

Inhibition of endothelial- and neuronal-type, but not inducible-type, nitric oxide synthase by the oxidized cholesterol metabolite secosterol aldehyde: Implications for vascular and neurodegenerative diseases

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The cholesterol ozonolysis products secosterol-A and its aldolization product secosterol-B were recently detected in human atherosclerotic tissues and brain specimens, and have been postulated to play pivotal roles in the pathogenesis of atherosclerosis and neurodegenerative diseases. We examined several oxidized cholesterol metabolites including secosterol-A, secosterol-B, 25-hydroxycholesterol, 5 β ,6 β -epoxycholesterol and 7-ketocholesterol for their effects on the activities of three nitric oxide synthases. In contrast to other oxidized metabolites, secosterol-A was found to be a potent inhibitor against the neuronal- and endothelial-type, but not the inducible-type nitric oxide synthase, with IC₅₀ values of 22 \pm 1 and 50 \pm 5 μ M, respectively. The calmodulin-binding regions of the neuronal- and endothelial-nitric oxide synthases contain lysine residues which are not present in the inducible-type nitric oxide synthase. Secosterol-A modifies proteins through the formation of a Schiff base with the lysine epsilon-amino group. It is possible that secosterol-A modifies lysine residues of constitutive nitric oxide synthases, leading to the inhibition of enzymatic activities. As nitric oxide is a critical signaling molecule in vascular function and in long-term potentiation, its reduced production through inhibition of constitutive nitric oxide synthases by secosterol-A may contribute to the development of atherosclerosis and memory impairment in particular neurodegenerative diseases.

Key Words: oxidized cholesterol metabolites, secosterol-A, endothelial-type NOS, neuronal-type NOS, inducible-type NOS

Oxidized cholesterol metabolites, 3 β -hydroxy-5-oxo-5,6-secocholestan-6-al (secosterol-A) and its aldolization product, 3 β -hydroxy-5 β -hydroxy-B-norcholestane-6 β -carboxaldehyde (secosterol-B), have recently been detected in human atherosclerotic tissues⁽¹⁾ and in human brain specimens (Fig. 1).^(2,3) Secosterol-A was previously reported to form through the cleavage of the $\Delta^{5,6}$ double bond of cholesterol by only ozone among the various reactive oxidants including singlet oxygen (¹O₂), superoxide anion, hydroxyl radical and ozone.⁽⁴⁻⁶⁾ On the basis of these previous studies, Wentworth and co-workers proposed that secosterol-A is formed in atherosclerotic plaques by oxidation of cholesterol with ozone, which is generated in the presence of leukocytes and immunoglobulins, and is converted to secosterol-B by an aldolization reaction.⁽¹⁾ Ozone could be formed by activated neutrophils through antibody (immunoglobulin)-

catalyzed oxidation of water with ¹O₂, which is generated from the reaction of H₂O₂ with HOCl.^(7,8) The concentration of secosterol-A was shown to increase significantly upon activation with phorbol myristate acetate (PMA) in atherosclerotic lesions collected from patients.⁽¹⁾ Brinkhorst *et al.*⁽⁹⁾ and Uemi *et al.*⁽¹⁰⁾ recently reported that both secosterol-A and -B are directly formed via Hock cleavage of cholesterol 5 α -hydroperoxide, which is formed from the reaction of cholesterol with ¹O₂. However, secosterol-B was formed more easily^(9,10) from 5 α -hydroperoxide under acidic conditions in organic solvents, whereas secosterol-A was either not formed at all or was a minor component in the aqueous buffer.⁽¹¹⁾ We also reported that secosterols are formed by the reaction of cholesterol with human myeloperoxidase (MPO) in the presence of its substrates H₂O₂ and Cl⁻.⁽¹²⁾ More recently we found that neutrophil-like differentiated human leukemia HL-60 (nHL-60) cells activated with PMA produced significantly increased levels of secosterol-A and its aldehyde-oxidation product. This secosterol-A increase was reduced in the culture of PMA-activated nHL-60 cells containing several ROS inhibitors and scavengers or in a culture of PMA-activated neutrophils isolated from MPO-deficient mice.⁽¹³⁾ These results suggest that secosterol-A is formed by an ozone-like oxidant generated with PMA-activated neutrophils through an MPO-dependent mechanism. Although the exact mechanism for the formation of secosterols *in vivo* remains unresolved, these compounds have been reported to be strongly cytotoxic against various types of cells.^(1,14-16) They also have the ability to covalently modify the amyloid- β peptide, α -synuclein and apolipoprotein C-II, accelerating their amyloidogenesis and fibrilization *in vitro*.^(2,3,17) Amyloidogenesis and/or fibrilization represent defining features of Alzheimer's, Parkinson's and related amyloid diseases as well as atherosclerosis.^(3,17,18) These results indicate that the formation of secosterols in inflamed tissues may contribute to the increased risks of a variety of diseases including atherosclerosis and neurodegenerative diseases such as sporadic Alzheimer's disease in subjects with hypercholesterolemia and inflammation.

