Molecular Medicine

Low-Dose Methotrexate Inhibits Methionine *S*-Adenosyltransferase *In Vitro* and *In Vivo*

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Methionine *S*-adenosyltransferase (MAT) catalyzes the only reaction that produces the major methyl donor in mammals. Low-dose methotrexate is the most commonly used disease-modifying antirheumatic drug in human rheumatic conditions. The present study was conducted to test the hypothesis that methotrexate inhibits MAT expression and activity *in vitro* and *in vivo*. HepG2 cells were cultured under folate restriction or in low-dose methotrexate with and without folate or methionine supplementation. Male C57BL/6J mice received methotrexate regimens that reflected low-dose clinical use in humans. *S*-adenosylmethionine and MAT genes, proteins and enzyme activity levels were determined. We found that methionine or folate supplementation greatly improved *S*-adenosylmethionine in folate-depleted cells but not in cells preexposed to methotrexate. Methotrexate but not folate depletion suppressed MAT genes, proteins and activity *in vitro*. Low-dose methotrexate inhibited *MAT1A* and *MAT2A* genes, MATI/II/III proteins and MAT enzyme activities in mouse tissues. Concurrent folinate supplementation with methotrexate ameliorated *MAT2A* reduction and restored *S*-adenosylmethionine in HepG2 cells. However, posttreatment folinate rescue failed to restore *MAT2A* reduction or *S*-adenosylmethionine level in cells preexposed to methotrexate. Our results provide both *in vitro* and *in vivo* evidence that low-dose methotrexate inhibits MAT genes, proteins, and enzyme activity independent of folate depletion. Because polyglutamated methotrexate stays in the hepatocytes, if methotrexate inhibits MAT in the liver, then the efficacy of clinical folinate rescue with respect to maintaining hepatic *S*-adenosylmethionine synthesis and normalizing the methylation reactions would be limited. These findings raise concerns on perturbed methylation reactions in humans on low-dose methotrexate. Future studies on the clinical physiological consequences of MAT inhibition by methotrexate and the potential benefits of *S*-adenosylmethionine supplementation on methyl group homeostasis in clinical methotrexate therapies are warranted.

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INTRODUCTION

Folate serves as a key metabolic carrier in one-carbon metabolism. 10-Formyl tetrahydrofolate is essential for *de novo* purine synthesis, and 5,10-methylenetetrahydrofolate assists in thymidine synthesis. In the form of 5-methyltetrahydrofolate (5-methylTHF), folate transfers the methyl groups to methionine synthase, generating methionine from homocysteine remethylation. Methionine can be further utilized for synthesis of the universal methyl donor *S*-adenosylmethionine.

Low-dose methotrexate (MTX) is one of the most common immunosuppressants used in rheumatic and other inflammatory conditions. MTX is a potent inhibitor of dihydrofolate reductase and thymidylate synthase, which can deplete folate and inhibit DNA synthesis. MTX also interferes with the enzymes methylenetetrahydrofolate reductase and methionine synthase, resulting in reduced cellular 5-methylTHF levels and decreased homocysteine remethylation (1,2). By decreasing 5-methylTHF, both folate depletion and antifolate treatments can impair 5-methylTHF–dependent methionine synthesis (3), reduce *S*-adenosylmethionine and affect methylation reactions (4). In HT29 colon cancer cells, high-dose MTX (in μmol/L) markedly decreases intracellular adenosine, *S*-adenosylmethionine and polyamine content (5). A previous study found that prolonged incubation of low-concentration MTX (1–10 nmol/L) inhibited *in vitro* monocyte chemotaxis and superoxide production; such inhibition was augmented by a low methionine condition, but was abolished by folinate and *S*-adenosylmethionine treatments (6). These findings suggested that, in addition to the inhibition of nucleotide syn-

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thesis, the efficacy of low-dose MTX may be linked to its inhibition of methionine synthesis and/or the depletion of *S*-adenosylmethionine.

MAT [ATP: L-methionine *S*-adenosyltransferase (EC 2.5.1.6)] catalyzes the only reaction that produces the major methyl donor and precursor of polyamine synthesis—*S*-adenosylmethionine (7). MAT proteins are encoded by two genes (*MAT1A* and *MAT2A*), and a third gene (*MAT2β*) regulates the *MAT2A*-encoded isoenzyme (7–9). The activity of MAT is regulated by the methionine concentration, but the Michaelis constants of this enzyme to methionine differ among these MAT proteins. The K_m is lowest for MAT II (expressed ubiquitously in all tissues), intermediate for MAT I and highest for MAT III (both expressed exclusively in the liver) (10–14). It is plausible that during antifolate treatments such as MTX, liver and nonhepatic tissues have distinctive responses to methionine depletion/supplementation with respect to *S*-adenosylmethionine synthesis and availability.

The liver plays an essential role in methyl group homeostasis. By decreasing 5-methylTHF, MTX could impair 5-methylTHF–dependent methionine synthesis and reduce *S*-adenosylmethionine. Although the efficacy of low-dose MTX in monocytes can be altered by folate and *S*-adenosylmethionine, the impacts of these metabolites on liver transmethylation in humans on low-dose MTX are less clear because the liver is not the main target for immunosuppressants. If *S*-adenosylmethionine depletion resulting from MTX treatments is mainly due to reduced folate-dependent methionine synthesis brought on by the inhibition of methylenetetrahydrofolate reductase and methionine synthase, then methionine supplementation would be equally, if not more, effective than folate supplementation to restore *S*-adenosylmethionine synthesis. However, our preliminary experiments suggested otherwise (15). Neither folinate nor methionine supplementation normalized *S*-adenosylmethionine levels in cells treated with low-dose MTX. These observations led to our postulation that MTX might suppress *S*-adenosylmethionine production by inhibition of MAT independent of folate depletion. The present study was conducted to test the hypothesis that MTX inhibits MAT expression and activity *in vitro* and *in vivo*.

