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Serum Total Antioxidant Activity in Relative Hypo- and Hypercholesterolemia

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Individuals with low serum cholesterol experience greater than expected age-adjusted mortality from non-atherosclerotic diseases, including cancer, respiratory and digestive illnesses, but the basis for these associations remains unclear. The current investigation considered the hypothesis that hypocholesterolemia is associated with reduced antioxidant reserve. Serum total antioxidant activity as well as concentrations of vitamin E, vitamin C, and thiols were compared in two groups of 24 subjects distinct in both mean low density lipoprotein (LDL) cholesterol (2.3 v. 4.9 mM) and mean total cholesterol (4.3 v. 7.0 mM). The low and high cholesterol groups were equivalent in gender mix, age, weight, and serum total protein. Results reveal that compared with the high group, the low cholesterol group had decreased total serum antioxidant activity ($p < .05$). Thiol concentrations were also lower in the low cholesterol group ($p < .05$). Group differences in serum total antioxidant activity and thiol concentration were larger among men than women. The two groups did not differ in vitamin C. Low cholesterol was associated with reduced absolute vitamin E levels, although the tocopherol: cholesterol ratio was the same in low and high cholesterol individuals. These data indicate that hypocholesterolemia may be associated with low serum antioxidant reserve, possibly increasing susceptibility to oxidative stress.

Keywords: Serum total antioxidant activity, vitamin E, vitamin C, thiols, cholesterol, gender

Abbreviations: LDL low density lipoprotein, HDL high density lipoprotein, ESR electron spin resonance, AAPH 2,2'-azobis (2-aminodinopropane) dihydrochloride

INTRODUCTION

Numerous epidemiologic studies have revealed associations between low serum cholesterol and mortality from cancer, respiratory diseases, digestive diseases, and residual medical causes.[1,2,3,4,5] Low cholesterol predates death by more than 5 years in the largest studies, and these relationships between low cholesterol and non-atherosclerotic disease mortality are stronger in men than women. Possible biological mechanisms for these phenomena have not been examined.

Free radical-induced oxidative damage, thought to play a role in carcinogenesis and most

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chronic degenerative and inflammatory disease processes,[6,7,8] is offset by antioxidants, several of which are transported in lipoprotein particles. Specifically, the fat-soluble antioxidants vitamin E (tocopherol), beta carotene, and ubiquinol circulate in the blood stream along with cholesterol and triglycerides in lipoprotein particles. Furthermore, Smith has suggested that cholesterol itself may serve as an antioxidant.[9] This preliminary communication examines the hypothesis that serum total antioxidant capacity varies as a function of serum cholesterol concentration.

METHODS

Subjects were 50 men and women between 25 and 60 years of age identified for inclusion if they had either low low density lipoprotein (LDL) cholesterol (≤2.8 mmol/l) or high LDL cholesterol (≥4.1 mmol/l). One subject in each group was excluded because of overlapping total serum cholesterol, yielding 24 low cholesterol subjects and 24 high cholesterol subjects. Other exclusion criteria were triglyceride concentration of >4.5 mmol/l, any serious illness, and current use of any vitamin preparation, antioxidant supplement, or cholesterol-lowering medication. The mean age (44 years) and weight (81 kg) did not differ significantly between the two cholesterol groups. There were six smokers in the low cholesterol group and four in the high cholesterol group; exclusion of smokers does not alter the findings of this paper. The protocol was approved by the Biomedical Investigational Review Board of the University of Pittsburgh.

Blood samples were drawn following a 12 hour fast and, after centrifugation and separation, serum aliquots were analyzed for standard lipid fractions, or frozen at –70°C for later antioxidant analysis. The average time lapse between sample collection and freezing was 90 minutes.

Lipid and Protein Measurements

Total cholesterol was determined enzymatically[10] by means of a bichromatic autoanalyzer. High density lipoprotein (HDL) cholesterol was determined after selective precipitation by heparin/manganese chloride and removal by centrifugation of very low density and low density lipoproteins.[11] Triglycerides were determined enzymatically according to the procedure of Bucolo *et al.*[15] LDL-C concentration was estimated using the Friedewald formula.[12] Protein concentration in serum samples was determined using the Bio-Rad Protein Assay Kit. During each assay, a standard curve was established using BSA.

