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DECREASED IN VIVO PROTEIN AND PHOSPHOLIPID METHYLATION AFTER IN VIVO ELEVATION OF BRAIN S-ADENOSYL-HOMOCYSTEINE

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SUMMARY

The administration of adenosine together with homocysteine resulted in a dose-related elevation of cerebral S-adenosyl-L-homocysteine without concomitant perturbation of S-adenosyl-L-methionine levels. The adenosine + homocysteine treatment also decreased the incorporation of labile and stable methyl groups into brain proteins. Brain [^{3}H]-phosphatidyl N,N-dimethylethanolamine and [^{3}H]-phosphatidylcholine were also significantly decreased while [^{3}H]-phosphatidyl-N-monomethylethanolamine remained unchanged. The data indicate that elevated brain S-adenosylhomocysteine can markedly and selectively inhibit the *in vivo* methylation of brain proteins and phospholipids.

INTRODUCTION

In recent years AdoMet utilizing methylations have been recognized as important in a wide range of biological processes including the chemotactic response (1), vesicle exocytosis (2), the biosynthesis of phospholipids (3), membrane fluidity (4), and ligand-receptor interactions (5). As reported previously, the intraperitoneal (I.P.) co-administration of equimolar Ado and Hcy elevates cerebral, hepatic, and blood levels of AdoHcy without significantly perturbing AdoMet levels (6,7). The elevated

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Abbreviations: Ado, adenosine; Hcy, D,L homocysteine; AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; PME, phosphatidyl-N-monomethylethanolamine; PMME, phosphatidyl-N,N-dimethylethanolamine; PC, phosphatidylcholine; PMT, S-adenosyl-L-methionine: phosphatidylethanolamine-N-methyltransferase (EC 2.1.1.17); MGI, methyl group incorporation; TCA, trichloroacetic acid.

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tissue AdoHcy levels, in turn, resulted in decreased histamine-N-methyltransferase (EC 2.1.1.8) and catechol-O-methyltransferase (EC 2.1.1.6) activities as assayed in vitro, as well as in a decrease, in vivo, in the catabolism of brain histamine, which occurs solely by transmethylation to 3-methylhistamine (8,9). In light of the fact that the methylation of proteins and phospholipids may participate in and/or mediate the regulation of neural activity (1,5,10,11), it became of interest to determine the effects of elevated cerebral AdoHcy levels on these two processes.

MATERIALS and METHODS

Animals. Adult male Swiss-Webster mice (24-32g) were from Charles River Laboratories (Portage, MI). They were fasted overnight prior to the experimental procedures which were conducted between 8:00 and 11:00 AM.

Drug treatment and tissue collection. Mice were injected intraperitoneally (I.P.) with an equimolar mixture of Ado and Hcy 40 min prior to sacrifice by immersion (head first) in liquid nitrogen. Brains were rapidly removed, refrozen in liquid nitrogen, and stored at -80°C until time of assay.

Determination of AdoMet and AdoHcy. AdoHcy and AdoMet disulfate di-p-toluenesulfonate were generously donated by Dr. Giorgio Stramentinoli (BioResearch Laboratories, Liscate, Italy). Amberlite CG-50 was from Accurate Chemical (Hicksville, NY) and Dowex 50W-X8 (200-400 mesh), from BioRad (Richmond, CA). AdoMet (methyl-[^{14}C]) (specific activity 57 mCi/mmol) and Ado-(8-[^{14}C]) (specific activity 54 mCi/mmol) were from New England Nuclear (Boston, MA). Radioactive AdoHcy (8-[^{14}C]) was prepared from L-Hcy thiolactone and Ado-(8-[^{14}C]) by high pressure liquid chromatography as previously described (12). AdoMet and AdoHcy levels were determined using a modified isotope-dilution/ion exchange/HPLC method (7).

