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Characterization of oxidative stress in blood from diabetic vs. hypercholesterolaemic patients, using a novel synthesized marker

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Abstract

In the present study, we extend our novel concept of designing and using exogenous markers for the characterization of oxidative stress (OS) and OS-associated diseases. The aim was to use such a synthetic compound as a tool for studying OS in blood from diabetic and hypercholesterolaemic (Hc) patients. The marker used N-linoleoyl tyrosine (LT) was constructed from tyrosine and linoleic acid (LA); both components are known to be easily oxidized upon exposure to different types of reactive oxygen/nitrogen species (ROS/RNS), and to generate specific oxidized products, depending on the type of oxidants present *in vivo*. Using the LT probe, we showed that the ratios of oxidized LT to total LT (Ox-LT/LT) is significantly higher in blood samples obtained from diabetic patients, than in Hc patients or healthy control subjects. LC/MS analysis revealed that blood from diabetic patients oxidizes the marker with predominant formation of Ox-LT hydroperoxide (LT-OOH) and epoxide (epoxy-LT), where the LA moiety is oxidized to hydroperoxide and to epoxide, respectively. Analysis of oxysterol levels in these samples (GC/MS) revealed that the blood of both diabetic and Hc patients contained significantly more oxysterols than blood of control subjects. Consumption of pomegranate juice by diabetic patients for 3 months suppressed their blood capacity to oxidize the LT and similarly also reduced their blood oxysterol/total cholesterol ratio by 93%. The use of an exogenous marker to characterize OS in blood samples yields important information on the extent of OS, and can provide a fingerprint for the early identification of different pathological conditions associated with OS.

Keywords: *Diabetes, hypercholesterolaemia, marker, oxidative stress, pomegranate juice*

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Introduction

Oxidative stre\ss (OS) is defined as an imbalance between pro-oxidants and antioxidants, in favour of the former, and has been implicated in the pathogenesis of various vascular disorders, including atherosclerosis, diabetes and hypertension. An

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excess of reactive oxygen/nitrogen species (ROS/RNS) is associated with changes in the structure and function of biomolecules, such as DNA, proteins and lipids (Halliwell & Gutteridge 1989, Diplock et al. 1998, Griffiths et al. 2002). Proteins can react with ROS/RNS to cause oxidation of their amino acids, deamination, decarboxylation and modifications in their aromatic rings (Brown et al. 1997, Dean et al. 1997, Davies et al. 1999, Headlam & Davies 2003), which may lead to changes in their three-dimensional structure and activity. Polyunsaturated fatty acids (PUFA) such as linoleic and arachidonic acid, constructing cells and tissues, are readily auto-oxidized under OS, react with ROS/RNS and thereby initiate other free radical reactions which affect cell function, generating changes in membrane density, fluidity, permeability and oxido/redox potential (Gutteridge 1995).

Organisms protect themselves from the toxicity of excess ROS and maintain an oxido/redox balance in different ways, including the use of endogenous and exogenous antioxidants, such as the enzymes superoxide dismutase, catalase and glutathione reductase, as well as other low-molecular-weight antioxidants or extracts. Among these are glutathione, tocopherols, ascorbic acid, carotenoids and polyphenols (Halliwell 1994, Evans & Halliwell 1999, Aviram 2000, Vaya & Aviram 2001, Dragsted 2003), including red wine (Fuhrman et al. 1995, Nigdikar et al. 1998), pomegranate juice (PJ) (Aviram et al. 2000, Kaplan et al. 2001, Aviram et al. 2004), and licorice root extract (Vaya et al. 1997, Belinky et al. 1998).

The identification of reliable biomarkers has become essential for the identification of imbalances in the oxido/redox system and for the prediction of pathological conditions early on in their development. Several modified endogenous compounds have been proposed as indicators of OS on proteins (chloro- or nitro-tyrosine; keto-proteins) (Hazen & Heinecke 1997, Davies et al. 1999, Winterbourn & Kettle 2000) and lipids (oxidized degradation products of PUFA) (Halliwell & Gutteridge 1989, Reis et al. 2003). The detection of biomarkers as indicators of OS, however, suffers from some limitations, such as insufficient specificity, accuracy and reliability (Halliwell 2002). The levels of endogenous biomarkers reflect a dynamic process, involving their formation, accumulation and removal, and in some instances may not reflect the oxidative process within the system, but rather alternative processes (Miller et al. 1998, Halliwell 2000, Griffiths et al. 2002). Other methods attempt to trap the reactive species involved, such as electron spin resonance (ESR), uses different synthetic probes to trap reactive species *in vitro* and *ex vivo* (Berliner et al. 2001, Utsumi & Yamada 2003).

