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Effect of folic acid on methotrexate induction of sulfotransferases in Rats

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Abstract

Our earlier investigation showed that MTX is an inducer of rat and human sulfotransferases. Here we report that folic acid treatment inhibited MTX induction of aryl sulfotransferase (AST-IV) in female rat liver and hydroxysteroid sulfotransferase (STa) in male rat liver. This is important for understanding the clinical mechanisms of MTX.

Keywords

methotrexate; folic acid; induction; sulfotransferase; STa; AST-IV

Introduction

Methotrexate (MTX) is an anti-proliferative and anti-inflammatory drug that has been used in cancer chemotherapy and for treatment of several autoimmune diseases for the last few decades [1; 2]. MTX is structurally very similar to folic acid (FA) and also uses FA receptors (FRs) and reduced folate carriers (RFCs) for transportation into cells. Anti-folate MTX is a strong inhibitor of dihydrofolate reductase (DHFR). MTX inhibition of DHFR prevents regeneration of tetrahydrofolate, resulting in the blockage of the folate cycle [3]. In the cell, MTX is polyglutamated (MTX-$Glu_{2–7}$) by folylpolyglutamate synthetase (FPGS), the same enzyme that catalyzes the polyglutamination of FA [4]. Both MTX and MTX-Glu are potent inhibitors of DHFR, the inhibition of which results in reduced folate pool [3]. The decrease in reduced folate pool indirectly inhibits thymidylate synthase (TS), leading to impaired production of pyrimidine nucleotide dTMP and dUMP. MTX-Glu may also directly inhibit TS [5]. MTX-Glu is poorly effluxed and exhibits prolonged retention and subsequently enhanced cytotoxicity [6; 7].

Reports on MTX induction of drug-metabolizing enzymes are limited. Early investigations from our laboratory demonstrated MTX induction of rat and human sulfotransferases (SULTs) [8; 9]. SULTs catalyze the sulfuryl group transfer from the universal sulfuryl group donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to nucleophiles, which may be endobiotics (i.e., hormones, monoamines, and bile acids) or xenobiotics [10–16]. Sulfation

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of hormones mostly results in their inactivation. Although the steroid-inactivating role of MTX has been shown in several studies [17], the detailed mechanism underlying this inactivation has not been elucidated. MTX induction of rat and human SULTs may be correlated with this role. However, the ability of MTX to induce SULTs is related to its transportation into and retention in cells. MTX is an antifolate and hence competes for FRs and other carrier-dependant mechanisms to enter cells.

In the present study, the role of FA treatment on MTX induction of SULTs was investigated in male and female rat liver. High-dose FA treatment completely inhibited the MTX induction of SULTs in rats of both sexes. This inhibition was demonstrated at mRNA expression, protein expression, and enzyme activity levels by RT-PCR, Western blotting, and enzymatic assays, respectively.

Materials and Methods

Chemicals

MTX was purchased from ICN Pharmaceuticals (Aurora, OH), and FA was obtained from VWR International (West Chester, PA). β-naphthol, [^{14}C]β-naphthol (4.7 mCi/mmol), *p*-nitro-phenyl sulfate (PNPS), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), and [1,2,6,7-^{3}H(*N*)] dehydroepiandrosterone ([^{3}H]DHEA, 60 Ci/mmol) were purchased from Sigma-Aldrich (St. Louis, MO). SDS-polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). Western blot chemiluminescence reagent kits (Super Signal West Pico Stable Peroxide and Super Signal West Pico Luminol/Enhancer solutions) were purchased from Pierce Chemical (Rockford, IL). Nitrocellulose membranes (Immobilon-P; Millipore Corporation, Bedford, MA) used during the Western blot procedure were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Total RNA extraction kit (RNeasy mini protection kit) was supplied by QIAGEN (Valencia, CA). One-step RT-PCR kit was purchased from Promega (Madison, WI). Antibodies against AST-IV and STa were generously provided by Dr. Michael W. Duffel (Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, IA). Antibodies against human DHEA-ST was purchased from Panvera (Madison, WI). Protein assay reagent was purchased from Bio-Rad. All other reagents and chemicals were of the highest analytical grade available.

