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Regulation of Methionine Adenosyltransferase Catalytic Activity and Messenger RNA in SH-SY5Y Human Neuroblastoma Cells

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ABSTRACT. The human neuroblastoma cell line SH-SY5Y was used to study the regulation of methionine adenosyltransferase (MAT II; E.C.2.5.1.6.) catalytic activity and transcript levels in cells of neuronal origin. The cells were exposed for 24 hr to a medium containing different concentrations of methionine (MAT substrate) as well as medium deficient of methionine. Furthermore, cells were treated with hydroxycobalamin, SAM, and the competitive MAT inhibitor cycloleucine. The MAT catalytic activity was inversely correlated to methionine concentrations, e.g. MAT V_{max} increased 2-fold in cells grown in methionine-deficient medium as compared with cells cultured under standard conditions. Interestingly, MAT K_m also increased from 9.04 ± 0.44 to 12.08 ± 0.83 in the methionine-deficient medium. Hydroxycobalamin caused an increase in activity at 40 μM while a decrease was observed at higher concentrations (100, 200, and 400 μM). Cycloleucine caused a significant inhibition of MAT catalytic activity, i.e. the inhibition was approximately 50% in the presence of 4 mM cycloleucine. The relevance of these results for the understanding of observations on MAT catalytic activity in brains of patients with Alzheimer's disease is discussed. BIOCHEM PHARMACOL 55;5:567–571, 1998. © 1998 Elsevier Science Inc.

KEY WORDS: SH-SY5Y neuroblastoma cells; methionine adenosyltransferase; S-adenosylmethionine; cycloleucine; vitamin B12; Alzheimer's disease

Methyltransferases are expressed in all tissues and these enzymes catalyze a wide variety of methylation reactions, including the methylation of nucleic acids, phospholipids, aminoacid side-chains of proteins and catecholamines among others. Most methyltransferases utilize S-adenosylmethionine (SAM)† as a methyl donor. The formation and availability of this substrate, therefore, constitutes a major determinant of the efficiency of multiple methylation pathways. SAM is formed from L-methionine and ATP by a magnesium-dependent enzyme, methionine adenosyltransferase (ATP:L-methionine S-adenosyltransferase, EC 2.5.1.6, MAT), which also displays tripolyphosphatase activity. MAT was first described by Cantoni and collaborators [1] and since then has been detected in both prokaryotic and eukaryotic cells. The structure of MAT shows a high degree of homology among the animal species. In mammals, three major enzyme isoforms have been characterized, MAT I, MAT II, and MAT III, respectively, which differ with respect to their tissue distribution and kinetic properties [2]. One of these isoforms, MAT II (the low-K_m MAT), which has a relatively high affinity for methionine (K_m 2–20 μM) [3], is the predominant isoform present in human brain and in most tumors, and this isoform is thought to be encoded by a gene distinct from the one encoding MAT I and MAT III [4]. Abnormally low erythrocyte and brain MAT activities in cases of schizophrenia and bipolar disorders have been observed [5–7]. With regard to central transmethylation mechanisms in connection with neurodegenerative diseases, low levels of vitamin B12 (cobalamin, B12) and SAM were found in cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD). Recently, we have also reported data on alterations of MAT activity in B12-deficient patients with Alzheimer's disease and senile dementia of the Alzheimer's type. An abnormally high affinity of MAT towards methionine was found in erythrocytes of the former, while abnormally high MAT V_{max} was found in the brain of the latter group [8]. The biochemical mechanism underlying the changes in MAT catalytic properties remains to be elucidated. Since methionine synthetase (EC 2.1.1.13), an enzyme catalyzing the methylation of homocysteine to methionine, requires methyl-B12 as a cofactor, we have speculated that the alterations in MAT activities in these B12-deficient patients with Alzheimer's disease could represent a form of adaptation mechanism, as a result of a low supply of methionine, in order to maintain the levels of SAM.

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† *Abbreviations:* ATP, adenosine triphosphate; AD, Alzheimer's disease; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; GAPDH, glyceraldehyde phosphate dehydrogenase; MAT, methionine adenosyltransferase; SAM, S-adenosyl methionine.

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Neuroblastoma cells have become an important model for studies related to neuronal function [9, 10]. The SH-SY5Y cell line represents an adrenergic clone of neural crest-derived human tumor cells that may be cultured using a simple monolayer technique. This cell line has been shown to exhibit several characteristics of neurons in the mature nervous system. For a review, see Biedler and coworkers [11] and Påhlman and coworkers [12].

