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Implications of N^{ε}-(Carboxymethyl)lysine in Altered Metabolism of Low Density Lipoproteins in Patients with Type 2 Diabetes and Coronary Artery Disease

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The aim of this study is to evaluate the role of the glycoxidation product, N^{ε}-(carboxymethyl)lysine (CML), on the metabolism of LDL which can give rise to increased risks of Coronary Artery Disease (CAD) in Type 2 Diabetes Mellitus (T2DM) patients. Polyclonal anti-CML antibodies, were used for measurement of serum CML. CML concentrations were measured by competitive enzyme-linked immunosorbent assay (ELISA) in the serum of 81 T2DM patients with CAD, 86 T2DM without CAD and 104 healthy subjects. LDLs from the study groups were isolated and labeled with the fluorescence dye, 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindo carbocyanine perchlorate (DiI). LDL from healthy subjects was subjected to oxidation and glycation. Receptor-mediated uptake of labeled native and modified-LDL was studied in human hepatocellular carcinoma (HepG2) cells. CML concentrations were significantly higher in T2DM with CAD patients than diabetic patients but without CAD (529.5±83.6 vs. 425.1±64.4 ng mL^{-1}, $p<0.001$). The uptake of DiI-LDL from type 2 diabetes patients with CAD was significantly lower than that for LDL from diabetic patients without CAD or glycated-LDL in HepG2 cells. This study demonstrated that CML may be largely responsible for the defective uptake of LDL of T2DM with CAD. Thus, CML may be used as endogenous biomarker for early detection of CAD in type 2 diabetes patients and may be considered as a new goal for the glycemic control in those patients.

Key words: N^{ε}-(Carboxymethyl)lysine, glycoxidation, type 2 diabetes mellitus, LDL metabolism, coronary artery disease

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INTRODUCTION

Coronary Artery Disease (CAD) is the major cause of mortality and morbidity in the industrialised world (Braunwald, 1997). The risk of Coronary Heart Disease (CHD) is up to four times higher in patients with diabetes in comparison to non-diabetic subjects (Haffner *et al*., 1998). In type 2 diabetes, lipid abnormalities are almost the rule. Therefore, T2DM has been defined as a coronary artery disease risk equivalent by the Adult Treatment Panel III (ATPIII) of the National Cholesterol Education Program (NCEP) (2001). Diabetes can lead to cardiovascular damage through a number of mechanisms, each of which in turn may accelerate or worsen the others. Many studies have indicated that hyperglycemia is an independent predictor for risk of Cardiovascular Disease (CVD) in T2DM (Kuusisto *et al*., 1994; Laakso, 1999). Potential mechanisms of how hyperglycaemia may induce vascular injury include an increased production of advanced glyccation end-products (AGEs) and excessive oxidative stress (Creager *et al*., 2003). It has been reported that AGE levels were increased in type 2 diabetic patients with coronary artery disease (Kiuchi *et al*., 2001) and microvascular complications (Sampathkumar *et al*., 2005).

Schleicher *et al*. (1981) found a two-fold increase in glycation of Apo-B in LDL from diabetic human subjects and were the first to suggest that increased glycation of lipoproteins *in vivo* might have significant metabolic consequences. It was hypothesized that the covalent bonding between lipid and glucose can play a role in *in vivo* LDL oxidation (Bucala *et al*., 1994). Not only was glycated LDL more susceptible to oxidation but when oxidized it has more potent atherogenic properties as suggested by Galle *et al*. (1998). N^{ε}-(carboxymethyl)lysine, a well characterized glycoxidation product formed during *in vitro* glycation and lipid peroxidation (Baynes and Thorpe, 2000) is the major immunological product determined on glycated proteins (Ikeda *et al*., 1996). In addition, CML has been found in atheromas by immunohistochemistry and chemical analysis (Schleicher *et al*., 1997) and has been identified in glucose-modified LDL and in macrophage foam cells of atherosclerotic plaques (Imanaga *et al*., 2000; Sakata *et al*., 2001). Serum CML levels have been found to be elevated in Type 1 Diabetes Mellitus (T1DM) patients (Berg *et al*., 1997). The significant association of non-CML AGEs levels with the severity of diabetic nephropathy and retinopathy, suggesting a role of non-CML AGE in the progression of microvascular complications in patients with T1DM. They also showed that CML may play a role in the initiation of diabetic retinopathy (Miura *et al*., 2003).

