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Nitrous Oxide Exposure Reduces Hepatic C_1-Tetrahydrofolate Synthase Expression in Rats

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C_1-tetrahydrofolate synthase (C_1-THF synthase) is a trifunctional enzyme which catalyzes the interconversion of one-carbon units attached to the coenzyme THF. Nitrous oxide (N_2O) inhalation is known to inactivate hepatic cobalamin-dependent methionine synthase leading to methionine deficiency and trapping of THF in the methyl-THF form. Liver tissue from rats exposed to N_2O for 48 hours exhibited a coordinate decrease in all three activities of C_1-THF synthase of approximately 25%. A corresponding 25% decrease in immunoreactive C_1-THF synthase was also observed after 48 hours. Thus, the decrease in the concentration of C_1-THF synthase accounted entirely for the decreases observed in the three activities. These results suggest that perturbations of hepatic THF pools by N_2O affect the level of C_1-THF synthase expression at a translational or pretranslational level.

One-carbon metabolism mediated by folate coenzymes plays an essential role in several major cellular processes including nucleic acid biosynthesis, mitochondrial and chloroplast protein biosynthesis, amino acid biosynthesis and conversions, and vitamin metabolism. The focus in our laboratory is on understanding the regulation of folate coenzyme interconversions, which allow the cell to divert the limited supply of coenzymes to satisfy the most immediate requirements of the cell. In eukaryotes, 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) are present on one polypeptide in the form of a trifunctional enzyme (see ref. 1 for review). This enzyme, termed C_1-THF synthase, is responsible for the interconversion of the one-carbon unit attached to the coenzyme tetrahydrofolate (THF). C_1-THF synthase from rat liver, recently purified and characterized in our laboratory (2), exhibits the typical trifunctional organization found for the other eukaryotic enzymes examined.

Prolonged exposure to nitrous oxide (N_2O) results in megaloblastic anemia in humans (3) and irreversible inactivation of methionine synthase in rats (4,5) due to oxidation of the active cob(I)alamin form of the enzyme (6). This direct effect on methionine synthase leads to secondary

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Abbreviations: THF, 5,6,7,8-tetrahydrofolate; N_2O, nitrous oxide; PMSF, phenylmethylsulfonylflouride.

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effects on folate metabolism, including a redistribution of hepatic folates towards the fully reduced form, 5-methyl-THF (7-10). N_2O exposure has been reported to affect the activities of other folate enzymes, including thymidylate synthase (11) and C_1-THF synthase (12). Perry *et al.* (12) reported that 10-formyl-THF synthetase activity increased, 5,10-methenyl-THF cyclohydrolase activity decreased, and 5,10-methylene-THF dehydrogenase activity was unchanged in livers of rats treated with N_2O. In view of the trifunctional nature of C_1-THF synthase in rat liver, we undertook a reinvestigation of the regulation of this enzyme in response to N_2O exposure. We report here that all three activities exhibited a small, but *coordinate* decrease after 48 hours exposure. These results were confirmed by an immunoassay of C_1-THF synthase which revealed changes in the concentration of the enzyme that paralleled those seen for the three activities.

Materials and Methods

Male Sprague-Dawley rats (150-200g) were exposed to air or N_2O/O_2 at a 1:1 ratio in 20 L metabolic chambers at a flow rate of 3 L/min (13). Lab chow and water were provided *ad libitum*. After sacrifice, livers were removed and homogenized in 4 volumes of Tris-SO_4 (pH 7.9), 50 mM KCl, 10 mM 2-mercaptoethanol, 1 mM PMSF and 3 mg/mL benzamidine with a polytron Tissumizer (Tekmar). This homogenate was centrifuged at 15,000g for 30 min and the supernatant fraction analysed as described below.

