S-Adenosylhomocysteine Metabolism in Different Cell Lines: Effect of Hypoxia and Cell Density

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Key Words

S-adenosylhomocysteine hydrolase • AdoMet/AdoHcy ratio • Methylation potential • Cell lines • Hypoxia • Cell density

Abstract

Background/Aims: The methylation potential (MP) is defined as the ratio of S-adenosylmethionine (AdoMet) to S-adenosylhomocysteine (AdoHcy). It was shown recently that hypoxia increases AdoMet/AdoHcy ratio in HepG2 cells (Hermes et al., Exp Cell Res 294: 325-334, 2004). In the present study, we compared AdoMet/AdoHcy ratio and energy metabolism in HepG2, HEK-293, HeLa, MCF-7 and SK-HEP-1 cell lines under normoxia and hypoxia. **Methods:** Metabolite concentrations were measured by HPLC. In addition, AdoHcy hydrolase (AdoHcyase) activity was determined photometrically. **Results:** Under normoxia HepG2 cells show the highest AdoMet/AdoHcy ratio of 53.4 ± 3.3 followed by MCF-7 and SK-HEP-1 cells with a AdoMet/AdoHcy ratio of 14.4 ± 1.1 and 21.1 ± 1.3 , respectively. The lowest AdoMet/AdoHcy ratios are exhibited by HeLa and HEK-293 cells (6.6 ± 0.7 and 7.1 ± 0.3). Hypoxia does

not significantly change the MP in MCF-7 and HeLa cells, but alters the MP in HepG2, HEK-293 and SK-HEP-1 cells. These alterations are dependent on the cell density. Under normoxia HepG2 cells exhibit AdoHcyase activity of $2.5 \pm 0.2 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$. All other cell lines show 3-5 times lower enzyme activity. Interestingly, hypoxia affects AdoHcyase activity only in HepG2 cells. **Conclusions:** Our data clearly show that the cell lines are characterized by different MP and different behavior under hypoxia. That implies that a lower MP is not necessarily associated with impaired transmethylation activity and cellular function.

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Introduction

The catalytic activity of S-adenosylmethionine (AdoMet)-dependent methyltransferases *in vivo* is not only a function of the concentration of AdoMet but also a function of the S-adenosylhomocysteine (AdoHcy) tissue level. Therefore, the ratio of AdoMet/AdoHcy, also termed methylation potential (MP), is frequently used as

Fig. 1. Metabolic pathway of S-adenosylhomocysteine (AdoHcy) formation and degradation. Since AdoHcy is a potent inhibitor of methyltransferases and since AdoHcyase controls the intracellular AdoHcy concentration, inhibition of this enzyme leads to marked changes in the methylation potential and thus in transmethylation reactions. ADA – adenosine deaminase; Ado-kinase – adenosine kinase; MEHT – 5-methylenetetrahydrofolate-homocysteine S-methyltransferase.

an indicator of cellular methylation capacity, whereby a decrease in this ratio predicts reduced cellular methylation activity [1-3]. AdoHcy is a competitive inhibitor of most AdoMet-dependent methyltransferases, because AdoHcy binds to the active site of these methyltransferases with a higher affinity than AdoMet [4]. Thus it is likely, that the increased intracellular concentrations of AdoHcy might be more effective in reducing cellular methylation capacity than decreased AdoMet levels. Therefore, efficient removal of AdoHcy is required to maintain the AdoHcy content at a low level to achieve values for the AdoMet/AdoHcy ratio > 10, reported for the most cells and tissues [5-8].

The transfer of an activated methyl group from AdoMet to numerous acceptors including nucleic acids, proteins and small molecules such as creatine, affects diverse aspects of gene regulation, metabolism and cellular function [9]. The product of these methylation reactions, AdoHcy is hydrolyzed solely by S-adenosylhomocysteine hydrolase (AdoHcyase, EC 3.3.1.1) in eukaryotic cells (Fig. 1). The reaction catalyzed by AdoHcyase is reversible producing adenosine (Ado), which has been suggested to play an important role in renal [10, 11], cardiovascular [12], and neuronal functions [13], and homocysteine which has been implicated in causing vascular disease [14]. Thus, AdoHcyase is an essential enzyme playing an important role in regulating processes like transmethylation [15], transsulfuration [16] and purine metabolism [12] (Fig. 1). The fact that deletion of the

AdoHcyase gene is associated with embryo lethality in mice shows the importance of this enzyme for biological transmethylation reactions [17].

