Ozonation of Cholesterol in the Presence of Ethanol: Identification of a Cytotoxic Ethoxyhydroperoxide Molecule

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ABSTRACT: Cholesterol ozonation was carried out in ethanol-containing aqueous or nonaqueous solvent, and the ozonized products were analyzed by chemiluminescence detection-HPLC with on-line electrospray MS (HPLC-CL-MS) and characterized on the basis of NMR and FABMS. After the ozonolysis of cholesterol in water/ethanol (aqueous system) as well as in chloroform/ethanol (nonaqueous system), a unique ethoxyhydroperoxide molecule (7α-ethoxy-3β-hydroxy-5α-B-homo-6-oxacholestane-5-hydroperoxide, termed "7α-ethoxy-5-OOH") appeared as main ozonation product. In addition to structural analysis, we confirmed the remarkable cytotoxicity of 7α-ethoxy-5-OOH toward human lung adenocarcinoma A549 cells and found that its cytotoxicity is superior to that of the commonly known autoxidized cholesterol (3β-hydroxycholest-5-ene-7-one). Hence, 7α-ethoxy-5-OOH is a toxic molecule of primary importance, arising during cholesterol ozonation in the presence of ethanol.

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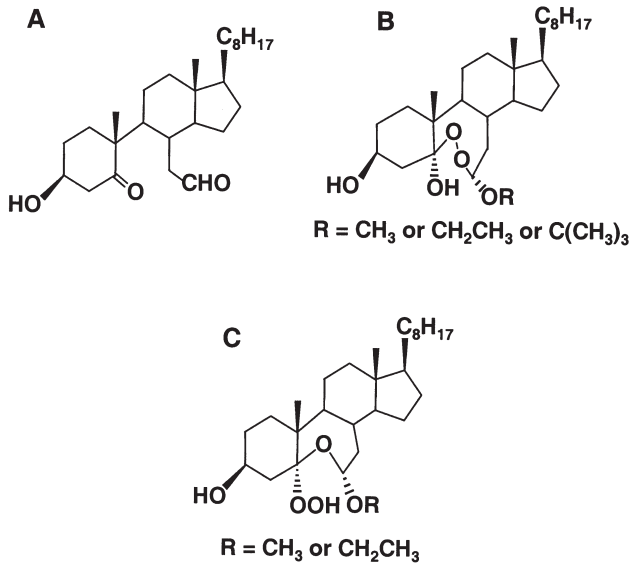

SCHEME 1

Ozone, a very powerful oxidant, is frequently used for disinfection, deodorization, and bleaching of wastewater and polluted air as well as foodstuffs (1). Since the primary targets of ozone are unsaturated lipids in cell membranes and foods, the chemistry of ozone reactions with lipids has been studied in detail (2–6). In particular, studies have focused on the ozonolysis of cholesterol that yields various oxygenated cholesterol derivatives (7–9).

In the case of ozonation of cholesterol in organic solvents, the reaction gave the expected ozonide, which was easily reduced to 3β-hydroxy-5-oxo-5,6-secocholestan-6-al as a major product (Scheme 1A) (8,10). The same compound also was formed in aqueous environments (7,9). By contrast, the ozonation of cholesterol in alcohol-containing solvent proceeded *via* a unique pathway that yielded a unique solvent-participated product (7). In 1988, Jaworski and Smith (11) investigated the reaction product of cholesterol with ozone in the presence of alcohol, and they proposed its structure to be an epidioxide, 7a-alkoxy-B-dihomo-6,7-dioxacholestan-3β,5-diol (Scheme 1B). This epidioxide structure was corrected to "alkoxy-hydroperoxide" (Scheme 1C; 7α-alkoxy-3β-hydroxy-5α-B-homo-6-oxacholestane-5-hydroperoxide) on the basis of recent NMR and X-ray studies (12–14).