In recent studies (Szuchman et al. 2006, Khatib et al. 2007, Vaya et al. 2007), we introduced novel markers for the characterization of OS in cells free, in cell lines and in primary astrocyte cells and mitochondria. These probes are constructed from several endogenous subunits connected together covalently. In Szuchman et al. (2006), the designed marker was created from linoleic acid (LA) covalently bound to tyrosine (Ty), producing N-linoleoyl tyrosine (LT). Exposure of LT to various types of ROS/RNS in cells free as well as in macrophage cell lines, results in the formation of selective and specific oxidized LT (Ox-LT), linked to the ROS/RNS type in question. Using this marker, it was possible to show that peritoneal macrophages taken from atherosclerotic apolipoprotein-deficient (E^0) mice showed specific and selective oxidation of Ox-LT at significantly higher levels than from similar experiments which used peritoneal macrophages harvested from control BalbC mice. In the present study, a further manifestation of the potential of using such a probe in characterizing

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the extent and type of OS in the blood of diabetic and hypercholesterolaemic patients vs. healthy control subjects, is described. The effectiveness of an intervention, such as supplementation of pomegranate juice (PJ), in decreasing blood capacity to oxidize the external marker and to accumulate oxysterols is also demonstrated. This may allow the marker's damage fingerprints to be associated with specific pathological conditions.

Materials and methods

Materials

LT was synthesized from Ty and LA (Figure 1), as described by Szuchman et al. (2006). N,O-bis(trimethylsilyl) acetamide (BSA) and butylated hydroxyanisole (BHA), were obtained from Sigma Chemical Co. (St Louis, MO, USA). 7-Ketocholesterol (7-keto), 7α and 7β-hydroxycholesterol (7α-OH, 7β-OH), 5 α, 6 α and 5 β,6 β-epoxy-cholesterol (α-epoxy, β-epoxy) and 19-hydroxycholesterol (19-OH), used as an internal standard, were purchased from Steraloids Inc. (Wilton, NH, USA). Pomegranate juice was donated by POM Wonderful USA. The fruits were processed as described in the section 'the effect of pomegranate juice on blood oxidation state in diabetic patients' below. All solvents were either spectrophotometric or high-performance liquid chromatography (HPLC) grade.

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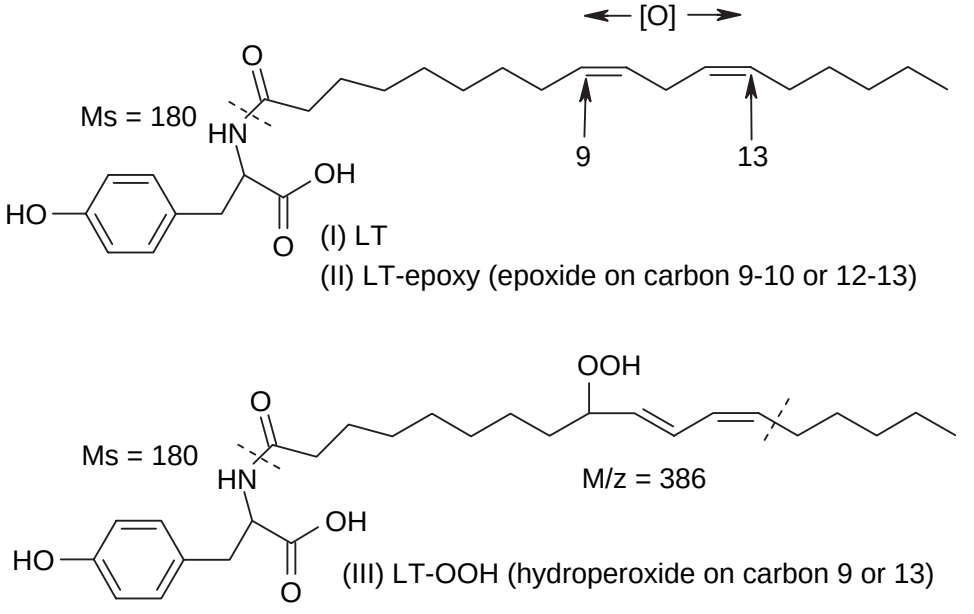

Figure 1. The structure of N-linoleoyl tyrosine (LT) and its epoxide and hydroperoxide. LT (I) has a molecular ion of m/z 442.6, monitored base on two fragments of m/z 180 (tyrosine) and 398.6 (M^{-1}-CO_2). Epoxidation of LT (II) occurred on either the double bond between carbon 9 and 10 or 12 to 13, with molecular ion of m/z 458.6 and monitored based on two fragments of m/z 396.6 and 180. The LT-OOH contains the hydroperoxide group on carbon 9 or 13 with molecular ion of 474.6 and monitored base on the two fragments of m/z 386.6 and 180.

