In Vivo Evidence for Increased Oxidation of Circulating LDL in Impaired Glucose Tolerance

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Oxidized LDL (oxLDL) is a key mediator in atherogenesis and a marker of coronary artery disease (CAD). Type 2 diabetes is associated with excessive cardiovascular morbidity and mortality. Because atherogenesis starts before diabetes is diagnosed, we investigated whether circulating oxLDL levels are increased in impaired glucose tolerance (IGT). OxLDL levels were measured in 376 subjects with normal glucose tolerance (NGT), 113 patients with IGT, and 54 patients with newly diagnosed type 2 diabetes. After correction for age and BMI, serum levels of oxLDL were significantly increased in IGT versus NGT subjects ($P = 0.002$). OxLDL levels were not associated with the following parameters of the oxidative/antioxidative balance in the blood: total antioxidant capacity, urate-to-allantoin ratio, and circulating phagocyte oxygenation activity. In stepwise multivariate analysis, LDL cholesterol ($P < 0.0005$) and triglycerides ($P < 0.0005$) were the strongest predictors of circulating oxLDL levels, followed by HDL cholesterol ($P = 0.003$), 2-h postchallenge C-peptide ($P = 0.011$), fasting free fatty acids ($P = 0.013$), and serum paraoxonase activity ($P = 0.035$). The strong correlation of oxLDL with LDL cholesterol and triglycerides indicates that LDL oxidation in IGT is preferentially associated with dyslipidemia. OxLDL increase may explain the high atherogenic potency of dyslipidemia in the prediabetic state. *Diabetes* 51:3102–3106, 2002

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CAD, coronary artery disease; CPOA, circulating phagocyte oxygenation activity; IGT, impaired glucose tolerance; IMT, intima-media thickness; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; oxLDL, oxidized LDL; PON, serum paraoxonase activity with paraoxon as substrate; RIAD, Risk Factors in Impaired Glucose Tolerance for Atherosclerosis and Diabetes; TAC, total antioxidant capacity.

Numerous in vitro studies revealed that oxidative modification of LDL enhances its atherogenicity (1). Recently, in accordance with the proposed causal role in atherosclerosis, the level of circulating oxidized LDL (oxLDL) has been shown to be a biochemical risk marker for coronary artery disease (CAD) (2,3).

Patients with type 2 diabetes and IGT are at high risk for macrovascular disease (4,5). In the Risk Factors in Impaired Glucose Tolerance for Atherosclerosis and Diabetes (RIAD) study, we showed an association between early disturbances in glucose tolerance and increased carotid intima-media thickness (IMT), a surrogate marker of early atherosclerosis (6). Whereas increased circulating levels of oxLDL have been found in diabetes (7), the presence of oxLDL in prediabetic states, e.g., IGT, has not yet been elucidated. Therefore, the objective of the present study was to investigate whether the degree of glucose intolerance is related to circulating levels of oxLDL and to parameters of oxidative stress.

In the present study, we show that circulating oxLDL levels in subjects with IGT are significantly increased when compared with subjects with NGT. LDL cholesterol and triglyceride levels were the strongest predictors of circulating oxLDL. In contrast, circulating oxLDL were not, or only weakly, associated with parameters of the oxidative/antioxidative potential in the circulation. It is suggested that higher oxidative modification of LDL particles in IGT is promoted particularly by diabetic dyslipidemia that favors the invasion of LDL particles into the subendothelial space followed by re-entry of modified lipoprotein into the circulation.

RESEARCH DESIGN AND METHODS

Subjects. A total of 543 subjects from the RIAD study with complete data set was included. In brief, middle-aged subjects (40–70 years) who were at risk for development of diabetes owing to a family history of diabetes, obesity, and/or hyper-/dyslipoproteinemia were examined. Clinically overt diabetes and medication affecting glucose tolerance (β-blockers, thiazide diuretics, and corticoids) were exclusion criteria. All subjects consented to participate in the study, which was approved by the local ethics committee.

