Reduced Plasma Peroxyl Radical Trapping Capacity and Increased Susceptibility of LDL to Oxidation In Poorly Controlled IDDM

Elaine C. Tsai, Irl B. Hirsch, John D. Brunzell, and Alan Chait

Oxidation of low-density lipoproteins (LDLs) has been postulated to play an important role in atherogenesis. Because oxidant stress may be increased and antioxidant defenses reduced in diabetes, the susceptibility of LDL to oxidative modification and total peroxyl radical trapping potential (TRAP) of plasma were evaluated in subjects with poorly controlled insulin-dependent diabetes mellitus (IDDM). The lag phase of conjugated diene formation after initiation of LDL oxidation by the addition of copper was shorter in diabetic subjects than in normal control subjects (126 ± 11 vs. 165 ± 15 min [means ± SE], $P < 0.05$). This could not be attributed to the presence of oxidation-susceptible, small, dense LDL particles in the diabetic subjects, whose lipoprotein particle distribution did not differ from the control subjects. However, the total TRAP of plasma, a measure of antioxidant defense, was reduced (626 ± 34 vs. 877 ± 41 μM, $P < 0.0001$) in diabetes. Of the plasma antioxidants measured, only uric acid and vitamin A were decreased in diabetes ($P < 0.01$), and both levels correlated with TRAP ($r = 0.75$, $P < 0.001$; $r = 0.54$, $P < 0.001$, respectively). The correlation between uric acid levels and TRAP persisted when the diabetes and control groups were analyzed separately. The reduced TRAP of plasma and the increased susceptibility of LDL to oxidative modification observed is consistent with a role for lipoprotein oxidation in the pathogenesis of atherosclerosis in IDDM. *Diabetes* 43: 1010–1014, 1994

Diabetes is associated with a markedly increased risk of atherosclerotic cardiovascular disease (1). However, the mechanism(s) by which diabetes enhances atherosclerosis and its complications has not been well established. Multiple cardiovascular risk factors, e.g., central obesity, insulin resistance, hypertension, a positive family history of premature atherosclerotic disease, and dyslipidemia coexist in subjects with non-insulin-dependent diabetes mellitus (NIDDM). The dyslipidemia is characterized by hypertriglyceridemia (because of accumulation of very-low-density lipoprotein [VLDL] and their remnants), low levels of high-density lipoprotein (HDL) cholesterol, and the presence of small, dense low-density lipoprotein (LDL) particles (2), similar to the atherogenic lipoprotein phenotype that is associated with an increased risk of cardiovascular disease in nondiabetic subjects (3). Subjects with insulin-dependent diabetes mellitus (IDDM) also have an increased risk of cardiovascular disease (4), despite the absence of the central obesity/insulin resistance syndrome and the presence of normal or even high levels of HDL cholesterol (5,6). Whether small, dense LDL particles typically are found in IDDM is unclear.

There has been considerable recent interest in the potential role of oxidatively modified lipoproteins in atherogenesis (7). Oxidized LDL has many biological properties that may promote atherogenesis, including stimulation of adhesion of monocytes to endothelial cells, monocyte chemotaxis, cytotoxicity, uptake by scavenger receptors resulting in the formation of macrophage-derived foam cells (7), and modulation of growth factor and cytokine gene expression (8). Several lines of evidence suggest that oxidation of lipoproteins may be increased in diabetes. First, oxidant stress may be increased in diabetes (9), in part because of generation of oxygen free radicals during protein glycation and glucose auto-oxidation (10,11). These oxygen radicals may facilitate LDL oxidation. Second, antioxidant defenses, such as vitamin C, may be reduced in diabetes (12,13). Third, the small, dense LDL particles that are typically observed in NIDDM (2) have an increased susceptibility to oxidative modification (14).

Therefore, this study was conducted to determine whether the susceptibility of LDL to oxidative modification was increased, antioxidant defenses were reduced, and small, dense LDL particles were present in subjects with IDDM in poor glycemic control.