This study focuses on non-enzymatic modifications of LDL including oxidation and glycation. It has been postulated that the uptake of LDL particles by LDL receptors is impaired in diabetes patients with coronary atherosclerosis, thereby decreasing its clearance from the blood circulation and increasing serum LDL concentrations. Alternatively, the uptake of these modified LDL particles by scavenger receptors on macrophages and vascular smooth muscle cells and by AGE receptors (RAGE) on endothelial cells, vascular smooth muscle cells and monocytes is enhanced and this, in turn, is centrally positioned to contribute to the pathogenesis of diabetic vascular complications. The purpose of this study was to evaluate the serum concentrations of the glycoxidation product, N^{ε}-(carboxymethyl)lysine and its influence on the metabolism of LDL and increased risks of CAD in T2DM patients. Moreover, the effect of CML contents of LDL in altering the recognition of LDL by its receptor was studied. The above effects on LDL uptake will be compared with those of Ox-LDL, glycated-LDL and LDL from T2DM patients with and without CAD.

MATERIALS AND METHODS

Study groups and events definitions: A total of 167 patients with type 2 diabetes were selected from patients who regularly attend the Cardiology and Endocrinology Clinics at the University of Malaya Medical Centre. Of these, eighty one patients suffered from Coronary Artery Disease (CAD) (mean age 46.8±14.6 years), whilst eighty six are without CAD (mean age 45.4±17.0 years). T2DM was considered to be present if the fasting blood glucose was $\geq$7.0 mmol L^{-1} and the 2 h blood glucose in the oral glucose tolerance test (OGTT) was $\geq$11.1 mmol L^{-1} (WHO, 1999).

The procedures used for evaluation of coronary artery disease were adapted from those previously described by Lamarche *et al*. (1995). The diagnosis of CAD was made clinically on the basis of a history of Myocardial Infarction (MI) and/or from a review of resting electrocardiogram (ECG) with Q wave changes suggestive of MI, coronary artery bypass grafting, positive exercise-tolerance test, or coronary angiography (cardiac catheterization). However, the selection of the appropriate test depends on the symptoms exhibited by the patient. T2DM patients without CAD were required to have normal ECG and no history or symptoms of CAD (i.e., history of myocardial infarction or angina pectoris) exhibited by the patient.

All diabetic patients were under dietary therapy and/or prescribed oral hypoglycemic agents such as

sulphonylureas and/or metformin. Subjects of age ≥70 years, those with known hepatic diseases, with peripheral vascular disease as determined by ankle-brachial index, or under antioxidants medications and/or insulin therapy were excluded from this study. Smokers and former smokers were also excluded and none of the patients had retinopathy, nephropathy, neuropathy, or erectile dysfunction. As a control, 104 age and sex matched healthy subjects (mean age 43.5±13.3 years) were recruited from Klang Valley, government bodies such as Police Force and private centers in Kuala Lumpur. Information was acquired through the distribution of questionnaires. Healthy control subjects were free from any vascular diseases or an increase in glucose concentrations or both. The exclusion criteria for patients were also applied for control group.

Blood samples were taken in the morning between 8:00 and 9:00 am after an overnight (12-14 h) fast. Written informed consent was obtained from all patients and control subjects prior to their inclusion in the study. The study was performed in accordance to the approval given by the Faculty Perubatan University of Malaya Medical Ethics Committee (FPUM MEC). The experimental protocols for anti-sera preparation were approved by the local Ethical Committee for Animal Experimentation in the Faculty of Medicine, University of Malaya.

CML and malondialdehyde measurements: Polyclonal anti-CML antibodies were developed in female New Zealand white rabbit according to the method described by Ikeda *et al.* (1996) and these antibodies were used for measurement of serum CML by competitive enzyme-linked immunosorbent assay (ELISA) in duplicates using CML-Bovine Serum Albumin (CML-BSA) as an adsorbed ligand antigen. The amount of plasma malondialdehyde (MDA), an index of lipid peroxidation, was determined by the thiobarbituric acid reactive substances (TBARS) method as previously described by Ohkawa *et al.* (1979).

