Biochemical and Histological Effects of Intermittent Carbon Monoxide Exposure in Cynomolgus Monkeys *(Macaca fascicularis)* in Relation to Atherosclerosis

RICHARD J. BING, M.D., F.A.C.C., J. S. M. SARMA, Ph.D., RONALD WEISHAAR, M.A., ANGELIKA RACKL, M.D., and GUNTER PAWLIK, M.D. Pasadena, Calif.

THE relationship of carbon monoxide (CO) exposure to the development of atherosclerosis has been a controversial subject. Astrup and co-workers exposed rabbits maintained on high cholesterol diet to continuous administration of CO for a period of approximately nine weeks (170 to 340 parts per million).[1] This has resulted in a cholesterol content of the aorta which was 2.5 times higher than in control rabbits not exposed to CO. In animals not receiving a cholesterol diet, moderate CO exposure (9 to 10 per cent COHb) induced arterial lesions indistinguishable from spontaneous arteriosclerosis.[2] Astrup and Kjeldsen deduced that this effect of CO was the result of hypoxia which produced arterial injuries through increased permeability of the endothelial membranes.[3] Malinow intermittently, for a period of 14 months, exposed cynomolgus monkeys to CO; some of these animals were maintained on a standard laboratory diet, others on a semipurified diet containing cholesterol.[4] No difference in plasma cholesterol level or in aortic or coronary atherosclerosis could be attributed to CO exposure.[4] Sarma and co-workers, working on isolated perfused human coronary arteries, found a high uptake of cholesterol by the arterial wall in CO-exposed vessels.[5] They also speculated that this was the result of increased vascular permeability. Topping expressed the opinion that the direct effects of CO-induced hypoxia on arterial metabolism may facilitate arterial deposition of cholesterol.[6]

It is the purpose of the present paper to report on the biochemical, hematological and histological aspects of carbon monoxide exposure in cynomolgus monkeys *(Macaca fascicularis)* maintained intermittently on CO for 12 months on a standard diet. This species was chosen since it represents an excellent experimental model for the development of atherosclerosis.[7] The animals were maintained on normal diet since increased incidence of vascular lesions has been mentioned as a sequence of CO exposure alone.[3] Particular emphasis was placed on the correlation of histological findings

From the Huntington Institute of Applied Medical Research, Pasadena, Calif.; the University of Southern California, Los Angeles, Calif.; and the California Institute of Technology, Pasadena, Calif. Supported by grants from the Council for Tobacco Research—U.S.A., Inc., and the Margaret W. and Herbert Hoover Jr. Foundation.

with biochemical studies, such as arterial cholesterol flux, mitochondrial function in heart muscle, and lipid and free fatty acid (FFA) levels in blood and tissue.

Materials and Methods

Eleven adult male cynomolgus monkeys obtained commercially (Primate Imports, Long Island, N.Y.) were quarantined for three months elsewhere and tuberculin tested. During the study period at this institution the animals were housed in individual cages and received identical diets, consisting of standard monkey chow supplemented by vitamins and fruit. Drinking water was provided ad libitum. Four animals were used as controls and breathed room air throughout the study period. The remaining seven animals were intermittently exposed to carbon monoxide (peak levels of 400 ppm in the inhaled air) for ten alternate half-hours of each day for about 12 months. Each of these animals was housed in an individual galvanized wire cage (24 in. wide × 34 in. high × 18 in. deep) surrounded by an outer Fiberglas cage (33 in. wide × 41 in. high × 28 in. deep) in a steel frame (custom built by Kirschner Scientific Co., Seattle, Wash.). The Fiberglas outer cage was equipped with a double-frame Plexiglas door with a gas-tight seal. Room air was forced through a mixing chamber and the animal cages and then exhausted into the outside atmosphere by means of an electric fan. Thus, a constant air current of about 3 cu. ft/min was maintained for each cage. During the time of CO exposure, pure CO gas (99.9 per cent) was introduced into the mixing chamber. The flow rate of CO was regulated by means of a dual gas regulator and a micrometer needle valve to achieve maximum CO levels of 400 ppm in the cages, as measured by a precalibrated infrared analyzer. The animals were automatically exposed to CO-containing air for 10 alternate half-hours starting at 8:00 P.M. each day. This system was assembled by the Engineering Department of the California Institute of Technology, Pasadena, Calif.

Measurement of COHb Levels in Monkey Blood. In order to measure carboxyhemoglobin (COHb) levels in the monkey's blood during carbon monoxide exposure, the animal was initially sedated with ketamine HCl (8 mg/kg) and placed in a restraining chair which in turn was placed in one of the Fiberglas cages; intermittent exposure of the animal to CO was initiated as described above. Periodic blood samples (1 to 2 ml) during the 10-hour exposure period were obtained through an indwelling catheter in the leg vein, and the COHb levels were determined on a precalibrated CO-oximeter. The COHb levels after the exposure were determined at a different time after the animal had been exposed to CO unrestrained for 10 alternate half-hours. The data from two animals were pooled.