Nitric oxide (NO), identified as an endothelium-derived relaxing factor (EDRF), is a critical signaling molecule in vascular function and homeostasis in mammals.⁽¹⁹⁾ NO is formed during the oxidation of L-arginine to L-citrulline, a reaction catalyzed by NO

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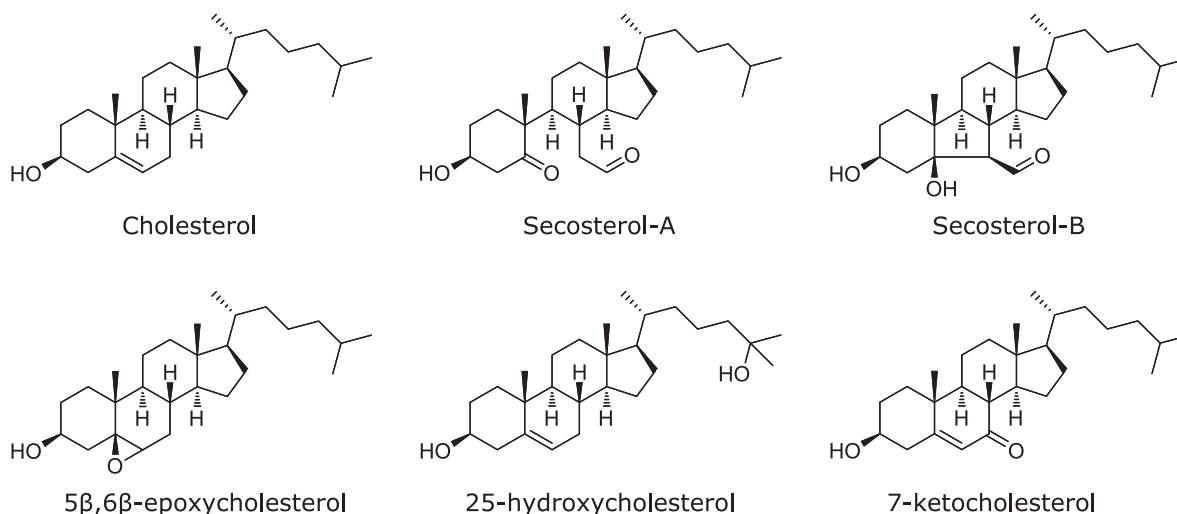


Fig. 1. Structures of cholesterol and its oxidized metabolites examined in this study.

synthases (NOSs, EC 1.14.13.39). Three NOS isoforms have been characterized and cloned: neuronal NOS (nNOS; type I), inducible NOS (iNOS; type II), and endothelial NOS (eNOS; type III).⁽²⁰⁾ Constitutive NOSs (eNOS and nNOS) are associated with the regulation of vascular tone in blood vessels and neurotransmission in the central and peripheral nervous tissues. In the immune system, inflammatory cytokines stimulate iNOS expression in macrophages and other cell types to provide defense against pathogens. Prolonged production of excess amounts of NO may damage normal tissues, thereby contributing to the development of chronic inflammatory diseases including atherosclerosis and neurodegenerative diseases.