In vivo protein methylation. L-(methyl-[^{3}H])-methionine (specific activity, 1 Ci/mmol) was from ICN Pharmaceuticals, Inc. (Irvine, CA) and L-([^{14}C]OOH)-methionine (specific activity, 60.7 mCi/mmol) was from Amersham-Searle (Arlington Heights, IL). For intraventricular (i.vt.) ([^{14}C]OOH)-methionine and [^{3}H]-methionine injections, the mice were lightly anesthetized with ether, a small piece of scalp removed for ease in identification of the injection site and 2 uCi ([^{14}C]OOH)- or 5 uCi ([^{3}H-methyl])-methionine was injected into the lateral cerebral ventricle in 10 ul of artificial cerebrospinal fluid (Merlis solution) 2, 30, and 60 min prior to sacrifice. In all cases, animals were treated with Ado+Hcy or saline/Tween 80 (controls), 40 min prior to sacrifice (7). Frozen brains were rapidly

weighed, and homogenized in 8.0 ml of ice-cold 5 mM sodium phosphate buffer (pH 6.8), Protein carboxymethylation was determined using the method of Kim (13), modified as follows: Two ml of tissue homogenate was centrifuged (27,000 x g for 30 min), after which the pellet was washed three times with 1.0 ml of 5 mM phosphate buffer (pH 6.8) and once with 1.0 ml ethanol.

To determine the total MGI, the pellet was resuspended in 1.0 ml of 5 mM phosphate buffer and a 0.1 ml aliquot counted in 10 ml of ACS (a tissue solubilizer-scintillation mixture; Amersham-Searle, Arlington Heights, IL). To determine the stable MGI, the washed, resuspended pellet was centrifuged (27,000 x g for 20 min), and the pellet resuspended in 1.0 ml of 0.2 M sodium phosphate buffer (pH 7.4). The mixture was heated in a water bath at 100°C for 5 min to hydrolyze methyl esters. The sample was cooled, 5 mg of bovine serum albumin (Miles Biochemicals, Elkhart, IN) added as a carrier, and protein was precipitated with 1.0 ml of 30% TCA. After centrifuging (27,000 x g for 10 min), the resulting precipitate was washed once with 1.0 ml of 15% TCA, once with 1.0 ml of ethanol, suspended in 1.0 ml water, and 0.25 ml were counted in 10 ml ACS to obtain values for the stable MGI. The difference between the total (see above) and the stable MGI represents the labile (carboxymethylation) MGI (14). Protein was determined according to Lowry (15).

In vivo phospholipid methylation. All mice were injected i.vt. with ([^{3}H-methyl])-methionine followed 20 min later with either Ado+Hcy (500 mg/kg of each) or saline/Tween 80 (20 ml/kg) I.P. After sacrifice, 60 min after ([^{3}H-methyl])-methionine, brains were processed as above for protein methylation. The first pellet was washed four times with 1.0 ml of 5 mM phosphate buffer (pH 6.8). To extract the phospholipids, the washed pellet was suspended in 2.0 ml of chloroform/methanol (2:1, v/v), the suspension vortexed for 60 sec, centrifuged (27,000 x g for 30 min) and the supernatant transferred to a screw-capped test tube. This extraction was repeated, and the 2 supernatants combined. The organic phase was washed twice with 2.0 ml of 0.1M KCl (in 50% MeOH), and the aqueous (top) phase aspirated. A 0.1 ml aliquot was counted in 5.0 ml ACS for total phospholipid methylation. The identification of the radioactive phospholipids was by thin-layer chromatography (16,17). The chloroform phase was dried under a stream of nitrogen, the tubes were capped, and stored overnight at -20°C. The residue was dissolved in 40 ul of a mixture containing 0.4 mg of each standard: L-α-phosphatidylethanolamine, β,γ-dipalmitoyl; L-α-phosphatidylethanolamine, N-methyl-β,γ-dipalmitoyl; L-α-phosphatidylethanolamine, N,N-dimethyl-β,γ-dipalmitoyl; L-α-lecithin, β,γ-dipalmitoyl; purchased from Calbiochem-Behring Co. (La Jolla, CA), in chloroform/methanol, 2:1, by vol. and 25 ul applied to silica gel 60 plates (without fluorescent indicator) (EM Laboratories, Elmsford, NY). Plates were developed (16 cm) in chloroform/propionic acid/n-propyl alcohol/water (2:2:3:1, by vol.) (16,17), dried at 100°C for 10 min, and sprayed with iodine (1% in ethanol) for detection of phospholipids. After the spots were outlined with pencil, the plates were heated as above to remove iodine, each lane was divided into 1 cm strips and was scraped into scintillation vials containing 10 ml ACS and the radioactivity determined by the channels ratio method. The identity of the phospholipids was further confirmed in the solvent systems chloroform/methanol/7 M

ammonia (60:35:5, by vol.) and chloroform/methanol/water (65:25:4, by vol.) by one-dimensional thin-layer chromatography (16,17).