Although AdoMet-dependent methylation reactions occur in different compartments of the cell, AdoHcyase has been described previously as a cytoplasmic enzyme [18, 19]. However, it has been shown that during embryogenesis of *Xenopus laevis* AdoHcyase is also temporally located in the nucleus of transcriptionally active cells [20]. The nuclear localization of AdoHcyase which coincides with high rates of mRNA synthesis may reflect the necessity to remove AdoHcy to maintain efficient cap methylation [20, 21]. Furthermore, inhibition of AdoHcyase results in intracellular accumulation of AdoHcy in HepG2 cells causing a significant decrease of the MP and a subsequent reduction of mRNA methylation and erythropoietin mRNA expression [3]. However, DNA methylation was not affected by the elevated cellular level of AdoHcy suggesting that some methyltransferases are more susceptible to the inhibitory effect of AdoHcy than others [3, 22].

Recent studies have shown, that AdoMet/AdoHcy metabolism is altered under several pathological conditions. AdoMet/AdoHcy ratio is reduced in patients with diabetes mellitus [23], uteroplacental insufficiency [24], vascular disease [25], and in ischemic rat kidney [8], whereas it is increased under hypoxia in human hepatocellular carcinoma cell line [3]. Since hypoxia is an important pathophysiological stress that occurs during

blood vessel injuries and tumor growth, we are interested in the effects of hypoxia on MP in different cell lines and the underlying mechanism.

In order to determine, whether proliferation activity and tissue origin affect MP, we examine the MP in two hepatoma cell lines, HepG2 and SK-HEP-1, two extra hepatic tumor cell lines, HeLa and MCF-7, and the human embryonic kidney cells, HEK-293, under normoxia and hypoxia.

Furthermore, we investigate AdoHcyase activities in the different cell lines under various conditions, because the activity of AdoHcyase plays a critical role in the control of AdoHcy tissue levels. In addition, we study if changes in AdoMet/AdoHcy metabolism are associated with alterations in energy metabolism, because previous data strongly support the interaction between energy metabolism and transmethylation reactions [8].

Materials and Methods

Materials

All cell lines studied were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, Germany). The media and reagents used for cell culture were provided by Invitrogen. AdoMet, AdoHcy, Ado, N⁶-methyladenosine, nucleoside phosphorylase and ammonium phosphate were obtained from Sigma. Ado deaminase was purchased from Boehringer Mannheim, xanthine oxidase from Roche and heptanesulfonic acid sodium salt from Fluka. HPLC grade methanol and acetonitrile were from Merck.

Cell culture

HepG2 [26, 27], MCF-7 [28] and SK-HEP-1 cells [29, 30] were cultured in RPMI 1640, HEK-293 [31] and HeLa cells [32] in minimum essential medium (MEM) containing glutamine. Medium was supplemented with 10% heat-inactivated newborn calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin and cells were grown in a humidified atmosphere containing 5% CO₂ at 37°C. For all experiments (normoxic and hypoxic conditions) cells were rinsed with PBS and then cultured with serumfree medium for 24 h. This allows us to compare our results to previous studies [3]. Hypoxia was established in an incubator where the oxygen partial pressure was lowered to 0.9 kPa (1% O₂ by vol.).

At the end of each incubation period, the cells were removed with trypsin or rinsed with medium (HEK-293 cells). Cell counts were determined with a hemocytometer, because the concentration of AdoMet, AdoHcy and Ado was standardized by that number and expressed as nanomoles per 1·10⁷ cells.

Metabolite determination

AdoMet, AdoHcy and Ado concentrations in cultured cells were measured in perchloric acid cell extracts. Cells were harvested by incubation with trypsin/EDTA or rinsed with medium (HEK-293) and then collected by centrifugation at 200 x g for 10 min. The cells were washed with PBS and lysed in 1.2 ml 0.6 N perchloric acid. The cell debris was removed by centrifugation (10 min, 20 000 x g). All samples and standards were supplemented with a known amount of N⁶-methyladenosine as internal standard. The supernatant was adjusted to a pH between 5.5 and 6.0 by adding 2 M K₂CO₃/1 M KH₂PO₄. The precipitated potassium perchlorate was discarded after centrifugation at 20 000 x g and the supernatant was applied onto solid-phase extraction column (BondElut, ICT, Bad Homburg, Germany). Elution of the compounds was performed with HCl (0.1 M) and eluate was analyzed by reversed-phase chromatography with UV-detection according to Delabar et al. [33] with following modifications. The binary solvent system consisting of solvent A (10 mM ammonium dihydrogenphosphate, 0.6 mM heptanesulfonic acid sodium salt in 3% methanol) and solvent B (like solvent A and in addition 10% acetonitrile) delivered the following gradient: Immediately after sample injection a linear gradient was started to increase solvent B to 15% with a transition rate of 1.2% /min and to 70% with a transition rate of 7.5% /min. Thereafter, solvent B was kept at 70% for 5 min. Before the next sample injection, solvent A was kept at 100% for 10 min to reequilibrate the system. Thus, the chromatogram was completed within 30 min. Remote control, data acquisition and quantification of peak areas were performed with the Peak Simple Software 3.12 by SRI Inc.

