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Methionine Synthase Inactivation by Nitrous Oxide during Methionine Loading of Normal Human Fibroblasts. Homocysteine Remethylation as Determinant of Enzyme Inactivation and Homocysteine Export[1]

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ABSTRACT

Nitrous oxide inactivates the enzyme methionine synthase by oxidation of enzyme bound cobalamin, which is formed from the cofactor methylcobalamin during the catalytic cycle. The resulting inhibition of homocysteine remethylation increases the homocysteine efflux and thereby the level of extracellular homocysteine, both in patients and cultured cells. In the present work we measured the kinetics of enzyme inactivation and homocysteine export rate in two human fibroblast cell lines exposed to nitrous oxide and cultured in the presence of low to supraphysiological concentrations (15–1000 μM) of methionine. Both the rate and extent of methionine synthase inactivation were reduced by increasing methionine concentration in the culture medium. In cells not exposed to nitrous oxide, methionine increased the homocysteine export rate in a dose-dependent manner. Nitrous oxide increased the export at low methionine concentrations, so that for treated cells the export was high and essentially independent of the extracellular methionine level. Neither methionine nor nitrous oxide significantly affected the amount of S-adenosylmethionine or folate in these cells. These data agree with methionine synthase as a low K_m and methionine conserving enzyme, highlight the importance of methionine synthase activity as a determinant of homocysteine export and point to the possibility of protecting the enzyme by reducing catalytic turnover through product inhibition.

The anesthetic agent nitrous oxide inactivates the cobalamin-dependent enzyme methionine synthase (N^5-methyl-THF-Hcy methyltransferase, EC 2.1.1.13.) (Nunn, 1987). The enzyme remethylates Hcy to methionine, and methyl-THF serves as methyl donor and is converted to THF in this reaction (Finkelstein, 1990). The inactivation may explain side effects from prolonged nitrous oxide exposure, like megaloblastic bone marrow changes and neurological damage, which resemble symptoms from cobalamin deficiency (Nunn, 1987). In addition, the inactivation induced by nitrous oxide may represent a useful experimental tool to delineate the role of methionine synthase in the metabolism of Hcy and related metabolites.

Hcy is exported into the extracellular medium under conditions of imbalance between production and further metabolism (Ueland and Refsum, 1989). This phenomenon is the biological basis for accumulation of Hcy in extracellular media like plasma and urine in patients with inborn errors of Hcy metabolism (Mudd *et al.*, 1989), with folate (Kang *et al.*, 1987) or cobalamin deficiency (Allen *et al.*, 1990; Brattström *et al.*, 1988; Stabler *et al.*, 1988) and after exposure to drugs like methotrexate (Refsum and Ueland, 1990) and nitrous oxide (Ermens *et al.*, 1991). In experimental studies, enhancement of cellular Hcy export has been shown to reflect inhibition of Hcy remethylation caused by methotrexate (Refsum *et al.*, 1991) and nitrous oxide (Christensen *et al.*, 1992). Methotrexate acts in an indirect manner by depleting intracellular reduced folate, including 5-methyl-THF (Baram *et al.*, 1987).

Exposure of cultured cells to nitrous oxide induces a time-dependent inactivation of intracellular methionine synthase and, in cells carrying out significant Hcy remethylation, there is a concurrent enhancement of Hcy export. This experimental system is suitable for the investigation of factors modulating nitrous oxide-induced inactivation of intracellular methionine synthase, as demonstrated for extracellular folate source in a recent publication (Christensen *et al.*, 1992).

Methionine loading refers to supplementation with excess methionine which is metabolized to Hcy *via* the transmethylation pathway (Brenton *et al.*, 1965; Finkelstein, 1990). Methionine loading of patients induces a drastic increase in plasma

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ABBREVIATIONS: methyl-THF, N^5-methyltetrahydrofolate; Hcy, homocysteine (form not specified); THF, tetrahydrofolate; PBS, phosphate-buffered saline; OPA, *o*-phthaldialdehyde.

Hcy, and this represents a test to diagnose defects in Hcy metabolism (Ueland and Refsum, 1989). We have studied recently a related phenomenon *in vitro* in various cell types cultured in the presence of increasing concentrations of methionine in the medium. High levels of methionine induced a marked enhancement of Hcy export from most cells (Christensen *et al.*, 1991).

