Formation of cholesterol ozonolysis products in vitro and in vivo through a myeloperoxidase-dependent pathway

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Abstract **3β-Hydroxy-5-oxo-5,6-secocholestan-6-al (secosterol-A) and its aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (secosterol-B) were recently detected in human atherosclerotic tissues and brain specimens, and they may play pivotal roles in the pathogenesis of atherosclerosis and neurodegenerative diseases. However, as their origin remains unidentified, we examined the formation mechanism, the stability, and the fate of secosterols in vitro and in vivo. About 40% of secosterol-A remained unchanged after 3 h incubation in the FBS-free medium, whereas 20% and 40% were converted to its aldehyde-oxidation product, 3β-hydroxy-5-oxo-secocholestan-6-oic acid, and secosterol-B, respectively. In the presence of FBS, almost all secosterol-A was converted immediately to these compounds. Secosterol-B in the medium, with and without FBS, was relatively stable, but ~30% was converted to its aldehyde-oxidation product, 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid (secoB-COOH). When neutrophil-like differentiated human leukemia HL-60 (nHL-60) cells activated with PMA were cultured in the FBS-free medium containing cholesterol, significantly increased levels of secosterol-A and its aldehyde-oxidation product, but not secosterol-B, were formed. This secosterol-A formation was decreased in the culture of PMA-activated nHL-60 cells containing several reactive oxygen species (ROS) inhibitors and scavengers or in the culture of PMA-activated neutrophils isolated from myeloperoxidase (MPO)-deficient mice. Our results demonstrate that secoterol-A is formed by an ozone-like oxidant generated with PMA-activated neutrophils through the MPO-dependent mechanism.**—Tomono, S., N. Miyoshi, H. Shiokawa, T. Iwabuchi, Y. Aratani, T. Higashi, H. Nukaya, and H. Ohshima. **Formation of cholesterol ozonolysis products in vitro and in vivo through a myeloperoxidase-dependent pathway.** ***J. Lipid Res.*** **2011.** 52: **87–97.**

Supplementary key words secosterol • neutrophils • myeloperoxidase • inflammation

A reactive species with a chemical signature similar to that of ozone has been proposed to be generated by the antibody-catalyzed oxidation of water with singlet oxygen during the oxidative burst of activated human neutrophils and in inflamed tissues (1, 2). The formation of an ozone-like oxidant from singlet oxygen by neutrophils can be catalyzed not only by antibodies but also by amino acids such as tryptophan, methionine, and cysteine (3). To prove the formation of an ozone-like oxidant by neutrophils, previous studies used chemical reactions, such as the conversion of indigo carmine to isatin sulfonic acid or the oxidation of vinylbenzoic acid to 4-carboxybenzaldehyde. However, these reactions are not sufficiently specific to ozone to conclude an ozone production by neutrophils (4, 5).

Further evidence for ozone formation in vivo was based on the detection and formation of the cholesterol ozonolysis products 3β-hydroxy-5-oxo-5,6-secocholestan-6-al (secosterol-A, also called atheronal-A) and its aldolization product 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (secosterol-B, also called atheronal-B) in human tissues. These secosterols were previously reported to be formed only by ozone among the various reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydroxyl

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Abbreviations: α, β-unsaturated-secosterol-B, 3β-hydroxy-B-norcholest-5-ene; DH, dansyl hydrazine; HOCl, hypochlorous acid; LPS, lipopolysaccharide; MPO, myeloperoxidase; nHL-60, neutrophil-like differentiated human leukemia HL-60; PA, 2-picolylamine; PEG, polyethylene glycol; ROS, reactive oxygen species; secoA-COOH, 3β-hydroxy-5-oxo-secocholestan-6-oic acid; secoB-COOH, 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid; secosterol-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secosterol-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde; SOD, superoxide dismutase; WT, wild-type.

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radicals, and ozone (6–9). Secosterols were recently detected in human atherosclerotic tissues (10) and in human brain specimens from patients with Alzheimer's disease (11). They were also detected in Lewy body dementia (12) by the analysis of their hydrazine derivatives with LC-MS. Higher concentrations of secosterol-A were detected in the diseased arteries of patients with atherosclerosis. This secosterol-A concentration was further increased upon activation with PMA, suggesting that human leukocytes within atherosclerotic tissues are activated to produce an ozone-like oxidant (10). However, it has been recently reported that secosterol-A and -B were generated in an ozone-independent manner via the Hock-cleavage of 5α-hydroperoxy cholesterol, which can arise from the singlet oxygen ene reaction with cholesterol (13, 14). Secosterol-B is formed easily under acidic conditions in organic solvents (13, 14), whereas secosterol-A is either not formed at all or is a minor component in the aqueous buffer (9). We reported recently that almost equal amounts of secosterol-A and -B were formed by the reaction of cholesterol with human myeloperoxidase (MPO) in the presence of its substrates hydrogen peroxide (H_2O_2) and Cl^- (15). On the other hand, five times more secosterol-B was formed than secosterol-A when cholesterol was incubated with hypochlorous acid (HOCl) and hydrogen peroxide. However, in both the reactions, immunoglobulin G (IgG) did not enhance the formation of secosterols, suggesting that singlet oxygen ($^1O_2$) and possibly another oxidant, but not an ozone-like oxidant, mediated the formation of secosterols (15).

Although the exact mechanism for the formation of secosterols in human tissues remains unidentified, secosterols have been reported to react with the amyloid β protein in Alzheimer's disease, resulting in the misfolding and aggregation of this protein (11, 16, 17), which accelerate amyloidogenesis. Secosterols can also accelerate α-synuclein fibrilization (12), which has been associated with Parkinson's disease and Lewy body dementia (18). Secosterols exert cytotoxic effects on various culture cells (10, 19, 20).

We recently reported a highly sensitive method for the quantification of secosterols based on derivatization with fluorescent dansyl hydrazine (DH) and detection by LC-MS/MS system, in which both secosterols were confirmed to be stable during the derivatization process (15). This sensitive method has allowed us to perform quantitative analysis of secosterols in cell culture and in animal tissues. In this study, we examined the molecular mechanism for the formation of secosterols in vivo. We demonstrate here that the formation of secosterol-A and -B are enhanced by the inflamed stimuli not only in the cultures of neutrophil-like differentiated HL-60 cells or mouse neutrophils, but also in plasma samples and the liver of mice after lipopolysaccharide (LPS) administration. In addition, we have studied the fate of secosterols added to the culture medium with and without FBS. Several products, including 3β-hydroxy-5-oxo-secocholestan-6-oic acid (secoA-COOH), 3β,5β-dihydroxy-B-norcholestane-6-oic acid (secoB-COOH), and 3β-hydroxy-B-norcholest-5-ene (α, β-unsaturated-secosterol-B), were newly identified as metabolites of secosterol-A and -B. On the basis of these findings, we have shown evidence suggesting that the formation of secosterols is mediated by an MPO-dependent system in vivo at least partly through an oxidant with the chemical signature of ozone.

