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Occurrence of cytotoxic 9-oxononanoyl secosterol aldehydes in human low density lipoprotein

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Running title: Acyl esters of secosterol aldehydes

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The following abbreviations are used: LDL, low density lipoprotein; secosterol-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secosterol-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde; C16:0-CE, cholesteryl palmitate; C18:1-CE, cholesteryl oleate; C18:2-CE, cholesteryl linoleate; 9-ONC, 9-oxononanoyl cholesterol; 9-ON-secoA, 9-oxononanoyl secosterol-A; 9-ON-secoB, 9-oxononanoyl secosterol-B; 5-OVA, 5-oxovaleroyl cholesterol; ROS, reactive oxygen species; DH, dansyl hydrazine; MRM, multiple reaction monitoring

Abstract

The reaction products of three major cholesteryl esters present in human low density lipoprotein (LDL), cholesteryl palmitate (C16:0-CE), cholesteryl oleate (C18:1-CE), and cholesteryl linoleate (C18:2-CE), treated with ozone were isolated and characterized. *In vitro* ozonization of C16:0-CE was found to form the palmitoyl ester of secosterol-A (3β-hydroxy-5-oxo-5,6-secocholestan-6-al) and its aldolization product secosterol-B (3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde). On the other hand, when C18:1-CE and C18:2-CE were oxidized by ozone, the aldehyde 9-oxononanoyl cholesterol (9-ONC) was formed as a primary product, which was then further oxidized to form 9-oxononanoyl secosterol-A (9-ON-secoA) and 9-oxononanoyl secosterol-B (9-ON-secoB). The compounds 9-ON-secoA and -B, but not 9-ONC, were found to exhibit strong cytotoxicity against human leukemia HL-60 cells. An LC-ESI-MS/MS method was developed for the detection of these cholesteryl ester ozonolysis products by derivatizing them with dansyl hydrazine. Using this method, we found for the first time that low concentrations of 9-ON-secoA and -B, but not palmitoyl secosterols, were present in human LDL. These novel oxidized cholesterol esters, 9-ON-secoA and -B, likely play important roles in the pathogenesis of several inflammatory disorders such as cancer, diabetes, atherosclerosis and neurodegenerative diseases.

Key words: LDL, cholesteryl ester, secosterol, LC-ESI-MS/MS, cytotoxicity.

INTRODUCTION

Low density lipoprotein (LDL) particles contain cholesteryl esters and triacylglycerols surrounded by a shell composed of phospholipids, free cholesterol and apolipoprotein B100. LDL particles are prone to oxidative modifications caused by enzymatic attack of myeloperoxidase and lipoxygenase, or by reactive oxygen species (ROS) such as hypochlorous acid (HOCl), phenoxyl radical intermediates or peroxynitrite generated in the innermost layer of the artery during inflammation and atherosclerosis. The peroxidation of fatty acid residues in phospholipids, cholesteryl esters, and triacylglycerols generates reactive aldehydes and truncated lipids. They play important roles in the early development of atherosclerosis through the recruitment of monocyte-derived macrophages into the arterial wall, and by promoting the intracellular accumulation of cholesteryl esters in these cells, resulting in the formation of foam cells [1]. In the necrotic core of an atherosclerotic lesion, dying foam cells can release their contents, oxidized cholesteryl esters and other lipid oxidation products, which then accumulate in the extracellular space.

Acyl chain oxidations of cholesteryl esters lead to the formation of corresponding lipid hydroperoxides, which then undergo carbon-carbon cleavage, yielding aldehydes which are still esterified to the parental lipid, termed a core-aldehyde [2]. 5-Oxovaleroyl cholesterol (5-OVA) and 9-oxononanoyl cholesterol (9-ONC) have been reported as two major core-aldehydes derived from the oxidation of cholesteryl arachidonate and of cholesteryl linoleate and oleate, respectively [2]. Some of them are thought to result from cellular damage, whereas others may play important physiological roles in a variety of cellular functions, including the regulation of cholesterol homeostasis. In addition, cholesterol is also endogenously oxidized enzymatically or non-enzymatically to form various oxidized cholesterols [3]. The enzymatic oxidations are mainly performed by the cytochrome P450 superfamily, resulting in the formation of 24*S*-hydroxy (24*S*-OH) cholesterol, 27-OH

cholesterol, and 25-OH cholesterol. The non-enzymatic oxidation products 7-ketocholesetrol, 7β-OH cholesterol, and 7α-OH cholesterol are mainly formed by the direct attack of various ROS on the ring structure. 7α-OH cholesterol and 25-OH cholesterol are formed via both enzymatic and non-enzymatic reactions. Secosterol-A (3β-hydroxy-5-oxo-5,6-secocholestan-6-al) and its aldolization product secosterol-B (3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde) have been recently reported to be major cholesterol oxidation products formed by cholesterol ozonolysis [4-6]. Elevated levels of secosterol-A and -B have been detected in human atherosclerotic plaques [7] and brain tissues of neurodegenerative diseases, such as Alzheimer's disease and Lewy body dementia [8, 9]. Secosterols exert strong cytotoxic effects toward various culture cells [7, 10-12] and have the ability to covalently modify proteins, such as the p53, amyloid-β peptide and α-synuclein to accelerate amyloidogenesis and fibrilization [9, 13-15]. We have reported the mechanism of secosterol-A and -B formation in phorbol myristate acetate (PMA)-activated HL-60 cell cultures, which was found to be myeloperoxidase-dependent [16]. Moreover, we revealed that secosterol-A can inhibit activities of endothelial nitric oxide synthase (NOS) and neuronal NOS, but not inducible NOS [17]. On the basis of these findings, secosterols have been postulated to play pivotal roles in the pathogenesis of atherosclerosis and neurodegenerative diseases. Cholesterol can be present in various tissues as its free form or acyl esters, the latter compounds are present in much higher concentrations than the free one, the ratio of free cholesterol and its acyl esters is estimated at 3:7 in the blood [18]. These cholesteryl esters can be oxidized similarly to free cholesterol, resulting in the formation of various oxidation products. However no studies have been reported concerning physiological levels and/or bioactivities of these compounds.