The diagnosis of IGT and diabetes was confirmed by an oral glucose tolerance test (OGTT) (75-g oral glucose challenge) according to World Health Organization guidelines and criteria: 2-h postchallenge plasma glucose concentration between 7.8 and 11.1 mmol/l for IGT and >11.1 mmol/l for diabetes. According to OGTT criteria, participants were grouped into subjects with NGT ($n = 376$), subjects with IGT ($n = 113$), and patients with newly diagnosed diabetes ($n = 54$). Their baseline clinical and biochemical characteristics are given in Table 1.

TABLE 1
Baseline clinical and biochemical characteristics of NGT, IGT, and diabetic (DM) subjects

Parameter	NGT	IGT	DM	*P* IGT/NGT	*P* DM/NGT
n	376	113	54		
Age (years)	57 ± 8	60 ± 8	60 ± 8	0.004	0.019
Sex (M/F)	173/203	39/74	31/23	—	—
BMI (kg/m^2)	26.4 ± 4.2	28.0 ± 3.9	28.8 ± 3.9	0.001	0.001
Total cholesterol (mmol/l)	5.64 ± 1.06	5.76 ± 0.98	5.42 ± 0.86	0.586	0.318
Triglycerides (mmol/l)	1.41 ± 0.76	1.68 ± 0.76	1.89 ± 0.97	0.006	0.000
HDL cholesterol (mmol/l)	1.56 ± 0.40	1.50 ± 0.47	1.32 ± 0.30	0.919	0.300
LDL cholesterol (mmol/l)	3.44 ± 0.98	3.48 ± 0.90	3.23 ± 0.81	0.489	0.000
HbA_{1c} (%)	5.4 ± 0.5	5.5 ± 0.5	6.1 ± 0.7	0.018	0.000
Plasma glucose (mmol/l)					
0 min	5.39 ± 0.54	5.84 ± 0.53	7.19 ± 1.05	0.000	0.000
120 min	5.56 ± 1.26	8.89 ± 0.92	13.10 ± 3.03	0.000	0.000
Free fatty acids (mmol/l)					
0 min	0.51 ± 0.24	0.66 ± 0.23	0.71 ± 0.29	0.000	0.000
120 min	0.09 ± 0.34	0.09 ± 0.07	0.12 ± 0.13	0.992	0.615
Proinsulin (pmol/l)					
0 min	4.15 ± 4.30	5.36 ± 3.96	9.62 ± 12.09	0.124	0.000
120 min	20.07 ± 24.22	29.30 ± 18.56	34.91 ± 32.89	0.000	0.000
Insulin (pmol/l)					
0 min	68.8 ± 71.4	84.1 ± 49.5	102.3 ± 65.7	0.102	0.003
120 min	259.3 ± 335.1	579.8 ± 555.4	572.5 ± 568.0	0.000	0.000
C-peptide (pmol/l)					
0 min	1,165 ± 536	1,416 ± 534	1,528 ± 606	0.000	0.000
120 min	3,649 ± 1,956	5,028 ± 1,416	4,586 ± 1,977	0.000	0.003

Data are means ± SD. 120 min represents values after oral glucose challenge.

Experimental protocol. Blood samples were centrifuged within 20 min at 4°C. Serum, citrate, and EDTA plasma was separated from cells immediately after centrifugation and stored at −80°C until analyzed. Whole blood chemiluminescence was assayed within 30 min.

Serum levels of total oxLDL particles were directly measured by a new sandwich enzyme-linked immunosorbent assay (within-day precision <3.5%, between-day precision <6.5%, within-person precision [n = 3] <6.5%; Mercodia, Uppsala, Sweden) based on monoclonal antibody mAB-4E6 used by Holvoet et al. (8).

Plasma glucose, HbA_{1c}, triglycerides, total cholesterol, HDL cholesterol, insulin, proinsulin, and C-peptide were measured as previously described (9). LDL cholesterol was calculated using the Friedewald formula.

