Serum Levels of Malondialdehyde in Type 2 Diabetes Mellitus Thai Subjects

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ABSTRACT

Objective: To investigate the serum MDA levels, as a biomarker of lipid peroxidation in T2D patients and the association with the other conventional cardiovascular risk factors (Glu, TC, TG, HDL-C, LDL-C, waist circumference, and BP).
Methods: Serum levels of malondialdehyde (MDA) are the most commonly used markers of this process measured as thiobabituric acid reaction substances (TBARS). The MDA-TBA adduct has a colorimetric measurement of 532 nm. A total of 50 T2D patients and a group of 40 healthy controls participated in this study.
Results: The MDA concentrations of the 40 healthy controls ranged from 0.699 to 2.684 ?mol/l. The serum levels of MDA concentration in type 2 diabetes patients were significantly elevated ($P<0.001$) compared with the healthy controls. MDA levels were significantly correlated ($P<0.05$) with conventional cardiovascular risk factors (age, Glu, LDL-C, HDL-C, TC, TG, UA, waist circumference, and blood pressure).
Conclusion: Serum levels of MDA were significantly increased in T2D patients. It may be a good marker for lipid peroxidation or an oxidative stress event that is implicated in various pathological conditions.

Keywords: Lipid peroxidation, malondialdehyde, oxidative stress, type 2 diabetes mellitus

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Type 2 diabetes mellitus (T2D) is recognized as one of the leading causes of morbidity and mortality in the world. T2D has been shown to increase free radical activity[1,2] and to be associated with the increased activity of free radical-induced lipid peroxidation and accumulation of lipid peroxidation products[3] as well as being associated with a substantially higher prevalence of atherosclerositic and cardiovascular mortality.[4] The mechanisms of the formation of free radicals in T2D may include not only increased non-enzymatic and auto-oxidative glycosylation, but also metabolic stress resulting from changes in energy metabolism, and the levels of inflammatory mediators.[4]

Lipid peroxidation is an autocatalytic free radical-mediated destructive process whereby poly-unsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides.[5,6] By-products of lipid peroxidation such as conjugated dienes and malondialdehyde (MDA) are increased in the circulation of T2D patients.

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MDA is generated as a relatively stable end product from the oxidative degradation of poly-unsaturated fatty acids (PUFA).[7-11] This free radical-driven lipid peroxidation has been causatively implicated in the aging process,[12,13] atherosclerosis,[14,15] Alzheimer's disease[16,17] and cancer.[18] Serum MDA has been used as a biomarker of lipid peroxidation and has served as an indicator of free radical damage.

MDA is a three-carbon dialdehyde that can exist in various forms in an aqueous solution. This method was most widely used for the reaction of MDA with thiobarbituric acid (TBA), when heated under acidic conditions. The TBA can react with a number of chemical species such as nucleic acids, amino acid, proteins, phospholipids, and aldehydes.[19] One molecule of MDA reacts with two molecules of TBA to form a stable pink to red color that absorbs maximally at 532 nm20 or fluorescence detection. These substances are termed thiobarbituric acid reacting substances (TBARS). The hypothesis of the current study is that elevated levels of malondialdehyde (as TBARS) are associated with increased cardiovascular risk in patients with T2D.

The aim of this study was to investigate the serum MDA levels, as a biomarker of lipid peroxidation in

T2D patients and the association with the other conventional cardiovascular risk factors (Glu, TC, TG, HDL-C, LDL-C, waist circumference, and BP).

MATERIALS AND METHODS

Subjects

A total of 50 T2D patients (12 male, 38 female) with a median age of 68.9 years (interquartile, 62-76 years) were recruited from the Tapho Primary Care Unit, Buddachinaraj Hospital. Also a group of 40 healthy controls (14 male, 26 female) with a median age of 65.5 years (interquartile, 58.8-73.7 years) was established from healthy volunteers. These subjects were apparently healthy as based on their medical history and a physical examination. For subjects exclusion criteria were smokers or taking supplements with antioxidant agents or alcohol consumption habits. All subjects gave written informed consent, and the Ethics Committee of the Naresuan University approved the study protocol.

Blood sampling and assays

Venous blood samples were taken without stasis after a 12-hour fast and a 30 minute rest in a supine position. Plasma glucose (Glu), serum total cholesterol (TC), triglycerides (TG), high density lipoprotein-cholesterol (HDL-C), BUN, Creatinine (CT), and uric acid (UA) were measured immediately after blood collection. Plasma Glu, serum TC, TG, HDL-C, UA, and the other biochemical variables were measured by standard enzymatic methods using a Hitachi 912 analyzer (reagents from Roche Diagnostics); low-density lipoprotein cholesterol (LDL-C) was estimated from the Friedewald's equation[21] Quality control and standardization was achieved throughout the analysis with internal and external quality assurance materials purchased from Roche Diagnostic.

Blood pressure was measured with the patients in the supine position after a 5 minute resting period with a Terumo digital blood pressure monitor, ES-P110. Blood pressure is given as the mean value of at lest two measurements of patients and the control subjects on the same day. For the determination of MDA, serum was stored at -70^{o}C without the addition of exogenous antioxidants before MDA analysis.