In this study, we have characterized the ozonolysis products of cholesteryl esters

and developed a sensitive and specific method to analyze these compounds in biological specimens. Using this method, we were able to detect 9-oxononanoly secosterol-A and -B (9-ON-secoA and 9-ON-secoB, respectively) for the first time in healthy human LDL. We also found that 9-ON-secoA and -B, but not 9-ONC, exert potent cytotoxicity in HL-60 cells, as reported for secosterol-A and -B. Our findings suggest that 9-ON-secoA and -B may play important roles in the development of several inflammation-related diseases such as cancer, diabetes, atherosclerosis and neurodegenerative disease.

MATERIAL AND METHODS

Materials

Cholesterol, cholesteryl palmitate (C16:0-CE), cholesteryl oleate (C18:1-CE), and cholesteryl linoleate (C18:2-CE) were purchased from Sigma-Aldrich, St. Louis, MO. Dansyl hydrazine (DH) and RPMI 1640 medium were purchased from Invitrogen (Carlsbad, CA) and Nissui Pharmaceutical Co., Ltd., Tokyo, Japan, respectively. All other chemicals were obtained from Wako Pure Chemical Industries, Osaka, Japan.

Preparation of secosterol-A and -B

Secosterol-A and -B and 3,4-^{13}C-secosterol-B, as an internal standard for LC-ESI-MS/MS analyses, were synthesized as reported previously [19]. The purity of the product was verified by TLC and ^{1}H-NMR. The stock solutions (10 mM) of secosterols were prepared in ethanol and stored at -20°C until use.

Preparation of secosterol esters

A solution of cholesteryl palmitate (500 mg, 0.8 mmol) was prepared in a mixture of chloroform/methanol (9:1) (100 ml). The solution was cooled to dry ice temperature and ozone (210 μmol/min) was bubbled through the sample for 10 min. The reaction mixture was evaporated under reduced pressure and stirred with Zn powder (650 mg, 10 mmol) in

water/acetic acid (1:9) (50 ml) at room temperature for 3 h and diluted with dichloromethane (100 ml). The mixture was then washed with water (3 × 50 ml), dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (EtOAc/*n*-hexane (1:6)) to give the compound (5-oxo-5,6-secocholestan-6-al-3β-palmitate, C16:0-secoA) as a white solid (104.7 mg, 20%). Data for C16:0-secoA: R_f = 0.4 (EtOAc/*n*-hexane (1:6)); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.61 (s, 1H, CHO-7), 5.38 (s, 1H, H-3), 3.06 (dd, *J*=14.4, 4.4 Hz, 1H, H-4); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 216.08 (C-5), 202.64, 172.99, 73.05, 56.12, 54.12, 52.33, 44.04, 43.27, 42.54, 42.09, 39.85, 39.47, 35.97, 35.75, 34.81, 34.48, 34.43, 31.93, 29.69, 29.67, 29.60, 29.45, 29.37, 29.24, 29.07, 28.01, 27.83, 25.15, 24.93, 23.75, 23.08, 22.80, 22.70, 22.55, 18.56, 17.64, 14.13, 11.52; DART-TOF/MS (positive) calcd for $C_{43}H_{77}O_4$ *m/z* $[M+H]^+$ 657.5822, found 657.5807. Aldolization of C16:0-secoA**.** A solution of C16:0-secoA (53.8 mg, 0.08 mmol) in acetonitrile/water (20:1) (100 ml) was added to L-proline (24 mg, 0.21 mmol) and stirred for 2 h in room temperature. The mixture was evaporated under reduced pressure and dissolved in EtOAc (50 ml) and washed with water (3 × 50 ml). The combined organic layers were dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (EtOAc/*n*-hexane (1:6)) to give the compound (5β-hydroxyl-B-norcholestane-6β-carboxaldehyde-3β-palmitate, C16:0-secoB) as a white solid (34.3 mg, 65%). Data for C16:0-secoB: R_f = 0.4 (EtOAc/*n*-hexane (1:6)); ^{1}H NMR (400 MHz, $CDCl_3$) δ: ^{13}C NMR (400 MHz, $CDCl_3$) δ: 9.68 (d, *J*=3.0 Hz, 1H, CHO-7), 5.13 (m, 1H, H-3), 2.51 (s, 1H, OH-5); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 203.83, 172.47, 83.66 (C-5), 70.15, 64.51, 56.04, 55.67, 51.38, 45.62, 44.67, 42.32, 39.65, 39.48, 39.25, 36.19, 35.62, 34.66, 31.93, 29.67, 29.64, 29.58, 29.45, 29.37, 29.23, 29.10, 28.35, 28.01, 27.96, 25.01, 24.66, 24.44, 23.82, 22.80, 22.70, 22.55, 21.57, 18.75, 18.34, 14.13, 12.50;

DART-TOF/MS (positive) calcd for $C_{43}H_{77}O_4$ *m/z* $[M+H]^+$ 657.5822, found 657.5869.

Using the above procedures, cholesteryl oleate (500 mg, 0.77 mmol) or cholesteryl linoleate (600 mg, 0.92 mmol) was oxidized with ozone to synthesize 5-oxo-5,6-secocholestan-6-al-3β-(9-oxononanoate) (9-ON-secoA) as a white solid (111.3 mg, 25%) or (33.2 mg, 6%). Data for 9-ON-secoA: R_f = 0.3 (EtOAc/*n*-hexane (1:3)); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.76 (t, *J*=2.0 Hz, 1H, CHO-9), 9.61 (s, 1H, CHO-7), 5.38 (s, 1H, H-3), 3.06 (dd, *J*=14.4, 4.4 Hz, 1H, H-4); ^{13}C NMR (400 MHz, $CDCl_3$) δ: 216.08 (C-5), 202.84 (C-9'), 202.64 (C-7), 172.84 (C-1'), 73.14 (C-3), 56.12, 54.13, 52.34, 44.04, 43.84, 43.26, 42.54, 42.11, 39.85, 39.46, 35.97, 35.74, 34.80, 34.45, 34.38, 28.94, 28.92, 28.79, 28.71, 28.00, 27.82, 25.31, 25.15, 24.79, 23.74, 23.07, 22.79, 22.54, 21.98, 18.56, 17.64; DART-TOF/MS (positive) calcd for $C_{36}H_{61}O_5$ *m/z* $[M+H]^+$ 573.4519, found 573.4410.

