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EFFECT OF METHIONINE SULFOXIMINE ON METHYLATION OF GUANINE RESIDUES IN ASTROGLIAL TRANSFER RIBONUCLEIC ACIDS

OTTO Z. SELLINGER AND ORCHID DER[1]
Laboratory of Neurochemistry
Mental Health Research Institute
University of Michigan Medical Center
Ann Arbor, Michigan 48109

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Culture-grown astrocytes derived from 3-day-old rat brain were incubated in the presence of [^{3}H]guanosine and of the convulsant agent L-methionine-*dl*-sulfoximine (MSO). The resulting [^{3}H]tRNA was purified from control and MSO-exposed cells at several time points during the incubation and was hydrolyzed to [^{3}H]guanine and four [^{3}H]methyl guanines which were separated by high pressure liquid chromatography. Three of the four [^{3}H]methyl guanines were more highly labeled in the [^{3}H]tRNA of the MSO-exposed cells, relative to that of the control cells throughout the entire incubation period. The findings extend to cultured astrocytes, the stimulatory effect of MSO on the methylation of neural tRNA guanines, previously observed both in vitro using [^{14}C]*S*-adenosyl-L-methionine and in vivo using [*methyl*-^{3}H]L-methionine.

INTRODUCTION

It is generally recognized that in non-neural cells post-transcriptional modifications of tRNA structure contribute in a large, but as yet incompletely understood, measure to make protein synthesis cell-specific. Since the processing of cerebral tRNA remains active throughout the first month of postnatal development (1–5) and since, as we have recently shown,

[1] Present address: Department of Pharmacology, The University of Wisconsin Center for Health Sciences, Madison, Wisconsin 53706.

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cerebral tRNA-modifying reactions are affected in vivo and their pattern of specificity altered by the convulsant agent L-methionine-*dl*-sulfoximine (MSO) (6), it appeared of interest to examine effects of MSO on the biosynthesis of neural tRNA. We chose to study this (7) in culture-grown astroglial cells (astrocytes) derived from the 3-day-old rat cerebral cortex (8), primarily because there is substantial cytological evidence that MSO affects the glial compartment of brain more significantly than the neuronal one (9, 10), and also because glutamine synthetase, an enzyme activity strongly inhibited by MSO in brain tissue (11), is now known to be an exclusively glial protein (12).

EXPERIMENTAL PROCEDURE

Astrocyte Culture. The culture procedure was as described previously (8), the cells being grown in plates 75 cm^2 containing 10 ml of growth medium (7). Briefly, forebrains of 3 to 4-day-old rats were dissected under sterile conditions, minced, and trypsinized in 0.25% trypsin in Earle's balanced salt solution (BSS). The suspension was centrifuged, and the pellet was washed once in BSS, and diluted to yield 10 plates per forebrain. Cells were grown in a medium composed of 10% fetal bovine serum in minimum essential medium, with either Earle's or Hanks' BSS. The medium was supplemented with 100 units penicillin, 100 μg streptomycin, and 100 units polymyxin per ml medium. Cells were grown in a National incubator, in an atmosphere of 95% air and 5% CO_2. The medium was changed on days 4, 8, and 11, and the cells harvested by days 17–19.

Exposure of Astrocytes to [^{3}H]Guanosine and MSO. At specified intervals (see Results) preceding the harvesting of the cells, the growth medium received 62.5 μCi of [^{3}H]guanosine (14.5 Ci/mM). Half the plates also received 10 mg of MSO/plate 2 hr before they were harvested and their RNA extracted. Under these conditions, the cells appeared unchanged and no evidence of toxicity was noted on inspection. A systematic study of the effect of MSO on cell viability was not intended in this study.

tRNA Extraction. The extraction procedure has been described in detail previously (7). Briefly, the medium was decanted and the cells rinsed with phosphate-buffered saline containing, in g/liter: $CaCl_2$ and $MgCl_2$, 0.1; KCl and KH_2PO_4, 0.2, NaCl, 8; and Na_2HPO_4, 215. Equal volumes of TNE buffer (0.01 M Tris HCl, pH 7.8, 0.1 M NaCl, 0.01 M Na EDTA) in diethylpyrocarbonate-treated water containing 10 mM $MgCl_2$ and β-mercaptoethanol, pH 8.2, were mixed with TNE-saturated phenol and used to transfer the cells into test tubes to which were added 5 ml of $CHCl_3$ followed by vigorous agitation and incubation, with shaking, at 60°C for 40 min. Three volumes of 95% ethanol were added to the aqueous phase resulting from a 9000 *g*, 15-min centrifugation of the above suspension, and the RNA was allowed to collect overnight at −20°C.

