THE STIMULATION OF CEREBRAL N^2-METHYL- AND N_2^2-DIMETHYL GUANINE-SPECIFIC tRNA METHYLTRANSFERASES BY METHIONINE SULFOXIMINE: AN IN VIVO STUDY*

C. E. Salas, W. G. Ohlsson and O. Z. Sellinger

Laboratory of Neurochemistry, Mental Health Research Institute, The University of Michigan Medical Center, Ann Arbor, Michigan 48109

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SUMMARY

The administration of a single convulsant dose or of multiple subconvulsant doses of L-methionine-dl-sulfoximine (MSO) to 18-day old rats results in a significant elevation of the specific activity of cerebral tRNA methyltransferases, as determined in an in vitro assay, using heterologous or species-homologous tRNAs as substrates. The increase was detectable as early as 90 min after MSO and persisted throughout the entire 5-6 h preconvulsant period. The ^{14}[C]-methyl tRNA was purified, and hydrolyzed to its constituent bases and their distribution was quantitated by high performance liquid chromatography. A marked increase in the formation of ^{14}[C]-N^2-methyl- and ^{14}[C]-N_2^2-dimethyl guanine was noted in the MSO-treated animals, demonstrating a specific stimulation by MSO in vivo of the cerebral N^2-methyl and/or N_2^2-dimethyl guanine-specific tRNA methyltransferases.

INTRODUCTION

Previous work from our Laboratory demonstrated that the administration of the convulsant agent L-methionine dl- sulfoximine (MSO) results in a marked decrease of the brain levels of the methyl donor molecule, S-adenosyl-L-methionine (SAM) (1) in both mice and rats. One possible mechanism to account for this depletion of SAM postulated that MSO stimulated the utilization of SAM by one or more of the very active brain methyltransferases (2,3). Support for this notion was recently derived from findings of a significant elevation of the activities of cerebral catechol-O- and histamine N-methyltransferases following MSO treatment (2). Moreover, regional effects of MSO were also demonstrated; thus, whereas in the rat, histamine N-methyltransferase remained unaffected by MSO in the hypothalamus and the

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midbrain it became significantly elevated in the brainstem, the hippocampus, the cerebellum and the cerebral cortex (2).

Since both homologous and heterologous methylation of transfer RNA are highly active processes in rat brain (4-7), it seemed possible that, as part of its general action on methyl transfer reactions, MSO might also affect the activity of cerebral tRNA methyltransferases.

In the present report we describe a significant stimulation by MSO *in vivo* of the total tRNA methyltransferase activity of rat brain cortex and we pinpoint a specific effect of the drug on the methyltransferases inserting methyl groups into the N-2 position of tRNA guanine residues. A preliminary account of some of these findings has appeared (8).

MATERIAL AND METHODS

S-adenosyl-L-[methyl-^{14}C] methionine (specific activity 45 mCi/mmol) was purchased from Research Products International (Elkhart, IN); *E. Coli* tRNA bulk was from Schwarz-Mann (Orangeburg, NY); *E. Coli* $tRNA^{Phe}$ and S-adenosyl-L-methionine were from Boehringer-Mannheim (Indianapolis, IN), and rat liver $tRNA^{Phe}$ was from Grand Island Biologicals (Grand Island, NY). The methylated bases used as standards were from Sigma (St. Louis, MO), and trifluoroacetic acid was from Matheson, Coleman & Bell (East Rutherford, NJ). μBondapak C_{18} columns (30 x 0.4 cm I.D.) were from Waters Associates (Milford, MA).

Sprague-Dawley albino rats (18-days old) were injected intraperitoneally with either saline or MSO (0.94 m moles/Kg for a single injection or 0.23 m moles/Kg every day for one week).

The animals were decapitated 90 min, 3 h or 5 h after the single injection or on the eighth day after beginning the chronic 7-day treatment. The brain cortices were rapidly excised, weighed and homogenized in a glass-teflon homogenizer in 3.5 vol of 10 mM Tris-HCl, pH 7.6, containing 1 mM dithiothreitol (DTT) and 1 mM $MgCl_2$. The homogenate was centrifuged at 165,000 x g for 150 min and the tRNA methyltransferases precipitated from the clear supernatant by addition of a saturated solution of ammonium sulfate pH 7.0 to 80% (w/v) saturation. The insoluble material was recovered by centrifugation and the pellet resuspended in 10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 1 mM DTT. This suspension was dialysed overnight against 1.5 l of the same buffer (one buffer change) and the dialysate fraction used as a source of tRNA methyltransferases.