Chemiluminescence Measurements of Total Antioxidant Reserves

A water-soluble azo-initiator, 2,2'-azobis (2-aminodinopropane) dihydrochloride (AAPH) was used to produce peroxyl radicals at a constant rate.[13] Oxidation of luminol by AAPH-derived peroxyl radicals was assayed by measuring the chemiluminescence using a Luminescence Analyzer 633 (Coral Biomedical Inc., San Diego, CA). Interaction of endogenous antioxidants in serum with AAPH-derived peroxyl radicals results in a quantifiable delay in the chemiluminescence response. The incubation medium contained: 0.05 M phosphate buffer pH 7.4 at 37°C, AAPH (50 mM), luminol (400 μM) and the serum sample (20μl).

High Performance Liquid Chromatography (HPLC) Detection of Vitamin E (α-tocopherol)

Extraction of α-tocopherol from serum samples (50μl) was performed using the procedure described by Lang *et al.*[14] α-Tocopherol was measured by HPLC using a Supelcosil LC-8 column (3 μm, 4.6 mm × 15.9 cm, Supelco). A Shimadzu LC-10A HPLC system was employed with an LC-10 pump and an RF-551-Fluorescence detector (292 nm excitation and 324 nm emis-

sion). The eluent was CH_3OH, flowing at 1 ml/min. Under these conditions the retention time for α-tocopherol was 12.0 min. The data acquired were exported from the RF-551 detector using Shimadzu EZChrom software.

Electron Spin Resonance (ESR) Measurements of Water-Soluble Antioxidants

Water-soluble antioxidants (e.g. ascorbate and reduced sulfhydryls) are capable of donating electrons to phenoxyl radicals, thus regenerating phenols at the expense of antioxidant (AH) oxidation:

$$Ph\text{–}O^{\bullet} + AH \rightarrow Ph\text{–}OH +$$

For hindered phenols whose phenoxyl radicals give well-resolved ESR signals, this regeneration process can be followed directly by ESR spectroscopy. In these measurements, the enzyme tyrosinase is used as a catalyst and VP-16 as a hindered phenol. Our previous experiments demonstrated that this oxidation system generates the VP-16 phenoxyl radical, which can be persistently detected by its characteristic ESR signal for 50–60 minutes.[15,16] The appearance of the ESR signal of semidehydroascorbyl radical is used to quantify ascorbate activity, and thiol concentration (GSH and protein sulfhydryls) is derived from the lag period after ascorbate consumption and before the reappearance of the VP-16 phenoxyl radical ESR signal. ESR measurements were performed using a JEOL-REFX-ESR spectrometer. Spectra of VP-16 were recorded at 335.5 mT—center field, 20 mW—power, 0.04 mT—filed modulation, 5 mT—sweep width, 500—receiver gain, 0.03 sec—time constant. Serum samples (5–20 μl) were added to the incubation medium containing VP-16 (0.7 mM) and tyrosinase (2.8 U/μl) and the final volume was adjusted to 60 μl with 0.05 M phosphate buffer (pH 7.4 at 25°C) containing 0.1 M NaCl and 100 μM DFO (time = 0).

Data Analysis

Each serum measure was submitted to two-factor analysis of variance: cholesterol group (low or high), gender (male or female). Analysis of covariance was employed to control for the influence of additional variables, as in analysis of thiol level across gender while controlling for body weight. When the interaction between group and gender was statistically significant, post hoc comparisons of means were conducted using Tukey's HSD test.[17] Relationships between serum antioxidants were examined by calculating Pearson correlation coefficients. In all analyses, statistical significance was defined as $p < 0.05$.

RESULTS

Table 1 provides lipid and protein concentrations, and indicates that the mean total and LDL-cholesterol concentrations in the low cholesterol group were substantially less than their high cholesterol counterparts (p's < 0.001). The two groups did not differ significantly in HDL-cholesterol, whereas triglycerides were slightly elevated in the high cholesterol group. Females had significantly greater HDL-cholesterol levels than males. A significant interaction between group and gender was noted for total cholesterol, due to somewhat lower cholesterol levels in men than women in the low group and somewhat higher cholesterol in men than women in the high group. Total protein concentration did not differ between the low and high subjects, or between men and women.

As revealed in Table 2, total antioxidant reserves were lower in the low cholesterol group, compared with the high group. In addition, men had higher total antioxidant reserve than women, and this gender difference persisted when weight, age, and HDL-cholesterol were entered in analysis as covariates. While the interaction between group and gender was not significant, the means in Table 1 suggest that the group difference is due

TABLE I Serum Lipids and Prtein by Cholesterol Group and Gender (Mean ± S.D.)

