Nitric Oxide Inhibits Methionine Synthase Activity in Vivo and Disrupts Carbon Flow through the Folate Pathway*

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Many of nitric oxide's biological effects are mediated via NO binding to the iron in heme-containing proteins. Cobalamin (vitamin B12) is structurally similar to heme and is a cofactor for methionine synthase, a key enzyme in folate metabolism. NO inhibits methionine synthase activity in vitro, but data concerning NO binding to cobalamin are controversial. We now show spectroscopically that NO reacts with all three valency states of cobalamin and that NO's inhibition of methionine synthase activity most likely involves its reaction with monovalent cobalamin. By following incorporation of the methyl moiety of [14C]methyltetrahydrofolic acid into protein, we show that NO inhibits methionine synthase activity in vivo, in cultured mammalian cells. The inhibition of methionine synthase activity disrupted carbon flow through the folate pathway as measured by decreased incorporation of [14C]formate into methionine, serine, and purine nucleotides. Homocysteine, but not cysteine, attenuated NO's inhibition of purine synthesis, providing further evidence that NO was acting through methionine synthase inhibition. NO's effect was observed both when NO donors were added to cells and when NO was produced physiologically in co-culture experiments. Treating cells with an NO synthase inhibitor increased formate incorporation into methionine, serine, and purines and methyl-tetrahydrofolate incorporation into protein. Thus, physiological concentrations of NO appear to regulate carbon flow through the folate pathway.

Vitamin B12 deficiency leads to pernicious anemia and subacute combined degeneration of the spinal cord (1). Pernicious anemia is characterized by megaloblastic erythropoiesis and is secondary to decreased activity of methionine synthase, one of the two mammalian enzymes that requires vitamin B12 (cobalamin) as a cofactor (2, 3). Methionine synthase catalyzes the transfer of the methyl group of 5-methyltetrahydrofolate to homocysteine via a methylcobalamin intermediate with cycling of cobalamin between the +1 valency state (i.e. cbl(I)) and the +3 valency state (i.e. cbl(III)) (2, 3). Methyltetrahydrofolate is the major intracellular storage form of folates, and its synthesis from 5,10-methylene tetrahydrofolate is essentially irreversible in vivo (2, 4) (Fig. 1). Thus, decreased methionine synthase activity leads to trapping of intracellular folates as 5-methyltetrahydrofolate, and the megaloblastic anemia of vitamin B12 deficiency is virtually indistinguishable from the megaloblastosis of folate deficiency (1).

Nitric oxide (NO) is produced by most cell types and regulates a diverse array of biological functions (5, 6). NO has been reported to inhibit methionine synthase activity in vitro (7–9), and it might be expected to bind to the cobalt in cobalamin because (i) NO binds tightly to the iron in heme (10); (ii) ferrous heme and cbl(III) are isoelectronic; and (iii) in both heme and cobalamin, the metal ion is coordinated to four in-plane nitrogen atoms of a tetrapyrrole ring and has two out-of-plane ligands (3). However, conflicting results have been published concerning NO binding to cobalamin: Bauer (11) reported that NO binds to divalent cobalamin (i.e. cbl(II)); Brouwer et al. (7) found that NO binds to both cbl(II) and (III); Firth et al. (12) found no evidence of NO binding to either cbl(II) or (III); and Rochelle and associates (13, 14) initially reported that NO binds to cbl(III) and oxidizes cbl(II) to cbl(III) but subsequently concluded that NO does not react with either species. There have been no studies of NO reaction with cbl(I), but nitrous oxide (N2O), another oxide of nitrogen, inhibits methionine synthase activity by oxidizing cbl(I) to cbl(II), and inhalation of this gas is the most common cause of acute megaloblastosis (1, 15). We found that NO reacts with all three oxidation states of cobalamin but that its mechanism of methionine synthase inhibition appears to be similar to that of N2O, i.e. oxidation of cbl(I) to cbl(II). We show that NO inhibits methionine synthase activity in vivo and that NO produced by three different pharmacological agents or produced physiologically by rat C6 glioma cells inhibits carbon flow through the folate pathway. Contrarily, an NO synthase inhibitor increased carbon flow through folates. Thus, the data suggest that NO may modulate folate-mediated one-carbon transfer reactions.