A luminescence-based technique was used for direct assessment of circulating phagocyte oxygenation activity (CPOA) (10). For this purpose, phagocytes in highly diluted heparinized whole blood were stimulated with zymosan, and luminol- or lucigenin-amplified chemiluminescence was recorded for 30 min. The results (integral counts over 30 min) are expressed as ratio of stimulated versus basal phagocyte activities.

To characterize the antioxidative potential in the circulation, three methods were applied. Total antioxidant capacity (TAC) was assayed by the delay of luminol-amplified chemiluminescence induced by 2,2′-Azobis(2-amidinopropane) hydrochloride (AAPH) in citrate plasma (11), the urate-to-allantoin ratio was estimated by high-performance liquid chromatography in heparinized plasma (12), and the serum activities of HDL-associated paraoxonase were determined photometrically using paraoxon as substrate (13).

Statistical analysis. Descriptive data were expressed as arithmetic means ± SD. Spearman's rank correlation coefficient was calculated between oxLDL levels, LDL cholesterol, and logarithm of triglyceride levels. For serum oxLDL levels, the association with selected metabolic and oxidative parameters was estimated using multiple linear regression analysis. The associations of glucose tolerance with metabolic and oxidative parameters were assessed by one-way ANOVA. Univariate and multivariate models were compared among

TABLE 2
Serum levels of oxidized LDL and parameters of the circulating oxidant/antioxidant status adjusted for age and BMI

	NGT	IGT	Diabetes (DM)	*P* IGT/NGT	*P* DM/NGT
n	376	113	54		
OxLDL (units/l)	70.4 (68.7–72.1)	76.7 (73.5–79.8)	75.2 (70.7–79.7)	0.002	0.153
Urate-to-allantoin ratio	6.1 (5.7–6.4)	6.1 (5.5–6.6)	6.2 (5.4–7.1)	1.000	0.984
TAC (μmol/l)	761 (744–778)	773 (742–804)	781 (736–826)	0.890	0.416
PON (units/ml)	0.27 (0.25–0.29)	0.29 (0.25–0.33)	0.28 (0.21–0.34)	0.497	0.801
CPOA Luminol-amplified	49.7 (47.1–52.1)	51.3 (46.7–55.8)	61.3 (54.7–67.9)	0.899	0.004
CPOA Lucigenin-amplified	7.1 (6.7–7.4)	7.7 (7.1–8.4)	7.5 (6.5–8.4)	0.265	0.833

Data are means (95% CI). CPOA is the ratio of stimulated versus basal circulating phagocyte oxygenation activity.

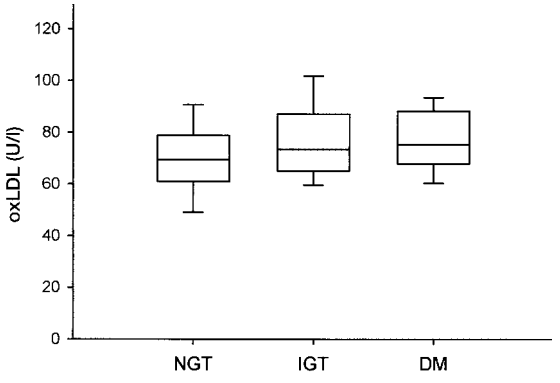

FIG. 1. Boxplots showing distributions of baseline levels of circulating oxidized LDL in NGT, IGT, and diabetic (DM) subjects.

each other to show the influence of complex effects. All analyses were conducted using the SPSS version 10.0 software.

RESULTS

Table 1 shows the baseline characteristics of subjects with NGT, IGT, and diabetes. Higher fasting insulin, proinsulin, and free fatty acid concentrations in IGT and diabetes have been considered as strong indicators of an insulin-resistant/hyperinsulinemic state. Furthermore, both subjects with IGT and diabetes showed significantly higher triglyceride levels. HDL levels in IGT and diabetes showed a tendency to be decreased when compared with NGT, but the differences were not statistically significant (Table 1). According to Boizel et al. (14), we estimated the triglyceride-to-HDL molar ratio as an indicator for LDL size. The values of 1.30 ± 0.89 for IGT and 1.59 ± 1.07 for diabetes were significantly higher when compared with NGT (1.05 ± 0.88), which is highly indicative of the occurrence of small dense LDL in these subjects.