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Separation of oxidized N-linoleoyl tyrosine (Ox-LT) products by LC/MS/MS

LT (Figure 1) was synthesized and analyzed by liquid chromatography/mass spectrometry (LC/MS), as described in Szuchman et al. (2006). The LC/MS was equipped with an HPLC model 2790 (Waters, MA, USA) and a Waters photodiode array detector (Model 996), connected to an MS (Micromass Quattro Ultima MS, UK). The HPLC column was a 3.5-μm C18 ODS XTerra (Waters) and the eluents were a gradient of solution A (0.1% acetic acid in acetonitrile) and solution B (0.1% acetic acid in DDW) as follows: starting with 40% A, changing to 60% A for 2 min, and then to 80% A for 10 min. Finally, the column was washed with a solution of 98% A.

MS/MS analysis of the oxidized products was performed in scan mode, using electrospray negative ions (ES^-). The source temperature of the MS was set at 150°C, with a cone gas flow of 24 l h^{-1} and a desolvation gas flow of 600 l h^{-1}. Peak spectra were monitored between 30 and 600 m/z. Collision-induced dissociation MS was performed, using collision energy of 25–30 eV and a capillary voltage of 3–3.5 kV. Multiple-reaction monitoring (MRM) was performed under the same conditions. A calibration curve of LT was run in each analysis set.

Analysis of cholesterol oxidation products (oxysterols)

Gas chromatography (GC)/MS analysis was performed by means of an HP gas chromatograph, model 5890 Series II (Waldbronn, Germany), fitted with an HP-5 trace analysis capillary column (column 0.32 mm I.D. 0.25 μm film thickness, 5% phenyl methyl silicone), with a mass selective detector, Model 5972, (Waldbronn) linked to an HP ChemStation data system. Dried extracts were subjected to the silylating reagent (BSA, 200 μl), and 1,4-dioxane (dried on 4 Å molecular sieves and passed through aluminium oxide) (200 μl) as solvent, and heated to 80°C for 60 min. The GC was operated in splitless mode for 0.8 min and then in a split ratio of 1:1. Helium was used as the carrier gas, at a flow rate of 0.656 ml min^{-1}, pressure 10.4 psi and a linear velocity of 31 cm s^{-1}. The MS transfer line was maintained at 280°C. The injector was set at 300°C, and the column was heated gradually, starting at 200°C, increasing to 250°C at 10°C min^{-1} and then to 300°C at 5°C min^{-1}; it was then maintained for an additional 15 min at 300°C.

Samples were detected by GC/MS in total ion monitoring (TIM) mode; the two to four most representative ions were selected for re-injection in single ion monitoring (SIM) mode. 19-OH was used as the internal standard (IS). For maximum sensitivity, the oxysterols were injected as their silyl ether derivatives, and the response factor for each oxysterol under the analytical conditions was calculated from the peak-area ratio (Vaya et al. 2000). The oxysterols 7α-OH, 7β-OH, 4β-OH, β-epoxy, α-epoxy, 3,5,6-trihydroxycholesterol (triol), 25-OH, 7-keto and 26-OH were selected for analysis as potential products during cholesterol oxidation (Addis et al. 1989, Dyer et al. 1997, Vaya et al. 2000). Oxysterols were separated, and the mean quantity of each oxysterol was calculated, using standard calibration curves.

Blood samples

Blood samples were collected from ten healthy subjects, six hypercholesterolaemic (Hc) patients and eleven diabetic patients (aged 20–50 years). The healthy control

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subjects were non-smokers, with no diabetes (glucose levels below 100 mg%), no hypertension and no coronary artery disease. Seventy-five per cent of the diabetic patients were treated with Glucophage® (metformin), and 50% with Gluben® (glybenclamide). The Hc patients were non-smokers, with no diabetes, hypertension, or coronary artery disease, and they were on statin therapy. Their total serum and low-density lipoprotein (LDL) cholesterol levels were above 280 mg dl^{-1} and 170 mg dl^{-1}, respectively, and their serum triglyceride levels were below 200 mg dl^{-1}.