Determination of Nucleotides

Adenine nucleotides were analyzed by HPLC using a Grom-Sil 120 ODS-3 CP column (5 µm, 125 x 4 mm i.d.; Sykam, München, Germany) and an UV detector (UVIS 200; Sykam) for absorbance recording at 254 nm. The column was eluted at 30°C with a flow rate of 1 ml min⁻¹ and a low pressure gradient. Eluent A consisted of 65 mM potassium phosphate, pH 4.6, and 5 mM tetrabutylammonium sulfate as ion-pair forming agent. Solvent B was solvent A + 40% (v/v) acetonitrile. Samples were prepared as described for metabolite determination. The perchloric acid supernatant was adjusted to a pH of 8.0 by adding 2 M K₂CO₃/1 M KH₂PO₄. The run was performed according to Hambrock et al. [34]. The chromatograms were quantified by peak area measurement by means of online computing integrator with the Peak Simple Software 3.12 by SRI Inc.

Determination of cell viability

Cell viability was monitored using a cell analyzer system (CASY Model TT, Schärfe System, Reutlingen, Germany). Cells were harvested by trypsination or rinsing with medium (HEK-293), resuspended and diluted in 10 ml CASY[®]-ton. The viability of the cells can be measured directly.

Table 1. Intracellular AdoMet, AdoHcy and Ado concentration in different cell lines under normoxic conditions. All cell lines were incubated for 24 h under normoxic conditions in serumfree medium. Thereafter, cells were harvested and extracted with perchloric acid. The metabolites were separated by HPLC as described in Materials and methods.

Cell line	n	AdoMet nmol/10 ⁷ cells	AdoHcy nmol/10 ⁷ cells	Ado nmol/10 ⁷ cells	MP
HepG2	25	1.76 ± 0.10	0.034 ± 0.002	0.033 ± 0.003	53.42 ± 3.25
HEK-293	16	1.42 ± 0.09	0.203 ± 0.015	0.055 ± 0.010	7.16 ± 0.3
MCF-7	8	2.14 ± 0.22	0.15 ± 0.02	0.35 ± 0.05	14.38 ± 1.07
HeLa	8	1.83 ± 0.25	0.29 ± 0.04	0.11 ± 0.01	6.57 ± 0.72
SK-HEP-1	9	2.35 ± 0.18	0.113 ± 0.01	0.036 ± 0.01	21.14 ± 1.29

Table 2. Influence of hypoxia on AdoMet, AdoHcy, Ado concentration and AdoMet/AdoHcy ratio in MCF-7 and HeLa cells. The cell lines were grown under normoxic conditions until they were nearly confluent and then incubated for 24 h under hypoxic conditions in serumfree medium. Cells were harvested by trypsin/EDTA and extracted with perchloric acid. After solid phase extraction, the metabolites were analyzed by HPLC as described in Materials and methods. Data are mean ± SEM for n determinations. *p<0.05 versus control of the corresponding cell line.

Cell line	n	AdoMet nmol/10 ⁷ cells	AdoHcy nmol/10 ⁷ cells	Ado nmol/10 ⁷ cells	MP
MCF-7	6	1.68 ± 0.13	0.13 ± 0.01	0.19 ± 0.03*	12.8 ± 1.1
HeLa	8	1.01 ± 0.16*	0.24 ± 0.03	0.21 ± 0.03*	4.55 ± 0.74

Preparation of total protein

Cells were harvested by incubation with trypsin/EDTA or suspended by rinsing with medium (HEK-293) and then collected by centrifugation at 200 x g for 10 min. The pelleted cells were washed with PBS and homogenized in Tris/HCl (20 mM, pH 7.2) by sonification. The homogenate was centrifuged at 20 000 x g for 15 min and supernatant was processed for immunoblot analysis and photometrical determination of enzyme activity as described below.

Protein quantification

The protein concentration was determined according to the method of Bradford using a protein assay kit (BioRad) with bovine serum albumin (BSA) as standard.

Immunoblotting

Expression of AdoHcyase in HepG2 cells was detected by immunoblotting. Total cell lysate was separated under reducing conditions by 12% SDS-polyacrylamid gel electrophoresis (PAGE). After electrophoresis the proteins were electroblotted onto a nitrocellulose membrane. The loading of equal amounts of protein was confirmed by staining the gel with 0.1% Coomassie Blue R 250 in 25% ethanol and 8% acetic acid. The membrane was blocked with 5% defatted milk powder in PBS overnight, then washed 3 times with Tween/PBS for 10 min. The first antibody (rabbit anti-bovine AcoHcyase; [35]) was carried out for 3 h in PBS+0.5% BSA at a dilution of 1:500. Subsequently, the membrane was washed and the second antibody (goat-anti-rabbit coupled to horseradish peroxidase, Dako) reaction was performed for 2 h in PBS+0.5% BSA at a dilution of 1:1500. The membrane was washed 3 times and the staining of the membrane was performed using 4-chloro-1-naphthol, H₂O₂ and ortho-toluidine. When color appeared on the membrane, the reaction was stopped by washing with water.