MATERIALS AND METHODS

Materials

Allopurinol, apocynin, cholesterol, 3,4-^{13}C-cholesterol, catalase (bovine), cell permeable catalase, covalently linked to polyethylene glycol (PEG-catalase), superoxide dismutase (SOD) (bovine), PEG-SOD, PMA, immunoglobulin G (bovine serum), *p*-toluenesulfonic acid, sodium azide, and LDL were purchased from Sigma, 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.

Synthesis of ozonolysis products of cholesterol

Secosterols and their metabolites were synthesized according to the method reported by Wentworth et al. (10). 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.

Fate of secosterols in culture media

One hundred pmol secosterol-A and -B were added into 1 ml of RPMI 1640 medium in the absence or presence of 10% FBS. After the incubation for 0-3 h, the media were spiked with stable isotopes labeled synthesized 3,4-^{13}C-secosterol-A or -B (5 nmol each) and 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 secosterols were analyzed as described below using a calibration curve prepared with several concentrations of standard compounds.

Formation of secosterols by HL-60 cells

Human leukemia HL-60 cells (RIKEN Cell Bank, Tsukuba, Ibaraki, Japan) were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin in an atmosphere of 95% air and 5% CO_2 at 37°C. HL-60 cells seeded at 5×10^5 cells/ml were differentiated into neutrophils by the treatment with 1.25% dimethylsulfoxide for 5 days (21). Then the medium was substituted for a fresh one containing either 10% FBS or no FBS. When the FBS-free medium was used, 6 μg/ml IgG and/or 30 μg/ml cholesterol were exogenously added into the medium, as FBS (10%) contained the compounds at these concentrations. The cells were then activated with 10 nM PMA for 0-3 h. When inhibitors of MPO (sodium azide, aminobenzohydrazide and salicylhydroxamic acid) were examined, they were added 1 h before the addition of PMA. Other inhibitors and chemicals were added just prior to the PMA activation. The harvested cells and the culture medium spiked with a stable isotope labeled synthesized 3,4-^{13}C-secosterol-A and -B (5 nmol or 10 pmol each in Figs. 2 and 3, respectively) were mixed with 25 ml chloroform-methanol (2:1). Secosterols were extracted and quantified by external or internal standard methods as shown in Figs. 2 and 3, respectively, as described below. The trace amounts of secosterol-A and -B (about 0.7 and 15 nM, respectively) were always detected in the cell-free culture media containing 10% FBS

(Fig. 2). Similarly, commercially available cholesterol also contained detectable amounts of secosterol-A and -B at the levels of ~7 and 200 μmol/mol cholesterol, respectively.

Animal experiments

Animals used in this study were kept according to guidelines for the care and use of laboratory animals at the University of Shizuoka, and all experiments were approved by the local animal ethical committee. *Mpo*$^{-/-}$ mice, backcrossed to a C57BL/6 background 10 times, were genotyped using polymerase chain reaction-amplified DNA from tail clippings (22). Wild-type (WT) male C57BL/6 control mice were obtained from the Japan SLC (Hamamatsu, Japan). Neutrophils were isolated from the bone marrow of the untreated WT and *Mpo*$^{-/-}$ mice (11 weeks of age) using the Percoll (Sigma) density gradient isolation method. Isolated neutrophils, with over 95% purity and viability confirmed by the trypan blue exclusion assay and the HE stain, were suspended in RPMI 1640 in the presence or absence of 10% FBS. After the PMA stimuli for 3 h, the cells and medium were harvested and then mixed with 4 ml chloroform-methanol (2:1) containing stable isotope labeled synthesized 3,4-^{13}C-secosterol-A and -B (10 pmol each). Secosterols extracted were derivatized and analyzed by LC-MS/MS as described below. The levels of secosterols were determined in the plasma samples of mice (6-7 weeks of age, n = 2-4 in each group), which had been treated with or without an intraperitoneal injection of 30 mg/kg body weight of *E. coli* LPS. At 6 or 22 h after LPS administration, the mice were euthanized, and blood samples were obtained with a heparinized syringe from the inferior vena cava. The plasma samples were stored at −80°C until analysis. 3,4-^{13}C-Secosterol-A and -B (10 pmol each) were added to 20 μl of plasma before being mixed with 100 μl chloroform-methanol (2:1). Then the secosterols were analyzed by LC-MS/MS as described below. Under these conditions, the recoveries of secosterol-A and -B (10 pmol each) were 100.1 and 101.3%, respectively.

Analysis of secosterols by LC-MS/MS

The secosterols -A, -B, and α, β-unsaturated-secosterol-B (structures, see Fig. 7) were analyzed after derivatization with DH as described in our previous study (15). Briefly, aliquots of extracted samples from the culture media or animal tissues were dissolved in 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. SecoA-COOH and SecoB-COOH (structures, see Fig. 7) were analyzed by LC-MS/MS after derivatization with 2-picolylamine (PA) (23). Briefly, aliquots of extracted samples were dissolved in acetonitrile (100 μl). Then, freshly prepared solutions of triphenylphosphine (10 mM) in acetonitrile (10 μl), 2,2′-dipyridyl disulfide (10 mM) in acetonitrile (10 μl) and PA (10 μg) in acetonitrile (10 μl) were successively added, and the mixture was incubated at 60°C for 10 min. After removal of the solvent in vacuo, the products were dissolved in 1 ml acetonitrile. A 10 μl aliquot from each individual sample was used for LC-MS/MS analysis. LC-MS/MS analyses, using nanospace SI-1 (SHISEIDO Co., Ltd., Tokyo, Japan) and API2000™ (Applied Biosystems, Forester City, CA) were performed with an electrospray ionization device running in a positive ionization mode. The derivatives were separated using a TSK-GEL ODS-100V column (150 × 2.0 mm, 3 μm TOSOH, Tokyo, Japan) with a linear gradient of 70% solvent A (0.1% formic acid in water) and 30% solvent B (acetonitrile containing 0.1% formic acid) to 100% solvent B in 20 min, followed by 100% solvent B for 20 min at the flow rate of 0.2 ml/min, and analyzed in MRM mode. The ion transitions monitored for DH derivatives of secosterol-A and -B were *m/z* 666.5/170.2, 236.1; those of 3,4-^{13}C-secosterol-A and -B were *m/z* 668.5/170.2, 236.1; that of α, β-unsaturated-secosterol-B was *m/z* 648.4/170.0, 365.1; and the PA-derivatives of secoA-COOH and secoB-COOH were *m/z* 525.4/109.1, 381.4.