RESEARCH DESIGN AND METHODS

Nineteen subjects with poorly controlled IDDM and 20 nondiabetic subjects were studied (Table 1). They were matched in age (31.7 ± 3.0 vs. 31.9 ± 1.8 years, means ± SE) and weight (body mass index [BMI] 23.3 ± 0.4 vs. 23.6 ± 0.5 kg/m^2). Duration of diabetes was 15 ± 2 years. Seventeen of the 19 diabetic patients were on multiple-dose insulin regimens (5 on ultralente plus regular insulin; 12 on NPH plus regular insulin) and 2 were on insulin pumps. Mean insulin dosage was 0.66 ± 0.05 U/kg. At least one high glycosylated hemoglobin (GHb) value (>9%) was documented in all diabetic patients before enrollment. Exclusion criteria were cigarette smoking; proteinuria >300 mg/24 h; hepatic, cardiac, or renal disease; untreated hypothyroidism; pregnancy; use of

From the Department of Medicine, Division of Metabolism, Endocrinology, and Nutrition, University of Washington, Seattle, Washington.

Address correspondence and reprint requests to Dr. Alan Chait, Division of Metabolism, Endocrinology, and Nutrition, RG-26, University of Washington, Seattle, WA 98195.

Received for publication 7 September 1993 and accepted in revised form 31 March 1994.

NIDDM, non-insulin-dependent diabetes mellitus; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; IDDM, insulin-dependent diabetes mellitus; BMI, body mass index; TRAP, total peroxyl radical trapping potential; GHb, glycosylated hemoglobin; AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; CV, coefficient of variation; PBS, phosphate-buffered saline.

estrogens, glucocorticoids, β-adrenergic blocking agents, diuretics, or lipid-lowering agents; and consumption of vitamin supplements at greater than the recommended daily allowance. Overt macrovascular disease was excluded by these criteria. Four of the diabetic patients had nonproliferative retinopathy, one had proliferative changes, but none had frank proteinemia (i.e., >300 mg/24 h). This study was approved by the University of Washington Human Subjects Review Committee.

Venous blood was collected after a 12- to 14-h overnight fast into tubes containing EDTA (1 mg/ml final concentration) for preparation of plasma. Plasma was stored under nitrogen at −70°C and used within 1 month for analysis of total peroxyl radical trapping potential (TRAP), measurement of the susceptibility of LDL to oxidative modification, and determination of lipoprotein distribution. GHb (15), fructosamine (Ro-TAG-fructosamine assay, Roche, Montclair, NJ), total carotene (16), vitamin A (retinol) (17), vitamin C (ascorbic acid) (18), vitamin E (α-tocopherol) (17), plasma lipoproteins (19), glucose, bilirubin, and uric acid (by standard techniques) were measured from the fresh blood samples drawn on the morning of the study. The injection of insulin was withheld until after the blood draw on the morning of the study to avoid possible hypoglycemic reactions due to fasting.

Measurement of TRAP. TRAP was measured as described by Wayner et al. (20). Plasma (50 μl), to which linoleic acid (4% vol/vol) had been added, was diluted in phosphate-buffered saline (PBS) in an oxygen electrode apparatus (YSI oxygen monitor system model 5300, Yellow Springs, OH) and equilibrated at 37°C. Oxidation was initiated by addition of the water-soluble thermolabile free radical generator, 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH; Polysciences, Warrington, PA; 4 mM final concentration) to initiate lipid peroxidation. Oxygen consumption was monitored during lipid peroxidation by an oxygen electrode (YSI dissolved oxygen monitor system model 18053/18172). After initiation of peroxyl radical formation from AAPH, oxygen content decreased slowly while plasma antioxidants were consumed. When the antioxidants were consumed, oxygen consumption increased rapidly. The time from the addition of AAPH to the onset of this rapid propagation phase was termed T_{plasma}. Trolox (4 μM final concentration), a water soluble vitamin E analog, was then added as an internal standard, leading to a second slow phase of oxygen consumption, followed by a second rapid propagation phase after its total depletion. The time from the addition of Trolox to the onset of the second propagation phase was termed T_{Trolox}. TRAP values were calculated from TRAP = $T_{plasma}/T_{Trolox} \times k$, where k was a constant that takes into account the concentration of Trolox and the plasma dilution factor (20). Intra-assay coefficient of variation (CV) was 9.5%, and interassay CV was 12%.