Enzymatic activity of AdoHcyase

The activity of AdoHcyase in total cell lysates was determined photometrically in a total volume of 500 µl in 50 mM potassium phosphate (pH 7.0) at room temperature. The reaction mixture contained total cell lysate, Ado deaminase (1U), nucleoside phosphorylase (0.09 U) and xanthine oxidase (0.08 U). Therefore, Ado generated by AdoHcy hydrolysis is processed to uric acid, which can be measured at 292 nm. The reaction was started by the addition of 50 µM AdoHcy [19].

Calculation and statistic

The energy charge (EC) of the adenylate system was calculated according to Atkinson and Walton [36], defined as

$$\frac{[ATP] + 0.5 \cdot [ADP]}{[AMP] + [ADP] + [ATP]}$$

Results are expressed as means ± SEM. Data were analyzed by Student t-test and Alternate t-test (InStat). A difference between groups was considered to be significant when p-value was <0.05.

Results

Comparison of methylation potential in different cell lines

HPLC analysis showed comparable AdoMet levels in all cell lines under normoxic conditions (Table 1). In contrast, the intracellular AdoHcy concentration strongly differs between the investigated cell lines (Table 1). The great differences in intracellular AdoHcy concentrations lead to various AdoMet/AdoHcy ratios in the cell lines studied. Highest AdoMet/AdoHcy ratios are shown by

Fig. 2. Correlation between cell density and AdoMet (A), AdoHcy (B), Ado (C)-concentrations and AdoMet/AdoHcy ratio (D) in HepG2 cells under hypoxia. The cells were grown under normoxia and then exposed to hypoxia (1% O₂) in serumfree RPMI 1640 for 24 h. Cells were harvested by trypsinization and centrifugation. After extraction with perchloric acid, metabolites were quantified by HPLC as described in Materials and methods.

HepG2 cells (53.4), lowest by HeLa (6.6) and HEK-293 (7.2) cells. MCF-7 and SK-HEP-1 cells exhibit a AdoMet/AdoHcy ratio of 14.4 and 21.1, respectively.

Since Ado is product and inhibitor of AdoHcyase, we also determined intracellular Ado concentration by HPLC. The Ado level varies between 0.033 nmol/10⁷ cells (HepG2 cells) and 0.35 nmol/10⁷ cells (MCF-7 cells) in the different cell lines (Table 1).

Effect of hypoxia on methylation potential

The investigated cell lines respond differentially to hypoxia. In MCF-7 and HeLa cells the MP is not changed significantly under hypoxia, although, the intracellular AdoMet concentration is significantly lowered in HeLa cells under hypoxia (Table 2).

However, hypoxia alters the MP in HepG2, HEK-293 and SK-HEP-1 cells and these alterations are dependent on the cell density (Figs. 2, 3, 4). As shown in Fig. 2A, there is no apparent relationship between cell density and AdoMet concentration, but a negative correlation between AdoHcy concentration and cell density ($r = 0.48$, $p < 0.05$; Fig. 2B) in HepG2 cell line. Thus, a strong correlation was found between AdoMet/AdoHcy ratio and cell density ($r = 0.73$, $p < 0.001$; Fig.

2D). When cells are grown at a cell density of 4-5·10⁵ cells/cm², exposure to hypoxia decreases AdoHcy level to 0.006 nmol/10⁷ cells, resulting in high AdoMet/AdoHcy ratio of 331. Similarly, a modest but significant correlation was found between cell density and Ado concentration ($r = 0.51$, $p < 0.05$; Fig. 2C). At high cell densities hypoxia reduces Ado concentration to 0.008 nmol/10⁷ cells, which is only about 25% of the amount determined in control cells.

Similar results were obtained for HEK-293 cell line under hypoxic conditions. This cell line also showed no apparent relationship between AdoMet concentration and cell density (Fig. 3A). However, a negative relationship between AdoHcy concentration and cell density was observed under hypoxia ($r = 0.80$, $p < 0.001$; Fig. 3B), resulting in a strong correlation between AdoMet/AdoHcy ratio and cell density ($r = 0.82$, $p < 0.001$; Fig. 3D). In HEK-293 cells, hypoxia leads to 7.5 fold increased MP from 7.16 (control) to 53 (hypoxia combined with high cell densities). Furthermore, under hypoxia the Ado concentration depends on cell density in HEK-293 cells ($r = 0.7$, $r < 0.01$; Fig. 3C).

