Improvement of Blood Oxygen Diffusion Capacity and Anginal Symptoms by Cholesterol Lowering with Simvastatin

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KEY WORDS: Oxygen, hemoglobin, blood oxygen transfer rate, Hb dissociation

ABSTRACT

Objective: To assess the hypothesis that a cholesterol-lowering regimen with simvastatin would improve red blood cell (RBC) oxygen diffusion, and that such improvement would correlate with the intensity of anginal symptoms and with exercise electrocardiogram (ECG) changes.

Methods: Desaturated venous blood samples from 18 hypercholesterolemic volunteer subjects with exertional chest pain were collected before and after they took 40 mg of simvastatin daily for 12 weeks. Blood samples were standardized to a hematocrit of 40% and a hemoglobin correction factor of 13.5 mg/dL; they were circulated in a closed-loop diffusion chamber to full oxygen saturation (O_{2Sat}) and then desaturated to about 70%. O_{2Sat} was measured in a continuous blood oxygen diffusion system of our own design. Plasma and RBC membrane cholesterol levels were determined. Assessment of exertional chest pain and exercise ECGs were performed in blinded fashion.

Results: Mean oxygen diffusion levels were 30.7% higher after treatment (P=0.03). With the exception of HDL, all cholesterol parameter levels were reduced, by between 32.5% and 43.5% (P<0.001). All but 1 subject reported significant improvement in chest pain symptoms. The exercise threshold for anginal symptoms increased by an average of 2.1-fold after treatment. Only 3 subjects had a positive exercise ECG before treatment and 1 of them had a significant improvement after treatment.

Conclusions: The plasma and RBC membrane cholesterol levels are inversely associated with the transmembrane O_2 diffusion rate of the RBC. As blood cholesterol levels decrease, blood oxygen diffusion increases, anginal pain decreases, and the threshold for angina increases.

INTRODUCTION

The reduction of cardiac events achieved by cholesterol lowering does not correlate well with the relatively small amount of physical regression in the amount of atherosclerotic plaque seen in the coronary arteries after treatment.[1,2] Furthermore, it has been shown that the transmembrane oxygen diffusion rate improves after cholesterol lowering.[3] We assessed the hypothesis that an effective cholesterol-lowering regimen with simvastatin (Zocor, Merck & Co., Inc., Whitehouse Station, NJ), a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, would improve red blood cell (RBC) oxygen diffusion, and that such improvement would correlate with the intensity of anginal symptoms and with exercise electrocardiogram (ECG) changes.

METHODS

Study Protocol

In this study, each patient served as his/her own control. The ECGs were read in blinded, randomized fashion. All subjects had documented stable angina pectoris, total plasma cholesterol levels greater than 200 mg/dL, and were not on cholesterol-lowering therapy. Each patient received a daily single 40-mg dose of simvastatin for 12 weeks. At the beginning and the end of the 12-week period, blood oxygen transfer rate, lipid profile, Bruce treadmill exercise test results, and clinical assessment of anginal symptoms, as well as patient angina self-assessment, were performed and recorded.

Subjects

Twenty-one subjects (7 men, 14 women) with documented stable untreated angina pectoris and total plasma cholesterol levels greater than 200 mg/dL were screened. All subjects gave written informed consent, and the Institutional Review Board of Human Subjects Committee of the University of Minnesota approved the study. By protocol, subjects would have been excluded if their condition worsened and necessitated medical intervention, if they suspended simvastatin treatment for more than a week during the last 4 weeks of the 12-week study period. Neither exclusion condition occurred.

Of the 21 subjects screened, all 21 entered the study but only 18 completed it (3 declined the follow-up assessment at week 12 and were therefore excluded). Out of the 18 subjects who completed the study, 1 was white and 17 Asian (15 Vietnamese, 2 Hmong).