In the present work we investigated the nitrous oxide effect on methionine synthase during methionine loading. The purpose of this study is 2-fold. First, this investigation may provide information on the role of methionine synthase in Hcy metabolism and export in the presence of normal level and excess extracellular methionine. The second question is whether availability of extracellular methionine may affect the sensitivity of the intracellular enzyme toward nitrous oxide. This question was motivated by our previous finding that low folate medium protects the enzyme against inactivation (Christensen *et al.*, 1992), and data on enzyme protection may point to strategies to prevent deleterious effects of nitrous oxide on cobalamin function in the clinical setting.

Materials and Methods

Chemicals. L-Methionine, DL-homocysteine, *S*-adenosyl-L-methionine, cyanocobalamin, folic acid, DL-5-methyl-THF (barium salt), dithioerythritol and tri-*n*-butylphosphine were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Mercaptoethanol (p.a.) and methanol (Gradient Grade) were from Merck (Darmstadt, Germany), sodium borohydride and bis(3,5,5-trimethylhexyl)phthalate from Fluka Chemie, AG (Buchs AG, Switzerland) and ammonium 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulfonate from Wako German Branch (Neuss, Germany). (±)-L-N^5-[methyl-^{14}C]methyl-THF (54 mCi/mmol; barium salt) was purchased from Amersham (Buckinghamshire, England). It was dissolved in 10 mM ascorbic acid and stored as 200-μl aliquots under nitrogen at −80°C until use. Nitrous oxide, supplied as a mixture of 50% N_2O, 25% N_2, 20% O_2 and 5% CO_2, and a mixture of 75% N_2, 20% O_2 and 5% CO_2, referred to as air, were obtained from AGA AB Norgas (Oslo, Norway). Bio-Rad AG 1-X8 resin, 200–400 mesh, chloride form, was from Bio-Rad Laboratories (Richmond, CA). The material was slurry-packed in 5-ml bed volume polypropylene columns [4.5 × 1.1 cm (inside diameter)] obtained from Pierce (Rockford, Il), and the material was covered with a polyethylene disc with pore size of 45 μm.

Cell lines and stock cultures. Normal human skin fibroblasts, referred to as skin fibroblasts, were derived from skin biopsy from a 14-month-old boy. These cells (GM05659B) were purchased from National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research (Camden, NJ). The MRC-5 cell line was isolated from normal lung of a human male fetus, and is referred to as lung fibroblasts. These cells (CCL 171) were obtained from the American Type Culture Collection (Rockville, MD).

Stock cultures of both cell lines were grown in Dulbecco's modification of Eagle's medium (Flow Laboratories, Irvine, Scotland) containing 10% heat-inactivated fetal calf serum and supplemented with nonessential amino acids (3.6 mM of each).

Cell culture conditions. To allow comparison with previous studies (Christensen *et al.*, 1992) on the effects of nitrous oxide on cultured cells, all experiments were performed with cells transferred to and cultured in variants of the RPMI 1640 medium (Flow Laboratories). The medium was supplied as methionine-free powder, which was dissolved and supplemented with 10% heat-inactivated fetal calf serum, various concentrations of methionine, 2 μg/ml of cyanocobalamin and 2.3 μmol/l of folic acid.

The fibroblasts were grown in 50-ml (25 cm^2) tissue culture flasks (Nunc, Roskilde, Denmark) with 5 ml of medium. The temperature was 37°C and the relative humidity 98%.

Nitrous oxide exposure and methionine loading of cultured cells. Two experimental protocols were used to create optimal conditions for the assessment of enzyme inactivation and Hcy export, respectively.

Determination of the initial kinetics of enzyme inactivation required concentrated cell homogenate. Cells were grown to mid-to-late logarithmic growth phase (in the presence of 30 μM methionine) corresponding to $> 10^5$ cells/ml. The medium was replaced with fresh medium containing 15, 30, 150 and 1000 μM of methionine, and the culture flasks were flushed carefully for 1 min with either 50% nitrous oxide or air (control) delivered at a rate of 2 l/min *via* a sterile Pasteur pipette, as described previously (Christensen *et al.*, 1992). The cells were harvested after 1.5, 3, 6, 12, 24, 36 or 48 hr of gas exposure.

