Effect of Fasting on Methionine Adenosyltransferase Expression and the Methionine Cycle in the Mouse Liver

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Summary The effect of fasting on mouse liver methionine adenosyltransferase (MAT I/III) expression and the regulation of methionine metabolism were investigated. The mRNA level, protein level, and activity of MAT I/III were increased by fasting for 10 or 16 h. In spite of the increase of MAT I/III activity, S-adenosylmethionine, the product of methionine due to MAT I/III, decreased. S-Adenosylhomocysteine, which is made from S-adenosylmethionine by its coupling to methyltransferase, increased as a result of fasting for 16 h. These results suggest that the total methylation reactions using S-adenosylmethionine are stimulated in the fasting mouse liver. However, the DNA methylation level was not changed by fasting for 16 h. Glutathione, which is made by the transsulfuration pathway from homocysteine, decreased due to fasting. Regulation of supplementation of S-adenosylmethionine may occur in the fasting mouse because MAT I/III activity increases and the flow to glutathione is decreased.

Key Words methionine adenosyltransferase, adenosylmethionine, fasting, methylation, glutathione

Methionine adenosyltransferase (MAT, EC 2.5.1.6) catalyzes the biosynthesis of S-adenosylmethionine (AdoMet) from methionine and ATP. Three forms of MAT (I, II, and III) have been identified in mammalian tissues. Liver-type MAT I and MAT III are the tetramer and dimer, respectively, of identical $\alpha 1$ subunits from the *MAT1A* gene (1, 2). Nonliver-type MAT II is present in the fetal liver, kidney, brain, testis, and lymphocytes (3, 4) and is a heteromer of the $\alpha 2$ and β subunits from the *MAT2A* and *MAT2B* genes, respectively (1, 5, 6). Hypermethionemic individuals with null *MAT1A* mutations have developed neurological problems, including brain demyelination (1). In *MAT1A* knockout mice, the absence of *MAT1A* in the liver leaves them more susceptible to injury; markers of an acute phase response are expressed, and increased proliferation is evident (7). At 3 mo, knockout mice are more susceptible to choline-deficient diet-induced fatty liver (7). For MAT II, a switch in gene expression from *MAT1A* to *MAT2A* in various human-derived liver cancer cell lines has been reported (8). Mato et al. report that as much as 48% of methionine metabolism occurs in the liver, and AdoMet is not only an intermediate metabolite in methionine catabolism but also an intracellular control switch that regulates essential hepatic functions such as regeneration, differentiation, and injury (9).

AdoMet contributes to transmethylation (10, 11),

transsulfuration to glutathione (GSH) (12), and, following decarboxylation, is a propylamine group donor in the biosynthesis of polyamines (13) (Fig. 1). AdoMet is an essential methyl donor in transmethylation, and S-adenosylhomocysteine (AdoHcy) is known as a potent inhibitor of many methyltransferases, including DNA methyltransferase (14). Increased plasma and lymphocyte AdoHcy levels are associated with increased DNA hypomethylation (15). The ratio of AdoMet to AdoHcy indicates the methylation potential.

Dietary factors such as folate, vitamin B₁₂, and choline change the DNA methylation of genes and lead to chromosomal instability (15, 16). Changes of MAT activity due to diet may affect the methylation reaction via the amounts of AdoMet or AdoHcy and may influence the risk of cancer and other diseases. Administration of a high-methionine diet increases MAT III activity in rat liver 4- to 5-fold (17). The activity of hepatic MAT I/III is decreased by vitamin B₁₂ deficiency in rats (18). Ethanol feeding or folate deficiency decreases the transcription level of *MAT1A* in micropigs (19). Fasting for 96 h in sheep results in a trend toward increasing MAT-specific activity (20). But the role of MAT changes to methionine metabolism, and the mechanism by which MAT is changed by dietary manipulation is not clear. In this study, we investigated the effect of fasting on MAT expression, regulation of the methionine cycle, and DNA methylation in the mouse liver.

MATERIALS AND METHODS

Reagents. All reagents used were of analytical grade and were purchased from Nacalai Tesque Ltd. (Kyoto,

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Abbreviations: MAT, methionine adenosyltransferase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; Hcy, homocysteine; GSH, glutathione.