LDL isolation and modification: EDTA-containing plasma samples were recovered and pooled into 26 separate pools (nine from type 2 diabetic patients with CAD, eight from type 2 diabetic patients without CAD and nine from healthy subjects). The pooled plasma samples were then used to isolate LDL, with each sample representing six or seven patients. LDL isolation, modification and cell culture procedures described in the next subsections were done with LDL prepared from aliquots of these plasma pools. LDL was isolated as previously described by Chung *et al.* (1986). Briefly, plasma (3 mL) from each pool was adjusted to a density of 1.21 g mL^{-1} by addition of 0.981 g of KBr. Then, isotonic saline (7 mL) with a density of 1.006 g mL^{-1} containing 1 mM EDTA was layered on top of the plasma in a Quick-seal polyallomer tubes (Sorvall instruments, Thermo Electron Corp., USA). LDL was isolated by density gradient ultracentrifugation at 4°C and 65000 rpm (397000 x g) for 3 h using a fixed-angle Sorvall T-880 rotor in a Sorvall ULTRA PRO® 80 Ultracentrifuge (Sorvall instruments, Thermo Electron Corp., USA). The LDL band was then carefully removed by tube slicing and dialyzed in the dark at 4°C against 150 mM NaCl solution supplemented with 0.26 mM EDTA (pH 7.4). The LDL-containing solutions were sterilized by filtration using 0.22 μm filter units. Afterwards, protein concentrations of isolated LDL were measured by the Bradford (1976) method and used in the subsequent experiments.

Copper sulfate was used as a catalyst for the oxidation based on the previously described method (Esterbauer *et al.*, 1989). AGE-LDL was prepared by a modification of the method described by Schmidt *et al.* (1993). Briefly, 1 mL native LDL solution (0.75 mg mL^{-1}) in PBS containing 0.01% EDTA was incubated with 100 mM butylated hydroxytoluene and glucose (0.2 M) in the presence of 200 mM $NaBH_3CN$ for 24 h at 37°C. All LDL preparations were extensively dialyzed against 1 L of 0.02 M phosphate-buffered saline (pH 7.4) to remove free glucose at 4°C with stirring for 48 h with 3 buffer changes at convenient intervals. At the end of the glycation process, the preparations were filter sterilized by passing through a 0.45 μm filter and protein concentrations were measured.

Measurement of LDL modification: Electrophoretic mobility of control and modified LDL was assessed by 1% agarose gel electrophoresis at pH 8.6 in sodium barbital buffer and LDL was visualized with Oil Red O staining (Noble, 1968). Relative Electrophoretic Mobility (REM) was calculated as the ratio of migration of modified LDL compared with control LDL. The degree of oxidative modification of LDL was estimated by measuring thiobarbiruric acid reactive substances (TBARS) (Ohkawa *et al.*, 1979).

Susceptibility of LDL to oxidation: Copper-catalyzed oxidation of LDL was performed as the formation of conjugated dienes (Esterbauer *et al.*, 1989). Briefly, 5.0 μM $CuSO_4$ was added to 1 mL LDL (200 μg mL^{-1}) followed by incubation at 37°C. Absorbance was read at 234 nm in Cary 50 UV-Vis spectrophotometer (Varian, Inc., USA) every 5 min for a maximum of 3 h or until the rapid phase of LDL oxidation reached a plateau. The lag phase, a

measure of the time during which LDL is protected from oxidation by antioxidants present in the LDL and the propagation phase, a measure of LDL oxidation, were determined and expressed in minutes from the addition of $CuSO_4$.

Uptake of DiI-labeled LDL: Native-LDL, Ox-LDL and AGE-LDL were labeled with the fluorescent lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo carbocyanine perchlorate (DiI) as described by Stephan and Yurachek (1993). Excess DiI was removed by dialysis in the dark at 4°C against 150 mM NaCl solution containing 0.26 mM EDTA (pH 7.4). Fluorescently labeled LDL (DiI-LDL) particles were passed through 0.22 μm filter units before using in the cell culture procedures. Human hepatocellular carcinoma (HepG2) cells were seeded on coverslips in 6-well plates at a density of 6×10^5 cells per well in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin). Cells were then incubated with 5% CO_2 in air in a humidified atmosphere at 37°C. After 2 days of cell culture, confluence was reached. On the day of the experiment, the medium was removed and cells were washed twice with serum-free DMEM. Thereafter, 500 μL serum-free DMEM containing 20 μg DiI-LDL was added to the cells and incubation was carried out for 2 h at 37°C. After incubation, the cells were rinsed three times in PBS, fixed in 5% formalin in PBS and mounted in PBS/glycerol. For an evaluation of cellular uptake, the cells monolayers were photographed under an Olympus BX-50/BX-FLA fluorescent microscope (Tokyo, Japan) equipped with a U-MNG filter set (excitation 530-550 nm, emission 590 nm) for visualization of the red fluorescence of DiI. For quantitative measurements of DiI-LDL preparations, cells seeded as above were incubated with DiI-LDL at 20 μg mL^{-1} serum-free DMEM at 4°C (binding studies) and 37°C (uptake studies) for 2 h (Teupser *et al*., 1996). Cells were washed three times with PBS and lysed by lysis buffer (1 g L^{-1} SDS in 0.1 M NaOH) for measuring the total amount of proteins in cultured cells using Bradford method (1976) and the relative binding and uptake of DiI-LDL into cells were measured on a fraction of the lysate with a Cary Eclipse microplate fluorescence reader (Varian, Inc., USA) with emission and excitation wavelengths set at 548 and 574 nm, respectively. All incubations were performed in duplicates. Standard solutions of DiI were prepared in isopropanol with a concentration range of 20 to 250 ng mL^{-1}. Inhibition studies were carried out by incubation of cells with 20 μg of DiI-LDL in the presence of 50-fold excess of unlabeled LDL.