Lipid Analysis. The lipid concentrations in the monkey's plasma were determined before, during, and at the end of the study period. The plasma lipids were extracted by the method of Folch et al.,[8] and the lipid classes were separated on a thin-layer chromatography (TLC) plate (silica gel G) using the double-development method of Freeman and West.[9] Cholesterol, cholesterol ester, triglyceride, diglyceride, and phospholipid fractions were eluted with a 2:1 chloroform–methanol mixture and dried under a stream of nitrogen. The cholesterol and cholesterol ester fractions were quantitated by the colorimetric method of Zak et al.[10] The plasma triglyceride and diglyceride concentrations were determined according to the method of Handel and Zilversmidt, which is based on the quantitative measurement of the glycerol moiety.[11] The phospholipids were quantitated by means of the organic phosphorous assay de-

scribed by McClare.[12] The optical density measurements in the above colorimetric determinations were carried out on a Beckman spectrophotometer (Kintrac VII, Beckman Instrument Company, Fullerton, Calif.).

Tissue and Plasma Free Fatty Acids. Levels of the following free fatty acids in plasma and heart muscle were measured: myristic ($C_{14:0}$), palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:0}$), linoleic ($C_{18:2}$), and arachidonic ($C_{20:0}$). These were determined by gas chromatography according to the technique of Ramadoos et al.[13] Following extraction with peroxide-free ether, the fatty acids were derivatized to their methyl esters and then separated using a Packard gas chromatograph. A 6-foot glass column (2 mm in diameter) packed with 10 per cent SP-222-PS was used, with nitrogen serving as the carrier gas. The temperature of the column was programmed from 130° to 190°C (3°C/min), with the inlet and detector at 190°C.

Clinical Laboratory Tests. Blood samples were examined in the hospital clinical laboratory for hematological and chemical changes. The parameters measured are summarized in Tables III and IV.

Measurement of Cholesterol Influx into the Animal's Arteries. The cholesterol influx into the animal's arteries was measured in control animals as well as in animals exposed to carbon monoxide for about 12 months. In the case of the CO-exposed group, the CO was turned off at least 24 hours prior to the experiment. Each animal was anesthetized initially with ketamine HCl (8 mg/kg), and the trachea was intubated. Later, deep anesthesia was accomplished with sodium pentobarbital (about 10 to 20 mg/kg body weight). The level of anesthesia was adjusted to maintain a uniform mean blood pressure of about 90 mm Hg. Respiration was maintained by means of an Air-Shields respirator. In principle, the technique outlined previously for evaluating cholesterol influx was followed.[14] About 13 ml heparinized blood was drawn from a saphenous vein and centrifuged at 2000 rpm (1400 G) for 15 minutes at 5°C to obtain plasma. The red cells were resuspended in saline to the original volume of blood and reinjected into the animal. Meanwhile, 200 μCi (1,2-^{3}H)-cholesterol in benzene solution was placed in a test tube and dried under a stream of nitrogen. About 5 ml plasma was then placed in the tube containing ^{3}H-cholesterol which was tightly capped and slowly rotated in a vertical plane in a chamber maintained at 37°C for 1.5 hour. The labeled plasma was then injected into the left femoral vein of the animal and 4-ml blood samples were collected from the right femoral vein at 2, 25, and 45 minutes after the injection. At the end of 45 minutes the chest was quickly opened and a portion of the thoracic aorta near the arch was removed and placed in an oxygenated Krebs–Ringer bicarbonate buffer (pH 7.4, 37°C) containing 6% BSA and 5 m*M* glucose for the measurement of its oxygen consumption rate. The heart was also removed and placed in ice-cold saline for the measurement of mitochondrial respiration and calcium uptake and binding by cardiac mitochondria. In addition, the remaining thoracic aorta, abdominal aorta, and the carotid, femoral, iliac, and cerebral arteries were removed and placed in cold saline for the measurement of tissue radioactivity and cholesterol content. Small portions of thoracic and abdominal aortas were placed in 10% buffered formalin for histological examination. Other organs, including the liver, kidney, adrenals, lungs, and the heart, were also fixed in formalin. The dry weight of the ocular lens was determined as an estimate of the animal's age.[15]

Analysis of Arterial Tissue. The arteries were dissected free of all connective tissue and adventitia and rinsed with several exchanges of saline. About 100 mg of each arterial tissue was digested in 1 ml Protosol at 55°C for 6 to 8 hours. The tissue radioactivity was then measured in the presence of Aquasol in a Packard Tricarb liquid scintillation spectrometer. In order to determine the cholesterol content of the aortic tissue, it was first crushed under liquid nitrogen into a fine powder and the lipids were extrated by the method of Folch et al.[8] The lipid classes were separated on a TLC plate, and the cholesterol and cholesterol ester fractions were eluted by a 2:1 chloroform–methanol mixture and quantitated by the method of Zak et al.[10]

Analysis of Blood Samples. The three heparinized blood samples obtained after the injection of labeled plasma were centrifuged at 1400 G for 15 minutes and plasma was separated. The lipids from 1 ml plasma were extracted and separated as above, and the cholesterol fraction was eluted with chloroform; the radioactivity of one half of this fraction was measured in the presence of Econofluor liquid scintillation solution. One fourth of the eluted fraction was used to quantitate cholesterol by the method of Zak et al.[10] The specific activity of plasma cholesterol was then calculated as the ratio of dmp/ml plasma to nmole cholesterol per ml plasma, where dpm stands for disintegrations per minute. The integrated average of the three specific activities was calculated using Simpson's rule, as described by Day et al.[16] The cholesterol influx into the arterial tissue was then calculated from the following formula:

$$\text{cholesterol influx (nmole/Gm)} = \frac{\text{dpm/Gm artery}}{\text{mean plasma cholesterol specific activity (dpm/nmole)}}$$

The plasma HDL cholesterol-to-total cholesterol ratios were measured by the use of heparin–manganese precipitation technique[17] by a commercial laboratory (Bio-Science Labs., Van Nuys, Calif.).