The *in vitro* "rate" methylation of tRNA was performed in the presence of 2.5 mM spermidine (4), except where noted otherwise.

The hydrolysis of [methyl-^{14}C] tRNA with trifluoroacetic acid and the quantitative analysis of the constituent [methyl-^{14}C] bases on μBondapak C_{18} using a high performance liquid chromatography procedure were as recently described elsewhere (9). Protein was determined by the procedure of Lowry et al. (10) using crystalline bovine serum albumin as standard.

Table 1. EFFECTS OF MSO, ETHIDIUM BROMIDE AND S-ADENOSYL-L-HOMOCYSTEINE ON CEREBRAL tRNA METHYLTRANSFERASES

ADDITION	tRNA Methyltransferase Activity*
None	1,402**
MSO, 10^{-4}M	1,351
MSO, 10^{-5}M	1,425
MSO, 10^{-6}M	1,370
Ethidium Bromide, 10^{-4}M	276
S-Adenosyl-L-Homocysteine, 10^{-4}M	186

* In 154,000 x g dialyzed supernatant of 18-day old rat cerebral cortex.

** DPM ^{14}[C]-Methyl tRNA/45 min.

RESULTS

Table 1 shows that MSO has no effect on the activity of cerebral tRNA methyltransferases *in vitro*, in contrast to 10^{-4}M S-adenosyl-L-homocysteine (SAH) and ethidium bromide which inhibit these enzymes markedly. The time course of the *in vitro* tRNA methylation of *E. Coli* tRNA (bulk) after, respectively, 90 min, 5 h or 1 week of MSO is shown in Figure 1. At all post-MSO times studied, tRNA methylation by the tRNA methyltransferases of the MSO-treated animals was significantly higher than control. Table 2 compares control and MSO tRNA methyltransferases (3 h) in terms of their ability to methylate different tRNAs, including the species-homologous rat liver $tRNA^{phe}$. The largest increase over control values was seen with *E. Coli* tRNA (bulk), followed by *E. Coli* $tRNA^{phe}$, rat liver $tRNA^{phe}$ and, last, *E. Coli* $tRNA^{glu}_2$.

The residue and site specificity of the methylation of *E. Coli* tRNA

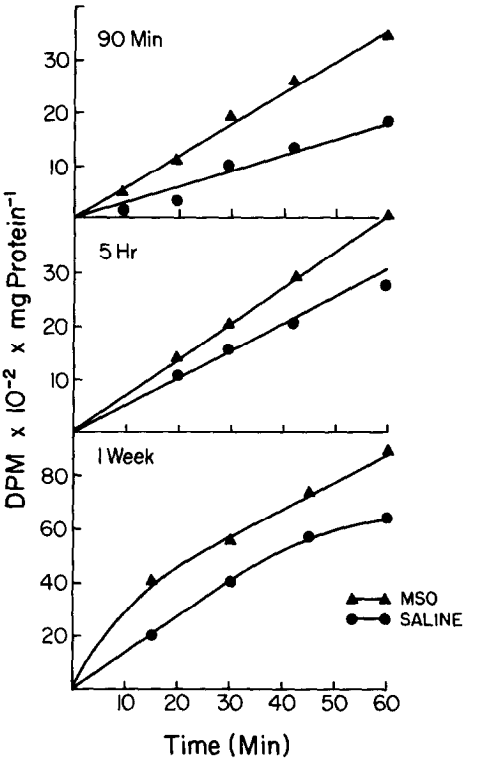

Figure 1. Time course of methylation of E. Coli $tRNA^{phe}$ using tRNA methyltransferases derived from control and MSO-treated animals. 40 µl aliquots were withdrawn at the intervals shown and precipitated with 10% TCA on Millipore filters. For details see Methods.