Other assays: Glucose, HDL cholesterol, triglyceride and total cholesterol concentrations were measured by enzymatic methods using Dimension Clinical Chemistry System (Dade Behring Inc., Newark, USA). LDL cholesterol concentrations were calculated using Friedewald's formula (Friedewald *et al*., 1972). Percent HbA_{1c} was determined by COBAS INTEGRA systems (Roche diagnostics, F. Hoffmann-La Roche Ltd., Basel, Switzerland) according to the manufacturer's instructions.

Statistical analysis: Data are expressed as Mean±Standard Deviation (SD). Chi-square (χ^2) test was applied for the analysis of categorical variables. Analysis of variance (ANOVA) was used to examine the differences among groups of the study followed by Bonferroni's Post-Hoc test to adjust the multiple comparisons. Differences in the medications administered by the two diabetic groups were examined using independent sample t-test. CML was correlated with CAD classical risk factors using univariate and multivariate correlation analysis. Logistic regression analysis was used to determine the magnitude of associations between CML and presence of CAD. For plasma pools comparisons, Kruskal-Wallis analysis was used and if the results showed that $p<0.05$, the Mann-Whitney U-test was used to adjust the multiple comparisons. The mean difference was judged to be significant if $p<0.05$. Statistical computations were calculated using SPSS 11.5 for Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics of study populations: Table 1 revealed the medications administered during the follow-up in both T2DM groups. There was no significant difference in the prevalence of each of the drugs used between diabetic patients with and those without coronary artery disease during the follow-up period. The clinical parameters of the two T2DM groups and healthy subjects group were shown in Table 2. Patients with

Table 1: Comparison of drugs received during the follow-up period in T2DM groups

Drugs	T2DM with CAD (N = 81)	T2DM without CAD (N = 86)	p-value*
Oral hypoglycemic agents	70 (86)	68 (79)	NS
ACE inhibitors	46 (57)	56 (65)	NS
Beta-blockers	40 (49)	35 (41)	NS
Insulin therapy	13 (16)	21 (24)	NS
Calcium channels blockers	56 (69)	67 (78)	NS
Statins	54 (67)	49 (57)	NS
Fibrates	4 (5)	3 (3)	NS
Aspirin	6 (7)	8 (9)	NS

Independent sample t-test was used for statistical analysis, values are presented as the number (%) of patients. NS: The difference is not significant

Table 2: Clinical and laboratory characteristics of the study populations

Characteristics	Healthy subjects	T2DM with CAD	T2DM without CAD	p-value*
Gender (Male/female)	104.00 (46/58)	81.00 (42/39)	86.00 (41/45)	NS
Age (years)	43.50±13.3	46.80±14.6	45.40±17.0	NS
Systolic blood pressure (mmHg)	113.00±19.8	119.00±11.6	116.30±12.7	0.03
Diastolic blood pressure (mmHg)	74.30±9.40	75.40±6.30	74.80±5.00	NS
Fasting blood glucose (mmol L^{-1})	5.08±0.88	6.05±0.99	5.76±1.08	<0.001
HbA_{1c} (%)	5.74±0.80	6.18±1.01	5.93±0.79	0.003
CML (ng mL^{-1})	407.80±77.2	529.50±83.6***	425.10±64.4	<0.001
MDA (µmol L^{-1})	1.94±0.58	3.02±0.93***	1.81±0.52	<0.001
Triglyceride (mmol L^{-1})	1.32±0.77	1.30±0.47	1.18±0.42	NS
Total cholesterol (mmol L^{-1})	5.34±0.90	4.76±1.03	4.92±0.88	<0.001
HDL-cholesterol (mmol L^{-1})	1.20±0.38	0.99±0.36	1.01±0.32	<0.001
LDL-cholesterol (mmol L^{-1})	2.94±0.83	3.29±0.92	3.41±0.87	0.001
Diabetes duration (years)	-	8.20±3.70	8.60±4.00	NS