Vascular diseases associated with hypercholesterolemia, diabetes and hypertension are characterized by endothelial dysfunction and reduced endothelium-mediated vasodilation. The inactivation and impaired generation of NO by reactive oxygen species play an important role in these disease states. For these reasons, we have studied the effects of several oxidized cholesterol metabolites on the activities of three isoforms of NOS *in vitro*. These included the metabolites 25-hydroxycholesterol, 5β,6β-epoxycholesterol, 7-ketocholesterol, secosterol-A and secosterol-B (Fig. 1). 25-Hydroxycholesterol is produced enzymatically by cholesterol 25-hydroxylase and acts as a potent inhibitor of cholesterol biosynthesis in different cell types.^(21,22) 5β,6β-Epoxycholesterol has been studied as a product of cholesterol autooxidation and lipid peroxidation⁽²³⁾ and has been shown to cause cytotoxicity in *in vitro* systems.^(24,25) 7-Ketocholesterol (3β-hydroxy-5-cholesten-7-one) is also a cholesterol autooxidation product and shows deleterious proinflammatory and pro-apoptotic effects *in vitro*.^(26,27) In the present study, the primary ozonolysis product of cholesterol, secosterol-A, was found to be a potent inhibitor of both nNOS and eNOS, but not of iNOS. We discuss possible pathophysiological roles of oxidized cholesterol metabolites in the development of atherosclerosis and neurodegenerative diseases in relation to the impairment of NO production mediated by inhibition of nNOS and eNOS with secosterol-A.

Materials and Methods

Materials. Secosterol-A and -B were synthesized according to Wentworth *et al.*⁽¹⁾ L-Arginine, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), (6R)-5,6,7,8-tetrahydro-

biopterin (BH₄), recombinant eNOS (from bovine), iNOS (from mouse macrophages), and Dowex® AG50W-X8 cation-exchange resin (mesh size, 100–200) were all purchased from Sigma Chem. (St. Louis, MO). Ultima Gold XR scintillation liquid was from PerkinElmer (Shelton, CA). Recombinant nNOS (from rat) was obtained from Cayman Chem. All other chemicals were obtained from Wako Pure Chem. Ind., Osaka, Japan.

Assay for NOSs activities. The NOS activities were determined by monitoring the conversion of L-[¹⁴C(U)]-arginine (Moravek, Brea, CA) to L-[¹⁴C(U)]-citrulline as previously described.⁽²⁸⁾ Recombinant enzymes of three isoforms of NOS (specific activity: eNOS 0.03, nNOS 2.9, iNOS 0.62 units/mg protein) (10 μl) were added to a reaction mixture (final volume of 100 μl) consisting of 50 mM HEPES buffer (pH 7.4), 1 mM NADPH, 4 μM FAD, 4 μM FMN, 10 μM BH₄, 1 mg/L (60 μM) calmodulin (CaM), 2.5 mM CaCl₂, 50 μM L-arginine, 0.5 μCi/ml of L-[¹⁴C(U)]-arginine (specific activity: 8.14–11.1 GBq/mmol) and varying concentrations of the test compound. After incubation for 30 min at 37°C, the reactions were terminated by the addition of 0.5 ml of 20 mM HEPES (pH 5.5) containing 2 mM EDTA and 2 mM EGTA. Samples were passed through a 1 ml column of Dowex AG50W-X8 (Na⁺-form) to remove unconverted L-[¹⁴C(U)]-arginine. L-[¹⁴C(U)]-Citrulline was eluted twice with 1 ml of distilled water, and the eluted fractions mixed with 10 ml of cocktail (Ultima Gold XR) were quantified using a liquid scintillation counter. A reaction mixture containing L-[¹⁴C(U)]-arginine without the enzyme was incubated as a control to determine background counts. The effects of oxidized cholesterol metabolites on the NOSs activities were examined in the presence and absence of the test compounds at various concentrations, which were prepared in 5% ethanol and a 10 μl aliquot was added into the reaction mixture.

Initial rates of citrulline formation were obtained by linear leastsquares data fitting of actual experimental data. Apparent K_m and V_{max} values at each inhibitor concentration were obtained from nonlinear regression fitting using the Michaelis-Menten equation. K_i and K_i' were obtained from the X-axis intercepts of plots of measured K_m/V_{max} and $1/V_{max}$ vs inhibitor concentration, respectively.

Statistical analysis. All experiments were repeated 3–8 times. Representative results are shown as means ± standard deviations (SD). For statistical comparison between two groups, an unpaired *t* test was used.