Because the Hcy export rate is dependent on cell density and decreases when growth reaches confluence (Christensen *et al.*, 1991), we also performed experiments with cells seeded at lower density. When the cells reached a density of 2 to 5 · 10^4 cells/ml (about 48 hr after seeding), the medium was replaced with fresh medium containing 15, 30, 150 and 1000 μM of methionine and exposed to air or nitrous oxide, as described above. The cells were harvested at intervals of 24 hr for 5 to 6 days.

Cell harvesting and counting. Samples of medium and the cells were harvested from two parallel flasks in each group. Medium was aspirated directly from the culture flasks with the anchorage-dependent fibroblasts. The cells were then washed gently with PBS, treated with trypsin (0.1 mg/ml) for 10 min, washed in PBS and centrifuged. The cell pellet was stored at −80°C until assayed for methionine synthase (Christensen *et al.*, 1992).

Cell counts were determined by using a Coulter Counter model ZM (Coulter Electronics Ltd., Luton, UK).

Intact cell assay. Methionine synthase activity in intact cells was assayed by determination of the incorporation of ^{14}C from N^5-[methyl-^{14}C]methyl-THF into cellular proteins. About 2 · 10^5 cells were seeded and cultured for 48 hr. Then the cells were washed with PBS and refed with 2 ml of a RPMI medium lacking methionine and folic acid, containing 0.2 mM L-Hcy thiolactone and 5 μM N^5-[methyl-^{14}C] methyl-THF. After 18 hrs, the cells were washed with PBS, and 500 μL of 5% trichloroacetic acid was added to the culture flasks. The precipitate was collected on Millipore filters (HAWP, 0.45-μm pore size) by using a Millipore filter manifold. The filters were washed 3 times with 3 ml of ice-cold trichloroacetic acid, placed in scintillation vials and the protein dissolved in 1 ml of 0.1 N NaOH before 15 ml of scintillation fluid was added.

This assay measured radiolabeled methionine incorporated into proteins, whereas the fraction converted into *S*-adenosylmethionine is not detected.

Determination of methionine synthase activity in cellular extract. This was performed by a slight modification (Christensen *et al.*, 1992) of the radioisotope method constructed by Weissbach *et al.* (1963). This assay measures the total methionine synthase activity. We also measured the activity after omission of cyanocobalamin from the assay mixture. This enzyme activity is referred to as holo activity.

Determination of Hcy and methionine in the culture medium. Hcy in the culture medium was determined by a modification of the method of Araki and Sako (1987), which measures both the major free fraction of Hcy and the small amounts of protein-bound Hcy in the culture medium.

The medium was treated with tri-*n*-butylphosphine and dimethylformamide for 30 min at 4°C and then derivatized with 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulfonate. The thiol adducts were separated by high-performance liquid chromatography on a 3-μm ODS Hypersil column (15 × 0.46 cm) which was eluted isocratically with 0.1 M phosphate buffer, pH 2.1 (Ubbink *et al.*, 1991), containing 4% acetonitrile. The flow rate was 2 ml/min, and the retention time for the Hcy derivative was 7.8 min.

Methionine in the medium was determined with an OPA method. The amino acids were derivatized with OPA in the presence of mercaptoethanol in saturated borate buffer, pH 9.5 (Krishnamurti *et al.*, 1984).

The derivatization procedure and the injection into a high-performance liquid chromatography column were carried out sequentially by a microprocessor controlled autosampler from Gilson, model 232. The methionine-OPA adduct was separated and quantitated by reversed-phase liquid chromatography and fluorescence detection (Krishnamurti *et al.*, 1984).

Determination of cellular *S*-adenosylmethionine. Cells ($5 \cdot 10^5$) from four parallel flasks were centrifuged and extracted in 500 μl of 0.6 N perchloric acid. The extract was analyzed on a cation-exchange column (Partisil 10 SCX, 0.46x25 cm, Whatman Inc, Clifton, NJ). The column was equilibrated and eluted with 120 mM ammonium formate, pH 3.5. The flow rate was 1.5 ml/min. The absorbance of the effluent was monitored at 260 nm. *S*-Adenosylmethionine eluted at 7.2 min in this system.