RESULTS

Fate of secosterol-A and -B in the culture medium with and without 10% FBS

We determined the stability and fate of secosterol-A and -B exogenously added to the culture medium in the presence and absence of 10% FBS. When we initially examined the stability of secosterols in culture media, we employed 10 pmol of 3,4-^{13}C-secosterol-A or -B as an internal standard, which resulted in a poor total recovery of compounds (20-40%) (data not shown). However, when 500 times more 3,4-^{13}C-secosterol-A or -B (5 nmol each) were added before lipid extraction with chloroform-methanol, their total recovery was markedly improved and reached between 75% and 100%. This increased recovery could be due to the replacement by excess 3,4-^{13}C-secosterol-A or -B of initially added secosterols, which were probably present as reversibly bound forms with medium components. Using these improved conditions, we found that almost all secosterol-A added to the FBS-containing medium disappeared very rapidly, and 60-80% of secosterol-A was immediately converted to secosterol-B (**Fig. 1A**). We also observed that smaller parts of secosterol-A were converted to the aldehyde-oxidation products of secosterols, secoA-COOH and secoB-COOH (18% and 3%, respectively). In contrast, secosterol-A added to the FBS-free medium disappeared relatively slowly compared with the FBS-containing medium (Fig. 1B). About 40%, 38%, and 17% of added secosterol-A were detected as an unchanged compound, secosterol-B and secoA-COOH, respectively, after 3 h incubation (Fig. 1B). Conversely, it was found that about 60% of the original amount of secosterol-B remained unchanged in 10% FBS-containing medium, but 20% and 0.8% of secosterol-B were converted immediately to secoB-COOH and α, β-unsaturated-secosterol-B, respectively (Fig. 1C). No further increase or decrease of these compounds was observed during a period of 3 h incubation (Fig. 1C). The stability and the fate of secosterol-B added to the FBS-free medium was similar to that observed in the 10% FBS-containing medium (Fig. 1D). No conversions of secosterol-B to secosterol-A were detected during incubation in the media, both with and without FBS (Fig. 1C, D). In addition to these metabolites, we also detected small peaks with *m/z* values corresponding to other secosterol metabolites, including dehydrated derivatives of secoA-COOH and secoB-COOH (data not shown). Our results indicate that secosterol-A is very rapidly converted mainly to secosterol-B and secoA-COOH by some components in FBS, and some parts of secosterol-B are converted to secoB-COOH by some constituents in the culture medium.

Formation of secosterol-A and -B by neutrophil-like differentiated HL-60 cells

On the basis of the above findings, we examined the molecular mechanism for the formation of secosterols by

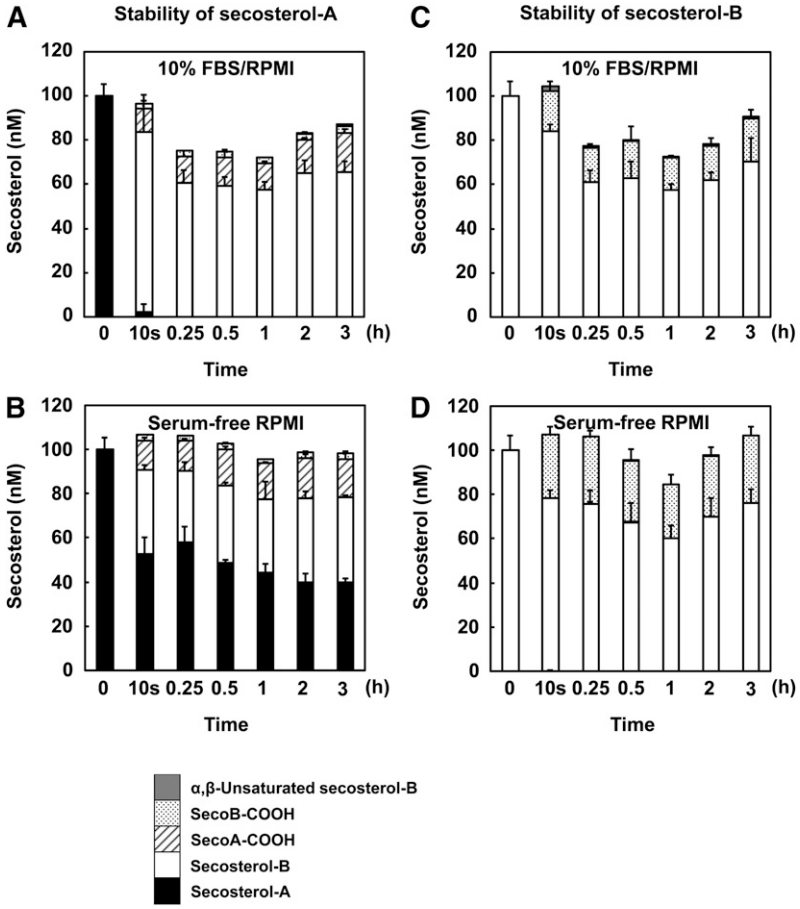

Fig. 1. Comparison of the stability and fate of secosterol-A and secosterol-B incubated in the culture medium with 10% FBS or without FBS. 100 pmol of synthesized secosterol-A (A and B) or secosterol-B (C and D) were incubated in 1 ml of the medium. At the indicated time, lipid fractions from harvested samples were extracted in the presence of excess 3,4-^{13}C-secosterol-A or -B (5 nmol), derivatized with DH or PA, and then analyzed as described in the text. Values are means ± SD (n = 3). DH, dansyl hydrazine; PA, 2-picolylamine; secosterol-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secosterol-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde; secoA-COOH, 3β-hydroxy-5-oxo-secocholestan-6-oic acid; SecoB-COOH, 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid; α, β-unsaturated-secosterol-B, 3β-hydroxy-B-norcholest-5-ene.

cultured neutrophil-like differentiated HL-60 (nHL-60) cells with or without PMA activation. When nHL-60 cells were incubated with 10 nM PMA for 3 h in the FBS-free medium into which cholesterol and IgG were added, significantly elevated levels of secosterol-A but not secosterol-B were formed (**Fig. 2A**, B). Moreover, increased levels of the aldehyde-oxidation products of secosterols, secoA-COOH and secoB-COOH, and α, β-unsaturated-secosterol-B were detected in the cholesterol-containing FBS-free medium (Fig. 2C-E). The formation of secosterol-A but not secosterol-B by the activated nHL-60 cells in the FBS-free medium occurred in a time-dependent manner as well as in the concentration-dependent manner of cholesterol or IgG added to the culture medium (**Fig. 3A, B**). No increased formations of secosterol-A were detected when BSA or heat-inactivated IgG were used in the place of IgG (Fig. 3C). Furthermore, heat-inactivated nHL-60 cells (activated with PMA) lost their ability to produce secosterol-A, even in the presence of IgG and cholesterol in the FBS-free medium (Fig. 3C). Additionally, levels of secosterol-A but not secosterol-B increased significantly (about 2-fold) when the FBS-free medium containing exogenous LDL was incubated with PMA-activated nHL-60 cells (data not shown). On the other hand, when the nHL-60 cells were incubated in the culture medium containing 10% FBS and upon activation with 10 nM PMA for 3 h, an increased level of secosterol-B but not secosterol-A was observed (Fig. 2A, B). When the nHL-60 cells were treated with PMA in the culture medium containing 10% FBS, the amounts of secosterol-B were also increased dose-dependently with increasing concentrations of PMA (up to 10 nM) and in a time-dependent manner (up to 3 h of incubation) (data not shown). Furthermore, the increased formations of secoA-COOH and secoB-COOH are clearly correlated with secosterol-A and secosterol-B formation, respectively (Fig. 2C, D). These results indicate that a major part of secosterol-A produced by the activated nHL-60 cells was immediately metabolized to its oxidation products and secosterol-B by components present in FBS.

