Toxic. in Vitro Vol. 6, No. 2, pp. 133–137, 1992
Printed in Great Britain.

0887-2333/92 $5.00 + 0.00

EFFECT OF NITROUS OXIDE ON FOLATE COENZYME DISTRIBUTION AND *DE NOVO* SYNTHESIS OF THYMIDYLATE IN HUMAN BONE MARROW CELLS

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(Received 21 November 1990; revisions received 7 May 1991)

Abstract—The effect of nitrous oxide on intracellular folate metabolism of the human bone marrow was studied *in vitro*. Bone marrow cells, obtained from healthy volunteers, were incubated with 5×10^{-8} M-[^{3}H]5-formyltetrahydrofolate (5-formylTHF) for 18 hr to label intracellular folate pools. Subsequently the cells were exposed to nitrous oxide for up to 10 hr, and the intracellular folate coenzyme levels were quantitated by HPLC. The dU suppression test was carried out on part of the bone marrow samples in order to measure folate-dependent synthesis of the DNA precursor thymidylate (dTMP). After 5 hr exposure to nitrous oxide the *de novo* dTMP synthesis of the bone marrow cells was significantly decreased ($P < 0.05$), and this reduced synthesis persisted at 10 hr. After both 5 and 10 hr of exposure to nitrous oxide the amount of 10-formylTHF was reduced ($P < 0.05$) while that of 5-methylTHF was increased ($P < 0.05$). At 10 hr the level of THF was also decreased ($P < 0.05$). This study shows that nitrous oxide exposure of human bone marrow cells causes a redistribution of the various folate coenzymes which supports the idea of 'functional cobalamin deficiency'. Moreover it seems probable that following prolonged exposure to nitrous oxide, not only folate-dependent dTMP synthesis but also *de novo* purine synthesis is reduced.

INTRODUCTION

Several clinical studies have shown that prolonged exposure to the anaesthetic gas nitrous oxide can cause bone marrow depression (Amess *et al.*, 1978; Chanarin *et al.*, 1985; Skacel *et al.*, 1983). This phenomenon may be explained by the fact that nitrous oxide specifically inactivates the methylcobalamin coenzyme of methionine synthase. This enzyme plays a crucial role in the cellular metabolism of reduced folates by conversion of homocysteine and 5-methyltetrahydrofolate (5-methylTHF), the major extracellular folate, to methionine and tetrahydrofolate (THF) (Fig. 1). Prolonged exposure to nitrous oxide therefore causes accumulation of 5-methylTHF at the expense of other reduced folates that form the quintessence of the 'methylfolate trap' hypothesis of functional cobalamin deficiency (Herbert and Zalusky, 1962), which recently has come under considerable criticism (Deacon *et al.*, 1990). As a result of the disturbed folate-dependent methylation reactions, impairment of the *de novo* synthesis of the DNA precursor thymidylate (dTMP) occurs. Obviously the rapidly dividing cells of the haematopoietic compartment are very dependent on the intact function of the methylcobalamin coenzyme.

The widespread use of nitrous oxide has evoked general interest in the possible toxicity of this anaesthetic gas. Several clinical studies on haematotoxic effects have therefore been performed (Amess *et al.*, 1978; Skacel *et al.*, 1983). However, metabolic research on nitrous oxide and its effects on intracellular folate metabolism has been limited to laboratory animals and cell lines (Boss, 1985; Wilson and Horne, 1986). This may be explained by the fact that studies on human material (*ex vivo*) have been hampered by the instability of the various folate coenzymes and even more by their low intracellular concentration (Duch *et al.*, 1983; Pheasant *et al.*, 1983; Wilson and Horne, 1986). The major objective of the present work was to determine the folate coenzyme distribution in fresh human bone marrow cells during exposure *in vitro* to nitrous oxide and to correlate these results with disturbances of folate-dependent dTMP synthesis.

Abbreviations: BSA = bovine serum albumin; dTMP = thymidylate; FCS = foetal calf serum; HBSS = Hanks' balanced salt solution; THF = tetrahydrofolate.

