Existence of 7α- and 7β-Hydroperoxycholest-5-en-3β-ols in Lipoproteins from Diabetic Patients and Normal Subjects

Ryoichi Sakamaki¹, Seishi Nagano¹, Shinji Yamazaki², Naoki Ozawa², Mitsuru Tateishi², Haruhiro Okuda³, and Tadashi Watabe³

¹First Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan. ²Drug Metabolism and Analytical Chemistry Research, Upjohn Pharmaceuticals Limited Tsukuba Research Laboratories, Ibaraki, Japan. ³Laboratory of Drug Metabolism and Toxicology, Tokyo College of Pharmacy, Tokyo, Japan.

We report evidence for the presence of 7α- and 7β-hydroperoxycholest-5-en-3β-ols (cholesterol 7-hydroperoxides, Ch 7α-OOH and Ch 7β-OOH, respectively) in human plasma lipoproteins in vivo, which had been reported to be markers of aging in rat skin. A comparative study was carried out focusing on the detection of Ch 7-OOHs in plasma lipoproteins from diabetic patients whose plasma has been suggested to be under high oxidative stress. Blood samples were collected from healthy volunteers (control) and diabetics with and without hypercholesterolemia. Ch 7-OOHs were isolated from low and high density lipoproteins (LDL and HDL, respectively) in the plasma of these subjects, identified, and determined by high-performance liquid chromatography with a chemiluminescence detector. The percent detection of Ch 7-OOHs in LDL from diabetics without hypercholesterolemia was similar to that in the control group. However, it was significantly higher in diabetics with hypercholesterolemia than in those without hypercholesterolemia. The percent detection of Ch 7-OOHs in HDL from diabetics without hypercholesterolemia was higher than both that in LDL from the same group and that in HDL from the control group. J Atheroscler Thromb, 1994; 1: 80-86.

Key words: Hypercholesterolemia, Lipid peroxidation, High-performance liquid chromatography (HPLC), Chemiluminescence detector

Clinically, atherosclerosis which has been shown to be increased in diabetic patients, is known to be a major cause of morbidity and mortality. The Framingham Heart Study showed that risks of coronary heart disease, cerebrovascular disease, and intermittent claudication in diabetic males aged 45 to 74 are two, three, and four times as high as those in non-diabetic males, respectively (1). Several lines of evidence indicate that modified low density lipoprotein (LDL), especially oxidized LDL, may play an important role in the development of atherosclerosis in vivo (2-4). The increase in lipid peroxidation in diabetic patients was suggested to be involved in glycation of LDL based on the finding that non-enzymatic glycation of LDL induced by incubation with a high concentration of glucose increases lipid peroxidation, as monitored by the formation of thiobarbituric acid-reacting substances (TBARS) in vitro (5). Although some reports have suggested by monitoring TBARS formation that lipid peroxidation is increased in the plasma (6) or erythrocytes (7) of diabetic patients, no direct evidence has yet been reported for the existence of a relationship between lipid
peroxidation and diabetes.

However, TBARS, good markers for lipid peroxidation in vitro, are merely degradation products of polyunsaturated fatty acid hydroperoxides (PUFA-OOHs) of lipids, and are known to be poor markers in vivo because those formed in lipoprotein in vivo have been suggested to be largely released into plasma and to be rapidly biotransformed (8). Actually, most TBARS formed in lipoprotein by lipid peroxidation in vitro are known to exist in the reaction media (8).

Recently, high-performance liquid chromatography (HPLC) with a chemiluminescence (CL) detector has been developed for detecting various lipid hydroperoxides and hydrogen peroxide at picomole levels (9).

Using HPLC-CL, Ozawa et al. showed the presence of 7α- and 7β-hydroperoxycholest-5-en-3β-ols (Ch 7α-OOH and Ch 7β-OOH, respectively) in rat skin which were stable and accumulated, and compared with PUFA-OOHs in vivo, were good markers for aging during the 1 to 45 weeks after birth, suggesting Ch 7-OOHs to be better markers for lipid peroxidation in vivo than TBARS and PUFA-OOHs (10). However, no evidence has yet been reported for the occurrence of Ch 7-OOHs in human lipoproteins in vivo (11), although hydroperoxides have been observed in human LDL oxidized with Cu²⁺ in vitro (12).

