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Plasma Oxysterols and Tocopherol in Patients with Diabetes Mellitus and Hyperlipidemia

Hiroshi Murakami[a], Naoki Tamasawa[a,*], Jun Matsui[a], Minoru Yasujima[b], and Toshihiro Suda[a]

[a]Third Department of Internal Medicine and [b]Department of Laboratory Medicine, Hirosaki University School of Medicine, Hirosaki, 036-8562, Japan

ABSTRACT: The plasma levels of free oxysterols (7-ketocholesterol; 7α-hydroxy-,7β-hydroxy-, 25-hydroxy-, and 27-hydroxycholesterol; and 5α,6α-epoxycholestanol) in patients with diabetes mellitus and hypercholesterolemia were determined using gas chromatography–mass spectrometry with selective ion monitoring. We studied 39 patients with diabetes mellitus, 20 nondiabetic patients with hypercholesterolemia, and 37 normal controls. Plasma cholesterol levels in diabetic and hypercholesterolemic patients showed no statistical difference. Plasma 7-ketocholesterol was significantly higher in patients with diabetes (31.6 ± 2.8 ng/mL) or hypercholesterolemia (52.3 ± 5.9) than in the control group (22.4 ± 1.2). The increased plasma cholesterol can be regarded as an oxidation substrate for the oxidant stress and the higher absolute levels of oxysterols in hypercholesterolemic plasma compared with the control plasma. This difference disappeared when 7-ketocholesterol was expressed in proportion to total cholesterol. The oxidizability of plasma cholesterol was evaluated by comparing the increased ratio of 7-ketocholesterol after $CuSO_4$ oxidation to the ratio before. We demonstrated that the patients with diabetes showed increased oxidizability (77.5%) compared with the control (36.6%) or hyperlipemic group (45.3%), which is likely due to the lower amounts of α-tocopherol in the diabetics. Measurement of oxysterols may serve as a marker for *in vivo* oxidized lipoproteins in diabetes and hyperlipemia.

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Oxidation of lipoproteins is believed to play an important role in atherosclerosis in patients with diabetes mellitus (DM) and hypercholesterolemia (HL) (1). When low density lipoprotein (LDL) undergoes oxidation *in vitro*, various changes in lipid composition occur, including a substantial loss of free and esterified cholesterol and the concomitant generation of oxidation products of cholesterol (oxysterols) (2,3). In fact, oxidatively modified lipoproteins in the circulating plasma are reported to contain an oxysterol-enriched subfraction (4,5), and oxysterols are considered to be the important lipid component in oxidized lipoproteins. Oxysterols have potent biological effects, some of which suggest a role in the initiation and/or progression of atherosclerosis (1,6). Further support for an atherogenic role for oxysterols comes from an epidemiological study of Indian migrants who were exposed to oxysterols in their diet: they had higher than expected morbidity and mortality from atherosclerosis but did not show the usual risk factors for this disorder (7).

In this study, we measured plasma free oxysterols in patients with DM, using gas chromatography–mass spectrometry (GC–MS) with selective ion monitoring (SIM). We also compared the oxidizability of plasma cholesterol among the patients on the basis of increased 7-ketocholesterol in response to *in vitro* incubation with $CuSO_4$ (8,9).

MATERIALS AND METHODS

Subjects. Subjects included 39 patients with DM, 20 non-diabetic patients with HL, and 37 normal controls. We defined HL as a total cholesterol level more than 220 mg/dL. Blood was collected in blood collection tubes containing heparin after overnight fasting. One microgram of butylated hydroxytoluene was added per milliliter of plasma, and samples were stored at –20°C until analysis.

Table 1 summarizes the clinical characteristics, including age, sex, body mass index, plasma lipid levels, and glycosylated hemoglobin (HbA1c), for each group of subjects. In the DM group, 18 patients were treated by dietary management, 11 with oral hypoglycemic agents (glibenclamide 1.25–7.5 mg/d), and 10 with insulin. Diabetic complications included nephropathy in 28 patients (microalbuminuria in 18 patients and macroalbuminuria in 10 patients), neuropathy in 10 patients, and retinopathy in 25 patients. The frequencies of diabetic complications were not affected by the presence or absence of hypercholesterolemia. None of the subjects was taking supplementary antioxidants such as probucol or α-tocopherol.

