Nitrosyl-Cobinamide, a New and Direct Nitric Oxide–Releasing Drug Effective *In Vivo*

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A limited number of nitric oxide (NO)-generating drugs are available for clinical use for acute and chronic conditions. Most of these agents are organic nitrates, which do not directly release NO; tolerance to the drugs develops, in part, as a consequence of their conversion to NO. We synthesized nitrosyl-cobinamide (NO-Cbi) from cobinamide, a structural analog of cobalamin (vitamin B_{12}). NO-Cbi is a direct NO-releasing agent that we found was stable in water, but under physiologic conditions, it released NO with a half-life of 30 mins to 1 h. We show in five different biological systems that NO-Cbi is an effective NO-releasing drug. First, in cultured rat vascular smooth muscle cells, NO-Cbi induced phosphorylation of vasodilator-stimulated phosphoprotein, a downstream target of cGMP and cGMP-dependent protein kinase. Second, in isolated *Drosophila melanogaster* Malpighian tubules, NO-Cbi–stimulated fluid secretion was similar to that stimulated by Deta-NONOate and a cGMP analog. Third, in isolated mouse hearts, NO-Cbi increased coronary flow much more potently than nitroglycerin. Fourth, in contracted mouse aortic rings, NO-Cbi induced relaxation, albeit to a lesser extent than sodium nitroprusside. Fifth, in intact mice, a single NO-Cbi injection rapidly reduced blood pressure, and blood pressure returned to normal after 45 mins; repeated NO-Cbi injections induced the expected fall in blood pressure. These studies indicate that NO-Cbi is a useful NO donor that can be used experimentally in the laboratory; moreover, it could be developed into a vasodilating drug for treating hypertension and potentially other diseases such as angina and congestive heart failure. Exp Biol Med 232:1432–1440, 2007

Key words: nitric oxide; hypertension; nitrosyl-cobinamide

Introduction

Nitric oxide (NO) has multiple physiologic effects, most notably relaxing vascular smooth muscle cells in the venous and arterial systems; thus, it is used clinically as a preload-reducing and afterload-reducing agent and an antihypertensive drug (1). Its major drawback for clinical use is that, as a gas, it is technically difficult to administer (2). NO is therefore commonly provided as an organic nitrate, such as nitroglycerin (glyceryl trinitrate), isosorbide dinitrate, or pentaerythritol tetranitrate. These compounds are not direct NO-releasing agents and require biotransformation to an active form; in the case of nitroglycerin, drug tolerance develops as a consequence of the biotransformation process (3–5). The only direct NO-releasing drug clinically available in the United States is sodium nitroprusside, but five cyanide ions are released for every NO molecule, limiting its use because of cyanide toxicity (6–9). It is therefore generally recognized that better NO-generating drugs are needed (10).

We recently studied the reactions of NO with cobalamin (vitamin B_{12}) and cobinamide; the latter is the penultimate precursor in the biosynthesis of cobalamin, lacking cobalamin's benzimidazole ribonucleotide moiety (11). The cobalt atom in cobalamin and cobinamide can exist in either a +3 or +2 valency state. Under ambient oxygenated conditions, the +3 valency state predominates, and we will refer to cobinamide^{+3} as "Cbi(III)" and to cobinamide^{+2} as "Cbi(II)." At neutral pH, NO does not bind to Cbi(III) but reduces it to Cbi(II), in the process of being

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oxidized to nitrite; NO then binds with relatively high affinity to Cbi(II) ($K_a \sim 10^{10}\ M^{-1}$), yielding nitrosylcobinamide (NO-Cbi) (11). The reaction of NO with Cbi(II) is fully reversible, and we hypothesized that NO-Cbi might be a good NO donor under physiologic conditions. We showed in our previous work that NO-Cbi releases NO directly, with a release rate (k_{off}) of 0.02 sec^{-1} in the presence of the NO scavenger myoglobin (11). We now present evidence that NO-Cbi is an effective NO-releasing agent at the cellular level (cultured rat vascular smooth muscle cells), at the organ level (isolated *Drosophila melanogaster* Malpighian tubules, mouse hearts, and mouse aortas), and at the organismal level (anesthetized mice, reducing blood pressure in the mice).

Materials and Methods

Materials. Cbi(III) was synthesized from hydroxocobalamin as described previously (12); at neutral pH, it exists as aquo-hydroxocobinamide. NO gas, >99.5% pure, was from Matheson (Montgomeryville, PA); it was bubbled through 1 *M* NaOH immediately prior to use to remove potential oxidized forms of NO. PAC-1 rat pulmonary artery smooth muscle cells were from A. Rothman (13); they maintain a differentiated phenotype through multiple subcultures. Canton-S *Drosophila melanogaster* were from the Bloomington Stock Center (Bloomington, IN), and 10- to 12-week-old male NIH Swiss and C57BL/6J mice were from Jackson Laboratories (Bar Harbor, ME). Mice were fed Teklad 7001 standard diet and were studied according to NIH Guidelines for the Care and Use of Laboratory Animals approved by the Institutional Animal Care and Use Committees of the University of California, San Diego and the VA San Diego Healthcare System. Nitroglycerin was from American Regent Laboratories (Shirley, NY), and the membrane-permeable cGMP analog 8-CPT-cGMP was from Biolog (San Diego, CA); adenosine, Deta-NONOate, dihydroethidium, hypoxanthine, prostaglandin F2α, sodium nitroprusside, and xanthine oxidase were from Sigma Chemical Co. (St. Louis, MO).