Statistical analysis. Statistical differences between drug-treated groups and corresponding vehicle-treated animals were evaluated by the Student's "t" test (two-tailed). P values less than 0.05 were considered not significant.

RESULTS

Effect of Ado+Hcy on Brain AdoMet and AdoHcy Levels, and on the Methylation Index. To determine the effects of Ado+Hcy on AdoMet and AdoHcy levels, mice were injected I.P. with Ado+Hcy (200 mg/kg or 500 mg/kg of each) and killed 40 min later (Table 1) at which time brain AdoHcy levels are maximally elevated (6,7). AdoMet levels remained unchanged, while AdoHcy levels increased in a dose-dependent manner by 500% and 1920% at 200 mg/kg and 500 mg/kg, respectively (Table 1;6,7). The methylation index (AdoMet/AdoHcy ratio), used as an index of transmethylation rates in brain tissue (18-20), was conversely, significantly decreased, -87% (200 mg/kg) and -96% (500 mg/kg) (Table 1).

Effect of Ado+Hcy on Phospholipid Methylation. Figure 1 shows that the co-administration of Ado+Hcy resulted in a significant decrease in the formation of [^{3}H]-PMME (-60%) and [^{3}H]-PC (-75%), these reactions being catalyzed by the same enzyme, PMT II. The brain levels of [^{3}H]-PME, whose formation is catalyzed by a different enzyme, PMT I (16,17), remained unchanged after Ado+Hcy treatment.

Effect of Ado+Hcy on Protein Methylation. Figure 2 shows a time related increase in both the stable (remaining after alkaline hydrolysis) and labile (carboxymethyl esters) MGI into proteins. Ado+Hcy significantly decreased stable MGI by 50% at 2 min and by 53% at 30 min, while at 60 min there was no longer any difference between the stable MGI in treated *vs.* control animals.

TABLE 1. Effect of Ado+Hcy on Mouse Brain AdoMet, AdoHcy and Methylation Index

Treatment	AdoMet[a]	% Change[b]	AdoHcy[a]	% Change[b]	Methylation Index[c]	% Change[b]
Saline/Tween 80	23.20 ± 1.73	--	2.32 ± 0.40	--	12.332 ± 2.29	--
Ado+Hcy 200 mg/kg	19.24 ± 1.57	-17	13.90 ± 1.55	+500[d]	1.583 ± 0.27	-87[d]
Ado+Hcy 500 mg/kg	23.80 ± 1.04	+3	46.47 ± 4.34	+1920[d]	0.541 ± 0.06	-96[d]

Mice were injected I.P. with saline/Tween 80 (20 ml/kg) or Ado+Hcy--200 mg/kg and 500 mg/kg 40 min prior to sacrifice.

a. Data are expressed as nmol/g ± S.E.M. in 5-7 animals.

b. Percent change values compared to vehicle-treated animals.

c. Methylation index: AdoMet/AdoHcy ratio as mean ± S.E.M. in 6 animals.

d. $p<0.001$, using students 't' test (two-tailed).