*To whom correspondence should be addressed at Third Department of Internal Medicine, Hirosaki University School of Medicine, Zaifu-cho, Hirosaki, 036-8562, Japan. E-mail: tmsw@cc.hirosaki-u.ac.jp

Abbreviations: Cholestane-3β,6α-diol, 5α-cholestane-3β,6α-diol; cholesterol, cholest-5-en-3β-ol; CV, coefficient of variation; DM, diabetes mellitus; 5α,6α-epoxycholestanol, 5,6α-epoxy-5α-cholestan-3β-ol; GC–MS, gas chromatography–mass spectrometry; HbA1c, glycosylated hemoglobin; HL, hypercholesterolemia; 7α-hydroxycholesterol, cholest-5-ene-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; 25-hydroxycholesterol, cholest-5-ene-3β,25-diol; 27-hydroxycholesterol, cholest-5-ene-3β,27-diol; 7-ketocholesterol, 3β-hydroxycholest-5-en-7-one; LDL, low density lipoprotein; SIM, selective ion monitoring; TC, total cholesterol; TG, triglyceride; TMS, ether, trimethylsilyl ether.

TABLE 1
Clinical Characteristics of Study Subjects[a]

	n	Age (yr)	Male/female ·	BMI (kg/m^2)	HbA1c (%)
Control	37	56.7 ± 3.1	17/20	22.3 ± 1.1	4.9 ± 0.03
DM	39	63.1 ± 1.5	14/25	22.9 ± 0.7	6.7 ± 0.25[b]
HL	20	53.2 ± 2.7	5/15	24.0 ± 0.7	5.2 ± 0.12

[a]Results are expressed as mean ± S.E.
[b]$P < 0.05$ vs. control. Abbreviations: BMI, body mass index; HbA1c, glycosylated hemoglobin; DM, diabetes mellitus; HL, hypercholesterolemia.

Measurement of plasma free oxysterols. Cholestane-3β,6α-diol dissolved in ethanol (22 ng/5 mL) was added to 2 mL of plasma as an internal standard. Plasma lipids were extracted with 20 vol of chloroform/methanol (2:1,vol/vol), and the separation of oxysterols from cholesterol was carried out in an early step in the procedure because it is important to prevent cholesterol autoxidation during sampling. The dried material was dissolved in 4 mL of hexane/dichloromethane(4:1, vol/vol) solution and applied to a packed silica column (800 mg; 0.8 cm i.d., Gasukuro Kogyo Inc., Tokyo, Japan). The column was washed out with 8 mL of hexane/dichloromethane (4:1, vol/vol) solution and 10 mL of dichloromethane, and the free oxysterols were then eluted with 8 mL of ethyl acetate. This fraction was dried and dissolved in 10 mL of chloroform and passed through another silica column (400 mg, 0.8 cm i.d.) to remove cholesterol completely. Then the oxysterols were eluted with 6 mL of ethyl acetate.

The average recovery rate of 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, cholestane-3β,6α-diol, and 25- and 27-hydroxycholesterol were 87.5 ± 2.8, 97.3 ± 8.7, 85.3 ± 9.3, 82.7 ± 9.3, 68.3 ± 8.9, and 82.7 ± 6.3%, respectively.

Analysis by GC–MS. Extracted oxysterols were derivatized to their trimethyl (TMS) ether derivatives by treatment with 350 mL pyridine/hexamethyldisilazane/trimethyl-chlorosilane (3:2:1, by vol) at 60°C for 30 min. After evaporation of the solvent under a nitrogen stream, the residue was dissolved in 10 mL hexane and analyzed by GC–MS, using a Shimadzu gas chromatograph (model GC-17A; Kyoto, Japan) equipped with a high-resolution capillary column, DB-1 (30 m × 0.25 mm i.d., J&W Scientific, CA), and a QP-5000 mass spectrometer with a Shimadzu Chemical Laboratory Analysis System & Software CLASS-5000. The oven temperature program was as follows: 150°C for 2 min, 20°C/min to 280°C, and then 5°C/min to 300°C and held for 10.0 min. Helium was used as the carrier gas at a flow rate of 0.8 mL He/min. The injector was operated in the splitless mode and was kept at 270°C, while the detector transfer line was heated at 280°C. The separator and ion source temperatures were 300°C, and the ionization energy was 70 eV.