In contrast to HepG2 and HEK-293 cells, the AdoMet concentration is dependent on cell density in SK-

Fig. 3. Influence of cell density on AdoMet (A), AdoHcy (B), Ado (C)-concentrations and AdoMet/AdoHcy ratio (D) in HEK-293 cells under hypoxic conditions. The cells were grown under normoxia and then exposed to hypoxia (1% O₂) in serum-free MEM with glutamine for 24 h. Cells were suspended by rinsing with medium and harvested by centrifugation. Metabolites were extracted with perchloric acid and analyzed by HPLC as described in Materials and methods.

Fig. 4. Plot of cell density and AdoMet (A), AdoHcy (B), Ado (C)-concentrations and AdoMet/AdoHcy ratio (D) in SK-HEP-1 cells under hypoxic conditions. The cells were grown under normoxia and then exposed to hypoxia (1% O₂) in serum-free RPMI 1640 for 24 h. Cells were harvested by trypsinization and centrifugation. Cells were extracted with perchloric acid and metabolites were analyzed by HPLC as described in Materials and methods.

Cell line	Experimental condition	n	AMP nmol/10 ⁷ cells	ADP nmol/10 ⁷ cells	ATP nmol/10 ⁷ cells	EC
HepG2	control	28	3.39 ± 0.20	11.55 ± 0.76	26.26 ± 0.76	0.78 ± 0.01
	hypoxia <2*10 ⁵ cells/cm ²	10	1.53 ± 0.32*	9.41 ± 1.00	29.24 ± 1.96	0.85 ± 0.04*
	hypoxia >2*10 ⁵ cells/cm ²	11	1.41 ± 0.20*	6.37 ± 0.71*	17.78 ± 1.40*	0.82 ± 0.01*
HEK-293	control	17	15.70 ± 1.31	23.08 ± 1.63	21.48 ± 2.30	0.54 ± 0.02
	hypoxia <2*10 ⁵ cells/cm ²	7	21.33 ± 2.84	16.34 ± 2.06*	13.14 ± 2.20*	0.42 ± 0.03*
	hypoxia >2*10 ⁵ cells/cm ²	6	6.27 ± 1.23*	7.73 ± 1.15*	7.10 ± 1.00*	0.52 ± 0.03
SK-HEP-1	control	7	3.00 ± 0.20	34.01 ± 3.09	68.53 ± 4.54	0.81 ± 0.01
	hypoxia <2*10 ⁵ cells/cm ²	3	1.60 ± 0.20*	25.16 ± 2.40	44.26 ± 2.74*	0.80 ± 0.01
	hypoxia >2*10 ⁵ cells/cm ²	3	2.03 ± 0.25*	31.32 ± 3.92	39.41 ± 6.93	0.84 ± 0.01*
MCF-7	control	8	6.56 ± 0.57	25.72 ± 3.44	46.87 ± 6.60	0.75 ± 0.01
	hypoxia	6	5.56 ± 0.83	18.65 ± 1.47	47.45 ± 3.59	0.79 ± 0.01*
HeLa	control	8	4.33 ± 0.49	35.40 ± 3.92	49.56 ± 5.63	0.75 ± 0.01
	hypoxia	5	7.41 ± 0.70*	30.40 ± 3.51	38.03 ± 4.87	0.70 ± 0.02*

Table 3. Endogenous adenine nucleotide levels and energy charge (EC) in the different cell lines under various oxygen tension. All cell lines were kept under normoxic or hypoxic conditions for 24 h in serumfree medium. Thereafter, cells were harvested and extracted with perchloric acid. Intracellular

adenine nucleotides were analyzed by HPLC as described in Materials and methods. Data are mean ± SEM for n determinations. *p<0.05 versus control of the corresponding cell line.

HEP-1 cells ($r = 0.9$, $p < 0.01$; Fig. 4A). At cell densities of $3 \cdot 10^5$ cells/cm² hypoxia increases AdoMet level from 2.35 nmol/10⁷ cells (control) to 7.8 nmol/10⁷ cells. However, AdoHcy and Ado concentrations are not significantly changed under hypoxia and the concentration of both metabolites is not dependent on cell density (Fig. 4B, 4C). Since the increased AdoMet concentrations are accompanied by unchanged AdoHcy levels under hypoxia, the AdoMet/AdoHcy ratio is increased from 21.1 (normoxia) to 74.2 (hypoxia) at high cell densities. As demonstrated in Fig. 4D, a strong positive correlation between the MP and cell density was found ($r = 0.97$, $p < 0.001$).