Despite recent progress in the structural elucidation of ozonized cholesterol, information on its formation and accumulation in food and biological samples has never been sought. In addition, little is known about the biological role of cholesterol ozonation products, and whether ozonized cholesterol is biologically more active than the commonly known oxysterols (e.g., 3β-hydroxycholest-5-ene-7-one, a major product of cholesterol autoxidation) has not been evaluated. The causes of such uncertainty are many, but foremost among them might be the lack of a suitable analytical method for measuring ozonized cholesterol directly. Hence, in this study, we report on an assay of cholesterol ozonation products and

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Abbreviations: CL, chemiluminescence; ESI, electrospray ionization; 7α-ethoxy-5-OOH, 7α-ethoxy-3β-hydroxy-5α-B-homo-6-oxacholestane-5-hydroperoxide; FAB, fast-atom bombardment mode; 7-OOH, 3β-hydroxycholest-5-ene-7-hydroperoxide; NBA, *m*-nitrobenzyl alcohol; TIC, total ion current.

its use as a tool to gain insight into the role of these compounds. Using a chemiluminescence detection-HPLC system with on-line MS (HPLC-CL-MS), which we previously developed to measure ozonized phospholipids sensitively and selectively (15), we found that 7α-ethoxy-3β-hydroxy-5α-B-homo-6-oxacholestane-5-hydroperoxide (termed "7α-ethoxy-5-OOH") is a major product of cholesterol ozonolysis carried out in an ethanol-containing aqueous or nonaqueous solvent. In addition, we confirmed that 7α-ethoxy-5-OOH is highly toxic for human lung tumor cells.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol and FBS were purchased from ICN Biomedicals Inc. (Aurora, OH). The 3β-hydroxycholest-5-ene-7-one and RPMI-1640 medium were obtained from Sigma (St. Louis, MO). Human lung adenocarcinoma A549 cells (TKG0184) were from the Cell Resource Center for Biomedical Research at Tohoku University School of Medicine (Sendai, Japan). All other reagents were of analytical grade.

Generation of ozone. An EO-301 ozonator (Okano Works, Osaka, Japan) was used for generating a 1.3% ozone-in-oxygen stream. Cholesterol sample solutions were bubbled at a flow rate of 100 mL/min under various conditions as described below.

Ozonation of cholesterol in chloroform/ethanol. Cholesterol (460 mg) was dissolved in 100 mL of chloroform/ethanol (1:1, vol/vol) and treated with ozone gas for 20 min under ice-cold conditions (7). After ozonation, the reaction mixture was dried and redissolved in 10 mL of chloroform. An aliquot portion (10 μL) was subjected to HPLC-CL-MS (detailed conditions as described below) for monitoring the yields of ozonized lipids.

Ozonation of cholesterol in water. Cholesterol (100 mg) was dissolved in 50 mL of acetone and then mixed with water (120 mL). The resultant mixture (170 mL) was concentrated to nearly 100 mL in a rotary evaporator. A 50-mL portion of the concentrated aqueous dispersion (containing 50 mg of cholesterol) was treated with ozone gas for 1 h at room temperature. After that, 50 mL of chloroform was added to the reaction mixture, shaken vigorously, and centrifuged at $1000 \times g$ for 10 min. The organic layer was collected, evaporated, and redissolved in 10 mL of chloroform. A 10-μL sample of the chloroform solution was subjected to HPLC-CL-MS.

Ozonation of cholesterol in water/ethanol. Cholesterol (50 mg) in 100 mL of water/ethanol (1:1) was ozonized for 1 h at room temperature. The ozonation products were extracted with chloroform and analyzed by HPLC-CL-MS.