In the present report, we provide evidence for the presence of Ch 7-OOHs in human LDL and high density lipoprotein (HDL) isolated from healthy volunteers (control), and diabetic subjects both with and without hypercholesterolemia (HC; total cholesterol > 250 mg/dl). In addition, a higher percent detection of hydroperoxides was observed in LDL and HDL from diabetics with and without HC, respectively, as compared with those in the corresponding plasma lipoproteins from controls.

### Materials and Methods

#### Materials

5α-Hydroperoxycholest-6-en-3β-ol (13) and 7β-hydroperoxychol-5-ene-3β-ol (Sitosterol 7β-OOH) (14) were prepared as previously reported. Ch 7α-OOH was prepared by rearrangement of 5α-hydroperoxycholest-6-en-3β-ol and recrystallized from hexane-ether as previous reported (15); mp 152-153.5°C; ¹H NMR δ 0.66 (3H, s, 18-H), 0.86 (6H, d, J = 6.6 Hz, 26-H, 27-H), 0.92 (3H, d, J = 6.6 Hz, 21-H), 1.00 (3H, s, 19-H), 3.62 (1H, m, 7-H), 4.16 (1H, m, 7-H), and 5.72 (1H, dd, J = 5.0 Hz, J = 1.9 Hz, 6-H); MS m/z [relative intensity (%)] 436 (M+., 2), 418 (3), 401 (3), 385 (65), 367 (100). Ch 7β-OOH was prepared by epimerization of Ch 7α-OOH and recrystallized from hexane-ether after chromatography on a silica gel thin layer plate as previously reported (16); mp 145-147°C; ¹H NMR δ 0.69 (3H, s, 18-H), 0.86 (6H, d, J = 6.6 Hz, 26-H, 27-H), 0.92 (3H, d, J = 6.6 Hz, 21-H), 1.05 (3H, s, 19-H), 3.59 (1H, m, 7-H), 4.13 (1H, m, 7-H), and 5.58 (1H, broad s, 6-H); MS m/z [relative intensity (%)] 436 (M+, 3), 418 (3), 401 (16), 385 (53), 367 (100).

Cholesterol esterase (EC 3.1.1.13), microperoxidase (MP-11) and isoluminol (6-amino-2, 3-dihydro-1, 4-phthalazinedione) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA), and [1α, 2α(n)-3H] cholesterol from Amersham International plc (Amersham, U.K.). Other chemicals used were of reagent grade.

#### Isolation of lipoproteins

Blood samples (20 ml) were collected from healthy volunteers (n = 12), and diabetic subjects with and without HC (n = 13 and 17, respectively) into test tubes containing EDTA (0.4 μmol). Clinical characteristics, including hemoglobin A₁c and plasma lipid levels, in each group of subjects are shown in Table 1. Patients with HC were treated with diet (n = 8), an oral hypoglycemic agent (n = 3) and insulin (n = 2), and those without HC, with diet (n = 10),

<table>
<thead>
<tr>
<th>Table 1. Clinical characteristics, including hemoglobin A₁c and plasma lipid levels, in control and diabetic subjects.</th>
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<tr>
<td>n²</td>
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<tr>
<td>Sex (male : female)</td>
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<tr>
<td>Age</td>
</tr>
<tr>
<td>Hemoglobin A₁c (%)</td>
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<tr>
<td>Triglyceride (mg/dl)</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
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<tr>
<td>LDL cholesterol (mg/dl)</td>
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<tr>
<td>HDL cholesterol (mg/dl)</td>
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<tr>
<td>Apolipoprotein B (mg/dl)</td>
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<td>Apolipoprotein A-I (mg/dl)</td>
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</table>

α: hypercholesterolemia; n: number of subjects; α Values are mean ± SD

*p < 0.05, **p < 0.01 control vs. diabetics with and without HC; *p < 0.05, **p < 0.01 diabetics without HC vs. diabetics with HC.
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oral hypoglycemic agent (n=4) and insulin (n=3). The numbers of diabetic patients with HC complicated with nephropathy, neuropathy, and retinopathy were three, two, and zero, respectively, and five, two, and one, respectively, in those without HC.