9-ON-secoA (69.7 mg, 0.12 mmol) was used to synthesize 5β-hydroxyl-B-norcholestane-6β-carboxaldehyde-3β-(9-oxononanoate) (9-ON-secoB) as a white solid (21.9 mg, 32%) as described above. Data for 9-ON-secoB: R_f = 0.4 (EtOAc/*n*-hexane (1:4)); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.76 (t, *J*=1.6 Hz, 1H, CHO-9'), 9.68 (d, *J*=2.8 Hz, 1H, CHO-7), 5.13 (m, 1H, H-3), 2.50 (s, 1H, OH-5); ^{13}C NMR (400 MHz, $CDCl_3$) δ: 203.84 (C-9'), 202.71 (C-7), 172.35 (C-1'), 83.65 (C-5), 70.17, 64.55, 56.03, 55.68, 51.49, 45.61, 44.66, 43.82, 42.29, 39.65, 39.48, 39.31, 36.19, 35.60, 34.55, 28.92, 28.82, 28.33, 27.99, 24.86, 24.66, 23.82, 22.78, 22.54, 21.95, 21.57, 18.75, 18.28, 12.49; DART-TOF/MS (positive) calcd for $C_{36}H_{61}O_5$ *m/z* $[M+H]^+$ 573.4519, found 573.4607.

Preparation of 9-oxononanoyl cholesterol. The authentic 9-oxononanoly cholesterol was prepared as previously reported [20].

Preparation of human LDL sample

This study was approved by the University of Shizuoka Ethical Committee. Ten ml each of whole blood from healthy volunteers (n=6) was obtained in a 15 ml centrifugation tube containing antioxidant (0.5 mg of 2,6-di-tert-butyl-4-methylphenol). The blood samples were placed for 60 min at room temperature, and then centrifuged at 3000 rpm for 10 min to obtain the serum fraction. LDL (*d* 1.006–1.063 g/ml) was isolated by sequential density ultracentrifugation from serum, dialyzed against 0.15M NaCl containing 1 mM EDTA, and stored at 4°C. For lipid extractions, the LDL (50 μg/ml protein; 1 ml) was mixed vigorously with 2 ml chloroform-methanol (2:1) for 1 min. After centrifugation at 3,000 rpm for 10 min, the organic phase was separated, washed with water twice, and evaporated to dryness *in vacuo.* The residue was analyzed for the detection of secosterol-A, secosterol-B, C16:0-secoA, C16:0-secoB, 9-ONC, 9-ON-secoA and 9-ON-secoB (structures, see Fig. 1) after derivatization with DH as described in our previous study [16, 19, 21]. Briefly, aliquots of extracted samples from the human LDL were dissolved in 100 μl of acetonitrile containing 0.5 mg/ml DH and 0.1 mg/ml *p*-toluensulfonic acid, and derivatized for 4 h at room temperature in darkness. The derivatized mixture was evaporated to dryness *in vacuo*, and the residue was finally dissolved in 1 ml acetonitrile. Then the DH derivatives of secosterols were analyzed by LC-ESI-MS/MS as described below. Under these conditions, the recovery rates of these compounds added into 1 ml of LDL (50 μg/ml protein) were more than 90%.

LC-ESI-MS/MS analyses

LC-ESI-MS/MS was performed on an Agilent 1200 series HPLC system using a TSK-GEL ODS-100V column (3 μm, 150 × 2.0 mm, TOSOH, Tokyo, Japan) and an Agilent G6410B triple quadrupole tandem mass spectrometer with an electrospray

ionization device running in positive ionization mode. The detector conditions were as follows: capillary voltage at 4000 V, source temperature at 300°C, drying gas flow at 7 l/min, nebulizer gas at 20 psi, and fragmentor at 200 V. The detection of compounds was performed using the multiple reaction monitoring (MRM) mode. The ion transitions were monitored for secosterol-A-DH and -B-DH (*m/z* 666.4>630.4, 365.3), 3,4-^{13}C-secosterol-A-DH and -B-DH (*m/z* 668.4>632.4, 236.0), C16:0-secoA-DH and B-DH (*m/z* 904.7>630.2, 365.2), 9-ON-secoA-*bis*DH and B-*bis*DH (*m/z* 534.5>630.2, 365.2), 9-ONC-DH (*m/z* 788.2>420.0, 369.2), C18:1-CE (*m/z* 668.6>369.3, 206.5), C18:2-CE (*m/z* 666.7>369.3, 206.0), C16:0-CE (*m/z* 642.5>369.3, 607.6), and cholesterol (*m/z* 369.0>161.2, 147.1). For the analyses of DH derivatives, solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. For the analyses of unoxidized cholesterol and cholesteryl esters, solvent A was 10 mM ammonium formate and solvent B was acetonitrile containing 30% isopropanol. Samples were separated using a linear gradient of 70% solvent A and 30% solvent B to 100% solvent B in 20 min and with 100% solvent B for an additional 40 min. The flow rate was 0.2 ml/min. The level of compounds were determined using 3,4-^{13}C-secosterol-B as the internal standard.

Assay for cytotoxicity

We have recently reported that secosterol-A, and -B, and their keto alcohol and acid derivatives induce cell death in several human cultured cells including acute promyelocytic leukemia HL-60, acute monocytic leukemia THP-1, acute T cell leukemia Jurkat, histiocytic lymphoma U937, lung carcinoma A549, umbilical vein cell EA.hy926 and rat pheochromocytoma PC-12 [29]. These reports indicated that HL-60 cells are relatively susceptible to secosterol-induced cell death compared to other cell type used in the experiment. For these reasons, we evaluated the cytotoxic effects of secosterols in

HL-60 cells (purchased from RIKEN Cell Bank Ibaraki, Japan), which were maintained in 10% FBS/RPMI 1640 supplemented with 50 units/ml penicillin and 50 µg/ml streptomycin and were grown in an atmosphere of 95% air and 5% CO_2 at 37ºC. The alamarBlue assay was carried out for quantitative analysis of cell viability. After culturing, cells were seeded in a 96-well plate, and then incubated with compounds at 37ºC for 24 h. Ten µl of an alamarBlue solution was added to each well, and the fluorescence was measured using an excitation at 560 nm and emission at 590 nm after incubation at 37ºC for 2–3 h in a humidified CO_2 incubator, according to the manufacturer's direction. The obtained values were compared with each of the controls incubated with vehicle only.