Determination of cellular folate. Folate in the cells or serum was assayed by using the Quantaphase folate radioassay produced by Bio Rad (Hercules, CA). We measured cellular folate with a procedure optimized for the determination of erythrocyte folate. The method is a radioligand displacement assay using ^{125}I-labeled folic acid and folate binding protein from bovine milk coupled to polymer beads as binding protein. The assay pH was adjusted to 9.2 so that folic acid and 5-methyl-THF are measured equally (Gregory, 1990).

The cells (about $1 \cdot 10^6$) were washed in ice-cold PBS, centrifuged and resuspended in 100 μl of PBS and 500 μl of 0.4% ascorbic acid was added.

Curve fitting and calculation of export rates. The logarithm of the cell number (N) and the amount of Hcy accumulated in the medium (y) were plotted *vs.* time of incubation, and the curves were fitted to polynomial functions. The Hcy export rate (v), given as the amount of Hcy exported per unit time and cell mass, was calculated from the equation

$$v = dy/dt/N.$$

The export rates were plotted against cell density (Christensen *et al.*, 1991).

Details on the curve fitting and construction of export rate curves have been published (Christensen *et al.*, 1991; Refsum *et al.*, 1991).

Determination of protein. Protein was determined by the method of Bradford (1976), by using bovine γ-globulin as standard.

Statistical analysis. Comparison between the activities remaining after exposure of cells to nitrous oxide and four concentrations of methionine was performed by using analysis of variance. In case significant differences were obtained, comparison between the separated groups was done by using Student's *t* test. All P values are given as two-tailed and corrected for multiple comparison by using the Bonferroni correction.

Results

Inhibition of Hcy remethylation in intact fibroblasts by nitrous oxide . Both the skin fibroblasts and lung fibroblasts used in this study incorporate significant amounts of radioactivity from N^5-[methyl-^{14}C]methyl-THF into proteins, and the incorporation was inhibited markedly by nitrous oxide (table 1). These data show that the intact fibroblasts carry out significant Hcy remethylation which is blocked in the presence of nitrous oxide.

Inactivation of methionine synthase at physiological or high levels of methionine. The kinetics of inactivation and residual activity of methionine synthase were investigated in fibroblasts cultured in the presence of 15 to 1000 μM methionine. In skin fibroblasts, the inactivation proceeded at a slightly higher rate (0.10 hr^{-1}) at low methionine compared to high methionine (0.06 hr^{-1}) (fig. 1; table 2). Similar results were obtained with the lung fibroblasts (table 2). The residual activities remaining after 48 hr of nitrous oxide exposure of

TABLE 1

Effect of nitrous oxide on intact cell methionine biosynthesis in human fibroblasts

(Skin fibroblasts $3 \cdot 10^5$ cells/ml) and lung fibroblasts ($1.9 \cdot 10^5$ cells/ml) were cultured in the absence and presence of nitrous oxide for 18 hr in a medium containing N^5-[methyl-^{14}C]methyl-THF and Hcy-thiolactone, as described under "Materials and Methods." The incorporation of radioactivity from N^5-[methyl-^{14}C]methyl-THF into acid precipitable material was determined. Values are mean of four determinations ± S.D.

Cell Line	Methyl Group Incorporated	
	−N_2O	+N_2O
	pmol/10^6 cells	*pmol/10^6 cells*
Skin fibroblasts	97 ± 5	28 ± 3
Lung fibroblasts	414 ± 65	62 ± 18

