

# Serum Oxidized Low Density Lipoprotein, Paraoxonase 1 and Lipid Peroxidation Levels during Oral Glucose Tolerance Test

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## Key words

- oxidized low density lipoprotein
- paraoxonase 1
- oral glucose tolerance test
- impaired glucose tolerance

## Abstract

Increasing evidence suggests that the postprandial state is a contributing factor to the development of atherosclerosis. To evaluate the effects of acute hyperglycemia on the oxidative stress, concentrations of serum-oxidized low density lipoprotein (oxLDL), paraoxonase 1 (PON1), and thiobarbituric acid reactive substances (TBARS) were measured in subjects with normal glucose tolerance (NGT) (n=35), impaired glucose tolerance (IGT) (n=25), and diabetic glucose tolerance (DGT) (n=20). In NGT group, the 2 hours' TBARS and oxLDL levels were not statistically different when compared to baseline, and 2 hours' PON1 activities were higher when compared to baseline (p<0.01). Subjects with IGT and DGT have higher 2 hours' serum TBARS and oxLDL levels than their baseline levels (p<0.01, for each). Baseline

oxLDL levels of both IGT and DGT groups were higher than NGT group (p<0.01 and p<0.01, respectively). While there were not any significant differences in 2 hours' versus baseline PON1 activities in the IGT group, the 2 hours' versus baseline PON1 activities in the DGT group were significantly lower (p<0.01). The postchallenge 2 hours' PON1 activities of both IGT and DGT groups were lower than NGT group (p<0.01 and p<0.01, respectively). Baseline oxLDL was positively correlated with 2 hours' glucose (r=0.613, p<0.01) in IGT and DGT groups. PON1 activities were correlated with HDL-cholesterol, total cholesterol, and fasting glucose (r=0.680, r=0.698 and r=0.431, respectively, for each p<0.01) in NGT. In conclusion, oxidative stress occurs at an early stage in diabetes, and protective effects of HDL against atherosclerosis may be dependent on the PON1 activities.

## Introduction

Diabetes is associated with accelerated atherosclerosis and subsequent cardiovascular disease [1]. The mechanisms underlying diabetes accelerated atherosclerosis are poorly understood. One of the proposed mechanisms, oxidative stress has been demonstrated to be increased *in vivo* in the diabetic state [2,3], which may contribute to the higher incidence of vascular disease in this population. Under oxidative stress, low density lipoprotein (LDL) and other serum lipoproteins including high density lipoprotein (HDL), are prone to lipid peroxidation [4]. Previous studies have shown that intra-arterial LDL oxidation is relevant to atherosclerosis [5,6] and oxidatively modified LDL (oxLDL) is believed to be one of the critical factors in atherogenesis [7]. The serum concentration of HDL has long been known to have an inverse correlation with the development of atherosclerosis [8–10]. Several

studies have reported that HDL had a protective effect against oxidative modification of LDL [11,12] and it has been previously shown that the antioxidant activity of HDL may relate, at least in part to the enzymes associated with HDL [13]. Among them, human serum paraoxonase (PON1) has raised special interest, which is believed to be important in the protection against LDL oxidation [14,15]. Studies have shown that serum PON1 activity is reduced in diabetic subjects [16,17]. Some of the studies reported so far lack either postchallenge plasma glucose or fasting plasma glucose in relation to atherosclerosis [18]. Taking into account the marked postprandial/postchallenge rise in blood glucose and its possible contribution to atherosclerosis due to oxidative stress, the aim of our study is to investigate the effects of glycemia on the serum oxLDL and PON1 concentrations at baseline and post challenge 2 hours' during oral glucose tolerance test (OGTT), and to see whether we can relate

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their contribution on other parameters such as thiobarbituric acid reactive substances (TBARS, as a lipid peroxidation marker) and plasma lipids related with diabetes and atherosclerosis.