RESULTS

Ozonolysis products of cholesteryl esters

Three major cholesteryl esters in human LDL, cholesteryl palmitate (C16:0-CE), cholesteryl oleate (C18:1-CE), and cholesteryl linoleate (C18:2-CE), were treated with ozone as described in the Materials and Methods section, and the major ozonolysis products were isolated and characterized by MS and NMR analysis (Fig. 1). The major reaction products of C16:0-CE with ozone were identified as C16:0-secoA and C16:0-secoB. On the other hand, when C18:2-CE and C18:1-CE were exposed to ozone, their primary ozonolysis product was identified as 9-ONC and two other ozonolysis products 9-ON-secoA and 9-ON-secoB were also isolated and characterized. These results suggest that ozonolysis of cholesteryl esters with saturated fatty acid results in the formation of secosterol esters with an unchanged fatty acid (Fig. 1). However, the reaction of ozone with cholesteryl esters containing unsaturated fatty acids (C18:2 and C18:1) first yielded cholesteryl esters with 9-oxononanoic acid, which was formed by the ozonolysis of C18:2 and C18:1. This core-aldehyde 9-ONC was then further oxidized to form 9-ON-secoA and

9-ON-secoB (Fig. 1).

Development of LC-ESI-MS/MS analysis of DNSL derivative of 9-ONC and secosterol esters

Authentic standards of C16:0-secoA, B, 9-ON-secoA, B, and 9-ONC were subjected to ESI-MS/MS analyses after derivatization with DH. Base ions of C16:0-secoA (B)-DH, 9-ON-secoE-A (B)-*bis*DH, and 9-ONC-DH, were detected at *m/z* 788.2 (*z*=1), 534.5 (*z*=2), and 904.7 (*z*=1), respectively, and their MS/MS spectra are shown in Fig. 2. To measure the endogenous levels of the ozonolysis products of cholesteryl esters, we optimized the analytical conditions for their LC-ESI-MS/MS-MRM analyses as described in the Materials and Methods section. 9-ON-secoA-*bis*DH, 9-ON-secoB-*bis*DH, 9-ONC-DH, C16:0-secoA-DH, and C16:0-secoB-DH were eluted at 29.8, 30.5, 41.4, 47.5, and 48.4 minutes, respectively (Fig. 3A). Detection limits of these compounds were ~1 fmol. In addition, cholesterol, C18:2-CE, C18:1-CE, and C16:0-CE were eluted at 35.2, 44.7, 49.5, and 50.5 minutes, respectively (Fig. 3B).

Detection of secosterol ester in human LDL

In order to determine the physiological levels of secosterols and their derivatives, human LDLs were isolated from six healthy volunteers (3 males and 3 females, aged 24.5±4.7), and analyzed for secosterols and their esterified derivatives. As shown in Table 1, 9-ONC, secosterol-A and secosterol-B were detected in all tested human LDL samples (n=6) at 182.7±75.8, 58.8±11.5, and 33.6±16.5 (pmol/mg LDL protein), respectively. Furthermore, we also detected 9-ON-secoA and 9-ON-secoB in all tested human LDL samples (n=6) at 16.5±5.4, and 11.3±3.9 (pmol/mg LDL protein), respectively (Table 1). However C16:0-secoA and C16:0-secoB were below the detection limits of the assay.

Levels of the cholesteryl esters C18:2-CE, C18:1-CE and C16:0-CE were at 2448±567, 540±279, and 48±24 nmol/mg LDL protein, respectively (Table 1). The level of cholesteryl arachidonate (C20:4-CE) was determined to be 155±58.7 nmol/mg LDL protein in human LDL (n=6). The secosterol esters derived from 5-OVA, another core-aldehyde derived mainly from C20:4-CE, were also below the detection limit in LDL from human healthy volunteers (data not shown). These results indicate that low concentrations of 9-ON-secoA and 9-ON-secoB are constitutively present in healthy human plasma LDL particles. In order to investigate the effects of ozone exposure on the concentrations of secosterols and their esterified derivatives, the samples of isolated human LDL were treated with ozone, and then analyzed for these compounds using our developed method. As shown in Fig. 4, treatment of human LDL with ozone resulted in increased levels of C16:0-secoA, and -B, which were below the detection limits in untreated LDL samples. The levels of 9-ONC detected in untreated LDL samples were markedly decreased by treatment with ozone, whereas the levels of 9-ON-secoA, but not 9-ON-secoB, were significantly increased in ozonized LDL, suggesting that 9-ONC is oxidized by ozone to yield 9-ON-secoA. Additionally, it was confirmed that secosterol-A, but not secosterol-B, was also significantly increased in ozonized LDL. Both 9-ON-secoA and secosterol-A were markedly increased (approximately 1200-fold) when the human LDL samples were exposed to ozone under the present experimental conditions.

Cytotoxic effect of secosterol esters on HL-60 cells

Secosterol-A and -B have been reported to be cytotoxic against several culture cell lines [7, 10-12, 29]. Here we evaluated the cytotoxic effects of secosterol esters on human promyelocytic leukemia HL-60 cells. As shown in Fig. 5, HL-60 cells viabilities were decreased in a dose- and time-dependent manner by the treatment with 9-ON-secoA and

9-ON-secoB, but not by the treatment with their parent compounds 9-ONC, C18:2-CE and C18:1-CE. The cytotoxic effects of 9-ON-secoA and -B were as strong as those of the unesterified secosterol-A and -B. When HL-60 cells were treated for 72 hours, IC_{50} values of secosterol-A and -B were 3.8 and 6.9 μM, respectively, whereas the IC_{50}s for 9-ON-secoA and -B were 4.0 and 4.5 μM, respectively. These results suggest that the aldehyde group formed by the oxidation of the 5,6 double bond on cholesterol is an important determinant of their cytotoxic activities.