NO-Cbi Synthesis. NO-Cbi was prepared fresh daily as described previously (11). Briefly, a 4 to 8 m*M* Cbi(III) solution in a stoppered tube was deoxygenated by bubbling argon through it for 5 mins; Cbi(III) was then reduced to Cbi(II) by adding a 5-fold molar excess of deoxygenated ascorbic acid. NO gas was bubbled through Cbi(II) for 3 mins, and excess unreacted NO was removed by bubbling argon through NO-Cbi(II) for 3 mins. Because Cbi(III) was already reduced to Cbi(II) at the time of NO addition, no nitrite was formed.

Spectral Analyses and Mass Spectrometry. NO-Cbi was diluted 100-fold in water, phosphate-buffered saline (PBS), or Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) to a final concentration of 50 μ*M*, and analyzed spectrophotometrically between 300 and 600 nm in a Kontron 860 spectrophotometer (Milan, Italy). For mass spectrometry, Cbi(II) and NO-Cbi were diluted in double-distilled water and analyzed by electrospray/ionization mass spectroscopy by injection at 100 μl/min into a LCQ classic ion-trap–type mass spectrometer (Thermo-Finnigan, San Jose, CA) operating at a capillary temperature of 160°C and a spray voltage of 5000 V. The mass spectrometry data were acquired in profile mode and analyzed on Xcalibur software.

Measurement of VASP Phosphorylation. Vasodilator-stimulated phosphoprotein (VASP) is phosphorylated in response to NO activation of the heme-containing enzyme soluble guanylate cyclase: the increased cGMP activates cGMP-dependent protein kinase (PKG), which phosphorylates VASP preferentially on Ser259 (14). We and others have used VASP phosphorylation as a measure of NO availability (15, 16). Briefly, PAC-1 cells treated with an NO-releasing agent were extracted rapidly in a sample buffer that contained sodium dodecyl sulfate (SDS), and the cell extracts were subjected to SDS-polyacrylamide gel electrophoresis. Phospho-VASP was assessed by immunoblotting using an antibody specific for VASP phosphorylated on Ser259 (17).

Measurement of Nitrite and Nitrate. NO has a short half-life, being oxidized to nitrite and nitrate in the presence of oxygen. Measuring nitrite and nitrate is therefore a common method for assessing NO production (13). We measured nitrite and nitrate in PAC-1 cell extracts by using a kit from Active Motif (Carlsbad, CA) based on an enhanced Griess reagent, as described previously (18).

Assessment of Superoxide Anion Production. Cellular production of superoxide anion was assessed by measuring dihydroethidium oxidation to ethidium (19, 20). Briefly, PAC-1 cells plated in 24-well culture dishes were incubated for 3 hrs with 5 μ*M* dihydroethidium and the indicated concentrations of NO-Cbi. The medium was removed, phenol red–free medium was added, and ethidium fluorescence was measured *in situ* by using a Photon Technologies Inc. (Toronto, CA) system as previously described (21). The excitation wavelength was 540 nm, and total light emission at >600 nm was measured for 15 secs. As a positive control, cells were treated with a superoxide-generating system consisting of 0.5 m*M* hypoxanthine and 1 mg/ml xanthine oxidase (22).

Measurement of Malpighian Tubule Secretion. Malpighian tubules are the osmoregulatory organs of *D. melanogaster*, corresponding to vertebrate kidneys; tubular fluid secretion is stimulated by NO and cGMP. We measured fluid secretion rates as described previously (12). Briefly, the Malpighian tubule pair of a fly was resected and suspended in mineral oil. The proximal end of the tubule was bathed in half-strength Schneider insect medium, to which could be added a secretagogue, and fluid secretion rates were assessed by measuring the size of drops formed at the ureteral end of the tubule.

Measurement of Coronary Flow in Isolated Mouse Hearts. Hearts isolated from NIH Swiss mice

anesthetized with sodium nembutal were perfused in Langendoff mode as described by Belke *et al.* (23), except the hearts were not paced externally and an intraventricular balloon was not used. Briefly, isolated hearts were placed in a 37°C water-jacketed chamber and perfused in a retrograde manner through the aorta with Krebs-Henseleit buffer at a constant pressure of 60 mm Hg. Coronary flow was measured by the timed collection of effluent dripping from the heart. After a 20-min equilibration period, baseline flow was measured, and then drugs were injected at a rate of 100 μl/min into the buffer stream by using a syringe pump. Drugs were infused for at least 3 mins prior to measuring flow for 10 mins; drug infusion was then halted, and flow was allowed to recover to baseline rates before infusion of the next drug. Adenosine was infused last to determine maximal coronary flow rates. Hearts were weighed at the end of each experiment, and coronary flow was expressed as ml/min/g wet weight.

Measurement of Mouse Aortic Ring Relaxation. After NIH Swiss mice were anesthetized with sodium nembutal, the thoracic aorta was removed, isolated from connective and adipose tissue, and cut into 3-mm-long segments. Two metal hooks were inserted into the lumen of the aortic rings: one held the rings in an organ bath that contained Krebs-Henseleit buffer, and the other was connected to a transducer to measure isometric tension. Resting tension was set at 0.5 g, and the rings were allowed to equilibrate for 60 mins; the bath solution was changed three times. The bath solution was changed to Krebs-Henseleit buffer that contained 40 m*M* KCl, and the rings were pretreated with 2 μ*M* prostaglandin $F_{2\alpha}$ to produce 0.5–1 g active tension. Once the tension reached a plateau, the rings were treated with the indicated concentrations of NO-Cbi and sodium nitroprusside.

