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EFFECTS OF 5'DEOXY-5'-METHYLTHIOADENOSINE ON THE METABOLISM OF S-ADENOSYL METHIONINE

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The treatment of transformed rat cells with micromolar amounts of 5'deoxy 5'methyl thioadenosine induces rapid effects on the rate of methylation of DNA concomitantly with alterations of intracellular pools of S-adenosyl methionine and S-adenosyl homocysteine. Pulse chase labelling experiments indicate that 5'deoxy 5'methylthioadenosine does not inhibit the degradation of S-adenosyl homocysteine but inhibits the comsumption of S-adenosyl methionine. In vitro transmethylation assays performed with heterologous DNA show that low doses of the thioether-nucleoside do not significantly affect the DNA methyltransferase activity of cellular extracts. The biological role of 5'deoxy 5'methylthioadenosine, a natural molecule formed during the synthesis of polyamines is discussed.

Metabolic pathways leading to the transfer of methyl groups to macromolecules and to the synthesis of spermine and spermidine share AdoMet as a common precursor. On one hand, through the transfer of its methyl group by methyltransferases, AdoMet leads to AdoHcy. On the other hand, after decarboxylation by AdoMet decarboxylase, the same molecule acts as a propyl donor for the synthesis of polyamines. During this last step, 5'methylthioadenosine is formed at the rate of one molecule per molecule of spermidine and two molecules per molecule of spermine synthesized (1,2). Both pathways are involved in gene expression, cell growth and differentiation. The role of 5'MTA was investiga-

Abbreviations and symbols : AdoMet : S-adenosylmethionine ; AdoHcY : S-adenosylhomocysteine ; 5'MTA : 5'deoxy 5'methylthioadenosine. AdoMet decarboxylase : 4.1.1.50 ; AdoHcy hydrolase : 3.3.1.1. ; Adenosine kinase : 2.7.1.20. Adenosine deaminase : 3.5.4.4. ; spermine synthase : 2.5.1.16 ; ribonuclease A : 3.1.27.5. Protease K : 3.4.21.14 ; histamine methyltransferase : 2.1.1.8 ; acetylserotonine methyltransferase : 2.1.1.4 ; protein methylase I : 2.1.1.23.

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ted by many authors both in vitro and in vivo. In vitro, 5'MTA was shown to inhibit AdoHcy hydrolase, Ado kinase and spermine synthase (3,4,5). No effect of 5'MTA was observed on Ado deaminase activity (4). Transmethylation of eucaryotic proteins and procaryotic DNA was markedly reduced when 5'MTA was added to in vitro assays (6,7). In vivo, 5'MTA was shown to inhibit cell proliferation (8) and to alter nucleolar morphology (9). Furthermore, postsynthetic methylation of DNA was reduced when human cells cultivated in vitro were treated with low amounts of 5'MTA (10). Thus, on the base of these observations, one could postulate that 5'MTA is able to inhibit methylation of DNA through an accumulation of AdoHcy induced by an inhibition of AdoHcy hydrolase. This hypothesis was studied in rat cells transformed by an avian sarcoma virus (B77).

MATERIALS AND METHODS

CHEMICALS AND CELLS

$[^{35}S]$ -labelled methionine (1150 Ci/mmol) and $[^{3}H]$ -labelled methylmethionine (50 Ci/mmol) were from the Radiochemical Center (Amersham, U.K.). $[^{14}C]$ -labelled thymidine (50 mCi/mmol) and $[^{3}H]$ labelled thymidine (51 Ci/mmol) were from C.E.A. (Saclay, France). AdoMet and AdoHcy were purchased from Boehringer (Mannheim, FRG). P-11 cellulose phosphate was from Whatman Ltd (Springfield, Mill. Maidstone, Kent). Sodium dodecylsulphate was from BDH Chemicals Ltd (Poole, U.K.). 5'MTA, ribonuclease A, protease K, and bovine serum albumin were from Sigma Chemical Co (St Louis, U.S.A.). Picofluor was from Packard Instruments Co. (U.S.A.).