Discussion

In this study, we reported for the first time the presence of 9-ON-secoA and -B in human LDL, which are probably generated by oxidation of cholesterol esters (C18:1-CE and C18:2-CE). The detection of these compounds was performed using a highly sensitive and specific method developed in our previous study [16], which was based on derivatization with DH and detection by LC-ESI-MS/MS. The levels of 9-ON-secoA and -B in human LDL were 16.5±5.4, and 11.3±3.9 (pmol/mg protein), which corresponded to 20-50% of the unesterified forms of secosterol-A and -B, respectively, detected in human LDL. The level of 9-ON-seco-A was markedly increased *in vitro* by the ozonolysis of C18:1-CE and C18:2-CE, which are two major cholesterol esters found in human LDL.

In the present study, we detected 9-ON-secoA and -B in human LDL isolated from healthy volunteers (n=6) at 16.5 and 11.3 pmol/mg LDL protein, respectively. These were 9.0 and 6.2% of 9-ONC, and 0.00055% and 0.00038% of the total amount of unoxidized cholesteryl oleate and cholesteryl linoleate in LDL, respectively. Secosterol-A and -B were also detected in the same samples at 55.8 and 33.6 pmol/mg LDL, respectively, which were 0.0021% and 0.0012% of unoxidized cholesterol. Free cholesterol seems to be 4–5-fold more readily oxidized to form secosterols than cholesteryl oleate and cholesteryl linoleate.

This might be due to the fact that cholesterol esters are present in the inner hydrophobic core of LDL particles whereas free cholesterol is present in the outer membrane. Further analyses are required to determine the pathological levels of secosterol esters in atherosclerotic plaques or the brain tissues of dementia patients.

The levels of three major cholesteryl esters, cholesteryl linoleate, oleate, and palmitate, in human plasma (n=6) were 2448±567, 540±279, and 48±24 nmol/mg LDL protein, respectively (Table 1). 9-ON-secoA and -B detected in human LDL are oxidized products of the two most abundant cholesteryl esters in LDL, cholesteryl linoleate and oleate. On the other hand, C16:0-secoA and C16:0-secoB formed by the ozonolysis of cholesteryl palmitate, were below the detection limits in human LDL isolated from healthy volunteers, which may be due to the fact that the amount of their respective parent compound was limited. However these derivatives may be formed and increased by inflammatory stimuli. Further studies are needed to analyze the association of these secosterol esters in human tissue with development of atherosclerosis and Alzheimer and Parkinson diseases.

With regard to the origin of secosterols in human LDL, it is still controversial whether ozone is necessary for the oxidative cleavage of the $\Delta^{5,6}$ double bond of the steroid structure to form secosterol-A, although secosterol-B was shown to be formed not only by aldolization of secosterol-A, but also by an ozone-independent pathway via Hock cleavage of 5α-hydroperoxycholesterol [22]. Uemi et al. suggested that secosterol-A is also formed via unstable cholesterol dioxetane in an ozone-independent pathway by the reaction of cholesterol with singlet oxygen [23]. On the other hand, Wentworth et al. previously demonstrated using an aqueous buffer system that secosterol-A was only formed by the reaction of cholesterol with ozone and not from the reaction of other various ROS such as singlet oxygen, superoxide anion, and hydroxyl radicals [24]. We have also previously

reported that secosterol-A was formed *in vivo* by a reaction with an ozone-like oxidant generated with PMA-activated neutrophils through an MPO-dependent mechanism [16]. Moreover, in the present study, we observed that secosterol-A, C16:0-secoA, and 9-ON-secoA, but not their aldolization products, secosterol-B, C16:0-secoB, and 9-ON-secoB, were markedly increased in human LDL treated with ozone (Fig. 4). Further studies are required to explore the mechanism for formation and elimination of free and esterified secosterols.

We also found that 9-ON-secoA and -B, but not 9-ONC, exert cytotoxic effects on HL-60 cells, which suggests that the aldehyde groups formed by the oxidative cleavage of the $\Delta^{5,6}$ double bond of the steroid structure is an important determinant of their cytotoxic activities (Fig. 5). Their cytotoxicities were as potent as unesterified secosterol-A and -B. Additionally, other reports also suggest these aldehyde groups are involved in proatherogenic roles such as protein aggregation, fibrilization, and induction of scavenger receptor (CD36 and SR-A) expression [9, 13-15, 25-28]. Alteration of their molecular polarities may cause translocation from the inner part to the outer surface of the LDL particle which could trigger several bioactivities.

In this study, we have reported for the first time the detection of the novel cytotoxic secosterol esters 9-ON-secoA and -B in human LDL, which are formed by oxidation of cholesteryl esters. As the aldehyde group in these secosterol derivatives is important for the execution of their bioactivities, further studies are needed to determine the association of the levels of free and esterified secosterols with human diseases caused by chronic inflammation such as cancer, diabetes, atherosclerosis and neurodegenerative disorders.

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Figure legends

Figure 1. Chemical structures and oxidation pathway of cholesteryl esters and their ozonolysis products.

Figure 2. MS/MS spectra of ozonolysis products formed from cholesteryl esters. Authentic standards of C16:0-secoA, -B, 9-ON-secoA, -B, and 9-ONC were derivatized with DH, then infused to ESI-MS/MS analyses. Collision-induced dissociation voltages for C16:0-secoA, B-DH, 9-ON-secoA, B-*bis*DH, and 9-ONC-DH were set at 29, 29, 13, 13, and 29V, respectively.

Figure 3. Typical chromatograms obtained by LC-ESI-MS/MS analyses. DH derivatives of secosterol-A, -B, 9-ON-secoA, -B, 9-ONC, C16:0-secoA, and -B (A) and cholesterol, C18:2-CE, C18:1-CE, and C16:0-CE (B) were subjected LC-ESI-MS/MS-MRM analyses. The ion transitions were monitored for secosterol-A-DH and -B-DH (*m/z* 666.4>630.4), 9-ON-secoA-*bis*DH and B-bisDH (*m/z* 534.5>630.2), 9-ONC-DH (*m/z* 788.2>369.2), C16:0-secoA-DH and B-DH (*m/z* 904.7>630.2), cholesterol (*m/z* 369.0>147.1), C18:2-CE (*m/z* 666.7>369.3), C18:1-CE (*m/z* 668.6>369.3), and C16:0-CE (*m/z* 642.5>369.3).