MATERIALS AND METHODS

Chemicals. 5-FormylTHF was obtained from Lederle (Etten-Leur, The Netherlands). The premixed gas cylinder containing 50% nitrous oxide, 20% oxygen, 25% nitrogen and 5% carbon dioxide was purchased from Hoekloos (Schiedam, The Netherlands). 3′,5′,7′[^{3}H] (6S) 5-formylTHF (sp. act. 40 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St Louis, MO, USA), Lymphoprep was obtained from Mycomed (Oslo, Norway) and foetal calf serum (FCS) was

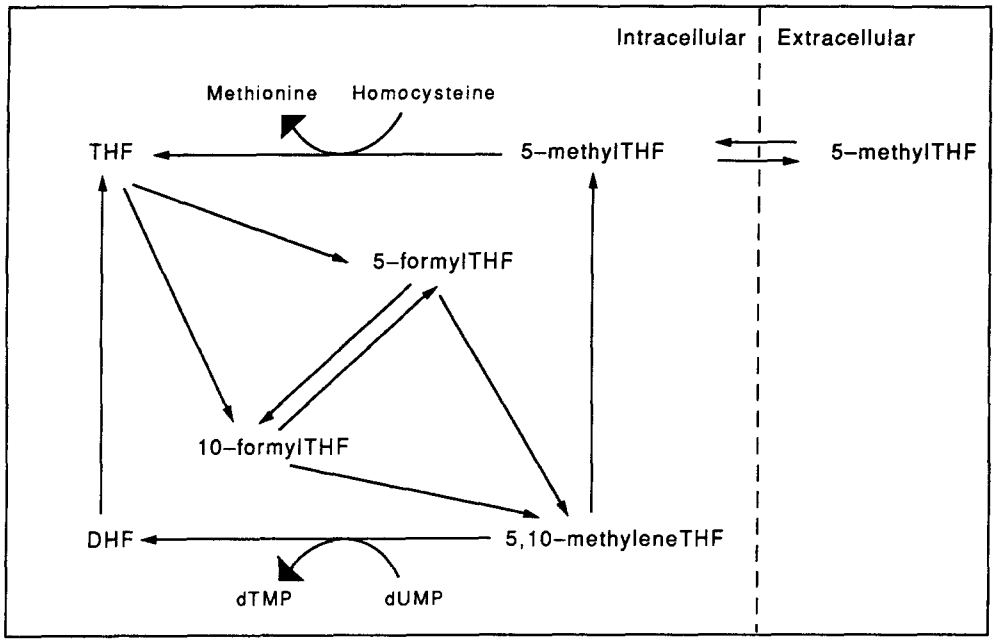

Fig. 1. Outline of the most important intracellular interconversions of reduced folates: DHF = dihydrofolate; THF = tetrahydrofolate.

from Flow Laboratories (Rickmansworth, Herts., England). Hog kidney hydrolase was prepared according to the method of McMartin *et al.* (1981), and pure human transcobalamin II was purified from Cohn's fraction III according to the method of Van Kapel *et al.* (1981). RPMI 1640 medium and Hanks' balanced salt solution (HBSS) were prepared in the laboratory.

Preparation of bone marrow cell suspension. Bone marrow aspirates were obtained after informed consent from five healthy volunteers. The samples were collected in tubes containing EDTA. The mononuclear cells were isolated by lymphoprep centrifugation and washed with HBSS containing 0.2% BSA. Monocytes and macrophages were removed by adherence to a large (150-cm^2) culture flask during a 1-hr incubation of the cells in HBSS with 10% FCS at 37°C. Subsequently the cell suspensions were divided into two. One part was used for the determination of the deoxyuridine (dU) suppression test value, and the other part was used for the radioactive labelling of the cellular folate pools.