Oxygen Consumption Rate of Aortic Tissue. The aortic tissue was dissected free of fat and connective tissue while submerged under the oxygenated buffer. About 100 mg aortic tissue was cut into small segments (about 0.5 mm) and its oxygen consumption rate was measured in a Yellow Springs oxygen monitor (Model 53) according to the method of Morrison et al.[18]

Mitochondrial Studies. Mitochondria were isolated using the method described by Sordahl and Schwartz,[19] with minor modifications. Following isolation, respiration was monitored using a Gilson Oxygraph equipped with a vibrating platinum electrode. Substrates used were glutamate (7.5 m*M*) plus oxaloacetate (1.5 m*M*), succinate (7.4 m*M*) with rotenone (5 μg/mg protein) added to inhibit NAD-linked respiration, and ascorbate (5 m*M*) plus N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) (0.2 m*M*). Active respiration was initiated by the addition of ADP. During the reaction the temperature was maintained at 30°C. Mitochondrial calcium uptake and binding were measured at 37°C, using the Millipore filter method described by Harigaya and Schwartz,[20] with some modifications.

Histological Examination of the Tissues. For histological studies, segments of thoracic and abdominal aorta and other organs were fixed in 10% buffered formalin. Histological sections were cut from either frozen tissues or those embedded in paraffin. The frozen tissues were stained with Oil Red O; tissues from the paraffin blocks were used for Verhoeff's Hematoxylin–Van Gieson Stain, Weigert's Hematoxylin–Van Gieson Stain, H & E, PAS-Alcian Blue, and Toluidine Blue.

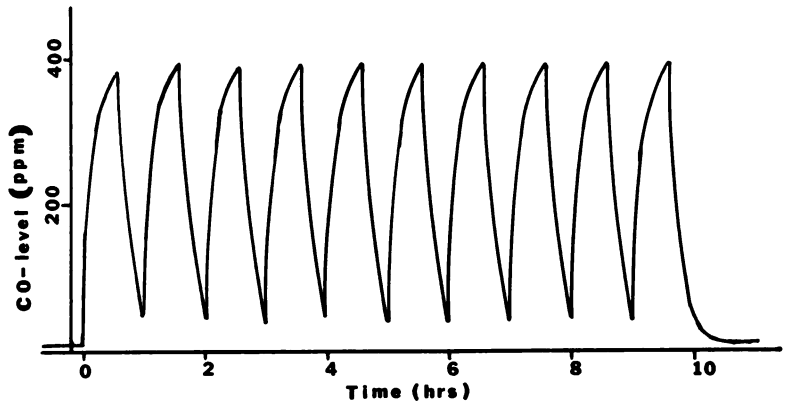

Fig. 1. Representative tracing of the variations in carbon monoxide level in the inspired air of CO-exposed monkeys.

Statistical Analysis. Statistical analysis was performed by using a two-way analysis of variance method, where applicable.[21] Other comparisons were made by means of a two-tailed two-sample *t*-test.[21] And if an *f*-test demonstrated a significant difference in variance, an approximate *t*-test was used.[21]

Chemicals. The monkey diet (code 5045) was obtained from Ralston Purina Co. (St. Louis, Mo.); 10% SP-222-PS was obtained from Supelco, Inc. (Bellefonte, Pa.). 1,2-^{3}H-Cholesterol, Protosol, Econofluor, and Aquasol were purchased from New England Nuclear (Boston, Mass.); $^4CaCl_2$ was obtained from ICN (Irvine, Calif.).

Results

Figure 1 shows a typical recording of the variations in carbon monoxide level in the cages during administration of CO over a period of 10 hours. Maximal levels of CO reached were 400 ppm. In Fig. 2 the buildup and disappearance of blood COHb levels in one monkey's blood during and after the 10-hour exposure to CO is shown. The highest COHb levels reached was about 23 per cent. COHb concentration returned slowly to normal over a period of 8 hours. No changes in blood pressure, heart rate, and electrocardiographic pattern were noticeable as a result of CO exposure.

Plasma Lipids and Free Fatty Acids. Table I illustrates the lipid concentration in plasma in control monkeys and in monkeys exposed to CO. Prolonged exposure to CO resulted in no significant changes in the plasma concentrations of free cholesterol, cholesterol esters, tri- and diglycerides, and phospholipids when the control and experimental groups were compared.

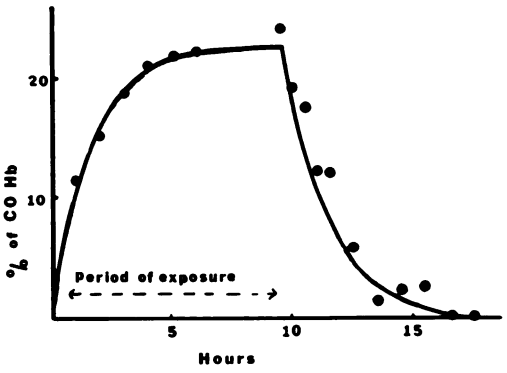

Fig. 2. Buildup and disappearance in blood of COHb levels during and after 10 hours of intermittent CO exposure of one monkey.