Figure 4. Effect of ozonolysis of human LDL on the formation of secosterol esters. Human LDL (50 μg protein/ml obtained from subject No. 4 shown in Table 1) was treated with (lower in each panels) or without (upper in each panels) ozone for 10 min in phosphate buffer (pH 7.4). Extracted lipid fractions were derivatized with DH, then subjected to LC-ESI-MS/MS. MRM chromatogram of 9-ON-secoA, B-bisDH (*m/z* 534.5>630.2); C16:0-secoA, B-DH (*m/z* 904.7>630.2); secosterol-A, B-DH (*m/z* 666.4>630.4); 9-ONC-DH (*m/z* 788.2>369.2).

Figure 5. Cytotoxic effects of ozonolysis products of cholesteryl esters on HL-60 cells.

HL-60 cells were treated with 9-ON-secoA (●), 9-ON-secoB (■), 9-ONC (△), C18:1-CE (○) and C18:2-CE (□) at indicated concentration for 24 h. Cell viabilities were determined by alamarBlue assay. Values are shown as the mean±SD (n=3).

Highlights

- Human LDL cholesteryl esters were treated with ozone and the products identified
- The products were also detected in human LDL samples by LC-ESI-MS/MS
- The products 9-oxononanoyl secosterol-A and -B were cytotoxic to HL-60 cells
- These products may play roles in the pathogenesis of inflammatory disorders.

Graphical abstract

Miyoshi N. et al.