Cells used were rat kidney cells transformed by avian sarcoma virus, type B77. They were grown as monolayers in Eagle's minimal essential medium (MEM) supplemented with 3 % $NaHCO_3$, 10 % tryptose phosphate broth (DIFCO Labs., Detroit, Mi., U.S.A.) and 10 % fetal calf serum at 37°C in a humid atmosphere of air/CO_2 (19/1, vol/vol). Viability of the cells was always larger than 90 % as judged by trypan blue staining.

LABELLING AND EXTRACTION OF DNA

Labelling medium was MEM medium containing 1.5 mg/ml of L-methionine (1/10th of the normal concentration) and supplemented with sodium formate at a final concentration of 20 mM to inhibit the incorporation of $[^{3}H]$ -labelled methyl groups into purines. At the end of the period of labelling, cells were rinsed and lysed. DNA was extracted with phenol-chloroform and treated with ribonuclease (50 µg/ml) and protease K (50 µg/ml). The extraction and enzymatic treatments were repeated twice. The specificity of labelling of cytosine was verified by thin layer chromatography as described by Munns (11).

ADOMET and ADOHCY ASSAY

Cells were labelled with $[^{35}S]$ -labelled methionine according to the same procedure as described for DNA. To assay AdoMet and AdoHcy,

the acidosoluble material was recovered and treated essentially as described by Eloranta et al. (12). The purity of the fractions was verified by thin layer chromatography on cellulose precoated plastic sheets with unlabelled AdoMet and AdoHcy as standards. The solvant was butan-1-ol/acetic acid/water (2/1/1, vol/vol).

CELL EXTRACTS

Cell extracts for testing DNA methyltransferase activities were prepared from NRK-B77 cells as described by Creusot et al. (13). In vitro methyl acceptance of Micrococcus lysodeikticus DNA, in the presence or in the absence of 5'MTA and AdoHcy was measured as described by the same authors (13).

RESULTS

EFFECT OF 5'MTA ON SYNTHESIS AND METHYLATION OF DNA IN VIVO

When cells were labelled for 1 hour in the presence of increasing amounts of 5'MTA, as described in the legend of Fig. 1A, no significant alteration of the rate of incorporation of $[^{14}C]$ -labelled thymidine could be detected when the culture medium was supplemented with the thioethernucleoside at a final concentration of 50 µM. However, the rate of incorporation of $[^{3}H]$ -labelled methyl groups was reduced by 54 % and 64 % when cells were treated with 5'MTA at final concentration of 10 and 50 µM respectively. Thus in these conditions, 5'MTA had a rapid effect on the rate of methylation of DNA.

EFFECT OF 5'MTA ON THE TRANSFER OF METHYL GROUPS TO DNA BY CELLULAR EXTRACTS IN VITRO

In order to test the effect of 5'MTA on the DNA methyltransferase activity of NRK-B77 cells, in vitro assays were performed as described in Materials and Methods. Results shown in Fig. 1B indicate that AdoHcy was a potent inhibitor of the reaction, when added to the assay at a final concentration as low as 5 µM, whereas 5'MTA had to be used at one hundred times higher concentration to achieve a similar level of inhibition.

EFFECT OF 5'MTA ON INTRACELLULAR POOLS OF ADOMET AND ADOHCY

The effect of 5'MTA on the rate of methylation of DNA in vivo could be due to an alteration of the metabolism of AdoMet. Therefore pulse labelling experiments and estimates of AdoMet and AdoHcy pools were performed to test this hypothesis. In a first set of experiments,