EXPERIMENTAL PROCEDURES

Generation of Cobalamin Derivatives and Absorption Spectra—Cbl(III)-OH (Sigma; acetate salt) was prepared in 0.1 M sodium phosphate, pH 7.0, in a spectrophotometer cell sealed with a rubber septum and deoxygenated by passing argon through the solution for 30 min. Cbl(II) and cbl(I) were produced from deoxygenated cbl(III) solutions using 300 μM dithiothreitol and zinc dust suspended in 10% aqueous ammonium chloride, respectively (16). Chemically pure grade NO gas (minimum 99% purity; Matheson Gas Products) was passed through 1 M NaOH immediately prior to use to remove contaminating nitrogen

The abbreviations used are: cbl(I), cbl(II), or cbl(III), cobalamin in the +1, +2, or +3 valency state, respectively; AdoMet, S-adenosylmethionine; BHK, baby hamster kidney cells; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; GS-NO, S-nitrosoglutathione; NAME, Nω-nitro-L-arginine-methyl ester; NO, nitric oxide; N2O, nitrous oxide; SNAP, S-nitroso-N-acetylpenicillamine; PAPAb NONOate, propylamine propylamine NONOate.

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NO Disrupts Carbon Flow through Folate Intermediates

FIG. 1. Folate-methionine reactions. Folic acid (FA) is reduced to tetrahydrofolate (THF) via dihydrofolate (DHF). Formate enters the folate pathway by combining with tetrahydrofolate to form 5,10-formyltetrahydrofolate, which can be reversibly converted to 5,10-methylenetetrahydrofolate and to 5,10-methylene tetrahydrofolate. The latter compound is converted irreversibly to 5-methyltetrahydrofolate, the major intracellular storage form of folates. Methionine (Met) can be converted to AdoMet, the main intracellular source of methyl groups for transmethylase reactions. A transmethylation cycle is completed when the methyl group of 5-methyltetrahydrofolate is transferred to homocysteine (Hcy) via a cobalamin intermediate and thereby regenerates free tetrahydrofolate. Methionine (Met) can be converted to AdoMet, the main intracellular source of methyl groups for transmethylation reactions. A transmethylation cycle is completed when the methyl group of 5-methyltetrahydrofolate is transferred to homocysteine (Hcy) via a cobalamin intermediate and thereby regenerates free tetrahydrofolate.

oxidation species; it was then added anerobically to cobalamin solutions. Similar results were obtained when NO was generated from the NO donor PAPA-NONOate (Cayman Chemical). Absorption spectra were obtained using a dual beam Kontron 980 spectrophotometer.

Cell Culture and Experimental Culture Medium—CS-54 rat pulmonary arterial smooth muscle cells, which maintain differentiated properties through multiple subcultures, were obtained from A. Rothman (University of California, San Diego) (17). HL-60 human promyelocytic leukemia cells and baby hamster kidney (BHK) fibroblasts were obtained from the American Tissue Culture Collection, and C6 rat glioma cells were obtained from M. Ellisman (University of California, San Diego). All four cell lines were routinely cultured as described previously: CS-54, BHK, and C6 cells in Dulbecco’s modified Eagle’s medium (DMEM) and HL-60 cells in RPMI 1640 medium (18–20). Both media were supplemented with 10% fetal bovine serum, which serves as a source of cobalamin.

Unless otherwise noted, experiments were performed using methionine-free DMEM supplemented with 200 μM S-homocysteine thiolactone (Sigma) and 0.2% bovine serum albumin (BSA). This medium, which will be referred to as experimental medium, was used because it allowed optimal incorporation of radioactive label into methionine residues of protein. It contains 9 μM folic acid but lacks purines and pyrimidines, and although cells did not grow in it because of lack of serum growth factors, they were fully viable in the medium for at least 24 h.