7-Ketocholesterol and 7β-hydroxycholesterol were purchased from Sigma Chemical Co. (St. Louis, MO), and 7α-hydroxycholesterol, 5α,6α-epoxycholestanol, 25-hydroxy- and 27-hydroxycholesterol, and cholestane-3β,6α-diol were obtained from Steraloids Inc. (Wilton, NH).

The selected ions used for analysis (*m/z*) and the retention times (min) of the compounds were as follows: 7α-hydroxycholesterol, 456 (M – 90), 14.78; 7β-hydroxycholesterol, 456 (M – 90), 16.19; 5α,6α-epoxycholestanol, 384, 16.64; cholesterol-3β,6α-diol, 458, 16.78; 7-ketocholesterol, 472 (M), 18.74; 25-hydroxycholesterol 131, 18.85; and 27-hydroxycholesterol, 456 (M – 90), 19.82.

Figure 1 presents the total ion chromatogram and mass chromatogram for each of the authentic compounds. Each of the oxysterols and the internal standard (cholestane-3β,6α-diol) were separated and quantified with adequate sensitivity. The detection limit for every oxysterol was under 0.02 ng/mL. In triplicate experiments, the within-day variation (coefficient of variation, CV) for 7α-hydroxy, 7β-hydroxy-, and 7-ketocholesterol was 16.2%, and the day-to-day variation (CV%) within one week was 11.9%.

In a preliminary study, we monitored the formation of oxysterols by $CuSO_4$-catalyzed oxidation of plasma. $CuSO_4$ was added to 2 mL of plasma at a final concentration of either 0.5 or 1.0 mM. Incubation was carried out for 24 h at 37°C. The oxidized plasma samples were subjected to polyacrylamide gel electrophoresis (LipoPhor kit, Jyoko, Tokyo, Japan), and the oxysterols were extracted and measured as described above. As shown in Figure 2, the LDL fraction was dispersed and then it migrated depending on the negative charge. Figure 3 presents the gas chromatograms of oxysterols in the same plasma. The C-7 position of the cholesterol ring was predominantly oxidized, and 7-keto-, 7α-hydroxy-, and 7β-hydroxycholesterol increased as a result of $CuSO_4$ induced oxidation in a dose-dependent fashion. Other oxysterols also increased, however, those increases were not so large as those for the C-7 oxidized products. This finding demonstrated that increases in electrophoretic mobility and in yields of oxysterol products are equally useful as indicators of oxidative modification of lipoproteins.

Next, the oxidizability of the plasma cholesterol was evaluated in response to incubation with $CuSO_4$. Plasma samples were selected randomly from each group (DM, $n = 16$; HL, $n = 8$; control, $n = 10$). After incubation of plasma samples with 100 μM $CuSO_4$ for 24 h at 37°C, oxysterols were measured as described above. The oxidizability of plasma cholesterol was expressed in terms of the ratio (%) of the amount of 7-ketocholesterol after the oxidation to the level before.

Statistical analysis. The values in the text and tables are given as means ± SE. Statistical analysis was performed using analysis of variance and the least significant difference to compare differences among three groups. $P < 0.05$ was considered to represent statistical significance.