## Materials and Methods

Subjects with normal (NGT), impaired (IGT), and diabetic (DGT) glucose tolerance participated in the study. The state of glycemia was classified according to the following criteria [19,20] after oral glucose tolerance test (OGTT) (75 g oral glucose challenge). NGT was defined when fasting serum glucose is <6.1 mmol/L and two-hours postchallenge serum glucose is <7.8 mmol/L (n=35). Subjects with fasting serum glucose below 7.0 mmol/L and two-hours postchallenge glucose levels between 7.8 and 11.1 mmol/L were identified as IGT (n=25) and subjects with postchallenge serum glucose  $\geq$ 11.1 mmol/L were identified as DGT (n=20). There were no specifics on evaluation of physical activity between groups. Exclusion criteria were the presence of hypertension, any cardiovascular complications or inflammatory diseases, smoking, exercise, and medications. All subjects showed no evidence of family history of diabetes. They were all on a weight maintaining diet containing at least 250g/day carbohydrates. All subjects gave their informed consent for the study. After a 12-hour fasting, subjects were challenged the equivalent of 75g anhydrous glucose dissolved in 250mL of water. Blood was drawn from an antecubital vein at baseline and two hours after glucose challenge. Blood samples were centrifuged within 20 minutes at 4°C. Serum was separated from cells immediately after centrifugation and stored at -80°C until analyzed. Litheparinized whole blood samples were used for HbA<sub>1c</sub> assay. Serum levels of total oxLDL particles were directly measured by a sandwich ELISA assay using the murine monoclonal antibody, m Ab-4E6 as capture antibody bound to microtitration wells and a peroxidase conjugated anti-apolipoprotein B antibody recognizing oxLDL bound to the solid phase (Mercadia Oxidized LDL ELISA, Uppsala, Sweden) [21]. The intraassay and interassay coefficients of variation were 5.1% and 9.3%, respectively. Data were expressed as units per liter (U/L).

Serum PON1 activity was measured using synthetic paraoxon (diethyl *p*-nitrophenyl phosphate) as substrate [22]. PON1 activity was determined by measuring the initial rate of substrate hydrolysis to *p*-nitrophenol, the absorbance of which was monitored at 412 nm in the assay mixture containing 20 mM paraoxon, 2 mM CaCl<sub>2</sub> and 20  $\mu$ L of plasma in 100 mM of Tris-HCl buffer (pH 8.0). The blank containing incubation mixture without serum was run simultaneously to correct for spontaneous substrate breakdown. Enzyme activity was calculated from E412 of *p*-nitrophenol (18290 M<sup>-1</sup> cm<sup>-1</sup>) and is expressed as units per liter. All chemicals were obtained from Sigma, St Louis, M.O. The intraassay and interassay coefficients of variation were 5.6% and 8.5%, respectively.

Lipid peroxidation was estimated by the modified thiobarbituric acid method described by Buege and Aust [23]. TBARS concentration was calculated using  $1.56 \times 10^{-5} \text{ M}^{-1} \text{ cm}^{-1}$  as moles per liter extinction coefficient. The intraassay and interassay coefficients of variation were 5.3% and 5.9%, respectively. The results were given as mmol/L.

Serum glucose, total cholesterol, triglyceride, HDL cholesterol, urea, creatinine were determined on a Hitachi Modular P analyzer using commercial kits (Roche Diagnostics, GmbH, Man-

**Table 1** Demographic details and biochemical characteristics of the subjects with normal (NGT), impaired (IGT) and diabetic (DGT) tolerance

	NGT subjects (n=35)	IGT subjects (n=25)	DGT subjects (n=20)
Age (years)	47.3 $\pm$ 13.74	50.3 $\pm$ 10.8	47.7 $\pm$ 9.3
Body Mass Index (kg/m <sup>2</sup> )	27.8 $\pm$ 5.3	27.1 $\pm$ 5.5	28.1 $\pm$ 5.4
Systolic blood pressure (mmHg)	95 $\pm$ 10	98 $\pm$ 9	99 $\pm$ 8
Diastolic blood pressure (mmHg)	75 $\pm$ 5	77 $\pm$ 7	77 $\pm$ 5
HbA <sub>1c</sub> (%)	5.24 $\pm$ 0.42	5.45 $\pm$ 0.34	5.78 $\pm$ 0.69
Urea (mmol/L)	5.0 $\pm$ 1.4	5.1 $\pm$ 0.9	6.4 $\pm$ 1.7
Creatinine ( $\mu$ mol/L)	72.3 $\pm$ 14.1	66.3 $\pm$ 8.8	69.8 $\pm$ 28.2
Total cholesterol (mmol/L)	5.12 $\pm$ 1.10	5.32 $\pm$ 1.03	5.38 $\pm$ 0.65
LDL cholesterol (mmol/L)	3.05 $\pm$ 0.82	3.21 $\pm$ 0.86	3.49 $\pm$ 0.71
HDL cholesterol (mmol/L)	1.43 $\pm$ 0.29	1.35 $\pm$ 0.35	1.22 $\pm$ 0.28
Triglycerides (mmol/L)	1.24 $\pm$ 0.40	1.25 $\pm$ 0.46	1.19 $\pm$ 0.52
Fasting glucose (mmol/L)	4.78 $\pm$ 0.85	5.07 $\pm$ 0.74	5.02 $\pm$ 0.51
Fasting insulin ( $\mu$ U/mL)	9.8 $\pm$ 4.9	10.2 $\pm$ 4.9	11.2 $\pm$ 2.3

Values are means  $\pm$  SD

nheim). LDL cholesterol was calculated using the Friedewald's formula if the triglycerides were less than 4.5 mmol/L.