Animals and Drug Treatment

Male and female Sprague-Dawley rats (Harlan, Indianapolis, IN), 10 to 11 weeks old and 200 to 300 g body weight, were housed at the Oklahoma State University animal resource facility and supplied with standard laboratory diet and water *ad libitum*. Animals were acclimatized for at least 1 week prior to use.

FA treatment—Twelve female rats were divided into four groups, each having three rats. Each group was treated with FA orally at dosages of 5, 50, or 125 mg/kg body weight as a homogeneous suspension in corn oil. The control group was treated only with vehicle. The treatment continued for 2 weeks.

FA and MTX treatment—Rats were divided into four groups, each having three rats of either sex. The groups were treated orally with MTX (1 mg/kg) and FA (125 mg/kg) either alone or in combination for 2 weeks. Drugs were administered as homogeneous suspensions in corn oil. The control group was treated with only vehicle.

The animals were sacrificed 24 h after the final drug treatment. Livers were collected, washed with sterile ice-cold NaCl (0.9%) (w/v) solution, and snap frozen. Samples were stored at −80°C until use.

Cytosol Preparation

Liver homogenates were prepared with 50 mM Tris buffer containing 0.25 M sucrose (pH 7.5) and centrifuged at 100000× g for 1 h at 4°C. Cytosol aliquots were collected and preserved at −80°C for enzymatic assay and Western blot analysis.

Enzyme Assays

Two different enzyme assay methods were used. The linearity of enzymatic reactions, which were incubated for 30 min, was verified using varying concentrations of protein and substrate.

PNPS assay—2-Naphthol sulfation activity in rat liver cytosols (AST-IV) was determined as previously described [18; 19]. Briefly, sulfation activity was determined in a reaction mixture containing 50 mM Tris buffer (pH 6.2) 5 mM PNPS, 20 μM PAPS, and 0.1 mM β-naphthol. Rat liver cytosols were used as the enzyme source in a total reaction volume of 250 μl.

Radioactive assay—DHEA sulfation activity in rat liver (STa) was determined using a radioactive assay method previously described [20]. Fifty micrograms of protein from rat liver cytosol was used for this assay in a reaction mixture containing [^{3}H] DHEA (diluted to 0.4 Ci/mmol; 2 μM final concentration) as a substrate and 20 μM PAPS as a cosubstrate. The data collected from the PNPS assay and radioactive assay of rat livers represent averages of results from three independent experiments from three rats. The data collected from the assay of cell cultures represent averages of two independent experiments. PAPS was eliminated from the controls in both assays.

Western Blot Analysis

Cytosol protein from liver (10 μg) was applied to a 12% (w/v) polyacrylamide gel and electrophoresed (Novex, San Diego, CA) at 200 V. Separated protein bands were transferred onto nitrocellulose membranes overnight at 40 V. All membranes were blocked in TBST (50 mM Tris (pH 7.5), 150 mM NaCl, 0.05% [v/v] Tween-20) containing 5% (w/v) dry milk for 1 h on a shaker at room temperature. Rat cytosol membranes were incubated with either rabbit anti-rat AST-IV or rabbit anti-rat STa antibodies at 1:5000 in TBST containing 5% (w/v) dry milk for 2 h on a shaker at room temperature. After incubation, all membranes were washed with TBST four times for 15 min and incubated in secondary antibody (horseradish peroxidase-conjugated Immuno-Pure goat anti-rabbit IgG; H+L) at 1:5000 in the same buffer for 2 h. The membranes were washed with TBST four times for 15 min and then with TBS three times for 5 min. Fluorescent bands were developed with 1 ml of substrate containing the same volume of each Super Signal West Pico Luminol Enhancer solution and Super Signal West Pico Stable Peroxidase solution at room temperature for 5 min. X-ray films were exposed to the membrane and then developed. Films were scanned, and densitometric analysis was performed with a Gel Documentation and Analysis System from Advanced American Biotechnology and with AAB software (Fullerton, CA).