Measurement of Mouse Blood Pressure. Pulse and systolic and diastolic blood pressures were measured noninvasively in the tail artery of mice by restraining mice on a warming platform maintained at ~38°C. The base of the tail was placed in a computer-controlled pneumatic cuff, and the distal tail was placed in a sensor assembly that consisted of a light-emitting diode and a photodiode detector (Model SC1000 Blood Pressure Analysis System, Hatteras Instruments, Cary, NC). Baseline measurements were made with the mice awake in a dark chamber. The mice were then anesthetized with 3% isoflurane in an induction chamber and returned to the platform where they received 1.5% isoflurane with oxygen (2 L/min) via a nose cone; isoflurane caused a small but stable drop in blood pressure. Drugs in a volume of 100 μl were injected intraperitoneally into the mice, and pulse and blood pressure were measured every 5 to 15 mins; five readings were taken at each time point.

Statistical Analyses. Unless stated otherwise, differences between groups were analyzed by a one-way ANOVA with a Dunnett post-test comparison to the control group. Values were expressed as mean ± SE ($P < 0.05$). Statistical comparison between dose-response curves was made by a two-way ANOVA with the Bonferroni correction performed *post hoc* to correct for multiple comparisons. Differences were considered to be statistically significant when P was less than 0.05.

Results

Analysis and Stability of NO-Cbi. To confirm NO-Cbi synthesis, we analyzed the product by spectrophotometry and mass spectrometry. The spectral analysis from 300 to 600 nm resembled that of Cbi(II) and was identical to the spectrum of NO-Cbi we published previously (Fig. 1a; [11]). The mass spectrum of NO-Cbi showed a species with a mass of 1037 Daltons that was not present in the spectrum of Cbi(II) (Fig. 1b); the latter had a mass of 1025 Daltons, with the difference between the two species corresponding to replacement of a water molecule on Cbi(II) by an NO group.

For NO-Cbi to be useful as a drug, it needs to be relatively stable in aqueous solution. From our previous work, we knew that the half-life of NO-Cbi is short when it is incubated with excess heme; the latter binds NO tightly and will effectively remove NO from solution (11). In addition to defining the kinetics of NO-Cbi dissolution, these studies showed that NO-Cbi's primary breakdown products are NO and Cbi(II): NO-Cbi $\rightleftharpoons$ NO + Cbi(II).

In the absence of an added NO scavenger, NO-Cbi was remarkably stable: its spectrum did not change over 16 hrs when kept at room temperature in water, and little spectral change occurred in PBS. These studies were conducted with samples open to air, indicating that the back reaction of NO with Cbi(II) is much faster than the reaction of NO with oxygen or the oxidation of Cbi(II) to Cbi(III). When NO-Cbi was added to DMEM that contained 10% FBS, the spectrum of NO-Cbi changed to that of Cbi(III) over about 3 hrs, yielding a half-life of ~90 min (Fig. 1). These data suggest that NO released from NO-Cbi reacted with a constituent of the culture medium not present in water or PBS. NO reacts readily with sulfhydryl groups in the presence of oxygen (24); this previous finding suggests that it was reacting with cysteines in the culture medium, and we found that adding 1 m*M* cysteine to PBS significantly reduced NO-Cbi's half-life. Thus, NO-Cbi is stable in aqueous solutions exposed to air but breaks down relatively quickly in the presence of an NO-reactive species such as heme or a sulfhydryl group; the resulting free Cbi(II) is oxidized to Cbi(III).

Effect of NO-Cbi on Cultured Cells. *Assessment of NO Release.* To determine whether NO released from NO-Cbi can have a physiologic effect in cultured cells, we assessed VASP phosphorylation in PAC-1 cells. VASP is phosphorylated under conditions of increased NO and increased cGMP, and because PAC-1 cells are vascular smooth muscle cells, they are a good model for studying NO