Effects of inhibitors, scavengers, and antioxidants on PMA-induced secosterol formation

To elucidate the underlying molecular mechanisms for the formation of secosterols, we examined the effects of various enzyme inhibitors, the scavengers of reactive species, and antioxidant enzymes on secosterol-A formation by PMA-activated nHL-60 cells in the serum-free culture medium to which cholesterol and IgG were exogenously added (**Table 1**). It was found that apocynin (a NADPH oxidase inhibitor), allopurinol (an inhibitor for a xanthine oxidase and a free radical signal generated by activated neutrophils), and MPO inhibitors (sodium azide, aminobenzohydrazide, and salicylhydroxamic acid) significantly inhibited the secosterol-A formation. Similarly, methionine and β-carotene (singlet oxygen scavengers) as well as vinylbenzoic acid (a scavenger for ozone and possibly other reactive species) significantly inhibited

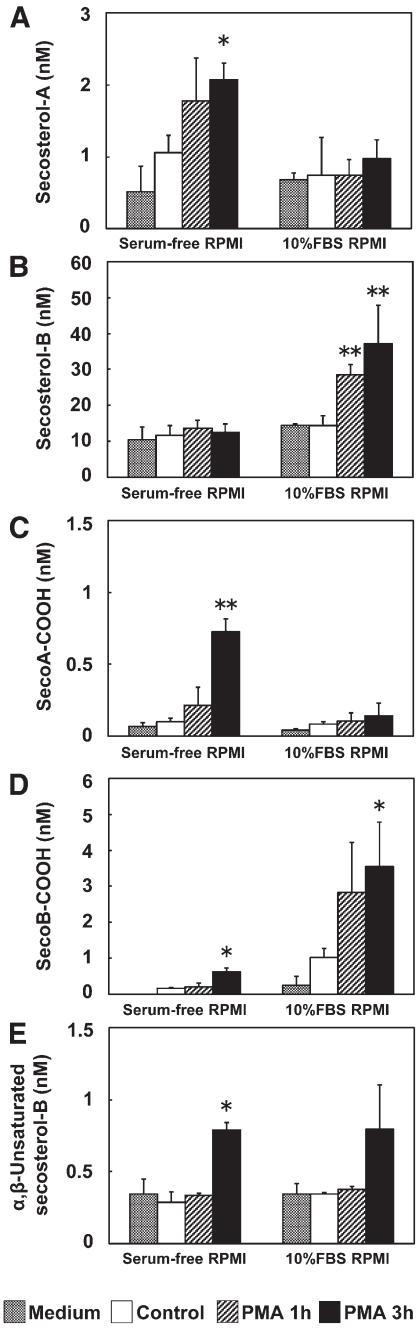

Fig. 2. Formation of secosterols and their oxidation products by nHL-60 cells in the presence or absence of FBS. nHL-60 cells were treated with 0.1% v/v ethanol (control) or 10 nM PMA in ethanol for 1 or 3 h in a serum-free medium containing 30 μg/ml cholesterol and 6 μg/ml IgG or in 10% FBS-medium. Lipid fractions extracted from the harvested cells and the medium were derivatized with DH or PA. Secosterol-A (A), secosterol-B (B), secoA-COOH (C), secoB-COOH (D), and α, β-unsaturated-secosterol-B (E) were analyzed as described in the text. Values are means ± SD (n = 5). Statistically significant: $*P < 0.05$, $**P < 0.01$ versus control. DH, dansyl hydrazine; nHL-60, neutrophil-like differentiated human leukemia HL-60; PA, 2-picolylamine; secoA-COOH, 3β-hydroxy-5-oxo-secocholestan-6-oic acid; secoB-COOH, 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid; secosterol-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secosterol-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde.

secosterol-A formation. Cell permeable catalase, covalently linked to polyethylene glycol (PEG-catalase) alone or PEG-catalase together with PEG-SOD added into the culture medium significantly decreased the amounts of secosterol-A formed by the activated nHL-60 cells, whereas neither PEG-SOD, SOD, nor catalase showed the inhibitory effects on its formation. Similar to the above findings, the formation of secosterol-B by the PMA-activated nHL-60 cells in the 10% FBS-containing medium was effectively inhibited by the same compounds as described above, including inhibitors for NADPH oxidase, xanthine oxidase, and MPO; the scavengers of reactive species such as methionine, β-carotene, and vinylbenzoic acid; PEG-catalase alone, and PEG-catalase plus PEG-SOD (Table 1). These results imply that several reactive species are involved in the formation of secosterols in the culture of PMA-activated nHL-60 cells.

Formation of secosterols by activated neutrophils obtained from wild-type and MPO-deficient mice

As our previous study (15) and the experimental results above suggested the involvement of MPO in the formation of secosterols by activated neutrophils, we employed neutrophils obtained from WT and MPO-deficient mice. Similar to the findings with the PMA-activated nHL-60 cells, cultured WT neutrophils upon activation with PMA significantly produced secosterol-A and -B in the medium containing no FBS (but containing exogenously added cholesterol and IgG) or 10% FBS, respectively (**Fig. 4A**, B). However, no increased formation of secosterol-A or -B was found when PMA-activated neutrophils from MPO-deficient mice were incubated under the same conditions as WT neutrophils (Fig. 4A, B).

Presence of secosterols in plasma and liver of WT and MPO-deficient mice

We determined the levels of secosterols in the samples of plasma and liver collected from WT and MPO-deficient mice. **Fig. 5** shows typical chromatograms obtained for the analyses of secosterols in mice plasma. The background levels of secosterol-A and -B were significantly lower in the plasma samples of MPO-deficient mice than WT mice (**Fig. 6A, B**). Similarly, the levels of secosterol-B detected in homogenates of the liver were also significantly lower in MPO-deficient mice than in WT mice (Fig. 6C). To examine the effect of an acute inflammation stimulus on the formation of secosterols, the mice were injected intraperitoneally with LPS. As shown in Fig. 6A and B, the levels of secosterol-A and -B in the plasma samples collected from WT mice were increased time-dependently after LPS injection, whereas the plasma levels of secosterols in MPO-deficient mice did not increase even after LPS injection.