10-formyl-THF synthetase, 5,10-methylene-THF dehydrogenase, and 5,10-methenyl-THF cyclohydrolase were assayed spectrophotometrically as described (14). Methionine synthase activity was determined as described (9) except that a potassium phosphate buffer was substituted for Tris-Cl. (L)-5-[$^{14}CH_3$]-tetrahydrofolate utilized in this assay was synthesized as described (15). C_1-THF synthase was quantified in liver homogenates by a solid-phase immunoassay (14) except that polyclonal antibodies specific for rat C_1-THF synthase (2) were utilized at a dilution of 1/2000 for development of slot-blotted and electro-blotted protein. A dilution series for each extract (0.25, 0.5, 1, 2, 4 μg) was analyzed by slot-blot immunoassay and quantitated by scanning densitometry. Slopes for each extract were calculated by linear regression analysis using the least-squares fit method.

Statistical comparisons were performed by analysis of variance techniques using the STAT-FAST statistical package on a Macintosh computer.

Results

Figure 1 shows a coomassie-stained SDS gel and the corresponding immunoblot of the liver extracts used in this study. Panel A illustrates that there are no obvious changes in major soluble proteins in liver tissue from rats exposed to N_2O for 24 and 48 hours compared to air breathing controls. Panel B shows that the polyclonal antibodies used in these experiments are specific for a single protein, C_1-THF synthase (2). Exposure to N_2O up to 48 hours does not result in the expression of any additional cross reactive proteins. Decreased immunostaining is apparent in the 48h N_2O-treated extracts (lanes 7-9). This decrease was quantitated by a solid-phase immunoassay. As seen in Table I, there is a significant, but *coordinate* decrease in the three enzymatic activities of C_1-THF synthase during the time course of exposure to N_2O and the decrease is approximately 25% after 48 hours. This decrease in activity correlates very closely with the decrease in immunoreactive C_1-THF synthase quantitated by slot-blot analysis. Thus, changes in the levels of the three activities of C_1-THF synthase can be

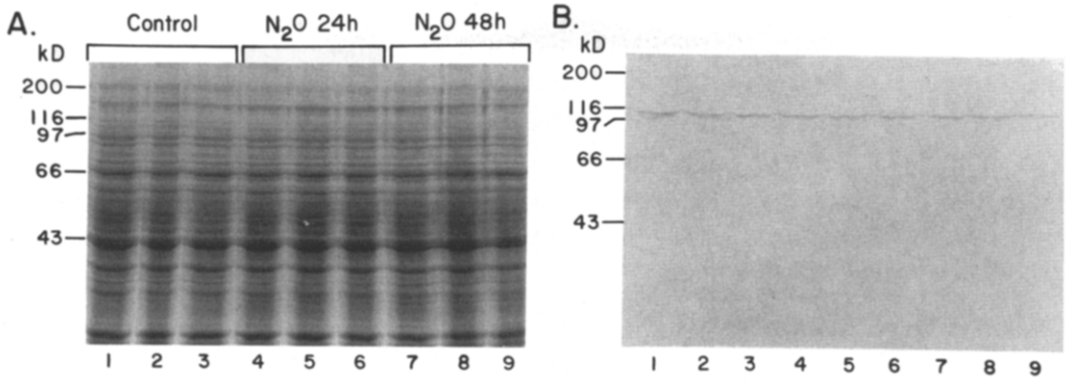

Fig. 1. (A) Coomassie blue stained SDS polyacrylamide gel of rat liver homogenates (20 μg protein per lane). (B) Western blot of a duplicate of gel in (A) transferred to nitrocellulose and stained with antisera specific for rat C_1-THF synthase. Extracts are from rats treated in groups of three with air for 48h (lanes 1-3), or N_2O/O_2 for 24h (lanes 4-6) or 48h (lanes 7-9).

explained entirely by a change in the concentration (*i.e.* expression) of the enzyme in response to N_2O exposure. Methionine synthase activity was undetectable at both 24 and 48 h (Table I), confirming the effectiveness of the N_2O-induced inactivation of that cobalamin-dependent enzyme.