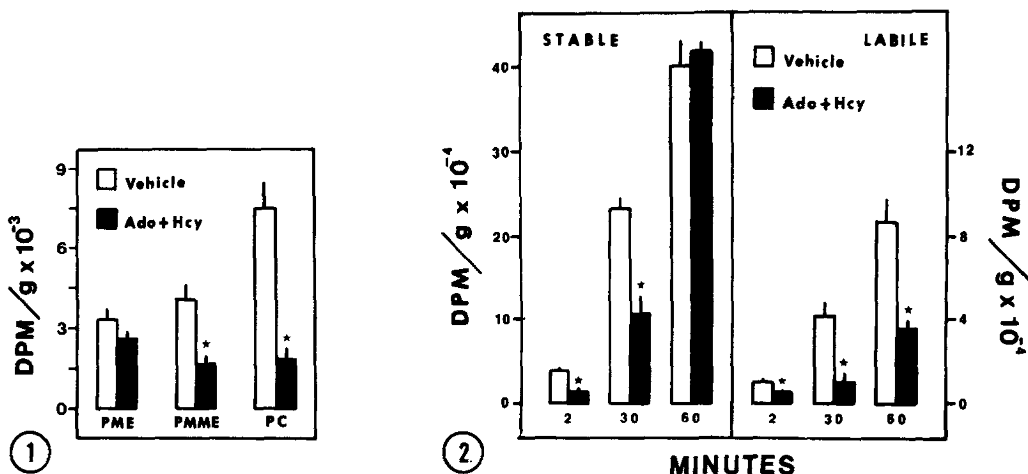

Figure 1. Effect of Ado+Hcy on Phospholipid Methylation in Mouse Brain. [^{3}H]-methyl methionine (5 uCi/10 ul) was injected into the lateral cerebral ventricle of mice 60 min prior to sacrifice. Ado+Hcy (500 mg/kg of each) or saline/Tween 80 (20 ml/kg) was injected intraperitoneally 40 min before death. Results are expressed in dpm/g x 10^{-3} $\pm$ S.E.M. in 5-7 mice. * Denotes a significant difference compared to corresponding vehicle-treated controls at the 0.05 level using the Student 't' test.

Figure 2. Effect of Ado+Hcy on Protein Methylation in Mouse Brain. [^{3}H]-methyl methionine (5 uCi/10 ul) was injected intraventricularly 2, 30 and 60 min prior to sacrifice. Ado+Hcy (500 mg/kg of each) or saline/Tween 80 (20 ml/kg) was injected intraperitoneally 40 min prior to death. Methyl group incorporation was determined as described in "Materials and Methods". Results are expressed in dpm/g x 10^{-4} $\pm$ S.E.M. in 5-7 mice. For statistical analysis, see Figure 1.

Furthermore, the administration of Ado+Hcy significantly decreased labile MGI (protein carboxymethylation), by 42%, 74%, and 60% at 2, 30, and 60 min, respectively.

DISCUSSION

We have previously shown that the co-administration of equimolar Ado+Hcy results in elevated AdoHcy levels in mouse brain (6,7). We established that the methylation index (AdoMet/AdoHcy) was reduced after Ado+Hcy (Table 1) supporting the idea that this ratio is a reflection of brain methylation activity (18,19), as suggested by others for several of the methyltransferase enzymes (20), including PMT I and II (21).

It is well established that PC can be synthesized by two distinct pathways in brain: 1) the conversion of free choline to CDP-choline and a phosphobase transfer to form 1,2-diacylglycerate (22), or 2) the stepwise methylation of phosphatidylethanolamine to PC using two AdoMet dependent methyltransferases (3,16,17).

As shown in Figure 1, there appears to be little inhibition by Ado+Hcy of [^{3}H]-PME formation perhaps because by 60 min labelling of PME may have begun to decay or because the newly formed AdoHcy fails to interact with and, hence inhibit PMT I. Since no inhibition of [^{3}H]-PME formation was noted after a 10 min [^{3}H-methyl]-methionine pulse (30 min prior to Ado+Hcy) (data not shown), the former option appears unlikely although further time studies need to be carried out to totally rule out this possibility. A likelier explanation is that since PMT I is an intrasynaptosomal enzyme (17,23), it is not accessible to the intact AdoHcy molecule which may be located for the most part extrasynaptosomally. This supposition is further borne out by studies which show the impermeable nature of membranes to AdoHcy (7,24-26). Further, the addition of AdoHcy did not alter the viscosity of erythrocyte membranes (4) or, by inference, PME levels. Kinetic studies with PMT I indicate that *in vitro* decreased PMT I activity does, in fact, occur simultaneously with a decrease in the methylation index (21). The inhibition by Ado+Hcy of [^{3}H]-PMME and [^{3}H]-PC formation reflects inhibition of PMT II. PMT II is thought to be located on the outside of the synaptosomal membrane (23) and hence more readily accessible to external AdoHcy. PMT II also has a high affinity for AdoMet and an even higher affinity for AdoHcy (21,27). The present findings support the results of Chiang and Cantoni (28,29) who found that