DISCUSSION

Currently there are two proposed mechanisms for the secosterol formation in vivo: *a)* the oxidation of cholesterol with an ozone-like oxidant to generate secosterol-A and its conversion to secosterol-B by aldolization (2, 10); and *b)* the formation of 5α-hydroperoxy cholesterol by the reaction of cholesterol with singlet oxygen and its Hock-

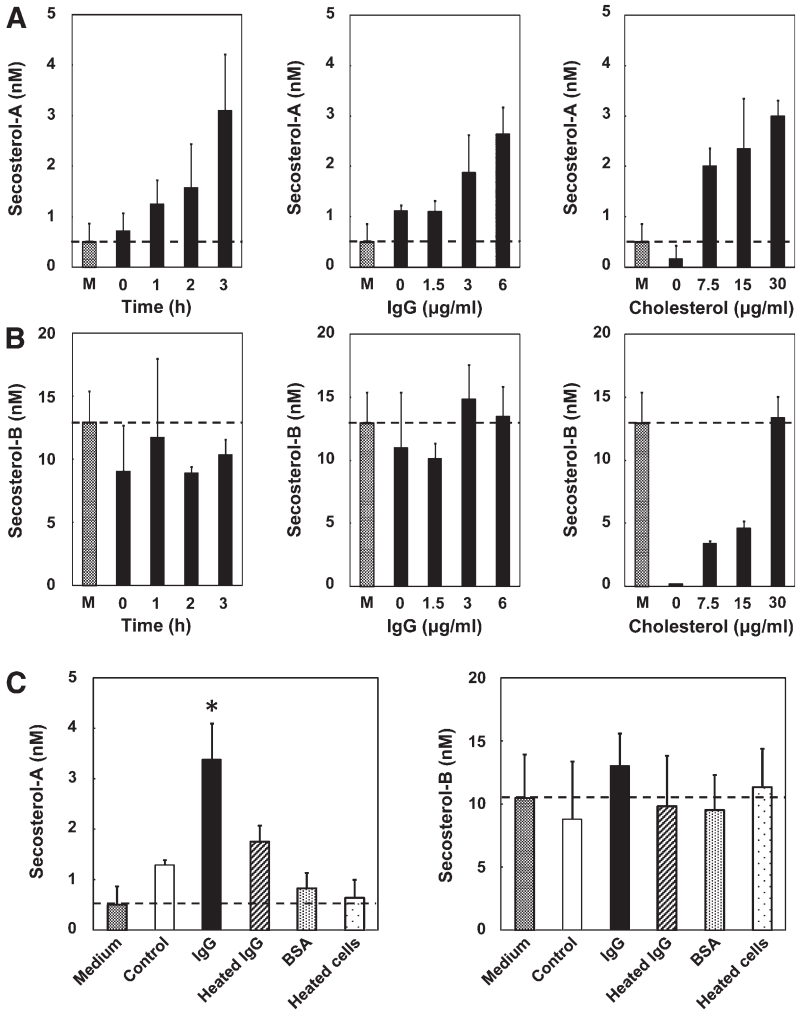

Fig. 3. Effects of incubation time and concentrations of IgG and cholesterol on the formation of secosterol-A and secosterol-B by PMA-activated nHL-60 cells. A, B: nHL-60 cells were incubated with 10 nM PMA in the presence of 30 μg/ml cholesterol or 6 μg/ml IgG for 3 h or indicated modified condition. Secosterols were extracted from the harvested cells and medium, and then analyzed as described in the text. The gray bar (M; medium) and dotted lines represent the levels of preformed secosterols detected in serum-free medium containing 30 μg/ml cholesterol or 6 μg/ml IgG. C: Comparison of formations of secosterol-A and secosterol-B by activated nHL-60 cells in the absence or presence of IgG (6 μg/ml), heat-inactivated IgG (6 μg/ml heated at 100°C for 5 min), and BSA (6 μg/ml). Heat-inactivated nHL-60 cells (100°C, 5 min) were also incubated in the FBS-free medium containing cholesterol and IgG in the presence of PMA. Values are means ± SD (n = 3). Statistically significant: $*P < 0.05$ when compared with control (C). Dotted lines represent the levels of preformed secosterols in the FBS-free medium containing cholesterol. nHL-60, neutrophil-like differentiated human leukemia HL-60; secosterol-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secosterol-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde.

cleavage to generate both secosterol-A and secosterol-B (13, 14). However, recently Wentworth et al. (9) reported that only ozone can react with cholesterol to form secosterol-A and that other oxidants, such as singlet oxygen, form secosterol-B but not -A as a major component. In the present study, we investigated the underlying molecular mechanism for the formation of secosterols by PMA-activated human neutrophil-like HL-60 cells in culture and in mice in vivo.

When we initially determined the stability and the fate of secosterols added to the culture medium, we could identify only 20-40% of the added compounds. However, the total recovery was markedly improved to 75-100% when 500 times more 3,4-^{13}C-secosterol-A or -B were added to the medium before lipid extraction with chloroform-methanol. This might be due to excess 3,4-^{13}C-secosterol-A or -B replacing the previously added secosterols, which could be present as reversibly bound forms with medium components. Using this method, it was found that secosterol-A added to the FBS-free medium decreased more slowly than in the FBS-containing medium, and 40% remained unchanged during 3 h incubation. Conversely, secosterol-A added to the FBS-containing medium immediately disappeared. In contrast, although ~30% of secosterol-B added to the FBS-free medium was converted to secoB-COOH, 60-70% of secosterol-B remained unchanged

TABLE 1. Effects of inhibitors for oxidant-generating enzymes, antioxidants, and scavengers of ROS on the formation of secosterols by PMA-activated nHL-60 cells