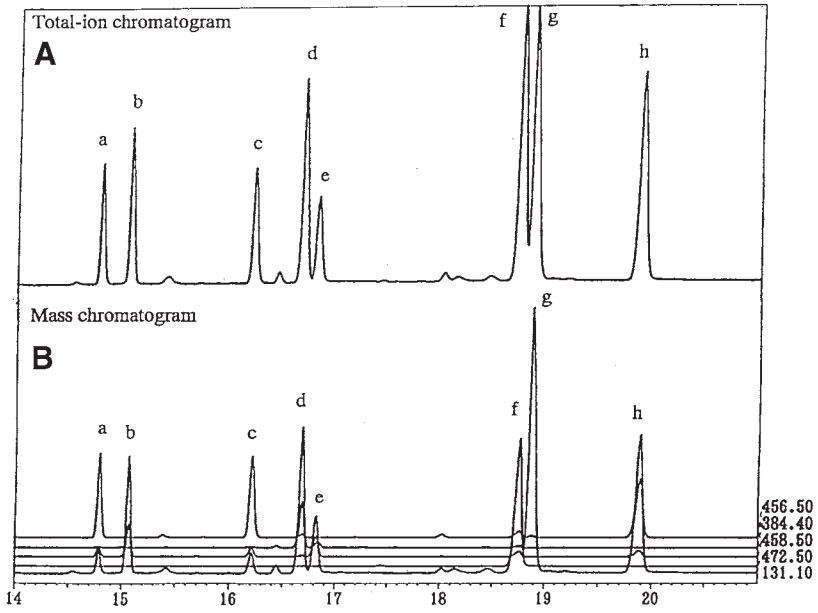

FIG. 1. Gas chromatography–mass spectrometry chromatograms of each oxysterol. Total-ion chromatogram (A) and mass chromatogram (B) are illustrated. The selective ion (*m/z*) of each oxysterol was as follows: 131, 25-hydroxycholesterol (g); 384, 5α,6α-epoxycholesterol (d); 456, 7α-hydroxycholesterol (a), 7β-hydroxycholesterol (c) and 27-hydroxycholesterol (h); 458, cholestane-3β,6α-diol (internal standard) (e); 472, 7-ketocholesterol (f); (b), cholesterol.

RESULTS

Table 2 presents the data on the oxysterol concentrations in each group. Total cholesterol (TC) levels in patients in the DM group showed no difference from those in the HL group (Table 3). There was no difference in oxysterol levels among the subgroups with age or body weight (data not shown). In the DM group, significantly higher absolute levels of 7-keto-, 25-hydroxy- and 27-hydroxycholesterol were observed compared with the control group. In the HL group, all oxysterols except 7α-hydroxycholesterol were significantly increased. 7-Ketocholesterol, the major autoxidation product of cholesterol, was highest in the HL group, and lowest in the control group. However, this difference disappeared when oxysterols were expressed in proportion to TC (Table 3). In the DM group, there was no significant correlation between the oxysterol levels and glycemic control. However, patients with long duration of diabetes showed a tendency to have high plasma oxysterol.

We compared the susceptibility of the plasma cholesterol of each group to oxidation by $CuSO_4$. Based on the results of the preliminary study, as described in Materials and Methods section, we focused on 7-ketocholesterol as the marker of cholesterol autoxidation. 7-Ketocholesterol level before and after the $CuSO_4$-oxidation in each group is indicated in Table 4. The mean increased rate of 7-ketocholesterol from before to after $CuSO_4$ oxidation was 77.5% (23.42 to 41.56 ng/mL) in DM, 45.3% (42.57 to 61.86 ng/mL) in HL, and 36.0% (23.31 to 31.70 ng/mL in the control group. α-Tocopherol levels relative to plasma triglyceride (TG) were also compared, and the value was lowest in the diabetic group as shown in Table 4.

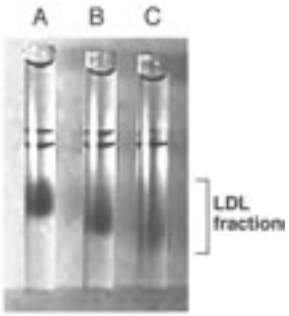

FIG. 2. $CuSO_4$ oxidation of plasma lipoproteins. $CuSO_4$ was added to plasma to a final concentration to 0 (A), 0.5 (B), or 1.0 (C) mM. Incubation was carried out for 24 h at 37°C. Then the oxidized plasma was analyzed by polyacrylamide gel electrophoresis.