ANOVA and Bonferroni-corrected repeated pair comparisons test were applied for statistical analysis, Values are Mean±SD. *Significantly different between groups if $p<0.05$. NS: The difference is not significant, ***$p<0.001$ vs. Healthy subjects and T2DM patients without CAD

Table 3: Multivariate correlation between CML and other risk factors of CAD in T2DM

Factors	β	p-value***
Fasting blood glucose	0.295	0.001
HDL-cholesterol	-0.240	0.010
LDL-cholesterol	0.165	0.070
HbA_{1c}	0.200	0.032
MDA	0.382	<0.001

β: Indicates partial regression coefficient, ***The difference is significant if $p<0.05$

Table 4: Logistic regression analysis of risk factors for coronary artery disease in T2DM patients

Factors	Odds ratio	CI 95%	p-value	Wald
Fasting blood glucose	1.10	0.78-1.50	0.59	0.29
LDL-cholesterol	0.75	0.50-1.13	0.16	1.94
HbA_{1c}	1.09	0.71-1.66	0.71	0.14
MDA	1.92	1.20-3.07	0.006	7.47
CML	1.01	1.01-1.02	<0.001	15.23

T2DM and CAD had significantly higher levels of CML ($p<0.001$) and MDA ($p<0.001$) than T2DM group without CAD. However, the difference in the fasting blood glucose, lipid profile and HbA_{1c} was not significant in both diabetic patients groups. There was no statistical significant difference in the durations of diabetes between type 2 diabetic patients with and without CAD groups, 8.2±3.7 vs 8.6±4.0 years, respectively.

Correlation between CML and other clinical parameters in T2DM groups: Both univariate (data not shown) and multivariate correlations between circulating CML and CAD risk factors were studied for diabetes patients with CAD. In multivariate analysis, the strongest association of CML was observed with MDA concentrations ($\beta = 0.382$, $p<0.001$) as shown in Table 3. Logistic regression analysis was conducted in the diabetic groups using CAD as the dependent variable and the risk factors, which had a significant association with CAD in the multivariate analysis as the independent variables. As shown in Table 4, only CML (Odds Ratio (OR): 1.01, 95% Confidence Interval (CI): 1.01-1.02, $p<0.001$) and MDA (OR: 1.92, 95% CI: 1.20-3.07, $p = 0.006$) had a significant association with CAD. However, the most significant association, even after adjustment for confounding factors, was shown by circulating CML concentrations with a Wald statistic of 15.23. In the diabetic populations with the absence of CAD complications, however, univariate correlation analysis demonstrated that only fasting blood glucose ($r = 0.23$, $p = 0.037$) and HbA_{1c} ($r = 0.26$, $p = 0.017$) were significantly associated with the concentrations of circulating CML.

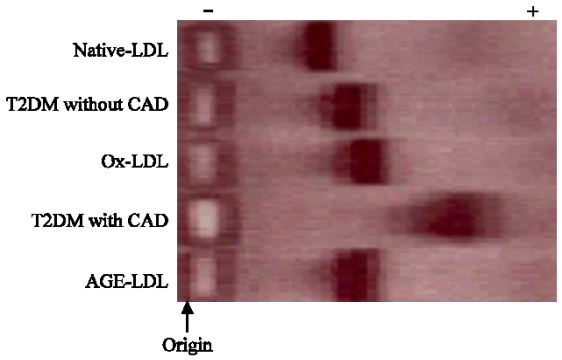

Fig. 1: Agarose gel electrophoresis of LDL preparations. Native LDL, Ox-LDL, AGE-LDL and LDL particles from both diabetes groups were subjected to 1% agarose gel electrophoresis and stained with Oil Red O

Degree of LDL modification: Figure 1 showed that electrophoretic mobilities of modified LDL preparations toward the anode were higher than those of unmodified LDL, indicating that modification had occurred. Thus, it is apparent that modification of LDL by these different ways was associated with an increase in the net negative charge with different degrees. AGE-LDL had lower effect on electrophoretic mobility than copper ion-induced LDL oxidation and this can be explained by the previous investigation (Posch *et al.*, 1999) that the *in vitro*