TABLE I

Lipid Concentrations in the Monkeys' Plasma During the Study Period*

Time after beginning of study	Free cholesterol		Cholesterol esters		Triglycerides†		Diglycerides‡		Phospholipids	
	Control group ($N = 4$)	CO-exposed group ($N = 7$)	Control group ($N = 4$)	CO-exposed group ($N = 7$)	Control group ($N = 4$)	CO-exposed group ($N = 7$)	Control group ($N = 4$)	CO-exposed group ($N = 7$)	Control group ($N = 4$)	CO-exposed group ($N = 7$)
Initial	0.68 ± 0.07	0.57 ± 0.04	1.41 ± 0.23	1.17 ± 0.10	0.65 ± 0.09	0.69 ± 0.13	—	—	—	—
4 Months	0.54 ± 0.04	0.54 ± 0.03	—	—	—	—	—	—	—	—
10 Months	0.45 ± 0.04	0.40 ± 0.03	1.31 ± 0.10	1.19 ± 0.07	0.75 ± 0.19	0.55 ± 0.09	0.024 ± 0.001	0.029 ± 0.003	20.3 ± 4.2	15.0 ± 2.0
12 Months	0.55 ± 0.08	0.60 ± 0.08	1.63 ± 0.18	1.74 ± 0.24	—	—	—	—	—	—

* Groups listed are described in text. The values for free cholesterol and cholesterol esters are expressed in μmole/ml plasma, for tri- and diglycerides in mg/ml plasma, and for phospholipids in μmole phosphate/ml plasma. Data represent the mean ± S.E.; N represents the number of animals in each group. No significant differences in free cholesterol, cholesterol esters, or triglycerides between the two groups were found (two-way analysis of variance). No significant differences in diglycerides or phospholipids were found between the two groups (two-tailed two-sample t-test).

† Measured against tripalmitin standard.

‡ Measured against dipalmitin standard.

TABLE II

Plasma and Myocardial Free Fatty Acid Levels*

Group	Myristic	Palmitic	Stearic	Oleic	Linoleic	Arachidonic
Plasma						
Control ($N = 3$)	6.82 ± 1.22	34.70 ± 0.90	11.85 ± 0.52	21.74 ± 0.70	10.91 ± 0.69	3.18 ± 0.13
Experimental ($N = 7$)	7.45 ± 1.77	43.90 ± 3.79	18.10 ± 2.48	26.15 ± 2.40	14.64 ± 2.88	3.03 ± 0.13
% Change from control	+ 9.2	+ 26.5	+ 52.7	+ 20.3	+ 34.2	− 4.7
Significance	NS	NS	$P < 0.05$	NS	NS	NS
Heart Muscle						
Control ($N = 4$)	2.66 ± 1.13	57.87 ± 8.66	—	47.43 ± 7.91	27.46 ± 7.59	11.36 ± 4.01
Experimental ($N = 6$)	4.68 ± 1.30	93.35 ± 13.63	—	67.48 ± 6.93	33.87 ± 6.17	11.61 ± 2.97
% Change from control	+ 75.6	+ 61.3	—	+ 42.3	+ 23.3	+ 2
Significance	NS	$P < 0.05$	—	$P < 0.05$	NS	NS

* Groups are as described in text. Plasma levels of free fatty acids are expressed as μg/ml plasma, and tissue levels as μg/Gm wet weight. Data represent the mean ± S.E. N represents the number of animals in each group. Significant increases were found to occur in plasma levels of stearic acid and in tissue levels of palmitic and oleic acids ($P < 0.05$). Analysis performed using a two-tailed two-sample t-test and also an f-test. NS = Nonsignificant.

TABLE III

Body Weight, Hematological Profile, and Plasma Electrolytes of Control and CO-Exposed Animals*

	Control group ($N = 4$)		CO-exposed group ($N = 7$)	
	Initial	Time of sacrifice	Initial	Time of sacrifice
Body weight (kg)	3.91 ± 0.11	5.36 ± 0.14	4.08 ± 0.10	4.81 ± 0.07
HCT	35.3 ± 1.2	37.4 ± 1.6	35.4 ± 0.9	40.1 ± 0.7
Hb	11.3 ± 0.5	11.5 ± 0.3	11.2 ± 0.1	12.4 ± 0.3
RBC	5.25 ± 0.47	5.08 ± 0.37	6.38 ± 0.23	5.94 ± 0.24
MCV	74 ± 2	73 ± 2	68 ± 1	67 ± 2
MCH	23.1 ± 0.7	23.1 ± 0.7	21.1 ± 0.6	21.1 ± 0.6
MCHC	31.3 ± 0.3	31.3 ± 0.2	31.1 ± 0.5	31.1 ± 0.5
WBC	12.0 ± 2.1	12.3 ± 1.1	10.0 ± 0.5	9.0 ± 0.9
Na^+	—	143 ± 2	—	145 ± 1
K^+	—	3.7 ± 0.1	—	3.8 ± 0.1
Cl^-	—	100 ± 2	—	107 ± 2
Ca^{2+}	—	9.2 ± 0.1	—	9.1 ± 0.1
Phosphorus	—	4.2 ± 0.1	—	3.7 ± 0.3

* Groups listed are described in text. HCT = hematocrit (%), Hb = hemoglobin (Gm/dl), RBC = red blood cell counts (mill/cm^3), MCV = mean corpuscular volume (μ^3), MCH = mean corpuscular hemoglobin ($\mu\mu g$), MCHC = mean corpuscular hemoglobin concentration (%), WBC = white blood cell counts (thous/cm^3); Na^+, K^+, Cl^- (meq/l.); Ca^{2+}, phosphorus (mg/dl). Data represent the mean ± S.E. When initial and final measurements were compared, a two-way analysis of variance evaluation was employed. For other comparisons, a two-tailed two-sample *t*-test was used (and where appropriate an *f*-test as well). Body weight was significantly increased in both groups; however, the weight gain was significantly more pronounced in the control animals. HCT and Hb also showed significant changes with time in both groups, and to the same extent in each group. Also, a significant increase in Cl^- was found in the CO-exposed animals.