As a first approach to investigating the basis of the defects in the central transmethylation mechanisms of B12-deficient patients with Alzheimer's disease, in the present study we have used the SH-SY5Y cell line to analyze some of the factors likely to influence the catalytic properties of MAT. Hence, the cells were exposed to substances with particular relevance for eukaryotic methionine turnover such as cobalamin, methionine, SAM, as well as the MAT inhibitor cycloleucine (1-aminocyclopentane-1-carboxylic acid).

MATERIALS AND METHODS

SAM-*p*-toluenesulfonate, L-methionine, hydroxycobalamin, cycloleucine, ATP (equine muscle disodium salt), and bovine serum albumin were obtained from Sigma, [methyl-^{3}H] methionine (191 mCi/mmol) from NEN Research Products, ion exchange resin (BioRad AG 50W-X8) from BioRad Laboratories and Ready-Safe™ liquid scintillation fluid from Beckman. All other chemicals used were of analytical grade.

The SH-SY5Y cells, which were kindly provided by Dr. Sven Påhlman, were cultured in Dulbecco's Modified Eagle's Medium (DMEM containing 200 μM methionine, basal medium) containing 1 g/L glucose and 10% fetal calf serum (FCS, Life Technologies). The cells were seeded at a density of 5×10^5 cells per 100 mm dish. At mid-exponential growth phase, the medium was replaced with a medium containing one of the tested substances, or DMEM lacking methionine but containing 1 g/L glucose and 10% FCS. The exposure of the cells to the methionine-deficient medium was preceded by twice rinsing the culture dishes with 5.0 mL of the referred medium. After 24 hr, the culture dishes were rinsed twice with 5.0 mL 0.05 M phosphate buffer (PBS), pH 7.5, and then dissociated from the plates by 2.0 mL of 0.05 M PBS, pH 7.5. In all experiments, cell contents from two dishes were used for MAT analyses. The cell suspension was centrifuged at $200 \times g$ for 10 min at room temperature. Thereafter, all procedures were performed at 4°. The cell pellet was resuspended in 10.0 mM TRIS/1.0 mM dithiotreithol (DTT)/0.5 mM EDTA/3.0 mM $MgCl_2$, pH 8.0, and lyzed by sonication (Branson sonifier cell disrupter model B15, output 6, duty cycle 30, three cycles of 10 sec each, with 50 sec intervals between the cycles). The lysate was centrifuged at $12{,}900 \times g$ for 10 min, and the supernatant was stored at −80° until analysis.

MAT activity was assayed by measuring the rate of formation of ^{3}H-SAM from ATP and L-[methyl-^{3}H]methionine [13]. Briefly, the reaction mixture in a total volume of 0.1 mL (pH 7.7) contained 20 μL of cell extracts (20 μg of protein) and final concentrations of: 30 mM $MgCl_2$, 26 mM KCl, 35 mM HEPES, 10 mM ATP, L-[methyl-^{3}H] methionine (16,000 cpm/nmol), and unlabeled methionine in the range of 2–80 μM. The MAT activities in extracts of cells treated with different drugs were routinely analyzed using a methionine concentration of 18 μM (2-fold MAT K_m). The blanks were prepared with water instead of ATP. After incubation at 37° for 60 min, the reaction was interrupted by the addition of 1.0 mL ice-cold 1.6 mM citric acid in 50% ethanol. After adsorption to an ion exchange resin (Dowex 50 W-X8 resin in the NH^{4+} form), ^{3}H-SAM was eluted with 2.0 mL of 3.0 M NH_4OH. Radioactivity in the effluent was counted with 10 mL scintillation fluid in a Packard counter. MAT activity was assayed in duplicate for each sample, and the apparent values of V_{max} (pmol SAM/mg protein/min) and the Michelis–Menten constant K_m (μM methionine) were determined by a Eadie–Hofstee plot. The protein concentrations were estimated by a modification of the Lowry procedure [14], using bovine serum albumin as a standard.

Total RNA was prepared from cells treated with the different drugs using the RNeasy preparation kit (Qiagen). Five μg of total RNA was separated on a 1.5% agarose gel containing formaldehyde [15] and transferred to a Hybond-N nylon membrane (Amersham Life Sciences). The membrane was hybridized with a random-prime-labeled cDNA probe corresponding to a 422 bp fragment of the coding region of the human MAT II (basepairs 118–540). As a loading control, the membrane was rehybridized with a 220 bp probe corresponding to glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. Densitometric analysis of the exposed films was performed with a Panasonic RM665 video camera and a quantitative image analysis processing software, Optilab/Pro 2.5 (Graftek).