Blood chemistry assays

Blood glucose was measured by means of an enzymatic kit (Roche). Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride concentrations were determined in serum, using diagnostic kits (Raichem).

Incubation of the LT marker in blood samples obtained from diabetic and Hc patients vs. healthy control subjects

Blood samples (1 ml) were collected in a glass tube containing 8 μl of marker (from a stock solution of 20 mM marker dissolved in DMSO) or DMSO (control) and left to coagulate at room temperature. After 1 h, blood was extracted twice with 3 ml of hexane:2-propanol (3:2 v/v) each, containing 10 ppm butylated hydroxyl anisol (BHA). The organic phase was collected and evaporated under nitrogen and samples were kept under argon at -20°C until analysis. Before analysis, samples were re-suspended with 20% methanol in acetonitrile and divided for LC/MS and GC/MS analyses. Heptadecanoeyl tyrosine (final concentration of 5 ppm, synthesized similarly to LT, unpublished data) was used as an internal standard for the LC/MS, and 19-OH was used as an internal standard for the oxysterols analysis in the GC/MS (25 ppm).

The effect of pomegranate juice (PJ) on blood oxidation state in diabetic patients

Pomegranates (donated by POM Wonderful USA) were picked by hand, washed and stored in tanks. The fruit was crushed and squeezed. The juice was filtered, pasteurized, concentrated and stored at -18°C. The antioxidant composition of the juice has been investigated elsewhere (Navindra et al. 2006). Six diabetic patients were instructed to consume 50 ml of PJ per day (containing 1.5 mmol of 50 ml of total polyphenols) for a period of 3 months. Blood samples were collected from these patients before (time zero) and after the 3 months of PJ consumption. The concentrated PJ was diluted 1:5 (v:v) with water daily to obtain single-strength PJ.

Statistical analysis

The relative amounts of the Ox-LT products were calculated as micromolar percentages of the unoxidized-LT in the sample, and the relative oxysterol amounts were calculated as micromolar percentages of cholesterol in the sample. The results were analyzed and found to be normally distributed and therefore statistical analyses were performed using the Student's *t*-test, when comparing the means of two groups. ANOVA was used when more than two groups were compared. Results are given as mean $\pm$ SEM.

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Results

LT was synthesized from Ty and LA, as described by Szuchman et al. (2006). LC/MS/MS analysis of LT and its oxidized products (Ox-LT, Figure 1) obtained under its exposure to different types of ROS/RNS were developed, using HPLC coupled to a diode-array detector and an MS detector set to ES^- mode with fragmentation of the molecular ions, using daughter ion and/or MRM methods. The LT marker revealed a molecular ion of m/z 442 (M-1), corresponding to LT: this marker was used throughout this study.

Formation of Ox-LT following its incubation with blood samples from diabetic or Hc patients vs. healthy subjects

The designed marker was incubated with fresh blood samples obtained from diabetic or Hc patients, as well as from healthy (control) subjects. The oxidized products were analyzed using GC/MS (oxysterols) and LC/MS (LT and Ox-LT). In Hc patients the total cholesterol (TC) and LDL cholesterol (LDL-Chol) in the serum were significantly higher than in the diabetic and control groups ($p < 0.01$; Table I), with no significant differences between them in the diabetic patients and the control group (Table I). HDL-Chol was lower in diabetics than in Hc and control groups, by 28% and 15%, respectively. The serum triglyceride levels of the diabetic patients were higher than in the Hc or control groups, by 22% and 60%, respectively. On the other hand, as expected, glucose level was significantly higher in the diabetic patients than in the Hc and control groups ($p < 0.001$; Table I).

Incubating the marker (160 μM) with blood (1 ml) from diabetic or Hc patients or controls (Figure 2A) immediately after its withdrawal revealed a significant increase in the oxysterol-to-cholesterol ratio in the diabetic and Hc blood vs. the control subjects (0.43 ± 0.3 and 0.43 ± 0.17 vs. 0.20 ± 0.09 μM/μM, respectively; $p < 0.05$ in diabetics/control and $p < 0.01$ in Hc/control, $n = 11$, 6, and 10). A detailed analysis of the different types of oxysterol distribution, showed 7-keto to be the major contributor to the increased oxysterol-to-cholesterol level in all three groups, whereas in the diabetic and Hc patients 7-keto was significantly higher than in the control subjects (0.31 ± 0.22 and 0.28 ± 0.13 vs. 0.13 ± 0.06 μM/μM, respectively; $p < 0.05$; Figure 2B). The major oxysterols distinguishing the blood of diabetics from that of Hc patients were 7α-OH and 7β-OH (0.035 ± 0.021 and 0.022 ± 0.015 μM/μM, respectively, in diabetics vs. 0.004 ± 0.003 and 0.004 ± 0.002 μM/μM in Hc and 0.009 ± 0.002 and 0.005 ± 0.002 in the controls; $p < 0.01$; Figure 2B). It was notable that α-epoxy cholesterol was characteristically higher in Hc patients than in diabetics or healthy