Extraction of Total RNA and RT-PCR

Total RNA was extracted from liver using RNeasy mini protection kit from QIAGEN according to the supplier's guidelines. The concentration and purity of the extracted RNA were checked spectrophotometrically by measuring 260/280 absorption ratios. The primer

pair for AST-IV was designed in our laboratory using Gene Fisher primer designing and Multialignment software. The 299-bp AST-IV cDNA was synthesized using the following primer pair: forward primer (FP), 5' GTGTCCTATGGGTCGTGGTA-3'; and reverse primer (RP), 5'-TTCTGGGCTACAGTGAAGGTA-3' (**GenBank accession no. X52883**) [8]. The 264-bp STa cDNA was synthesized using the following primer pair: FP 5'-TCCTCAAAGGATATGTTCCG-3'; RP 5' CAGTTCCTTCTCCATGAGAT-3' (**GenBank accession no. M33329**) [8]. The specificity of all primers was tested using BLAST, the National Center for Biotechnology Information Open Reading Frame software. cDNA synthesis from 1 μg of total RNA from liver was performed in a 50-μl reaction mixture. The concentrations of the different ingredients were used according to the supplier's protocol. For controls, 500 bp cDNA of rat β-actin was synthesized from the same amount of RNA from respective sources. The primer pair—FP 5'- GATGTACGTAGCCATCCA-3'; RP 5'-GTGCCAACCAGACAGCA-3'— used for the synthesis of rat β-actin cDNA was designed in our laboratory using the software mentioned above.

Statistical Analysis

Student's *t* test was performed to calculate the statistical significance of differences between the means of control and treated groups.

Results

2-Naphthol and DHEA sulfation activities in the livers of female rats following MTX and FA treatment

Treatment with different dosages of FA did not produce significant changes in either 2-naphthol or DHEA sulfation activities in the livers of female rats (Fig. 2). The main isoforms in rat liver are AST-IV and STa, which catalyze 2-naphthol and DHEA sulfation, respectively. Moreover, female rat liver expresses higher amounts of STa protein than AST-IV protein. This was confirmed by the present Western blot experiment (Fig. 2), which revealed no significant changes in either protein level after FA treatment.

2-Naphthol and DHEA sulfation activities in the livers of male and female rats following MTX and FA treatment

MTX treatment at 1 mg/kg for 2 weeks significantly increased 2-naphthol sulfation activity (~2 fold, $p<0.001$) in female rat liver and DHEA sulfation (STa) activity (~3 fold, $p<0.001$) in male rat liver (Fig. 3). FA (125 mg/kg) completely inhibited MTX (1 mg/kg) induction of both enzyme activities in the livers of both sexes, restoring activities to control levels (Fig. 3). FA treatment alone had no significant effect on SULTs, except in male rat liver, where it moderately increased STa activity (Fig. 3).

Western blot and RT-PCR analyses of AST-IV and STa expression in the liver of male and female rats following MTX and FA treatment

MTX treatment (1 mg/kg for 2 wks) significantly increased STa and AST-IV protein levels in the livers of male and female rats, respectively (Fig. 4). FA treatment (125 mg/kg for 2 wks) completely inhibited the MTX induction of the expression of both proteins, as demonstrated by Western blot (Fig. 4A) and densitometry analysis (Fig. 4C). RT-PCR results (Fig. 4B) also demonstrated that MTX treatment significantly increased STa and AST-IV mRNA expression in male and female rat liver. FA treatment completely inhibited this MTX-induced increase, as shown by RT-PCR densitometry analysis (Fig. 4C). These results indicate that SULTs protein and mRNA expression and expression inducibility in rat liver are sex dependant. The Western blot and RT-PCR results are consistent and can be correlated with the corresponding enzyme activity results (Fig. 3)