Hydrolysis of tRNA and Separation of its Guanine and Methyl Guanines. A tRNA fraction was obtained following suspension of the centrifuged RNA pellet in 0.01 M Tris HCl, containing 0.01 M $MgCl_2$, 0.001 M EDTA, and 0.05 M NaCl, pH 7.5 (buffer A), and chromatography on DEAE-cellulose (13). The tRNA fraction was eluted with 0.7 M NaCl. Following dialysis to remove NaCl and lyophilization, the tRNA was hydrolyzed according to Klagsbrun (14). Separation of the guanine from 1-methyl-, 7-methyl-, N^2-methyl, and N^2_2-dimethyl guanine was accomplished using HPLC (7, 15).

tRNA Aminoacylation. The rat liver or brain aminoacyl–tRNA synthetases were prepared as described (7). Aminoacylation of astrocyte tRNA was carried out in a reaction volume of 1 ml containing, in order of addition: 1 μCi [^{3}H]lysine or phenylalanine; 0.4 M Tris HCl, pH 7.5; 20 mM (for lysine) or 50 mM (for phenylalanine) KCl; 10 mM (for lysine) or 20 mM (for phenylalanine) $MgCl_2$; 1 mM (for lysine) or 4 mM (for phenylalanine) ATP; 0.6 mM CTP; 2 mM β-mercaptoethanol; 0.3–0.5 A_{260} units of tRNA; and 0.4–0.8 mg of aminoacyl–tRNA synthetase protein. Incubation was for 10 min at 37°C. The procedure of Yang and Novelli (16) was used to isolate and quantitate the [^{3}H]aminoacyl–tRNA.

RESULTS

RNA Yield and tRNA Aminoacylation. Growing astrocytes for 17 days yielded 1.4–1.5 A_{260} units per culture dish. The aminoacylating capacity of the tRNA extracted from ten control and ten MSO-exposed dishes is compared in Table I. Lysine acceptance was between 60- and 100-fold higher than phenylalanine acceptance when the hepatic tRNA–aminoacyl synthetase was used; acceptance values were lower when the tRNA–aminoacyl synthetase preparation was derived from brain tissue.

Incorporation of [^{3}H]Guanosine into Astroglial RNA and tRNA. The labeling of total RNA as a function of the exposure time (1–8 hr) to [^{3}H]guanosine and to MSO (2 hr) is shown in Figure 1, while Figure 2 depicts the specific radioactivity (sra) of the [^{3}H]tRNA fraction. Figure 1 indicates a negligible amount of total RNA labeling during the initial 3–3.5 hr, while Figure 2 illustrates a gradual increase in the sra of [^{3}H]tRNA between 2.5 and 3 hr, followed by a steep labeling spurt in both control and MSO-exposed cells between 3 and 4 hr. The figures also show a uniformly higher incorporation into total RNA in control cells and higher than control [^{3}H]tRNA sra values in the MSO-exposed cells during the second half of the [^{3}H]guanosine incorporation process.