Radioactive labelling of intracellular folate pools. The cells were labelled with [^{3}H]5-formylTHF according to the method of Baram *et al.* (1987). A suspension of 2×10^6 cells/ml in RPMI 1640 (without folic acid or cobalamin), containing 10% dialysed FCS, 50 nM-[^{3}H]5-formylTHF and 40 pM transcobalamin II was prepared. After incubation at 37°C, under an atmosphere containing 5% CO_2 for 18 hr, the cells were washed with HBSS containing 0.2% BSA and resuspended (at 2×10^6 cells/ml) in RPMI 1640, without folic acid or cobalamin, containing 10% dialysed FCS. After resuspension, 6×10^6 cells were used for direct determination of total intracellular [^{3}H]folate content and for HPLC separation of the folate coenzymes (see below).

Exposure of bone marrow cells to nitrous oxide. Both radioactively labelled and unlabelled cell suspensions were divided between four culture flasks (6×10^6 cells in 6 ml RPMI 1640 per flask). From each cell suspension two flasks were exposed to nitrous oxide by flushing for 15 min with a filtered mixture released from a premixed cylinder. Subsequently, the culture flasks were closed and incubated at 37°C for 5 or 10 hr. The two other flasks were flushed with a mixture of air and 5% CO_2 for 15 min and also incubated for 5 or 10 hr at 37°C. Before and after incubation, cell viability (trypan blue exclusion) and cell concentrations were checked. Total cell loss never exceeded 10%.

Quantitation of folate coenzyme pools. After incubation, cells were washed with HBSS containing 0.2% BSA and counted, and a sample was drawn from each flask for measurement of total intracellular [^{3}H]folate content. A standard mixture of unlabelled folates was added to the remaining cells. Subsequently, folates were extracted according to Baram *et al.* (1987), using a 90-sec boil of the cell pellet in 2 ml 2% mercaptoethanol–2% ascorbic acid (pH 6.0). After treatment for 1 hr with hog kidney hydrolase in order to break down folate polyglutamates, 2 ml of the extraction solution was added before an additional 90-sec boil. Folates were further purified and concentrated on C_{18} SEP-PAK cartridges, and then separated by reverse phase HPLC essentially as described by Allegra *et al.* (1986), with the following modifications. The prepared folate

Table 1. Effect of nitrous oxide exposure on the total intracellular [^{3}H]folate and the distribution of the various folate coenzymes in human bone marrow cells *in vitro*

Treatment	Total [^{3}H]folate (pmol/10^6 cells)	Folate coenzymes (% of total folate)				
		$N^{10}F$	N^5F	DHF	THF	MTHF
Pre-incubation	302 ± 126	23 ± 4	7 ± 1	3 ± 2	8 ± 2	59 ± 5
Control (5 hr)	256 ± 125	25 ± 6	9 ± 2	3 ± 3	9 ± 2	53 ± 5
N_2O (5 hr)	238 ± 85	19 ± 6*	8 ± 1	1 ± 1	7 ± 2	65 ± 8*
Control (10 hr)	260 ± 101	25 ± 5	9 ± 2	4 ± 3	10 ± 2	52 ± 8
N_2O (10 hr)	242 ± 97	16 ± 4*	7 ± 3	1 ± 1	5 ± 2*	71 ± 6*

$N^{10}F$ = 10-formylTHF N^5F = 5-formylTHF DHF = dihydrofolate THF = tetrahydrofolate MTHF = 5-methylTHF

Values are means ± SD for five bone marrow samples, and those marked with an asterisk differ significantly (Wilcoxon signed rank test for paired observations) from the corresponding control value (*$P < 0.05$).