Discussion

The present investigation demonstrates that FA treatment completely inhibited the MTX induction of STa in male rat liver and AST-IV in female rat liver. This finding was demonstrated at enzyme activity, protein expression, and mRNA expression levels by enzymatic assay, Western blotting, and RT-PCR, respectively. Hormonal induction and regulation of SULTs is well known. Earlier investigations from our laboratory showed that MTX induces rat and human SULTs [8; 9]. MTX is an apoptosis inducer and is cytotoxic. The rescuing property of folate and folinic acid against MTX toxicity has been reported [21–23].

MTX is structurally similar to FA, so it competes with FA for FRs and RFCs [24; 25]. The inhibitory effect of FA on MTX induction of SULTs suggests two possibilities: (1) FA effectively out-competes MTX to bind FRs and/or RFC, thereby blocking MTX entry into cells; and (2) high-dosage FA treatment significantly down regulates FR expression in the apical (luminal) surface of epithelial cells [26], thereby effectively blocking MTX entry into cells.

In the present study, drugs were administered orally; thus, intestinal epithelial cell membranes represent the first site to be exposed to the drugs. RFCs are highly expressed in small intestine [27]. FRα-mediated transport of MTX is also evident [28]. Reports on low folate induced upregulation of FR support the inverse relationship between FA concentration and FR expression [29]. In either case, MTX concentration in circulation probably decreases, resulting in low MTX levels in liver. In the present investigation, the FA-mediated restoration of SULTs mRNA and protein content indicates that less MTX was available to induce SULTs gene expression in liver.

The MTX-mediated induction of SULTs we observed in this investigation and reported earlier [8; 9] could be correlated with the steroid inactivating role of MTX [30]. The anti-proliferative role of MTX may be partly attributed to its steroid inactivating properties possibly via SULTs induction and increased steroid sulfation. The anti-proliferative role of MTX may also be attributed to the inhibitory effects of MTX on DHFR, TS, and other pathways leading to folate cycle block and growth arrest. It is well known that steroids play important roles in cellular growth and differentiation. It is also known that, besides FRs in the folate pool, FR expression is under the control of hormonal regulation. Estrogen (17-β estradiol, E2) represses FRα expression, whereas anti-estrogen tamoxifen (TAM) reverses this repression [30]. This suggests that an inverse relationship exists between FRs and estrogen receptors (ERs), and subsequently active steroid levels. In the present investigation, MTX induction of SULTs might result in inactivation of steroids [30], leading to increased FR expression, which subsequently leads to increased MTX transport and intracellular MTX concentration. Such positive feedback may lead to the increased susceptibility of MTX to intracellular polyglutamination and sustained retention, which is crucial for its cytotoxicity [31]. But when FA is administered with MTX, SULTs induction is completely repressed, resulting in probable restoration of steroids and subsequent decreased expression of FR. Decreased FR expression results in decreased transport of MTX. Thus, FA manifests its protective response over MTX, as evidenced by several investigations [32; 33].

This work suggests that FA can prevent the induction of SULTs by MTX in an animal model. This information may contribute to the understanding of the biological functions of SULTs and the mechanisms of MTX cytotoxicity. The knowledge may also help in the better clinical use of this drug. It would be interesting to investigate the possible physiological consequences of SULTs induction by MTX and whether this induction is related to the anti-proliferative and anti-cancer properties of MTX.