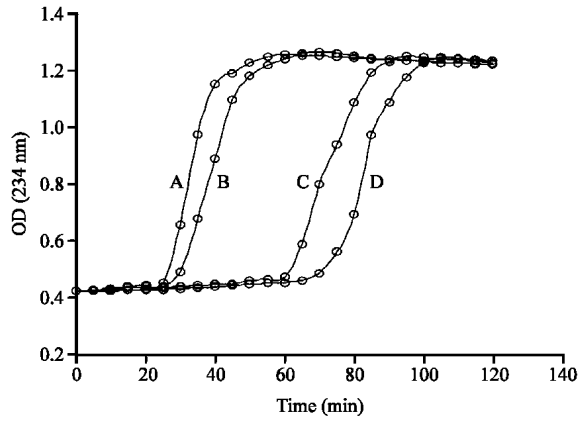

Fig. 2: Representative curves showing the kinetics of copper-mediated oxidation of AGE-LDL from healthy subjects (A), T2DM patients with CAD (B), T2DM patients without CAD (C) and native-LDL (D)

glycation procedures did not lead to oxidative modifications of LDL. In addition, the LDL preparations from T2DM without CAD revealed a slight increase in the electrophoretic mobility on agarose gel compared to control at baseline. However, relative electrophoretic mobility was significantly enhanced in LDL particles from T2DM with CAD patients compared to T2DM patients lacking CAD (Fig. 1). In addition, *in vitro* modified-LDL showed higher concentrations of MDA than native LDL with highest amounts in Ox-LDL (Table 5). MDA contents of LDL molecules from both diabetic patients were also shown in Table 5.

Susceptibility of LDL to oxidation: The susceptibility of LDL, obtained from the three populations of the study, to *in vitro* copper-catalyzed oxidation was compared (Fig. 2). The lag time to oxidation in type 2 diabetics with CAD was significantly shorter than that in type 2 diabetic patients without CAD or control subjects ($p<0.005$). There was no significant difference in the lag time in LDL isolated from diabetic patients without CAD and control subjects, 79.7±1.8 vs 83.2±3.23 min, respectively. Although the lag time was shorter in AGE-LDL (52.0±2.3 min) than LDL from diabetics with CAD (54.7±3.3 min), the difference was not significant. Furthermore, the time to achieve maximum rate of propagation of the copper-mediated oxidation of LDL was shorter in LDL of T2DM with CAD than LDL obtained from diabetic patients without CAD (V_{max}, 54.1±2.2 vs 92.4±2.6 min, $p<0.003$), indicating an increased susceptibility to copper-mediated oxidation of LDL in diabetes patients with CAD.

Table 5: Characteristics of LDL from diabetic patients and LDL modifications from healthy subjects

Characteristics	TBARS (nmol MDA mg^{-1} protein)	Relative Electrophoretic Mobility (REM)
Native-LDL	1.14±0.18	1.00
Ox-LDL	10.36±1.03*	1.75
AGE-LDL	8.51±0.69	1.53
T2DM with CAD	15.63±1.86**	2.35
T2DM without CAD	6.72±1.31	1.42

TBARS values are means±SD (n = 8), Mann-Whitney U-test was used for comparison of numerical variables, *$p<0.05$ vs AGE-LDL and LDL of T2DM without CAD, **$p<0.001$ vs Ox-LDL, AGE-LDL and LDL from T2DM without CAD

Table 6: Uptake of fluorescent-labeled native and modified lipoproteins

	N-LDL	Ox-LDL	AGE-LDL	p-value*
Binding	336±5‡	206±9	210±9	<0.0001
Uptake	1109±49	842±76	795±43	<0.0001
Internalization	773±49	635±72	584±43	<0.0001

Kruskal-Wallis test was used for statistical analysis; Data are shown as Mean±SD, *Significant difference between groups if $p<0.05$, ‡: Values are expressed as ng LDL mg^{-1} protein

Uptake of fluorescent DiI-LDL by HepG2 cells: Fluorescence microscopy was carried out to evaluate the cellular uptake of DiI-LDL particles. As shown in Fig. 3, DiI-labeled LDL from healthy subjects was taken up efficiently by HepG2 cells and was localized in the intracellular vesicles. Both Ox-LDL and AGE-LDL showed a significant DiI-LDL uptake but to a lesser degree. LDL particles from T2DM with CAD revealed negligible bound to and internalized by LDL receptors and similar results were obtained from uptake studies. There was a similarity in the distribution of labeled LDLs in the cytoplasm between diabetics without CAD and *in vitro* modified-LDL (i.e., AGE-LDL and Ox-LDL) from healthy individuals. Competition studies were performed in the presence of an excess (50-fold) amount of unlabeled LDL. Unlabeled LDL competed for DiI-LDL uptake, indicating that DiI-LDL from controls was not incorporated into the cells and unlabeled-LDL was internalized into the cells via the LDL receptor.