Table I. Serum lipids and glucose concentrations in hypercholesterolaemic (Hc) patients ($n = 6$), diabetic patients ($n = 11$) and healthy control subjects ($n = 10$). $^\star p < 0.001$.

Subjects	TC (mg dl^{-1})	LDL-C (mg dl^{-1})	HDL-C (mg dl^{-1})	TG (mg dl^{-1})	Glucose (mg dl^{-1})
Controls	164 ± 26	94 ± 19	52 ± 11	93 ± 22	75 ± 13
Hc patients	$284 \pm 17^\star$	$199 \pm 18^\star$	61 ± 5	122 ± 20	95 ± 7
Diabetics	160 ± 38	92 ± 28	44 ± 12	149 ± 116	$241 \pm 123^\star$

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TC, triglyceride.

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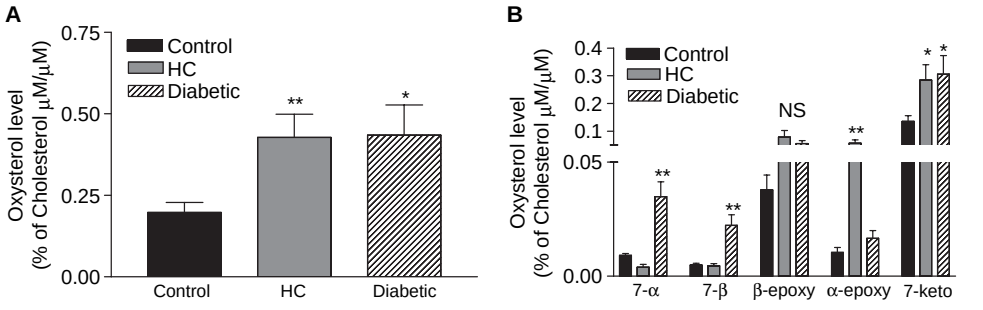

Figure 2. Total oxysterols levels (A) and oxysterols distribution (B) in blood from hypercholesterolaemic (Hc) patients, diabetic patients and healthy control subjects. * $p<0.05$, ** $p<0.005$.

subjects (0.056 ± 0.030 vs. 0.016 ± 0.011 in diabetics and 0.010 ± 0.007 in the control; $p<0.01$).

Analysis of the Ox-LT products showed a four-fold increase in the Ox-LT/LT ratio in blood from the diabetics vs. Hc patients and control groups (4.61 ± 3.18 vs. 1.15 ± 2.11 and 1.02 ± 0.41 µM/µM, respectively, $p<0.01$; Figure 3A), with no significant differences between their amounts in Hc patients and controls. The major contributor to this Ox-LT increase in diabetic blood were the linoleoyl hydroperoxide and the linoleoyl epoxide moieties (2.47 ± 1.93 and 2.14 ± 1.25 in the diabetics, respectively, vs. 0.50 ± 1.16 and 0.63 ± 1.13 in the Hc patients and 0.25 ± 0.27 and 0.77 ± 0.14 µM/µM in the controls, respectively; Figure 3B).

The effect of PJ consumption by diabetic patients on their blood oxidative status, as assessed by the marker oxidation pattern

Diabetic patients consumed 50 ml daily of the potent antioxidant nutrient PJ for 3 months and then their blood samples were collected before and after the PJ consumption period. Analysis of the oxysterol-to-cholesterol ratio in these blood samples revealed a reduction of 94% in the total oxysterol content in diabetics who had consumed PJ daily during 3 months (Figure 4). GC/MS analysis of the different types of oxysterols showed that, in the blood, 7-keto, 7β-OH and α-epoxy cholesterol were the most affected by PJ consumption (by 97%, $p<0.01$; 93%, $p<0.05$; and