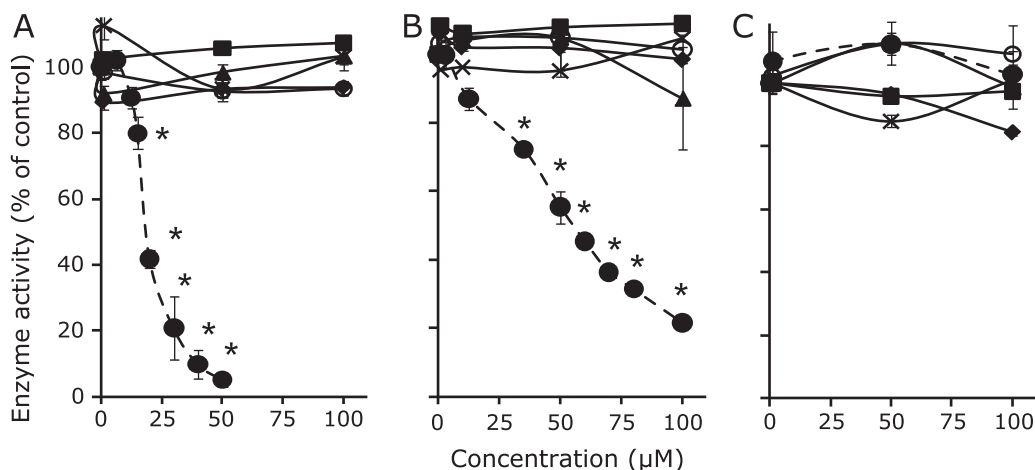


Fig. 2. Effects of oxidized cholesterol metabolites on the enzymatic activities of three isoforms of NOSs, (A) nNOS, (B) eNOS and (C) iNOS. A reaction mixture containing a recombinant NOS, necessary cofactors, 50 μM L-arginine (including L-[^{14}C (U)]-arginine) and a test compound at the indicated concentration was incubated for 30 min at 37°C. Results are shown as the means \pm SD normalized to a value of 100% for the control (the absence of compounds) ($n = 3-6$). Significantly different when compared with the control, $*p < 0.01$. cholesterol (\times), 25-hydroxycholesterol (\blacklozenge), 5 β ,6 β -epoxycholesterol (\blacksquare), 7-ketocholesterol (\blacktriangle), secosterol-A (\bullet) and secosterol-B (\circ).

Results

Effects of cholesterol and various oxidized cholesterol metabolites on the activities of three NOS isoforms.

Cholesterol and its oxidized metabolites including 25-hydroxycholesterol, 5 β ,6 β -epoxycholesterol, 7-ketocholesterol, secosterol-A and secosterol-B were examined for their effects on the activities of three NOS isoforms *in vitro*. As shown in Fig. 2 A and B, only secosterol-A exhibited potent inhibitory effects on nNOS and eNOS activities in a concentration-dependent manner, with IC_{50} values of 22 ± 1 and 50 ± 5 μM , respectively. Interestingly, secosterol-A at the same tested concentrations (1–100 μM) did not affect the iNOS activity (Fig. 2C). Cholesterol and other oxidized cholesterol metabolites exhibited no inhibitory effects on the activities of three NOS isoforms even at the highest concentration (100 μM) (Fig. 2 A–C).

Kinetics by substrate-dependent constitutive NOS by secosterol-A. To elucidate the mechanism for inhibition of constitutive NOSs (nNOS and eNOS) by secosterol-A, a series of enzyme assays were carried out in the presence of varying concentrations of its substrate L-arginine and the inhibitor secosterol-A. Depending on the secosterol-A concentration, the presence of an excess amount of L-arginine only partially recovered the activities of nNOS and eNOS inhibited by secosterol-A (Fig. 3A and 4A). Double reciprocal (Lineweaver-Burke) plots of constitutive NOSs activities vs L-arginine concentrations in the absence and presence of varying amounts of secosterol-A are shown in Fig. 3B and 4B. The kinetics parameters obtained from these plots are shown in Table 1. The pattern of secosterol-A-mediated inhibition of the rat nNOS activity indicates that the inhibition was noncompetitive or a mixed-type. Here, a K_i value of 10 μM for the catalytic site and a K_i' value of 1 μM for the allosteric site were observed. These results suggest that secosterol-A can bind more easily with the enzyme-L-arginine complex than the apo-enzyme. The inhibition of the bovine eNOS activity by secosterol-A followed a similar pattern as that of the rat nNOS activity (Fig. 4B), with the K_i and K_i' values being 50 and 23.3 μM , respectively.