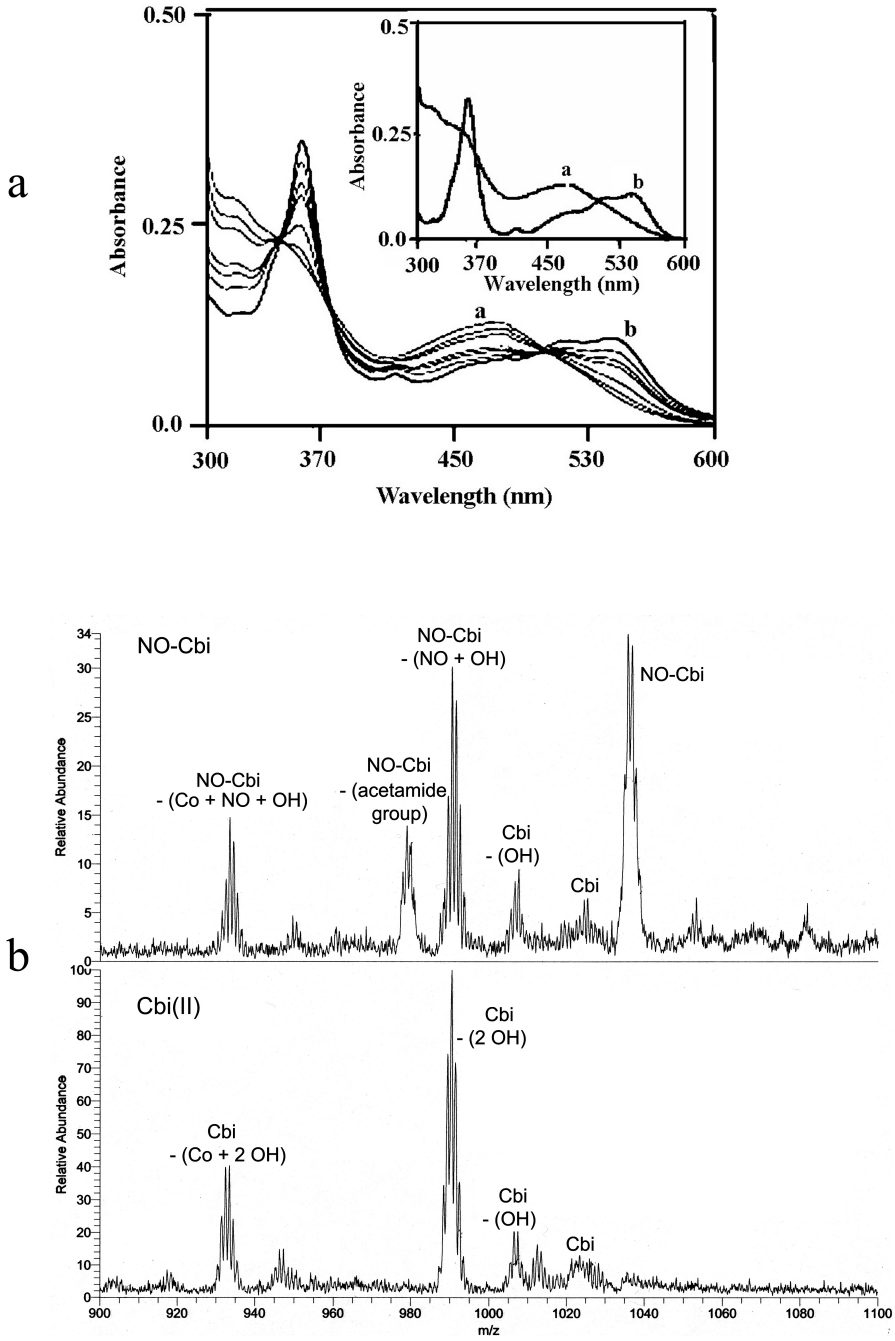

Figure 1. Spectrophotometric and mass spectroscopic analysis of NO-Cbi. (a) NO-Cbi synthesized as described in the Materials and Methods was diluted 100-fold into DMEM that contained 10% FBS, transferred to a cuvette, and analyzed immediately by spectrophotometry between 300 and 600 nm (curve a in inset and main figure). Spectra were obtained every 30 mins for 3 hrs, generating the family of curves in the main figure; curve b is the 3-h spectrum, and in the inset, curve b is the spectrum at 16 hrs. The cuvette was open to air and kept at room temperature. (b) NO-Cbi (upper spectrum) and Cbi(II) (lower spectrum) were subjected to mass spectrometry as described in the Materials and Methods. A species with a mass of 1037 Daltons present in the NO-Cbi spectrum (labeled "NO-Cbi") was not present in the Cbi(II) spectrum; cobinamide (Cbi) had a mass of 1025 Daltons. Other species in the NO-Cbi and Cbi(II) spectra were products of the ionic dissolution and were labeled presumptively on the basis of their molecular masses: the hydroxyl groups (OH) and NO molecule had been coordinated to the cobalt atom, the acetamide group was from the corrin ring, and the cobalt atom itself (Co) was probably lost. For both panels, the experiments were repeated twice with similar results.

effects (15, 16). We found over a concentration range of 1 to 30 μ*M* that NO-Cbi induced VASP phosphorylation and that 10 μ*M* NO-Cbi yielded similar results as 100 μ*M* 8-CPT-cGMP (Fig. 2a). The effect of NO-Cbi could be observed at 15 mins, was maximal at 30–60 mins, and declined over the ensuing 4 hrs (Fig. 2b).

We confirmed NO release by NO-Cbi by showing increased nitrite and nitrate in the culture medium of NO-