Table 2 shows values for oxLDL levels and parameters of the oxidant/antioxidant status in the circulation adjusted for age and BMI. Serum oxLDL levels were significantly higher in IGT subjects ($P = 0.002$) when compared with NGT subjects. In diabetic patients, oxLDL levels showed a trend to be increased, but the difference to subjects with NGT was not statistically significant. To demonstrate the variation of oxLDL levels within the three groups, the data are given by boxplots showing medians as well as 5th, 25th, 75th, and 95th percentiles (Fig. 1).

The total antioxidant capacity, the urate-to-allantoin ratio, and the serum paraoxonase activity did not differ among the three groups (Table 2). Of note, only diabetic patients, not IGT subjects, showed a significant enhanced luminol-amplified circulating phagocyte oxygenation activity when compared with normoglycemic control subjects ($P = 0.004$).

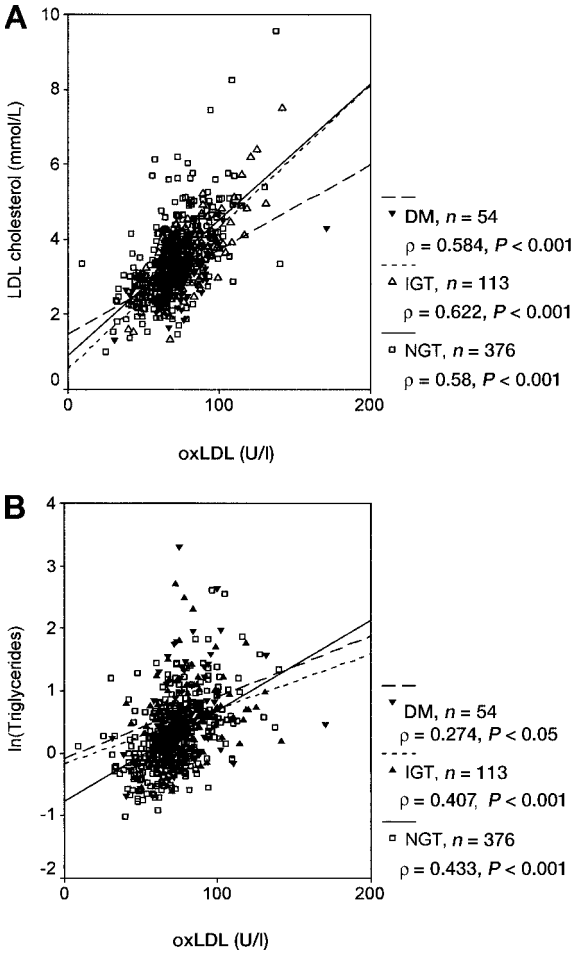

FIG. 2. Relationship between baseline oxLDL levels and baseline LDL cholesterol levels (*A*) and ln(triglycerides) levels (*B*), respectively. Spearman's rank correlation coefficients (ρ) are given. DM, diabetic subjects.

Table 3 shows the relationship between serum oxLDL levels and parameters of the glucose and lipid metabolism and oxidative/antioxidative balance, respectively, for the entire study population. Univariate analysis showed LDL cholesterol ($P < 0.005$, Fig. 2), ln(triglycerides) ($P < 0.005$, Fig. 2), HDL cholesterol ($P < 0.005$), C-peptide (120 min after oral glucose challenge; $P < 0.005$), and fasting free fatty acids ($P = 0.007$) to be correlated with oxLDL. Multivariate analysis revealed LDL cholesterol ($P < 0.005$)

TABLE 3
Multiple linear regression analysis of relationship between circulating oxLDL levels and metabolic parameters