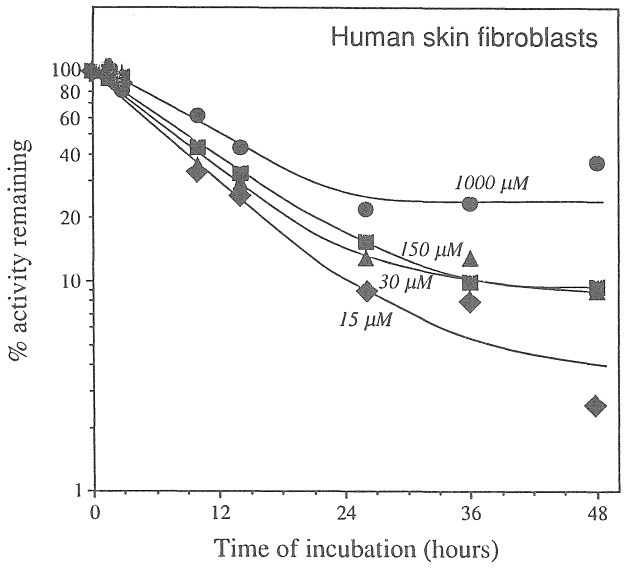

Fig. 1. Methionine synthase inactivation by nitrous oxide in human skin fibroblasts cultured in the presence of various concentrations of methionine. The cells were grown to $1.05 \cdot 10^5$ cells/ml and then were transferred to a medium containing 15 (♦), 30 (▲), 150 (■) and 1000 μM (●) methionine. The corresponding Hcy export rate curves are show in figure 2 A and B, which also show the cell density during the experiment. The results are given as percentage of activity remaining, which is the activity of the cells exposed to nitrous oxide in percentage of the activity of control cells. Each value is the average of duplicate measurements. The experiment was repeated 3 times.

both skin and lung fibroblasts were significantly higher at high methionine medium compared with low methionine medium (table 2). Thus, high extracellular methionine protects in part methionine synthase against inactivation induced by nitrous oxide.

The methionine synthase activity also was measured in the fibroblasts seeded at low density and cultured for 5 to 6 days in the presence of increasing concentrations of methionine, with and without nitrous oxide. Again, the residual activity remaining after inactivation of methionine synthase by nitrous oxide was relatively high (45%) at 1000 μM concentration of methionine and low (10–20%) at 15 μM methionine. Notably, the absolute activity in the absence of nitrous oxide was essentially independent of the methionine concentration (data not shown).

We also measured the methionine synthase activity in the absence of added cyanocobalamin, *i.e.*, holo activity. This activity was regularly 10 to 15% of the total activity (measured in the presence of added cyanocobalamin), and the inactivation kinetics and residual activities mirrored the data obtained for

TABLE 2

Inactivation of methionine synthase by nitrous oxide in human fibroblasts cultured in the presence of various concentrations of methionine

The experimental design was as described in the legend to figure 1. The values for the inactivation rate are obtained from typical experiments, and the complete curves for the skin fibroblasts are shown in figure 1. The residual activities after 48 hr of nitrous oxide exposure are obtained from a separate experiment based on six parallel determinations. These data are given as means ± S.D.

Cell Line	Methionine in Medium	Initial Rate of Inactivation[a]	Residual Activity After 48 hr
	μM	hr^{-1}	% of control
Skin fibroblasts	15	0.10	8.4 ± 1.0[a]
	30	0.09	10.0 ± 4.7[b]
	150	0.08	12.1 ± 3.9[c]
	1000	0.06	26.6 ± 5.5
Lung fibroblasts	15	0.20	10.0 ± 1.0[a,d]
	30	0.17	11.3 ± 2.5[a,e]
	150	0.14	16.3 ± 2.8[a]
	1000	0.09	44.7 ± 7.3

[a] Significantly different from 1000 μM, $P < .0005$.
[b] Significantly different from 1000 μM, $P < .001$.
[c] Significantly different from 1000 μM, $P < .005$.
[d] Significantly different from 150 μM, $P < .005$.
[e] Significantly different from 150 μM, $P < .05$.