Treatment	Concentration	Secosterol-A (nM) Formed in FBS-free Medium	% Inhibition[a]	Secosterol-B (nM) Formed in 10% FBS Medium	% Inhibition[a]
Medium alone		0.5 ± 0.4		14.5 ± 0.3	
nHL-60 without PMA treatment		0.8 ± 0.4		20.3 ± 2.2	
nHL-60 with PMA		3.2 ± 1.1[b]		35.8 ± 4.4[b]	
+ apocynin	100 μM	1.0 ± 0.4[c]	91.7	13.9 ± 2.2[d]	> 100
+ allopurinol	100 μM	0.9 ± 0.1[c]	95.8	15.2 ± 3.5[c]	> 100
+ SOD	100 U/ml	3.1 ± 0.3	4.2	39.2 ± 2.2	< 0
+ catalase	1000 U/ml	2.2 ± 0.3[c]	41.7	26.9 ± 14.7	57.4
+ SOD + catalase	100 U / 1000 U/ml	2.8 ± 0.1	16.7	42.5 ± 7.1	< 0
+ PEG-SOD	100 U/ml	2.8 ± 0.5	16.7	42.9 ± 7.0	< 0
+ PEG-catalase	200 U/ml	1.4 ± 0.2[c]	75.0	17.2 ± 2.0[c]	> 100
+ PEG-SOD + PEG-catalase	100 U / 200 U/ml	1.8 ± 0.7[c]	58.3	21.9 ± 0.8[c]	89.7
+ sodium azide	200 μM	0.5 ± 0.4[c]	> 100	15.6 ± 2.9[c]	> 100
+ aminobenzohydrazide	100 μM	0.2 ± 0.1[d]	> 100	18.1 ± 2.9[c]	> 100
+ salicylhydroxamic acid	100 μM	1.8 ± 0.5[c]	58.3	21.6 ± 2.1[c]	91.6
+ methionine	100 μM	0.7 ± 0.3[c]	> 100	7.1 ± 0.1[d]	> 100
+ β-carotene	50 μM	0.5 ± 0.3[c]	> 100	4.6 ± 1.1[d]	> 100
+ vinylbenzoic acid	100 μM	0.6 ± 0.4[c]	> 100	24.9 ± 2.5[c]	70.3

nHL-60 cells were activated with 10 nM PMA for 3 h in the indicated medium in the presence or absence of test compounds. Formation of secosterol-A or secosterol-B was determined in the FBS-free medium containing 30 μg /ml cholesterol and 6 μg/ml IgG, or in the 10% FBS medium, respectively. Values are means ± SD (n = 3). nHL-60, neutrophil-like differentiated human leukemia HL-60; PA, 2-picolylamine; PEG, polyethylene glycol; ROS, reactive oxygen species; SOD, superoxide dismutase.

[a]Percentage of inhibition representing relative inhibitory effect was calculated for the following equation:

$$\%\text{inhibition} = 100 \times \left(1 - \frac{A - C}{B - C}\right)$$

where A is the amount of secosterol formed by the PMA-activated cells in the presence of a test compound; B is the amount of secosterol formed by the PMA-activated cells in the absence of a test compound; and C is the amount of secosterol presented in the cell culture without PMA treatment.

[b]$P < 0.05$ comparing with and without PMA activation.

[c]$P < 0.05$ comparing the presence and absence of a test compound.

[d]$P < 0.01$ comparing the presence and absence of a test compound.

regardless of the presence or absence of FBS. Moreover, we detected small peaks with *m/z* values corresponding to dehydrated products of secoA-COOH and secoB-COOH (5-oxo-secocholestan-6-oic acid and 5β-hydroxy-B-norcholestane-6-oic acid, respectively). About 20% of secosterol-A added to the 10% FBS-containing medium remained unidentified. Secosterol-A could be converted to the above minor or unidentified metabolites, or it could be bound to proteins present in the FBS. Further studies are needed to identify the fate of secosterol-A in vivo.

We next examined whether secosterol-A and -B are formed by nHL-60 cells in culture (Fig. 2). In the case of the experiments with the FBS-free medium, we added both cholesterol and IgG in the same concentrations as those present in 10% FBS. It was found that significantly increased amounts of secosterol-A and its oxidation product secoA-COOH, but not secosterol-B, were formed by the incubation of activated nHL-60 cells. Similarly, the formation of secosterol-A was also observed with activated neutrophils isolated from WT mice (Fig. 4). Under these conditions, the formation of secosterol-A was dependent on the concentrations of cholesterol and IgG added exogenously (Fig. 3). Several enzyme inhibitors and scavengers for different reactive species also inhibited the nHL-60 cell-mediated formation of secosterol-A in the FBS-free medium, implying that the activated cells generate superoxide, hydrogen peroxide, hypochlorous acid, and singlet oxygen, all of which are required to form secosterol-A (Table 1). On the basis of these findings, we can speculate that secosterol-A was formed in the FBS-free medium by the reaction of exogenously added cholesterol with an ozone-like oxidant(s) generated from other reactive species (superoxide, hydrogen peroxide, hypochlorous acid, and singlet oxygen) in the presence of IgG. In addition, the greater amount of secosterol-B formation observed in PMA-activated nHL-60 cells or WT mice neutrophil cultures containing 10% FBS could be mainly derived from a Hock-cleavage of 5α-hydroperoxy cholesterol, as it cannot be explained merely by the aldolization of generated secosterol-A.

In addition to our findings showing that secosterol-A or -B added to the culture medium were immediately oxidized to form secoA-COOH or secoB-COOH (Fig. 1), we found for the first time that secoA-COOH and secoB-COOH were formed in the PMA-activated nHL-60 cells culture (Fig. 2). SecoA-COOH has been reported to induce the fibrilization of apolipoprotein C-II (24) and α-synuclein (12). As no data have been available concerning the presence of these oxidized secosterols in vivo, further studies are needed to establish the levels of metabolites of secosterols in in vivo samples.

We previously reported that almost equal amounts of secosterol-A and -B were formed by the reaction of choles-

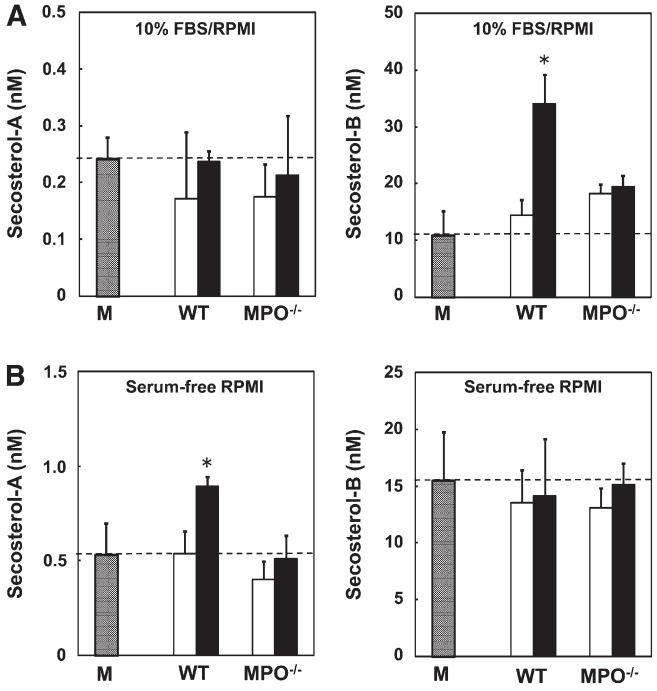

Fig. 4. Formation of secosterols by neutrophils isolated from WT or MPO-deficient mice. Isolated neutrophils were incubated with (closed bars) or without (open bars) 10 nM PMA in the medium without FBS but containing 30 μg /ml cholesterol and 6 μg/ml IgG (A) or containing 10% FBS (B). Values are means ± SD (n = 3). Statistically significant: $*P < 0.05$ compared the amounts formed by the cells without PMA activation. Dotted lines represent the levels of preformed secosterols in the medium used in each assay. M, medium; MPO, myeloperoxidase; WT, wild-type.