	Low Cholesterol Group			High Cholesterol Group			Low v. High	Males v. Females
	Total	Men	Women	Total	Men	Women		
N	24	12	12	24	12	12	—	—
Total cholesterol (mM)	4.31 ± .67	4.02 ± .73	4.61 ± .46	6.98 ± .89	7.17 ± 1.03	6.80 ± .71	$p < .001$	N.S.
LDL cholesterol (mM)	2.32 ± .29	2.26 ± .37	2.38 ± .19	4.87 ± .67	4.98 ± .81	4.76 ± .47	$p < .001$	N.S.
HDL cholesterol (mM)	1.40 ± .42	1.10 ± .29	1.70 ± .30	1.27 ± .23	1.17 ± .12	1.32 ± .27	$p = .08$	$p < .001$
Triglycerides (mM)	1.30 ± .98	1.45 ± 1.16	1.15 ± .78	1.84 ± .77	2.21 ± .84	1.48 ± .48	$p < .05$	$p < .05$
Total protein (mg/dl)	6.35 ± .47	6.36 ± .48	6.35 ± .47	6.58 ± .54	6.75 ± .60	6.41 ± .44	N.S.	N.S.

primarily to less total antioxidant reserve in low, relative to high, cholesterol men.

With respect to individual antioxidants, serum concentration of thiols was lower in the low, compared with the high, cholesterol subjects, and was higher in men than women across cholesterol groups. This gender difference was not due to the weight, age, and HDL-cholesterol differences between men and women. Although the interaction between group and gender was only marginally significant ($p = 0.1$), the data suggest that, as with total antioxidant reserves, men in the low and high cholesterol groups differed in thiol concentration, whereas women in the two groups did not. Thiols per mg protein and vitamin C concentration were similar in the low and high cholesterol groups. As expected, the mean concentration of vitamin E in the low cholesterol group was substantially lower than that of the high group. When vitamin E was standardized for total cholesterol, the resulting tocopherol: cholesterol ratio did not differ between groups or across gender.

Pearson correlation coefficients were calculated between total antioxidant reserves and individual antioxidants. Neither vitamin C nor vitamin E were signficantly related to total antioxidant activity. Thiols, however, were well correlated with total antioxidants, $r = .639$, $p < 0.001$, as depicted in Figure 1.

TABLE II Serum Antioxidants[1] by Cholesterol Group and Gender (Mean ± S.D.)

	Low Cholesterol Group			High Cholesterol Group			Low v. High	Males v. Females
	Total	Men	Women	Total	Men	Women		
Total antiox. act. (min/ml)	500 ± 93	543 ± 93	457 ± 74	553 ± 104	624 ± 59	482 ± 91	$p < .05$	$p < .001$
Thiols (μM)	661 ± 133	699 ± 159	624 ± 93	742 ± 172	846 ± 185	640 ± 73	$p < .05$	$p < .001$
(μmol/mg prot)	10.4 ± 2.2	11.0 ± 2.5	9.9 ± 1.8	11.3 ± 2.6	12.6 ± 2.9	10.0 ± 1.4	N.S.	N.S.
Vitamin C (μM)	44.0 ± 35.5	42.8 ± 35.4	45.2 ± 37.1	50.3 ± 42.2	51.0 ± 33.2	49.7 ± 51.2	N.S.	N.S.
Vitamin E (μM)	12.9 ± 3.4	10.9 ± 2.1	14.9 ± 3.3	20.8 ± 7.1	23.0 ± 6.2	18.6 ± 7.6	$p < .001$	N.S.
(μmol/mmol TC)	3.01 ± .70	2.75 ± .54	3.25 ± .73	3.01 ± 1.04	3.21 ± .77	2.78 ± 1.28	N.S.	N.S.

TC = total cholesterol

(1) Compared with non-smokers, the 10 smokers tended to have lower levels of each measured antioxidant, though none of the differences reached statistical significance. Exclusion of smokers does not alter the findings presented.

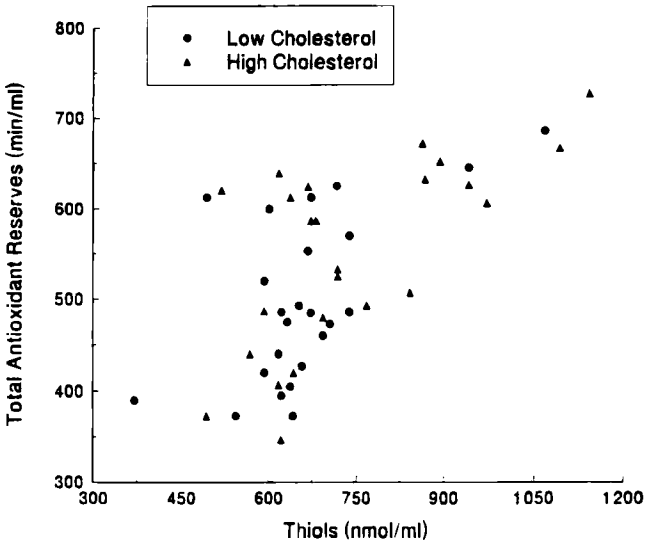

FIGURE 1 Scatter plot depicting relationship between serum thiols and total antioxidant reserve. Pearson correlation coefficient (r) for entire sample equals .639 ($p < .001$).