(bulk) were examined next. The results of this analysis are shown in Table 3. The Table also compares the distribution (as % of the total recovered activity) of the ^{14}[C]-methyl bases obtained following methylation by the control and the experimental tRNA methyltransferases. The results indicate that the 51% increase in overall methylation noted in row 1 of Table 2 reflects an average between the highly significant 2.5-4.8-fold increases in N^2-methyl and N_2^2-dimethyl guanine methylation and the virtually unchanged methylations of guanine in the 1 position and of those of adenine, cytosine and hypoxanthine. The findings thus clearly demonstrate a highly specific effect of MSO on the N^2-methyl and the

Table 2. EFFECT OF MSO *IN VIVO* ON THE ABILITY OF CEREBRAL tRNA METHYLTRANSFERASES TO METHYLATE DIFFERENT tRNAs *IN VITRO*

tRNA	Control (dpm/mg)	MSO* (dpm/mg)	Δ (%)
1. *E.coli* tRNA (bulk)	8,179	12,369	+ 51.0
2. *E.coli* $tRNA^{phe}$	9,038	12,419	+ 37.4
3. *E.coli* $tRNA^{phe}$	8,022	10,222	+ 27.4
4. *E.coli* $tRNA^{glu_2}$	16,500	17,190	+ 4.0
5. Rat liver $tRNA^{phe}$	3,140	3,913	+ 24.6

*0.94 mmoles/Kg, 3 h before death
tRNA methyltransferases were from 18-day old rat cerebral cortex. Tubes 1, 2, 4 and 5 contained 2.5 mM spermidine, tube 3 contained 15 mM putrescine. [tRNA]: 100 µg in tube 1, 20 µg in tubes 2-5.
Incubation: 60 min.
Values refer to the radioactivity (dpm) in tRNA/mg of brain protein.

N_2^2-dimethyl guanine-specific tRNA methyltransferases of rat cerebral cortex. In quantitative terms, the effect of MSO on N_2^2-dimethyl guanine formation was twice as great as that affecting the formation of N^2-methyl guanine. Although this imbalance resulted in a 2 to 1 ratio of specific radioactivities (N_2^2-dimethyl guanine to N^2-methyl guanine), the % increase in the contributions of these two newly methylated bases to the total methyl base content of *E. Coli* tRNA (bulk) remained approximately the same (15.5% for N^2-methyl guanine vs 13.3% for N_2^2-dimethyl guanine).

DISCUSSION

Since MSO failed to alter the activity of brain tRNA methyltransferases when added to the *in vitro* incubation mixture, the possibility that it interacts directly with any of its components, e.g. by mimicking spermidine or by

Table 3. THE EFFECT OF MSO IN VIVO ON CEREBRAL tRNA METHYLTRANSFERASES: CHANGES IN THE DISTRIBUTION OF [METHYL^{-14}C] BASES IN E.Coli tRNA

[Methyl^{-14}C] Base	Control (dpm/mg)	MSO* (dpm/mg)	MSO / Control	Control (%)	MSO (%)	Δ (%)
N^2-methyl guanine	1,915	4,814	2.51	23.4	38.9	+ 15.5
N_2^2-dimethyl guanine	501	2,402	4.79	6.1	19.4	+ 13.3
1-methyl adenine	1,348	1,157	0.85	16.5	9.4	
5-methyl cytosine	1,597	1,571	0.98	19.5	12.7	
1-methyl guanine	1,491	1,401	0.94	18.3	11.3	
1-methyl hypoxanthine	1,327	1,024	0.77	16.2	8.3	

*0.94 mmoles/Kg, 3 h before death
tRNA methyltransferases were from 18-day old rat cerebral cortex. The incubation mixture contained 100 µg of E.coli tRNA (bulk) and 2.5 mM spermidine.
Values refer to the radioactivity (dpm) in the methylated base/mg of brain protein and to the percent of the total recovered radioactivity, respectively.
Incubation: 60 min.

changing the conformation of tRNA methyltransferase proteins, may be ruled out. Previously, MSO (10^{-3}-10^{-8}M) was found to have no effect on the activity of partially purified rat brain histamine N-methyltransferase (2). Preincubation of MSO with tRNA, the enzyme fraction or SAM at 37° C for 10 min equally failed to change the rate of [methyl^{-14}C] tRNA formation. By contrast, MSO administered in vivo either acutely or chronically, clearly increased the specific activity of cerebral tRNA methyltransferases (Fig. 1). These findings cannot be explained by differences in SAH levels since all brain extracts were extensively dialyzed; moreover, previous work from this Laboratory reported a decrease in brain SAH levels following MSO (11).