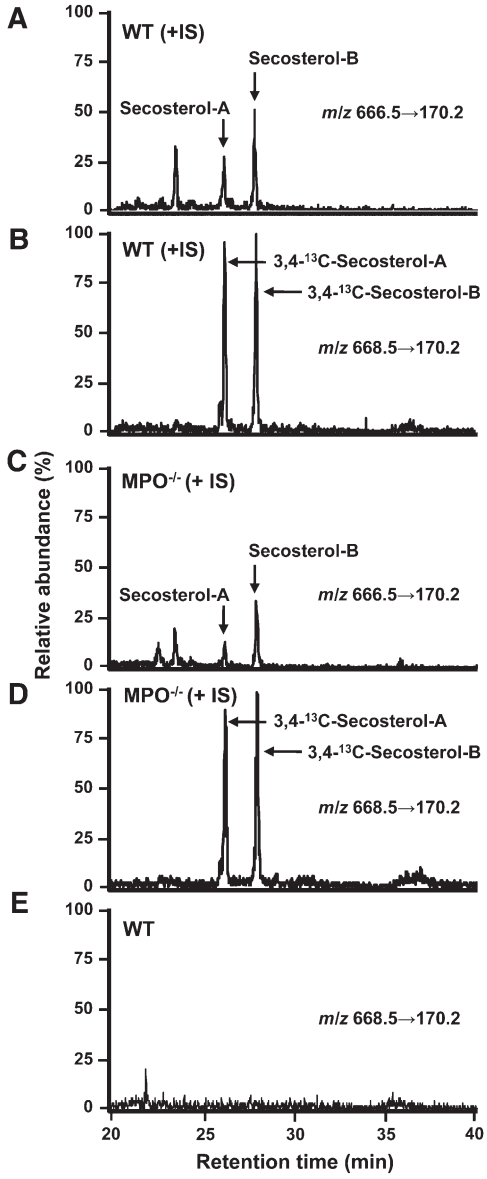

Fig. 5. LC-MS/MS analyses of secosterol-A and secosterol-B in mice plasma. Typical chromatograms obtained by monitoring the ion transition of *m/z* 666.5/170.2 for the DH derivatives of secosterol-A and secosterol-B extracted from a plasma sample of WT mouse (A) and MPO-deficient mouse (C) and 668.5/170.2 for those of internal standards (IS) (3,4-^{13}C-secosterol-A and -B) (B, D). No interfering peaks were detected when the derivatized extract of WT mouse plasma without addition of IS was analyzed for 668.5/170.2 (E). DH, dansyl hydrazine; MPO, myeloperoxidase; secosterol-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secosterol-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde; WT, wild-type.

terol with human MPO in the presence of its substrates hydrogen peroxide and Cl^- (15). The results in this study also indicate that inhibitors of MPO significantly attenuated the formation of secosterols by the PMA-activated nHL-60 cells. These data strongly suggest that MPO is involved in the formation of secosterols. To elucidate the role of MPO, we examined the formation of secosterols by neutrophils isolated from WT and MPO-deficient mice (Fig. 4). It was found that similar to the results obtained with the PMA-activated nHL-60 cells, increased amounts of secosterol-A and -B were formed when PMA-activated neutrophils isolated from WT mice were cultured in the medium without and with 10% FBS, respectively. Interestingly, these increased formations of secosterols were not observed when neutrophils isolated from MPO-deficient mice were cultured under the same conditions. Furthermore, low levels of secosterol-A and -B were detected in the plasma samples and liver obtained from WT mice, whereas their levels were much lower in those obtained from MPO-deficient mice (Fig. 6). Also interestingly, upon the induction of acute inflammation by LPS injection, the plasma levels of both secosterol-A and -B were increased significantly in the WT mice, whereas no increase was found in the MPO-deficient mice. These results clearly indicate that MPO is required for the formation of secosterols in vivo.

We have also shown that the formation of secosterol-A but not -B due to the reaction of cholesterol with PMA-activated nHL-60 cells in the FBS-free medium is enhanced by IgG, but it is decreased by the various enzyme inhibitors and scavengers for different reactive species and by neutrophils from MPO-deficient mice. These results support the notion proposed by Babior et al. (1) and Nieve and Wentworth (2) that a reactive species with a chemical signature similar to that of ozone is generated by activated human neutrophils. On the basis of our results and those of Babior et al. (1) and Nieve and Wentworth (2), we can speculate the following reaction mechanism for the

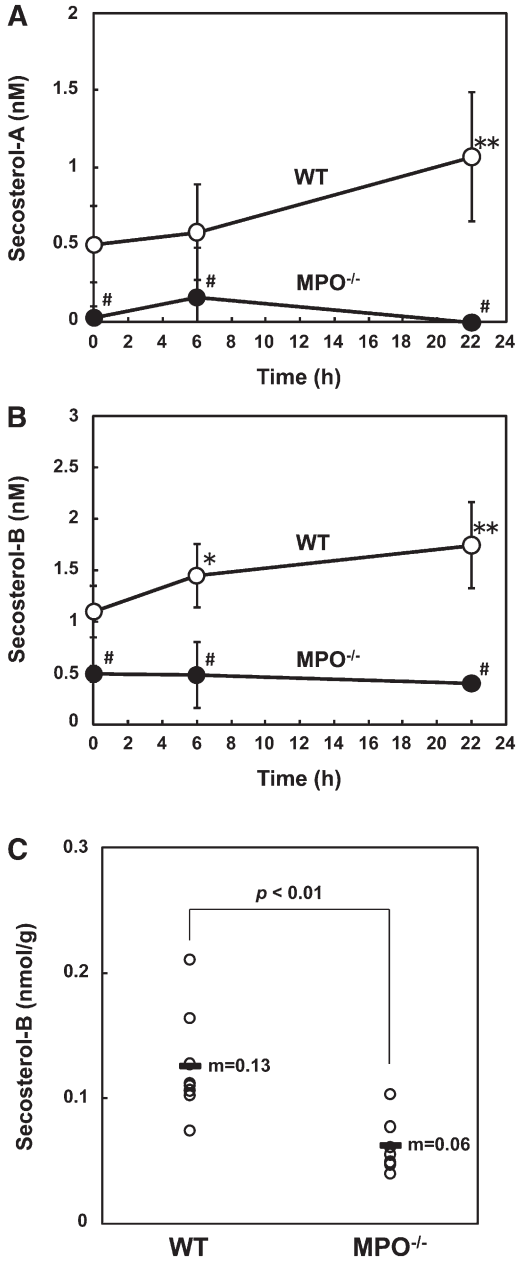

Fig. 6. Levels of secosterols detected in plasma samples and in the liver of WT or MPO-deficient mice. A, B: The levels of secosterol-A and secosterol-B in plasma samples of mice collected at 0, 6 and 22 h after the intraperitoneal injection of LPS (30 mg/kg BW). Values are means ± SD (n = 2-4 in each time point). Statistically significant: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the levels detected in untreated mice; $^{\#}P < 0.01$ compared with the levels between WT and MPO-deficient mice at each time point. C: The levels of secosterol-B in the liver samples collected from untreated WT (n = 8) and MPO-deficient (n = 7) mice. Median (m) values are presented. LPS, lipopolysaccharide; MPO, myeloperoxidase; secosterol-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secosterol-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde; WT, wild-type; BW, body weight.