DISCUSSION

Oxidation of cholesterol gives rise to a large number of oxidation products (oxysterols) (10). Oxysterols such as 7α-hydroxycholesterol (11) and 27-hydroxycholesterol largely are formed enzymatically and may be involved in elimination of cholesterol from macrophages and endothelial cells (12). On the other hand, a free radical-mediated process (autoxidation) can lead to the formation of a variety of oxysterols, e.g., epoxy, keto-, and hydroxy-derivatives of cholesterol (10). These oxys-

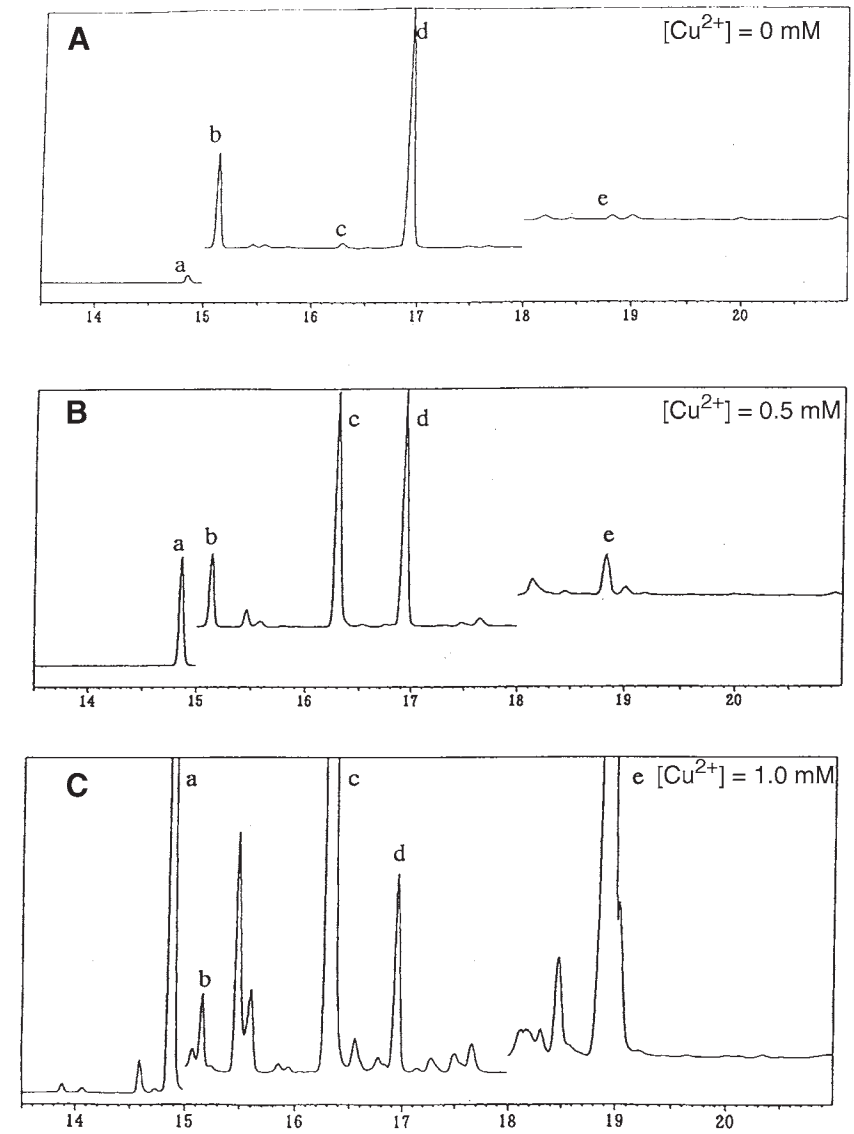

FIG. 3. $CuSO_4$ oxidation of plasma cholesterol. Total-ion chromatograms of plasma oxysterols before and after oxidation with 0, 0.5, or 1.0 mM $CuSO_4$. (a) 7α-Hydroxycholesterol; (b) cholesterol; (c) 7β-hydroxycholesterol; (d) cholestane-3β,6α-diol (internal standard); (e) 7-ketocholesterol.

terols have various biological activities (13), including cytotoxicity (8,10), inhibition of DNA synthesis (14), inhibition of cholesterol synthesis (15), and effects on cell membrane structure and function (16).