All values are given as means ± SEM. Analyses of differences in the means of more than two populations and the influence of independent variables on the experimental results were evaluated by factorial analyses of variance (ANOVA). The associations between two parameters were evaluated by Student's *t*-test for unpaired samples (*t*-test), and Fisher's PLSD test.

RESULTS

MAT Activities in Cells Exposed to Different Methionine Concentrations

The kinetic curves of MAT activity in cells cultured under standard conditions (mean values of 5 experiments) and in methionine-deficient medium (mean values of 3 experiments) are shown in Figs. 1a and 1b. In extracts of SH-SY5Y cells grown in basal medium containing 200 μM methionine, the values of MAT V_{max} and MAT K_m estimated by the Eadie–Hofstee method were 113.00 ± 8.42 pmol/mg protein/min and 9.04 ± 0.44 μM methionine, respectively (Figs. 1c and 1d). The cells were also

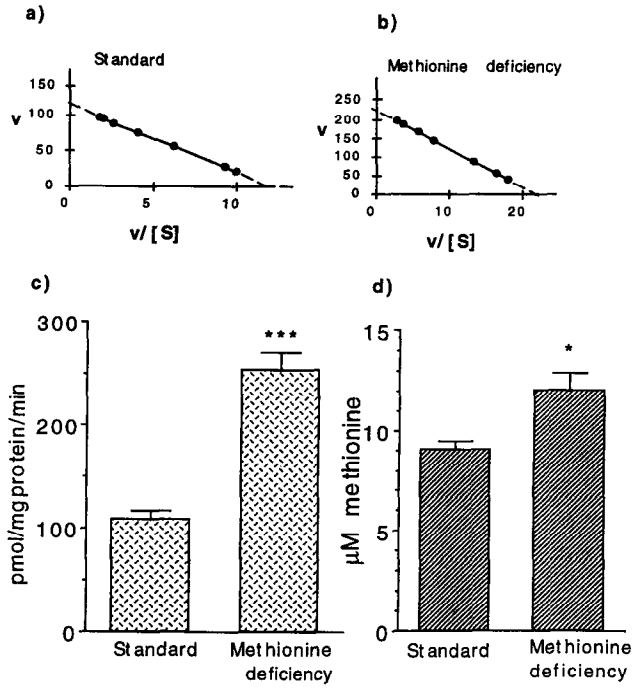

FIG. 1. (a–b) Kinetic curves (Eadie–Hofstee plot) of MAT activity in SHSY-5Y cells cultured under standard conditions (Fig. 1a, $N = 5$) and in cells exposed for 24 hr to a methionine-deficient medium (Fig. 1b, $N = 3$). The estimated mean values of MAT V_{max} and MAT K_m (mean ± SEM) towards methionine are shown in Figs. 1c and 1d, respectively. ***$P < 0.0001$ and $P < 0.05$ in comparison with the control group, Student's unpaired *t*-test.

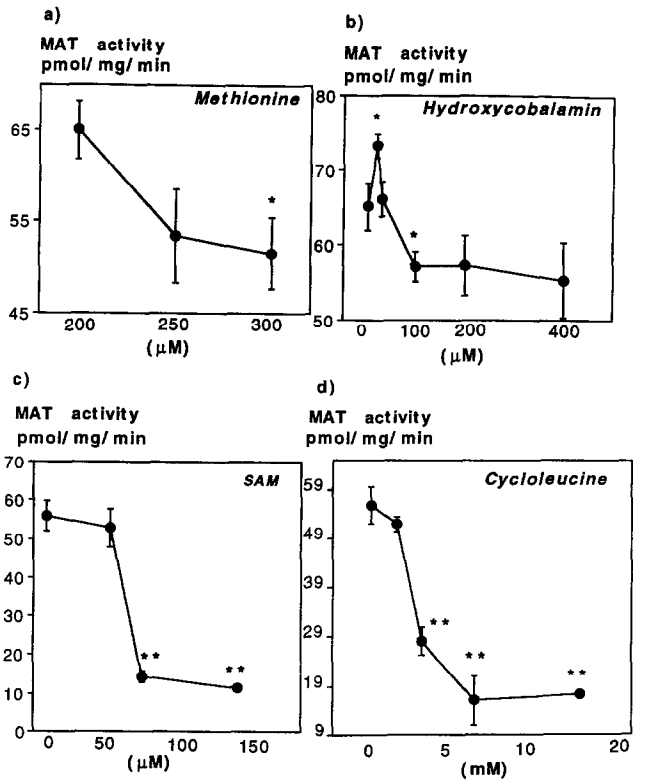

FIG. 2. (a–d) Effects on MAT catalytic activities in SH-SY5Y cells treated for 24 hr with different concentrations of (a) methionine, (b) hydroxycobalamin, (c) SAM, and (d) cycloleucine. The data points represent mean values ± SEM. Statistical significance was calculated by factorial ANOVA, followed by Fisher's PLSD test. *$P < 0.05$ and **$P < 0.01$.