DISCUSSION

Fifteen years ago, Kark and colleagues speculated that the association between low cholesterol and cancer might be attributable to low levels of vitamin A, which is a derivative of the antioxidant beta-carotene.[18,19] However, little research followed. The current investigation considered the related hypothesis that serum total antioxidant reserves vary with cholesterol concentration. The data reveal that, compared to those with high cholesterol, low cholesterol individuals had reduced serum total antioxidant activity. Furthermore, this difference was greater in men than women, paralleling the epidemiologic findings.

The measurement of serum total antioxidant activity utilized chemiluminescence to detect depletion of antioxidants by AAPH-derived peroxyl radicals.[13,20] Similar to that of other investigators,[21,22,23] the technique measures traditional antioxidants—low molecular weight compounds either consumed in the diet or regularly synthesized and recycled by reducing enzymes—as well as larger compounds, such as thiol groups on albumin, whose function may be as "sacrificial antioxidants."[24] Thiols, when specifically measured, were also found to differ between the low and high cholesterol groups. This observation, combined with the close correlation between thiols and total antioxidant reserves, suggests that the reduced serum antioxidant activity in individuals with hypocholesterolemia may be largely attributable to fewer thiols. The difference in thiols per mg protein did not reach statistical significance, suggesting that the reduced thiol concentration of low cholesterol subjects may be due, in part, to somewhat lower protein concentration. Also, several important extracellular antioxidants, such as urate, were not measured in this preliminary study.

A secondary finding was greater antioxidant reserve in men compared to women. This could be due in part to the greater total cholesterol contrast between low and high men compared to women in our sample. However, other investigators have also found increased total serum antioxidant activity in men compared to women.[25] Men have higher resting metabolic rates and are generally more physically active than women,[26] both key

determinants of oxidative stress, and therefore the greater antioxidant capacity in men could represent an adaptive response.

With respect to limitations of this investigation, samples were frozen at –70°C within 90 minutes of collection whereas immediate separation and freezing is preferred to minimize spontaneous degradation of antioxidants. However, because all samples were handled identically, the lag time should not create a bias. Also, no dietary data are available. However, dietary antioxidant intake does not necessarily affect serum total antioxidant activity[22] and in any case, to the extent that individuals with low cholesterol may be expected to consume healthy diets, rich in vitamins, diet habits would bias against the hypothesized association. Finally, the assays measured serum, not intracellular, antioxidant levels whereas oxidative injury leading to non-atherosclerotic disease may predominantly involve intracellular oxidation. Nonetheless, extracellular antioxidants participate in the protection of cell membrane lipids, and clinical studies indicate that extracellular (serum) antioxidant levels predict health outcomes.

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References

[1] D. R. Jacobs, H. Blackburn, M. Higgins, D. Reed, H. Iso, G. McMillian, J. Neaton, J. Nelson, J. Potter, B. Rifkind, J. Rossouw, R. Shekelle and S. Yusuf (1992) Report of the conference on low blood cholesterol: mortality associations. *Circulation*, **86**, 1046–1060.

[2] J. D. Neaton and D. Wentworth (1992) Serum cholesterol, blood pressure, cigarette smoking, and death from coronary heart disease. *Archives of Internal Medicine*, **152**, 56–64.

[3] E. N. Meilahn and Re Ferrell (1993) 'Naturally-occurring' low blood cholesterol and excess mortality. *Coronary Artery Disease*, **4**, 843–853.

[4] D. R. Jacobs, M. F. Muldoon and L. Rastam (1995) Low blood cholesterol, nonillness mortality, and other nonatherosclerotic disease mortality: a search for causes and confounders. *American Journal of Epidemiology*, **141**, 518–522.

[5] R. B. D'Agostino, A. J. Belanger, W. B. Kannel and M. Higgins (1995) Role of smoking in the U-shaped relation of cholesterol to mortality in men. *American Journal of Epidemiology*, **141**, 822–827.