Effect of MSO on Incorporation of [^{3}H]Guanosine into Guanine and Methyl Guanine Residues of Glial tRNA. Following hydrolysis of [^{3}H]tRNA at each time period indicated in Figure 2, its [^{3}H]guanine residues were separated from its [^{3}H]methyl guanine residues by HPLC (15). Figure 3 illustrates the distribution of the radioactivity at the two early time points among four individual [^{3}H]methyl guanines and in their sum ($\sum$ mG). Initially, [^{3}H]methyl guanines represented a much higher proportion of the [^{3}H]tRNA formed in the MSO-treated than in the control cells, relative to [^{3}H]guanine. This "hypermethylating" effect of MSO was particularly evident at the level of the two *N*-methylated guanines, which accounted for 10% of the total radioactivity in the MSO-treated, as against 1.6% in the control, cells at 1.5–1.75 hours. Conversely, at the same time, [^{3}H]1-methyl guanine represented 2.9 and 0.6% of the

[^{3}H]tRNA formed in the control and the MSO-exposed cells, respectively. These findings thus reveal not only marked differences in the early labeling pattern of the individual [^{3}H]tRNA methyl guanines between control and MSO-treated astrocytes, but they also confirm the previously described ability of this agent to alter the specificity of neural tRNA methyltransferases (6, 17).

By 8 hr of incubation all four tRNA guanine residues were affected by MSO (Table II). Particularly striking in this respect was the more than twofold increase over control values in 1-methyl guanine and N_2^2-dimethyl guanine radiactivity.

DISCUSSION

The exposure of the astrocytes to [^{3}H]guanosine and, in the case of the experimental group of cells, to 1 mg/ml MSO for 2 hr as well, resulted in the labeling profile for total cellular RNA shown in Figure 1. Several reasons may account for the observed temporal delay (3–3.5 hr) in RNA labeling, most of which can only be speculated upon, since very little is known about glial transport, metabolism, and the tRNA precursor properties of guanosine. It is known, however, that brain has an active system of enzymes involved in nucleoside interconversions and phosphorylation (18) and that it is rich in a guanosine–inosine-specific phosphorylase (19, 20) which is distinct from the nucleoside phosphorylase utilizing adenosine. It is not known whether in neural cells, as is likely to be the case in Novikoff hepatoma cells (21), this enzyme exists in more than one

TABLE I
AMINOACYLATION OF ASTROGLIAL tRNA BY LIVER AND BRAIN tRNA AMINOACYLTRANSFERASES

	Liver enzyme[a]		Brain enzyme[a]	
Amino acid	Control tRNA[b]	MSO tRNA[b]	Control tRNA[b]	MSO tRNA[b]
Lysine	12.9	16.8	8.4	11.5
Phenylalanine	0.21	0.18	0.06	0.05

[a] Values (averages of two separate experiments) are in pmol/A_{260}/mg protein. Liver and brain enzyme refer to tRNA aminoacyltransferase preparations partially purified (7) from adult rat liver and brain.

[b] Control and MSO tRNA refer to tRNA isolated, respectively, from normal astrocytes and from astrocytes exposed for 2 hr to 1 mg/liter of MSO.

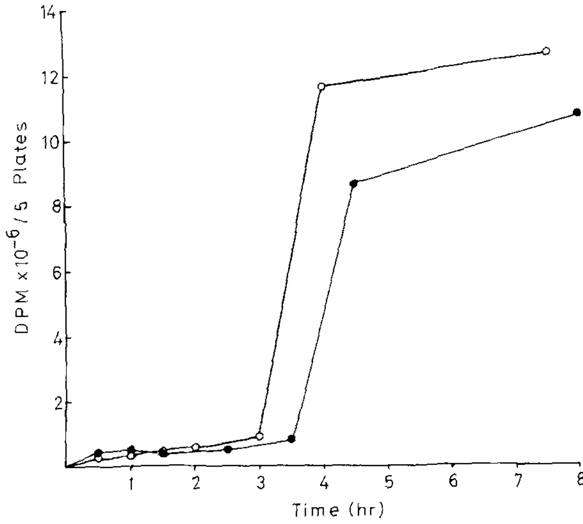

Fig. 1. The incorporation of [^{3}H]guanosine into astrocyte ribonucleic acids. Astrocytes grown for 17 days were incubated in the presence of [^{3}H]guanosine and MSO, as described in Experimental Procedure. At the times indicated and for the most part with half-hour displacements between the control and the MSO-exposed cells (for reasons of ease of manipulation), the cells of 10 plates were subjected to RNA extraction and the determination of its radioactivity. ○ = control cells; ● = cells exposed to MSO for 2 hr.