HPLC analysis. For the HPLC-CL-MS system (15), an ODS column (TSK-gel 80Ts, 5 μm, 4.6 × 250 mm; Tosoh, Tokyo, Japan) was used. The column was eluted using a binary gradient, consisting of the following HPLC solvents: A (water) and B (methanol). Ammonium acetate (0.1 mM) was added to both mobile phases A and B. The gradient profile was as follows: 0–10 min, 90% B; 10–15 min, 90–100% B linear; 15–30 min, 100% B. The flow rate was adjusted to 1 mL/min, and the column temperature was maintained at 40°C. At the postcolumn, the eluent was split. One of the eluents (flow rate, 0.95 mL/min) was mixed with the hydroperoxide-specific CL reagent (a mixture of cytochrome *c* and luminol in 50 mM borate buffer, pH 10) (16,17) and sent to a JASCO 825-CL detector (Japan Spectroscopic Co., Tokyo, Japan). The flow rate of CL reagent was 0.9 mL/min. The other column eluent (flow rate, 0.05 mL/min) was sent to a Mariner electrospray ionization (ESI)/time-of-flight mass spectrometer (Applied Biosystems, Farmington, MA). Parameters for MS were positive-ion measurement mode, a spray voltage of 1900 V, a nozzle potential of 150 V, and a nozzle temperature of 150°C. The flow rate of nebulizer gas was 0.3 mL/min. Full scan spectra were obtained by scanning ions between *m/z* 300 and 900 at 4 s/scan.

Identification procedures. By using the HPLC technique, ozonized cholesterol was isolated and purified from the sample reaction mixture, and its chemical structure was characterized by the following spectroscopic procedures. The mass of the purified ozonation product was determined using a JEOL-JMS-700 mass station (JEOL, Tokyo, Japan) in fast-atom bombardment mode (FAB). FAB-MS measurement was performed in the positive and negative mode with *m*-nitrobenzyl alcohol (NBA) as a matrix. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 600 spectrometer (Varian, Palo Alto, CA) at 600 MHz for ^{1}H NMR and at 150 MHz for ^{13}C NMR using $CDCl_3$ as a solvent. The IR spectrum was assayed using JEOL JIR-5500.

Cytotoxicity of ozonide. Lung adenocarcinoma A549 cells were seeded on 96-well culture plates at densities of 1×10^4 cells/well in 100 μL of RPMI-1640 medium containing 10% FBS, penicillin G (100 units/mL), and streptomycin (100 μg/mL). After incubation at 37°C in a 5% CO_2 incubator for 24 h, the culture medium was replaced with 100 μL of FBS-free medium containing test samples at various concentrations. Twenty-four hours later, 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution was added to each well for evaluating cell viability (18). After 3 h of incubation, cells were washed with 0.2 mL of PBS, resuspended in 200 μL of 2-propanol containing 0.04 N HCl, and left in the dark for 10 min. The plates were measured by a microplate reader (Elx 800; Bio-Tek Instruments Inc., Winoski, Vermont) with a wavelength of 570 nm and a reference wavelength of 630 nm.

Statistical analysis. The data were expressed as the mean ± SD. Statistical comparisons were made with Student's *t*-test.

RESULTS AND DISCUSSION

Among three different reaction conditions, we first tested the chloroform/ethanol system. Figure 1A shows the typical total ion current (TIC) chromatogram, when ozone gas-exposed cholesterol in chloroform/ethanol (1:1) was subjected to HPLC-CL-MS. After ozonation, cholesterol itself was completely decomposed, and two large peak components ***1*** and ***2*** appeared as the main ozonation products in the TIC chromatogram (Fig. 1A). Peaks ***1*** (retention time = 18 min) and ***2*** (25

min) gave clear ESI/MS spectra (ions at *m/z* 497 for ***1*** and 434, 416 for ***2***) (Figs. 1B and 1C). In the CL chromatogram, both ***1*** and ***2*** gave intense chemiluminescence peaks (retention time of 18 min for ***1*** and 25 min for ***2***) (Fig. 1D), indicating that both peak components might possess hydroperoxide groups.