METHODS

Chemicals and Cell Culture

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. HepG2 human hepatocellular carcinoma cell line (FIRDI, Hsinchu, Taiwan) was grown in minimum essential α medium (α-MEM) with 10% (v/v) bovine calf serum. The medium was replaced every 72 h.

Effects of Folate Restriction and MTX Treatment on Folate Status and *S*-Adenosylmethionine Production

The experimental conditions were based on previous experiments from our group and others. To study the impacts of low-dose MTX and compare them to the impact of folate-depletion conditions on folate and *S*-adenosylmethionine status, HepG2 cells were either cultured in folate-restricted medium (10 nmol/L folinate for 144 h) (16) or treated with 50 nmol/L MTX for 72 h (17). Control cells were cultured in regular α-MEM medium that contained 2.2 μmol/L folic acid in all experiments (Gibco Invitrogen, Carlsbad, CA, USA). In arthritis patients taking low-dose MTX for immunosuppression, intracellular MTX concentration was approximately 34 nmol/L (18–51 nmol/L) in erythrocytes (16,18). In the folate restriction experiments, cells were cultured in αMEM with 10 nmol/L folinate with 10% folate-depleted fetal calf serum that was prepared by dialyzing serum against 10 volumes of phosphate-buffered saline (PBS) at 4°C for 24 h with buffer changes every 4 h (16,19). These cells were harvested for further analysis of folate, *S*-adenosylmethionine and MAT activity, as previously described (16,20,21).

Effects of Folate and Methionine Supplementations on Intracellular *S*-Adenosylmethionine Production in MTX Pretreated Cells

The experimental conditions were based on our previous experiments. To investigate whether folate or methionine supplementation could ameliorate the *S*-adenosylmethionine depletion caused by MTX, cells were pretreated with 50 nmol/L MTX for 72 h and then supplemented with either folinate (100 nmol/L) or methionine (500 μmol/L) for 48 h. In a separate experiment, cells were concomitantly cotreated with MTX and folinate (100 nmol/L) or methionine (500 μmol/L) for 72 h. The control cells were cultured in the regular α-MEM media containing 100 μmol/L methionine. After these treatments, cells were collected for intracellular *S*-adenosylmethionine determinations as previously described (4,16,22).

Effects of Methotrexate on MAT Expression and Activities *In Vivo*

The animal experiment was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University. To study the impacts of MTX on MAT expression and activity, 12 male C57BL/6J mice (3 wks of age) were obtained from the National Lab of Animal Care (NLAC, Taipei, Taiwan). The mice were fed an amino acid–defined diet containing 2 mg folic acid/kg diet (23) with 1% succinyl sulfathiazole (Dyets Inc., Bethlehem, PA, USA). All mice were housed in specific pathogen-free filtered cages (2 mice per cage) at 22°C and experienced a 12-h light/darkness cycle (0700 to 1900 h). After adaption at the facility for 1 wk, the mice were equally divided into 2 groups by body weight. MTX treatment (0.3 mg MTX/kg/2 d, dissolved in PBS with 0.01% dimethyl sulfoxide [DMSO]) was freshly prepared prior to injection and began when mice were 4 wks old. The mice were injected intraperitoneally once on alternate days with either PBS (0.01% DMSO) (control group, n = 6) or low-dose MTX (MTX group, n = 6)

for 4 wks. This dose was within the range (0.1–0.5 mg/kg/d) of those used in previous studies in rodent arthritis models. MTX treatment at the dose of 1 mg MTX/kg/2 d (0.5 mg/kg/d) significantly decreased the arthritis score and increased locomotion in the mouse collagen arthritis model (24). At the dose of 0.75 mg/kg body weight per wk (0.11 mg/kg/d), MTX intraperitoneal injections significantly reduced paw volume in the adjuvant-induced arthritis (25). At a dose of 0.3 mg/kg/2 d (0.15 mg/kg/d), MTX significantly decreased the arthritic score and improved histological and radiological scores in rats with collagen-induced arthritis (26). Furthermore, the dose used in the present study (0.15 mg/kg/d in mice) was comparable to the clinical use of low-dose MTX in treating human rheumatoid arthritis. MTX was generally administered to rheumatoid arthritis patients at a dosage of 7.5–15 mg/wk (18,27,28). Considering the normal life expectancy of mice, the 4-wk duration reflected approximately 3 years of MTX treatment in humans. The median duration of MTX treatment in our human subjects from previous studies was approximately 36 months (95% CI 45.7–77.8) (27–30), and the mean weekly dose was 12.5 mg (95% CI 10.5–12.4 mg) in arthritis patients (31). The mice were sacrificed at the age of 8 wks and tissue samples were stored at –80°C for analyses of levels of *MAT* gene and protein expression and MAT activity.

Effects of MTX on Glucocorticoid-Induced *MAT2A* Expression

To investigate whether MTX suppresses glucocorticoid-induced *MAT2A* expression, the HepG2 cells were synchronized in serum-free media for 16 h, then treated with either MTX (50 nmol/L for 72 h), dexamethasone (100 nmol/L for 72 h), actinomycin D (5 μg/mL for 12 h just before cells were harvested), dexamethasone combined with MTX (cotreated for 72 h) or dexamethasone combined with actinomycin D (dexamethasone treatment for 72 h; at 60 h, actinomycin D was added for an additional 12 h just before cell harvest). The performance of these experiments was based on previous experimental conditions (17,32).

Effects of Concomitant and Post-MTX Folinate Supplementation on *MAT2A* Gene Expression

Because concomitant folinate supplementation with MTX effectively restored *S*-adenosylmethionine levels, we next investigated whether concurrent folinate supplementation could rescue MTX-induced *MAT2A* gene inhibition in HepG2 cells. *MAT2A* gene expression levels were compared between cells that received concurrent MTX (50 nm for 72 h) and folinate treatments (100 nmol/L for 72 h) and cells pretreated with the same dose of MTX followed by folinate supplementation for various time periods.