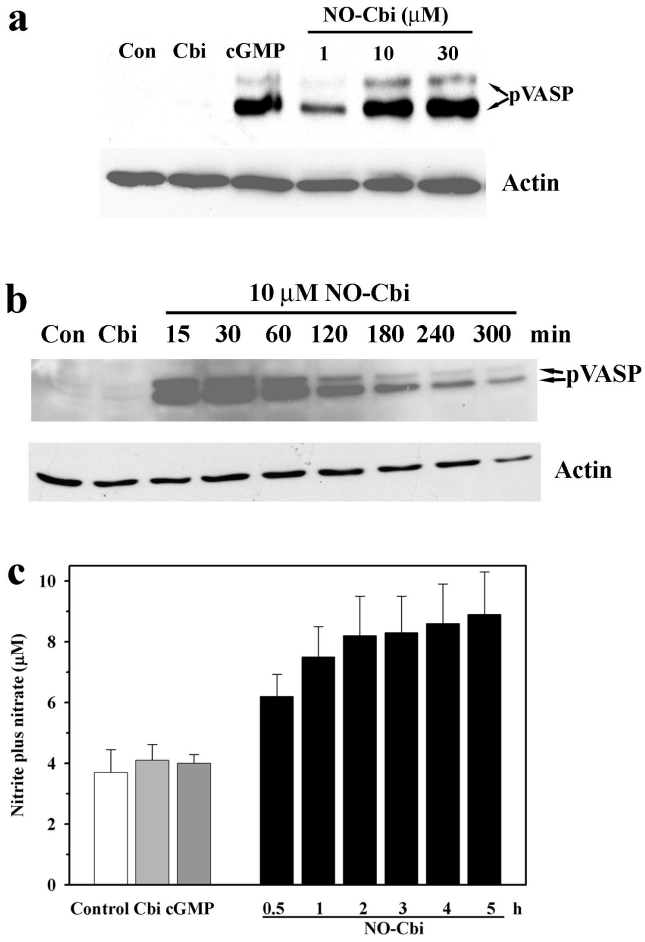

Figure 2. Effect of NO-Cbi on cultured cells. PAC-1 rat vascular smooth muscle cells were treated with the indicated concentrations of NO-Cbi for 1 hr or 10 μ*M* NO-Cbi for the indicated times. (a and b) The cells were extracted *in situ*, and the extracts were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for VASP phosphorylated on Ser239 (upper blots). Actin served as a loading control (lower blots). Dual bands of phosphorylated VASP were observed because VASP can be phosphorylated on Ser157 in addition to Ser239; the former phosphorylation caused the protein to migrate with a slightly higher apparent molecular mass. Controls (con) were cells treated with water. As indicated, the effects of 30 μ*M* Cbi(III) (Cbi) and 100 μ*M* 8-CPT-cGMP (cGMP) are shown for comparison. Similar results were obtained in two other experiments. (c) Cell extracts were clarified by centrifugation, and nitrite and nitrate in the extracts were measured by an enhanced Griess reagent method. Results are the mean ± SEM of three independent experiments; at time points of 1, 2, 3, 4, and 5 hrs the results for the NO-Cbi–treated samples were statistically different from those of the control samples ($P < 0.05$).

Cbi–treated cells (Fig. 2c). The increased amount of nitrite and nitrate accounted for 55% of the amount of NO added to the cells as NO-Cbi. This suggests either that NO-Cbi did not break down entirely to NO and Cbi(II) or that a substantial amount of NO was lost; NO could diffuse out of the medium into the surrounding air or react with heme-containing or sulfhydryl-containing compounds in the cells or medium. Because the nitrite/nitrate levels showed minimal increases after 3 hrs, it would appear that all of the NO-Cbi was broken down but that some NO was lost. Our previous work that showed that NO-Cbi breaks down rapidly in the presence of heme (11) and the present studies that show that NO released from NO-Cbi reacts readily with cysteine are also consistent with this explanation. Measuring nitrite and nitrate accumulation also provided an estimate of the half-life of NO-Cbi, which we calculated to be between 30 mins and 1 hr.

Cbi(III), Cbi(II), and ascorbate had no effect on VASP phosphorylation or nitrite/nitrate levels when added to PAC-1 cells (Figs. 2a–c; only Cbi(III) data are shown).

Assessment of Superoxide Generation. Some NO-releasing compounds generate superoxide anion intracellularly (20, 25). To determine whether NO-Cbi increased cellular superoxide generation, we treated cells with dihydroethidium, which is oxidized by superoxide anion to ethidium; the latter can be detected by measuring fluorescence (19). We found no increase in cellular fluorescence in NO-Cbi–treated cells when compared to that of cells treated with dihydroethidium only. Treating cells with the combination of hypoxanthine and xanthine oxidase increased fluorescence by about 2-fold.

Effect of NO-Cbi on *D. melanogaster* Malpighian Tubule Secretion. Malpighian tubules are an excellent model for studying the effects of a drug on an intact organ system (26). We found that 1 m*M* NO-Cbi stimulated fluid secretion to a similar extent as 100 μ*M* 8-CPT-cGMP but to a somewhat lesser extent than 500 μ*M* Deta-NONOate, which releases two NO molecules (Fig. 3). The reason for the lesser efficacy of NO-Cbi compared to that of Deta-NONOate is probably related to cysteine in the Schneider medium that bathed the tubules; no effect of NO-Cbi was observed in full-strength Schneider medium, and Schneider medium that was less than half strength did not support tubule secretion. Deta-NONOate may not be affected as much by cysteine in the medium, because it has a slower NO release rate and the intact drug may be transported intracellularly before much NO is released. Neither Cbi(III) nor Cbi(II) significantly affected tubular secretion (Fig. 3).