To determine the chemical structures of compounds ***1*** and ***2***, these components were isolated by HPLC, and their structures were determined using FAB-MS and NMR. Peak ***2*** was identified as the ethoxy-hydroperoxide, 7α-ethoxy-3β-hydroxy-5α-B-homo-6-oxacholestane-5-hydroperoxide (7α-ethoxy-5-OOH, Scheme 2): (–) FAB-MS (70 eV, NBA) $[M - H]^-$ *m/z* 479; (+) FAB-MS (70 eV, NBA) $[M - CH_3CH_2OH - H_2O + H]^+$ *m/z* 417; ESI-MS *m/z* 434, 416; m.p. 133–135°C; IR (KBr) 3275, 1169, 1142, 1068, 1041, 1016, 987, 953 cm^{-1}. ^1H NMR ($CDCl_3$:δ): 2.00 (2H, *m*, 7a-H), 2.65 (1H, *d*, 4α-H), 3.99 and 3.58 (2H, AB *x* 3 *m*, $O\underline{C}H_2CH_3$), 3.89 (1H, *m*, 3α-H), 4.74 (1H, *t*, 7-H), 10.26 ppm (1H, *s*, 5-OOH). ^{13}C NMR ($CDCl_3$:δ): 15.1 ($OCH_2\underline{C}H_3$), 63.9 ($O\underline{C}H_2CH_3$), 66.8 (C-3), 100.7 (C-7), 111.8 ppm (C-5). In the ^1H NMR measurement, an important signal at δ10.26, characteristic of the hydroperoxide group, was clearly confirmed (Fig. 2). Considering the structure of 7α-ethoxy-5-OOH, the unknown ESI/MS spectrum of peak 2 from the HPLC-CL-MS analysis (Fig. 1C) would correspond to the fragment ions of 7α-ethoxy-5-OOH: $[M - CH_3CH_2OH]^+$ *m/z* 434 and $[M - CH_3CH_2OH - H_2O]^+$ *m/z* 416. On the other hand, peak component ***1*** (*m/z* 497 in the ESI/MS analysis) was speculated to be a novel ozonized cholesterol. The amount of component ***1*** available in the present study was not sufficient to carry out NMR and other identification procedures. Therefore, the structure of component ***1*** could not be determined.

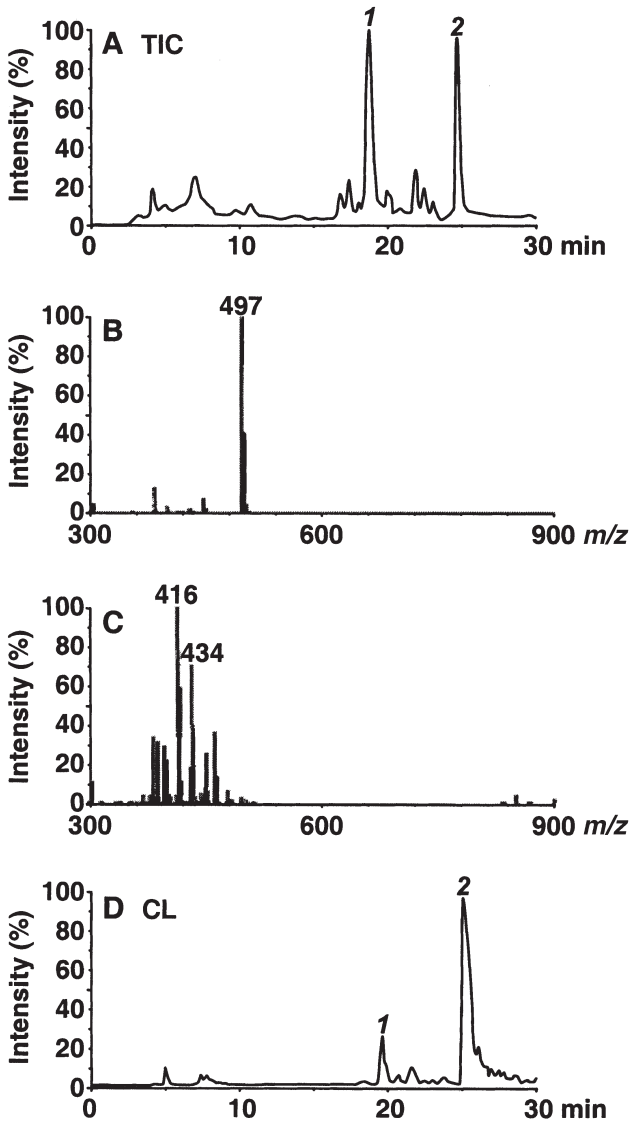
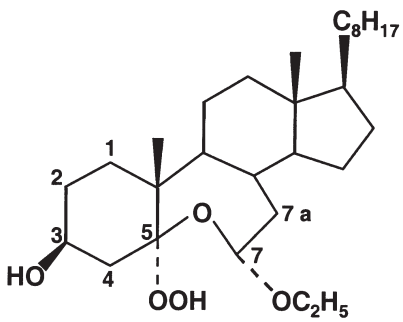