In Table II, average levels of various free fatty acids in plasma and heart muscle from the control animals and monkeys exposed to CO are presented as determined before to sacrifice of the animals. All plasma FFA showed an increase in the CO-exposed group, with the exception of arachidonic acid. However, only the changes in stearic acid levels were statistically significant. A similar increase was shown in tissue levels of FFA (Table II). The rise was significant for palmitic and oleic acids.

Average changes in weight and hematologic profile are shown in Table III. All animals (control and experimental) gained weight; this weight gain was greater in the controls than in the experimental group. Hematocrit and hemoglobin were significantly increased in both groups, and to the same degree. There was also a significant increase in Cl^- in the CO-exposed animals compared to the controls (Table III). The dry weights of the ocular lenses were similar in all animals (76 ± 2 mg control; 75 ± 1 mg experimental series), indicating uniform age.

Table IV illustrates that in both groups average values of liver and renal function remained relatively normal. There was, however, a significant increase in BUN in the CO-exposed animals. This finding, coupled with an increased level of Cl^- in the blood and a normal level of creatinine, may be an indication of ca-

TABLE IV

Liver and Renal Functions of Control and CO-Exposed Monkeys*

Group	Total bilirubin	AP	SGPT	SGOT	LDH	Pro-Time	Creatinine	BUN
Control ($N = 4$)	0.2 ± 0.1	269 ± 23	32 ± 14	46 ± 5	560 ± 20	96 ± 2	1.1 ± 0.1	16 ± 1
CO-exposed ($N = 7$)	0.2 ± 0.1	268 ± 27	43 ± 6	43 ± 3	421 ± 47	100 ± 0	1.2 ± 0.1	24 ± 1

* Groups are as described in text. Total bilirubin, creatinine, and blood urea nitrogen (BUN) in units of mg/dl. Alkaline phosphatase (AP), serum glutamic-oxaloacetic transaminase (SGOT), lactate dehydrogenase (LDH), and serum glutamic-pyruvic transaminase (SGPT) in units of mU/ml; Pro-Time (prothrombin time) in units of %. Data represent the mean ± S.E. N represents the number of animals in each group. BUN was significantly increased with CO-exposed animals (two-tailed two-sample t-test).

tabolism. Total blood protein, albumin, and glucose were not affected by chronic CO exposure (total protein, control and experiment, 7.6 ± 0.2 and 7.3 ± 0.1, respectively; albumin, control and experiment, 4.0 ± 0.1 and 4.0 ± 0.1, respectively; and glucose, control and experiment, 61 ± 7 and 66 ± 6, respectively).

Cholesterol content in the aorta (thoracic and abdominal) as well as cholesterol flux into the walls of various arteries are shown in Table V. There was a slight increase in cholesterol content of the aortas of animals exposed to CO, though this increase is not statistically significant. Equally, cholesterol influx into the arterial walls of various vessels did not differ significantly in the control and experimental series. The greatest influx in both groups took place in the cerebral arteries, followed by iliac and femoral vessels. Aortic oxygen consumption and high-density lipoprotein cholesterol/total cholesterol ratios also did not differ in the two groups (Table V).

Previous studies in vitro had shown that CO inhibits electron transport in mitochondria.[22] However, in the present study chronic repeated exposure to CO failed to influence mitochondrial function. In Table VI the respiratory control index, ADP/O ratio, and QO_2 states III and IV are represented. No significant differences in mitochondrial functions were present, though a small increase in state III respiration was noted in the experimental animals. Also, as shown in Figs. 3 and 4, chronic CO exposure failed to influence mitochondrial calcium uptake or binding.

Histological studies on the arteries of CO-exposed animals do not demonstrate any structural damage or fat deposition. In a previous publication, Malinow et al., working on monkeys, found no influence of CO on gross pathology or lipid accumulation in blood vessel walls.[4] In the pres-

TABLE V

Aortic Cholesterol Contents, Arterial Cholesterol Influx Rates, Aortic O_2 Consumption Rates, and Plasma HDL Cholesterol-to-Total Cholesterol Ratios of Control and CO-Exposed Monkeys*

	Aortic cholesterol content				Arterial cholesterol influx						Aortic O_2 consumption rate	Plasma HDL chol./ total chol. ratio
	Thoracic aorta		Abdominal aorta									
Exp. no.	Chol.	Chol. esters	Chol.	Chol. esters	Thoracic aorta	Abdominal aorta	Carotid artery	Femoral artery	Iliac artery	Cerebral arteries		
Control group ($N = 4$)												
1	4.76	0.75	3.69	0.56	6	8	4	9	7	21	190	0.58
2	4.78	0.85	3.36	0.69	5	9	8	15	12	—	200	—
3	3.96	0.62	2.78	0.97	7	9	6	6	7	40	268	0.52
4	2.30	0.62	2.40	0.80	15	12	3	24	13	36	195	0.56
Mean ± S.E.	3.95 ±0.58	0.71 ±0.06	3.06 ±0.29	0.76 ±0.09	8 ± 2	10 ± 1	5 ± 1	14 ± 4	10 ± 2	32 ± 6	213 ± 18	0.55 ± 0.02
CO-exposed group ($N = 7$)												
1	1.75	0.23	10.30	0.87	6	8	4	11	14	26	202	0.51
2	5.53	0.76	3.87	0.84	4	7	8	15	8	28	249	0.56
3	6.76	0.80	8.56	0.60	17	10	10	19	18	—	—	—
4	3.72	1.15	2.16	0.49	6	7	4	14	14	32	234	0.46
5	3.72	1.15	2.16	0.49	7	7	8	17	11	24	270	—
6	5.16	0.78	4.30	0.78	16	10	10	22	26	—	241	—
7	3.75	0.54	3.54	0.79	6	15	8	35	18	37	209	0.52
Mean ± S.E.	4.34 ±0.61	0.77 ±0.12	4.98 ±1.20	0.69 ±0.06	9 ± 2	9 ± 1	7 ± 1	19 ± 3	16 ± 2	29 ± 2	234 ± 10	0.51 ± 0.02