Energy metabolism under normoxia and hypoxia

To determine if changes in energy metabolism play an important role in the hypoxic regulation of AdoMet/AdoHcy metabolism, we next investigated the influence of hypoxia on AMP, ADP and ATP concentrations, because transmethylation and energy metabolism are linked by AdoHcyase. Under control conditions, AMP, ADP and ATP concentrations differ between the cell lines investigated (Table 3). Since HEK-293 cells exhibit 3-5 times higher AMP levels the EC is much lower in these cells (0.54) compared to all other cell lines examined (0.75-0.81).

Fig. 5. Cell viability in different cell lines under normoxic and hypoxic conditions. The cell lines were incubated for 24 h under normoxia or hypoxia in serumfree medium. Then, cells were harvested and diluted in CASY[®]ton. Viability was quantified by CASY[®] cell counter + analyzer system as described in Materials and methods. Data represent averages ± SEM for 3-12 experiments.

Under hypoxia, changes in adenine nucleotide concentrations can be observed in HepG2, HEK-293 and SK-HEP-1 cells. A significant decrease in AMP levels was measured in HepG2 and SK-HEP-1 cells independent of cell density. In contrast, the cellular AMP concentration was lowered in HEK-293 cells only when cells were grown at high cell densities ($>2 \times 10^5$ cells/cm²). Furthermore, HEK-293 and HepG2 cells exhibit a strong negative relationship between cell density and ATP or ADP levels (data not shown). Significant reduced ATP levels were also observed in SK-HEP-1 cells under hypoxia, when cells were grown at low cell densities. Interestingly, under hypoxia, there were no significant changes in intracellular ADP and ATP levels in MCF-7 and HeLa cells. Thus, the EC is decreased in HeLa and HEK-293 cells under hypoxia, whereas it is elevated in all other cell lines by hypoxia.

Viability of the cells under normoxia and hypoxia

Under normoxic conditions all cell lines investigated showed a viability of about 90% when cells were cultured in serumfree medium for 24 h. The viability slightly changed when HepG2 and HEK-293 cells were incubated under hypoxia for 24 h. However, the viability of all other cell lines did not change significantly under hypoxia compared to normoxia (Fig. 5).

Enzymatic activity of AdoHcyase under normoxia and hypoxia

Under control conditions HepG2 cells showed a AdoHcyase activity of 2.5 ± 0.2 nmol min⁻¹mg⁻¹ protein. All other cell lines showed a 3-5 times lower enzyme activity (Table 4). Photometrical determination revealed a AdoHcyase activity of 0.9 ± 0.06 (HEK-293), 0.6 ± 0.04 (MCF-7), 0.7 ± 0.07 (HeLa) and 0.6 ± 0.05 nmol min⁻¹mg⁻¹ protein (SK-HEP-1), respectively.

Since hypoxia changed MP, AdoHcy and Ado concentration in three cell lines, the influence of hypoxia on the activity of the AdoHcy metabolizing enzyme was also studied. Our data show, that hypoxia lowers AdoHcyase activity about 40% compared to normoxic control in HepG2 cells, whereas enzyme activity is independent of O₂ concentration in all other cell lines.

Western blot analysis of AdoHcyase expression

The protein expression of AdoHcyase was only studied in HepG2 cells, because under hypoxia a reduced enzyme activity was only observed in this cell line. Western blot analysis of total cellular extracts with the

Fig. 6. Western blot analysis of AdoHcyase expression in HepG2 cells. Cells were grown under normoxia and then exposed to normoxia (1) and hypoxia (2) for 24 h in serumfree RPMI 1640. Total cellular protein was separated by SDS-PAGE and electroblotted onto nitrocellulose membrane. AdoHcyase was detected immunologically by a specific antibody.

anti-bovine AdoHcyase antibody [35] revealed a single band with a molecular weight of 42 kDa (Fig. 6). Exposure to hypoxia for 24 h clearly reduced the AdoHcyase expression. Since the reported molecular mass of the subunit of human placenta is 47 kDa [37], AdoHcyase was purified from HepG2 cells and subjected to SDS-PAGE. Immunoblot analysis again showed one band at 42 kDa (data not shown), suggesting that the molecular weight of HepG2 AdoHcyase is lower than that of human placenta.

Discussion

The ratio of AdoMet/AdoHcy concentrations, also termed as methylation potential (MP), is referred to as the methylation index within the cell, since elevated levels of AdoHcy inhibit most AdoMet-dependent methyltransferases [22]. In this study, we analyzed whether various cell lines differing in proliferation activity and tissue origin have different MP and energy metabolism. Moreover, we studied the impact of hypoxia on AdoMet/AdoHcy and energy metabolism. Since AdoHcyase controls the intracellular AdoHcy level the activity of AdoHcyase was also determined.