HbA<sub>1c</sub> determination is based on the turbidimetric inhibition immunoassay (TINIA) for hemolyzed whole blood on a Hitachi Modular P analyzer (Roche Diagnostics GmbH, Mannheim). Serum insulin levels were determined by solid-phase-two-site chemiluminescent immunometric assay (Immulite, Euro/DPC Ltd.).

## Statistical Analysis

All data are expressed as means  $\pm$  SD. The baseline levels of the groups were compared with unpaired student's *t*-tests, ANOVA, and Tukey HSD. The comparison of postchallenge 2 hours' levels with baseline was performed by paired student's *t*-tests. Spearman's rank correlation coefficients (*p*) were used to express the relation between PON1 activities and oxLDL levels and lipids, as well as glucose. Statistical significance was defined as *p* < 0.05.

## Results

Demographic details and basic biochemical characteristics of the subjects with NGT, IGT and DGT groups are given in **Table 1**. There were no significant differences between the groups in terms of age, BMI, blood pressure. Serum total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, glucose, insulin, and HbA<sub>1c</sub> did not differ significantly between the groups at baseline.

**Table 2** and **Fig. 1** show baseline and postchallenge 2 hours' serum TBARS, oxLDL, PON1 and glucose levels in NGT, IGT and DGT groups. As expected, 2 hours' serum glucose levels were found to be higher than baseline in all three groups (*p* < 0.01). OxLDL levels at baseline were higher in DGT than in IGT and NGT (*p* < 0.05 and *p* < 0.001, respectively). Patients with IGT have also

**Table 2** Baseline and postchallenge 2 hours serum thiobarbituric acid reactive substances (TBARS), oxidized low density lipoprotein (oxLDL), paraoxonase 1 (PON1) and glucose levels in normal (NGT), impaired (IGT) and diabetic (DGT) groups

	Parameters	Baseline	2 hours
NGT (n = 35)	TBARS (nmol/mL)	4.61 ± 0.51	4.72 ± 0.60
	oxLDL (U/L)	40.3 ± 7.1	43.3 ± 5.5
	PON1 (U/L)	53.8 ± 14.5	72.4 ± 15.8 <sup>a</sup>
	Glucose (mmol/L)	4.78 ± 0.85	5.50 ± 1.34 <sup>a</sup>
IGT (n = 25)	TBARS (nmol/mL)	4.82 ± 0.47	5.25 ± 0.55 <sup>a</sup>
	oxLDL (U/L)	45.5 ± 5.2 <sup>b</sup>	57.3 ± 7.5 <sup>a,b</sup>
	PON1 (U/L)	55.9 ± 17.2	53.5 ± 15.3 <sup>b</sup>
	Glucose (mmol/L)	4.87 ± 0.74	8.80 ± 0.98 <sup>a,b</sup>
DGT (n = 20)	TBARS (nmol/mL)	4.78 ± 0.65	5.55 ± 0.70 <sup>a</sup>
	oxLDL (U/L)	63.3 ± 6.3 <sup>b,c</sup>	85.5 ± 10.5 <sup>a,b,c</sup>
	PON1 (U/L)	54.2 ± 14.6	40.3 ± 15.7 <sup>a,b,c</sup>
	Glucose (mmol/L)	5.02 ± 0.51	12.13 ± 1.13 <sup>a,b,c</sup>

Values are means ± SD

<sup>a</sup>Comparison with baseline levels

<sup>b</sup>Comparison with NGT

<sup>c</sup>Comparison with IGT

p < 0.05, statistical significance

higher baseline oxLDL levels than NGT ( $p < 0.01$ ). The 2 hours' oxLDL and TBARS in the DGT and IGT groups were found to be higher in comparison to NGT group ( $p < 0.01$ , for each comparison). Subjects with DGT also have lower 2 hours' PON1 activities than with both IGT and NGT ( $p < 0.01$  and  $p < 0.01$ , respectively) and 2 hours' PON1 activities in IGT were significantly lower than in NGT ( $p < 0.01$ ).

The differences between 2 hours' and baseline TBARS and oxLDL levels were not found to be statistically significant in NGT group. In this group however, 2 hours' PON1 activity was found to be increased than its baseline levels ( $p < 0.01$ ).