Measurement of LDL oxidation. LDL was isolated by discontinuous density gradient ultracentrifugation after a single spin (2.5 h at 50,000 rpm at 10°C) in a vertical rotor (Beckman VTI-50, Fullerton, CA) by the method of Chung et al. (21). The LDL fraction was aspirated and passed over a Sephacryl S-300 column to desalt and remove the EDTA, and its cholesterol content was measured with the use of a cholesterol oxidase kit (Boehringer Mannheim, Indianapolis, IN). LDL purity was confirmed by the absence of apolipoprotein AI and albumin. LDL oxidation was measured by the method of Esterbauer et al. (22) in which 1.66 μM copper was added to a spectrophotometer curvette containing LDL (250 μg cholesterol/ml in PBS), and the kinetics of conjugated diene formation were monitored by changes in absorbance at 234 nm at 37°C. Three characteristic phases (lag, propagation, and decomposition) were observed, from which the lag time, rate, and amount of conjugated diene formation during LDL oxidation were calculated (22). In a separate experiment, lag phase of LDL-conjugated diene formation was determined immediately and after 4 weeks of storage of plasma at −70°C in three control and four diabetic subjects. No significant differences were observed (data not shown). These findings are consistent with previously published findings (23), where plasma was stored for up to 6 months before oxidation of LDL for oxidation studies. A computer program (Kaleidagraph, Synergy Software, Reading, PA) was used to obtain the best tangent to the slop of propagation, from which lag time and rate were calculated. Intra- and interassay CVs for the lag phase were 5.5 and 11.8%, respectively.

Lipoprotein particle distribution pattern. Lipoprotein particles were separated by nonequilibrium density gradient ultracentrifugation in a Sorvall TV-865B vertical rotor for 90 min at 65,000 rpm at 10°C, as described previously (24). Lipoprotein fractions were removed by using a Beckman fraction recovery system with a proportionating pump that allowed a downward flow collection of lipoprotein fractions. The cholesterol content of each of the 38 fractions collected was measured with the use of a cholesterol assay kit (Boehringer Mannheim, Indianapolis, IN).

Statistical analysis. Statistical calculations were performed using StatView software package (version 4.01). Values are given as means ± SE. Comparisons between diabetic and control groups were made using the nonparametric Mann-Whitney test. Linear regression analysis was used for detecting relationships between TRAP values, conjugated diene lag phase, and various plasma antioxidant levels. Multivariate analysis using a multiple regression model was performed between TRAP and various antioxidants in both groups combined and in individual groups alone. All correlations were performed within individual groups of IDDM or control subjects and also in both groups combined.

TABLE 1
Characteristics of study subjects

	IDDM	Control	*P* value
n	19	20	
Gender (M/F)	12/7	12/8	
Age (years)	31.7 ± 3.0*	31.9 ± 1.8	NS
BMI (kg/m^2)	23.3 ± 0.4	23.6 ± 0.5	NS
GHb (%)	12.2 ± 0.4	5.7 ± 0.1	<0.0001
Fasting plasma glucose			
(mM)	13.5 ± 1.3	5.1 ± 0.1	<0.0001
(mg/dl)	(243.3 ± 23.5)	(91.9 ± 1.4)	
Fructosamine (μM)	418 ± 65	254 ± 19	<0.0001
Duration (years)	15 ± 2	N/A	
Insulin dosage (U/kg)	0.66 ± 0.05	N/A	
Retinopathy	5 of 19	N/A	
Nephropathy	0 of 19	N/A	
Neuropathy	3 of 19	N/A	
Peripheral vascular disease	0 of 19	0/20	
Coronary artery disease	0 of 19	0/20	

Data are means ± SE. Insulin dosage is the mean dose at the time of the study. Retinopathy was nonproliferative in 4 of 5 subjects, based on formal examination by ophthalmologists. Nephropathy was defined as urinary protein excretion <300 mg/24 h. Neuropathy was based on clinical exam findings: vibration and microfilament testing, orthostatic hypotension, and resting tachycardia. Peripheral vascular disease was defined by the symptoms of claudication and history of arterial occlusive diseases.