Determination of *MAT* Gene Expression

Total RNA was isolated from the livers (expressing *MAT1A*) and kidneys (expressing *MAT2A*) of the mice or MTX-treated cells with TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). The integrity of RNA was checked by electrophoresis. A total amount of 2 μg RNA was treated with DNase I for eliminating DNA, and then RNA was converted to cDNA by reverse transcription–polymerase chain reaction (PCR) by use of Moloney murine leukemia virus RNA reverse transcriptase (Promega, Madison, Wisconsin, USA) and oligo-dT as primers. Human *MAT 2A* and *18S* gene expression levels were determined by quantitative real-time PCR ABI7000 using commercial TaqMan probes (Applied Biosystems Inc., Foster City, CA, USA). Mouse gene expression levels (Mus *MAT 1A*, Mus *MAT 2A*, Mus *18S*) were determined by quantitative real-time PCR by using SYBR Green reagents (Applied Biosystems). The expression of *MAT* genes was calculated by normalizing the threshold cycle value of the target gene to that of the control housekeeping gene (*18 sRNA*) (20).

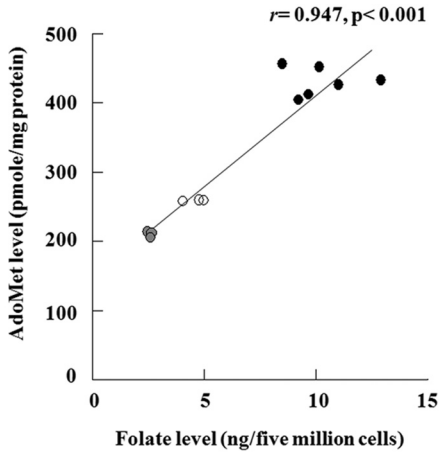

Figure 1. *S*-adenosylmethionine (AdoMet) levels were highly correlated with the intracellular folate levels in HepG2 cells ($r = 0.947$, $P < 0.001$, n = 12). Control (●): cultured in α-MEM. FA def (○): folate restricted (10 nmol/L) for 144 h. MTX (●): treated with 50 nmol/L MTX for 72 h (n = 3 in each treatment).

Determination of MAT Protein Expression

After the incubation period, cells were harvested, washed and pelleted. At termination, mouse tissues were immediately frozen at –70°C until analyses. Cell pellets or mouse tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer (25 mmol/L Tris·HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) with protease inhibitor (CalBiochem, Darmstadt, Germany). The lysates were centrifuged and the supernatants were used for Western blot assay. A total amount of 60 μg protein (measured by the bicinchoninic acid method) was used for SDS polyacrylamide gel electrophoresis (SDS-PAGE) and further immunoblotting. Protein lysates from tissue or cells were denatured and separated on a 12% SDS-PAGE gel by using a Minigel apparatus, and then transferred onto a polyvinylidene difluoride membrane by using a transfer cell (Bio-Rad, Hercules, CA, USA). After blocking with Tris-buffered saline (TBS) containing 10% skim milk, the lysates were incubated with rabbit polyclonal

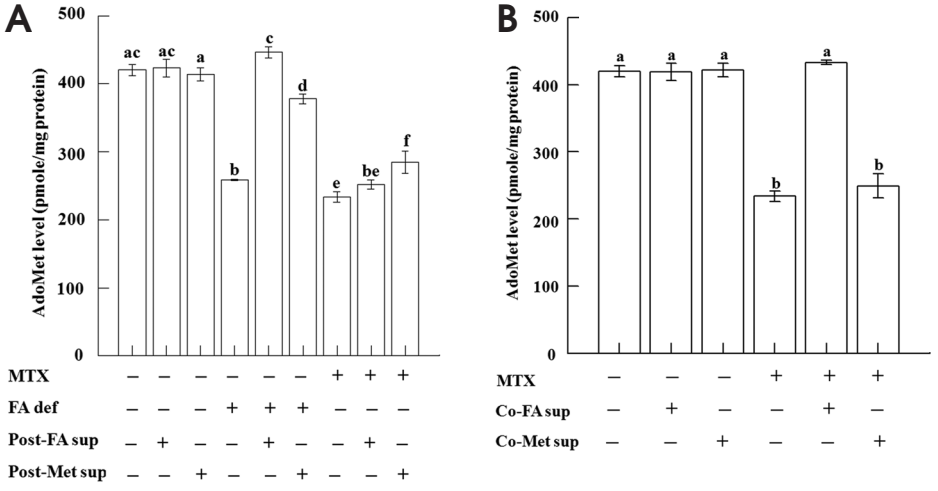

Figure 2. Folinate or methionine supplementation restored *S*-adenosylmethionine levels in folate-depleted cells but not in MTX pretreated cells. (A) Effects of folate or methionine supplementation on intracellular *S*-adenosylmethionine (AdoMet) levels in folate-depleted or MTX pretreated HepG2 cells ($P > 0.05$). MTX: treated with 50 nmol/L MTX for 72 h, FA def: folate restricted (10 nmol/L) for 144 h. Post-FA sup: supplemented with 100 nmol/L folinate for additional 48 h in MTX pretreated cells, Post-Met sup: supplemented with 500 μmol/L methionine for additional 48h in MTX pretreated cells. (B) Effects of cosupplemented MTX with folinate or methionine on intracellular *S*-adenosylmethionine (AdoMet) levels in HepG2 cells. Co-FA sup: cosupplemented MTX with 100 nmol/L folinate for 72 h, Co-Met sup: cosupplemented MTX with 500 μmol/L methionine for 72 h. Data are shown as mean ± SE. The values with different letters were significantly different (n = 4 for each treatment).

antibody (1:1000) against MAT II (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in HepG2 cells or mouse kidney samples. A mouse monoclonal antibody was used for the determination of MAT I/III (Abnova Inc., Taipei, Taiwan) in mouse liver samples. Membranes were washed three times with TBS containing 0.1% Tween 20 and then covered with horseradish peroxidase–linked anti–rabbit IgG for MAT II or anti–mouse IgG for MAT I/III (1:5000) at room temperature. The immunoblots were visualized by use of an enhanced chemiluminescence kit (New England Biolabs, Beverly, MA, USA). The expression level of MAT protein was calculated by normalizing the quantity of target protein to that of the internal control β-actin.