The effects of CaM and BH₄ concentrations on the secosterol-A-mediated inhibition of constitutive NOSs activities. We next examined the possibility whether secosterol-A interfered with the binding of cofactors CaM and BH₄ to the constitutive

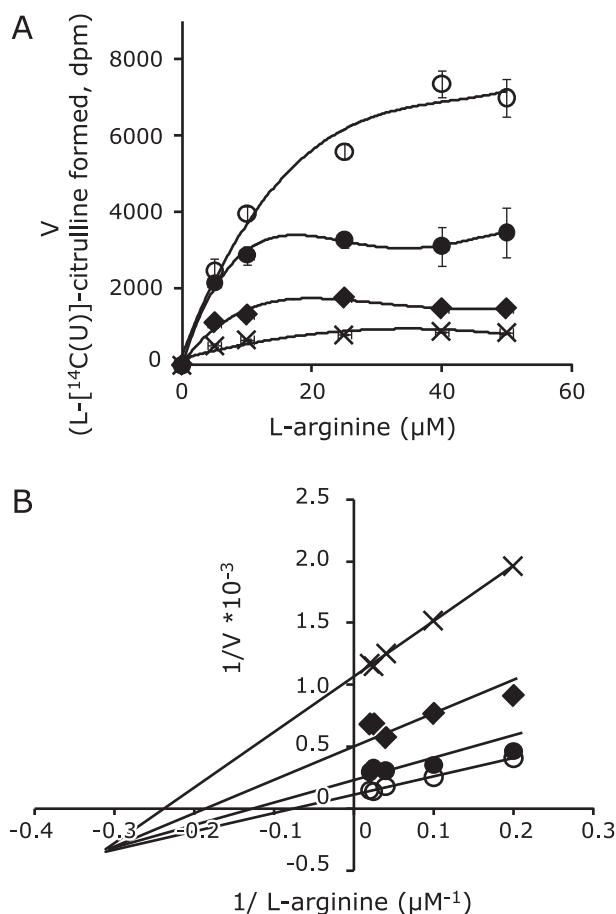


Fig. 3. Effect of secosterol-A on L-arginine-dependent nNOS activity. (A) nNOS activity both in the absence (\circ) and presence of 20 (\bullet), 30 (\blacklozenge) or 40 μM (\times) secosterol-A; (B) A double reciprocal (Lineweaver-Burke) plot.

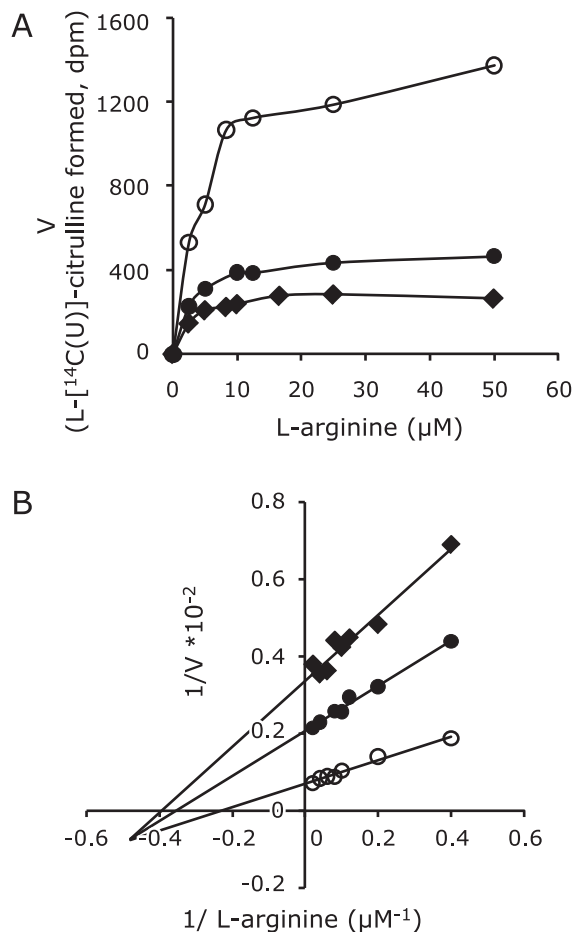


Fig. 4. Effect of secosterol-A on L-arginine-dependent eNOS activity. (A) eNOS activity both in the absence (○) and presence of 50 (●) or 100 μM (◆) secosterol-A; (B) A double reciprocal (Lineweaver-Burke) plot.