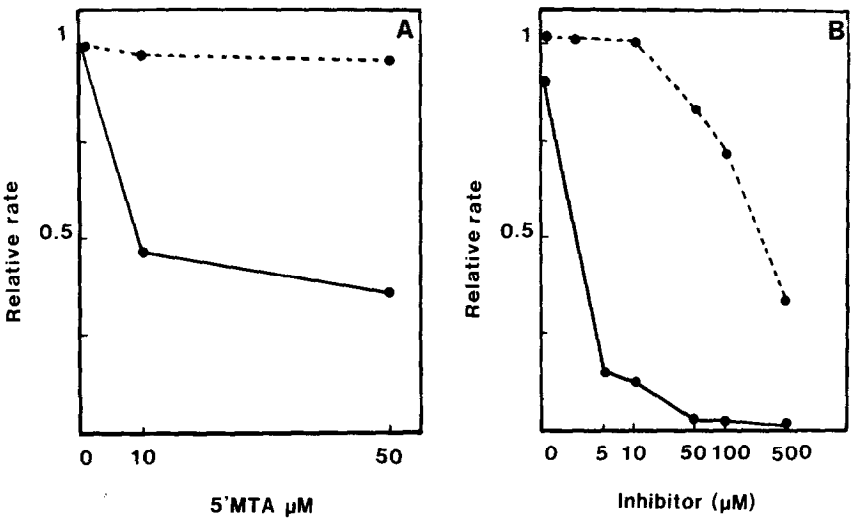

Fig. 1. Effects of 5'MTA on the rate of methylation of DNA in vivo and in vitro.

1A : Exponentially growing cells were treated with 5'MTA at final concentrations of 5 and 50 µm for 2 hours. 1 hour before the end of the treatment, cells were labelled with tritiated thymidine at a final concentration of 50 µCi/ml ●– – –●.

Cells were treated with 5'MTA as above and labelled 1 hour before the end of the treatment with $[^{14}C]$ -labelled thymidine and $[^{3}H]$ -labelled methyl methionine at final concentrations of 1.5 µCi/ml and 100 µCi/ml respectively. The rate of methylation was evaluated from the ratio of $^{3}H/^{14}C$ labels incorporated into DNA ●——●.

1B : In vitro assays were performed as described in Materials and Methods in the presence of increasing amounts of AdoHcy or 5'MTA. Results are expressed as relative rates of acceptance of labelled methyl groups by micrococcus lysodeikticus DNA.
●——● in the presence of AdoHcy
●– – –● in the presence of 5'MTA

cells were labelled with $[^{35}S]$ -labelled methionine in the presence of 5'MTA as described in the legend of Table 1. Results obtained indicate that, in such conditions, the pool of $[^{35}S]$ -labelled AdoMet increased whereas the pool of $[^{35}S]$ -labelled AdoHcy decreased. Thus, it can be concluded that, under the same experimental conditions, methylation of DNA and metabolism of AdoMet are markedly affected by 5'MTA. These effects could be due either to a direct inhibition of the degradation of $[^{35}S]$ -labelled AdoMet into AdoHcy, or to an inhibition of the degradation of $[^{35}S]$ -labelled AdoHcy by AdoHcy hydrolase. In order to follow the fate of labelled AdoMet and AdoHcy, exponentially growing cells were pulse labelled for 1 hour with $[^{35}S]$ -labelled methionine. The culture medium was then removed and cells were rinsed with pre-

Table 1. Effects of 5'MTA on [^{35}S]-AdoMet and [^{35}S]-AdoHcy intracellular pools Pulse labelling experiment.

Treatment with 5'MTA (μM)	AdoMet dis/min/mg	AdoHcy dis/min/mg	AdoMet / AdoHcy
0	215 867	16 315	13,23
10	223 996	12 903	17,36
50	323 436	12 654	25,56

Cells were treated for 2 hours with 5'MTA. One hour before the end of the treatment, [^{35}S]-methionine (specific activity 1150 Ci/mmole) was added to the culture medium at a final concentration of 20 μCi/ml. [^{35}S] AdoMet and [^{35}S]-AdoHcy were assayed as described in Material and Methods. Results are expressed as amounts of [^{35}S]-labelled AdoMet and AdoHcy per mg of protein.

warmed medium. 5'MTA was then immediately added and cells were kept for 1 hour in the presence of the thioethernucleoside. Labelled AdoMet and AdoHcy were then assayed as previously described. Results presented in Table 2 indicate that the treatment of cells with increasing amounts of 5'MTA induced an accumulation of prelabelled AdoMet and a decrease of the concentration of prelabelled AdoHcy. Therefore, it can be concluded that in our experimental conditions, 5'MTA did not inhibit the degradation of AdoHcy, but reduced the degradation of AdoMet into AdoHcy.