Determination of MAT Activity

Harvested samples from cells or animal tissues were homogenized in an ice-cold buffer consisting of 0.154 mol/L KCl/50 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 7.4). MAT activity was determined by quantifying *S*-adenosylmethionine production by 300 μg of cellular protein. The reaction mixture consisted of 80 mmol/L Tris-HCl/50 mmol/L KCl (pH 7.4), 5 mmol/L ATP, 40 mmol/L $MgCl_2$ and cellular protein of enzyme solution (20). Methionine (5 mmol/L) was added to initiate the enzyme reaction after preincubation of the cellular protein and the reaction mixture for 3 min. The reaction was carried out at 37°C for 1 h and then terminated by 10% ice-cold perchloric acid. MAT activity was calculated as the *S*-adenosylmethionine production (the concentrations of these reactions were 0.4–1.0 nmol/L in the cell experiments and 18–30 nmol/L in the tissues) after subtracting the baseline *S*-adenosylmethionine level (<0.01 nmol/L) from the supernatant fraction without adding methionine, and expressed as nanomoles *S*-adenosylmethionine formed per milligram protein per hour (20).

In separate experiments, we investigated whether MTX treatments inhibit MAT activity in cell-free protein lysates prepared from untreated cells and healthy animal tissues. Protein lysates extracted from normal mouse liver or HepG2 cells were preincubated with various concentrations of MTX (500–2500 μmol/L) at room temperature for 20 min, then the assay reagents were added for determination of MAT activity as previously described (33).

Statistical Analyses

Comparisons of means between the control and the MTX treatment groups or the control and the folate-deficient groups were determined by using the Student *t* test. Results are expressed as mean ± standard deviation. The expression level of *MAT2A* and concentrations of *S*-adenosylmethionine among cells treated with MTX for different periods with and without folinate supplementation were examined by analysis of variance. All statistical analyses were performed with SYSTAT 11.0 for Windows™ (Systat Software Inc., Richmond, CA, USA). For all analyses, the results were considered statistically significant if *P* values were <0.05.

RESULTS

Folate Restriction and MTX Treatment Both Reduced Intracellular Folate and *S*-Adenosylmethionine in HepG2 Cells

Both folate restriction and MTX treatment significantly reduced intracellular folate concentrations in HepG2 cells. Under the experimental conditions, folate restriction for 144 h reduced the intracellular folate level by approximately 55%, and MTX treatment for 72 h decreased intracellular folate by 75% compared with the control cells under the same conditions (data not shown). Intracellular *S*-adenosylmethionine was reduced approximately 40% by folate restriction and approximately 51% in MTX-treated cells (data not shown). The levels of intracellular *S*-adenosylmethionine were highly correlated with the in-

tracellular folate contents ($r = 0.947$, $P < 0.001$, n = 12) (Figure 1).

Folinate or Methionine Supplementation Restored *S*-Adenosylmethionine Levels in Folate-Depleted Cells but Not in MTX-Pretreated Cells

Folinate (post-FA sup+) or methionine (post-Met sup+) supplementation alone did not alter *S*-adenosylmethionine levels in HepG2 cells (Figure 2A). In folate-restricted cells (FA def+), folinate supplementation alone successfully restored intracellular *S*-adenosylmethionine to a normal level similar to that of the folate-replete control cells (Figure 2A). Excessive methionine also drastically improved *S*-adenosylmethionine status in these cells, although it was slightly less effective than folinate. In contrast, folinate supplementation failed to improve the *S*-adenosylmethionine status in cells pretreated with MTX (MTX+). No significant improvement in *S*-adenosylmethionine levels was observed in these cells after they were supplemented with folinate for 48 h (MTX+ and post-FA sup+) (Figure 2A). Methionine supplementation was slightly more effective than folinate on *S*-adenosylmethionine production, but excessive methionine for as long as 48 h failed to normalize *S*-adenosylmethionine levels (MTX+ and post-Met sup+) (Figure 2A).

To establish reliable experimental conditions and determine the optimal duration of methionine supplementation for *S*-adenosylmethionine production, we incubated these cells with excessive methionine for various time periods in a pilot experiment. *S*-adenosylmethionine levels reached a plateau within 90 min and remained at that level for at least 48 h when the incubation time was prolonged. In the experiments in which cells were pretreated with the same dose of MTX, the *S*-adenosylmethionine level remained suppressed even 48 h after loading with excessive methionine.

These results suggested that the mechanism by which MTX caused *S*-adenosylmethionine depletion was not simply due to folate deficiency or methionine depletion, and that an additional mechanism was likely involved. These observations provided initial evidence that MTX may inhibit MAT; and MTX-induced *S*-adenosylmethionine depletion was investigated further.

Effects of Concomitant Treatments with Folinate or Methionine on *S*-adenosylmethionine Status

When methionine was supplemented concomitantly (co-Met sup) with MTX treatment, intracellular *S*-adenosylmethionine remained reduced (Figure 2B), supporting our postulation that impaired methionine synthesis is not the main cause for MTX-induced *S*-adenosylmethionine depletion. However, when folinate was supplemented concomitantly (co-FA sup) with MTX, intracellular *S*-adenosylmethionine was restored to a normal level similar to that of the untreated control cells (Figure 2B). The impacts of concomitant and post-MTX folinate supplementations were investigated further.

MTX Inhibited MAT Activity and Protein and Gene Expressions *In Vitro* Independent of Folate Depletion

Because a high concentration of methionine could not restore the *S*-adenosylmethionine level, we investigated whether MTX reduces *S*-adenosylmethionine synthesis by the inhibition of MAT activity and/or MAT expression. In HepG2 cells, folate restriction significantly induced *MAT 2A* gene expression by approximately 50% compared with the controls ($P = 0.005$, n = 6) (Figure 3A). No significant difference was found in MAT II protein expression (Figure 3B) or MAT enzyme activity (Figure 3C) between folate replete and depleted cells. These results supported our postulation that the inhibition of *S*-adenosylmethionine synthesis by MTX was independent of folate depletion.