As compared with baseline, both IGT and DGT subjects had higher 2 hours' TBARS and oxLDL concentrations ( $p < 0.01$ , for each comparison). While there were not any significant differences in 2 hours' versus baseline PON1 activities in the IGT group, the 2 hours' versus baseline PON1 activities in the DGT group were significantly lower ( $p < 0.05$ ). Although there were no significant differences at the baseline, the postchallenge 2 hours' PON1 activities of both IGT and DGT groups were found to be lower than NGT group ( $p < 0.01$ , for each comparison). The decrease is 36% in IGT and 45% in DGT group.

A significant positive correlation was found between baseline oxLDL and 2 hours' glucose in IGT and DGT groups ( $r = 0.613$ ,  $p < 0.01$ ), however, there was no significant correlation among lipid parameters, PON1 and oxLDL in these groups. In NGT group, baseline PON1 activities were significantly correlated with HDL-cholesterol, total cholesterol and fasting glucose levels ( $r = 0.680$ ,  $r = 0.698$  and  $r = 0.431$ , respectively; for each  $p < 0.01$ ).

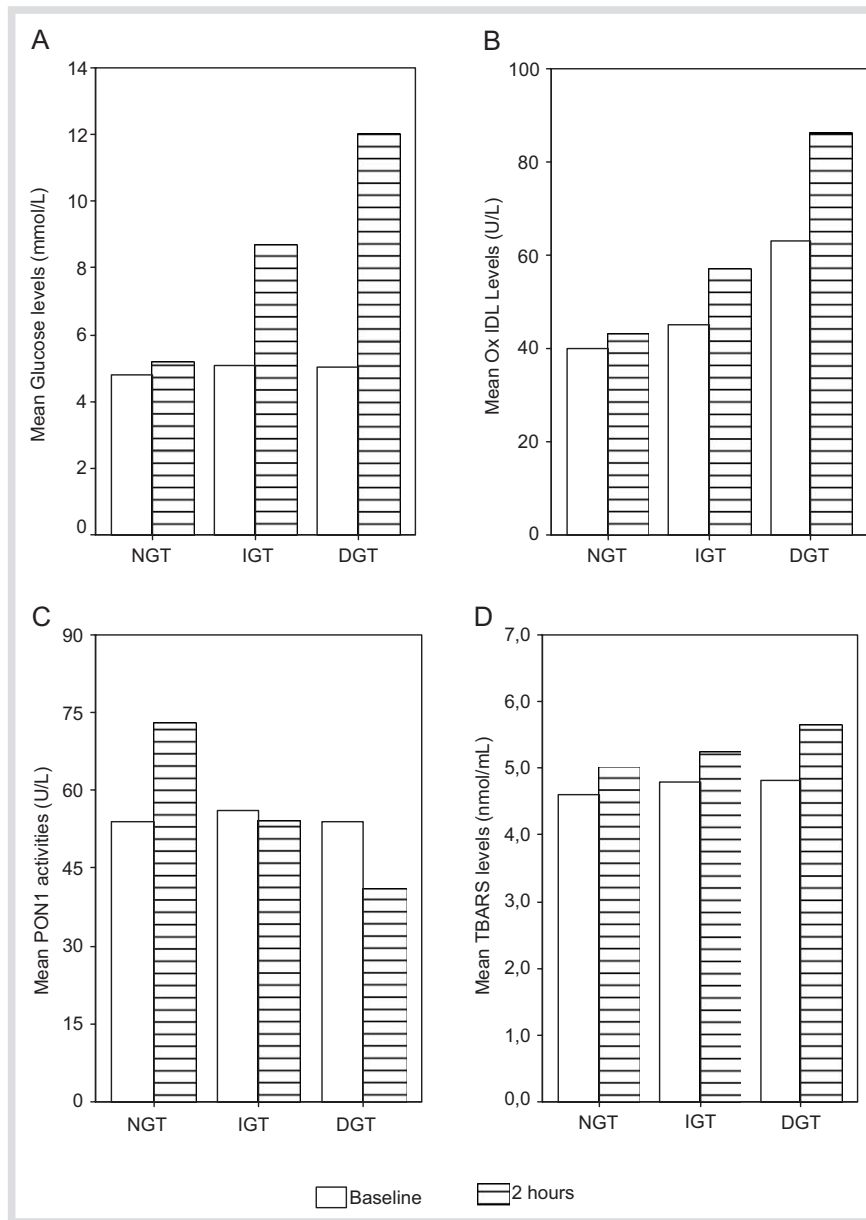
## Discussion

Although many studies indicate the importance of postprandial glucose levels there is not much data [24] comparing plasma oxidizability and its possible contribution to atherosclerosis among persons with NGT, IGT, and DGT. Furthermore, it has not been shown whether postchallenge glycemic status during OGTT influences LDL oxidizability *in vivo*.