Abbreviations

SULT	sulfotransferase
STa	rat hydroxysteroid sulfotransferase a
AST-IV	rat aryl sulfotransferase IV
MTX	methotrexate
FA	folic acid
FR	folate receptor

Acknowledgments

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References

1. Preet Singh Y, Aggarwal A, Misra R, Agarwal V. Clin. Rheumatol. 2007; 26:84. [PubMed: 16636937]
2. Hider SL, Bruce IN, Thomson W. Rheumatology (Oxford). 2007; 46:1520. [PubMed: 17586865]
3. Widemann BC, Balis FM, Adamson PC. Clin. Chem. 1999; 45:223. [PubMed: 9931044]
4. Goldman ID, Zhao R. Semin. Oncol. 2002; 29:3. [PubMed: 12571805]
5. Takemura Y, Jackman AL. Anticancer Drugs. 1997; 8:3. [PubMed: 9147608]
6. Galivan J, Pupons A, Rhee MS. Cancer Res. 1986; 46:670. [PubMed: 2416430]
7. Kremer JM, Galivan J, Streckfuss A, Kamen B. Arthritis Rheum. 1986; 29:832. [PubMed: 2427090]
8. Maiti S, Chen G. Arch. Biochem. Biophys. 2003; 418:161. [PubMed: 14522587]
9. Chen X, Baker SM, Chen G. J. Appl. Toxicol. 2005; 25:354. [PubMed: 15986412]
10. Wang LQ, James MO. Curr. Drug Metab. 2006; 7:83. [PubMed: 16454694]
11. Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, McManus ME. Toxicol. Sci. 2006; 90:5. [PubMed: 16322073]
12. Runge-Morris M, Kocarek TA. Curr. Drug Metab. 2005; 6:299. [PubMed: 16101570]
13. Chapman E, Best MD, Hanson SR, Wong CH. Angew. Chem. Int. Ed. Engl. 2004; 43:3526. [PubMed: 15293241]
14. Coughtrie MW. Pharmacogenomics J. 2002; 2:297. [PubMed: 12439736]
15. Duffel MW, Marshal AD, McPhie P, Sharma V, Jakoby WB. Drug Metab. Rev. 2001; 33:369. [PubMed: 11768773]
16. Glatt H, Engelke CE, Pabel U, Teubner W, Jones AL, Coughtrie MW, Andrae U, Falany CN, Meinl W. Toxicol. Lett. 2000; 112:113–341.
17. Davies NM, McLachlan AJ, Day RO, Williams KM. Clin. Pharmacokinet. 2000; 38:225. [PubMed: 10749518]
18. Chen G, Battaglia E, Senay C, Falany CN, Radominska-Pandya A. Protein Sci. 1999; 8:2151. [PubMed: 10548061]
19. Chen G, Chen X. J. Biol. Chem. 2003; 16:16.
20. Chen G, Zhang D, Jing N, Yin S, Falany CN, Radominska-Pandya A. Toxicol. Appl. Pharmacol. 2003; 187:186. [PubMed: 12662902]
21. Assaraf YG. Cancer Metastasis Rev. 2007; 26:153. [PubMed: 17333344]
22. Jansen G, van der Heijden J, Oerlemans R, Lems WF, Ifergan I, Scheper RJ, Assaraf YG, Dijkmans BA. Arthritis Rheum. 2004; 50:2130. [PubMed: 15248210]
23. McGuire JJ. Curr. Pharm. Des. 2003; 9:2593. [PubMed: 14529544]
24. Gangjee A, Kurup S, Namjoshi O. Curr. Pharm. Des. 2007; 13:609. [PubMed: 17346178]