Analysis of oxysterols in biological materials is not easy because of the presence of various compounds having structures closely related to cholesterol and because of great differences between their concentrations and the concentration of cholesterol. At present, most analytical methods for oxysterols are based on GC–MS with SIM (17). A substantial portion of the oxysterols in plasma is present as fatty acyl esters like cholesterol (10). Most oxysterols with an established enzymatic origin are esterified to a great extent, whereas other oxysterols are less esterified (17). In order to determine the total or esterified oxysterols by GC, a hydrolysis step is necessary (18). However, the reaction conditions have to be chosen carefully, otherwise, cholesterol autoxidation products induced artifactually could completely mask the endogenous oxysterol levels (19). In our method, plasma free oxysterols were separated from free and esterified cholesterol, and the analytical procedures were practical and highly reliable. Each molecular species was analyzed using a GC-quadrupole mass spectrometer with SIM, and sufficient sensitivity for plasma oxysterols was demonstrated.

The oxidation of LDL may possibly take place during its circulation in the blood. Actually, the lipoprotein particles enriched in oxidatively modified lipids and proteins are proposed

TABLE 2
Plasma Levels of Free Oxysterols (ng/mL) in Study Groups[a]

	n	7α-Hydroxy-cholesterol	7β-Hydroxy-cholesterol	5α,6α-Epoxy-cholestanol	7-Keto-cholesterol	25-Hydroxy-cholesterol	27-Hydroxy-cholesterol
Control	37	36.53 ± 3.52	2.85 ± 0.16	5.85 ± 1.11	22.42 ± 1.20	0.07 ± 0.01	4.77 ± 0.50
DM	39	33.51 ± 2.60	2.84 ± 0.19	8.84 ± 1.15	31.59 ± 2.78[a]	0.20 ± 0.01[a]	8.30 ± 0.50[a]
HL	20	41.93 ± 5.59	3.56 ± 0.24[a,b]	12.72 ± 2.03[a]	52.33 ± 5.85[a,b]	0.19 ± 0.02[a]	10.05 ± 0.80[a,b]

[a]Results are expressed as mean ± SE. [a]$P < 0.05$ vs. control; [b]$P < 0.05$ vs. DM; for abbreviations see Table 1.

TABLE 3
Plasma Lipids and α-Tocopherol of Study Groups[a]

Group	No.	TC (mg/dL)	7KC (ng/ML)	T.OXY (ng/mL)	7KC/TC (× 10^{-4})	T.OXY/TC (× 10^{-4})	TG (mg/dL)	αToc (μg/mL)	αToc/TG (μg/mg TG%)
Control	37	168.5 ± 6.0	22.42 ± 1.2	72.58 ± 4.3	0.15 ± 0.011	0.46 ± 0.03	85.0 ± 4.1	19.2 ± 1.8	0.23 ± 0.03
DM	39	226.5 ± 10.0[a]	31.59 ± 2.8[a]	85.27 ± 5.1	0.15 ± 0.019	0.41 ± 0.04	121.1 ± 16.6[a]	28.1 ± 1.8[a]	0.32 ± 0.03
HL	20	244.9 ± 10.7[a]	52.33 ± 5.9[a]	120.78 ± 9.5[a,b]	0.19 ± 0.028	0.45 ± 0.05	101.9 ± 9.9	34.5 ± 3.6[a]	0.34 ± 0.03

[a]Results are expressed as means ± SE. [a]$P < 0.05$ vs. control; [b]$P < 0.05$ vs. DM. Abbreviations: 7KC, 7-ketocholesterol; T.OXY, total oxysterol (which includes 7α-, 7β-, 25-, 27-hydroxy-, 5α,6α-epoxy-, and 7keto-cholesterol); αToc, α-tocopherol. For other abbreviations see Table 1.