Fig. 1. Methionine metabolic pathway. Enzymes are shown by number in the pathway. 1, Methionine adenosyltransferase EC 2.5.1.6: MATI/III. 2, Methyltransferase EC 2.1.1-containing glycine *N*-methyltransferase EC 2.1.1.20: GNMT, liver, kidney, pancreas, phosphatidylethanolamine *N*-methyltransferase EC 2.1.1.17: PEMT2, and DNA (cytosine-5)-methyltransferase EC 2.1.1.37: Dnmt. 3, *S*-Adenosylhomocysteinase EC 3.3.1.1: SAHH. 4, Betaine-homocysteine *S*-methyltransferase EC 2.1.1.5: BHMT. 5, 5-Methyltetrahydrofolate-homocysteine *S*-methyltransferase, methionine synthase EC 2.1.1.13: MS. 6, Glycine hydroxymethyltransferase EC 2.1.2.1: GHMT. 7, 5,10-Methylene-tetrahydrofolate reductase (EADH₂) EC 1.7.99.5: MTHFR. 8, Dihydrofolate reductase EC 1.5.1.3: DHFR. 9, Cystathionine β -synthase EC 4.2.1.22: CBS.

Japan) unless otherwise stated. Phosphocellulose paper (P81) and DEAEcellulose paper (DE81) were obtained from Whatman International Ltd. CpG methylase was obtained from New England Biolabs. [α - 32 P]dCTP, L-[methyl 3 H]methionine, and S-adenosyl-L-[methyl 3 H]methionine were purchased from Amersham Biosciences.

Animal treatments. Male mice (*ddY* strain, 4 wk old, 20–25 g) were purchased from Japan SLC and maintained with laboratory food (MF, Oriental Yeast Co., Ltd., Japan) and water provided ad libitum on a 8:00 to 20:00 light/20:00 to 8:00 dark cycle. In the fasting experiment, diet was withheld from some animals (3 or 6 mice for the 16 h fasting experiment or 3 mice for the time course experiment) for 10 or 16 h starting at 18:00. Tissues were collected from all animals, frozen immediately in liquid nitrogen, and stored at -80°C . Food was not withheld from the control animals, and tissues were collected at the same time as those from

RNA preparation and hybridization analysis. Total RNA was extracted using the acid guanidine thiocyanate-phenol-chloroform method (21). The RNA (20 μ g) was separated by electrophoresis on a 0.9% agarose gel containing 2.2 M formaldehyde (22) and transferred to a nylon membrane (Nytran Plus, Schleicher & Schuell, Germany). The MAT1A/P1 fragment (23), MAT1A/P2 fragment (23), MAT2A fragment (the 1,023 to 1,868 bp fragment of cDNA, AB070266), MAT2B fragment (the 199 to 1,034 bp fragment of cDNA, AB070267), and 18S rRNA fragment were labeled with 32 P using a Random Prime DNA Labeling Kit Ver. 2.0 (Takara Shuzo Co., Ltd., Japan) and were used as probes. Hybridization was performed in a solution of PerfectHyb (Toyobo Co., Ltd., Japan). The membranes were washed according to the manufacturer's instructions and then autoradiographed with Fuji RX film at -70°C using intensifying screens. The expression of MAT mRNA was quantified using an imaging analyzer (FLA 2000, Fuji Film Co., Ltd., Japan), and the ratio of the expression of each mRNA-to-18S rRNA signal was calculated and compared.

Measurement of MAT activity and western blot analysis. MAT activity was assayed as described previously (24). Briefly, liver homogenate with 0.25 M Tris HCl pH 7.4, 4 mM 2-mercaptoethanol was centrifuged and the supernatant was added to the assay mixture (100 μ L) containing 0.1 M Tris HCl pH 8.2, 20 mM $MgCl_2$, 150 mM KCl, 5 mM ATP, 5 mM 2-mercaptoethanol, and 5 mM or 20 μ M (185 kBq) L-[methyl 3H]methionine. The reaction mixture was incubated for 30 min at 37°C, and the reaction was stopped with 10 μ L of 2 M $HClO_4$ containing 5 mM methionine. After centrifugation, 50 μ L of the supernatant was spotted onto Whatman P81 phosphocellulose paper, washed in 5 mM sodium phosphate buffer pH 7.0, and transferred to a counting vial containing 0.4 mL of 1.5 M NH_4OH . The radioactivity was measured with a liquid scintillation counter. Western blot analysis was performed using the method described previously (25), with anti-mouse MAT I/III rabbit serum.