Measurement of 54Cl Methyltetrahydrofolate Incorporation into Protein—CS-54 cells were transferred from growth medium into the experimental medium and were shaken gently for 20 min at 37 °C in the absence or presence of 200 μM PAPA-NONOate or 2 mM NOS-nitro-l-arginine-methyl ester (NAME, Alexis Biochemicals). The cells were then incubated for 2 h with 2.5 μCi of 5-14Cl-methyltetrahydrofolic acid (Amersham Pharmacia Biotech; 57 mCi/mmol, final medium concentration 53 μM). At the end of the incubation, cells were extracted for 20 min in ice-cold 5% trichloroacetic acid; the extracts were heated to 80 °C for 30 min and cooled on ice for 30 min, and precipitated protein free of DNA and RNA was collected on glass microfiber filters as described previously (21). Radioactivity on the filters was quantitated by liquid scintillation counting, and the assay was linear with time from 1 to 3 h and with cell number from 2 to 5 × 10⁶.

Measurement of Rates of Methionine and Serine Synthesis—Rates of methionine and serine synthesis were measured as described previously following 14Clformate incorporation into methionine and serine in protein (22–24) (Fig. 1). Briefly, HL-60 cells at a density of 1 × 10⁶/ml or subconfluent BHK or CS-54 cells were transferred to the experimental medium and were shaken gently for 2 h with 20 μCi of 14Clformate (Moravek Radiochemicals; 52 mCi/mmol, final medium concentration 190 μM). The cells were extracted as described above for measuring 14Clmethyltetrahydrofolate incorporation into protein, but after heating and cooling the extracts, they were centrifuged at 800 × g; the protein pellet free of DNA and RNA was washed three times in 10% trichloroacetic acid, resuspended in 6 N ultrapure HCl, and heated at 120 °C for 20 h. The HCl was evaporated, and the residue containing free amino acids was resuspended in H2O. Methionine was separated from serine by thin layer chromatography in a phenol/ethanol H2O/NH4OH (65:20:20:2) system, and appropriate spots were cut out with radioactivity quantitated by liquid scintillation counting. The assays were linear with time from 1 to 3 h and with cell number from 1 to 5 × 10⁶.

Measurement of Rates of de Novo and Salvage Purine Nucleotide Synthesis—To be strictly comparable with the above experiments, cells were incubated in experimental medium during measurement of purine nucleotide synthesis; however, as described later, similar results were obtained when the medium contained methionine. In the experiments shown in Fig. 5, homocysteine was omitted from the experimental medium.

Cells were preincubated at 37 °C for 30 min in the absence or presence of drugs as described above for measuring rates of methionine and serine synthesis, and then rates of de novo and salvage purine nucleotide synthesis were measured as described previously (21, 25, 26). Briefly, for de novo purine nucleotide synthesis, cells were incubated with 10 μCi of 14Clformate for 1 h and then extracted in 0.4 N perchloric acid; the cell extracts were heated at 100 °C for 70 min, and the resulting free purine bases were isolated by Dowex 50 ion exchange chromatography. Radioactivity incorporated into the bases was quantitated by liquid scintillation counting, and the data are expressed ascpm incorporated/h/10⁶ cells. The assay was linear with time from 15 min to 2 h and with cell number from 1 × 10⁶ to 5 × 10⁶.

To measure purine nucleotide synthesis by the salvage pathway, cells were incubated with 10 μCi of [14C]guanine (ICN, 56 mCi/mmol, final medium concentration 180 μM) for 1 h and then washed three times in phosphate-buffered saline plus 0.5% BSA. The cells were dissolved in 0.1 N NaOH, and radioactivity in the lysate was measured by liquid scintillation counting. The assay was linear with time and cell number, and we have shown previously that this method follows the incorporation of guanine into guanine nucleotides with <10% of the radioactivity remaining as free intracellular guanine (25, 26).