FIG. 1. Ozonation products of cholesterol in chloroform/ethanol system. Cholesterol (460 mg) in chloroform/ethanol (1:1) was exposed to ozone gas for 20 min and analyzed by HPLC-CL-MS. (A) Total ion current (TIC) chromatogram; (B) mass spectrum of peak ***1*** (18 min) detected in chromatogram A; (C) mass spectrum of peak ***2*** (25 min) in chromatogram A; (D) chemiluminescence (CL) chromatogram.

In our opinion, the ozonation that takes place in aqueous environments (rather than in organic solvent) better reflects the reaction of lipids with ozone gas that actually occurs in food and biological samples. However, little is known about cholesterol ozonation in aqueous systems, except for the study by Gumulka and Smith (7). Thus, we then investigated the reaction of cholesterol with ozone in water/ethanol or in water alone. Figure 3 shows the typical TIC and CL chromatograms of the ozonized cholesterol in water/ethanol (1:1). Peaks ascribed to component ***1*** (retention time = 18 min) and 7α-ethoxy-5-OOH (25 min) were clearly detected in TIC and CL chromatograms, together with another peak component ***3*** (20 min) that produces strong chemiluminescence (Fig. 3). Peak ***3*** showed the molecular ion $[M - H_2O]^+$ *m/z* 400, and the same retention time as the synthetic reference 3β-hydroxycholest-5-ene-7α-hydroperoxide (7α-OOH) and 3β-hydroxycholest-5-ene-7β-hydroperoxide (7β-OOH) (19). Hence, Peak ***3*** was tentatively identified as a mixture of isomers of 7α-OOH and 7β-OOH, which are the well-known oxidized cholesterol. However, the formation of 7α-OOH and 7β-OOH during ozonation has not been reported in the past. Therefore, if such epimeric 7-hydroperoxides are actually formed, this mechanism is interesting. Further study would be needed to elucidate whether 7α-OOH and 7β-OOH arise from ozone action on cholesterol. In the case of cholesterol ozonation in water, component ***1*** and 7α-ethoxy-5-OOH were absent entirely (data not shown). Thus, component ***1*** and 7α-ethoxy-5-OOH would appear to be principal products during ozonation in the presence of alcohol (ethanol).