In contrast to the folate-depleted cells in which *MAT 2A* was significantly induced, in MTX-treated cells at 72 h *MAT 2A* gene expression was decreased (Figure 4A). MAT protein expression was inhibited by approximately 50% at 72 h (Figure 4B), and MAT activity was inhibited by 33%, 52% and 61% at 24 h, 48 h and 72 h, respectively (Figure 4C). Taken together, these results demonstrated that low-dose MTX at 50 nmol/L inhibited MAT activities in HepG2 cells in a time-dependent manner.

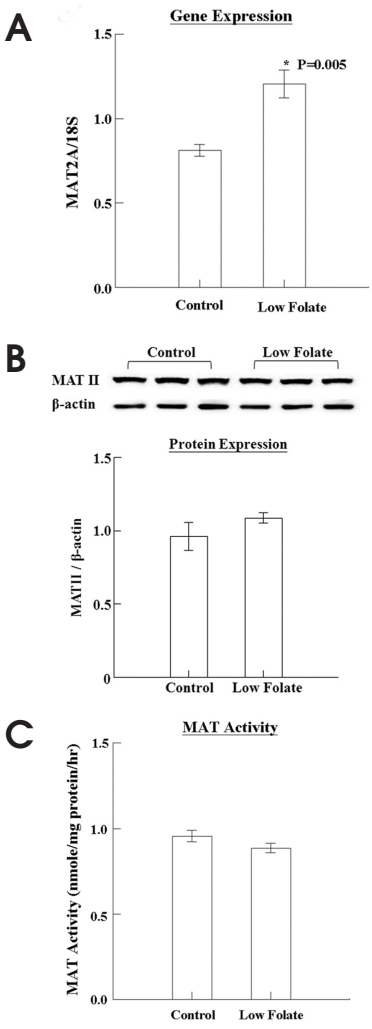

Figure 3. Effects of folate restriction on MAT expression and enzyme activity in HepG2 cells. (A) MAT2A mRNA. (B) MAT II protein. (C) MAT enzyme activity (n = 4 for each treatment). Data are shown as mean ± SE. Folate restriction significantly induced MAT 2A mRNA expression by 50% compared with controls ($P = 0.005$, n = 3 per group). No significant difference was found in MAT II protein expression levels or MAT enzyme activity between folate replete and depleted cells.

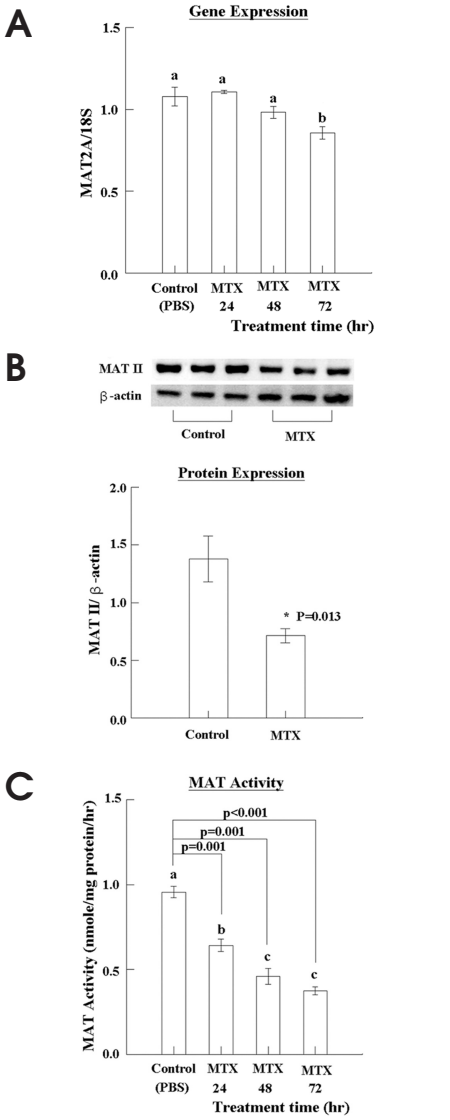

Figure 4. Effect of MTX treatments on MAT expression and enzyme activity in HepG2 cells. (A) MAT2A mRNA. (B) MAT II protein. (C) MAT enzyme activity. Data are shown as mean ± standard error (SE). MAT2A mRNA and protein were significantly decreased in MTX-treated cells at 72 h ($P < 0.05$). MAT activity was inhibited by 33%, 52%, and 61% at 24 h, 48 h and 72 h, respectively ($P \leq 0.001$).

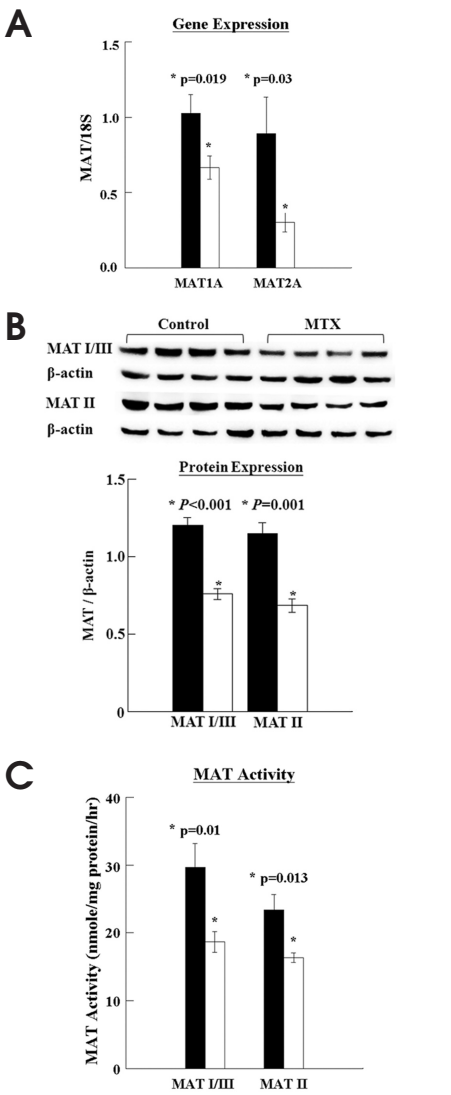

Figure 5. MTX treatment significantly reduced MAT expression and enzyme activity *in vivo*. (A) mRNA expression. MAT1A (from the liver) and MAT2A (from the kidney). (B) MAT I/III protein (liver) and MAT II (kidney) expressions, and (C) MAT enzyme activities. Data are shown as mean ± SE. Controls are shown in filled bar and MTX in open bar (n = 5–6 per group).