exposed for 24 hr to three higher concentrations of L-methionine; 250, 300, and 400 μM, respectively. Compared to cells cultured under standard conditions, a tendency to a decrease in MAT catalytic activity was observed at these concentrations of methionine. In the presence of 300 μM methionine, the MAT activity decreased by approximately 20% as compared with controls ($P < 0.05$, Fig. 2a). On the other hand, when the cells were exposed for 24 hr to a methionine-deficient medium, the V_{max} value was more than 2-fold that of cells cultured under standard conditions, i.e. 255.13 ± 18.83 pmol/mg protein/min (Figs. 1a–1d). In addition, a significant increase in the MAT K_m towards methionine (12.08 ± 0.83 μM) was observed in the cells exposed to the methionine-deficient medium (Figs. 1c and 1d).

Effects of Hydroxycobalamin, S-adenosylmethionine, and Cycloleucine, on MAT Activity

In another set of experiments, the neuroblastoma cells were exposed for 24 hr either to the vitamin B12 analogue hydroxycobalamin (40, 100, 200, 400 μM), SAM (50, 75, 150 μM), or the MAT inhibitor cycloleucine (2, 4, 8, 16 mM). Hydroxycobalamin caused a biphasic alteration of MAT catalytic activity at the concentrations investigated ($F < 0.01$, ANOVA factorial). Treatment with 40 μM hydroxycobalamin resulted in increased MAT activity as compared with control cells (Fig. 2b). In contrast, at 100, 200 and 400 μM of hydroxycobalamin, a decrease in the activity of MAT was observed. This effect was statistically significant at 100 μM. SAM, on the other hand, induced a dose-related decrease in MAT activity (Fig. 2c) at the three concentrations applied. At these concentrations, 50, 75 and 150 μM, respectively, SAM induced a pronounced decrease in MAT activity ($F < 0.005$, ANOVA factorial) which was statistically significant at the two higher concentrations ($P < 0.005$, Fisher's test). Cycloleucine, which is a competitive inhibitor of MAT, induced a dose-related inhibition of the catalytic activity (Fig. 2d). A substantial decrease in MAT activity was observed following treatment of the cells with cycloleucine at concentrations of 2, 4, 8, and 16 mM in a dose-dependent fashion ($F < 0.05$, ANOVA factorial). It was not possible to calculate an accurate IC_{50} for cycloleucine, since a substantial number of the cells died at treatments with the two higher concentrations (8 and 16 mM, respectively).

Northern Blotting

A strong band was observed in several independent blots that corresponded to approximately 4 (±0.2) kb, which is

FIG. 3. Northern blot analysis using a probe for MAT II. Five mg of total RNA from cells cultured under (a) standard conditions and cells treated with (b) methionine-deficient medium, (c) a high concentration of methionine (400 μM), (d) SAM (75 μM), and (e) hydroxycobalamin (400 μM), was loaded into each lane. The visualized band has a size of approximately 4 kb. Hybridization with a probe for glyceraldehyde dehydrogenase (GAPDH) was used as a loading control.

in good agreement with the findings of Horikawa and Tsukada [16] (Fig. 3). They found two bands in human kidney, 3.4 and 3.8 kb, respectively. However, in the SH-SY5Y cell line the shorter transcript was not detected. The mRNA levels seemed to be well correlated with the catalytic activity. A 5-fold upregulation of the transcript was observed after treatment with methionine-deficient medium, whereas a high methionine (400 μM) concentration caused a decrease in the MAT II mRNA. Treatment with 75 μM SAM caused a decrease in mRNA as compared with control cells, whereas treatment with 400 μM hydroxycobalamin did not seem to affect the transcript levels.