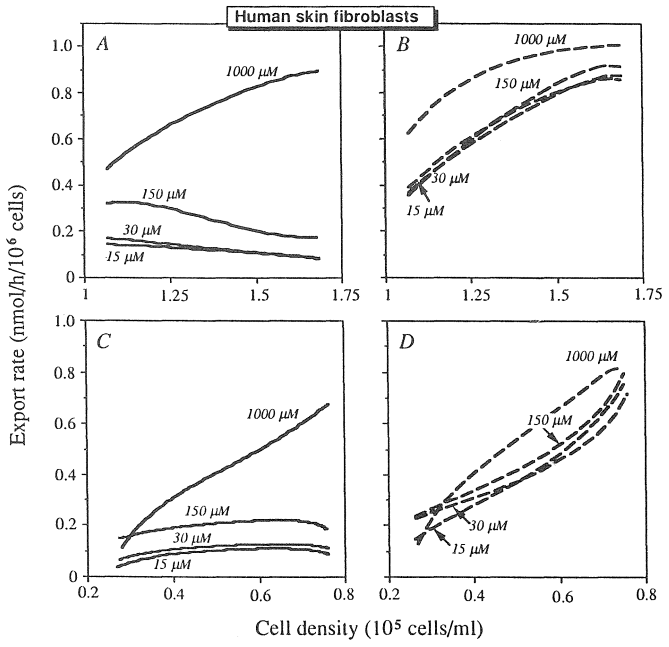

Fig. 2. Effect of methionine in the culture medium on Hcy export from human skin fibroblasts exposed to air or nitrous oxide. The concentrations of methionine (15–1000 μM) are indicated on the graph. The experiment was performed with $1.05 \cdot 10^5$ cells/ml and cultured for an additional 48 hr (A and B) and with $0.25 \cdot 10^5$ cells/ml and cultured for 5 days (C and D). Results from cells exposed to air are given on A and C (——) and from cells exposed to nitrous oxide on B and D (– – –). The experiment was repeated 3 times.

the total activity (data not shown). However, the residual holo activity approached the background of our methionine synthase assay, and this precluded accurate determinations.

Hcy response to methionine loading. The Hcy export rates were investigated in skin and lung fibroblasts cultured in the presence of 15 to 1000 μM methionine. The export was determined under the conditions (high cell density) used for measurement of inactivation kinetics (figs. 2, A and B and 3, A-D) and with cell seeded at low density cultured for 5 to 6 days (figs. 2, C and D and 3, E-H). The latter design was motivated by the observation (Christensen *et al.*, 1991) that Hcy export rates are related to cell density and growth phase.

Methionine loading increased the Hcy export rate of both cell lines seeded at high or low density (figs. 2, A and C and 3). Export from cells cultured at high density decreased immediately after seeding, whereas the export for cells seeded at low density increased or was stable for at least one doubling time.

Effect of nitrous oxide on the Hcy export during methionine loading. The Hcy export rates were determined for skin and lung fibroblasts cultured in the presence of 15 to 1000 μM methionine during nitrous oxide exposure. The experimental design (high and low density) was the same as that of the experiments described in the preceding paragraph.

Methionine loading of cells exposed to nitrous oxide induced no or only a slight increase in Hcy export rate, as demonstrated for the skin fibroblasts in figure 2, B and D. This reflects that nitrous oxide enhanced the Hcy export from cells cultured at low concentrations of methionine in the medium, but not at high (1000 μM) methionine (figs. 2 and 3).

Nitrous oxide also caused a change in the relation between cell density and Hcy export rate. In the skin fibroblasts treated with nitrous oxide, the Hcy export rate increased as a function of cells growth (fig. 2). Similarly, in nitrous oxide-treated lung fibroblasts, the Hcy export rate showed an initial increase, and the decline in export rate at high cell density was delayed compared with cells cultured without nitrous oxide (fig. 3).

Methionine, *S*-adenosylmethionine and folate. The methionine concentrations in the culture medium were followed during cell growth. The percentage of reduction was highest for cells cultured for up to 6 days in the presence of low medium methionine. When the initial concentration was higher than 30 μM, the level decreased by less than 20%. Largest decrease was observed for lung fibroblasts grown at 15 μM methionine for 6 days. At this time point methionine in the medium was reduced by 70% (data not shown).

Fibroblasts cultured in the presence of various concentrations of methionine for 48 hr contained essentially the same amount of *S*-adenosylmethionine. Nitrous oxide did not affect the *S*-adenosylmethionine concentration. The concentrations of *S*-adenosylmethionine were about 400 pmol/10^6 both in the skin and in the lung fibroblasts (table 3).

We also determined the intracellular folate content in skin fibroblasts cultured in the presence of 15 to 1000 μmol/l of methionine and the absence and presence of nitrous oxide. Neither methionine in the culture medium nor nitrous oxide exposure affected the intracellular folate concentration, which was 33.6 ± 5.3 (mean ± S.D.) pmol/10^6 cells in these cells (data not shown).

Discussion

The inactivation of human and animal methionine synthase by nitrous oxide has been investigated extensively *in vivo* (referenced in Christensen *et al.*, 1992) and isolated cells in culture have been used in only a few studies (Boss, 1985; Christensen *et al.*, 1992; Rosenblatt *et al.*, 1984).