RESULTS

Glycemic control. As shown in Table 1, the diabetic patients remained poorly controlled with very high GHb levels (12.2 ± 0.4%).

Lipid and lipoproteins. No differences were noted in lipid and lipoprotein levels between the diabetic and control groups, except for VLDL cholesterol, which was higher (0.5 ± 0.1 mM) in the control subjects than in subjects with IDDM (0.3 ± 0.1, $P < 0.05$) (Table 2). The distribution of lipoprotein particles by density gradient ultracentrifugation did not differ

TABLE 2
Lipid profile of control and IDDM subjects

	IDDM	Control	*P* value
Total cholesterol	5.0 ± 0.2	4.7 ± 0.2	NS
	(193 ± 7)	(182 ± 6)	
LDL	3.1 ± 0.1	2.8 ± 0.1	NS
	(118 ± 6)	(109 ± 5)	
HDL	1.6 ± 0.1	1.4 ± 0.1	NS
	(63 ± 4)	(54 ± 4)	
VLDL	0.3 ± 0.1	0.5 ± 0.1	<0.05
	(12 ± 2)	(17 ± 2)	
Triglyceride	0.8 ± 0.1	0.9 ± 0.1	NS
	(70 ± 8)	(83 ± 8)	
Apolipoprotein B	(89 ± 4)	(81 ± 3)	NS
Lipoprotein (a)	(16 ± 3)	(24 ± 5)	NS

Data are means ± SE in mM (mg/dl); NS, not significant.

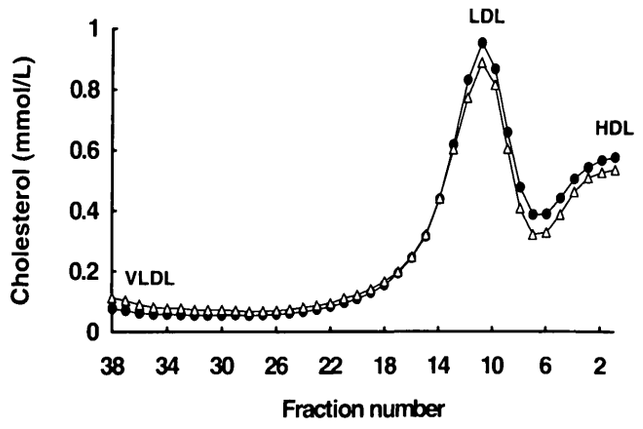

FIG. 1. Lipoprotein particle distribution in control and diabetic subjects. Plasma lipoproteins were separated by nonequilibrium density gradient ultracentrifugation. Fractions of decreasing density were collected and assayed for their cholesterol content. The positions of VLDL, LDL, and HDL are indicated. ●, IDDM subjects; △, control subjects.

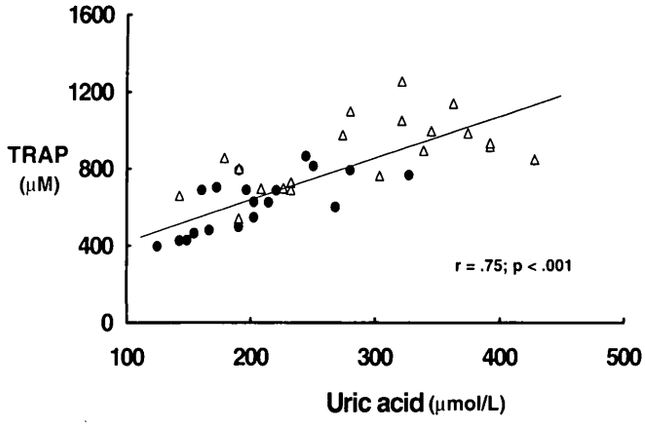

FIG. 2. Relationship between uric acid and TRAP of plasma. A significant correlation ($r = 0.70$, $P < 0.001$) persists between uric acid and total TRAP when the diabetic group was analyzed separately. In a multiple regression model, uric acid remains significantly correlated to TRAP even after adjustment for vitamins C and E, carotene, and bilirubin ($P < 0.001$). ●, IDDM subjects; △, control subjects.

between the two groups (Fig. 1). This is in contrast to the well-described finding of small, dense LDL particles in subjects with NIDDM (2).