Study Drug

Simvastatin (supplied by Merck Sharp & Dohme Research Laboratories, West Point, Pa) was given orally at a single daily dose of 40 mg—a dose that has been shown to decrease plasma cholesterol levels by 20% to 40%.[4]

Clinical Assessment

Subjects were assessed at week 0 and week 12. The blood oxygen transfer rate, as well as the cholesterol concentration in whole blood, plasma, RBCs and RBC membranes were measured. Assessment of exertional chest pain and exercise ECGs were performed in blinded fashion at the time of the assessment (week 0 or week 12).

All subjects had a history of chest pain and were evaluated by a nurse to confirm anginal symptoms. The exercise ECG test was performed according to the Bruce protocol (MAX-1 Marquette advance exercise system).[5] Most of the subjects in this study had moderate angina pectoris; thus, the endpoint of the stress test was time to angina symptoms or maximum exercise time. Ten exercise tests at week 0, and 8 at week 12, were terminated by request of the subject because of fatigue before anginal symptoms appeared. All exercise tests were

performed by an exercise laboratory technologist and supervised when necessary by a physician. The exercise test analysis was performed independently by 2 investigators (A.H. and N.T.), who were blinded to the order of the exercise tests. The exercise test result was considered positive when ST-segment changes of ≥ 1 mm were observed. Additionally, 1 investigator (N.T.) performed blinded side-by-side comparisons of the exercise tests for week 0 (before treatment) and week 12 (after treatment), each subject acting as his/her own control. The overall exercise ECG grading indicated whether the initial exercise test result (week 0) was better, worse, or the same as the subsequent test result (week 12), with respect to ST segment depression and T wave changes.

At week 0, and again at week 12, trial subjects completed an angina pectoris questionnaire to elicit the character and localization of the pain, its frequency, duration and severity, as well as a quality-of-life questionnaire covering functional status, well-being, and an overall self evaluation of health. At week 12, the subjects were also asked to compare their angina pectoris parameters to their week 0 recollections for a self-perceived status of health. Each subject was asked to give a numerical score: a positive change in score indicating an improvement in health perception, a negative change in score indicating a decline in health perception.

Blood Oxygen Transfer Rate Assessment

All desaturated blood samples were standardized to a hematocrit of 40% and a hemoglobin correction factor of 13.5 mg/dL. Hematocrit standardization was achieved by spinning the blood sample to 2500 rpm for 10 minutes, separating the plasma from the packed cells, and combining a 40% volume of the cells with a 60% plasma aliquot. The blood samples were circulated in a closed-loop diffusion chamber to full oxygen saturation (O_{2Sat}) and then desaturated to about 70%. O_{2Sat} was measured in a continuous blood oxygen diffusion system of our own design. Oxygen diffusion, defined as the net transfer of oxygen from inside the RBC to outside the RBC, was represented by the change in total oxygen content of discrete blood samples in a controlled time interval.

In vivo oxygen diffusion through a capillary of radius 100 μm from a partial pressure of oxygen (pO_2) of 100 to a pO_2 of 40 takes a fraction of a second (0.4 to 0.6 sec).[6] In this time, the hemoglobin carried in the RBC can release 1 to 2 molecules of oxygen. Whereas pO_2 measurement indirectly defines the oxygen content status of blood and oxygen saturation measurement directly defines the oxygen content status of blood, a more consistent and precise method of assessing not only the oxygen content status but also the oxygen diffusion capacity is measurement of the net total oxygen content transfer and the rate of transfer. To measure accurately the net transfer of oxygen, our system measures the total content of oxygen at full saturation (98%) and calculates the rate of diffusion over time in the blood. This process is accomplished by controlling gas exchanger surface area and time of exposure to the blood in the system, thereby the flow of oxygen from the blood sample. Lengthening the time it takes the blood oxygen to be released from the RBC in the system allows measurement of oxygen transfer rates at the different segments of the oxygen diffusion curve. In experimental settings, the average time necessary to bring a blood sample from 98% oxygen saturation to about 70% (the range at which most oxygen is transferred in the human body to the tissues) is 15 minutes.[3] One hundred milliseconds of in vivo capillary

oxygen diffusion time is equivalent to 3 minutes of experimental time.