By using isolated cells as an experimental system for studying the effect of nitrous oxide has several attractive features. First, the interaction with the target enzyme, methionine synthase, takes place in intracellular environments. Secondly, the level of intracellular metabolites can be modulated by changing the composition of the culture medium. This makes it possible to

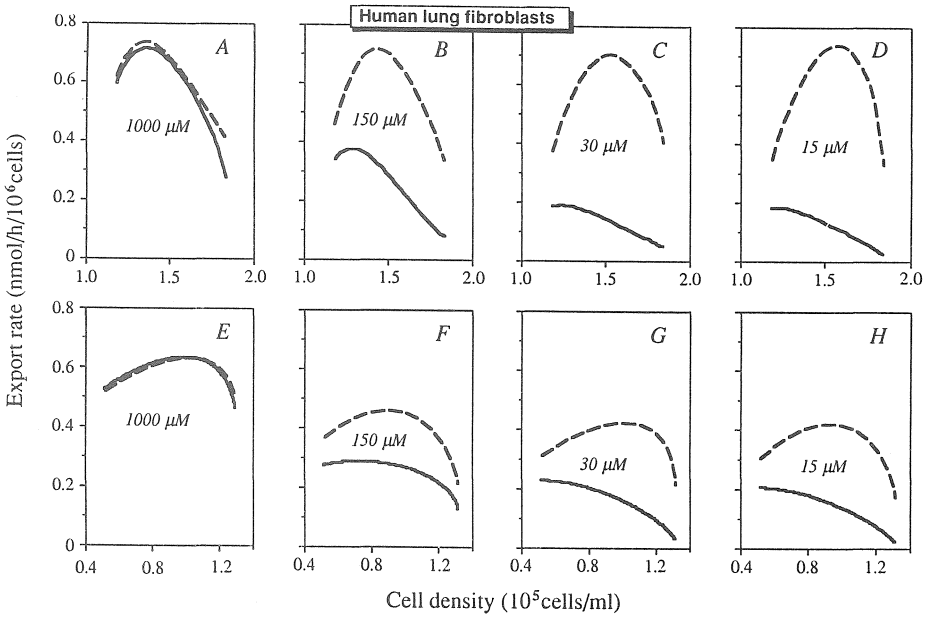

Fig. 3. Effect of nitrous oxide on Hcy export from human lung fibroblasts cultured in the presence of various concentrations of methionine. The concentrations of methionine (15–1000 μM) are indicated on the graph. The experiment was performed with 1.15 · 10^5 cells/ml and was cultured for 48 hr (A-D) and with 0.5 · 10^5 cells/ml and was cultured for 6 days (E-H). Results from cells exposed to air are given as ——, and from cells exposed to nitrous oxide as – – –. The experiment was repeated 4 times.

TABLE 3

Effect of methionine and nitrous oxide on the cellular content of *S*-adenosylmethionine in human fibroblasts

The fibroblasts were cultured for 48 hr to a cell density of 1 · 10^5 cells/ml and then transferred to a medium with different concentrations of methionine and cultured for an additional 48 hr in the absence or presence of nitrous oxide. Values are mean of four determinations ± S.D.

Cell Line	Methionine in Medium	S-Adenosylmethionine	
		$-N_2O$	$+N_2O$
	μM	pmol/10^6 cells	pmol/10^6 cells
Skin fibroblasts	15	460 ± 166	449 ± 22
	30	329 ± 70	394 ± 59
	150	570 ± 36	372 ± 76
	1000	409 ± 88	340 ± 66
Lung fibroblasts	15	378 ± 19	397 ± 19
	30	349 ± 21	310 ± 19
	150	368 ± 22	397 ± 19
	1000	426 ± 32	417 ± 37

investigate whether the intracellular availability of substrates or cofactors of the methionine synthase reaction affects the sensitivity of the enzyme in intact cells toward nitrous oxide-induced inactivation. We have tested this idea and investigated the effect of extracellular folic acid and 5-methyl-THF on the inactivation of methionine synthase in intact cultured cells (Christensen *et al.*, 1992). A rapid and extensive inactivation was observed at high folate medium, whereas low folate medium protected the enzyme.

In the present paper we investigated the effect of nitrous oxide on human fibroblasts cultured in the presence of varying methionine concentrations. The Hcy export increased as a function of increasing levels of methionine in the medium, and nitrous oxide profoundly affected this relationship.

At low (physiological) methionine concentrations (≤30 μM), methionine synthase was rapidly and extensively inactivated by nitrous oxide (fig. 1; table 2). This finding agrees with control cells having low Hcy export which was increased markedly upon nitrous oxide exposure (fig. 2). This increase is probably due to inhibition of Hcy remethylation.