elevated hepatic AdoHcy levels inhibited hepatic [^{3}H]-PC formation. Yet, since our procedure for administration of Ado+Hcy (as opposed to deazaadenosine administration (28,29)), insured increases in AdoHcy levels without perturbations in AdoMet levels and in the absence of any deaza-adenosylhomocysteine, it is possible to relate PMT II inhibition directly to the elevated AdoHcy levels generated by the Ado+Hcy administration.

To date there are three different enzymes known which are responsible for the N-methylation of either lysyl, arginyl or histidyl residues (referred to in this paper as stable MGI), or the O-methylation of carboxyl groups (referred to as labile MGI) (10,30), the latter reaction being catalyzed by protein methylase II (31). As shown (Figure 2), treatment with Ado+Hcy markedly decreased the labile MGI at all times tested and the stable MGI at the two earlier time points. Of interest is the positive correlation between the observed *in vivo* inhibition and *in vitro* kinetic studies. Assuming a 70% volume of distribution (32) at the higher dose of Ado+Hcy (500 mg/kg), this should result in intracellular AdoHcy concentrations of 0.75 uM, which is well within the range of Ki values (6.5×10^{-9} M to 6×10^{-6} M) determined for protein methyltransferases I-III (10,20,33). The *in vivo* inhibition of labile MGI (42-60%) (Figure 2) is within the expected values. The decreased methylation ratio, as mentioned, infers that the inhibition of stable or labile MGI is directly caused by the high intracellular AdoHcy levels. We do not find the failure of stable MGI to be reduced at 60 min as surprising, for all the available protein N-methylation sites may have become irreversibly occupied by 30 min. Another possibility is that the ([^{3}H-methyl])-methionine, administered 20 min before Ado+Hcy, may have methylated all sites before AdoHcy levels had

increased sufficiently to inhibit this process. That these differences in stable and labile MGI are due to incorporation of only the methyl group of methionine, and not of the intact methionine molecule, is supported by preliminary experiments in which there were no differences in incorporation of ([^{14}C]OOH)-methionine into cerebral proteins comparing control and Ado+Hcy-treated mice (data not shown).

With the recent evidence of increased protein O-methylation simultaneous with increased neurosecretion in the pituitary and parotid glands (34,35) and in the adrenal medulla (2), the ability to slow the rate of this methylation by raising endogenous AdoHcy levels may be of importance for modulation of neural activity in general.

In addition, the demonstration of a role of the stepwise methylation of the membrane phospholipids in membrane fluidity (4) and in ligand-receptor interactions (5) again reflects the importance of controlling endogenous AdoHcy levels.

The notion that AdoHcy is a neural modulator is further expanded by preliminary evidence from several laboratories including ours (unpublished observations) which shows that AdoHcy possesses sedative (36) as well as anticonvulsant (37) properties.

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REFERENCES

1. Borchardt, R. T. (1980) J. Med. Chem. 23, 347-357.
2. Diliberto, E. J. Jr., Viveros, O. H., and Axelrod, J. (1976) Proc. Natl. Acad. Sci. 73, 4050-4054.
3. Bremer, J., and Greenberg, D. M. (1961) Biochim. Biophys. Acta. 46, 205-216.
4. Hirata, F., and Axelrod, J. (1978) Nature 275, 219-220.