MTX Significantly Reduced MAT Activity and MAT Protein and Gene Expression *In Vivo*

Results from animal experiments indicated that both *MAT1A* (from the liver) and *MAT2A* (from the kidney) could be inhibited by low-dose MTX treatments. *MAT1A* gene expression was inhibited by approximately 35% in mouse liver and the *MAT2A* gene was reduced by 66% in the kidney (Figure 5A). Furthermore, MAT protein was significantly reduced in mice that received low-dose MTX. MTX decreased the expression of MAT I/III protein (liver) by 31% and inhibited the expression of MAT II by 28% (kidney) (Figure 5B). In MTX-treated mice, MAT activity was inhibited by approximately 37% in the liver and by 28% in the kidney (Figure 5C). Taken together, *in vivo* experiments showed that low-dose MTX significantly reduced *MAT* gene expressions, MAT protein levels, and MAT activity. These results supported our postulation that MTX directly inhibits MAT expression and that the *S*-adenosylmethionine depletion associated with MTX was due to MAT inhibition.

MTX Does Not Affect MAT Activity in Cell-Free Protein Lysates

In separate experiments, fixed amounts of total proteins were prepared from untreated cells or healthy animal livers. Then the MAT-inhibition phenomena were observed in MTX-treated cells, and further investigated in cell-free lysates obtained from mice. MTX (up to 2500 μmol/L) did not inhibit MATII (cell-free protein lysates obtained from untreated HepG2 cells, Figure 6A) or MATI/III (cell-free protein lysates obtained from normal mouse liver, Figure 6B).

MTX Inhibited Glucocorticoid-Induced MAT Gene Expression

Low-dose MTX and glucocorticoids are both commonly used disease-modifying antirheumatoid drugs (DMARDs) for treating patients with rheumatoid arthritis; yet the impacts of clinical use of DMARDs on MAT function remain to be established. Previous studies revealed that certain glucocorticoids can induce *MAT2A* mRNA expression (32). In both hepatoma cell lines and primary cultures of adult rat hepatocytes, triamcinolone and dexamethasone can increase *MAT2A* mRNA expression in a time- and dose-dependent manner that can be blocked by actinomycin D (32). In the present study the dexamethasone-induced *MAT2A* mRNA expression was abolished by low-dose MTX treatment in HepG2 cells (Figure 7). This experiment provided additional evidence that low-dose MTX treatments

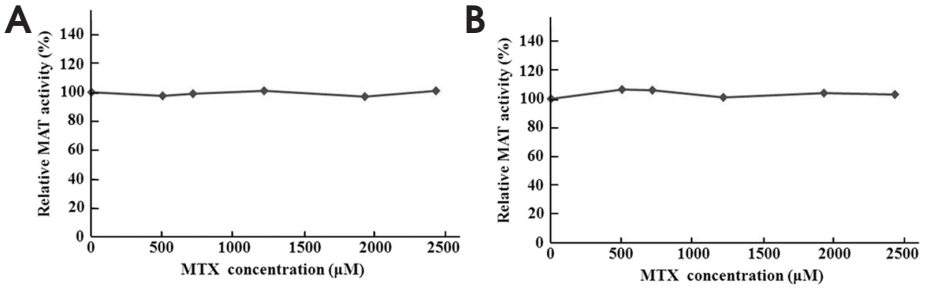

Figure 6. Effects of MTX treatment on MAT activity in cell free protein lysates. Cell free protein lysates from (A) untreated HepG2 cells or (B) healthy mice liver tissue were treated with MTX (from 500 to 2500 μmol/L), and MAT activity was determined as described in Materials and Methods. MTX did not alter MAT activity in cell-free lysates.

transcriptionally inhibit *MAT2A* gene expression.

Only Concomitant Folinate Supplementation Can Alleviate *MAT2A* Inhibition by MTX Treatments

Folinate supplementation alone (100 nmol/L for 72 h) did not affect *MAT2A* gene expression compared with untreated control cells (Figure 8A),

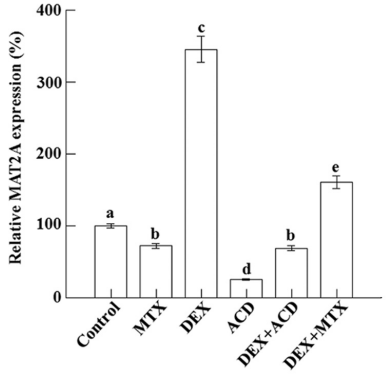

Figure 7. MTX treatment suppressed dexamethasone-induced *MAT2A* gene expression. MTX: cells were treated with 50 nmol/L MTX for 72 h. DEX: treated with 100 nmol/L dexamethasone for 72 h. ACD: treated with 5 μg/mL actinomycin D for 12 h before harvest. DEX + ACD: treated with DEX for 72 h, at 60 h, the 5 μg/mL actinomycin D was added for additional 12 h. DEX + MTX: the cells were cotreated with 50 nmol/L MTX and 100 nmol/L DEX for 72 h. Data are shown as mean ± SE (n = 3). The values with different letters are significantly different ($P < 0.05$).

whereas low-dose MTX inhibited *MAT2A* expression in a time-dependent manner (Figures 8A, B). MTX treatment at 50 nmol/L for 72 h inhibited *MAT2A* gene expression by 26% compared with untreated cells. In cells pretreated with same dose of MTX for 72 h, folinate supplementation (100 nmol/L for 72 h) could not restore *MAT2A* gene expression to the normal level (Figure 8A). In contrast, concomitant folinate rescue along with MTX significantly improved *MAT2A* expression and successfully restored the *MAT2A* level to that of the untreated cells (Figure 8A). No difference in *MAT2A* expression was found between cells that received concomitant MTX and folinate treatments and the control, untreated cells at 24, 48 and 72h. Concomitant folinate rescue was less effective in restoring *MAT2A* mRNA expression when cells were preexposed to MTX (Figure 8B).