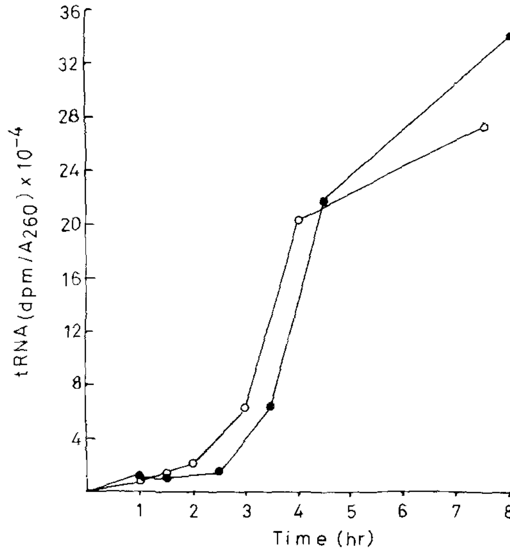

Fig. 2. The specific radioactivity of [^{3}H]tRNA. The [^{3}H]tRNA fraction was obtained from the [^{3}H]RNA depicted in Figure 1 and its radioactivity and A_{260} content were determined, as indicated. ○ = control cells; ● = cells exposed to MSO for 2 hr.

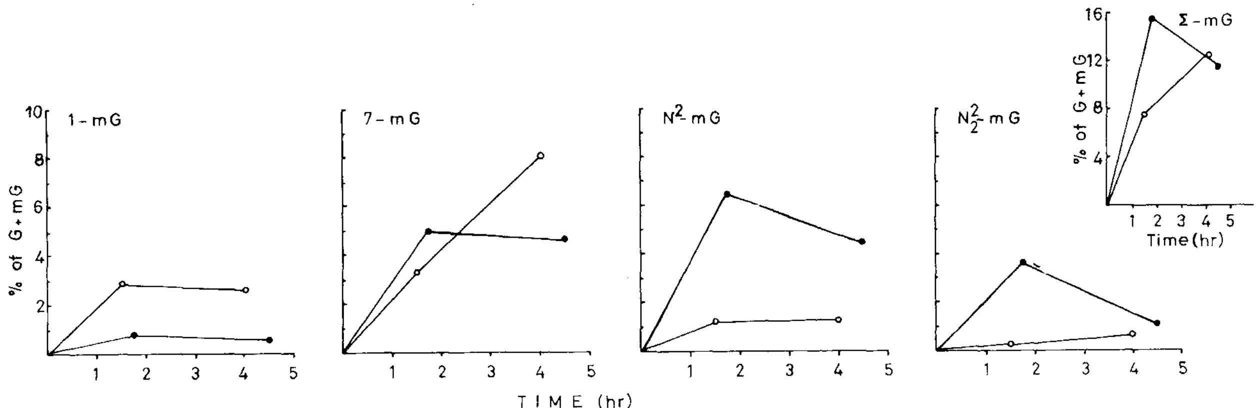

FIG. 3. The distribution of radioactivity among the [^{3}H]methyl guanines of [^{3}H]tRNA. The [^{3}H]tRNA obtained from control and MSO-exposed cells (note early time of 1.5 hr for control and 1.75 hr for MSO-exposed cells) was hydrolyzed and its [^{3}H]guanine separated from its [^{3}H]methyl guanines by high-pressure liquid chromatography (17). The radioactivity in each [^{3}H]methyl guanine is expressed as percentage of the dpm in [^{3}H]guanine + [^{3}H]methyl guanines. The dpm in the sum of the [^{3}H]methyl guanines is shown in the inset panel. 1-mG: 1-methyl guanine; N^2- and N^2_2-mG = N^2- and N^2_2-dimethyl guanine, $\sum$ mG = all four methyl guanines, G = guanine. ○ = control cells; ● = cells exposed to MSO for 2 hr.