DISCUSSION

The affinity of MAT II towards methionine in the cultured SH-SY5Y cells was similar to that reported previously on cultured human cells [3]. Furthermore, the K_m value (9.04 μM) was very similar to that reported by our group for human brain tissue ($K_m \approx 11.0$ for human parietal cortex, [8]). The highly significant increase in MAT V_{max} observed in extracts of SH-SY5Y exposed to a medium deficient in methionine (Fig. 1) suggests that methionine or its metabolites are involved in the regulation of MAT. A direct interaction of such compounds with the MAT enzyme protein seems unlikely in this case, since the enzyme activity assay is performed in a standardized solution where the test compounds have been washed away and the cell content has been highly diluted in the extract. However, it might be speculated that the regulating mechanism could involve: 1) an alteration in enzyme protein biosynthesis at the transcriptional or translational level; 2) activation of a preexisting enzyme by posttranslational modification, e.g. phosphorylation; or 3) a decreased degradation of MAT. Caboche [17] and Jacobsen and coworkers [18] have reported findings similar to ours with respect to the influence of methionine on MAT II catalytic activity. These authors showed that transformed human fibroblasts cultured in methionine-deficient medium had an elevated MAT activity. Moreover, they demonstrated that increased MAT activity was prevented by treatment with protein synthesis inhibitors. Hence, changes in MAT V_{max} may be a consequence of regulation of MAT biosynthesis. This seems likely, since changes in MAT V_{max} caused by treatment of the cells with low or high methionine concentrations were parallel with the changes in MAT mRNA levels.

The alterations in K_m values towards methionine that were observed after treatment of the cells with a methionine-deficient medium cannot be entirely explained without additional molecular and biochemical experiments, e.g. purification and characterization of MAT isoform. The presence of two distinct MAT enzymes with only slight differences in K_m towards methionine cannot be completely excluded, e.g. a form with higher K_m may in fact be induced by exposure to medium low in methionine.

With regard to SAM, it has been shown in several investigations to be a powerful regulator of MAT II. Feedback inhibition of MAT by its product SAM has been described, for example for human erythrocytes and oncogenically transformed human and rat cells [3, 16–18]. This was also confirmed in the present study: SAM caused a substantial reduction of both MAT II catalytic activity and mRNA. It is therefore attractive to speculate that the effect caused by different concentrations of methionine on MAT catalytic activity could reflect a feedback regulation of MAT by its product SAM.

A biphasic effect on MAT catalytic activity was observed by treating the cells with the vitamin-B12 analogue hydroxycobalamin, which increased MAT V_{max} at the lower dose while causing a decrease at the higher doses. Similar results have been reported by other investigators, e.g. effects on MAT in cultured baby hamster kidney cells after exposure to vitamin-B12 [19, 20]. However, at the mRNA level, we did not observe any alterations after hydroxycobalamine treatment (Fig. 3). Our group previously reported that MAT V_{max} was abnormally high in brains from patients with AD and senile dementia of Alzheimer's type [8]. In the light of the present *in vitro* study, it is interesting to hypothesize that these individuals, as a consequence of their B12 deficiency, had a lowered methionine synthesis rate, and therefore a decreased formation of SAM, which in turn would explain their changes in MAT activity. On the other hand, it is possible that AD patients have other metabolic disturbances, causing alterations in MAT activity, e.g. increased turnover of SAM.

Concerning cycloleucine, Lombardini and Talalay [21] were the first to report on the inhibitory effect of this compound on MAT activity. This competitive inhibitor of MAT caused an inhibition of approximately 50% of MAT activity at the dose of 8 mM, which is in agreement with published data of the inhibitory potency of this methionine analogue on MAT catalytic activity [22]. It is noteworthy that cycloleucine concentrations above 8 mM resulted in morphological changes of the cells, dissociation from the culture dishes, and cell death. These effects may either indicate a crucial function of MAT or reflect nonspecific actions of this drug. It has been suggested that the neurotoxic effect of cycloleucine *in vivo* is due to a degeneration of myelin, presumably by dysregulating the mechanism of utilization of SAM on membrane phosphatidylcholine biosynthesis [23].

In conclusion, in this study human neuroblastoma cells

have been used to study regulation of MAT catalytic activity and mRNA in a CNS-derived cell line. Manipulations of the levels of MAT substrate (methionine) and its product (SAM), as well as the cofactor in enzymatic methionine biosynthesis, vitamin B_{12} resulted in profound and parallel changes in SH-SY5Y MAT catalytic activity and mRNA. The decrease in MAT catalytic activity in cells exposed to SAM, and to high concentrations of methionine, may be explained by a SAM-induced feedback inhibitory effect on the transcription of MAT.

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