LDL oxidation. Three parameters were derived from the kinetics of conjugated diene formation when LDL was oxidized in the presence of copper. Only the lag time was significantly decreased in IDDM relative to control subjects (126 ± 11 vs. 165 ± 15 min, $P < 0.05$). Neither propagation rates nor maximum conjugated diene levels differed between the two groups (data not shown). No significant correlations were detected between parameters of LDL oxidation and glycemic indexes, either in individual groups or the combined group. The decreased lag time indicates increased susceptibility of LDL to oxidation in IDDM.

Plasma antioxidant content. Plasma levels of uric acid and vitamin A were significantly lower in IDDM than in control subjects (Table 3). However, carotene and vitamins C and E did not differ between the two groups. This, again, is in contrast to the well-documented finding of lower than normal levels of vitamin C in NIDDM (12,13).

Plasma TRAP. A highly significant decrease in TRAP values was observed in IDDM compared with control subjects (626 ± 34 vs. 877 ± 41 μM, $P < 0.0001$). This indicates that the overall plasma antioxidant defense to lipid peroxidation was impaired in this group of poorly controlled IDDM subjects.

Because a decrease in plasma antioxidant defense might contribute to the increased susceptibility of LDL to oxidation, plasma vitamins C and E, carotene, vitamin A, uric acid, bilirubin, protein (total), and albumin were analyzed for possible relationships to TRAP. Only uric acid correlated significantly with TRAP values (Fig. 2), either in both groups combined ($r = 0.75$, $P < 0.001$) or in individual groups ($r = 0.70$, $P < 0.001$, IDDM; $r = 0.81$, $P < 0.005$, control). This significant relationship persisted even in a multivariate model adjusted for vitamins C and E, carotene, and bilirubin, in either the combined group ($P < 0.001$) or individual groups alone ($P < 0.01$, IDDM or control). Vitamin A levels were found significantly correlated with TRAP values ($r = 0.54$, $P < 0.001$) only for both groups combined and not in either IDDM or control group alone. A significant correlation existed between TRAP and plasma albumin concentrations ($r = 0.41$, $P = 0.01$) in the combined group and in the control group alone (mean 44 ± 1 g/l; $r = 0.6$, $P < 0.005$) but not in the IDDM group (mean 42 ± 1 g/l; $r = 0.10$, $P < 0.5$). No significant correlation was observed between TRAP and total plasma protein levels. Neither vitamins C and E nor carotene levels correlated with TRAP. All indexes of glycemic control (GHb, fasting plasma glucose, and fructosamine) were inversely correlated with TRAP values, although the statistical significance of this correlation was lost when analysis was carried out in either group alone, most likely because of clustering of data within each group.

TABLE 3
Plasma antioxidants

	IDDM	Control
Vitamin C (μM)	39 ± 5	41 ± 3
Vitamin E (μM)	24.6 ± 1.6	24.9 ± 1.4
Vitamin E/cholesterol	0.47 ± 0.02	0.53 ± 0.02
Vitamin A (μM)	1.55 ± 0.07*	1.95 ± 0.11
Carotene (μM)	2.25 ± 0.3	2.27 ± 0.2
Uric acid (μM)	202 ± 12*	273 ± 24
Bilirubin (μM)	11.3 ± 1.7	12.1 ± 1.7

Data are means ± SE. *$P < 0.01$.

DISCUSSION

Lipoprotein oxidation, which has been postulated to play an important role in atherogenesis (7,8), is likely to occur in the interstitial space of the artery wall rather than in plasma. Therefore, evaluation of lipoprotein oxidation in vivo is difficult and has to be performed by indirect methods (25). The most commonly used method is measurement of the lag time of conjugated diene formation after initiation of oxidation by the addition of copper ions to LDL isolated from plasma (22). Using this method, we were able to demonstrate a reduction in the lag phase of LDL oxidation in subjects with IDDM, consistent with increased susceptibility of their LDL to oxidative modification.