extracts were dissolved in 400 µl 2% mercaptoethanol–2% vitamin C (pH 6.0). From this extract 200 µl was injected onto a Chromsphere C18 column, 200 × 3 mm (Chrompack Inc., Bridgewater, NJ, USA). The elution conditions were as follows: 0–15 min, isocratic with 10 mM-ammonium phosphate, 5 mM-Pic A, 12.5% methanol, pH 5.5; 15–49 min, linear gradient from 12.5 to 22% methanol in same buffer. The flow was 0.4 ml/min. The retention times of the folates were as follows: 10-formylTHF, 21.2 min; THF, 24.2 min; 5-formylTHF, 28.9 min; DHF, 31.9 min; 5-methylTHF, 37.9 min. Samples were collected at 30-sec intervals. The amount of radioactive folates was determined by liquid scintillation counting. Unlabelled folate standards were detected at 290 nm with a spectrophotometer (Spectroflow 757, Kratos) and used for identification of the radioactive folate peaks. Background counts in the HPLC eluate were ± 100 dpm; fractions of folate peaks contained 100–2000 dpm above the background. Before and after analysis of radioactive extracts, unlabelled standard folate mixtures (the same as mentioned above) were injected onto the HPLC system. Calculation of intracellular radioactive folate pools was based on the recovery of the unlabelled folate standards in the cell extract, and the amount of radioactivity in the peaks. Although present in small amounts, 5,10-methyleneTHF could not be quantitated accurately in the present study.

Table 2. Effect of nitrous oxide on *de novo* synthesis of thymidylate (dTMP) as measured by the deoxyuridine suppression test

Treatment	dU value†
Pre-incubation	5.5 ± 1.0
Control (5 hr)	6.2 ± 2.7
N_2O (5 hr)	11.8 ± 5.4*
Control (10 hr)	6.3 ± 2.3
N_2O (10 hr)	12.5 ± 4.7*

†Results are expressed as the percentage ratio of [^{3}H]thymidine incorporation with dU to that without dU.

Values are means of triple incubations of five bone marrow samples ± SD. Values marked with asterisks differ significantly (Wilcoxon signed rank test for paired observations) from the corresponding control value (*$P < 0.05$).

Deoxyuridine suppression test. This test was used to evaluate the impairment of the *de novo* synthesis of dTMP as a consequence of the disturbance of folate metabolism. [^{3}H]Thymidine incorporation into DNA is measured with and without the pre-incubation of cells with deoxyuridine (dU). If the folate-dependent dTMP synthesis from dUMP (see Fig. 1) is normal, the pre-exposure to dU will suppress [^{3}H]thymidine incorporation to less than 10% of the control incubations without dU. However, in the case of disturbed folate metabolism, the decreased *de novo* dTMP synthesis favours the utilization of [^{3}H]thymidine for incorporation into DNA.

The results of this assay are expressed as the percentage ratio of the [^{3}H]thymidine incorporation with and without dU in similar cell suspensions. The test was carried out essentially as described by Metz *et al.* (1968), with some modifications. Briefly, after incubation cells were washed with HBSS containing 0.2% BSA and counted. 10^6 cells with or without 0.1 mmol/dU/litre were incubated for 1 hr at 37°C, followed by another 1-hr incubation in the presence of 0.3 µCi [^{3}H]thymidine (sp. act. 25 Ci/mmol; Amersham International plc, Bucks., UK). All results were the mean of triple incubations.

Statistical evaluation. The effects of treatment with nitrous oxide were tested against the control values with the Wilcoxon signed rank test for paired observations. Results are presented as mean ± SD.

RESULTS

Effect of treatment on total [^{3}H]folate content

During the first 5 hr after the pre-incubation with [^{3}H]5-formylTHF some loss of intracellular [^{3}H]folate occurred. Table 1 shows that this reduction was caused mainly by a decrease in intracellular [^{3}H]5-methylTHF. The total [^{3}H]folate levels of the cells exposed to nitrous oxide tended to be lower than those of control cell suspensions although the differences were not statistically significant.

Effect on intracellular folate coenzyme distribution

After an initial decrease in the level of 5-methylTHF, the folate coenzyme distribution of the control cell suspensions remained unchanged during the experiments. Exposure to nitrous oxide caused

significant decreases ($P < 0.05$) in the level of 10-formylTHF after 5 and 10 hr. Moreover a 50% decrease in THF was observed after 10 hr of exposure to nitrous oxide ($P < 0.05$). In contrast with this, there was a time-dependent increase in the 5-methyl-THF pool, up to 36% after 10 hr of nitrous oxide exposure ($P < 0.05$).