The gas mixtures used in the gas exchanger were prepared in the following manner: To obtain atmospheric gas pressures, the chamber was flushed with room air. To obtain an oxygen-depleted environment, the chamber was first flushed with nitrogen gas. Once the gas exchanger was flushed and filled with the respective gases, access was closed, thereby allowing the enclosed gas to remain until the end of the test.

Continuous Blood Oxygen Diffusion System

The diffusion system consists of 4 components: the gas exchanger, the Biotrend oxygen saturation and hematocrit monitoring system (Medtronic Inc, Minneapolis, Minn), the Masterflex 7550-60 peristaltic pump (Cole-Parmer Instrument Co, Vernon Hills, Ill), and a personal IBM computer (IBM, New York, NY). The gas exchanger allows for blood sample oxygenating and deoxygenating conditions comparable to those found in vivo. The Biotrend system provides a means of measuring optically inline blood oxygen saturation through a sensing cuvette connected fiberoptically to a microprocessor-based monitoring device. The peristaltic pump allows for circulation of blood samples in a closed loop between the gas exchanger and the Biotrend system using Tygon microbore tubing (Norton Company, Akron, Ohio). Tygon tubing has extremely low gas permeability. The personal IBM computer is used for continuous monitoring of gas diffusion and for analysis of the data output. The total blood sample volume contained in the diffusion system during testing was 13.5 mL.

Gas Exchanger

The gas exchanger consists of a 2 mm-long Silastic medical-grade silicone tube (Specialty Manufacturing, Saginaw, Mich) with an inside diameter of 0.22 cm and an outside diameter of 0.30 cm. This tube is enclosed in a stoppered 500-mL Erlenmeyer flask with a thermometer. Gas exchange within the flask is regulated by inflow and outflow glass tubes inserted through the rubber stopper. Blood samples are introduced and evacuated from the chamber by 2 separate inflow and outflow stainless-steel ports connected in a loop to the Silastic tube. Oxygen diffusion takes place across this silicone tubing. The gas exchanger rests on a heater to maintain the blood sample at 37˚C. Water is allowed to stand in the bottom of the exchanger to saturate the gases with water vapor.

Cholesterol Determinations

After all the subjects underwent a 12-hour fast, we obtained venous blood samples in sodium heparin-containing tubes. Cholesterol content measurement in whole blood and RBCs was performed using the method of Abell et al.[7] Plasma and RBC membrane cholesterol measurements were assayed using the Sigma Diagnostics Cholesterol Enzymatic Assay (Sigma Diagnostics, St. Louis, Mo), based on the method described by Allain et al.[8]

Statistical Analysis

Results are expressed as the mean plus or minus the standard error of the mean (SEM). Study data were analyzed using the Kendall rank-correlation coefficient ([])[9] and a 2-sided Student *t* test. A two-sided *P* value less than 0.05 was considered statistically significant.

RESULTS

The demographic characteristics of the study subjects are displayed in Table 1.

All cholesterol parameter levels were reduced by between 32.5% and 43.5%, ($P<0.001$), except for HDL, which remained significantly unchanged

Table 1. Demographic Baseline Characteristics of Subjects

Race, n	-
White	1
Vietnamese	15
Hmong	2
Sex, n	
Male	7
Female	11
Age (mean ± SEM), y	
All	54.3 ± 0.45
Male	52.4 ± 0.97
Female	55.5 ± 0.78

(Table 2). However, the cholesterol levels did not strongly correlate with blood oxygen diffusion, (R^2=0.11).

The mean oxygen diffusion levels were 30.7% higher after treatment, *P*=0.026 (Figure 1, Table 3).

All but 1 subject reported significant improvement in anginal symptoms. The threshold for anginal symptoms increased by an average of 2.1-fold (114%, *P*<0.00001) after treatment (Table 4). Only 3 subjects (from a total of 18) had a positive exercise ECG before treatment. One subject had a significant improvement in the exercise ECG test after treatment (Table 4).