NOSs. In the presence of 60 μM CaM, secosterol-A at 40 μM concentration reduced the nNOS activity to 22% when compared to the activity without secosterol-A. The addition of excess CaM at 300 and 600 μM in the presence of 40 μM secosterol-A restored the nNOS activity to 63 and 86%, respectively (Fig. 5A). On the other hand, the addition of excess CaM did not restore the eNOS activity inhibited by the presence of 100 μM secosterol-A (Fig. 5A). In contrast, in the presence of 10 μM BH₄, 40 and 100 μM secosterol-A significantly decreased the nNOS and eNOS activities to 23 and 41% of the activities without secosterol-A, respectively (Fig. 5B). The addition of 50 and 100 μM BH₄ did not restore the activities of constitute NOSs inhibited by secosterol-A (Fig. 5B).

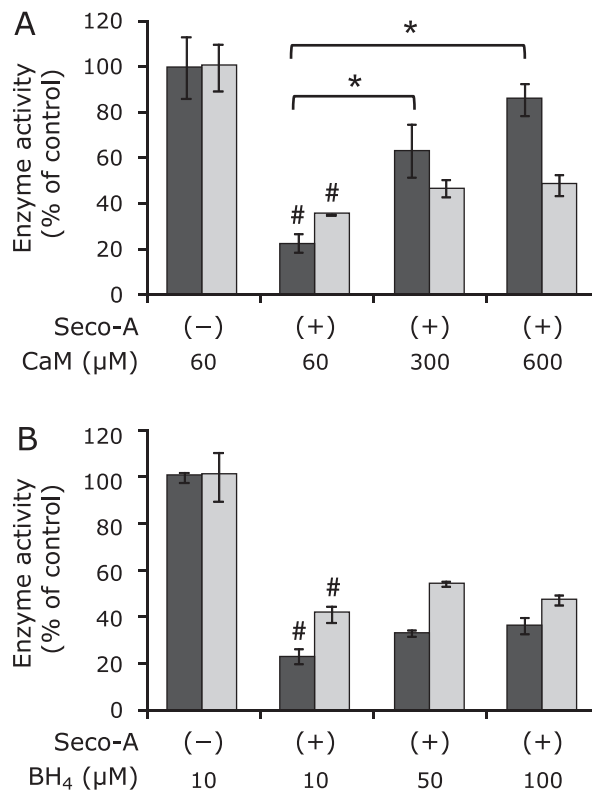


Fig. 5. Effects of increased calmodulin (CaM) or BH₄ concentrations on constitutive NOSs inhibition by secosterol-A. Addition of increasing concentrations of CaM (60–600 μM) (A) or BH₄ (10–100 μM) (B) to the reaction mixture containing nNOS (closed bars) or eNOS (open bars). nNOS and eNOS activities were determined in the presence of secosterol-A (Seco-A) at concentrations of 40 and 100 μM, respectively. Results are shown as the means ± SD normalized to a value of 100% for the control (the absence of secosterol-A) (*n* = 3). #significantly different (*p* < 0.05) when compared with the control (the absence of secosterol-A). *significantly different (*p* < 0.05) when compared with the reaction in the presence of 60 μM CaM.

As Fig. 6A shows, the addition of excess amounts of CaM only partially recovered the activity of nNOS in the presence of varying concentrations of secosterol-A. Double reciprocal (Lineweaver-Burke) plots of nNOS activities versus CaM concentrations in the absence and presence of secosterol-A are shown in Fig. 6B. The secosterol-A-mediated inhibition of rat nNOS activities was noncompetitive or a mixed-type of inhibition, with a *K_i* value of 1 μM for the catalytic site and a *K_i'* of 30 μM for the allosteric site. This observation suggests that secosterol-A binds more easily with the apo-enzyme than the enzyme-CaM complex.

Table 1. Kinetic parameters for the inhibition of constitutively active NOSs by secosterol-A

Isoform	Secosterol-A (μM)	<i>K_m(app)'</i> (μM)	<i>V_{max}(app)'</i> (μmol·L ⁻¹ ·30 min ⁻¹)	<i>K_i(app)</i> (μM)	<i>K_i(app)'</i> (μM)	IC ₅₀ (μM)
nNOS (Rat)	0	15	10000	—	—	—
	40	4.1	909	10	1	22 ± 1
eNOS (Bovine)	0	4.3	1400	—	—	—
	50	2.8	480	50	23	50 ± 5

K_m(app)', *K_i* and *K_i'* were obtained based on the results in Fig. 3 and 4. *K_i(app)* for the catalytic site and *K_i(app)'* for the allosteric site. Values of IC₅₀ were calculated from $IC_{50} = 10^{(\log(A/B) * (50 - C) / (D - C) + \log(B))}$. A and B represent the highest and lowest concentrations of an inhibitor, respectively. C and D represent the % inhibition in the presence of B or A, respectively.