25. Matherly LH, Hou Z, Deng Y. Cancer Metastasis Rev. 2007; 26:111. [PubMed: 17334909]
26. Kane MA, Portillo RM, Elwood PC, Antony AC, Kolhouse JF. J. Biol. Chem. 1986; 261:44. [PubMed: 3941083]
27. Strum WB. J. Pharmacol. Exp. Ther. 1981; 216:329. [PubMed: 6109773]
28. Matherly LH, Goldman DI. Vitam. Horm. 2003; 66:403. [PubMed: 12852262]
29. Zhu WY, Bunni M, Priest DG, DiCapua JL, Dressler JM, Chen Z, Melera PW. J. Exp. The.r Oncol. 2002; 2:264.
30. Corrigan CJ, Shiner R, Shakur BH, Ind PW. Clin. Exp. Allergy. 2003; 33:1090. [PubMed: 12911783]
31. Clarke L, Waxman DJ. Arch. Biochem. Biophys. 1987; 256:585. [PubMed: 3619447]
32. van Ede AE, Laan RF, Rood MJ, Huizinga TW, van de Laa MA, van Denderen CJ, Westgeest TA, Romme TC, de Rooij DJ, Jacobs MJ, de Boo TM, van der Wilt GJ, Severens JL, Hartman M, Krabbe PF, Dijkmans BA, Breedveld FC, van de Putte LB. Arthritis Rheum. 2001; 44:1515. [PubMed: 11465701]
33. Dijkmans BA. Br. J. Rheumatol. 1995; 34:1172. [PubMed: 8608361]

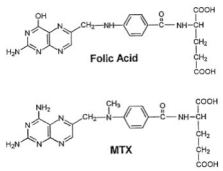

Figure 1.
Chemical structures of methotrexate (4-amino-N^{10}-methylpteroyl-L-glutamic acid, MTX) and folic acid (pteroyl-L-glutamic acid, FA).

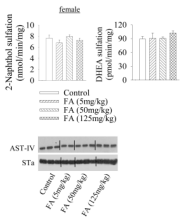

Figure 2.

2-Naphthol and DHEA sulfation activities in female rat liver following FA treatment for 2 weeks. (A) Histograms showing 2-Naphthol and DHEA sulfation activities following FA treatment. (B) Western blots showing the effects of FA on AST-IV) and STa in liver. FA was administered orally to rats as a homogeneous suspension in corn oil. Control animals were treated with only vehicle. Fifty micrograms of liver cytosolic protein was used in the PNPS and radioactive assays to measure 2-naphthol and DHEA sulfation activities, respectively. Ten micrograms of cytosolic protein was subjected to 12% bis-acrylamide–SDS gel electrophoresis for subsequent Western blotting.

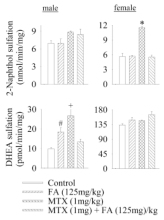

Figure 3.

2-Naphthol and DHEA sulfation activities in male and female rat liver following MTX and FA treatment. Drugs were administered orally to rats either alone or in combination as homogeneous suspensions in corn oil. Control animals were treated with only vehicle. Fifty micrograms of liver cytosolic protein was used in the PNPS and radioactive assays to measure 2-naphthol and DHEA sulfation activities, respectively. Values are means ± SEM, $^{\#}P<0.05$; $^{+}P<0.01$; $^{*}P<0.001$.