The AdoHcy levels varied significantly between the cell lines investigated, whereas the AdoMet concentrations

AdoHcyase activity nmol/min*mg protein	HepG2 (n = 15 / 8)	HEK-293 (n = 10 / 7)	MCF-7 (n = 5 / 5)	HeLa (n = 3 / 3)	SK-HEP-1 (n = 4 / 3)
normoxia	2.53 ± 0.21	0.86 ± 0.06	0.59 ± 0.04	0.66 ± 0.07	0.56 ± 0.05
hypoxia	1.56 ± 0.45*	0.78 ± 0.04	0.47 ± 0.1	0.51 ± 0.03	0.41 ± 0.03

Table 4. Enzymatic activity of AdoHcyase in different cell lines under normoxia and hypoxia. Cells were exposed to normoxia or hypoxia for 24 h. The AdoHcyase activity was measured photometrically in total cellular lysates as described in Materials and methods. Data are mean ± SEM for n determinations. *p<0.05 versus normoxic control of the corresponding cell line.

varied only moderately. Thus, the values for the AdoMet/AdoHcy ratio (Table 1) showed marked differences under normoxic conditions. This means that, dependent on the cell line, AdoMet/AdoHcy ratios between 5 and 80 are consistent with the physiological cell function [5-8, 38]. Previous studies suggest that a decreased AdoMet/AdoHcy ratio is necessarily associated with impaired methylation activity. In contrast, our observations suggest that cellular functions were not impaired even when the cell line showed an AdoMet/AdoHcy ratio of 5. Lower values could not be observed under physiological conditions in any cell line investigated. However, inhibition of AdoHcyase leads to accumulation of AdoHcy, resulting in an AdoMet/AdoHcy ratio of 1.4 and decreased mRNA methylation and gene expression in HepG2 cells [3]. Interestingly, an effect of AdoHcyase inhibition on mRNA methylation and gene expression was only observed when AdoMet/AdoHcy ratio was decreased to values <2 [3]. Recent studies report of a human with AdoHcyase deficiency resulting in 150-fold elevated AdoHcy plasma levels and an AdoMet/AdoHcy ratio of 0.6. This leads to biochemical and physiological abnormalities but is consistent with survival [39].

To examine the effects of proliferation activity and tissue origin on AdoMet and AdoHcy levels we chose cell lines from different tissues with similar doubling times. HepG2, MCF-7 and HeLa cells have a cell cycle of 50-60 hours, while SK-HEP-1 and HEK-293 cells double all 25-30 hours. Our results show that the AdoMet and AdoHcy levels were independent of doubling time. The marked variations of AdoHcy levels might be tissue specific and depend on AdoHcyase activity.

The intracellular AdoHcy pool reflects the cumulative balance among the activities of the multiple methyltransferases and the rate of hydrolysis and/or synthesis by AdoHcyase. The specific activity of this enzyme was 2-4 times higher in HepG2 cells than in all other cell lines investigated (Table 4). This coincides with

the low AdoHcy concentrations in HepG2 cells. Under normal physiological conditions, it appears that AdoHcyase is responsible for controlling the AdoHcy levels as suggested by previous studies [4, 40]. Since it is unlikely that AdoHcy is transported across the plasmamembrane [41, 42], differences in AdoHcy transport among the different cell types can be ruled out. Moreover, the rate of removal of Ado and homocysteine controls intracellular AdoHcy levels [43]. Determination of Ado deaminase activity revealed a high enzymatic activity, for instance in the HepG2 cell line (data not shown). This could be one explanation for the low Ado level in the different cell lines, except MCF-7 cells.

AdoMet is not only the major donor of methyl groups for most transmethylation reactions, but serves also as the source of propylamine moieties for polyamine biosynthesis and is essential to maintain the cellular structure and function [44, 45]. Under normoxic conditions the AdoMet concentration was approximately the same in the cell lines investigated, except HEK-293 cell line, suggesting that in these cell lines AdoMet synthesis and the rates of the various methyltransferase reactions are similar and independent of proliferation activity. Furthermore, these cell lines, except HEK-293 cells, exhibit similar energy charges. The energy charges of 0.75-0.81 are within the physiological range obtained by in vivo experiments in different human and rat tissues [8, 46]. In contrast, HEK-293 only exhibited an energy charge of 0.54. However, previous studies have shown, that the energy charge can be reduced to much lower values without apparent impairment of normal cellular function [47].

In order to induce hypoxia in the cell lines, O₂ concentration was lowered to 1%. Limitation in availability of oxygen is a stress important in both biomedical and environmental contexts and necessitates rapid adaptive changes in metabolic organization, for instance in activation of anaerobic ATP-generating pathways like

glycolysis [48]. Our results show that these cell lines respond with a heterogeneous MP to hypoxia. While MCF-7 and HeLa cells appear to be insensitive to hypoxia in term of the MP, hypoxia alters the MP in HepG2, HEK-293 and SK-HEP-1 cells. Interestingly, these alterations are dependent on the cell density. During hypoxia the concentration of AdoHcy decreases as the cell density increased. This phenomenon could be explained by the observation that the pericellular pO_2 is much lower than the pO_2 in the incubation gas in confluent monolayers [49]. Thus, it is likely that the hypoxic stimulus is more severe in cells cultured at high cell densities compared to those cultured at low cell densities.