The determinants of the lag phase of LDL oxidation are not known with certainty. Oxidation of LDL proceeds rapidly after consumption of endogenous antioxidants such as vitamin E and depletion of antioxidants such as vitamin C and uric acid from the aqueous milieu of the lipoproteins (26). However, no correlation was found between lag time of

conjugated diene formation and TRAP, which is a measure of the overall antioxidant capacity of plasma. A poor correlation between LDL lag time and LDL antioxidant content has been demonstrated previously (26), suggesting that factors other than antioxidants also are involved in determining the lag time of oxidation. One possible determinant of susceptibility of LDL to oxidation is glucose. The auto-oxidation of glucose is associated with generation of oxygen free radicals (11). High levels of glucose can enhance the oxidation of LDL in vitro (11,27). Therefore, it is conceivable that the hyperglycemia of diabetes might result in LDL being seeded with small amounts of lipid peroxidation products in vivo, thereby rendering them more susceptible to subsequent oxidation in vitro. However, a recent study suggests that glucose inhibits LDL oxidation in vitro (28). We were unable to demonstrate significant correlations between indexes of glycemic control and any of the parameters of LDL oxidation in control or diabetic subjects, possibly because of clustering of data within each group.

Susceptibility of LDL to oxidation also is related to the size and density of the lipoprotein particles; small, dense subfractions of LDL have shorter lag times of oxidation than more buoyant subfractions (14,29). Small, dense LDL is typically found in subjects with NIDDM (2). Because the distribution of lipoproteins by nonequilibrium density gradient ultracentrifugation in our IDDM subjects did not differ from that observed in the nondiabetic control subjects, the reduced resistance of LDL to oxidative modification that we observed in IDDM could not be accounted for by small, dense LDL. Recently, LDL isolated from subjects with NIDDM also was shown to have increased susceptibility to oxidation, measured by a different technique (28). However, the potential role of glycemic control, antioxidants, and LDL particle distribution could not be evaluated from that study.

Conceivably, the increased oxidation is a result of, rather than the cause of, vascular disease. Yet the diabetic subjects had no overt macrovascular disease or frank nephropathy, and only 5 of 19 had retinopathy. Thus, in our study, there was little clinical evidence of vascular disease to support this possibility. However, to adequately address this cause-and-effect issue, it would be necessary to study newly diagnosed patients with IDDM.

An alternate explanation for our finding of increased susceptibility of LDL to oxidative modification in subjects with IDDM is that antioxidant defenses are reduced in diabetes, possibly as a result of increased oxidant stress. The reduced TRAP of plasma in subjects with IDDM is consistent with this hypothesis. The total TRAP of plasma is contributed to by vitamins E and C, β-carotene, uric acid, bilirubin, protein-bound thiols (including non-lipoprotein thiols), and probably other as yet unknown compounds (30). Whereas conjugated diene formation assesses the resistance of LDL oxidation, contributed to primarily by fat-soluble antioxidants, the TRAP assay gives a collective measurement of the total antioxidant capacity in plasma. Thus, the contribution of various water-soluble antioxidants, such as ascorbic acid, bilirubin, uric acid, and protein-bound thiols, also are reflected in the TRAP values. It is not known whether this measurement is relevant to oxidation of LDL under in vivo conditions. However, LDL in the artery wall would be bathed in an ultrafiltrate of plasma, which would include the components that are being assessed in the TRAP assay.