In our study, postchallenge 2 hours' serum TBARS and oxLDL levels of the IGT and DGT groups were found to be higher than their baseline levels. Postchallenge oxLDL increased ~20 U/L in diabetes and ~10 U/L in IGT, but only ~3 U/L in NGT. In addition, baseline oxLDL levels of both IGT and DGT groups were higher than NGT group. Previous studies have shown that LDL isolated from the plasma of patients with diabetes and coronary heart disease were more susceptible to oxidation *in vitro* than LDL from normal subjects [25,26]. However in their work, Schwenke et al. [27] have found conflicting results with the above studies. They studied *in vitro* LDL oxidizability and found no evidence for increased LDL oxidizability for subjects with known diabetes. But LDL oxidizability of newly diagnosed diabetics in their study appeared to be higher. Our results of the newly diagnosed diabetics were consistent with their results. Kopprasch et al. [28] have shown that oxLDL levels were significantly higher in IGT subjects when compared to NGT subjects, but the increase in oxLDL levels in their diabetic group when compared to NGT group was not statistically significant. They have interpreted their data based on diabetic dyslipidemia rather than oxidative/antioxidative balance that particularly influences the level of circulating oxLDL. Our data about lipid parameters on both groups have not confirmed their interpretation about dyslipidemia. But more recently, Kopprasch et al. [29] have demonstrated that total cholesterol and LDL cholesterol did not differ significantly in their NGT, IGT and DM groups. In this study, they confirmed their previous finding [28] of increased levels of circulating oxLDL in IGT subjects, and far from their previous interpretation they have demonstrated significantly elevated oxLDL levels in newly diagnosed DM patients as we have shown in our study.

At issue is whether oxidative stress occurs at an early stage in diabetes, preceding the appearance of complications, or whether it is the result of prior tissue damage. Considering the postchallenge 2 hours' status of our parameters, this question may be answered as oxidative stress occurring as a consequence of acute hyperglycemia at an early stage in diabetes, even at the stage of impaired glucose tolerance. In the present study we have also demonstrated that 2 hours' serum TBARS and oxLDL levels were not statistically different in NGT group when compared with baseline. However, the 2 hours' PON1 activities were significantly higher than baseline, in this group. The second finding about PON1 activities was that they were not statistically different at baseline in all three groups.

We assume that NGT subjects have not been susceptible to hyperglycemia related oxidative stress and as their HDL cholesterol levels were slightly higher than the IGT and DGT groups, their higher PON1 activities may efficiently protect them from possible oxidative susceptibility. In fact postchallenge PON1 increased ~20 U/L in NGT but remained nearly the same in IGT and decreased to ~14 U/L in DGT. In our study, PON1 activities were significantly correlated with HDL-cholesterol, total cholesterol and fasting glucose in NGT group. Only small increases in HDL concentrations have been shown to greatly reduce atherogenicity and this effect has been confirmed to be related to increased PON1 activities [29]. Mackness et al. [30,31] have reported that serum PON1 activity was low in insulin dependent diabetics and this group extended their finding to noninsulin dependent diabetics. Our baseline PON1 levels which were found to be statistically indifferent from each other in all three groups were not consistent with the above studies. However, the lower postchallenge 2 hours' levels both in IGT when compared



**Fig. 1** Means of Glucose (1-A), Oxidized LDL (1-B), PON1 (1-C), and TBARS (1-D) in subjects with normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and diabetic glucose tolerance (DGT) at baseline and 2 hours after glucose loading.

to NGT and in DGT when compared to both NGT and IGT could reasonably be explained on the basis of acute hyperglycemia and related oxidative stress again. The elevated postchallenge 2 hours' levels of TBARS and oxLDL in these groups may be explained as a confirmatory basis. Nevertheless the mechanism through which PON1 protects against oxidative damage and consequent development of atherosclerosis is not entirely clear. Many studies have suggested that PON1 degrades proinflammatory native and oxidized phospholipids, including platelet-activating factor and thus protects plasma lipoproteins and plasma membranes from reactive oxygen species-mediated damage [32,33].

As a result, the protective effects of HDL against atherosclerosis may not just be dependent on the levels of HDL cholesterol in the blood but rather on the PON1 levels relative to the concentration of oxLDL. This effect is determined by both genetic and environmental factors and one of the most important factors is diabetes, which increases oxidative stress at even impaired glucose tolerance state [34]. However, further studies are needed to

elucidate the conflicting data on oxLDL and PON1 levels of the subjects with IGT and newly diagnosed diabetes mellitus.

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