DISCUSSION

The relationship between elevated plasma cholesterol—especially low-density lipoprotein (LDL) cholesterol—and atherosclerosis has been known for many years. More recently, reduction of LDL cholesterol by means of surgery[10] or drugs[4,11] has been shown to reduce the risk of death and of cardiovascular events in patients with coronary heart disease. However, the reduction of cardiovascular events achieved by cholesterol lowering does not correlate well with the relatively small amount of physical regression in the amount of atherosclerotic plaque seen in the coronary arteries after treatment.[1] In the Program on the Surgical Control of the Hyperlipidemias (POSCH) trial,[1] the clinical event rate for atherosclerotic coronary artery disease mortality or confirmed nonfatal myocardial infarction was reduced in the partial ileal bypass treatment group when compared to the control group, even when adjusted for overall disease assessment (ie, the magnitude of change in the severity of coronary artery disease as graded by a panel of blinded readers). Similarly, in the Familial Atherosclerosis Treatment Study (FATS),[2] average vessel stenosis in patients in the control group increased by 2.1%, while in patients in the intensively treated groups vessel stenosis decreased by less than 1% (lovastatin and colestipol group, 0.7%; niacin and colestipol group, 0.9%), but, concomitantly, these intensive therapies reduced the incidence of cardiovascular events by 74%.

Our study demonstrated that the

Table 2. Lipid Levels in Both Groups Before and After Treatment*

	Before	After	% Change	*P*
Total Plasma Cholesterol	258 ± 3.19	174 ± 2.17	-32.5%	<0.001
LDL	170 ± 4.44	99 ± 2.13	-42.1%	0.001
HDL	41.5 ± 0.65	43 ± 0.49	4.1%	0.623
Triglycerides	314 ± 11.02	177 ± 4.96	-43.5%	0.014
RBC Membrane Cholesterol	71.7 ± 1.18	48 ± 0.91	-33.0%	0.001
RBC Cholesterol	131 ± 2.09	77 ± 1.23	-40.9%	<0.001
Blood Cholesterol	347 ± 4.28	233 ± 2.58	-32.7%	<0.001

*All values are expressed in mg/dL as mean ± SEM. Probability values between groups were calculated with Student unpaired *t* tests.

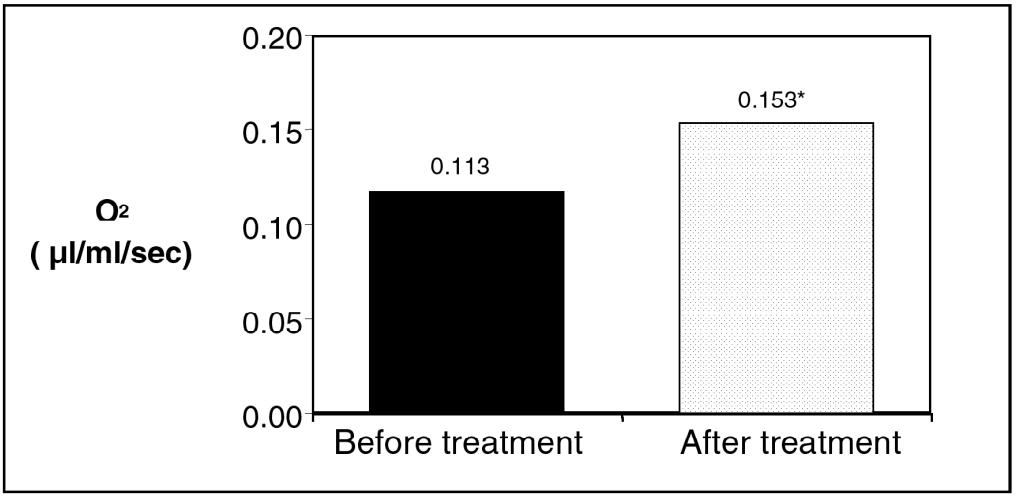

Figure 1. Blood oxygen diffusion rate before and after 12 weeks of simvastatin therapy. µL/mL/sec = microliter of O_2 per milliliter of blood per second; *P<0.026 vs. before treatment.