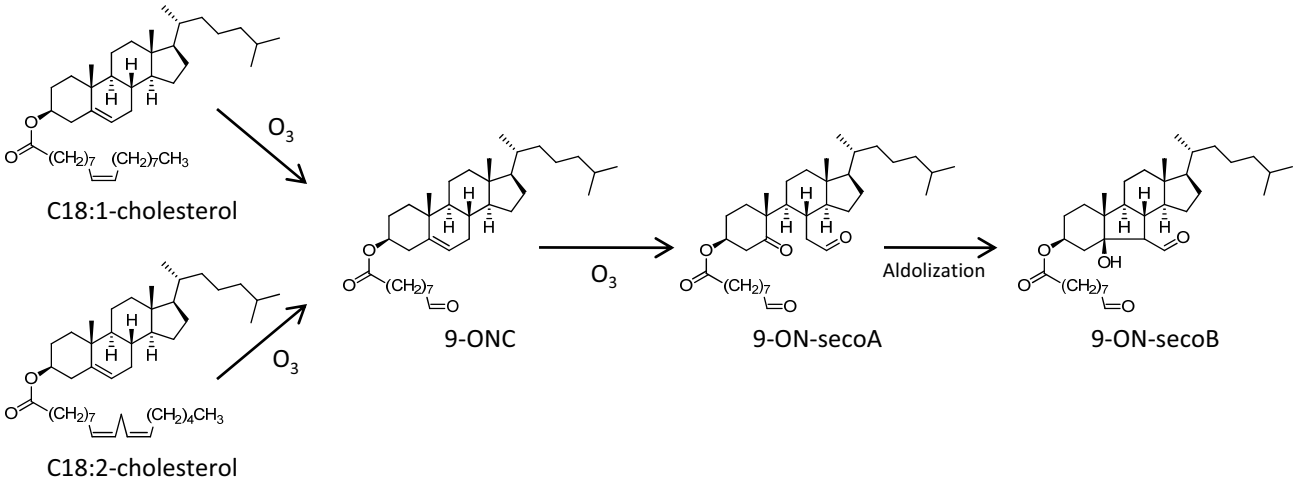

Figure 1, Miyoshi *et al.*

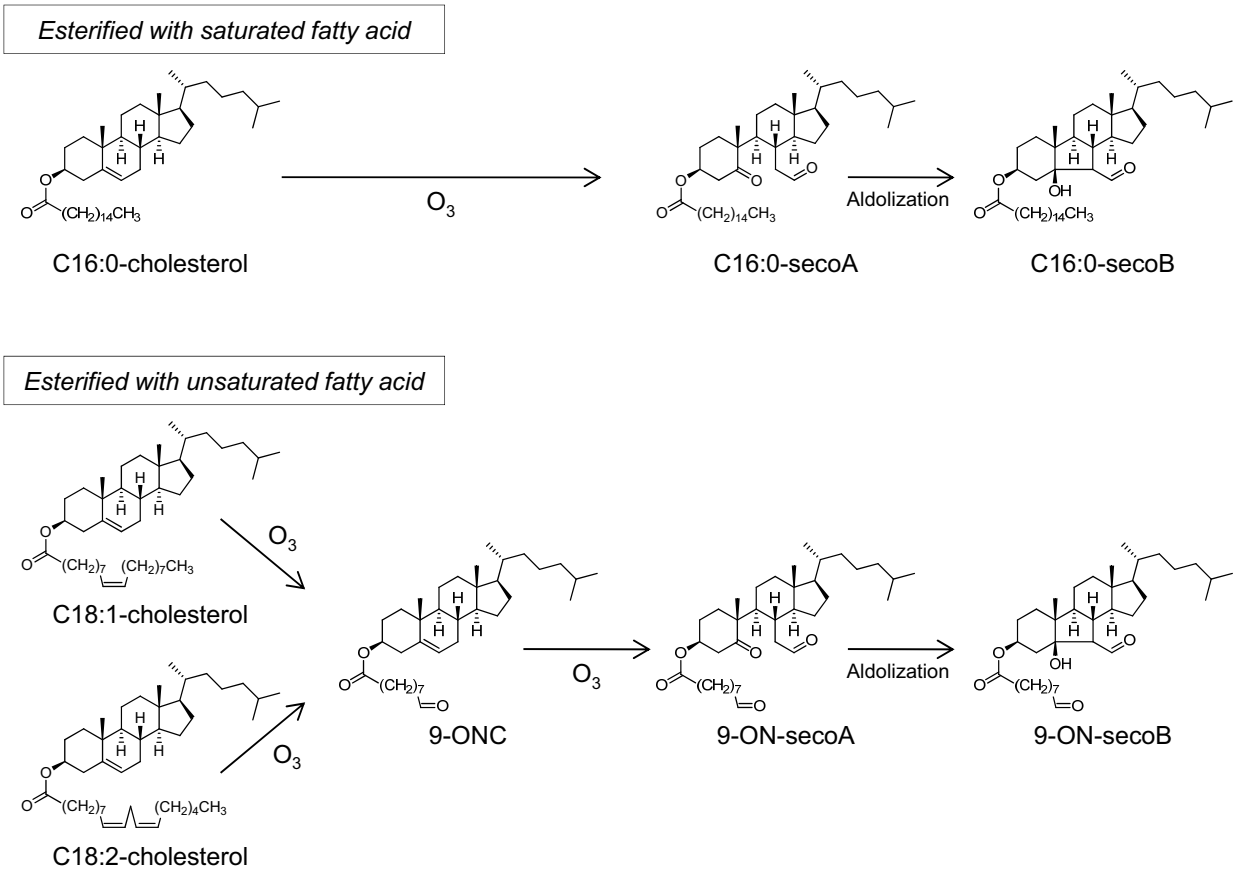

Figure 2, Miyoshi *et al.*

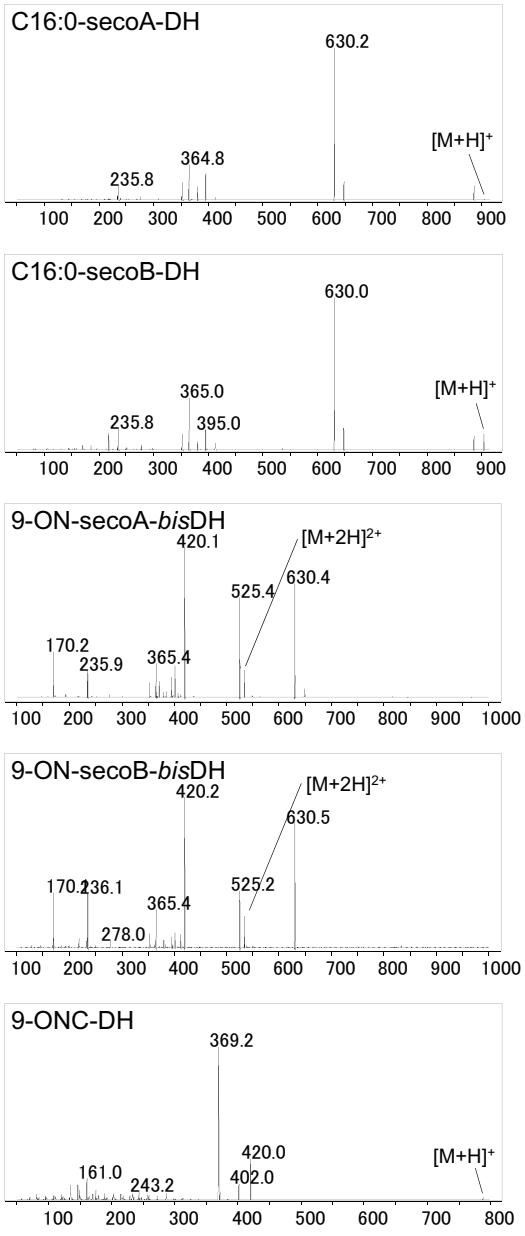

Figure 3, Miyoshi *et al.*

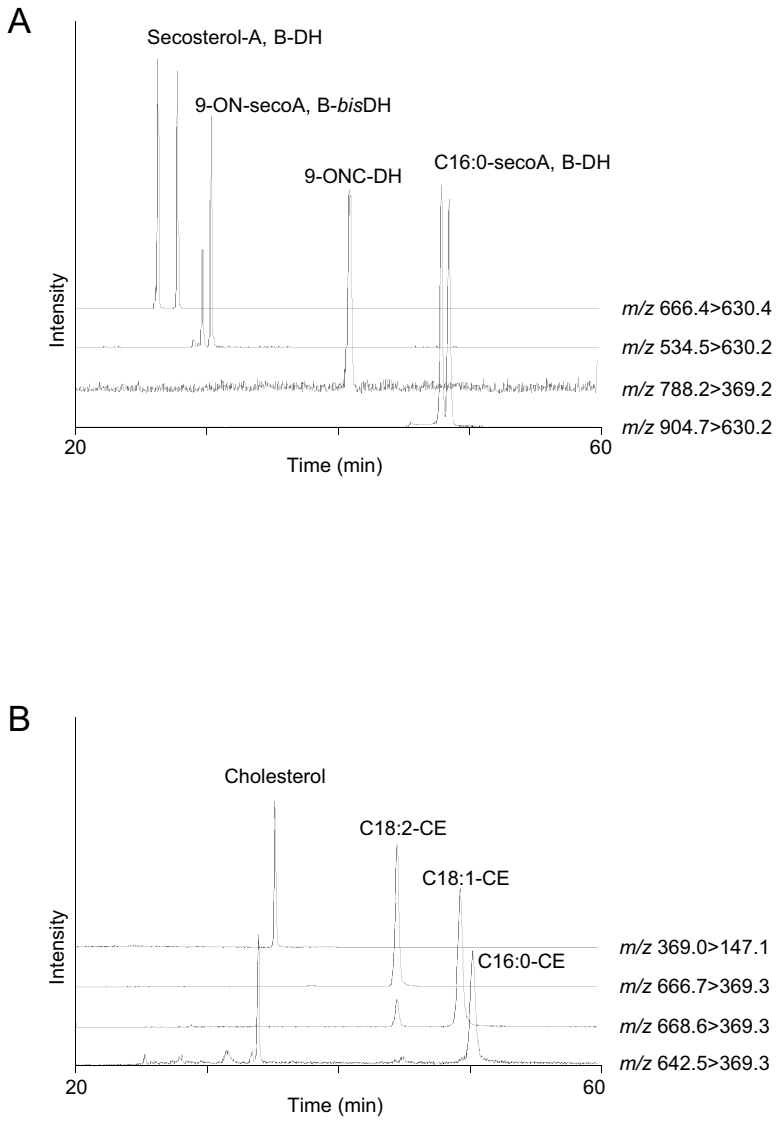

Figure 4, Miyoshi *et al.*

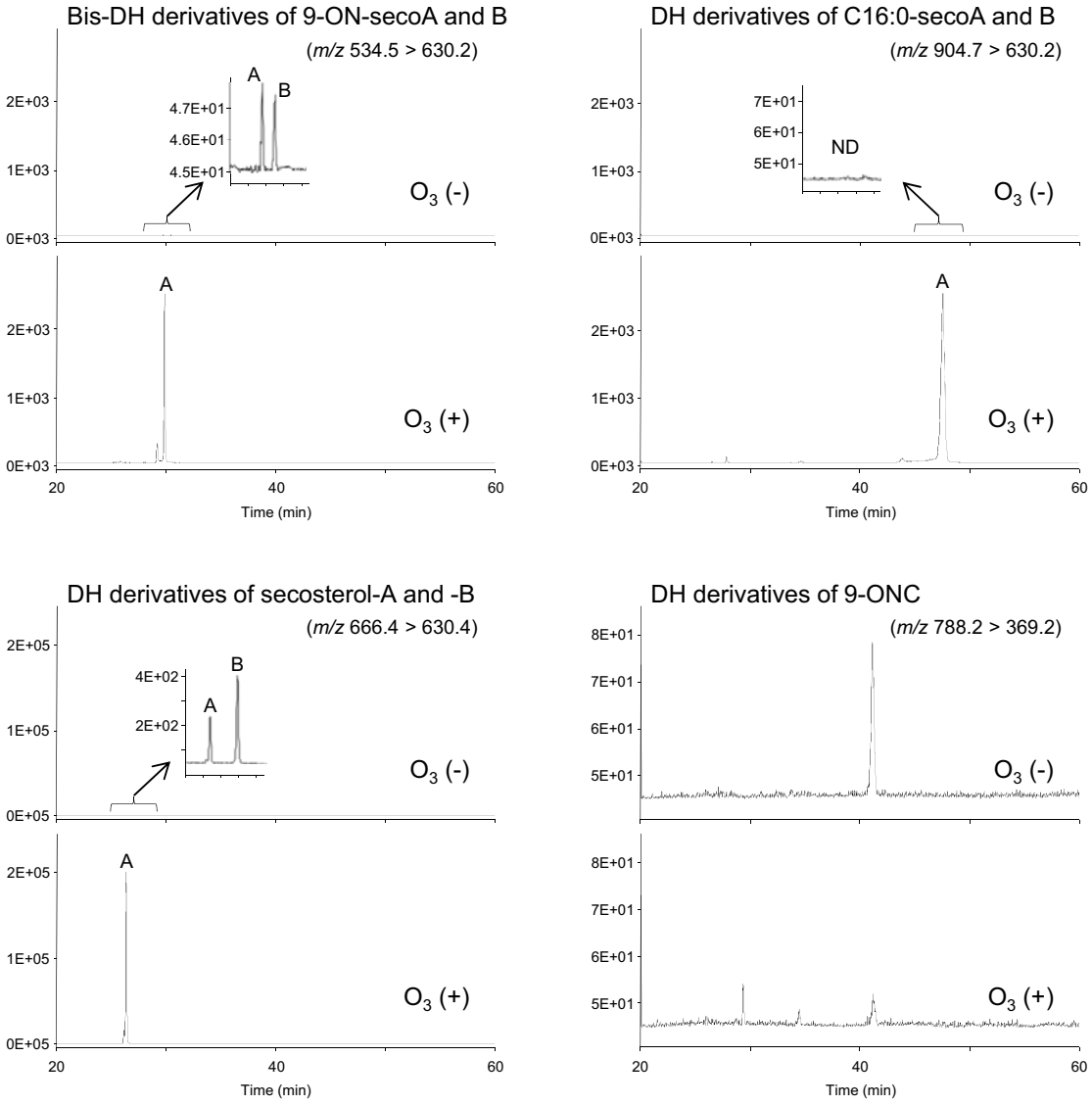

Figure 5, Miyoshi *et al.*

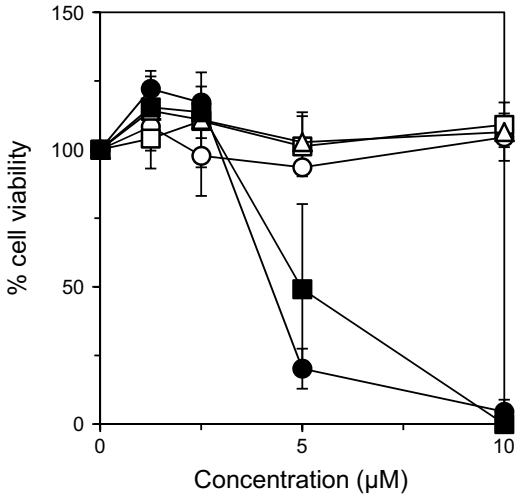

Table 1. Levels of oxidized and unoxidized steroidal compounds in human LDL.

Subject No.	Secosterol-A	Secosterol-B	9-ON-secoA	9-ON-secoB	9-ONC	C16:0-CE	C18:2-CE	C18:1-CE	Cholesterol
	(pmol/mg LDL protein)					(nmol/mg LDL protein)			