5. Hirata, F., Strittmatter, W. J., and Axelrod, J. (1979) Proc. Natl. Acad. Sci. 76, 368-372.
6. Schatz, R. A., Wilens, T. E., and Sellinger, O. Z. (1980) Trans. Am. Soc. Neurochem. 11, 244.
7. Schatz, R. A., Wilens, T. E., and Sellinger, O. Z. (1981) J. Neurochem. In press.
8. Schwartz, J. -C., Pollard, H., Bischoff, S., Rehault, M. C., and Verdiere, M. (1971) Eur. J. Pharmacol. 16, 326-335.
9. Schwartz, J. -C., Barbin, G., Garbarg, M., Pollard, H., Rose, C., and Verdiere, M. (1976) Adv. Biochem. Psychopharm. 15, 111-126.
10. Kim. S., Galletti, P., and Paik, W. K. (1979) In Biochemical and Pharmacological Roles of Adenosylmethionine and the Central Nervous System (Zappia, V., Usdin, E., and Salvatore, F., eds.) pp.37-50.
11. Gagnon, C., and Heisler, S. (1979) Life Sci. 25, 993-1000.
12. Schatz, R. A., Vunnam, C. R., and Sellinger, O. Z. (1977) Life Sci. 20, 375-384.
13. Kim, S., Galletti, P., and Paik, W. K. (1980) J. Biol. Chem. 255, 338-341.
14. Kim. S. (1977) In The Biochemistry of Adenosylmethionine (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H. G., and Schlenk, F., eds.) pp. 415-434, Columbia University Press, New York.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1955) J. Biol. Chem. 193, 265-275.
16. Hirata, F., Viveros, O. H., Diliberto, E. J., and Axelrod, J. (1978) Proc. Natl. Acad. Sci. 75, 1718-1721.
17. Crews, F. T., Hirata, F., and Axelrod, J. (1980) J. Neurochem. 34, 1491-1498.
18. Baudry, M., Chast, F., and Schwartz, J. -C. (1973) J. Neurochem. 20, 13-21.
19. Schatz, R. A., Vunnam, C. R., and Sellinger, O. Z. (1977) Neurochem. Res. 2, 27-38.
20. Cantoni, G. L., Richards, H. H., and Chiang, P. K. (1979) In Transmethylation (Usdin, E., Borchardt, R. T., and Creveling, C. R., eds.) pp. 155-164, Elsevier/North-Holland, New York.
21. Hoffman, D. R., Uthus, E. O., and Cornatzer, W. E. (1980) Lipids 15, 439-446.
22. Kennedy, E. P., and Weiss, S. B. (1956) J. Biol. Chem. 222, 193-197.
23. Crews, F. T., Hirata, F., and Axelrod, J. (1980) Neurochem. Res. 5, 983-991.
24. Duerre, J. A., Miller, C. H., and Reams, G. G. C. (1969) J. Biol. Chem. 244, 107-111.
25. Walker, R. D., and Duerre, J. A. (1975) Can. J. Biochem. 53, 312-319.
26. Knudsen, R. C., and Yall, O. (1972) J. Bacteriol. 112, 569-575.
27. Hoffman, D. R., and Cornatzer, W. E. (1978) Fed. Proc. 37, 1833.
28. Chiang, P. K., and Cantoni, G. L. (1979) Biochem. Pharmacol. 28, 1897-1902.
29. Chiang, P. K., and Cantoni, G. L. (1980) Biochem. Biophys. Res. Comm. 94, 174-181.
30. Paik, W. K., and Kim, S. (1980) Protein Methylation, Biochemistry: A series of monographs 1, 1-209, (Meister, A, ed.) John Wiley and Sons, New York.

31. Kim, S. (1973) Arch. Biochem. Biophys. 157, 476-484.
32. Cizek, L. J. (1954) Am. J. Physiol. 179, 104-110.
33. Casselas, P., and Jeanteur, P. (1978) Biochim. Biophys. Acta. 519, 255-268.
34. Diliberto, E. J. Jr., and Axelrod, J. (1974) Proc. Natl. Acad. Sci. 71, 1701-1704.
35. Strittmatter, W. J., Gagnon, C., and Axelrod, J. (1978) J. Pharmacol. Exp. Ther. 207, 419-424.
36. Fonlupt, P., Roche, M., Cronenberger, L., and Pacheco, H. (1980) Can. J. Physiol. Pharmacol. 58, 160-166.
37. Fonlupt, P., Roche, M., Andre, A. C., Cronenberger, L., and Pacheco, H. (1980) Can. J. Physiol. Pharmacol. 58, 493-498.