this result appears to be compatible with the general assumption that mRNA capping and subsequent reduced export from the nucleus into the cytosol is largely impaired when high intracellular concentrations of AdoHcy inhibit AdoMet dependent methylation of mRNA [34, 35]. The major finding of the present study is that an increase in AdoHcy tissue content in the rat kidney lowers stimulated EPO expression only under CO exposure whereas AdoHcy-hydrolase expression remains unaltered. Therefore, this finding suggests that (i) the mRNA levels of the genes investigated respond differentially to changes in MP, and (ii) the MP must be lowered to values below 10 to influence transmethylation reactions in the rat kidney. The calculated intracellular AdoHcy concentrations are in the range of 5 -10 mol/l which is in the range of K_i values of RNA methyltransferases [1]. Published data obtained under a variety of experimental conditions and using different models demonstrate the importance of AdoHcy as a major predictor of reduced methylation capacity [1]. The increased AdoHcy concentration was mainly associated with global DNA hypomethylation [18-20]. On the other hand, short term changes in MP or transient Hcy elevation do not change global DNA methylation suggesting that the differential gene expression is not associated with changes of methylation pattern of the DNA [22, 36]. Therefore, we suggest that the lowered EPO expression during HHcy might be the result of inhibition of mRNA methylation. This observation raises the question whether moderate HHcy may contribute to anemia in patients with end stage renal failure. In addition, increased plasma levels of AdoHcy may also contribute to disturbed transmethylation reactions in end stage renal failure [16, 37, 38].

We are aware that the results of the experiment of HHcy may not be quantitatively extrapolated to human renal physiology, since the major difference between rat and human with respect to Hcy is that in rats Hcy is mainly present in the unbound form [39], while in human it is mainly protein-bound [40]. However, the enzymes metabolizing Hcy are the same in rats and human.

In summary, the present data support the importance of AdoHcy as the primary determinant of reduced methylation capacity affecting gene expression. Moreover, our results with experimental HHcy indicate inhibition of mRNA methylation by AdoHcy in the model of stimulated renal EPO production.

Acknowledgments

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