Spectrofluorometeric results are shown in Table 6 and 7. Cells incubated with native DiI-LDL showed a significant increase in lipoprotein uptake when compared to all modified lipoproteins ($p<0.0001$). However, no statistically significant difference was present between Ox-LDL and AGE-LDL in uptake ($p = 0.17$), binding $p = 0.43$) and internalization ($p = 0.13$). Other experiments demonstrated negligible DiI uptake (binding and internalization) in HepG2 cells treated with DiI-LDL isolated from T2DM with CAD patients, while cells have taken and internalized DiI-LDL isolated from T2DM patients without CAD, DiI-Ox-LDL and DiI-AGE-LDL but to a lesser degree when compared to native DiI-LDL.

Table 7: Uptake of fluorescent-labeled LDL from healthy and T2DM groups

	Healthy controls	T2DM without CAD	T2DM with CAD	p-value*
Plasma pools (n)	9	8	9	-
Binding (ng LDL mg^{-1} protein)	336±5	217±10	109±8	<0.0001
Uptake (ng LDL mg^{-1} protein)	1109±49	812±56	217±8	<0.0001
Internalization (ng LDL mg^{-1} protein)	773±49	595±54	108±11	<0.0001

Kruskal-Wallis test was used for statistical analysis; Values are Mean±SD; *Significant difference between groups if p<0.05

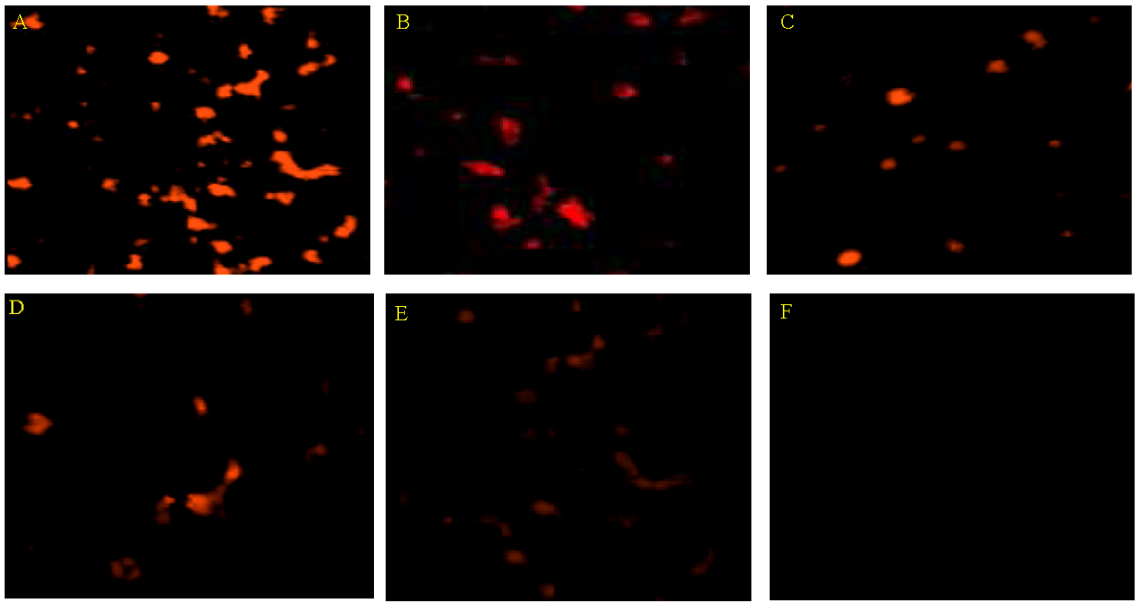

Fig. 3: DiI-labeled lipoproteins uptake by human Hepatoma (HepG2) cells. Cells were cultured for 2 days and treated with DiI-lipoproteins for 2 h at 37°C. Bright areas on the photographs represent DiI-LDL taken by the cells. Native-LDL (A), DiI-LDL from diabetics without CAD (B), DiI-Ox-LDL (C), DiI-AGE-LDL (D), DiI-LDL from type 2 diabetic patients with CAD (E) and unlabeled LDL-treated cells (F). The results are representative of seven independent experiments. Magnification, x400

DISCUSSION

Type 2 diabetes is a complex chronic disease with increasing macrovascular complications. Previous studies have indicated that peroxidation of lipids, together with glycation, may be a source of CML in diabetes and atherosclerosis (Fu *et al*., 1996). It has been shown that T2DM patients with renal or retinal complications had significantly higher CML levels compared to diabetic patients without these complications. However, no significant different was seen between diabetes patients with and without macroangiopathy including CHD and stroke (Wautier *et al*., 2003). Recently, Hirata and Kubo (2005) have reported that high CML levels were directly proportional to the severity of both retinopathy and nephropathy in patients with type 2 diabetes.