* Groups listed are described in text. Aortic cholesterol content and arterial cholesterol influx in units of nmole/Gm tissue. Aortic O_2 consumption rate in units of μl/Gm/hr. Data represent the mean ± S.E. N represents the number of animals in each group. No significant changes were found (two-way analysis of variance test).

TABLE VI

Mitochondrial Respiration of Control and CO-Exposed Monkeys*

Substrate	R.C.I.	ADP/O Ratio	QO_2 (state III)	QO_2 (state IV)
Glutamate–oxaloacetate				
Control ($N = 4$)	7.5 ± 1.7	2.6 ± 0.1	106 ± 16	17 ± 6
CO-exposed ($N = 7$)	6.9 ± 0.9	2.6 ± 0.1	126 ± 15	20 ± 3
Succinate (Rotenone)				
Control ($N = 4$)	3.1 ± 0.5	1.7 ± 0.1	110 ± 10	42 ± 9
CO-exposed ($N = 7$)	3.2 ± 0.3	1.6 ± 0.1	142 ± 18	45 ± 3
Ascorbate TMPD				
Control ($N = 4$)	1.4 ± 0.1	—	169 ± 11	118 ± 10
CO-exposed ($N = 7$)	1.4 ± 0.1	—	220 ± 25	153 ± 14

* Groups are as described in text. R.C.I. = Respiratory control index (ratio of state III respiration to state IV respiration); ADP/O ratio = ratio of nanoatoms of ADP phosphorylated to nanoatoms of oxygen consumed; QO_2 = oxygen consumption in nanoatoms/min/mg mitochondrial protein during active (state III) and resting (state IV) respiration. Data represent the mean ± S.E. N represents the number of animals in each group. No significant changes were found using two-tailed two-sample t-test.

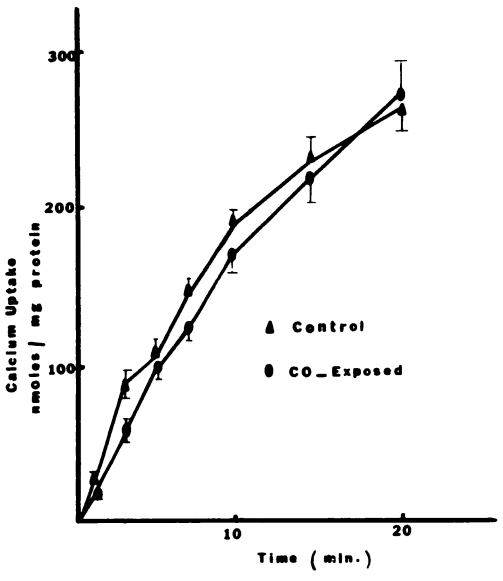

Fig. 3. Effect of chronic intermittent CO exposure on calcium uptake by cardiac mitochondria. Data represent the mean ± S.E. No diminution in calcium uptake was observed in the experimental animals.

ent study, stains with Oil Red O revealed no increase in neutral fats in the arterial wall. As a matter of fact, one of the controls showed a higher degree of Oil Red O stainable material than the experimental animal. Other stains (Verhoeff's Hematoxylin–Van Gieson Stain, Weigert's Hematoxylin–Van Gieson Stain, H & E, PAS-Alcian Blue, and Toluidine Blue) revealed no pathology.

Discussion

The results presented show that, in general, prolonged and periodic exposure to carbon monoxide resulting in carboxyhemoglobin (COHb) levels which are above those seen in moderate to heavy smokers[3] has no effect on plasma lipids, with the exception of some FFA (Tables I and II). Also, only minor changes in plasma electrolytes (Table III) and in liver and renal function (Table IV) were

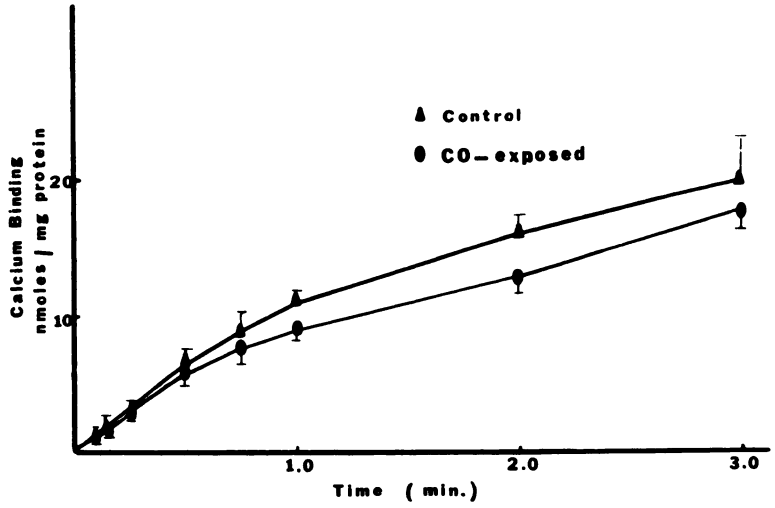

Fig. 4. Effect of chronic intermittent CO exposure on calcium binding by cardiac mitochondria. Data represent the mean ± S.E. No interference with calcium binding was observed.

found. A moderate increase in hemoglobin and hematocrit is noted, but in general the blood picture remains normal (Table III).