We were unable to demonstrate differences in the levels of vitamins C and E between the diabetic and control subjects, nor was a relationship between TRAP values and the levels of these antioxidants in plasma observed. The observation that uric acid levels, not accountable by gender differences, were lower in subjects with IDDM, and its significant correlation with TRAP values whether analyzed either in the combined or individual groups, suggests that this antioxidant may be an important determinant of the resistance of LDL to oxidative modification. Vitamin A, but not carotene, levels were also reduced in the subjects with IDDM. The positive relationship between vitamin A and TRAP in the combined group is somewhat surprising, but the likelihood of a cause-and-effect process is lessened by the loss of significant correlations in the individual diabetes or control groups alone. Vitamin A is not believed to be a powerful antioxidant, although a reduction of plasma vitamin A levels in patients with diabetes has been observed previously (31). The positive relationship observed between serum albumin and TRAP raises the question of whether protein-bound thiols, which were not measured directly in this study, might also be playing a role in the reduced antioxidant defenses observed in the patients with diabetes.

Reduced antioxidant defenses in poorly controlled subjects with IDDM may thus, in part, account for the increased susceptibility of LDL from subjects with IDDM to oxidative modification in vitro. Although LDL is separated from water-soluble antioxidants such as uric acid and vitamin C during its isolation, seeding of LDL with small amounts of early lipid peroxidation products (8) would render the lipoprotein more susceptible to oxidative modification in vitro. Because peroxyl radical trapping capacity also is likely to be reduced in the milieu of the artery wall, where lipoproteins appear to be oxidized during atherogenesis, antioxidant therapy may be of value in reducing the markedly accelerated atherosclerosis seen in diabetes. This hypothesis should be tested by a clinical trial.

ACKNOWLEDGMENTS

This study was supported in part by National Institutes of Health Grant DK-02456, the Clinical Nutrition Research Unit (DK-35816), and the Diabetes Endocrinology Research Center (DK-17047). Part of this study was performed at the University of Washington's Clinical Research Center (RR-00037).

The authors wish to thank John Hokanson for helpful suggestions in multivariate analysis, and Julie Kirk and Pat Burns for assistance in manuscript preparation.

REFERENCES

1. Kannel WB, McGee DL: Diabetes and cardiovascular disease: the Framingham Study. *JAMA* 241:2035–2038, 1979
2. Barakat HA, Carpenter JW, McLendon VD, Khazanie P, Leggett N, Heath J, Marks R: Influence of obesity, impaired glucose tolerance, and non-insulin-dependent diabetes on low-density lipoprotein structure and composition: possible link between hyperinsulinemia and atherosclerosis. *Diabetes* 39:1527–1533, 1990
3. Austin MA, Breslow JL, Hennekens CH, Buring JE, Willett WC, Krauss RM: Low-density lipoprotein subclass patterns and risk of myocardial infarction. *JAMA* 260:1917–1921, 1988
4. Krolewski AS, Kosinski EJ, Warram JH, Leland OS, Busick EJ, Asmal AC, Rand LI, Christlieb AR, Bradley RF, Kahn CR: Magnitude and determinants of coronary artery disease in juvenile-onset, insulin-dependent diabetes mellitus. *Am J Cardiol* 59:750, 1987
5. Nikkila EA: High-density lipoproteins in diabetes. *Diabetes* 30 (Suppl. 2):82–87, 1981
6. Eckel RH, Albers JJ, Cheung MC, Wahl PW, Lindgren FT, Bierman EL: High-density lipoprotein composition in insulin-dependent diabetes mel-