Effect on deoxyuridine suppression test

During the experiments the capacity of the control cell suspensions for *de novo* dTMP synthesis hardly changed as measured by the dU test values which were always below 10%. However, in all experiments exposure to nitrous oxide caused an increase of the dU test values ($P < 0.05$) which indicates a decreased *de novo* dTMP synthesis. After 10 hr of nitrous oxide exposure, four of the five bone marrow samples gave dU test values that were above the upper limit of the normal range (10%).

DISCUSSION

Nitrous oxide impairs methionine synthase activity by oxidation of its cobalamin coenzyme. The effects of this 'functional cobalamin deficiency' may be explained by the 'methylfolate trap hypothesis' (Herbert and Zalusky, 1962). This states that during cobalamin deficiency (either nutritional or drug-induced) all folate coenzymes become trapped as 5-methylTHF, the major folate transport form. Finally this disturbance of the folate metabolism results in impairment of *de novo* synthesis of dTMP. Over the years the methylfolate trap hypothesis has been supported by the results of several biochemical studies on animal tissues. The present paper for the first time provides information on disturbances of the folate coenzyme distribution of human bone marrow cells during nitrous oxide exposure. However, it should be noted that in this study possible effects on the level of polyglutamation of the folates cannot be detected (Lumb *et al.*, 1985).

Apart from an initial fall in 5-methylTHF, possibly due to transmembrane transport to the folate-free medium, the folate coenzyme distribution of the control cell suspensions remains stable. Exposure to nitrous oxide induces an accumulation of 5-methylTHF at the expense of the other folate forms, the most important of which is the reduction of 10-formylTHF which is an indispensable coenzyme for both AICAR- and GAR-transformylase. These two enzymes are involved in the *de novo* synthesis of purines. Our results therefore support earlier suggestions that besides impaired dTMP synthesis, disturbance of purine metabolism may contribute to the haematotoxic effect of nitrous oxide (Boss, 1985). In addition, the decrease in intracellular THF, which plays a key role in the overall metabolism of folate, points to the derangement of other folate-dependent pathways.

Levels of 5,10-methyleneTHF, the coenzyme of dTMP synthase, could not be measured because of its low intracellular concentration. Therefore we chose the dU suppression test to measure folate-dependent *de novo* synthesis of dTMP. Exposure *in vitro* to nitrous oxide for 5 hr reduced the *de novo* dTMP synthesis of the bone marrow cells. These results concur with studies on anaesthesia patients in which nitrous oxide exposures of 5–6 hr induced abnormal values in the dU suppression test (Kano *et al.*, 1984).

Interestingly, the continued derangement of the folate metabolism that occurs during nitrous oxide exposure for 5–10 hr is not accompanied by further reduction of the *de novo* dTMP synthesis. Possibly, the relatively small pool of 5,10-methyleneTHF is already depleted after 5 hr of nitrous oxide exposure. If this is so, continued exposure to nitrous oxide would mainly affect other folate-dependent pathways, especially *de novo* purine synthesis. Although the present paper confirms earlier investigations in animals *in vivo*, the *in vitro* effects of nitrous oxide on the folate coenzyme distribution are smaller. The most probable explanations for this are the shorter exposure period (Lumb *et al.*, 1985) and/or the use of a lower concentration of nitrous oxide (50% N_2O) in comparison with that used in animal studies (70% N_2O; Wilson and Horne, 1986).

In conclusion, this study demonstrates that the specific redistribution of folate coenzymes during nitrous oxide exposure of human bone marrow cells is in agreement with the theory of 'functional cobalamin deficiency'. Moreover, this study suggests that the haematotoxic effects of nitrous oxide may be based on reductions in synthesis of both dTMP and purine.

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