1	53.2 ± 7.4	48.0 ± 6.6	18.6 ± 1.6	7.6 ± 1.8	264 ± 64.7	77.5 ± 25.9	2179 ± 810	596 ± 307	2506 ± 369
2	52.5 ± 15.1	53.1 ± 16.4	19.2 ± 13.6	10.4 ± 3.6	288 ± 7.7	74.8 ± 27.5	3089 ± 463	736 ± 145	2780 ± 480
3	55.5 ± 1.5	42.8 ± 5.4	18.0 ± 2.4	7.3 ± 1.4	175 ± 27.4	34.2 ± 2.6	2847 ± 207	928 ± 81	2617 ± 308
4	62.6 ± 19.9	18.5 ± 1.7	11.8 ± 1.6	13.7 ± 0.4	127 ± 25.0	32.7 ± 1.9	1964 ± 152	296 ± 38	2278 ± 171
5	72.9 ± 13.4	21.9 ± 5.5	12.9 ± 3.2	16.0 ± 1.4	119 ± 12.0	31.4 ± 0.8	2006 ± 3	270 ± 19	2998 ± 187
6	56.4 ± 0.7	17.3 ± 1.1	18.6 ± 3.2	12.5 ± 5.0	125 ± 32.7	34.5 ± 0.9	2604 ± 640	418 ± 231	3123 ± 186

Mean±SD of triplicated analyses are shown.