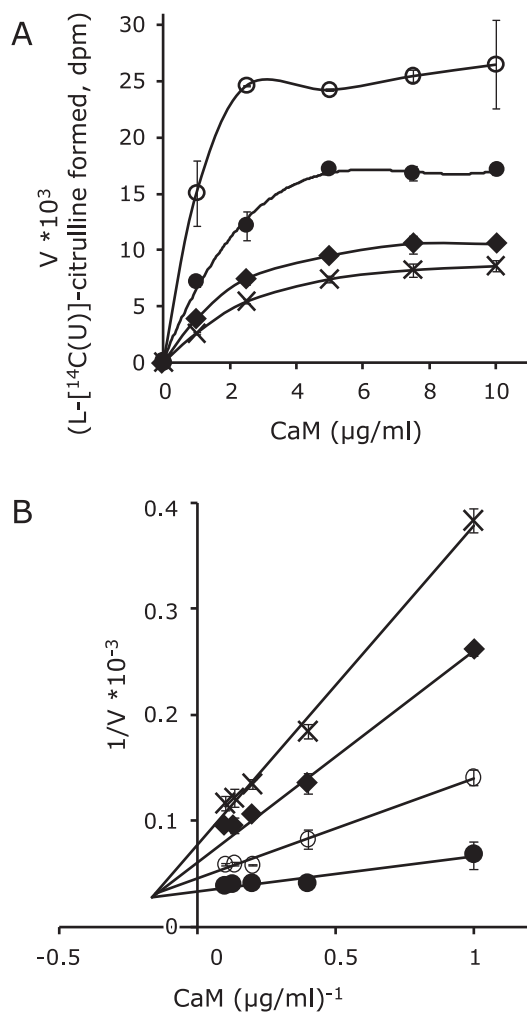


Fig. 6. Effect of secosterol-A on CaM-dependent nNOS activity. (A) nNOS activity both in the absence (○) and presence of 25 (●), 30 (◆) or 40 μM (×) secosterol-A; (B) A double reciprocal (Lineweaver-Burke) plot.

Discussion

The present results show that although cholesterol and its oxidized metabolites such as 25-hydroxycholesterol, 5β,6β-epoxycholesterol, 7-ketocholesterol and secosterol-B do not affect the enzymatic activities of NOSs, only secosterol-A at relatively low concentrations strongly inhibits the enzymatic activities of eNOS and nNOS, but not iNOS. This inhibition by secosterol-A of rat nNOS activity was partially restored by the addition of increased amounts of CaM, but not BH₄. In contrast, the inhibition by secosterol-A of bovine eNOS was not ameliorated by adding increased amounts of CaM and BH₄. These results suggest that the binding sites of CaM, but not that of BH₄, in nNOS may be important for secosterol-A inhibitory activity. Further studies are required to elucidate the mechanisms by which secosterol-A inhibits only constitutive NOSs, but not inducible NOSs.

Although the exact mechanisms for the inhibition of constitutive NOSs by secosterol-A is unresolved, it is likely that secosterol-A interacts with eNOS and nNOS and modifies their conformations. Previous studies^(2,3) have shown that hydrophobic aldehydes, including secosterol-A, modify proteins through the formation of a Schiff base with the lysine epsilon-amino group or N-terminal amino group. We compared the amino acid sequences of three NOSs isoforms (bovine eNOS, rat nNOS and mouse iNOS)