Co-culture of HL-60 Leukemic Cells with C6 Glioma Cells—To six-well cluster dishes that were either empty or contained ~1 × 10⁶ rat C6 glioma cells that had been grown to confluence were added 1 × 10⁶ HL-60 cells in 1 ml of experimental DMEM. To half of the cultures was added 10 μM hemoglobin (as an NO scavenger), and after a 30-min equilibration period, the cells were incubated with 14Clformate as described above. Rates of methionine, serine, and de novo purine synthesis were measured in HL-60 cells only by recovering the cells with three gentle washes with phosphate-buffered saline; we showed by cell counting that >95% of the HL-60 cells were recovered and by microscopic examination that the monolayer of C6 cells was left intact. As described previously for other cell types (27), we found in preliminary experiments that C6 glioma cells excrete only a small amount of newly synthesized purines into the culture medium and that the amount of purines excreted was <5% of the amount of purines synthesized by the HL-60 cells during the incubation period. Thus, it is unlikely that the C6 cells contributed significantly to the measurement of purine synthesis by HL-60 cells.

RESULTS

NO Binds to All Three Valency States of Cobalamin—To determine if NO binds to cobalamin, we performed spectral studies of NO reactions with all three oxidized states of cobalamin. We found that NO caused a small but definite change in the spectrum of cbl(III)-OH (Fig. 2a), similar to that found by Brouwer et al. (7). These spectral changes on adding NO to cbl(III) were considered too small to infer NO binding to cbl(III). We therefore confirmed NO ligation to this species by showing that NO-exposed cbl(III) was reduced by dithiothreitol.
at a rate markedly slower than that of non-NO-exposed cbl(III) (Fig. 2a, inset); thus, a truly unique cbl(III)-NO species had been formed. In agreement with Brouwer et al. (7), we found no effect of NO on the visible spectra of CH₃-cbl(III) or deoxyadenosyl-cbl(III) (data not shown).

To determine if NO binds to cbl(II) and cbl(I), we generated these two species from cbl(III)-OH using dithiothreitol to produce cbl(II) and zinc dust suspended in ammonium chloride to produce cbl(I) (16). Adding NO to cbl(II) changed its spectrum, indicating formation of cbl(II)-NO (Fig. 2b); passing argon through this solution yielded back the spectrum of cbl(II). Adding NO to cbl(I) (Fig. 2b, inset) immediately changed the spectrum to that of cbl(II); a longer exposure yielded the spectrum of cbl(II)-NO. Thus, NO binds to both cbl(II) and cbl(III), and it rapidly oxidizes cbl(I) to cbl(II). As discussed later, the following three observations concerning NO's reactions with cobalamins i.e. lack of binding to CH₃-cbl(III), weak and reversible binding to cbl(II), and instantaneous reaction with cbl(I) suggest a mechanism whereby NO inhibits methionine synthase activity.

**NO Inhibits Methionine Synthase Activity in Vivo**—Inhibition of methionine synthase activity in cell extracts and in purified enzyme preparations by high NO concentrations (7–9) does not predict whether physiological concentrations of NO inhibit the enzyme in vivo. We therefore assessed methionine synthase activity in vivo by measuring [¹⁴C]methyltetrahydrofolate incorporation into protein in CS-54 pulmonary arterial smooth muscle cells. Although this assay cannot distinguish
between incorporation of radioactivity into protein directly via methionine insertion as an amino acid residue or indirectly via protein methylation by S-adenosylmethionine, it is strictly dependent on methionine synthase activity (Fig. 1). Using this assay, we found that 200 μM PAPA-NONOate significantly decreased incorporation of radioactivity into protein, from 625 ± 65 cpm/h/10⁶ cells in the absence of the drug to 408 ± 43 cpm/h/10⁶ cells in the presence of the drug (means ± S.D. of three independent experiments performed in duplicate; p < 0.05, Student’s t test). Similar results were found in C6 glioma cells. Thus, NO inhibits methionine synthase activity in vivo.