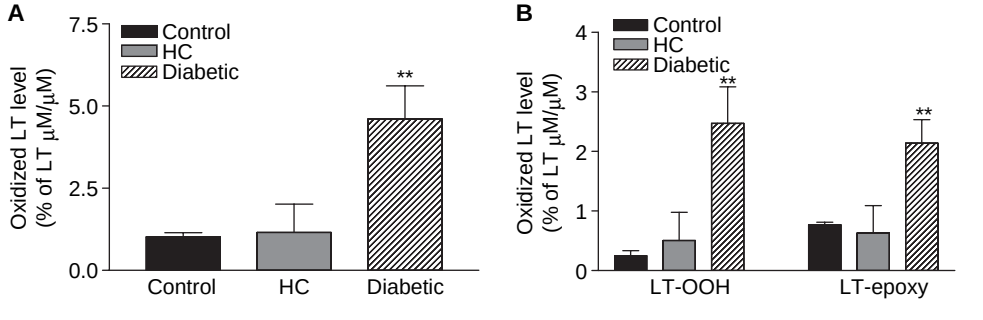

Figure 3. Total oxidized N-linoleoyl tyrosine (LT) level (A) and oxidized LT (Ox-LT) products distribution (B) in blood from hypercholesterolaemic (Hc) patients, diabetic patients and healthy control subjects. **$p<0.005$. The total amount of Ox-LT was calculated as the sum of LT-epoxy and LT-OOH base on the area under the peak of their fragments monitored in multiple-reaction monitoring (MRM).

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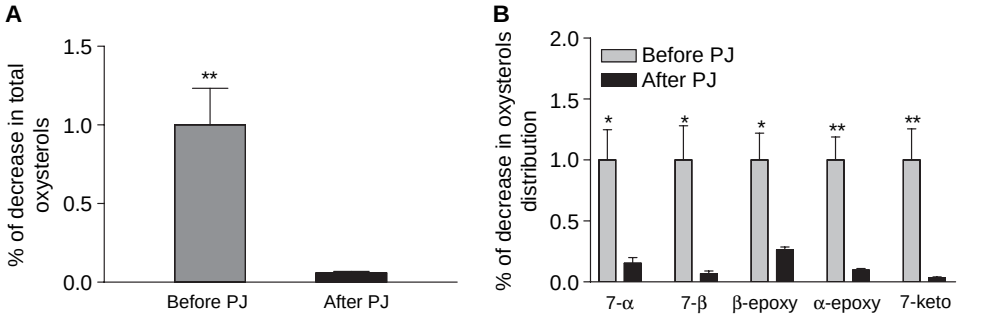

Figure 4. Percentage decrease in total oxysterol level (A) and percentage decrease in oxysterol distribution (B) in blood from diabetic patients before and after pomegranate juice (PJ) consumption. $^{*}p < 0.05$, $^{**}p < 0.005$.

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90%, $p<0.01$, respectively; Figure 4B). Similarly, incubation of the blood with LT for 1 h immediately after its withdrawal revealed a reduction in Ox-LT level by 93% (level of LT-OOH suppressed by 97% and that of LT-epoxide by 91%) (Figure 5).

PJ consumption resulted in a non-significant reduction in serum glucose and C-peptide levels (14.5% and 16.6% reduction, respectively), and had no effect on HbA_{1C} or insulin levels (Table II). These results indicate that, despite the high sugar content of PJ, the serum diabetic parameters did not worsen, even improving after PJ consumption.

Discussion

Various methods are available to measure oxidative damage in lipids, proteins and DNA in body fluids and tissue samples. Other methods aim to identify and quantify the reactive species (RS) involved (Pryor 2000, Griffiths et al. 2002, Halliwell & Whiteman 2004). For example, the ESR method uses different synthetic probes to trap RS, mostly in vitro and ex vivo (Berliner et al. 2001, Utsumi & Yamada 2003), whereas using a similar approach, aromatic molecules, such as salicylate and phenylalanine, are used to trap RS and characterize them (Kaur et al. 1996, Kaur et al. 1997, Themann et al. 2001). Some of these existing methods are limited, in