formation of secosterol-A by PMA-activated nHL-60 cells in the FBS-free medium: *i*) the activated cells produce superoxide anion by NADPH oxidase and/or xanthine oxidase; *ii*) superoxide is converted to hydrogen peroxide by SOD; *iii*) hydrogen peroxide is used by MPO to generate hypochlorous acid; *iv*) hypochlorous acid in the presence of hydrogen peroxide generates singlet oxygen; *v*) IgG catalyzes the formation of an ozone-like oxidant in the presence of singlet oxygen and hydrogen peroxide; and *vi)* an ozone-like oxidant reacts with cholesterol to form secosterol-A (**Fig. 7**).

On the other hand, increased amounts of secosterol-B but not -A were formed when the PMA-activated nHL-60 cells and neutrophils isolated from WT mice were cultured in the medium containing 10% FBS (Figs. 2 and 4). Furthermore, higher levels of secosterol-B than -A were detected in the samples of plasma and liver collected from WT mice compared to those from MPO-deficient mice (Fig. 6). The secosterol-B levels were also significantly increased in the plasma samples of LPS-treated WT mice (Fig. 6). The origin of secosterol-B formed under these conditions is currently unknown, although there are two possibilities as described above: *a)* the conversion of secosterol-A to secosterol-B by aldolization (2, 10), and *b)* the direct formation of secosterol-B by the Hock-cleavage of 5α-hydroperoxy cholesterol (13, 14). On the basis of our results, the former pathway seems to be more important than any other. However, it is clear that under inflammatory conditions, increased amounts of secosterol-B could be formed and may exert adverse effects as discussed below.

It is widely accepted that oxysterols resulting from the chemical or enzymatic oxidation of cholesterol play pivotal roles in the development of cardiovascular diseases and atherosclerotic lesions (25–28). They might also be involved in the development of important degenerative diseases, such as Alzheimer's disease (29), osteoporosis (30–32), and age-related macular degeneration (32). In addition, it has been reported that secosterols induced apoptotic cell death at μM concentrations in several cultured cells, such as T- and B-cells, macrophage, cardiomyoblasts, and abdominal aortic endothelial cells (10, 19, 20). We also observed that secosterols are much more strongly cytotoxic (more than 10-fold) compared with five known toxic oxysterols, including 7β-hydroxycholesterol, 7-ketocholesterol, 5β,6β-epoxycholesterol, and 25-hydroxycholesterol for 48 h incubation in HL-60 cells (Tomono et al., unpublished observations). Further studies are warranted to elucidate the roles of cholesterol ozonolysis products, including their metabolites, in the development of atherosclerosis, neurodegenerative diseases, and other oxidative stress-related disorders, especially in the context of their reactions with tissue components, such as proteins, nucleic acids, and lipids, and their resulting effects on the biological functions of these components.

In conclusion, we demonstrated that secosterols are formed by PMA-activated human neutrophil-like cells as well as activated neutrophils obtained from WT mice. These activated neutrophils produce increased amounts of secosterol-A in the medium without 10% FBS, to which cholesterol was added exogenously. This secosterol-A formation was enhanced significantly by IgG, and the experiments with some enzyme inhibitors and scavengers of ROS imply that the reaction requires the generation of superoxide, hydrogen peroxide, hypochlorous acid, and singlet oxygen. On the basis of

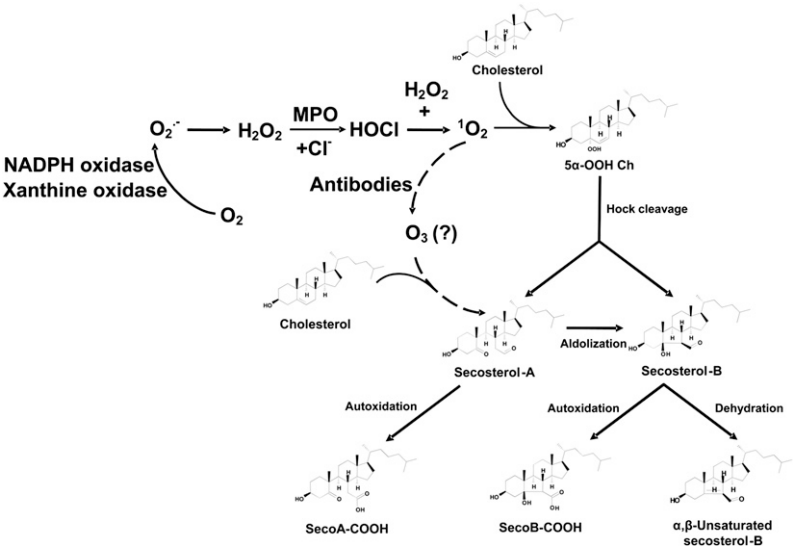

Fig. 7. Proposed mechanisms for the formation of secosterol-A, secosterol-B, and their oxidation products by activated neutrophils. α, β-unsaturated-secosterol-B, 3β-hydroxy-B-norcholest-5-ene; HOCl, hypochlorous acid; MPO, myeloperoxidase; secoA-COOH, 3β-hydroxy-5-oxo-secocholestan-6-oic acid; secoB-COOH, 3β-hydroxy-5β-hydroxy-B-norcholestane-6-oic acid; secosterol-A, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al; secosterol-B, 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde.

these findings, we conclude that secosterol-A, at least in the medium without 10% FBS, is formed by an ozone-like oxidant, which is generated by the catalysis of IgG in the presence of singlet oxygen and hydrogen peroxide. In addition, we clearly demonstrated that MPO plays an important role in the formation of secosterols. Possibly, hypochlorous acid generated by MPO is needed to generate singlet oxygen and, therefore, an ozone-like oxidant in the presence of hydrogen peroxide. As MPO has been implicated in the pathogenesis of atherosclerosis and neurodegenerative diseases, such as Alzheimer's disease (33, 34), further studies are needed to elucidate the biological roles of the MPO-mediated formation of secosterols and their further oxidation products in the development of these diseases.

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