NO Decreases Rates of Methionine and Serine Synthesis in Cultured Mammalian Cells—Decreased cellular methionine synthase activity should decrease not only methionine synthesis from homocysteine, but because of trapping of folates as 5-methyltetrahydrofolate, it should also decrease carbon flow through the folate pathway and thereby decrease serine synthesis (2, 22) (Fig. 1). To test this hypothesis, we measured [14C]formate incorporation into methionine and serine in HL-60 human leukemic cells and BHK fibroblasts; as described previously, this assay measures in vivo rates of methionine and serine synthesis and carbon flux through the folate pathway (22, 23). We found that three different NO donors, SNAP, PAPA-NONOate, and GS-NO, at concentrations known to have physiological effects (28, 29), decreased methionine and serine synthesis in both cell types (Fig. 3, a and b; the effect of GS-NO is not shown but was similar to the other two NO donors). Methionine synthesis was decreased by 75–90% in HL-60 cells and by 70–80% in BHK cells, and serine synthesis was decreased by 70–80% in both HL-60 and BHK cells (Fig. 3, a and b). Neither acetylpenicillamine nor oxidized glutathione, end products of SNAP and GS-NO, had any effect on methionine or serine synthesis. The inhibition of methionine and serine synthesis by the three NO donors was not due to generalized toxicity of the drugs or to inhibition of protein synthesis, because over the course of the experiments, neither cell viability as measured by trypan blue exclusion, nor rates of protein synthesis as measured by [3H]leucine incorporation into protein were significantly affected.

NO Decreases Rates of de Novo Purine Nucleotide Synthesis in Cultured Mammalian Cells—Trapping folates as 5-methyltetrahydrofolate should also decrease purine nucleotide synthesis by the de novo pathway, since two steps in the pathway require 10-formyltetrahydrofolate (Fig. 1). We found that the three NO donors reduced rates of de novo purine nucleotide synthesis by 60–75% in HL-60 cells (Fig. 4a) and 45–70% in BHK cells (Fig. 4b) (again, only the data for SNAP and PAPA-NONOate are shown). In HL-60 cells, PAPA-NONOate inhibited purine synthesis at a concentration as low as 10 μM and exhibited maximal inhibition at 200 μM (data not shown). The inhibition of de novo purine synthesis by PAPA-NONOate was rapidly reversible; when the drug was washed away from BHK...
cells prior to adding [14C]formate, rates of purine synthesis returned toward values observed in untreated cells (Fig. 4b,
inset). Although these experiments were performed in methionine-free experimental medium supplemented with 200 μM DL-homocysteine, similar results were obtained when the cells were incubated in experimental medium containing physiological concentrations (i.e. 10–30 μM) of methionine. As was observed in the methionine and serine synthesis experiments, neither acetylpenicillamine nor oxidized glutathione decreased rates of de novo purine synthesis in HL-60 or BHK cells.

**Fig. 4. Effect of NO donors on de novo and salvage purine nucleotide synthesis in HL-60 and BHK cells.** HL-60 cells (a) and BHK cells (b) were incubated with NO donors as described in the legend to Fig. 3. b (inset), cells were incubated in the absence (left bar) or presence (middle bar and right bar) of 200 μM PAPA-NONOate; cells were washed two times with phosphate-buffered saline prior to adding fresh medium with (middle bar) or without (right bar) PAPA-NONOate. At the end of the incubation period, rates of purine nucleotide synthesis by the de novo pathway (a and b, filled bars) and by the salvage pathway (b, stippled bars) were measured as described under “Experimental Procedures.” The data are the mean ± S.D. of at least three independent experiments performed in duplicate.
m-cysteine (circles) or containing 200 μM MDL-homocysteine thiolactone (triangles) or 100 μM cysteine (squares). PAPA-NONOate was added at the indicated concentrations, and 30 min later, rates of de novo purine nucleotide synthesis were measured as described under “Experimental Procedures.” The data are the mean ± S.D. of at least three independent experiments performed in duplicate.