Although Kilhovd *et al*. (1999) found that serum AGEs levels increase in patients with coronary heart diseases and T2DM, little data are available on the relationship between serum CML and macrovascular complications in individuals with T2DM. The present study showed that both CML, a major product of the oxidative modification of glycated proteins and MDA levels were found to be markedly elevated in T2DM patients suffered from CAD when compared to either diabetics without CAD complications or healthy control subjects. There was similarity between the HbA_{1c} concentrations among the study groups, indicating that glycation alone is not responsible for CAD development in diabetics. In addition, multivariate regression analysis between CML concentrations and other traditional risk factors revealed highest significant association of circulating CML with MDA, suggesting increased systemic oxidative stress in T2DM patients with CAD (Table 3). For optimal comparison, we attempted to study the correlations between CML and the presence of CAD

in T2DM patients using logistic regression analysis. To our knowledge, this is the first study to show the significant predictive power of high serum CML concentrations to CAD in diabetics and this association between CML and CAD is independent of other risk factors (Table 4).

LDL cholesterol is a well-known risk factor for CHD and is now recognized as the primary target of lipid lowering therapy (Adult Treatment Panel III, 2001). Studies in susceptibility of LDL to oxidation and lipid peroxidation products formation revealed that oxidative modification is significantly higher in LDL isolated from T2DM patients with CAD than diabetic patients without CAD and healthy control subjects. Additionally, AGE-LDL showed similar LDL oxidizability to LDL isolated from patients with T2DM and CAD to provide clear evidence that both glycation and oxidation processes are strongly associated with enhanced oxidative stress in diabetes with complications rather than diabetes alone (Fig. 2). Evidence in support of our observations includes the fact that CML is formed by the copper-induced oxidation of LDL, which might be derived from lipid peroxidation products rather than from carbohydrates (Requena *et al*., 1996). In fact, glycated-LDL has been reported to be more susceptible to metal ion-catalyzed oxidative modification than native LDL (Sakurai *et al*., 1991).

It has been shown that nonenzymatic glycation of proteins including LDL may be closely correlated to lipid peroxidation in the arterial intima as explained by colocalization of CML in the cytoplasm of macrophage-derived foam cells in atherosclerotic lesions (Imanaga *et al*., 2000). These investigators and others (Moro *et al*., 1999; Kawamura *et al*., 1994) provide a compelling line of evidence which allows us to consider carboxymethylated-LDL more atherogenic than classic oxidized and glycated-LDL particles. Using HepG2 cells which abundantly express LDL binding sites (Dashti *et al*., 1984) and nonradioactive mechanisms for studying of altered metabolism of modified LDL particles (i.e., Ox-LDL and AGE-LDL) and comparing them with LDL separated from diabetes groups (i.e., T2DM with and without CAD) provide an *in vitro* model for the evaluation of LDL receptor activity.

In this report, we have provided several lines of evidence that support the argument for the associated effect of CML-modified proteins including LDL in the clearance and catabolism of LDL by HepG2 cells. We investigated the uptake (i.e., binding and internalization) of LDL molecules using DiI fluorescent dye. This reagent has been used in many studies to measure uptake and trafficking of LDL in various model systems (Stephan and Yurachek, 1993; Ghosh and Webb, 1994). Our measurements of LDL receptor activity shows that LDL obtained from diabetics without CAD, DiI-AGE-LDL and DiI-Ox-LDL has similar uptake and distribution in the cytoplasm as explained by both qualitative and quantitative measurements. These LDL particles did not appear to be defective in terms of their ability to bind to the LDL receptor rather, their interaction with the LDL receptor was reduced when compared to native LDL as explained by competition studies. The significant uptake of glycated-LDL and oxidized-LDL explains that either glycation or oxidation did not completely abolish LDL binding to the LDL receptor. On the other hand, the binding and uptake of LDL of T2DM with CAD patients by HepG2 cells were completely impaired, indicating that LDLs obtained from T2DM with CAD patients may have increased atherogenic potential in hyperglycemia.

It is concluded from these results that the glycoxidative effect of CML may be largely responsible for the defective LDL uptake by LDL receptor rather than AGE-LDL or Ox-LDL. The results of this study not only confirms the previous observations that glycoxidized-LDL is present *in vivo* but rather, it shows that glycoxidized-LDL with CML-epitopes could be found in higher concentrations in patients with T2DM and CAD than diabetes patients without such complications. Thus, CML may play a crucial role in the development of coronary artery disease in patients with T2DM. Finally, CML may be considered as a marker of interest for early detection of coronary artery disease in type 2 diabetic patients and may serve as an important target for the glycaemic control in those patients.

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