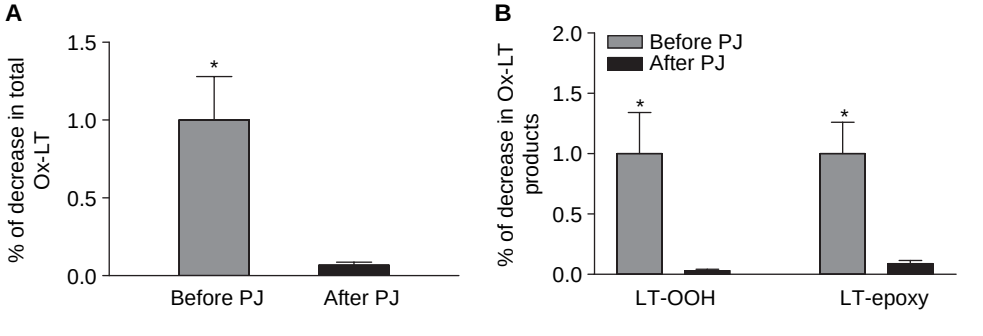

Figure 5. Percentage decrease in total oxidized N-linoleoyl tyrosine (LT) level (A) and percentage decrease in oxidized LT (Ox-LT) products (B) in blood from diabetic patients before and after pomegranate juice (PJ) consumption. $^{*}p<0.05$.

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Table II. Serum lipids, glucose, insulin, C-peptide and haemoglobin concentrations and biochemical parameters in diabetic patients ($n=6$) before and after pomegranate juice (PJ) consumption, $^{\star}p<0.05$.

	TC (mg dl^{-1})	HDL-C (mg dl^{-1})	TG (mg dl^{-1})	Glucose (mg dl^{-1})	Insulin (pmol l^{-1})	C-peptide (pmol l^{-1})	HbA_{1C} (%)
Before PJ	187.5±13.7	41±3	327±79	223.6±29.3	95.75±12.6	1083.5±169.7	8.3±0.8
After PJ	170.0±8.4*	39±3	302±64	191.0±19.3*	95.5+22.3	903.0±173.8	8.0±0.3

TC, total cholesterol; HDL, high-density lipoprotein; TC, triglyceride.

specificity for example, some measure uncharacterized RS products, and the application of yet others in vivo is questionable (Halliwell 2002).

The present study describes a continuation of the approach of using exogenous probes (Szuchman et al. 2006, Khatib et al. 2007, Vaya et al. 2007), which may overcome some of the limitations reported with existing endogenous markers. The present marker is composed from Ty and LA, both of which are sensitive to ROS/RNS, and their reaction products with oxidants have been extensively investigated: each separately (Aviram & Fuhrman 1998, Halliwell 2000, Heller et al. 2000, Pryor 2000, Vaya et al. 2000, Burkitt 2001, Szuchman et al. 2003), and as a couple (Szuchman et al. 2006, Vaya et al. 2007). The two components of the marker, Ty and LA, represent major families of the body's building blocks, the proteins and the unsaturated fatty acids, but their combination into one molecule results in an exogenous molecule for organs. We previously synthesized this marker (LT) and studied its ability to characterize OS cells free and in macrophages (Szuchman et al. 2006), and in primary astrocytes (Vaya et al. 2007). The LT active sites, i.e. the aromatic ring in the Ty, the two double bonds and the allylic positions in the LA, all remained intact. We thus hypothesized that the type of Ox-LT formed as a result of the reaction between LT and the RS could be used as a damage fingerprint, characterizing the specific OS. In the present study, the level of OS in the blood of diabetic patients was significantly higher than that observed in Hc patients and in healthy (control) subjects on the basis of Ox-LT formed. The Ox-LT/LT was higher in blood of diabetics than in Hc patients and healthy subjects, suggesting an enhanced oxidative capacity in the diabetics' blood. This finding is in agreement with findings demonstrating that patients with diabetes show increased plasma oxidizability, compared with hyperlipidaemic and control groups (Murakami et al. 2000).

Cardiovascular diseases are the leading cause of mortality and morbidity in patients with diabetes mellitus; dyslipidaemia associated with this condition contributes significantly to the increased risk of atherosclerosis in these patients. It has been well documented that oxidized LDL plays an important role in the pathogenesis of atherosclerosis, and that circulating levels of oxidized LDL are increased in patients with diabetes (Baynes 1991, Kawamura et al. 1994, Lyons & Jenkins 1997, Imanaga et al. 2000).