	Univariate analysis		Multivariate analysis	
	R^2	P	F	P
LDL cholesterol	0.392	<0.0005	391.7	<0.0005
ln(triglycerides)	0.182	<0.0005	75.5	<0.0005
HDL cholesterol	0.071	<0.0005	8.8	0.003
C-peptide/120-min OGTT	0.046	<0.0005	6.5	0.011
Free fatty acids/0-min OGTT	0.036	0.007	6.2	0.013
Paraoxonase activity	0.023	0.959	4.5	0.035

Univariate analysis was performed using age- and BMI-adjusted values. Because of the skewed distribution of triglycerides, these data were logarithmically transformed to fit the linear model. The multivariate model explained 53.6% of the variation of oxLDL.

and ln(triglycerides) to be the strongest predictors of circulating oxLDL levels (Table 3). The multivariate model used explained 53.6% of the variation of oxLDL.

The TG-to-HDL molar ratio was significantly correlated with oxLDL in the total population ($\rho = 0.433$, $P = 0.000$) as well as within the groups when analyzed separately (NGT: $\rho = 0.422$, $P = 0.000$; IGT: $\rho = 0.366$, $P = 0.000$; diabetes: $\rho = 0.276$, $P < 0.05$).

DISCUSSION

In the present study, we first demonstrated increased levels of circulating oxLDL in subjects with IGT compared with NGT. Oxidative modification of lipids and proteins is a common part of inflammatory diseases including diabetes. While increased oxidation products have been found in frank diabetes (7,15), oxidative damage of lipoproteins in early stages of diabetes, particularly in IGT, has not been elucidated yet. Recently, circulating oxLDL have been shown to be a useful parameter for identifying CAD (2,7,8) and also to be a marker for differentiating the degree of severity of acute coronary syndromes (3). OxLDL are suggested to play a key role in atherogenesis, and diabetes is associated with accelerated atherosclerosis and atherosclerotic complications including CAD (4,5). The examined subjects were recruited from the RIAD study that started in 1996 as a prospective study including 40- to 70-year-old subjects at a high risk for diabetes (6). Previously, in a subgroup of the RIAD study population, we found a positive correlation between carotid IMT, an early surrogate marker of atherosclerosis, and postchallenge hyperglycemia under prediabetic conditions (6), suggesting that atherogenetic processes could already start in subjects with IGT.

The origin of oxLDL in circulation is yet unknown. Theoretically, oxLDL could be formed within the circulation enzymatically and/or by metal ion-catalyzed reactions (1). Alternatively, oxLDL could be formed in the subendothelial space of the vessel wall. During circulation, LDL particles enter and re-emerge from the subendothelium.

As estimated by stable isotope lipoprotein kinetic studies performed in normo- and hypercholesterolemic subjects, the in vivo retention time of LDL particles has been shown to be directly associated with the number of circulating oxLDL particles (16).

In the present study, we investigated whether oxLDL levels in the circulation were related to parameters of the oxidative/antioxidative balance in the blood. Due to their function in host defense, phagocytes are provided with a high capacity to enzymatically produce reactive oxygen species. Luminol-amplified CPOA, which characterizes mainly phagocyte myeloperoxidase activity (17), was significantly increased in diabetic patients but not in IGT subjects. While, to our knowledge, studies with IGT subjects were not previously performed, studies investigating the oxygenation activities of blood cells in diabetic patients showed inconsistent results. Using different methods to evaluate oxidant production either unchanged (18) or enhanced phagocyte activities have been found (19). In the present investigation, an association between CPOA and circulating oxLDL levels in the entire population has not been found, suggesting that circulating phagocytes are not responsible for intravascular LDL oxidation.