In HepG2 and HEK-293 cells the increased MP under hypoxia is the result of decreased AdoHcy levels. An enhanced AdoHcy hydrolysis can be ruled out because the activity of AdoHcyase is only slightly affected or even decreased by hypoxia (HepG2). Therefore, the decreased AdoHcy levels are probably due to a lowered transmethylation activity. In this study we show for the first time a regulation of AdoHcyase enzyme activity by hypoxia. Interestingly, we have shown previously that hypoxia decreases AdoHcyase mRNA expression in HepG2 cells, suggesting that reduced protein expression and enzyme activity are at least in part caused by decreased mRNA levels [3].

The increased AdoMet/AdoHcy ratio in SK-HEP-1 cells seems to be the result of enhanced AdoMet levels. In MCF-7 and HeLa cells hypoxia reduces the AdoMet levels, whereas all other cell lines showed increased AdoMet concentrations under hypoxia which are not or only slightly (SK-HEP-1 cells) influenced by cell density. Reduced AdoMet concentrations accompanied by a decrease in the AdoMet/AdoHcy ratio were found in rat liver and primary rat hepatocytes exposed to hypoxia [50-52]. This effect was caused by a reduction in MAT mRNA expression and activity during hypoxia [50] and could be one explanation for our observation made in MCF-7 and HeLa cells. Since hypoxia did not significantly influence ATP levels, a lowered availability of the substrates ATP and methionine was probably not responsible for the reduced AdoMet concentration in these cell types. The explanation mentioned above could not be applied to the three other cell lines. It is unlikely that the increased AdoMet levels observed in HepG2, HEK-293 and SK-HEP-1 under hypoxia are due to increased AdoMet synthesis, because the ATP levels are significantly lowered under hypoxia. This may indicate that the activity of methyltransferases utilizing AdoMet

is reduced. This suggestion is supported by the decreased AdoHcy level. Therefore, we suggest that a higher MP is not necessarily accompanied by enhanced transmethylation activities.

Previous studies have shown that hypoxia or ischemia lowers the energy charge and the recovery of normal values of the energy charge is achieved by degrading the AMP formed during periods of high ATP hydrolysis [36]. Adenine nucleotide degradation accompanied by a transient accumulation of Ado has been observed in ischemic rat tissue [8]. In cultured cells the rapid decrease of ATP levels following hypoxia is well known, although varying degrees of subsequent degradation of the nucleotides have been reported [53, 54]. In the present experiments, ATP degradation during hypoxia appears to be dependent on cell density in HepG2 and HEK-293 cells. Thus, cell density must be an important factor in biotechnological maintenance of cell culture. Furthermore, ADP and AMP levels are not enhanced or even reduced during ATP hydrolysis. As a consequence, the sum of the adenine nucleotides differs greatly between various experimental conditions. One possible explanation could be a limited availability of Ado, because under hypoxia the Ado concentration is decreased when cells are grown at high cell densities. The degradation of AMP and ADP leads to maintain the equilibrium among the three adenine nucleotides since the energy charge values remained similar or higher to those observed under normoxia. Thus, the energy charge is not dependent on the cell density. These observations showed that hypoxic cells adapt with reduced functional capacity that allows preservation of ATP mainly done by reducing activities of ATP using enzymes [55].

Determination of cell viability showed that hypoxia did not change (MCF-7, HeLa, SK-HEP-1) or only slightly changed (HepG2 and HEK-293) viability. Therefore, the elevated AdoMet/AdoHcy-ratio and decreased adenine nucleotide concentrations under hypoxic conditions are not caused by increased cell death.

Interestingly, under hypoxia a negative correlation was found between ADP/ATP concentrations and cell density in HepG2 and HEK-293 cells. This discovery, together with the fact that under hypoxia Ado and AdoHcy levels also correlate with cell density suggests that alterations in energy metabolism and AdoMet/AdoHcy metabolism may affect each other.

In summary, under normoxic conditions cell lines exhibit marked differences in the MP which are consistent with the physiological cell function. The alterations in the

MP of HepG2, HEK-293 and SK-HEP-1 cells during hypoxia are dependent on the density of cells in culture. However, hypoxia only slightly altered the energy charge independent of the cell density. Taken together, our results suggest that the MP is only a general indicator for a given cell type and not a particular indicator for the transmethylation activities of cells.

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