The three NO donors had no effect on purine nucleotide synthesis by the folate-independent salvage pathway as measured by [8-14C]guanine incorporation into guanylates (Fig. 4b; similar data were obtained for HL-60 cells). The latter data indicate that NO did not decrease the availability of phosphoribosylpyrophosphate, the major rate-limiting substrate for purine nucleotide synthesis, whose intracellular concentration is tightly regulated (26, 30).

Homocysteine Diminishes NO’s Inhibition of de Novo Purine Nucleotide Synthesis—If NO decreased rates of de novo purine nucleotide synthesis by inhibiting methionine synthase, then measuring purine synthesis in the absence of homocysteine, the substrate of methionine synthase, should increase the potency of inhibition by NO donors. We performed these experiments in CS-54 cells and found, in the absence of NO, an approximate 30% decrease in de novo purine synthesis when homocysteine was omitted from the medium (Fig. 5, compare circles with triangles). These data are consistent with our previous finding that starvation for an essential amino acid reduces de novo purine synthesis (21) and indicate that homocysteine can, at least in part, replace methionine. On adding PAPA-NONOate at increasing concentrations, purine synthesis decreased rates of [14C]formate incorporation into methionine, serine, and purine nucleotides (21–26, 36–38) to show that NO inhibited carbon flow through folate intermediates. NO’s effects on carbon flow through folate intermediates have been reported in human and rodent cells, including vascular smooth muscle cells. NO’s inhibition of carbon flow through folate intermediates was

**DISCUSSION**

NO plays a critical role in many different physiological processes including blood pressure regulation, platelet aggregation, neurotransmission, and macrophage cytotoxicity (5, 6). Many of NO’s effects (e.g. blood pressure regulation and platelet aggregation) are mediated via NO binding to the iron in the heme prosthetic group of guanylate cyclase, which markedly activates the enzyme and thereby increases the intracellular concentration of the second messenger cGMP (32, 33). NO has a remarkably high affinity for ferrous heme with a binding constant on the order of 10^{12} to 10^{14} M^{-1}, and NO also binds to ferric heme (34). Iron and cobalt are transition metals adjacent to the heme corrin ring of cobalamin and are both substituted tetrapyrrole rings (35). Thus, it is not surprising that NO binds to the cobalt in cobalamin, and we have shown that NO reacts with all three valency states of cobalamin. We used differences in rates of reduction of NO-exposed and non-NO-exposed cbl(III) to infer that spectral changes observed on NO binding to cbl(III), although small and thus dismissed as trivial in some studies (12, 14), are real and reflect NO ligation. Furthermore, we report for the first time that NO reacts rapidly and irreversibly with cbl(I).

Further spectroscopic and EPR/FTIR studies showing NO ligation to cbl(II) and cbl(III) will be published separately.