The present study shows that while the blood of diabetics has a higher potential to oxidize endogenous elements, such as linoleic acid, than that of Hc patients, their oxysterols level are about the same, and both significantly higher than healthy subjects. The major oxysterol contributing to this elevation in diabetics and Hc patients is 7-keto. This finding is in agreement with others which show that plasma 7-keto was significantly higher in patients with Hc and diabetes than in a control group (Murakami et al. 2000). The present study also demonstrated that 7α-OH and 7β-OH

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were the most characteristic markers for oxidation in diabetic blood, relative to Hc patients and controls, while α-epoxy cholesterol characterizes Hc patients. In a recent study, Rimner et al. (2005) demonstrated that 7-keto, as well as 7β-OH and 7-epoxy cholesterol, are the major contributors to the high (two-fold) oxysterol level found in plasma from patients with stable coronary artery disease, compared with a control group. The primary 7-oxygenated product of cholesterol, i.e. 7-hydroperoxycholesterol, seems to be the most cytotoxic oxygenated lipid present in oxidized LDL and in biological membranes (Smith 1987, Chisolm et al. 1994). This oxysterol is rapidly decomposed to 7α-OH, 7β-OH or 7-keto, and all are present at relatively high concentrations in foam cells and fatty streaks (Brown et al. 1997, Vaya et al. 2000). It is generally believed that most, if not all of the 7β-OH is derives from a non-enzymatic origin (Salonen et al. 1997), and its presence specifically characterizes the existence of oxidized LDL, and of OS in general (Bjorkhem & Diczfalusy 2002). This suggests that the elevation and differences in 7β-OH level in diabetics vs. Hc patients, characterize the increased OS elevation in diabetics.

Analysis of the LT moiety of the marker showed a more than four-fold increase in the Ox-LT/LT ratio in the blood of diabetics vs. Hc patients or the control blood. In addition, the LA subunit was more susceptible to oxidation when incubated with blood from diabetics or Hc patients than the Ty subunit. The linoleoyl moiety was converted mainly to hydroperoxide (LT-OOH) and to epoxide (epoxy-LT) in the blood with no oxidation of the Ty residue. Using the designed marker, we further examined its potential to characterize alterations in oxidative status in blood obtained from diabetic patients before and after the consumption of PJ for 3 months. PJ is known to contain high levels of potent antioxidants, such as hydrolyzed and non-hydrolyzed tannins (mostly punicalagin isomers) (Navindra et al. 2006) with a high capacity to scavenge free radicals, to inhibit LDL oxidation in vitro and in vivo, and to inhibit atherosclerotic plaque formation (Gil et al. 2000, Aviram et al. 2002, Aviram et al. 2004). In the present study, PJ significantly reduced the total oxysterol level in blood (Figure 4). The levels of all oxysterols were reduced, with the most pronounced reduction being in the 7-keto and 7β-OH levels.

PJ consumption did not result in any significant reduction in the amounts of cholesterol, glucose level or HbA_{1C} in the serum of diabetic patients (Table II), although it did noticeably reduce their blood oxysterol accumulation (Figure 4) and oxidative capacity, according to their oxidative effect on the LT marker (Figure 5). HbA_{1C} is the result of a modification in haemoglobin, due to reactions between glucose and amino-group side chains on the protein, and its concentration is proportionally increased in diabetic patients (Cohen & Wu 1994, Wolffenbuttel et al. 1996). The decrease in the oxidizability of diabetics' blood could be attributed the ability of PJ to decrease or eliminate ROS/RNS, despite its inherently high glucose concentration. Consumption of PJ by diabetic patients for only 3 months resulted in the inhibition of their blood's capacity to oxidize LT by 93%, as measured in the serum, to levels below that detected in the healthy subjects.

We therefore conclude that the designed marker LT is sufficiently sensitive to detect specific oxidative-state changes in blood from patients under OS, and to identify the preferential susceptible marker subunit to OS. The LT allowed us to evaluate the beneficial effect of nutritional antioxidants (PJ) and, similarly, may further provide data on the effectiveness of various interventions aimed at reducing the oxidizability of cells, tissues or organs. Thus the concept of using exogenous markers aimed at

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characterizing various aspects of OS in vitro and in vivo seems to be feasible. Such exogenous markers are continuously under development; Khatib et al. (2007) constructed a marker containing in addition to the linoleic acid and tyrosine representing PUFA and proteins, a third component, the nucleic acid 2-deoxyguanosine, representing the DNA. Vaya et al. (2007) investigated in addition to the above markers, a third probe build from linoleic acid, tyrosine and cholesterol as an additional component. These markers were incubated with cells (astrocytes, neurons) or mitochondria evaluating the effect of heme oxygenase 1 upregulation on OS status and on sterol levels. Currently, in vivo experiments (animals) are in progress using the above markers and other new synthetic markers, exploring their pharmacokinetic behaviour and types of oxidation they undergo when neurological diseases are developing (unpublished data).

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