Effect of NO-Cbi on Coronary Flow in Isolated Mouse Hearts and on Mouse Aortic Ring Relaxation. Basal coronary flow measured in isolated perfused mouse hearts was 10.8 ± 0.5 ml/min/g wet weight ($n = 7$ hearts). Infusing nitroglycerin (12.0 ± 0.5 μ*M*) into hearts increased coronary flow 34% to 15.3 ± 1.0 ml/min/g wet weight, whereas infusing cobinamide (7.1 ± 0.4 μ*M*) into hearts increased coronary flow more than 2-fold to 22.8 ± 1.2 ml/min/g wet weight; the latter value was similar to the maximal coronary flow of 24.6 ± 1.8 ml/min/g wet weight obtained with adenosine (61.7 ± 1.1 μ*M*). To increase coronary flow to a level equivalent to that observed with cobinamide, the nitroglycerin concentration had to be increased to 770 ± 21 μ*M*, which was about 100-fold greater than the cobinamide concentration (Fig. 4a). Cobinamide concentrations as low as 20 n*M* significantly increased coronary flow.

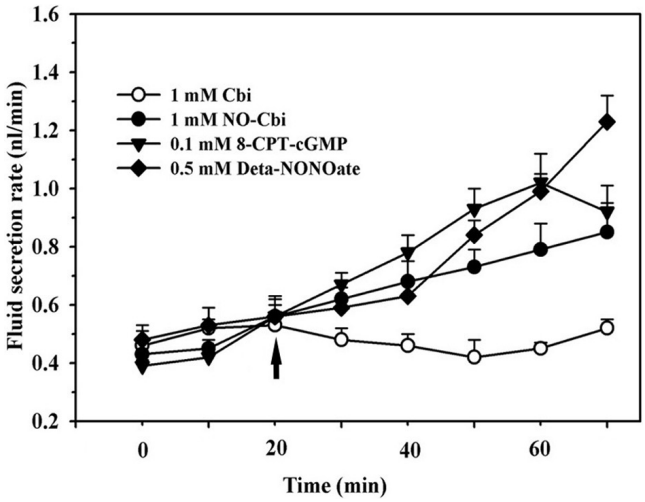

Figure 3. Effect of NO-Cbi on *D. melanogaster* Malpighian tubule secretion. Tubular secretion rates of Malpighian tubules isolated from *D. melanogaster* were measured as described in the Materials and Methods by using half-strength Schneider medium. Basal tubular secretion was measured at 0, 10, and 20 mins; at 20 mins (arrow) 1 m*M* Cbi(III) (Cbi, open circles), 1 m*M* NO-Cbi (filled circles), 0.5 m*M* Deta-NONOate (diamonds), or 100 μ*M* 8-CPT-cGMP (inverted triangles) was added. Results are the mean ± SD of three independent experiments performed on Malpighian tubule pairs from 10 flies. At the 70-min time point (i.e., 50 mins after drugs were added), rates of tubular secretion were 1.6 times higher in the NO-Cbi–treated tubules than in the Cbi(III)-treated tubules ($P < 0.01$ for the difference between the two groups using a one-tailed *t* test). Results observed with 1 m*M* Cbi(II) and 5 m*M* ascorbic acid were similar to those observed for 1 m*M* Cbi(III).

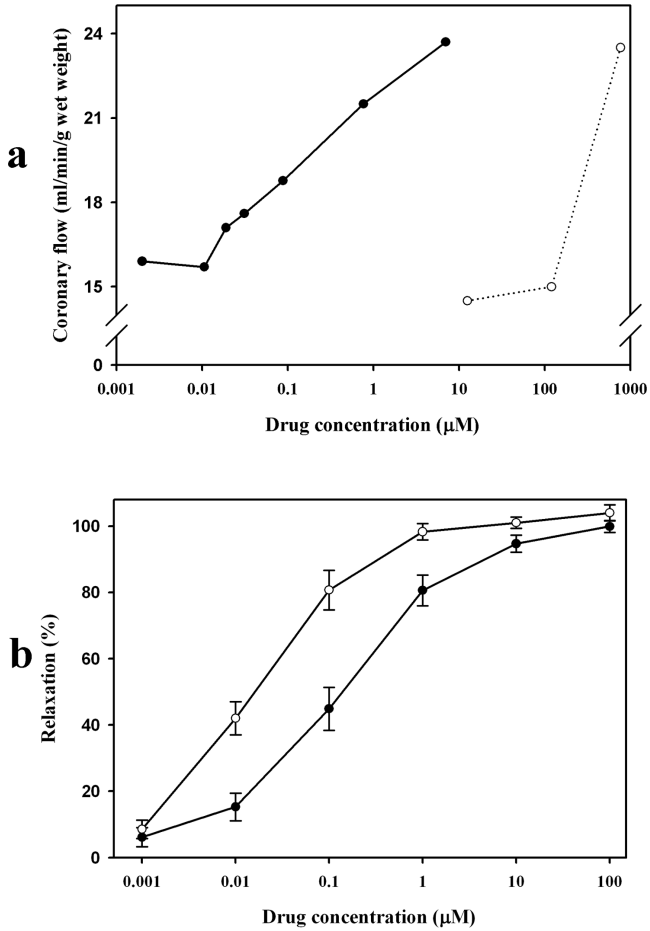

Figure 4. Effect of NO-Cbi on coronary flow in isolated mouse hearts and on mouse aortic ring relaxation. Hearts and aortas were removed from NIH Swiss mice anesthetized with sodium nembutal, and (a) coronary flow was measured in the hearts by using a Langendoff preparation, and (b) relaxation of aortic rings was measured after constriction of the rings was induced by prostaglandin F2α. The hearts and aortic rings were treated with a range of concentrations of NO-Cbi (filled circles), (a) nitroglycerin (open circles, dotted line), and (b) sodium nitroprusside (open circles). Coronary flow was measured in the same hearts for NO-Cbi and nitroglycerin after an appropriate washout period between drugs, and aortic ring relaxation was measured on the same aortic rings for NO-Cbi and sodium nitroprusside. The data in (a) are the means of two independent experiments performed on two separate hearts in each experiment, and the data in (b) are the means ± SEM of two independent experiments performed on five separate aortic rings in each experiment.