litus. *Diabetes* 30:132–138, 1981
7. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL: Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320:916–924, 1989
8. Witztum JL, Steinberg D: Role of oxidized low-density lipoprotein in atherogenesis. *J Clin Invest* 88:1785–1792, 1991
9. Lyons TJ: Oxidized low-density lipoproteins: a role in the pathogenesis of atherosclerosis in diabetes. *Diabetic Med* 8:411–419, 1991
10. Gillery P, Monboisse JC, Maquart FX, Borel JP: Glycation of proteins as a source of superoxide. *Diabetes Metab* 14:25–30, 1988
11. Hunt JV, Smith CC, Wolff SP: Autoxidative glycosylation and possible involvement of peroxides and free radicals in LDL modification by glucose. *Diabetes* 39:1420–1424, 1990
12. Stankova L, Riddle M, Larned J, Burry K, Menashe D, Hart J, Bigley R: Plasma ascorbate concentrations and blood cell dehydroascorbate transport in patients with diabetes mellitus. *Metabolism* 33:347–353, 1984
13. Cunningham JJ, Ellis SL, McVeigh KL, Levine RE, Calles-Escandon J: Reduced mononuclear leukocyte ascorbic acid content in adults with insulin-dependent diabetes mellitus consuming adequate dietary vitamin C. *Metabolism* 40:146–149, 1991
14. Chait A, Brazg RL, Tribble DL, Krauss RM: Susceptibility of small, dense low-density lipoproteins to oxidative modification in subjects with the atherogenesis lipoprotein phenotype: pattern B. *Am J Med* 94:350–356, 1993
15. Abraham EC, Perry RE, Stallings M: Applications of affinity chromatography for separation and quantification of glycosylated hemoglobins. *J Lab Clin Med* 102:187–197, 1983
16. Nino HV, Shaw W: Vitamins. In *Fundamentals of Clinical Chemistry*. Tietz NW, Ed. Philadelphia, Saunders, 1976, p. 545–547
17. Bieri JG, Tolliver TJ, Catignani GL: Simultaneous determination of α-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am J Clin Nutr* 32:2143–2149, 1979
18. Lee W, Hamernyik P: Ascorbic acid in lymphocytes: cell preparation and liquid chromatographic assay. *Clin Chem* 28:2165, 1982
19. Lipid Research Clinic Programs. In *U.S. Department of Health and Human Services, Manual of Laboratory Operations: Lipid and Lipoprotein Analysis*. 2nd ed. Washington, DC, U.S. Govt. Printing Office, 1982, p. 63–77
20. Wayner DD, Burton GW, Ingold KU, Locke S: Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. *FEBS Lett* 187:33–37, 1985
21. Chung BH, Wilkinson T, Geer JC, Segrest JP: Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J Lipid Res* 21:284–291, 1980
22. Esterbauer H, Striegl G, Puhl H, Rotheneder M: Continuous monitoring of in vitro oxidation of human low-density lipoprotein. *Free Radical Res Commun* 6:67–75, 1989
23. Princen HM, van Poppel G, Vogelezang C, Buytenhek R, Kok FJ: Supplementation with vitamin E but not β-carotene in vivo protects low-density lipoprotein from lipid peroxidation in vitro: effect of cigarette smoking. *Arterioscler Thromb* 12:554–562, 1992
24. Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson JE, Krauss RM, Beaudrie K, Cone JT: Single vertical spin density gradient ultracentrifugation. In *Methods in Enzymology, Plasma Lipoproteins: Preparation, Structure, and Molecular Biology*. Segrest JP, Albers JJ, Eds. Orlando, FL, Academic, 1986, p. 181–209
25. Chait A: Methods for assessing lipid and lipoprotein oxidation. *Curr Opin Lipidol* 3:389–394, 1992
26. Esterbauer H, Jurgens G: Mechanistic and genetic aspects of susceptibility of LDL to oxidation. *Curr Opin Lipidol* 4:114–124, 1993
27. Kawamura M, Heinecke JW, Chait A: Glucose-dependent lipid peroxidation of low-density lipoprotein (Abstract). *Clin Res* 40:102A, 1992
28. Babiy AV, Gebicki JM, Sullivan DR, Willey K: Increased oxidizability of plasma lipoproteins in diabetic patients can be decreased by probucol therapy and is not due to glycation. *Biochem Pharmacol* 43:995-1000, 1992
29. deGraaf J, Hak-Lemmers HL, Hectors MP, Demacker PN, Hendriks JC, Stalenhoef AF: Enhanced susceptibility to in vitro oxidation of the dense low-density lipoprotein subfraction in healthy subjects. *Arterioscler Thromb* 11:298–306, 1991
30. Wayner DD, Burton GW, Ingold KU, Barclay LR, Locke SJ: The relative contributions of vitamin E, urate, ascorbate, and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochem Biophys Acta* 924:408–419, 1987
31. Basu TK, Tze WJ, Leichter J: Serum vitamin A and retinol- binding protein in patients with insulin-dependent diabetes mellitus. *Am J Clin Nutr* 50:329–331, 1989