AdoMet, AdoHcy, GSH, and amino acid measurement. AdoMet and AdoHcy in the liver were measured by the HPLC method (26), using a Waters M-600 pump equipped with a Waters 2487 Dual λ Absorbance Detector operating at 254 nm and a TSK gel ODS-80TM column (25 cm $\times$ 4.6 mm, Tosoh Co., Kyoto, Japan). GSH was measured by the microtiter plate assay (27). For the amino acid measurements, liver homogenate with 0.4 M HClO₄ was centrifuged, and the supernatant was neutralized with 4 M KOH. After centrifugation, the supernatant was concentrated with a Speed Vacuum Concentrator (Savant, USA) and resuspended to 1/10 vol. 0.1 M HCl. After that, the amino acids were measured with the PTC-amino acid method by HPLC with a Pico-Tag column (15 cm $\times$ 3.9 mm, Waters) (28).

DNA methylation. Genomic DNA was prepared from

Fig. 2. Effect of fasting on MAT mRNA expression in the mouse liver. (A) Effect of 16 h of fasting on MAT mRNA expression for MAT I/III and MAT II. (B) Effect of fasting time on MAT mRNA expression for MAT I/III. Northern hybridizations were performed with the specific probe for each MAT transcript. The mean±SE of the ratio of each MAT mRNA to the level of the 18S rRNA signal is shown as an arbitrary unit. **p*<0.05, ***p*<0.01, and ****p*<0.001: significantly different from each normal group.

mouse liver according to a previously described method (29). The reaction mixture (30 μ L) containing 0.5 μ g DNA, 3 μ M *S*-adenosyl-L-[methyl 3 H]methionine (74 kBq), 3 units of CpG methylase, 10 mM Tris HCl at pH 7.9, 120 mM NaCl, 10 mM EDTA, and 1 mM dithiothreitol was incubated at 30°C for 1 h. The methylated DNA was isolated from the reaction mixture by filtering 15 μ L on Whatman DE81 ion exchange paper. After washing, the radioactivity remaining on the paper was measured in a liquid scintillation counter (30).

Other methods and statistical analysis. Protein concentration was measured by the dye-binding method using a protein assay (Bio-Rad, Japan). Bovine serum albumin was used as the standard. Groups in all experiments were compared using the non-paired Student's *t*-test.

RESULTS

MAT expression in the fasting mouse liver

MAT 1A mRNA increased in the fasting mouse liver. The *MAT1A* gene has two promoters in the 5' region (23). The p1 promoter gives rise to the P1 transcript ini-

tiated at +1, and the p2 promoter gives rise to the P2 transcript initiated at -156. Both the P1 and P2 transcripts have the same translation initiation site. However, because the -107 to +145 nucleotide region is missing from the mature P2 transcript, the P1 and P2 transcripts are detected separately by the transcript-specific probes (MAT1A/P1 and MAT1A/P2). As shown in Fig. 2A, both MAT1A/P1 and P2 mRNA increased after 16 h of fasting. MAT2A mRNA decreased, and MAT2B mRNA did not change expression level. Figure 2B shows the effect of fasting time on the expression of MAT1A/P1 mRNA, which is the major transcript in the liver. MAT1A/P1 mRNA started to increase at 10 h. The protein level of MAT I/III from the *MAT1A* gene increased after 16 h but not after 10 h of fasting (Fig. 3). The MAT activity, measured with 5 mM substrate, increased after 16 h of fasting. The enzyme activity with 5 mM substrate indicates activity of MAT I/III because the *K*_m value of MAT I/III for methionine is 23 μ M to 1 mM for MAT I and 215 μ M to 7 mM for MAT III. The activity with 20 μ M substrate shows MAT II activity (the *K*_m value for methionine is 4 to 10 μ M for MAT II).

Fig. 3. Effect of fasting on MAT protein level and activity. (A) Western blot analysis. (B) MAT activity. Each data point is shown as mean±SE. ** $p<0.01$: significantly different from the normal group.

Table 1. Methylation potential and GSH level in 16 h-fasting-mouse liver.

Groups	AdoMet (nmol/g liver)	AdoHcy (nmol/g liver)	AdoMet/AdoHcy ratio	Total GSH (μmol/g liver)	Incorporation of methyl group (dpm/0.25 μg DNA)
Normal	83.9±4.0	6.3±1.5	19.6±5.8	4.24±0.17	9,278±697
Fasting	63.2±3.2**	15.2±2.4*	5.1±1.3*	2.90±0.11**	13,682±2,137

Each datum is mean±SE (n=6).
* $p<0.05$, ** $p<0.01$: significantly different from normal group.

Table 2. Amino acid levels in 16 h-fasting-mouse liver.