To 4 ml of plasma separated from the samples by centrifugation at 1,500 × g for 15 min at 4°C were added 40 μl of ethanol containing 10% (w/v) butylated hydroxytoluene (BHT) and 1.5% (w/v) 2,5-dimethylfuran (DMF) as antioxidants. LDL and HDL were isolated from 4 ml of the plasma by ultracentrifugation by the method of Hatch and Lees (17).

Extraction and partial purification of Ch 7-OOHs

Ch 7-OOHs were extracted by the method of Ozawa et al. (10) with slight modifications as follows: to 1.5-2.0 ml of the above lipoprotein fraction were added 10 ml of chloroform-methanol (2:1, v/v) containing 0.1% (w/v) BHT, 0.015% (w/v) DMF and Sito 7β-OOH (100 pmol), an internal standard, and the mixture was agitated and centrifuged at 2,000×g for 15 min. The organic layer was separated and applied to a Bond Elut® Sil column (22×40 mm, Analytichem International, Harbar City, CA, USA). The solvent was evaporated in vacuo at room temperature to dryness from the organic phase obtained by elution of the column with 20 ml of chloroform. The residue obtained was dissolved in 0.2 ml of tert-butanol containing 5% (w/v) Triton X-100, and then incubated at 37°C for 15 min with cholesterol esterase (10 units) in 1.8 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA. After incubation, Ch 7-OOHs were extracted with 3 ml of chloroform, and the extract was applied to a Bond Elut® NH₂ column (10×18 mm, Analytichem International). After eluting the column with 3 ml of ethyl acetate, the eluate was evaporated in vacuo at room temperature to dryness for subsequent analysis by HPLC-CL.

HPLC-CL measurements of Ch 7-OOHs

The above sample from the Bond Elut® NH₂ column was dissolved in the chromatographic mobile phase (100 μl) described below, and an aliquot (20 μl) of the sample solution was subjected to HPLC (pump : 880-PA, JASCO, Tokyo, Japan; mobile phase : methanol/water, 90:10 (v/v); flow rate : 1 ml/min) or a chiral phase column (Chiralcel OD, 4.6×250 mm, Daicel Chemical Industries, Tokyo, Japan; mobile phase : acetonitrile/water, 80:20 (v/v); flow rate : 0.8 ml/min) and a chemiluminescence detector (825-CL, JASCO, chemiluminescence reagent : isoluminol, 10 μg/ml, and microperoxidase, 20 μg/ml, in 50 mM borate buffer, pH 9.5; flow rate : 1.2 ml/min). HPLC was carried out at room temperature.

Spectroscopy

'1H NMR spectra were recorded in CDCl₃ on a Brucker Model AM-300 spectrometer with Si(CH₃)₄ as an internal standard. Mass spectra were obtained at 70 eV for the ionization voltage with a Hewlett Packard Model 5988A spectrometer equipped with a Hewlett Packard Model 5890 gas chromatography system (column ; DB-17, 0.25 mm×10 m, oven temperature 200 to 280°C, 10°C/min, injection port temperature ; 270°C, and flow rate of carrier gas ; 0.9 ml He/min).

Statistical analysis

Statistical analyses were performed using Student’s unpaired t-test and the χ²-test to compare differences between groups as appropriate.

Results

Four peaks due to hydroperoxides selectively detectable with the CL detector appeared in chromatograms obtained by HPLC-CL of partially purified neutral lipid fractions from plasma LDL and HDL in some of healthy volunteers and diabetic subjects with and without HC. Three of the hydroperoxide peaks were identified on two different columns eluted with solvent mixtures consisting of different components as Ch 7α-OOH, Ch 7β-OOH, and Sito 7β-OOH, an internal standard, by co-HPLC with the corresponding authentic specimens. Ch 7α-OOH, Ch 7β-OOH, and Sito 7β-OOH were eluted at retention times of 10.0, 12.2, and 15.0 min from the chiral phase column and of 16.2, 17.1, and 19.3 min from the reverse phase column, respectively (Fig. 1). The peaks identified as Ch 7α-OOH and Ch 7β-OOH always appeared as sharp singlets in chromatograms obtained on both columns without splitting off from the co-chromatographed authentic specimens. The hydroperoxide peak eluted behind the internal standard from the both columns was unidentified, but found in most of samples examined.