SCHEME 2

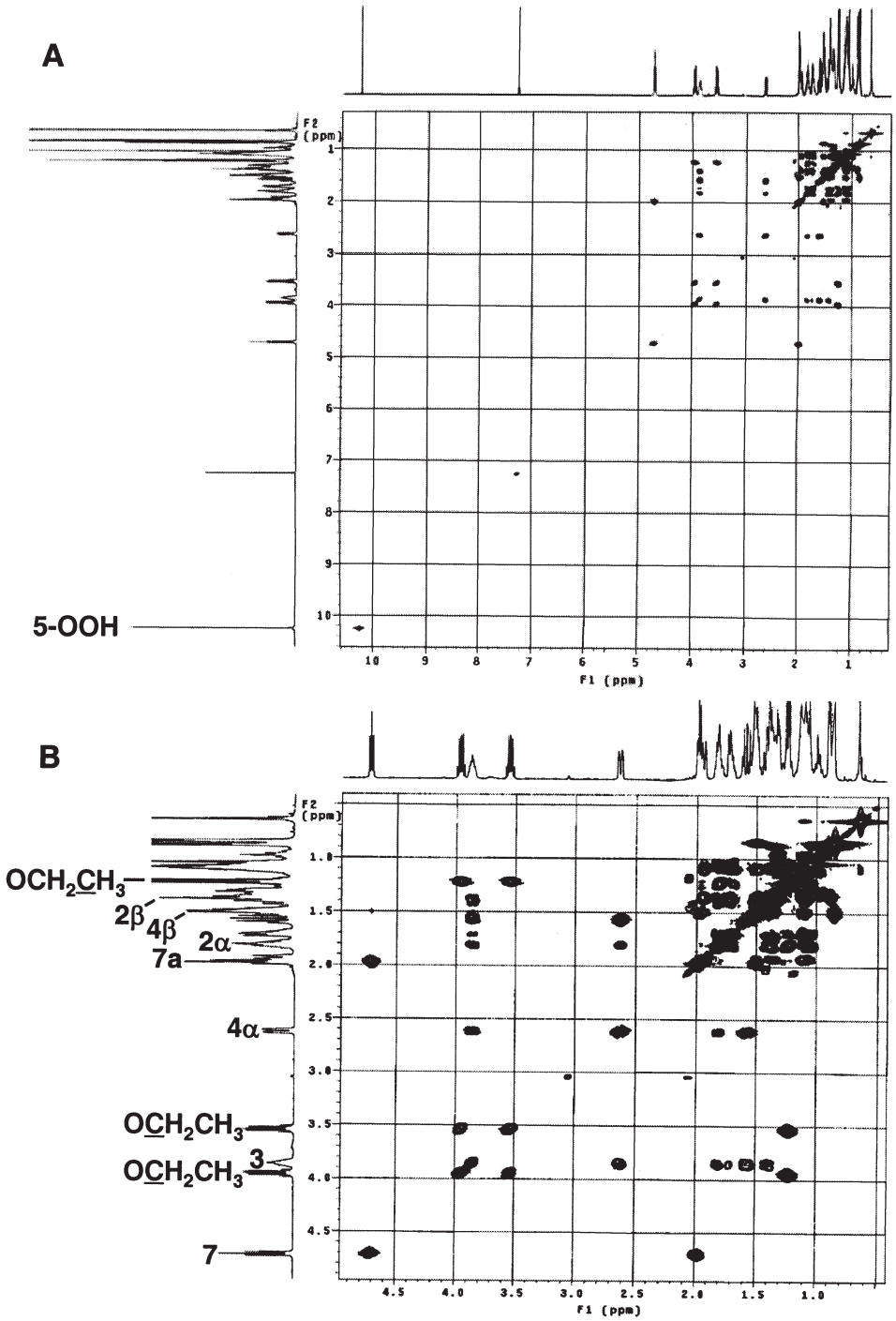


FIG. 2. (A) ^{1}H-^{1}H correlation spectroscopy 2-D NMR spectrum of 7α-ethoxy-3β-hydroxy-5α-B-homo-6-oxacholestane-5-hydroperoxide (7α-ethoxy-5-OOH). (B) An expanded spectrum between 0 and 5.0 ppm.

In considering the present results, it could be rationalized that 7α-ethoxy-5-OOH are formed by the Criegee mechanism (14) during cholesterol ozonation in the presence of ethanol. A possible 7α-ethoxy-5-OOH formation pathway is shown in Scheme 3. The ozonation of cholesterol gives a primary ozonide ***4***, and the ozonide is converted into carbonyl oxide intermediate ***5***. The intramolecular partial capture of ***5*** by the 6-carbonyl oxygen yields the dipolar intermediate ***6***. Ethanol can readily react with the intermediate ***6*** to form 7α-ethoxy-5-OOH. In the presence of water, the intermediate ***6*** reacts with H_2O, which gives secoaldehyde ***7***.