After thawing the samples, measurements of MDA in term of TBARS were performed for each of the 90 samples by the modified method of Wong et al.[22] Briefly, a 50 µL of TEP standards, serum specimen, and quality control specimen was pipetted into the 13-ml polypropylene test tube. Also 0.75 ml of phosphoric acid (0.44 mol/l) solution was added into the respective tubes and vortex-mixed. Then 0.25 ml of TBA (42 mmol/l) was added to each tube. Distilled water (0.50 ml for reagent blank, 0.45 ml for TEP standards, plasma, samples, and quality control samples) was added to adjust the final volume to 1.5 ml. The test tubes were capped tightly and heated at 100^{o}C for 60 minutes after which the samples were cooled in an ice water bath (0^{o}C). The optical density of the pink chromogen was read at 532 nm in a double-beam spectrophotometer (UV-610 Shimadzu, Japan). The within-run and between-run precisions of MDA assay were analyzed in two pooled-control sera. The replicate daily analyses were CVs of 6.22% and 6.11% for within-run precision assay, and 6.62% and 7.14% for between-run assay with the MDA concentration averaging (SD) 2.233 (0.139), 4.208 (0.257) and 2.250 (0.149), 4.428 (0.316) µmol/L. The %recovery for this MDA determination in serum sample and TEP standard were 102.21% and 95.13% respectively. TEP working standard solutions (5, 10, 15, 20 µmol/l) were prepared by using 1,1,3,3-Tetraethoxypropane (TEP, Sigma Chemical) stock standard solution dissolved in an ethanol solution. These working standards were prepared bi-weekly and were stored at 4^{o}C as described in Wong et al.[22]

Statistical analysis

Statistic analysis was performed using the SPSS computer program version 11.0 (SPSS, Chicago, IL). All results are presented as median and interquartile ranges (Q1-Q3). Mann-Whitney U test was used to estimate differences between groups. Spearman rank correlation was used to assess the correlation between MDA and other variables of traditional cardiovascular risk factors. All analyzes were undertaken using $\alpha <$ 0.05 (two-tailed) as the significant statistic.

RESULTS

Clinical characteristics of the healthy controls and T2D patients are shown in Table 1. The median level for serum MDA concentrations in healthy controls was 1.652 µmol/L (2.5^{th}/97.5^{th} percentile = 0.699-2.684 µmol/L). In this study, the age in both groups were not significantly different (P = 0.059). 29 (58%). T2D patients had elevated MDA ($\geq$ 2.68 µmol/l), The serum MDA levels, a product of lipid peroxidation as the oxidative stress marker from 50 T2D patients (12 men, 38 women, age range 50-86 years) were significantly higher than the MDA levels from healthy controls ($P<$ 0.001). The conventional cardiovascular risk factors (Glu, TC, TG, LDL-C, UA, waist circumference, and systolic blood pressure) were also significantly higher ($P<0.05$) in T2D patients than healthy controls (Table 1). HDL-C levels were also significantly lower in T2D patients ($P<0.001$). The serum MDA levels were significantly correlated ($P<$ 0.05) with the conventional cardiovascular risk factors (age, Glu, TC, TG, LDL-C, HDL-C, UA, waist circumference, and blood pressure) using the Spearman rank correlation test (Table 2).

DISCUSSION

Increasing evidence in experimental and clinical studies suggest that oxidative stress plays the major role in the pathogenesis of T2D. Free radicals are formed disproportionately in DM by glucose degradation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation which may play an important role in the development of complications in T2D patients. The levels of MDA (as TBARS) were significantly elevated in T2D patients.[11,23-25] In the present study, the serum MDA levels were also significantly elevated in T2D patients. The present study found free radical activity is increased in the type 2 diabetic mellitus[1,2] which leads to a higher incidence of atherosclerotic and cardiovascular disease.[4] The absolute level of atherogenic lipoproteins in the circulation is a major factor in the risk of cardiovascular diseases, and this oxidative

TABLE 1. Clinical and chemical characteristics in Thai elderly type 2 diabetic patients and healthy control groups. The values are shown as medians and interquartile (Q1-Q3).