The CL detector, whose sensitivity was influenced by the solvent used as a mobile phase, showed higher sensitivities to the steroid hydroperoxides eluted from the chiral phase column than from the reverse phase column. Therefore, determination of Ch 7-OOHs in plasma lipoproteins was carried out on the chiral phase column. The intensity of CL emitted from Ch 7α- and 7β-OOHs with the chemiluminescence reagents in the detector differed markedly, and was recorded as peaks with areas of different components as Ch 7α-OOH, Ch 7β-OOH, and Ch 7α- and 7β-OOHs always appeared as sharp singlets in chromatograms obtained on both columns without splitting off from the co-chromatographed authentic specimens.

Sito 7α-OOH and Ch 7β-OOH always appeared as sharp singlets in chromatograms obtained on both columns without splitting off from the co-chromatographed authentic specimens. The hydroperoxide peak eluted behind the internal standard from the both columns was unidentified, but found in most of samples examined.

The CL detector, whose sensitivity was influenced by the solvent used as a mobile phase, showed higher sensitivities to the steroid hydroperoxides eluted from the chiral phase column than from the reverse phase column. Therefore, determination of Ch 7-OOHs in plasma lipoproteins was carried out on the chiral phase column. The intensity of CL emitted from Ch 7α- and 7β-OOHs with the chemiluminescence reagents in the detector differed markedly, and was recorded as peaks with areas in the ratio 2:1 in chromatograms, with detection limits of 2 and 4 pmol, respectively, on the chiral phase column.

The antioxidants BHT and DMF, added as a mixture to the lipoprotein fractions containing EDTA were effective in retarding the formation as artifacts of Ch 7-OOHs and their fatty acid esters from cholesterol and its esters throughout their extraction from the lipoprotein fractions, incubation with cholesterol esterase, and partial purification, whereas addition of BHT alone increased the
artificial formation of Ch 7-OOHs. This was confirmed in the present study as reported previously (10) using \(^{3}H\) cholesterol which was added to the solvent used for the extraction of lipids from plasma. No detectable levels of \(^{3}H\) Ch 7-OOHs were formed from \(^{3}H\) cholesterol as checked by the previously reported method (10), including the separation of radioactive hydroperoxides diluted with unlabeled Ch 7-OOHs from radioactive cholesterol by HPLC monitored with a UV detector, followed by liquid scintillation counting of the chromatographic fractions. Ch 7-OOHs in the partially purified fractions separated from cholesterol before HPLC-CL were stable enough to be stored at 4°C for at least 2 weeks without decomposition, even in the presence of the antioxidants.

Table 2 shows the mean values of concentrations of Ch 7-OOHs in lipoproteins of subjects whose plasma contained the hydroperoxides. The ratio of the concent-

[Table 2]

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Hydroperoxide</th>
<th>Control</th>
<th>Diabetics without HC</th>
<th>Diabetics with HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>Ch 7α-OOH</td>
<td>7.6 ± 0.7</td>
<td>6.7 ± 2.4</td>
<td>6.6 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Ch 7β-OOH</td>
<td>7.6 ± 1.7</td>
<td>13.9 ± 8.2</td>
<td>10.8 ± 8.3</td>
</tr>
<tr>
<td>HDL</td>
<td>Ch 7α-OOH</td>
<td>6.7 ± 0.4</td>
<td>10.4 ± 3.7</td>
<td>4.9 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Ch 7β-OOH</td>
<td>10.6</td>
<td>19.1 ± 9.6</td>
<td>9.1 ± 3.5</td>
</tr>
</tbody>
</table>

HC: hypercholesterolemia; Values are mean ± SD in Ch 7-OOHs-positive subjects. The number of positive subjects in control, diabetics with HC and diabetics without HC were 4, 9 and 3 respectively.
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A

B

Fig. 2. Detection of Ch 7-OOHs in lipoproteins from controls, and diabetics with and without hypercholesterolemia. A, from LDL; B, from HDL. LDL and HDL were isolated from controls (n=12), diabetics (DM, n=17), and diabetics with hypercholesterolemia (DM+HC, n=13).

**Percent detection of Ch 7-OOHs in LDL from DM+HC was significantly higher than in that from DM (p <0.01). *Percent detection of Ch 7-OOHs in HDL from DM was higher than in that from controls (p <0.05).