We used the well established method of following [14C]formate incorporation into methionine, serine, and purine nucleotides (21–26, 36–38) to show that NO inhibited carbon flow through the folate pathway in several different types of human and rodent cells, including vascular smooth muscle cells. NO’s inhibition of carbon flow through folate intermediates was
probably secondary to its inhibiting methionine synthase activity because (i) other workers have shown that NO is a potent inhibitor of methionine synthase activity in vitro (7–9); (ii) we found that NO donors decreased [14C]methyltetrahydrofolate incorporation into protein; and (iii) we showed that homocysteine reduced the effectiveness of PAPA-NONOate as an inhibitor of de novo purine nucleotide synthesis. We cannot exclude the possibility, however, that NO-decreased purine nucleotide synthesis by additional mechanisms, e.g. NO, is known to inhibit ribonucleotide reductase (39), which could decrease DNA synthesis and thereby cause purine nucleotides to accumulate; the latter could inhibit de novo purine synthesis by a feedback mechanism. At the concentrations used, the NO donors did not appear to exhibit generalized toxicity, because they had no significant effect on [3H]leucine incorporation into protein or [8-14C]guanine incorporation into purine nucleotides. There are at least 16 enzymes that require cobalamin as a co-factor in bacteria and lower organisms, whereas in mammalian systems only two enzymes, methionine synthase and methyl-malonyl-coenzyme A mutase, are known to require cobalamin (3). In addition to regulating the generation of free tetrahydrofolate from 5-methyltetrahydrofolate, methionine synthase is one of four enzymes involved in regulating the cycling of methyl groups between methionine, S-adenosylmethionine (AdoMet), S-adenosylhomocysteine, and homocysteine (2) (Fig. 1). AdoMet is the source of methyl groups for a large number of biological transmethylation reactions including those that methylate DNA, RNA, and protein (40); in addition, AdoMet provides propylamine for polyamine synthesis (40). In the reaction catalyzed by methionine synthase, the methyl group of 5-methyltetrahydrofolate is transferred to cobalamin, generating a methyl-cbl(III) intermediate (3). Cleavage of the methyl-cobalt bond occurs heterolytically, generating cbl(I), which, because of its highly reactive oxidation state, can be oxidized to cbl(II) (3). A human methionine synthase reductase has been cloned recently that uses AdoMet to regenerate methyl-cbl(III) from cbl(II) (41). Thus, the methionine synthase reaction is complex, and because of its importance to several metabolic pathways, it is likely to be highly regulated. Our data suggest that NO may serve as a physiological regulator of methionine synthase, because we found that endogenously produced NO inhibited methionine, serine, and purine nucleotide synthesis and that an NO synthase inhibitor increased [14C]methyl-tetrahydrofolate incorporation into protein and increased [14C]formate incorporation into methionine, serine, and purine nucleotides.

There are at least two possible mechanisms whereby NO’s interaction with cobalamin could inhibit methionine synthase...
activity and thus interfere with carbon flow through the folate pathway. First, NO could bind to the methyl ligand binding site of cbl(III), thereby preventing or diminishing formation of the CH₃-cbl(III) intermediate in the methionine synthase reaction. Second, NO could oxidize cbl(I) to cbl(II) and therefore interfere with the cbl(II) → cbl(III) cycle that is an essential part of the methionine synthase reaction. We found no evidence that NO could compete with the methyl group in CH₃-cbl(III), and the binding equilibrium constant of cbl(II) with RCH₃ (where R represents H or deoxyadenosyl) is about 10⁻¹³ M⁻¹ (42) as opposed to weak binding of NO to cbl(II). Thus, it appears that direct reactions of NO with cbl(III) or cbl(II) derivatives [(CH₃)-cbl(III) or CH₃-cbl(II)] play no significant role in enzyme inhibition. If NO inhibits methionine synthase by reacting with cbl(I), then our data would predict that NO will have little or no effect on the methyl-malonyl-coenzyme A mutase reaction, which involves only cbl(II) and cbl(III) oxidation states. Although NO, like N₂O, appears to inhibit methionine synthase activity by interacting with cbl(I), the nature of enzyme inhibition may be quite different between the two gases. While N₂O damages the protein by producing ‘OH radical, with depletion of cobalamin being a secondary cause, NO should prevent only regeneration of the CH₃-cbl(III) prosthetic group.

NO is a potent vasodilator and inhibitor of platelet aggregation; therefore, NO is generally thought to be anti-atherogenic (6, 43). The NO-mediated inhibition of methionine synthase we observed suggests that pharmacologically administered NO could raise the intracellular (and thus the serum) concentration of homocysteine, and because of homocysteine’s known atherogenic properties, NO’s effect on the vasculature may be more complex than previously realized (44). In addition, it will be interesting to determine whether prolonged NO exposure, like prolonged N₂O exposure, leads to megabloblastosis.

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