Histological examination of the animals, maintained on intermittent chronic exposure to CO but not on an atherogenic diet, reveals no atherosclerosis. Oil Red O-stainable material in the aortic wall is not increased, and special stains fail to demonstrate an increase in mucopolysaccharides. These findings are in general agreement with those of Malinow and co-workers who placed cynomolgus monkeys on an atherogenic diet but were unable to detect any increase in atherosclerosis on CO exposure.[4] In the present series, cholesterol influx into the arterial wall of various vessels does not differ in the control and experimental series (Table V). This, as previously stated, represents only a one-way flux rather than the net accumulation of cholesterol.[14] Cholesterol flux does however correlate well in vitro with factors which increase vascular permeability, such as endothelial damage or high perfusion pressure.[22]

Our results, as well as those of Malinow et al.,[4] differ from those of Kjeldsen and co-workers,[24] who exposed rabbits continuously to 180 ppm CO for about two weeks. Myocardial lesions including those in mitochondria were found by these workers, and so were varying degrees of injury in the blood vessels.[24] In another report, Astrup et al. found that the combination of high cholesterol with CO exposure visibly increased aortic atheromatosis and significantly increased the content of total cholesterol in the aortic tissue.[1] The role of tissue hypoxia resulting from diminished oxygen capacity in blood and a shift of the oxyhemoglobin dissociation curve to the left in the genesis of these lesions was suggested.[8] It is also known that CO interferes directly with tissue metabolism.[22] Astrup and Kjeldsen also furnished evidence that CO, as well as hypoxia, increases endothelial membrane permeability.[3] Sarma et al. also found an increased uptake of cholesterol in human coronary arteries perfused with blood containing low to high concentration of CO (15 to 80 per cent carboxyhemoglobin).[5] These experiments, how-

ever, were performed in vitro, and cholesterol flux was determined during exposure to CO. In the present series of experiments we could not find a statistically significant increase in cholesterol influx or cholesterol content of this arterial wall (Table IV). It is possible that the difference between the findings of Astrup et al.[1] and our group may be due to species differences and the fact that our animals were maintained on a standard diet and were periodically exposed to CO. Temporary exposure to CO also had no influence on oxygen consumption of the aortic tissue, suggesting normal aortic mitochondrial function.

A rise in plasma and tissue concentration of FFA (Table II) confirms the data of Turner and Topping.[25] Stearic acid (plasma) and palmitic and oleic acids (tissue) were elevated significantly in the experimental group. This may be explained on the basis of the rise in carboxyhemoglobin which, according to Topping,[6] leads to adrenal hypoxia. A resulting increase in plasma catecholamine levels could then be responsible for the rise in FFA levels. We, as well as Turner and Topping,[25] found no elevation in plasma triglycerides (Table I).

Tissue FFA levels were also elevated, as shown in Table II. CO is known to affect mitochondrial respiratory chain activity of all mammalian tissues at the terminal cytochrome oxidase. Chance et al. also demonstrated interference with electron transport by CO.[22] Such an inhibition of the electron transfer system could explain the increase in FFA tissue levels, were it not for the finding illustrated in Table VI that mitochondrial respiratory function is not diminished. However, the finding of Whereat, who postulated that hypoxia causes an increase in mitochondrial NADH/NAD^+ ratio and a resultant increase in the synthesis of lipids, may explain our findings.[26]

As shown in Table V, no significant difference in the ratios of HDL cholesterol to total cholesterol in plasma was found between the control and CO-exposed groups. This observation is important in view of the recent findings by Kannel et al. that cholesterol in the low-density lipoprotein fraction is atherogenic, whereas cholesterol in the high-density lipoprotein fraction is protective.[27] It has also been shown by Sarma et al. that isolated pig coronary arteries perfused with isologous plasma in the presence of added HDL took up significantly lesser amounts of cholesterol from the plasma than those of the paired controls.[28] Our results therefore suggest that under the present experimental conditions CO exposure does not increase the risk of development of atherosclerosis which may follow changes in HDL cholesterol to total cholesterol ratio.

Summary

The data in this report do not suggest any association between periodic carbon monoxide exposure and the development of atherosclerosis in cynomolgus monkeys. Animals were exposed to 200 to 400 ppm CO in the inspired air for 10 alternate half hours daily for approximately 12 months.

These conclusions are reached from both histological and biochemical studies (aortic cholesterol content, arterial cholesterol influx, aortic oxygen consumption, as well as plasma triglyceride concentrations and HDL cholesterol to total cholesterol ratios).

A rise in plasma and tissue free fatty acids (FFA) was observed in the experimental group exposed to carbon monoxide. However, the increase in FFA tissue levels was not believed to be due to any inhibitory effect of CO on the electron transfer system since mitochondrial respiratory function was not depressed.