Variables	T2D patients (n= 50)	Healthy control group (n=40)	*P*-value
BMI	25.40	23.52	0.012
Q1-Q3	22.80-27.40	21.26-25.31	
Weight (Kg)	60.3	57.9	0.682
Q1-Q3	53.80-66.2	51.9-66.3	
Waist circumference (cm)	89.2	83.4	<0.001
Q1-Q3	85.0-94.2	78-89	
Systolic Blood Pressure (mmHg)	134.8	120.5	0.008
Q1-Q3	121.3-154	112.5-138.3	
Diastolic Blood Pressure (mmHg)	77.3	75	0.013
Q1-Q3	71.0-93.0	70-80.8	
Glucose (mg/dl)	118.7	81.3	<0.001
Q1-Q3	96.0-147	76.8-90.0	
BUN (mg/dl)	13.3	11	0.002
Q1-Q3	11.2-16.3	9.3-12.9	
Creatinine (mg/dl)	0.91	0.84	0.19
Q1-Q3	0.73-1.09	0.74-0.96	
Uric acid (mg/dl)	6.0	4.8	0.002
Q1-Q3	4.8-6.8	4.1-5.7	
Total Cholesterol (mg/dl)	202.5	179.3	<0.001
Q1-Q3	185.5-229	165.4-192.7	
Triglycerides (mg/dl)	171.7	126	<0.001
Q1-Q3	122.3-342	102.5-170.5	
HDL-Cholesterol (mg/dl)	45.7	59.8	<0.001
Q1-Q3	38.1-51.9	52.3-68.1	
LDL-Cholesterol (mg/dl)	114.1	93.6	<0.001
Q1-Q3	90.4-133.6	82.0-93.6	
MDA level (μmol/L)	2.750	1.652	<0.001
Q1-Q3	2.216-3.420	1.296-2.050	

modification of blood lipids further increased this risk.[26] Oxidized LDL particles are more readily internalized by macrophages and thereby enhance foam cell formation which, in the vascular wall, favors smooth muscle cell proliferation, increases platelet adhesion and the anticoagulant activity of the endothelium.[27] Increased lipoperoxide levels have been correlated to atherosclerosis, Alzheimer's disease, and cancer, as well as the aging process. These consequences of oxidative stress can promote the development of complications in diabetes mellitus patients. T2D patients have shown, increased lipid peroxidation and decreased levels of reduced glutathione, glutathione reductase, glutathione peroxidase, glutathione and G6PDH.[19]

Lipid peroxidation is an autocatalytic free-radical-mediated destructive process whereby PUFA undergo degradation to form lipid hydroperoxides. These latter compounds decompose to form a wide variety of products: low-molecular mass hydrocarbons, hydroxyl aldehydes and fatty acids, ketone, alkenals, and alkanals, including MDA.[28] Many laboratory techniques have been used to quantify MDA. The most commonly used methods are: (i) gas chromatography;[29] (ii) analysis of age-related pigments (e.g. lipofuscin, uroid) based on characteristic fluorescence and emission spectra;[30] (iii) spectrophotometry of conjugated dienes at 233 nm;[31] (iv) colorimetry and fluorimetry of MDA and other chromogens based on the thiobarbituric acid (TBA) reaction;[32,33] and (v) the measurement of various products by high-performance liquid chromatography (HPLC).[34,35] The methods most widely used are based on the reaction of MDA with TBA, in which one molecule of MDA reacts with two molecules of TBA to form a stable pink to red color that absorbs maximally at 532 nm.[32,33] The acid hydrolysis process in the colorimetric method has been used with different acids. Wong, Knight, Koschsour, Londero and Neilson all used orthophosphoric acid, Sinnhuber used hydrochloric acid, whereas Carbonneau used perchloric acid. Seljeskog et al. tested 8 different acids at three different concentrations for the initial acidifying step of samples. The stronger the ability of an acid to oxidize, the more bound MDA will be liberated and more MDA may be produced by PUFA oxidation. Then, our study separated the TCA from the acid hydrolysis step to decrease nonspecific color reaction and to reduce the turbidity interference from serum protein after the acid hydrolysis step (the boiling solution was cooled) by adding TCA

TABLE 2. Spearman rank correlation between serum malondialdehyde (MDA) levels with other cardiovascular risk factors.

	MDA	
	r	(*P*-value)
Plasma glucose	0.450	0.005
Serum total cholesterol	0.295	0.005
Serum triglyceride	0.220	0.038
Serum LDL-C	0.259	0.014
Serum HDL-C	-0.234	0.027
Serum uric acid	0.346	0.001
Waist circumference	0.304	0.004
Systolic blood pressure	0.210	0.048
Diastolic blood pressure	0.19	0.010

(50 µL, 100%).[41] Our study used serum and found TBARS from 40 healthy subjects with a median value of 1.652 µmol/L for men and women with 2.5th/97.5th percentile = 0.699-2.684 µmol/L. These results were clo se to previous researchers as determined by gas chromato-graphy-mass spectrophotometry, 1.30 ± 0.07 µmol/L42 and 1.67 ± 0.16 µmol/L.43 However, meas urement by either spectrophotometry or fluormetry induces various interfering compounds, thereby decreasing the specificity of the method.[44,45] Wong et al.[22] reported an improved liquid chromatographic technique for measu-ring MDA in plasma with reliable reference values. By this spectrophotometic method we tried to reduce some interferences including separated TCA from the acid hydrolysis step and precipitated the protein after the acid hydrolysis step.

In conclusion, increased oxidative stress caused an increased lipid peroxidation, measured as serum malondialdehyde (TBARS) concentration. T2D patients were significantly higher in MDA concentration than the healthy controls (P<0.001). A significant correlation was found with the traditional cardiovascular risk factors (age, Glu, TC, TG, LDL-C, HDL-C, UA, waist circumference, and blood pressure).

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