Amino acid	Normal (nmol/g liver)	Fasting (nmol/g liver)
Asp	412±61	482±61
Glu	1,946±381	1,603±266
Ser	319±42	342±29
Gly	3,146±225	2,823±278
Thr	156±34	178±31
Ala	1,710±382	477±77*
Met	107±14	119±25
Leu	151±73	182±42
Lys	376±69	348±24

Each datum is mean±SE (n=6).
* $p<0.05$: significantly different from normal group.

The activity with 20 μM methionine did not change after 16 h of fasting.
Effect of fasting on hepatic metabolites and DNA methylation

After 16 h of fasting, AdoMet decreased and AdoHcy increased significantly (Table 1). The AdoMet/AdoHcy ratio, which shows the methylation potential, decreased to one fourth. However, in vitro incorporation of the methyl group into DNA by CpG methylase did not change, showing that the DNA methylation level did not change in the fasting mouse liver. The level of GSH, which is synthesized by the γ-glutamyl cycle as a byproduct of the transsulfuration pathway, was decreased by fasting (Table 1). Alanine is known to decrease due to fasting (31). As shown in Table 2, alanine decreased after 16 h of fasting, but the methionine level in the liver did not change. Free homocysteine (Hcy) was not detected in the livers of either control or fasting mice.

DISCUSSION

In this study, we investigated the effect of fasting on MAT expression and the regulation of the methionine cycle in mice. MAT1A mRNA level and activity increased in mouse liver after 16 h of fasting. The AdoMet/AdoHcy ratio decreased as a result of fasting, but the DNA methylation level did not change.

The enzymes in the methionine metabolic pathway may be regulated to maintain the levels of methionine and AdoMet in the fasting mouse liver; MAT I/III activity increased and the methionine level remained constant in fasting mouse liver (Fig. 3 and Table 2). Mudd and Poole have estimated that about 53% of the available homocysteine is converted to cystathionine during each cycle in males fed a basal diet (32). GSH, which is the most abundant low-molecular-weight thiol, is made by the transsulfuration pathway. In 3-mo-old MAT1A knockout mice, the plasma methionine level increases 776%, and hepatic AdoMet and GSH are reduced by 74 and 40%, respectively (7). This result and our results for GSH in the fasting mouse liver (Table 1) indicate that when AdoMet is low, the transsulfuration pathway may down-regulate to maintain the level of AdoMet. GSH deficiency contributes to oxidative stress, which plays a key role in aging and the pathogenesis of many diseases (33). Thus, long-term fasting may contribute to oxidative stress.

AdoMet, which is made by MAT in the methionine cycle, is essentially a methyl donor for DNA methylation. DNA methylation is the covalent addition of a methyl group to the 5-carbon position of cytosine. In normal situations, 60 to 90% of all CpG sequences in the genome are methylated. Unmethylated CpG sequences are mainly clustered in CpG-rich sequences, termed CpG islands, within the promoter regions of genes (34). Methylation of CpG islands inhibits gene transcription by directly inhibiting the binding of transcription factors to their *cis*-acting sites on DNA (35). As a consequence of folate deficiency, cellular AdoMet is depleted, which in turn induces DNA hypomethylation and potentially induces proto-oncogene expression, leading to cancer. In addition, DNA hypomethylation has been shown to lead to chromosomal instability in embryonic stem cells that are null for the DNA methyltransferase 1 gene (36). Many reports indicate that dietary modifications change DNA methylation levels (16, 37). Therefore, we studied the DNA methylation level of fasting mouse liver. In this study, no increase in DNA methylation occurred in fasting mouse liver. However, other methylation reactions appeared to increase, as indicated by the finding that AdoHcy, the demethylated product of the methyltransferase reaction, increased in the fasting mouse liver (Table 1). We searched the database about the relation between glycine N-methyltransferase, phosphatidylethanolamine N-methyltransferase, or guanidinoacetate N-methyltransferase expression and fasting. But no published reports exist on the relation between methyltransferase and fasting. At the present time, the reason why

AdoHcy increases in fasting mouse liver has not been established.

AdoHcy can negatively affect methylation potential in humans. Data from several recent studies suggest that hyperhomocysteinemia is accompanied by significant increases in plasma and intracellular AdoHcy (38). Hyperhomocysteinemia is a marker of vascular diseases (38). Thus, the inhibition of methylation reactions, in addition to the depression of the GSH level, is related to many diseases. Understanding the effects of diets on MAT expression and the methionine cycle is important for maintaining health. The mechanism of the regulation of MAT expression by fasting and other dietary changes is unclear. Further investigations are needed.

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