DISCUSSION

5'MTA, a sulfur containing nucleoside, is produced during the synthesis of spermine and spermidine. This molecule was shown to be present in very low amounts in cells and, when added to the culture medium, to be actively transported across the cellular membrane (14,15). Final concentrations of 5'MTA as low as 10 and 50 μM had a rapid effect on the rate of incorporation of methyl groups into the DNA of transformed rat cells. This effect was dose dependent and could be observed after 2 hours of treatment only. Such an effect was reported by Woodcock et al. (10) on human cell lines. 5'MTA was shown in human erythrocytes and lymphocytes (3,4) to inhibit AdoHcy hydrolase in vitro. Since AdoHcy is a potent inhibitor of transmethylation reactions,

Table 2. Effects of 5'MTA on the fate of prelabelled pools of AdoMet and AdoHcy

Treatment with 5'MTA (μM)	AdoMet dis/min/mg	AdoHcy dis/min/mg	AdoMet / AdoHcy
0	72 652	16 217	4,48
10	81 072	11 631	6,97
50	216 243	14 096	15,34

Cells were pulse labelled with [^{35}S]-methionine (specific activity 1150 ci/mmole) at a final concentration of 140 μCi/ml, for 1 hour. The chase was performed for 1 hour in the presence of 5'MTA. Results are expressed as amounts of [^{35}S]-labelled AdoMet and AdoHcy per mg of protein.

the question raised whether the effect observed here could be due to its accumulation through an inhibition of AdoHcy hydrolase. But results observed in the pulse chase labelling experiments show that the pool of AdoMet increased whereas the degradation of AdoHcy was not affected. Therefore the accumulation of AdoMet does not seem to be due to a feedback inhibition of its degradation induced by the accumulation of AdoHcy. Thus, the treatment of cells by low amounts of 5'MTA induces a rapid effect on the intracellular concentration of AdoMet and on the transmethylation activity of the cell.

The effect detected on the rate of methylation of DNA could then be explained by a direct effect of 5'MTA on the DNA methyltransferase activity as described by several authors in vitro. Zappia et al. described the effect of 5'MTA on histamine methyltransferase and acetylserotonine methyltransferase (16). The protein methylase I of Krebs II ascites was shown to be inhibited by 5'MTA and the same inhibitory activity on E. Coli B modification enzyme was reported (6,7). In this last experiment, AdoHcy did not inhibit the transmethylation reaction at equimolar levels. In our case, when 5'MTA was tested for its ability to inhibit the transfer of methyl groups to heterologous DNA by an extract of NRK-B77 cells, the amount required to achieve the same level of inhibition as the one obtained with AdoHcy was greater by several orders of magnitude. Since 5'MTA is present in very low amounts in cells

(1), it seems unlikely that, in vivo, transmethylating enzymes are the prime target of this thioethernucleoside. The growth of BHK-21 cells and SV-3T3 cells was markedly affected when this nucleoside was added to the culture medium (8,17), as well as the growth of stimulated lymphocytes (18) and murine lymphoid cells (19). The mechanism of this inhibitory activity remains unclear. In BHK-21 cells, the capacity of the degradation of 5'MTA was shown to exceed that of the uptake mechanism (15). However, the treatment of 3T3 fibroblasts transformed by SV40, with 5'MTA at a final concentration as low as 10 μM was able to inhibit cell growth (8). In this system, the inhibitory effect was not enhanced by inhibitors of adenosine deaminase. This result suggests that the action of 5'MTA is not mediated through its degradation into adenine by phosphorylase. In NRK-B77 cells, the action of 5'MTA through the production of adenine seems unlikely, as adenine and adenosine are known to inhibit AdoHcy hydrolase, the activity of which is not impaired, as shown here.

The interest in 5'MTA as an inhibitor of methylation of DNA was underlined by Woodcock et al. (10). This natural non toxic molecule is able to interfere with transmethylation reactions without inducing chromosome damage as does 5 aza-cytidine. The production of 5'MTA is directly correlated with the synthesis of spermidine and spermine. The metabolism of polyamines was shown to be early modified during normal and malignant growth. Thus, one can reasonably ask the question whether this metabolic pathway could interfere with transmethylation mechanisms and then influence the expression of genes. Work is in progress to investigate the role of 5'MTA in normal and transformed cells.

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