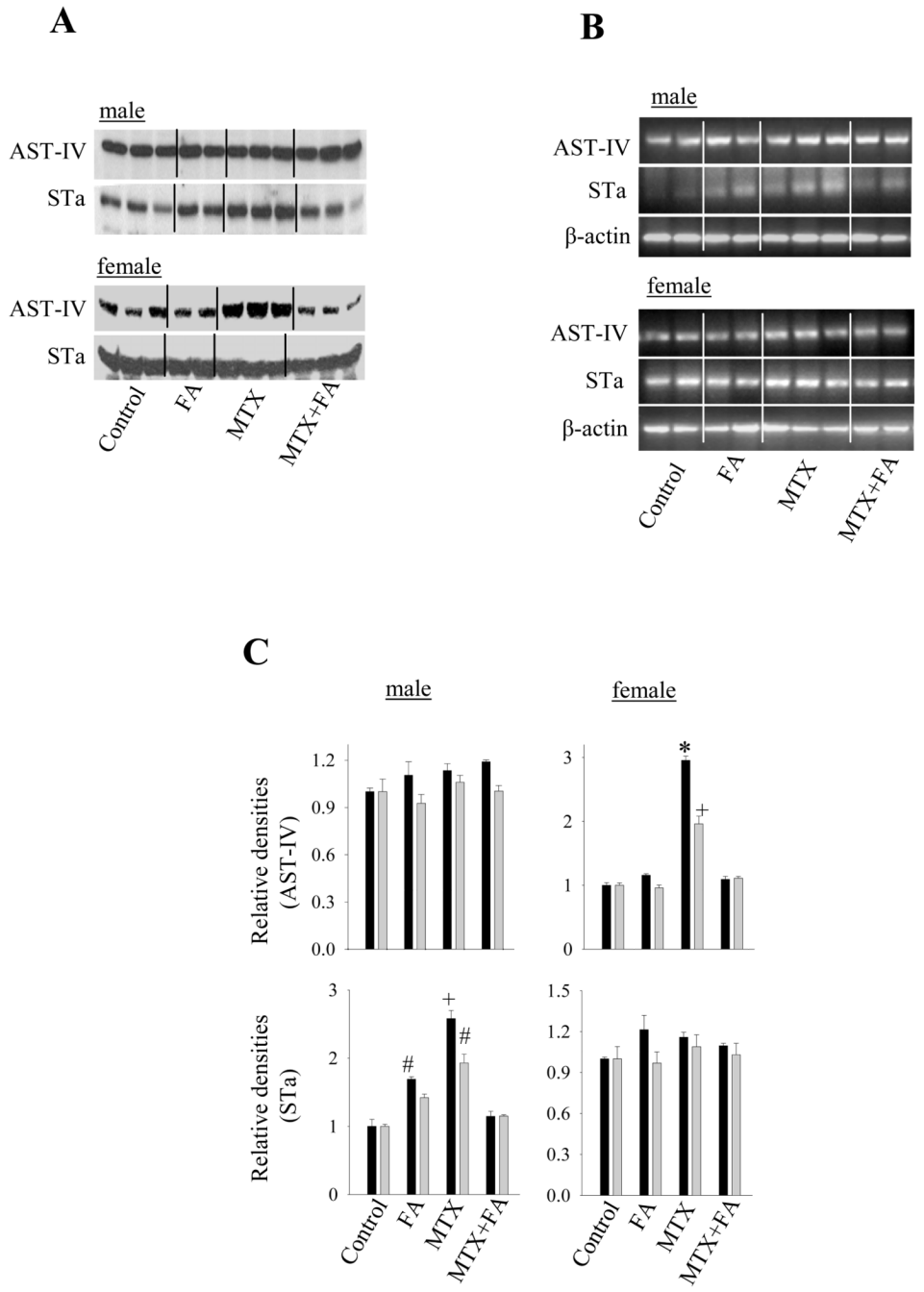

Figure 4.

Western blot and RT-PCR analyses of AST-IV and STa protein and mRNA expression in male and female rat liver following MTX and FA treatment. (A) Representative Western blot showing the effects of MTX and FA on AST-IV and STa protein expression. (B) RT-PCR analysis showing the effects of MTX and FA on AST-IV and STa mRNA expression. Densitometry analysis data are shown in (C). Drugs were administered orally to rats either alone or in combination as homogeneous suspensions in corn oil. Control animals were treated with only vehicle. Ten micrograms of cytosolic protein was subjected to 12% bis-acrylamide–SDS gel electrophoresis for the Western blot experiment. For the RT-PCR experiment, cDNA was synthesized from 1 μg of total RNA from liver in the presence of a

gene-specific primer. The reaction was carried out in a total reaction mixture volume of 50 μl; 10 μl of product was subjected to 2% agarose gel electrophoresis. The semi-quantitative densitometry data in (C) are expressed as relative to the corresponding control value using Advanced American Biotechnology Gel Documentation System and its related software. The solid bars in (C) represent Western blot results and the grey bars represent RT-PCR results. Values are means ± SEM, $^{\#}P<0.05$; $^{+}P<0.01$; $^{*}P<0.001$.