Table 3. Blood Oxygen Diffusion Capacity Before and After Treatment*

	Before	After	% Change	*P*
Oxygen Transfer Rate	0.117 ± 0.002	0.153 ± 0.003	30.7%	0.026

*Values are expressed in microliters of oxygen per milliliters of blood per second (µL/mL/sec) as mean ± SEM. Probability values between groups were calculated at a single time point, with Student unpaired *t* tests.

blood oxygen transfer rate did indeed improve significantly when cholesterol was lowered with simvastatin. The correlation between cholesterol and oxygen diffusion in our study was relatively weak, suggesting that the physiologic mechanisms underlying the disparity between symptomatic improvement and angiographic changes are quite possibly multifactorial. Other possible physiologic causes of this dissociation phenomenon are now being explored by other investigators. For example, it has been hypothesized that endothelium-dependent L-arginine/nitric oxide vasodilation is impaired in hypercholesterolemia, resulting in unopposed β-adrenergic-mediated vasoconstriction. Assessment of the mechanism of this impairment involves treatment approaches of cholesterol lowering to improve endothelial function and, thereby, lessen abnormal vessel constriction,[12,13,14] and L-arginine oral supplementation to improve vasodilation.[15]

Because symptoms of angina pectoris improve whether cholesterol is lowered by surgery[1] or drugs,[2] cholesterol reduction, per se, may be a factor in anginal symptoms remission, even though pleiotropic effects of the statin drugs may contribute to the final result. However, the mechanism by which cholesterol lowering improves oxygen transport is controversial. Kon et al[16] studied the kinetics of oxygen egress from human RBCs and found that the ratio of the rate of oxygen dissociation from oxyhemoglobin and the rate of oxygen disappearance from the medium (as it was consumed by baker's yeast) depended on hemoglobin content as well as on the amount of membrane cholesterol present. Koyama and Araiso[17] studied the time course of oxygen passage through 4 phospholipid bilayers between alveolar air and lung erythrocytes. They used nanosecond fluorometry and concluded that the high viscosity of the

Table 4. Self Perception of Health and Exercise ECG Results Before and After Treatment*

	Before		After		Before vs. After
Subject	Self-perception of Health	Exercise ECG	Self-perception of Health	Exercise ECG	% Change
1	2.0	-	4.5	-	125.0%
2	2.0	-	0.0	-	-100.0%
3	3.0	-	3.0	-	0.0%
4	3.0	-	5.0	-	66.7%
5	3.0	-	6.0	-	100.0%
6	3.0	-	7.0	-	133.3%
7	2.0	+	8.0	+	300.0%
8	2.0	+	8.0	+	300.0%
9	3.0	++	9.0	+	200.0%
10	3.0	-	8.5	-	183.3%
11	3.0	-	5.0	-	66.7%
12	3.0	-	7.0	-	133.3%
13	3.0	-	6.0	-	100.0%
14	3.0	-	8.0	-	166.7%
15	3.0	-	6.0	-	100.0%
16	2.0	-	8.0	-	300.0%
17	4.0	-	7.0	-	75.0%
18	4.5	-	4.0	-	-11.1%
Mean	2.9 ± 0.04	-	6.1 ± 0.14		114.0%

*All values are expressed as self-perceived ability to perform daily activities (from 0 = Unable to 10 = Best Able). Mean ± SEM. Probability values between groups were calculated with Student unpaired *t* tests. Exercise ECG results: negative (-); positive (+).

phospholipid bilayers seemed to reduce the rate of oxygenation of the hemoglobin solution. Swartz[18] used electron paramagnetic resonance (EPR) oximetry to study gradients between intracellular and extracellular oxygen concentrations in mammalian cells; he concluded that "cells appear to have a heretofore unrecognized mechanism that affects the entrance or availability of oxygen in the intracellular compartment" and that "under some circumstances, free diffusion of oxygen into cells does not occur."