Discussion

In this report, it is shown that the folate interconverting activities of C_1-THF synthase are coordinately reduced approximately one quarter after 48 hours of exposure to N_2O. This reduction in the trifunctional activities correlates well with the level of C_1-THF synthase expression as

Table I. Effect of Nitrous Oxide on C_1-THF Synthase Activity and Concentration

Exposure time	Methionine synthase	Formyl-THF Synthetase	Methylene-THF Dehydrogenase	Methenyl-THF Cyclohydrolase	Immunoactivity
hours		*nmol/min/mg*			*peak heigth/μg*
0	0.10 ± 0.01	32.0 ± 3.6	38.3 ± 3.2	60.9 ± 5.1	28.0 ± 1.5
24	ND[a]	28.0 ± 1.0 (87.5)[b]	35.3 ± 0.6 (92.2)	54.2 ± 1.8 (89.0)	26.4 ± 1.1 (94.3)
48	ND	21.0 ± 2.6[c,d] (65.6)	29.7 ± 3.1[c,d] (77.5)	47.7 ± 1.8[c,d] (78.0)	20.5 ± 0.8[c,d] (73.2)

Enzyme activity determinations were performed in duplicate or triplicate on each of three animals per time point. Values reported are the average ± standard deviation. Immunoactivity determinations were performed on a dilution series (0- 4 μg protein) of each extract. Values reported represent the slope ± standard deviation.

[a]Not detected

[b]Percent of 0 h time point

[c]Significantly different from 0 h time point ($p<0.01$)

[d]Significantly different from 24 h time point ($p<0.05$)

determined by immunoassay. This observation contrasts a previous report by Perry *et al.* (12) which showed a decrease by one third in 5,10-methenyl-THF cyclohydrolase activity, a three-fold increase in 10-formyl-THF synthetase activity and an unchanged level of 5,10-methylene-THF dehydrogenase activity after 48 hours of a 1:1 mixture of N_2O/O_2. The trifunctional enzyme activities we observed for control animals (32 mU/mg for synthetase, 38 mU/mg for dehydrogenase and 61 mU/mg for cyclohydrolase) are similar to values for normal rat liver reported by a number of other investigators (16-18).

It is difficult to explain the differences in our results and those of Perry *et al.* (12). The levels of the three activities of C_1-THF synthase reported in that earlier work are 10- to 40-fold lower than our values, perhaps due to different enzyme assay conditions. The effectiveness of the N_2O treatment used by Perry *et al.* is also difficult to ascertain since neither the flow rate of N_2O/O_2 used nor the resulting methionine synthase levels were reported. Comparison of work from the groups of Tephly (13) and Stokstad (19) indicate the importance of a sufficiently high dose (*i.e.* flow rate) of N_2O/O_2 in obtaining inactivation of methionine synthase and the resulting THF pool perturbations.

N_2O treatment inactivates methionine synthase in rats causing a methionine deficiency and a redistribution of hepatic folates toward the 5-methyl form (7-10). One effect of these perturbations of folate coenzyme pools in rat liver appears to be a repression of C_1-THF synthase expression. In the yeast *Saccharomyces cerevisiae*, the trifunctional enzyme is repressed by the simultaneous presence of adenine, histidine, methionine and pantothenic acid (14). Omission of any one of these nutrients, or drug-induced folate starvation, results in derepression of C_1-THF synthase. Although these observations in yeast do not appear to correlate with the effects of N_2O on hepatic C_1-THF synthase in rat, the differences between the two studies should be emphasized. A true folate deficiency (as in the yeast experiment) probably has very different metabolic effects than the 'functional' folate deficiency caused by treating rats *in vivo* with N_2O. One major difference is the increase in 5-methyl-THF observed in N_2O treatment which is not seen in true folate deficiency (20). 5-methyl-THF may be an important metabolite which directly or indirectly reduces the level of C_1-THF synthase. While the signal(s) which modulate C_1-THF synthase expression are unknown, folate derivatives such as 5-methyl-THF are potential candidates.

Finally, these results are consistent with the few reports in the literature concerning regulation of C_1-THF synthase. In yeast, control of C_1-THF synthase appears to be at a pretranslational level as changes in the activities and concentration of the enzyme correspond with changes in the levels of its mRNA (14). Wasserman *et al.* (21) also observed a correlation between C_1-THF synthase enzyme activity and its transcript levels in chicken liver. The lack of differential changes in the three activities and the clear correlation with immunoreactive protein levels observed in our experiments also suggest translational or pretranslational control of C_1-THF synthase, at least in response to N_2O exposure.

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