It is generally difficult to characterize the structure of ozonized cholesterol, and caution is required when judging whether ozonized cholesterol possesses a hydroperoxide group. Consequently, a simple and reliable method for characterizing ozonized cholesterol has been highly desired. As

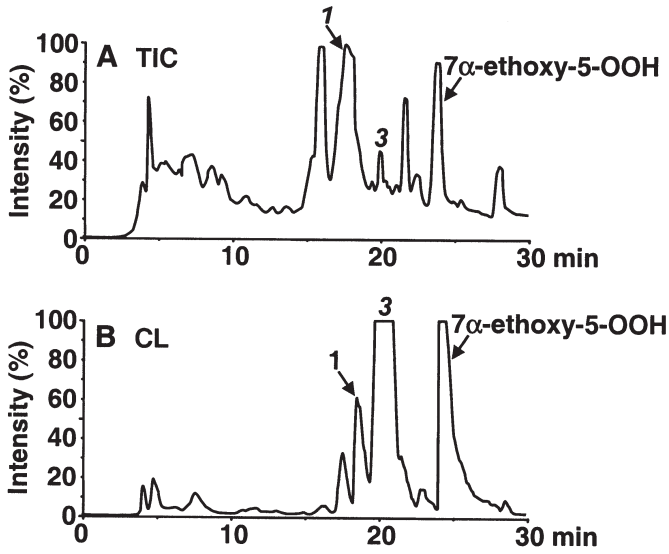
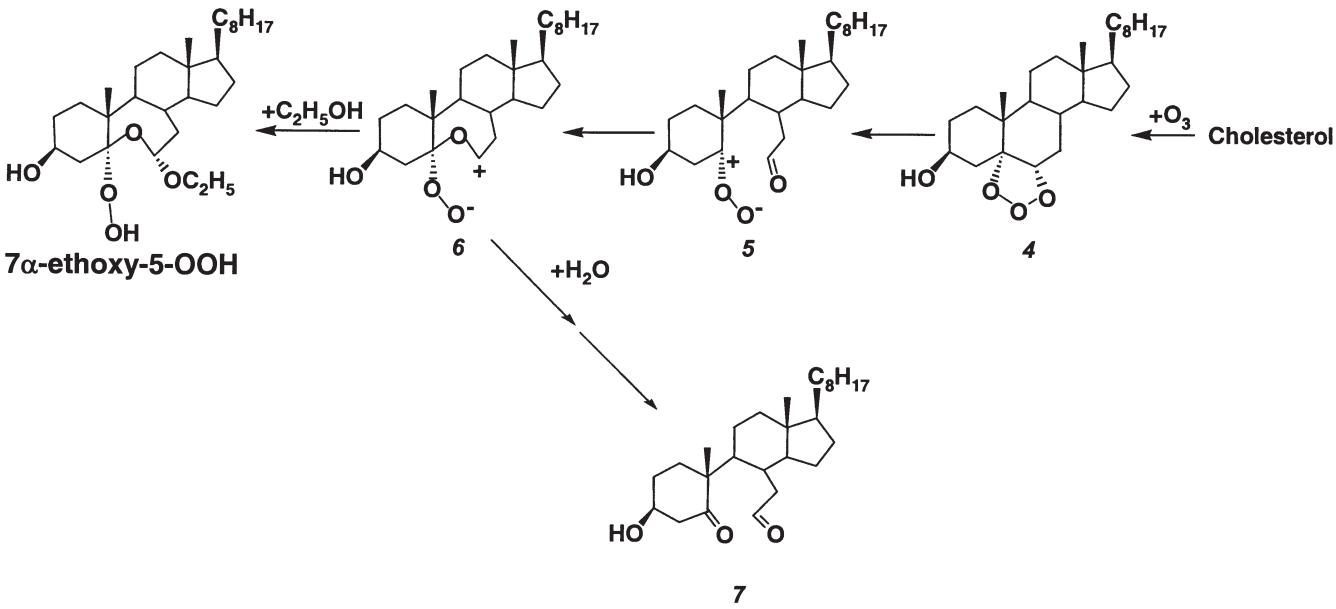


FIG. 3. Ozonation products of cholesterol in water/ethanol system. Cholesterol (50 mg) in water/ethanol (1:1) was exposed to ozone gas for 1 h and then analyzed by HPLC-CL-MS. (A) TIC chromatogram; (B) CL chromatogram. For abbreviations see Figure 1.

shown in Figures 1 and 3, we succeeded in detecting 7α-ethoxy-5-OOH by using the HPLC-CL-MS system. This analytical system can easily identify the cholesterol ozonides bearing a hydroperoxide moiety, because detection by CL is highly specific for the hydroperoxide group (15–17,19–23).