A significant counterpart of CPOA constitutes the complex antioxidative system in the blood (20). To characterize this system, we measured the TAC and the urate-to-allantoin ratio, respectively. The water-soluble antioxidant uric acid (urate at pH 7.4) is an important part of the antioxidative defense in human plasma. In our previous in vitro studies, urate has been shown to significantly inhibit lipoprotein oxidation (21). Allantoin is formed by nonenzymatic oxidation of urate. Thus, the urate-to-allantoin ratio is suggested to be a parameter of free radical reactions in vivo (22). In the present investigation, no differences in TAC and urate-to-allantoin ratio between NGT, IGT, and diabetic subjects could be found suggesting that essential parts of the antioxidative status did not differ among the groups.

In addition, concentration of circulating oxLDL may be modulated by HDL-associated enzymes. We measured the activity of serum paraoxonase activity with paraoxon as substrate (PON) that is able to protect LDL from oxidative modification by destroying oxidized phospholipids (23).

No differences in the PON activities among the three groups have been found. In other studies, serum PON activity was lower in diabetic than in control subjects, and associations between low PON activities and late complications have been observed (24,25). Possibly, the recruitment of diabetic patients (newly diagnosed diabetes in our study versus clinical diabetes in the others) accounts for differences in PON activities. In multivariate analysis, PON activity was the only parameter of the oxidative/antioxidative balance that was independently associated with oxLDL levels. However, PON was only a weak predictor of circulating oxLDL levels.

In the present study, univariate and multivariate analysis revealed concentrations of plasma triglycerides and LDL cholesterol to be the strongest predictors of circulating oxLDL levels. In this context, LDL cholesterol represents the LDL pool size and triglyceride represents the LDL particle size. Individuals with IGT and diabetes show an atherogenic lipoprotein phenotype that is characterized by high triglycerides, low HDL cholesterol and, in particular, a preponderance of small dense LDL particles (26). An association between small LDL particles and atherosclerotic disease has been found in several studies (27). In these subjects, the plasma triglyceride level is among the major determinants of LDL particle size. Insulin resistance is associated with a higher flux of free fatty acids from the splanchnic circulation to the liver, causing increased production of VLDL particles and hypertriglyceridemia, respectively (28). The concomitant reduction of lipoprotein lipase activity in the peripheral tissues and the liver as well as the action of the cholesterol ester transfer protein result in LDL particles enriched with triglycerides and depleted with cholesteryl esters, respectively. In consequence of this, LDL particles show a higher affinity for hepatic lipase. During hydrolysis of triglycerides, the LDL particles shrink and become more dense. The LDL pool size, compositional changes in LDL particles, and a decrease in LDL particle size favor the exposure of LDL to the subendothelium and oxidative modification of both lipids and apolipoproteins, respectively (29). Cholesterol-depleted small dense LDL particles have been shown to be more prone toward lipid peroxidation in vitro than buoyant LDL (30). In these

studies, only the amount of free cholesterol per particle was associated with LDL oxidizability (30). Recently, we could demonstrate that an entity of small, more dense, α-tocopherol–poor LDL particles (LDL_2, Svedberg units [S_f] 0–7) is particularly prone to oxidation in vivo and in vitro (16,31). The present data obtained from IGT and diabetic subjects are consistent with these former investigations and the model discussed above. Of note, the normoglycemic control subjects were selected dependent on their degree of glucose intolerance alone, not on their lipoprotein phenotype. Consequently, the NGT group comprised both normo- and hyperlipidemic subjects.

Although the oxLDL level only in IGT showed a statistically significant increment when compared with control subjects, our interpretation and conclusion of the present data are that the metabolic situation of IGT and newly diagnosed diabetes is associated with the diabetic dyslipidemia that particularly influences the level of circulating oxLDL.

In summary, elevated levels of circulating oxLDL have been found in subjects with IGT. The oxLDL levels were not, or only weakly, related to the oxidative/antioxidative balance in the blood. The close association between oxLDL levels and plasma LDL cholesterol and triglycerides, respectively, favors the hypothesis that dyslipidemia particularly promotes the oxidation of LDL in the subendothelium followed by re-entry of the modified lipoprotein into circulation.

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