In summary, folinate rescue could not restore *S*-adenosylmethionine levels or *MAT2A* expression in cells preexposed to MTX. However, at the same treatment dose, concomitant folinate supplementation with MTX treatment could effectively normalize *MAT2A* mRNA expression and maintain optimal *S*-adenosylmethionine status.

DISCUSSION

MTX is a potent inhibitor of numerous enzymes in folate and methionine metabolism, including dihydrofolate reductase, thymidylate synthase and methylenetetrahydrofolate reductase. MTX also reduces methionine synthase activity due to folate depletion (1,2). Here, we have demonstrated novel findings that an additional key enzyme in the transmethylation cycle—MAT—is inhibited by MTX. At a dose comparable to clinical use in treating human inflammation, MTX inhibits MAT gene and MAT I/III and MAT II protein expression, as well as MAT enzyme activity both *in vitro* and *in vivo* independent of folate depletion. The evidence for these findings was as follows. First, MTX treatment *in vitro* inhibited MAT gene expression, which was the opposite of folate depletion alone, which induced MAT gene expression. Second, MTX treatment decreased MAT protein expression and reduced MAT activity, which also was different from folate depletion. Third, although MTX treatments caused folate depletion and *S*-adenosylmethionine reduction, folate and methionine supplementation could not restore the *S*-adenosylmethionine level in MTX-pretreated cells. Fourth, low-dose MTX inhibited the expressions of *MAT1A* and *MAT2A* genes and MATI/II/III protein, as well as MAT enzyme activities in mouse liver and kidney. Fifth, MTX inhibited glucocorticoid dexamethasone-induced *MAT2A* expression. The inhibition of MAT by low-dose MTX raised concerns as to the adverse effects of *S*-adenosylmethionine depletion and perturbed methylation reactions in the clinical use of MTX.

An interesting observation from this study was that the timing of folinate rescue determined its efficacy on *S*-adenosylmethionine production and *MAT2A* gene expression. Concomitant administration of folinate with the MTX treatment maintained the optimal *S*-adenosylmethionine status, but at the same dose folinate supplementation was unable to ameliorate the *S*-adenosylmethionine status in MTX pretreated cells. If MTX directly inhibited MAT, how did folinate rescue *S*-adenosylmethionine synthesis when it was coadministered with MTX? The observation that concomitant methionine supplementation was completely ineffective in restoring

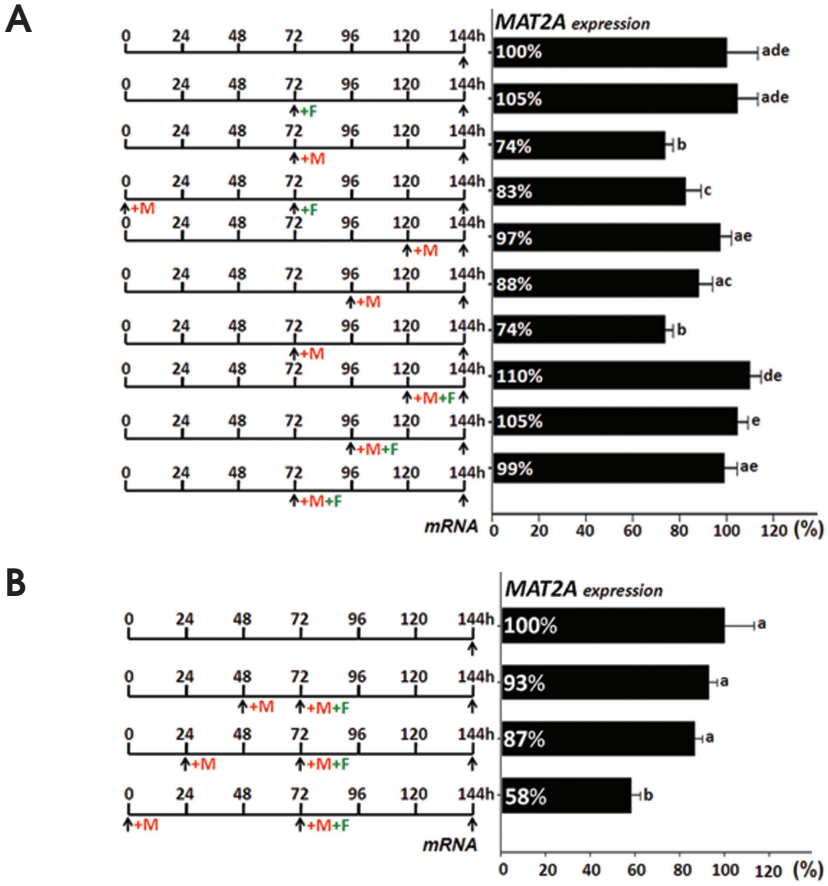

Figure 8. Concurrent folinate supplementation ameliorated MAT2A mRNA reduction by MTX. HepG2 cells received folinate (100 nmol/L, +F), MTX (50 nmol/L, +M), concurrent folinate supplementation with MTX (+M+F), or folinate supplementation after MTX exposure for different time periods. Cells were harvested and *MAT2A* gene expression levels were determined as described in Materials and Methods. Data are shown as mean ± SE. The values with different letters are significantly different (n = 3).