Both sodium nitroprusside and NO-Cbi induced relaxation of isolated mouse aortic rings (Fig. 4b). Nitroprusside was more effective than NO-Cbi: EC_{50} values (concentrations that induced half-maximal relaxation) were 31 ± 7 n*M* for nitroprusside and 195 ± 12 n*M* for NO-Cbi (Fig. 4b).

Thus, cobinamide was much more potent than nitroglycerin in increasing coronary blood flow in isolated mouse hearts but was somewhat less potent than sodium nitroprusside in relaxing mouse aortic rings.

Effect of NO-Cbi on Blood Pressure and Heart Rate in Mice. To determine the effect of NO release from NO-Cbi in an intact mammal, we measured the blood pressure and pulse of mice given intraperitoneal injections of NO-Cbi. We found that a single intraperitoneal injection of 0.8 μmol NO-Cbi rapidly reduced the systolic and diastolic blood pressure of mice and that ~45 mins was required for the blood pressure to return to control values (Fig. 5a; only the mean arterial pressure is shown). NO-Cbi reduced blood pressure more potently than an equimolar amount of nitroglycerin (Fig. 5a; the late decrease in blood pressure by nitroglycerin may be because the drug can potentially release three NO molecules with different kinetics). Concomitantly with the decreased blood pressure, pulse rates of the mice increased, and again the effect of NO-Cbi was more pronounced than that observed with nitroglycerin (Fig. 5b). For NO-Cbi, pulse rates remained more than 10% above control values until 75 mins; this result suggests that NO continued to be released until about this time and that reflex tachycardia compensated for NO-induced hypotension in the mice. Cbi(III), Cbi(II), and ascorbate had no significant effect on blood pressure or heart rate (Fig. 5a and b). Because of the compensatory tachycardia, it is difficult to estimate the half-life of NO-Cbi in the mice, but it appeared to be between 30 and 60 mins.

To determine whether repeated injections of NO-Cbi were effective, we administered 0.6 μmol NO-Cbi three

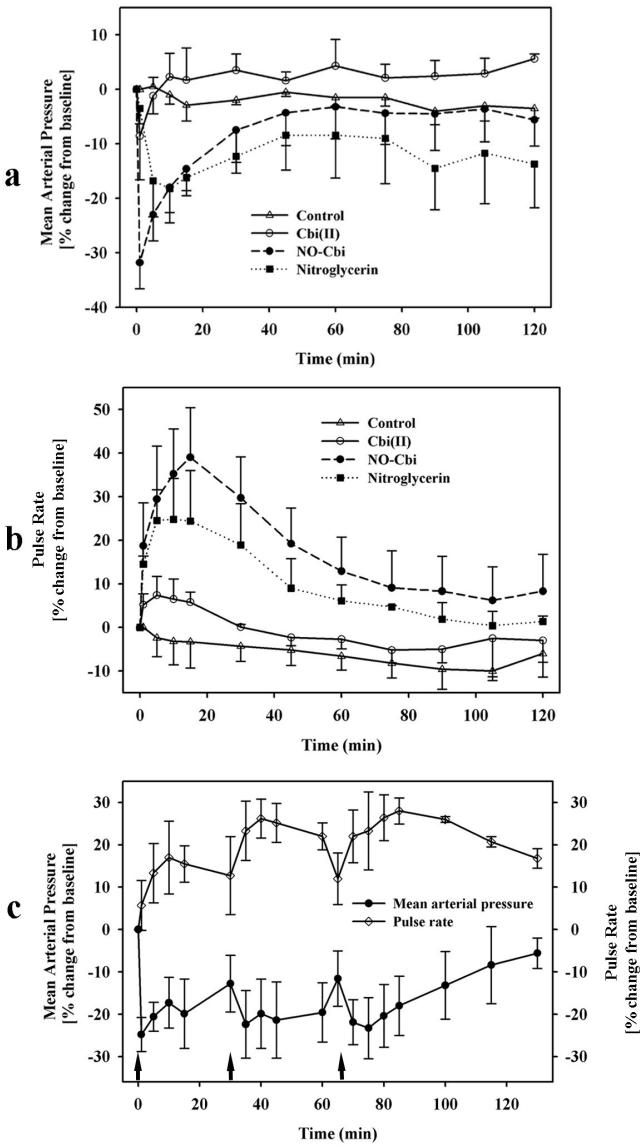

Figure 5. Effect of NO-Cbi on blood pressure and heart rate in mice. (a and b) Mice were anesthetized with 1.5% isoflurane gas, and after a stable blood pressure and pulse were achieved (about 10 mins), they were given 100-μl intraperitoneal injections of 8 m*M* Cbi(II) (open circles, solid line), 8 m*M* NO-Cbi (filled circles, dashed line), or 8 m*M* nitroglycerin (squares, dotted lines). Control refers to anesthetized mice who received 100 μl water (triangles, solid line). (c) One hundred microliters of 6 m*M* NO-Cbi was injected at 0, 32, and 64 mins (arrows). Systolic and diastolic blood pressures and pulse rate were measured at the indicated times on the tail artery by using a noninvasive tail cuff. The percent changes in (a and c) mean arterial pressure and (b and c) pulse rate are shown. Data in (a) and (b) are the mean ± SD of results from five mice for NO-Cbi studied on separate days and from four mice for the other conditions. The blood pressure and pulse rate for the NO-Cbi–treated mice were significantly different ($P < 0.01$) from those of the control mice assessed at 1 and 15 mins, respectively. Data in (c) are the mean ± SD of results from three mice studied on different days.