TABLE 4
Plasma Oxysterols After $CuSO_4$-Oxidation[a]

			Before oxidation		After oxidation				
Group	No.	TC (mg/dL)	7KC-n (ng/mL)	7KC-n/TC (× 10^{-4})	7KC-a (ng/mL)	7KC-a/TC (× 10^{-4})	αToc (μg/mL)	TG (mg/dL)	αToc/TG (μg/mg TG%)
Control	10	178.1 ± 10.5	23.31 ± 1.58	0.13 ± 0.01	31.70 ± 3.06	0.18 ± 0.02	25.0 ± 2.9	77.5 ± 6.0	0.34 ± 0.05
DM	16	244.3 ± 7.9[a]	23.42 ± 2.24	0.10 ± 0.01	41.56 ± 5.03	0.18 ± 0.02	29.7 ± 1.9	144.5 ± 25.2[a]	0.27 ± 0.04
HL	8	270.9 ± 17.8[a]	42.57 ± 8.00[a,b]	0.17 ± 0.03[b]	61.86 ± 7.47[a,b]	0.24 ± 0.04	42.2 ± 7.9[a,b]	134.1 ± 21.5	0.35 ± 0.06

[a]Results are expressed as means ± SE. [a]$P < 0.05$ vs. control; [b]$P < 0.05$ vs. DM. Abbreviations: 7KC, 7-ketocholesterol; T.OXY, total oxysterol (which includes 7α-, 7β-, 25-, 27-hydroxy-, 5α,6α-epoxy-, and 7keto-cholesterol); αToc, α-tocopherol. For other abbreviations see Table 1.

to exist in the plasma on the basis of measurements of small amounts of plasma cholesterol oxides (4), fragmented apolipoprotein B (20), and thiobarbituric acid-reactive substances (21). Hodis *et al.* (4) reported that circulating oxidatively modified lipoproteins in the monkey contain an oxysterol-enriched subfraction (53% of cholesterol as oxysterols), and oxysterols are important lipid components of oxidized LDL (22,23).

We evaluated the extent of cholesterol oxidation by assessing the plasma free oxysterol levels. In our study, the plasma free 7-ketocholesterol levels in patients with DM and HL were significantly higher than those in normal subjects. However, there was no difference between the groups when expressed in proportion to total cholesterol level (Table 3). The increased plasma levels of cholesterol can be regarded as an oxidation substrate to oxidation stress, by endothelial cells and activated monocytes (24). This may explain the higher absolute levels of oxysterols in hypercholesterolemic plasma compared with the control plasma.

DM has been proposed to be a state of increased free radical activity and high oxidant stress (25,26). This may include increased nonenzymatic glycosylation, which can also be associated with generation of oxygen free radicals (27,28). Although the factors determining the susceptibility of LDL to oxidation are not thoroughly understood (29), $CuSO_4$ oxidation of lipoproteins has been widely used to examine LDL oxidation (8,11). As demonstrated in the present study, free oxysterols, particularly C-7 oxidized oxysterols, are major products of $CuSO_4$ oxidation of plasma lipids. In this study, we compared plasma levels of 7-ketocholesterol and 7-ketocholesterol/TC after $CuSO_4$ oxidation (100 μM) with the basal level (Table 4). We found that the rate of 7-ketocholesterol/TC in the DM group was higher than that in other groups, likely owing to the lowest amounts of α-tocopherol in expression per plasma TG (Table 4). The small number of data from these groups did not allow statistical difference to be identified, however, our results may indicate one reason for the susceptibility of plasma cholesterol to $CuSO_4$ oxidation in diabetics in our study. However, reports on the levels of antioxidants as defense mechanisms (i.e., superoxide dismutase, vitamin E, or C, etc.) in diabetic patients are still contradictory: both increases and decreases of antioxidant activity are reported (30). Oxidizability has also been postulated to increase in small, dense LDL, which is commonly found in diabetic patients with hypertriglyceridemia (29). The patient's age, sex, type of treatment, and diabetic complications had no correlation with plasma oxysterol levels (data not shown).

In conclusion, plasma free oxysterol levels were measured using a GC–MS SIM method. Plasma oxysterol levels were high in patients with DM and HL. Our study suggests the possibility that oxysterols may serve as a marker for *in vivo* lipoprotein oxidative modification (31).

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