Percent detection of Ch 7-OOHs in LDL from each group is shown in Fig. 2. The percent detection of Ch 7-OOHs in LDL from diabetics without HC was not higher than that in LDL from the control group, whereas in diabetics with HC, that in LDL was higher than that in LDL from those without HC (p <0.01). On the other hand, the percent detection of Ch 7-OOHs in HDL from diabetics without HC was higher than that in HDL from the control group (p <0.05).

Percent detection of Ch 7-OOHs in diabetics with and without HC was not related to the patient’s age, sex, clinical treatment, diabetic complication, duration of diabetes or triglyceride levels.

Discussion

Various oxidation products of cholesterol have been reported to exist or to be formed in human plasma lipoproteins both in vivo (18) and in vitro (19-22), such as 7α- and 7β-hydroxycholest-5-en-3β-ols, 3β-hydroxycholest-5-en-7-one, 5, 6α-epoxy-5α- and 5, 6β-epoxy-5β-cholestan-3β-ols, cholesa-3, 5-dien-7-one, cholest-4-en-3-one, and 25-hydroxycholest-5-en-3β-ol (11, 18-23). Ch 7α- and 7β-OOHs have been demonstrated to be precursors of 7α- and 7β-hydroxycholest-5-en-3β-ols and 3β-hydroxycholest-5-en-7-one in vitro (24). However, these cholesterol 7-oxidation products are also formed by cytochrome P-450 (25, 26) or subsequent dehydrogenation, and their formation does not directly reflect the peroxidation of cholesterol. Therefore, it is important to determine the precursor, Ch 7-OOHs, directly linked to the cholesterol 7-oxidation products in relation to diabetes, which has been suggested to be caused by hyperoxidative stress (6). In addition, no evidence has
yet been presented for a relationship between diabetes and the 7-hydroxy and -oxo derivatives of cholesterol. Recently, 7-OOHs were isolated from human LDL oxidized with Cu^{2+} in vitro and identified with authentic specimens (12). However, nothing is known of the existence of Ch 7α- and 7β-OOHs in human plasma lipoproteins in vivo, although Ozawa et al. have demonstrated their existence in rat skin by HPLC-CL, and reported that hydroperoxides are good markers for aging (10).

Based on previous studies using rat liver microsomes in the presence of ADP-Fe^{3+} and an NADPH-generating system (27), oxidation at the 7-position of cholesterol is most likely to be initiated by peroxidation of lipid PUFA residues (LH). Lipid peroxyl radicals (LOO·) may initiate the formation of an allylic carbon radical of cholesterol which readily affords Ch 7-OOHs via cholesterol 7-peroxyl radicals with formation of 7β-hydroperoxide being preferential to that of 7α-hydroperoxide (Fig. 3). Under the same conditions, cholesterol has been demonstrated to yield 5, 6α-epoxy-5α- and 5, 6β-epoxy-5β-cholestan-3β-ols via 6-alkylperoxy-5-radicals (28-30).

Earlier studies carried out by Sato et al. demonstrated the TBARS value of LDL to be higher in diabetic patients than in normal subjects, suggesting that diabetic patients were under higher oxidative stress (6). Based on these studies, two interesting studies have recently been reported using human plasma LDL and HDL. One of these subjects under the conditions used (31). In contrast, the latter study found no difference in TBARS value in LDL between both groups of subjects.

The present study was carried out to investigate the suggested higher oxidative stress in diabetic patients. We determined 7-OOHs as markers for lipid peroxidation in their plasma lipoproteins, as our unpublished data indicated that there was little difference in TBARS value in LDL between normal and diabetic subjects. Ch 7-OOHs, however, were detected in LDL and HDL from diabetic patients, but also from normal subjects. The ratio of Ch 7-OOHs to apolipoprotein B levels in LDL, as well as that of the hydroperoxides to apolipoprotein A-I levels in HDL, were not higher in diabetics than in normal subjects (data not shown). Therefore, the data in the present study provide no conclusive evidence for a relationship between diabetes and enhanced lipid peroxidation in plasma lipoproteins. However, we provide the first evidence for the presence of Ch 7-OOHs in human lipoproteins in vivo, and the percent detection of Ch 7-OOHs in LDL and HDL were significantly higher in diabetics with and without HC, respectively. These results suggest that diabetic patients may be under higher oxidative stress. A further study will be necessary to confirm the above suggestion by investigating a larger number of cases.

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