times to mice at 32-min intervals and found a decrease in blood pressure and an increase in heart rate with each injection (Fig. 5c). Administering 0.8 μmol NO-Cbi repeatedly caused profound hypotension.

Discussion

NO is an extremely potent vasodilator. It is a strong activator of soluble guanylate cyclase, and the consequent increase in intracellular cGMP activates PKG. In vascular smooth muscle cells, PKG phosphorylates multiple target proteins, leading to decreased intracellular calcium and vasorelaxation (27). As an endogenous regulator of vascular tone, NO is an attractive agent to treat hypertension and congestive heart failure. Its use, however, has been limited because of its mode of delivery. Organic nitrates, the most commonly used NO-generating drugs, can induce rapid tolerance: tolerance develops almost uniformly with nitroglycerin and less frequently with pentaerythrityl tetranitrate (28, 29). The mechanism of tolerance is complex and multifactorial, but recent studies with nitroglycerin point to the importance of the generation of the reactive oxygen species superoxide and peroxynitrite during the drug's biotransformation and to the inhibition of mitochondrial aldehyde dehydrogenase (ALDH2) (3–5).

NO-Cbi does not require biotransformation but rather directly releases NO (11). We found it was an effective NO-releasing agent in a variety of model systems and that it has several favorable features. First, it is easy to synthesize from Cbi(III), which itself is easily made from hydroxocobalamin (12); the latter is commercially available. Second, NO-Cbi is stable in aqueous solution, and storage does not present a hazard as in the case of nitroglycerin. Third, the non-NO product of NO-Cbi is Cbi(II), which, under physiologic conditions, is oxidized to Cbi(III); as discussed below, Cbi(III) appears to be relatively nontoxic, at least at the doses probably needed to treat hypertension and congestive heart failure.

We showed previously that Cbi(III) can act as an effective NO scavenger in cultured cells and *D. melanogaster* (12, 18). We now show that Cbi(II) fully saturated with NO is a good NO donor. Thus, Cbi(III) and NO-Cbi serve two different biological functions, which is similar to hemoglobin and oxyhemoglobin serving as an oxygen acceptor and donor, respectively.

Cobinamide lacks the dimethylbenzimidazole ribonucleotide tail coordinated to the lower axial position of cobalt in cobalamin. This makes cobinamide chemically different from cobalamin in several important ways, one of which is that cobinamide is considerably more water soluble than cobalamin[1] and has a higher affinity for ligands (11). Thus, the binding affinity of NO is about 100-fold greater for cobinamide than for cobalamin ($K_a \sim 10^8\ M^{-1}$ for cobalamin), and nitrosyl-cobalamin releases NO much faster than NO-Cbi (11). Nitrosyl-cobalamin has been shown to inhibit the growth of cultured cancer cell lines through nitrosylation of sulfhydryl groups of the Apo2L/TRAIL receptor DR4 (30, 31). It might have similar effects as we observed for NO-Cbi, but with a shorter half-life.

[1] M. Balasubramanian and G. R. Boss, unpublished observations, 2006.

Commercially produced cobalamin is isolated from bacteria. Because cobinamide is the penultimate precursor in cobalamin biosynthesis, it is not surprising that cobalamin-containing vitamins are contaminated with cobinamide (32). Cobinamide is found in human serum and bile, indicating that it is likely absorbed across intestinal epithelial cells (32–34). It binds relatively poorly to intrinsic factor, but, at least in the case of hog ileum, is absorbed independently of intrinsic factor (35, 36). Whether NO-Cbi would be stable in the gastrointestinal tract and absorbed will require further study.

Cobinamide given at a dose 40 times that of cobalamin was not toxic to baby chickens nor was it toxic to rats when infused at a rate of 2 μg/hr for 14 days (37, 38). We found that cobinamide inhibited the growth of cultured cells but only at concentrations greater than 50 μ*M* and that the growth inhibition could be reversed fully by adding equimolar amounts of cobalamin (12). Cobinamide does not inhibit either of the two mammalian cobalamin-dependent enzymes, methionine synthase and methylmalonyl CoA-mutase, as we and others have found (12, 38). These data suggest that at relatively high concentrations cobinamide may interfere with cobalamin metabolism. Because only nanomolar concentrations of NO are required to regulate blood pressure (39), it is unlikely that a toxic dose of cobinamide would be reached when NO-Cbi is used.

The current studies provide evidence that NO-Cbi is a good NO-releasing drug in several model systems. It can therefore be used in the laboratory as an NO donor, but more importantly, we feel it has potential for use in humans because it is a direct NO-releasing agent and, at the doses required, is unlikely to be toxic.

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