At high methionine medium, methionine synthase was inactivated slowly and a high residual activity remained after prolonged exposure (fig. 1; table 2). The observation that there is essentially no effect on Hcy export in spite of significant inactivation in methionine synthase (fig. 1; table 2) suggests that this enzyme is responsible for the metabolism of only a minor fraction of the total amount of Hcy formed in cells cultured in the presence of excess methionine.

The difference in the Hcy export rates in the absence and presence of nitrous oxide (fig. 3) probably reflects the Hcy remethylation catalyzed by methionine synthase, and suggests that this enzyme remethylates a significant fraction of Hcy at low methionine concentrations. This agrees with the idea of methionine synthase as a low K_m enzyme important for methionine conservation (Finkelstein, 1990). The high maximal export rate reached at high concentrations of methionine or in the presence of nitrous oxide (figs. 2 and 3) may reflect metabolic processes draining Hcy during methionine excess. The high K_m Hcy degrading enzyme, cystathionine β-synthase (Finkelstein, 1990; Fowler, 1982), is a likely candidate for this function.

The observation of a high Hcy export rate essentially independent of methionine level from cells treated with nitrous oxide (figs. 2 and 3) resembles the response observed for methionine-dependent murine lymphoma cells (Christensen *et al.*, 1991). In cells with no or low methionine synthase activity, the Hcy export is probably determined by a constant rate of Hcy formation from *S*-adenosylhomocysteine (Svardal *et al.*, 1986a,b) and the capacity of the high K_m enzyme cystathionine β-synthase (Finkelstein, 1990).

Formation of *S*-adenosylhomocysteine catalyzed by *S*-adenosylhomocysteine hydrolase is an alternate route of homocysteine disposal (Ueland, 1982). This reaction requires the presence of a significant amount of the cosubstrate adenosine. Because cellular adenosine level is kept low through the action adenosine deaminase, probably only a minor portion of Hcy is converted into *S*-adenosylhomocysteine (Ueland, 1982).

Studies on isolated methionine synthase from rat liver and *Escherichia coli* show that the enzyme inactivation by nitrous oxide is dependent on catalytic turnover, and occurs only in the presence of all components required for enzyme catalysis (Frasca *et al.*, 1986). Nitrous oxide oxidizes enzyme bound cobalamin, which is transiently formed during the catalytic

cycle (Chanarin *et al.*, 1985). Our results with cultured cells agree with this model. High medium folate (Christensen *et al.*, 1992) probably increases the cellular pool of methyl-THF (Bunni *et al.*, 1988) and thereby increases the flux through methionine synthase, whereas high concentrations of extracellular methionine may protect methionine synthase by reducing the catalytic activity of enzyme. Methionine may act through product inhibition of methionine synthase (Burke *et al.*, 1971). An indirect effect *via* intracellular *S*-adenosylmethionine (Finkelstein, 1990) or folate is unlikely because the content of these metabolites in the human cultured fibroblasts (data not shown) and living rats (Horne, 1989) was not influenced by methionine supplementation (data not shown).

Other mechanisms for the effect of methionine and nitrous oxide should be considered. Synthesis of new enzyme may be enhanced in the presence of high levels of methionine, or the intracellular enzyme may become reactivated, in spite of the fact that inactivation is irreversible *in vitro* (Frasca *et al.*, 1986). The possibility that methionine or nitrous oxide directly affect the Hcy transporter cannot been ruled out. Notably, methionine affects glutathione efflux from isolated rat liver cells *in vitro* (Aw *et al.*, 1984).

Feeding monkeys (Scott *et al.*, 1981) or fruit bats (Van der Westhuyzen *et al.*, 1982) a diet rich in methionine protected these animals against the neurological impairment induced by prolonged nitrous oxide exposure. Administration of folate to fruit bats aggravated the effect. The authors proposed that methionine supplementation acts by ameliorating the methyl group deficiency (Scott *et al.*, 1981; Van der Westhuyzen *et al.*, 1982). The results from previous (Christensen *et al.*, 1992) and present experiments with cultured cells suggest an alternative explanation. Administration of folate or methionine to whole animals may modulate the susceptibility of methionine synthase to nitrous oxide-induced inactivation.

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