(UniProtKB/Swiss-Prot P29473, P29476 and P29477, respectively). There are 12 lysine residues which are present in both eNOS and nNOS, but not in iNOS. Interestingly, four out of these 12 lysine residues in the rat nNOS (K717, K725, K732, K743) are localized near or within the CaM-binding region (725–745) and two (K751 and K754) are present in the BH₄-binding region (750–769). The same amino acid sequences are found in the bovine eNOS (K488, K496, K499, K506, K518 and K521) near or within the CaM- or BH₄-binding regions, whereas these lysine residues are replaced by other amino acids (Q496, R504, R510, F507, S529 and R532, respectively) in the mouse iNOS. We speculate that secosterol-A reacts with one or more lysine residues in these regions to modify their conformations, leading to the inhibition of the binding of the cofactors, and thereby reducing the activities of eNOS and nNOS. As these lysine residues are also well conserved in human eNOS and nNOS (UniProtKB/Swiss-Prot P29474, P29475), it is expected that secosterol-A may inhibit human constitutive NOSs in a similar fashion as the bovine and rat homologues. Further studies are needed to elucidate the inhibition mechanisms of constitutive NOSs by secosterol-A, but not by other cholesterol oxidized metabolites. Although there have been no published studies concerning the comparison of the reactivity with proteins among various oxysterols, our preliminary data show that secosterol-A can react with proteins such as bovine serum albumin to form the adduct more easily than secosterol-B (unpublished data).

Multiple lines of investigation indicate that NO produced by eNOS is atheroprotective and that NO deficiency is critically involved in the pathogenesis of hypercholesterolemia-induced vascular disease.⁽²⁹⁾ The eNOS protein has been shown to be associated with caveolae as a result of myristoylation on the glycine adjacent to the leading methionine and thiopalmitoylation on the cysteine at positions 15 and 26.^(30,31) Caveolae are specialized, lipid-ordered plasma membrane microdomains enriched in cholesterol, glycosphingolipids, sphingomyelin, and lipid anchored membrane proteins, and they contain a variety of signal-transduction molecules. Membrane cholesterol is essential for normal caveolae function. The concentration of secosterol-A in the atherosclerotic lesions of patients displaying an atherosclerosis disease state have been reported to be in the range of 6.8 and 61.3 pmol/mg of plaque (7–60 μM). Significant amounts of secosterol-A (up to 200 pmol/mg of plaque, ~200 μM) were also found to be formed in atherosclerotic lesions upon activation with PMA.⁽¹⁾ As the IC₅₀ value for eNOS was 50 ± 5 μM, it is possible that the secosterol-A formed in caveolae reacts directly with the caveolae-associated eNOS to inhibit enzymatic activity.

The brain contains the highest levels of cholesterol in mammalian organs. High levels of total cholesterol in serum have been shown to be a risk factor in the pathology of the central nervous system associated with the development of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.^(32,33) Cholesterol may be oxidized to generate secosterols during inflammation and oxidative stress. Low levels of secosterol-A and -B have been detected in human and rat brain samples at combined concentrations of up to 400 pg/mg of wet brain (~1 μM concentration). However, as these secosterols are very reactive, free forms may disappear rapidly from the tissues by forming adducts with proteins. Consequently, much higher concentrations of secosterols may actually be continuously generated in inflamed tissues. On the basis of these facts, it is plausible that nNOS in the brain could also be inhibited by secosterol-A. As NO plays an important role in long-term potentiation, a form of synaptic plasticity that is a putative mechanism of memory formation in the hippocampus, reduced NO production through inhibition of nNOS by secosterol-A may contribute to impairment of memory during the development of neurodegenerative diseases.

It is also interesting to note that secosterol-A did not affect the enzyme activity of iNOS. Secosterol-A could be formed during

inflammation and oxidative stress under which iNOS may also be expressed simultaneously. As both secosterol-A and excess NO are cytotoxic, it is possible that both compounds may exert detrimental effects against normal tissues and induce inflammation-related tissue damage.

In conclusion, we have shown that the oxidized cholesterol metabolite secosterol-A is a potent inhibitor of eNOS and nNOS, but not iNOS *in vitro*. Since secosterol-A has been reported to be present and/or formed in human atherosclerotic tissues and in human brain specimens, is strongly cytotoxic against various types of cells, and can covalently modify various proteins including amyloid- β peptide and apolipoprotein, the impairment of NO production mediated through inhibition of eNOS and nNOS by secosterol-A may contribute to the development of atherosclerosis and particular neurodegenerative diseases.

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Abbreviations

BH ₄	(6R)-5,6,7,8-tetrahydrobiopterin
MPO	myeloperoxidase
NOS	nitric oxide synthase
nNOS	neuronal NOS
iNOS	inducible NOS
eNOS	endothelial NOS
secosterol-A	3 β -hydroxy-5-oxo-5,6-secocholestan-6-al
secosterol-B	3 β -hydroxy-5 β -hydroxy-B-norcholestane-6 β -carboxaldehyde

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