Next, the cytotoxic effect of 7α-ethoxy-5-OOH on human lung adenocarcinoma A549 cells was investigated because the biological role of the cholesterol ozonation products has never been evaluated. We used 3β-hydroxycholest-5-ene-7-one as a reference compound. Treating A549 cells with 7α-ethoxy-5-OOH (1–20 μg/mL), we confirmed that the compound significantly reduced the number of viable cells (Fig. 4). Especially, 20 μg/mL of 7α-ethoxy-5-OOH resulted in an approximately 70% reduction in the viability of lung tumor cells. In contrast, 3β-hydroxycholest-5-ene-7-one was less toxic, even at 20 μg/mL. These results clearly indicated that ethoxyhydroperoxide (7α-ethoxy-5-OOH) arising during cholesterol ozonation is a cytotoxic molecule and that its cytotoxicity is greater than that of 3β-hydroxycholest-5-ene-7-one. This implies that ozonized cholesterol may be more toxic than autoxidized cholesterol.



SCHEME 3

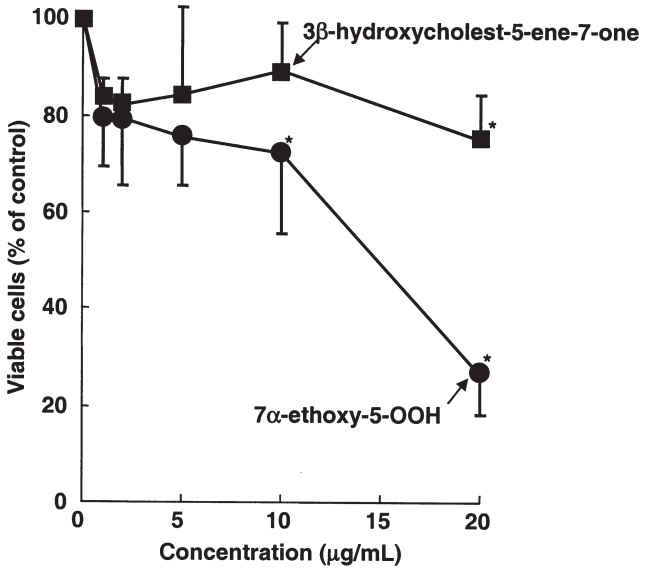


FIG. 4. Effects of increasing concentrations of 7α-ethoxy-3β-hydroxy-5α-B-homo-6-oxacholestane-5-hydroperoxide (7α-ethoxy-5-OOH) and 3β-hydroxycholest-5-ene-7-one on the viability of human lung adenocarcinoma A549 cells. Cells were incubated in medium containing 7α-ethoxy-5-OOH (●) or 3β-hydroxycholest-5-ene-7-one (■) at concentrations from 0 (control) to 20 μg/mL for 24 h. The viable cells were then assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are expressed as mean ± SD; $n = 6$. $^*P < 0.05$ as compared to control cells.

As for the sterilization of foods, the use of a combination of different disinfection procedures (i.e., treatment with ozone together with alcohol or UV light) was recently recommended to enhance bactericidal effect (24,25). If one is applying ozone/ethanol treatment for food disinfection, 7α-ethoxy-5-OOH may be formed from food cholesterol. This compound would be toxic due to its highly reactive hydroperoxide group, thereby influencing the quality and flavor of food. Our HPLC-CL-MS system might be used to elucidate compounds formed by ozone-sterilization of foods, since CL detection enables easy identification of the hydroperoxide structure.

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