Khan and colleagues[19] studied oxygen gradients in Chinese hamster ovary (CHO) cells and their mutants, in which plasma membrane cholesterol varied significantly between cell lines and also was altered by various biochemical means. They found that the oxygen gradient was greater in cell lines with increased content of cholesterol in the plasma cell membrane and additionally increased with the incorporation of additional cholesterol in the plasma membrane. Oxygen gradients decreased when the cholesterol was depleted from the plasma membrane. They concluded that the concentration of cholesterol in the plasma membrane can be an important factor for the magnitude of the oxygen gradient observed across the cell membrane. In contrast, also using EPR techniques, Subczynski and Hyde[20] found that high concentrations of cholesterol decreased membrane permeability coefficients by 3- to 5-fold; they concluded that oxygen concentration differences across membranes are negligible at physiologic conditions, except in protein-rich membranes where lipids are packed so closely that solubility and diffusion of oxygen are severely reduced.

Table 5. Oxygen Diffusion Rates in Younger vs. Older Subjects with Nearly Identical Mean Plasma Cholesterol Values*

	Younger	Older	*P*
Age, y	21.4 ± 0.11	57.7 ± 1.40	<0.001
Total Plasma Cholesterol	191.5 ± 1.80	192.5 ± 0.80	0.868
Oxygen Release Rate	0.200 ± 0.003	0.135 ± 0.004	0.005
Mean % Change Older vs. Younger Group		-78.6%	0.005

*Values are expressed as mean ± SEM.

Although other studies by our group suggested that hypercholesterolemia impairs oxygen transport, they also suggested that oxygen transport is influenced by other factors. In one of these previous studies,[21] we assessed blood oxygen release rates before and for 72 hours after open-heart surgery in 11 patients. At 48 hours after surgery, the mean oxygen release rate dropped to 11.6% below baseline values ($P<0.05$), followed by a recovery to near-normal values at 72 hours—a typical acute-phase response. In contrast to the inverse correlation between plasma cholesterol and oxygen release seen in unstressed individuals, the postoperative plasma cholesterol in these 11 patients exhibited an acute-phase response similar to that seen in the oxygen release rate.

More recently, we compared oxygen transport data between 11 University of Minnesota athletes (off season—not in peak condition) and 11 older adults who had volunteered to be screened as possible candidates for a cholesterol-lowering study. Subjects were selected so that mean cholesterol values for the 2 groups were nearly identical. We found that the oxygen desaturation rate was significantly lower ($P<0.001$) in the older adult group (Table 5). These data suggest that age, or possibly age and relative state of physical conditioning, markedly affect the oxygen release rate of cells. Both of the above studies suggest that some factor or factors other than cholesterol are associated with these oxygen transport effects.[21,22] We did not measure 2,3-diphosphoglycerate, but it is known to modulate the position of the oxygen dissociation curve, shifting it to the right in high concentrations and to the left in low concentrations.[22]

The ethnic distribution in the population of the current study deserves comment. Our selection criteria called for subjects who had exertional chest pain, but were not being treated with statin drugs. Because statin drugs are now widely available for the treatment of hyperlipidemia, few whites in our community with angina pectoris are not already receiving this treatment. Due to their relative unfamiliarity with health care options in the United States, recent emigrants from Vietnam and Laos were more likely than whites to meet our selection criteria and benefit from simvastatin treatment.

While the stress test was able to identify coronary heart disease in only a portion (16.6%) of the angina sufferers in our study, 1 out of 3 (33%) of those with a positive test showed improvement. The other 2 experienced no significant change. While both chest pain and a positive stress test denote a deficit in tissue oxygenation, the exertion test attempts to quantify the degree of limitation under specific physical demands. The stress test has a sensitivity (ie, patients with a disease who have an abnormal test) of 50% and a specificity (ie, patients free of disease who have a normal test) of 90%.[23]

ACKNOWLEDGMENTS

Supported in part by an unrestricted grant from Merck & Co., Inc.,

Whitehouse Station, NJ. Simvastatin provided by Merck Sharpe & Dohme Research Laboratories, West Point, Pa. We are grateful to James Boen, PhD, for assisting with data analysis; Leslie Macho, RN, for doing the study subject's physical examinations; and Lynn Lau, for the blood drawing.

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