S-adenosylmethionine indicated that concomitant folinate rescue on *S*-adenosylmethionine production was independent of folate-dependent methionine synthesis. We speculated that this was due to the competition between folinate and MTX in their uptake and polyglutamation. Folate receptors have a lower affinity for MTX (0.15–1.7 μmol/L) (34–36) but a high affinity for folic acid (~1 nmol/L) and reduced folates, including folinate (5-formyltetrahydrofolate) and 5-methylTHF (10–40 nmol/L). In clinical conditions, folates have been coadministered with some antifolate drugs to reduce toxicity, and natural folate has been shown to diminish the activity of agents that undergo polyglutamation by suppression of the formation of these agents at the level of folylpolyglutamate synthetase (FPGS) (37). Folinate is a better substrate of FPGS than MTX. As a potent competitor of glutamylation (38), folinate at low concentrations could prevent formation of cytotoxic polyglutamates of MTX. We speculate that when high levels of folinate are coadministered with MTX, they may compete with each other for folate receptors and FPGS; as a result, the inhibition of MAT is alleviated by blocking MTX uptake/retention. This assumption is partly supported by our observation that folinate supplementation rescued *S*-adenosylmethionine production and *MAT* mRNA expression only when it was concurrently administered with MTX; the same high dose folinate failed to restore *S*-adenosylmethionine once those cells had already been exposed to MTX due to MAT inhibition.

Although a single dose of concomitant folinate supplement coadministered with MTX treatment could successfully maintain optimal *S*-adenosylmethionine levels *in vitro*, it was clear that folinate could not rescue *S*-adenosylmethionine production if those cells had already been exposed to MTX. In other words, once MTX had entered and become polyglutamated in the cell, a high dose of folinate was ineffective in alleviating the inhibition of MAT by MTX treatment. It should be noted that clinical low-dose MTX used in immunosuppression is a long-term regimen in that MTX is polyglutamated and gradually accumulates in the cells over a period of time (39). Low-dose MTX is usually given once weekly as an oral dose started at around 7.5 mg weekly and is increased by 2.5 mg over few a months as needed. Folate rescue is usually taken at 5–10 mg weekly or as two tablets of Pregaday daily. Many rheumatologists avoid applying folate rescue on the same day as the MTX, because higher doses of folate may reduce the efficacy of MTX. Folate rescue is commonly recommended to be taken 1–2 d after the MTX (40–43).

Our findings in a cell line and in animal models may or may not reflect what happens in humans. If clinical use of MTX indeed inhibits MAT action in humans, as we have demonstrated in the animal experiments, then *S*-adenosylmethionine–dependent methylation reactions could be impaired in patients continuously taking long-term low-dose MTX. Such perturbations may not be fully rescued by folate supplements. The effects of the clinical use of MTX on *S*-adenosylmethionine and transmethylation reactions during chronic inflammation must be considered more carefully, and studies should be conducted to investigate this in humans.

Studies are also required to evaluate the efficacy of the coadministration of

folinate with MTX in each regimen, specifically for *S*-adenosylmethionine synthesis and methylation reactions under clinical conditions. Low-dose MTX, a traditional folate antagonist, and DMARD administered weekly either alone or as combination therapy, are widely used in treating human rheumatoid arthritis (44). In the blood and liver of humans receiving low-dose MTX treatments, MTX is predominantly in the polyglutamated form with hepatic folate stores depleted compared with baseline specimens (22). Our finding that MTX inhibited MAT activity implied that patients on long-term low-dose MTX are susceptible to hepatic *S*-adenosylmethionine deficiency that may not be rescued by folinate supplementation. Direct inhibition of MAT by MTX could be more harmful than we previously realized because MAT is the required enzyme that catalyzes the only reaction that produces the *S*-adenosylmethionine (7) needed for global methylation reactions and polyamine synthesis. Humans with mutations in the *MAT1A* gene that result in a MAT I/III deficiency have persistent hypermethioninemia without elevated homocysteine (45). Although MTX-treated humans may not have elevated methionine due to the inhibition of methionine synthase, chronic depletion of *S*-adenosylmethionine and perturbed methylation status in the liver by MAT inhibition are of concern. Increased risk of liver pathogeneses has been reported in the case of extreme MAT deficiency in humans and mouse models. In MAT I/II–deficient patients, the irregular formation of *S*-adenosylmethionine in the liver leads to low delivery of *S*-adenosylmethionine from the liver to plasma and tissues (46). In animal models of alcoholic liver injury, depletion of the hepatic *S*-adenosylmethionine level could induce global DNA hypomethylation and increase *c-myc* expression and genome-wide DNA strand breaks (47). These changes are considered to lead to predisposition to pathological conditions of the liver and malignant degeneration (47). Chronic hepatic *S*-adenosylmethionine deficiency in the *MAT1A* knockout (–/–) mouse model was shown to predispose the organ to further injury, spontaneous development of steatohepatitis and hepatocellular carcinoma (48–50). By inhibiting MAT, long-term exposure to MTX might increase the susceptibility to liver injury and/or promote the progression of existing liver diseases. More importantly, because polyglutamated MTXs tend to stay in the hepatocytes and directly inhibit MAT in the liver, the efficacy of folinate supplements with respect to rescuing *S*-adenosylmethionine synthesis and normalizing the methylation reactions would be limited. Direct *S*-adenosylmethionine supplementation might be an option for optimizing *S*-adenosylmethionine status as well as methylation homeostasis in patients taking MTX. However, well-designed clinical trials would be required.

CONCLUSION

In conclusion, we have provided novel *in vitro* and *in vivo* evidence that MTX directly inhibited MAT mRNA expression and reduced MAT I/II/III protein levels, which significantly decreased MAT activity in different tissues. Future studies on the clinical physiological consequences of MAT inhibition by MTX and the potential benefits of *S*-adenosylmethionine supplementation on methyl group homeostasis in clinical MTX therapies are warranted.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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