The effect of MSO could be readily demonstrated with E. Coli tRNA bulk and E. Coli and rat liver $tRNA^{phe}$, but not with E. Coli $tRNA^{glu_2}$ (Table 2). The effect was most marked when E. Coli tRNA bulk was used, most probably because of its 5-fold excess over the amounts of the individual species tRNAs

(see Legend, Table 3); the large excess of substrate thus available afforded all the tRNA methyltransferases present, including those with low substrate affinities (12) with optimal opportunities for substrate recognition. Moreover, spermidine (0.1-20 mM) was found to have no effect in shifting the optimal activity of tRNA methyltransferase in MSO-treated as compared to control animals (data not shown).

A somewhat surprising finding was the rather substantial methylation of rat liver $tRNA^{phe}$ by the cerebral tRNA methyltransferases. These results are analogous to those of Simon et al. (13) who reported appreciable methylation of rat liver tRNA (bulk) by adult rat brain tRNA methyltransferases. It should also be noted that the methylation of partially methyl-deficient rat liver tRNA by normal rat liver tRNA methyltransferases was recently reported (12,14) and that we recently described a highly active homologous tRNA methylation process in immature rat brain in which brain tRNAs (and/or tRNA precursors) are methylated *in vitro* by homologous brain tRNA methyltransferases in the absence of exogenous tRNA (4,5).

The specific radioactivity of each [methyl-^{14}C] base formed in *E. Coli* tRNA bulk is shown in Table 3. The results clearly indicate that the MSO-elicited increase in tRNA methylation was confined to N^2-methyl guanine and N_2^2-dimethyl guanine residues. On the basis of the observation shown in Table 2 that *E. Coli* $tRNA^{phe}$ is more susceptible to MSO than $tRNA^{glu_2}$ and also from the fact that these two tRNAs are methylated by two different rat liver N^2-guanine tRNA methyltransferases (15-18) (Enzyme I methylates in position 10 from the 5' OH end requiring a s^4UAGCUC sequence; Enzyme II methylates in position 27 from the 5' OH end and requires a CUCG sequence), we conclude that MSO certainly, although perhaps not exclusively, affects a brain tRNA methyltransferase analogous to enzyme I.

An alternative explanation of the effect of MSO on N^2-guanine methylation in *E. Coli* $tRNA^{phe}$ would be that part of the MSO-elicited increment involves a site other than guanylate 10. Enzyme II has been partially purified and

characterized in the presence of different E. Coli tRNAs (15-17) and $tRNA^{leu_3}$, $tRNA^{leu_4}$ and $tRNA^{fmet}$ were found to be the preferred substrates while $tRNA^{phe}$, although it also has a G in position 27, acted as a poor substrate for the enzyme. While it is, therefore, unlikely that methylation of E. Coli $tRNA^{phe}$ by the control tRNA methyltransferases (Table 2) involved residue 27, it is conceivable that access to this site by a type II enzyme may become less encumbered as a result of the administration of MSO.

With regard to the marked effect of the MSO treatment on N_2^2-dimethyl guanine formation, satisfactory explanations are more difficult to find. Since, in eukaryotes guanine 10 is methylated by a N^2-mono methyl guanine tRNA methyltransferase (16,17) and guanine 27 by one or more enzymes which can both mono- and dimethylate, it is possible that methylation of guanine-27 to N_2^2-dimethyl guanine by both control and experimental enzymes was exclusively via a guanine-27 (Type II) specific methyltransferase. Alternatively, if, as in the in vivo system of KB cells used by Munns et al. (19), formation of N^2-methyl guanine and N_2^2-dimethyl guanine is not synchronous, the latter being substantially faster than the former, it is possible that portion of the MSO-elicited N_2^2-dimethyl guanine increment is the result of increases in the methylation of pre-existing N^2-methyl guanine sites by "an activity (which) adds the second methyl group in some cases" (17). The much higher net increase in N_2^2-dimethyl- than in N^2-methyl guanine formation (Table 3) suggests that this possibility is worth exploring.

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