[6] B. N. Ames, M. K. Shigenaga and T. M. Hagen (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Science*, **90**, 7915–7922.

[7] B. Halliwell (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet*, **344**, 721–724.

[8] J. P. Kehrer and C. V. Smith (1994) Free radicals in biology: Sources, reactivities, and roles in the etiology of human diseases. In *Natural Antioxidants in Human Health and Disease*. Academic Press, San Diego, CA, pp. 25–62.

[9] L. L. Smith (1991) Another cholesterol hypothesis: cholesterol as antioxidant. *Free Radical Biology and Medicine*, **11**, 47–61.

[10] C. C. Allain, L. S. Poon, C. S. Chan, W. J. Richmond and P. C. Fu (1974) Enzymatic determination of total serum cholesterol. *Clinical Chemistry*, **20**, 470–475.

[11] G. R. Warnick and J. J. Albers (1978) A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. *Journal of Lipid Research*, **19**, 65–76.

[12] W. T. Friedewald, R. I. Levy and D. S. Frederickson (1972) Estimation of the concentration of low density lipoprotein cholesterol in plasma without the use of the preparative ultracentrifuge. *Clinical Chemistry*, **18**, 499–503.

[13] E. Niki (1990) Free radical initiators as source of water-or-lipid-soluble peroxyl radicals. *Methods in Enzymology*, **186**, 100–108.

[14] J. K. Lang, K. Cohil and L. Packer (1986) Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates and subcellular fractions. *Annals of Biochemistry*, **157**, 106–116.

[15] V. E. Kagan, J. C. Yalowich, B. W. Day, R. R. Goldman and D. A. Stoyanovsky (1994) Ascorbate is the primary reductant of the phenoxyl radical of etoposide (VP-16) in the presence of thiols both in cell homogenates and in model systems. *Biochemistry*, **33**, 9651–9660.

[16] T. G. Gantchev, J. E. van Lier, D. A. Stoyanovsky, J. C. Yalowich and V. E. Kagan (1994) Interactions of the phenoxyl radical of an antiumor drug, etoposide (VP-16), with reductants in solution and in cell and nuclear homogenates. ESR and HPLC measurements. *Methods in Enzymology*, **234**, 643–654.

[17] R. E. Kirk (1968) *Experimental Design: Procedure for the Behavioral Sciences*. Wadsworth Publishing Co, Belmont, CA.

[18] J. D. Kark (1981) Retinol, carotene, and the cancer/cholesterol association. *Lancet*, 1371–1372.

[19] J. D. Kark (1982) Serum retinol and the inverse relationship between serum cholesterol and cancer. *British Medical Journal*, **284**, 152–284.

[20] J. T. Uotila, A. L. Kirkkola, M. Rorarius, R. J. Tuimala, T. Metså-Ketelå (1994) The total peroxyl radical-trapping ability of plasma and cerebrospinal fluid in normal and preeclamptic parturients. *Free Radical Biology & Medicine*, **16**, 581–590.

[21] N. J. Miller, C. Rice-Evans, M. J. Davies, V. Gopinathan, and A. Milner (1993) A novel method for measuring antioxidant capacity and its application to montoriing the antioxidant status in premature neonates. *Clinical Science*, **84**, 407–412.

[22] C. Calzada, M. Bizzotto, G. Pganga, J. J. Miller, K. R. Bruckdorfer, A. T. Diplock and C. A. Rice-Evans (1995) Levels of antioxidant nutrients in plasma and low density lipoproteins: A human volunteer supplementation study. *Free Radical Research*, **23**(5), 489–503.

[23] B. Frei, R. Stocker and B. N. Ames (1988) Antioxidant defenses and lipid peroxidation in human plasma. *Proceedings of the National Academy of Science*, **85**, 9748– 9752.

[24] J. M. Gutteridge and B. Halliwell (1994) *Antioxidants in Nutrition, Health and Disease.* Oxford University Press, Oxford.

[25] S. R. Maxwell, C. H. Thorpe and T. P. Whitehead (1993) An enhanced chemiluminescent assay for antioxidant capacity in biological fluids. In: Galteau MM, Siest G, Henry J, eds. *Biologie prospective. Comptes rendus du 8 colloque de Port-a-Mousson*, John Libbey Eurotext, Paris, 519–22.

[26] P. J. Arciero, M. I. Goran and E. T. Poehlman (1993) Resting metabolic rate is lower in women than in men. *Journal of Applied Physiology*, **75**, 2514–2519.