References

1. Astrup, P., Kjeldsen, K., and Wanstrup, J.: Enhancing influence of carbon monoxide on the development of atheromatosis in cholesterol-fed rabbits. *J. Atheroscl. Res.* 7:343 (1967).
2. Wanstrup, J., Kjeldsen, K., and Astrup, P.: Acceleration of spontaneous changes in rabbit aorta by a prolonged moderate carbon monoxide exposure. *Acta Pathol. Microbiol. Scand.* 75:353 (1969).
3. Astrup, P., and Kjeldsen, K.: Carbon monoxide, smoking and atherosclerosis. *Med. Clin. North America* 58:323 (1973).
4. Malinow, M. R., McLaughlin, P., Dhindsa, D. S., Metcalf, J., Ochsner, A. J., Hill, J., and McNulty, W. P.: Failure of carbon monoxide to induce myocardial infarction in cholesterol-fed cynomolgus monkeys *(Macaca fascicularis). Cardiovasc. Res.* 10:101 (1976).
5. Sarma, J. S. M., Tillmanns, H., Ikeda, S., and Bing, R. J.: The effect of carbon monoxide on lipid metabolism of human coronary arteries. *Atherosclerosis* 22:193 (1975).
6. Topping, D. L.: Metabolic effects of carbon monoxide in relation to atherogenesis. *Atherosclerosis* 26:129 (1977).
7. Kramsch, D. M., and Hollander, W.: Occlusive atherosclerotic disease of the coronary arteries in monkey *(Macaca fascicularis)* induced by diet. *Exp. Mol. Pathol.* 9:1 (1968).
8. Folch, J., Lees, M., and Stanley, H. S.: A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497 (1957).
9. Freeman, C. P., and West, D.: Complete separation of lipid classes on a single thin layer plate. *J. Lipid Res.* 7:324 (1966).
10. Zak, B., Dickerman, R. C., White, E. G., Burnett, H., and Cherney, P. J.: Rapid estimation of free and total cholesterol. *Amer. J. Clin. Pathol.* 24:1307 (1954).
11. Van Handel, E., and Zilversmit, D. B.: Micromethod for the direct determination of serum triglycerides. *J. Lab. Clin. Med.* 50: 152 (1957).
12. McClare, C. W. F.: An accurate and convenient organic phosphate assay. *Analyt. Biochem.* 39:527 (1971).
13. Ramadoos, C. S., Uyeda, K., and Johnston, J. M.: Studies on the fatty acid inactivation of phosphofructokinase. *J. Biol. Chem.* 251:98 (1976).
14. Bing, R. J., Sarma, J. S. M., and Chan, S. I.: Inhibition of cholesterol uptake by the arterial wall in the intact animal. *Artery* 5:14 (1979).
15. Malinow, M. R., and Corcoran, A.: Growth of the lens in howler monkeys *(Alouatta caraya). J. Mammalogy* 47:58 (1966).
16. Day, A. J., Wahlquist, M. L., and Campbell, D. J.: Differential uptake of cholesterol and of cholesterol esters by atherosclerotic intima *in vivo* and *in vitro. Atherosclerosis* 11:301 (1970).
17. Warnick, G. R., and Albers, J. J.: A comprehensive evaluation of the heparin–manganese precipitation procedure for estimating high density lipoprotein cholesterol. *J. Lipid Res.* 19:65 (1978).
18. Morrison, A. D., Orci, L., Berwick, L., Perrelet, A., and Winegrad, A. I.: The effects of anoxia on the morphology and composite metabolism of the intact aortic intima-media preparation. *J. Clin. Invest.* 59:1027 (1977).
19. Schwartz, A.: Methods in Pharmacology. New York, Appleton-Century-Crofts, 1971, p. 247.
20. Harigaya, S., and Schwartz, A.: Rate of calcium binding and uptake in normal animal and failing human cardiac muscle. *Circ. Res.* 25:781 (1969).
21. Sokal, R. R., and Rohlf, F. J.: Biometry. San Francisco, W. H. Freeman and Co., 1960.
22. Chance, B., Erecinska, M., and Wagner, M.: Mitochondrial response to carbon monoxide toxicity. *Ann. New York Acad. Sci.* 174:193 (1970).
23. Sarma, J. S. M., Tillmanns, H., Ikeda, S., Grenier, A., Colby, E., and Bing, R. J.: Lipid metabolism in perfused human and dog coronary arteries. *Amer. J. Cardiol.* 35:579 (1975).
24. Kjeldsen, K., Thomsen, H. K., and Astrup, P.: Effect of carbon monoxide on the myocardium. Ultrastructural changes in rabbit after moderate, chronic exposure. *Circ. Res.* 34:339 (1974).
25. Turner, D. M., and Topping, D. L.: The effect of tobacco smoke and some of its constituents on triglyceride secretion in the squirrel monkey. *Res. Commun. Chem. Pathol. Pharmacol.* 12:85 (1975).
26. Whereat, A. F.: Is atherosclerosis a disorder of intramitochondrial respiration? *Ann. Int. Med.* 73:125 (1970).
27. Kannel, W. B., Castelli, W. P., and Gordon, T.: Cholesterol in the prediction of atherosclerotic disease. *Ann. Int. Med.* 90:85 (1979).
28. Sarma, J. S. M., Tschurtschenthaler, G. V., and Bing, R. J.: Effect of high density lipoproteins on the cholesterol uptake by isolated pig coronary arteries. *Artery* 4: 214 (1978).

Address for reprints: Richard J. Bing, M.D., Huntington Memorial Hospital, 100 Congress Street, Pasadena, Calif. 91105.