TABLE II
LATE EFFECT OF MSO ON INCORPORATION OF [^{3}H]GUANOSINE INTO ASTROGLIAL tRNA[a]

Astrocytes	A_{260}[b]	^{3}[H]-tRNA (10^{-6})	sra[c] (10^{-5})	Radioactivity (dpm): Guanine	1-mG (10^{-4})	7-mG (10^{-4})	N^2-mG (10^{-4})	N_2^2-mG (10^{-4})	Recovery (%)[d]
Control	2.72	1.98	7.27	175 (90.8)	2.94 (1.52)	6.94 (3.60)	5.43 (2.81)	2.41 (1.24)	97.5
MSO	2.92	2.90	9.94	256 (87.9)	6.91 (2.38)	13.6 (4.71)	9.13 (3.14)	5.53 (1.90)	100.2
$\left(\frac{\text{MSO}}{\text{Control}}\right) \times 100$	—	+46.2	+36.6	+46	+123	+95	+67	+123	—

[a] MSO was present for 2 hr, [^{3}H]guanosine for 7.5 and 8 hr, respectively, in control and MSO groups. The MSO values are not corrected for the 30-min difference in exposure time to [^{3}H]guanosine between the two cell groups. For details, see Experimental Procedure. The results refer to data obtained in three different experiments. The values are the means. Differences between experiments were under 20%.
[b] As tRNA, before acid hydrolysis.
[c] sra = specific radioactivity in dpm/A_{260}.
[d] dpm × [(guanine + methyl guanines), after HPLC]/([^{3}H]tRNA, before hydrolysis) × 100. The numbers in parentheses refer to percent of G + mG, set to 100%

intracellular location, i.e., in the soluble cell sap and in the plasma membrane.

The formation of glial RNA from [^{3}H]guanosine was recently reported by Yanagihara (22), who found it to be higher than in neurons and who also noted a 6.5-fold higher uptake of [^{3}H]guanosine by bulk-isolated glial cells, relative to neuronal perikarya. The labeling of astrocytic tRNA (Figure 2), recently examined in the 3-day-old rat brain in vivo (7), began sluggishly, but from 3 to 3.5 hr onward its specific radioactivity (sra) increased by more than threefold in 1 hr. The effect of MSO appeared to be biphasic in nature, i.e., [^{3}H]tRNA formation was lower than in control cells up to 3.5 hr, becoming higher in the second half of the labeling period and reaching 20% higher than control sra values by 8 hr. It is not possible at this time to fully assess the reasons for the biphasic effect of MSO since it is not possible to correlate its action on the [^{3}H]guanosine incorporating process to its inracellular concentration. It is likely, however, that MSO entered the astrocytes with no difficulty since, using [^{3}H]MSO, we showed its entry into the brain to be relatively unencumbered under a number of different experimental conditions (23, 24). On the cellular level, a glial locale for its early action, i.e., during the 3 hr following its administration to rodents, has been suggested. We showed an apparent concentration of [^{3}H]MSO in bulk-isolated astrocytes during the first hour following its intrathecal administration to 18-day-old rats (25), while, more recently, Phelps (9) reported an accumulation of glycogen in astrocytes following MSO, a finding further explored by Berel et al. (26) and Hevor and Gayet (27).

The early effects of MSO on the methylation of the four individual tRNA guanines were of three different sorts: higher than control for N^2-methyl and N^2_2-dimethyl guanine, lower than control for 1-methyl guanine and both higher and lower than control for 7-methyl guanine (Figure 3). By 8 hr, however (Table II), the sra of the [^{3}H]tRNA formed in the MSO-exposed cells had overtaken the control value by 36%, and the radioactivity of all [^{3}H]methyl guanines was higher by an average of 102%. These results complement our previous findings (6, 17) of an effect of MSO on rat brain guanine tRNA methyltransferases. It is of interest that Brunke et al. (28) recently reported that the administration of the carcinogen 9,10-dimethyl-1,2-benzanthracene specifically stimulates the N^2-guanine tRNA methyltransferase of rat mammary gland tumors, while Glazer and Hartman (30) reported this enzyme to be the primary target of 5-azacytidine and dihydro-5-azacytidine action in murine leukemia L1210 cells. Whether glial N^2-guanine tRNA methyltransferase is a primary target of MSO action awaits elucidation.

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