Oxidation of Cholesterol by Amyloid Precursor Protein and β-Amyloid Peptide*

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Alzheimer’s disease (AD) is characterized by accumulation of the neurotoxic peptide β-amyloid, which is produced by proteolysis of amyloid precursor protein (APP). APP is a large membrane-bound copper-binding protein that is essential in maintaining synaptic function and may play a role in synaptogenesis. β-Amyloid has been shown to contribute to the oxidative stress that accompanies AD. Later stages of AD are characterized by neuronal apoptosis. However, the biochemical function of APP and the mechanism of the toxicity of β-amyloid are still unclear. In this study, we show that both β-amyloid and APP can oxidize cholesterol to form 7β-hydroxycholesterol, a proapoptotic oxysterol that was neurotoxic at nanomolar concentrations. 7β-Hydroxycholesterol inhibited secretion of soluble APP from cultured rat hippocampal H19–7/IGF-IR neuronal cells and inhibited tumor necrosis factor-α-secretase activity but had no effect on β-site APP-cleaving enzyme 1 activity. 7β-Hydroxycholesterol was also a potent inhibitor of α-protein kinase C, with a $K_i$ of ~0.2 nm. The rate of reaction between cholesterol and β-amyloid was comparable to the rates of cholesterol-metabolizing enzymes ($k_{cat} = 0.211 \text{ min}^{-1}$). The rate of production of 7β-hydroxycholesterol by APP was ~200 times lower than by β-amyloid. Oxidation of cholesterol was accompanied by stoichiometric production of hydrogen peroxide and required divalent copper. The results suggest that a function of APP may be to produce low levels of 7-hydroxycholesterol. Higher levels produced by β-amyloid could contribute to the oxidative stress and cell loss observed in Alzheimer's disease.

Several lines of evidence have implicated a role for cholesterol in Alzheimer’s disease (AD). Elevated serum cholesterol is associated with increased risk for AD (1, 2). Feeding cholesterol and copper to rabbits produces some of the pathological signs reminiscent of Alzheimer’s disease, including amyloid-like plaques, and interferes with the ability of rabbits to perform difficult memory tasks (3). The e4 mutant in the gene for apolipoprotein E (apoE), an important cholesterol transport protein associated with low density lipoprotein (LDL), has been shown to be a risk factor for Alzheimer’s disease, and cholesterol-lowering drugs reduce the prevalence of AD. Cholesterol can also regulate the generation of the toxic β-amyloid peptide in affected brain regions and can inhibit β-amyloid clearance. Aberrations in cholesterol transport produced by redistribution of presenilin have been shown to increase cellular levels of β-amyloid (4).

Much attention has also focused on β-amyloid, a neurotoxic peptide produced by proteolysis of APP. APP fragments can regulate cell excitability, synaptic transmission, and long term potentiation. Soluble β-amyloid inactivates specific voltage-dependent K⁺ channels (5) and activates others (6). The parent protein, APP, is an integral membrane copper-binding protein (7) found in the CNS and in a variety of other cells, including epithelial cells. At least eight alternatively spliced forms of APP are known (8, 9). Zou et al. (10) suggested that monomeric β-amyloid behaves as an antioxidant, whereas oligomerized β-amyloid loses its antioxidant activity. It was shown that cholesterol and monomeric β-amyloid protect against apoptosis (11).

Copper also may be a factor in AD. Copper levels are increased in cerebral cortex of APP knock-out mice (12), suggesting that APP could be a regulator of copper homeostasis (13). Dietary copper reduces β-amyloid production (14). APP binds copper with high affinity and reduces Cu(II) to Cu(I) (13, 15, 16). It has been suggested that APP may function as a redox-active metalloprotein (17) or as an antioxidant, because of its ability to chelate copper (18, 19), and it was predicted that removal of β-amyloid would lead to more oxidative stress (18, 19). The APP-Cu(I) complex undergoes site-specific and random fragmentation in the presence of H₂O₂ (20). Down-regulation of APP reduces copper neurotoxicity (21), suggesting that APP may generate toxic quantities of Cu(I). It was proposed that this redundant might deplete intracellular glutathione, paradoxically causing oxidative stress. Conversely, copper supplementation decreases β-amyloid formation in Chinese hamster ovary cells (22).

Another characteristic of Alzheimer’s disease is oxidative stress. β-Amyloid produces oxidative stress by three mechanisms: by the radical nature of β-amyloid at Met35, which can react with oxygen in a metal-independent fashion (23), by inducing receptor mediated pro-inflammatory signaling pathways, or by interacting with mitochondrial or endoplasmic reticulum membranes to impair the oxidation chain, thereby potentially compromising Ca²⁺ uptake and Ca²⁺ regulation of mitochondria and the endoplasmic reticulum. β-Amyloid was also reported to promote oxidative stress directly by catalytically producing H₂O₂ from cholesterol (24). Oxidized proteins have been found in brains of Alzheimer’s patients (25).

However, despite intensive research, the precise relationship between APP, copper, and cholesterol, the mechanism by which β-amyloid causes cell death and synaptic dysfunction, and the biochemical role of cholesterol and oxidative stress in AD are still unclear. To gain further insight as to how β-amyloid...
causes oxidative stress, we examined the interaction between APP and cholesterol and found that β-amyloid and APP possess oxidative activity toward cholesterol and produces 7-hydroxy-cholesterol, a toxic oxyester.

**MATERIALS AND METHODS**

**Materials—**Synthetic cholesterol derivatives were obtained from Steraloids, Inc. and Sigma Chemical Co. Cholesterol-5α-hydroperoxide and 3β-hydroxycholesterol-4-ene-6α-hydroperoxide were synthesized by photooxidation of cholesterol (28). Antibodies were obtained from Santa Cruz Biotechnology and Upstate Biotechnology. Precast isoelectric focusing gradient strips were obtained from Invitrogen. Purified proteins and PKC isozymes were obtained from Sigma. Apolliprotein E was obtained from Calbiochem. Fresh synthetic human β-amyloid 1–42 (Sigma) was dissolved in 50% Me2SO and stored in a glass vial at −80 °C until use. The concentration and purity of β-amyloid were monitored by HPLC.

**Enzymatic Oxidation of Cholesterol—**[7-3H]Cholesterol (Amersham Biosciences) in toluene solution (2 μl) was evaporated and redissolved in 2 μl of ethanol in a 0.5-ml capped polypropylene centrifuge tube. Varying amounts of β-amyloid 1–42 or purified APP were added, and the volume was brought to 15 μl with deionized water. The final concentration of Tris-HCl (pH 7.4) was 100 mM; Triton X-100, 0.66%; CuCl2, 2 μM; EDTA, 0.1 μM; Me2SO, 0.3–5%. The centrifuge tubes were sealed and incubated at 37 °C for 2 h and immediately stored at −20 °C until analysis. In some experiments, the reaction was carried out in a suspension of rat hippocampal neurons in 1× phosphate-buffered saline, in the absence of Me2SO, Triton, Tris, and Tween, and the volume was increased to 100 μl. In these experiments, the reaction products were extracted with CHCl3, evaporated under nitrogen, and redissolved in 15 μl of ethanol prior to HPLC analysis. Old solutions of cholesterol were found to contain unidentified contaminants that inhibited the reaction. Therefore, the cholesterol radioactive source vials were stored at −80 °C and used within 2 weeks of purchase.

**HPEC—**Samples (10 μl) were injected onto a Microsphere RP 300 5-μm C18 reversed-phase HPLC column equilibrated with solvent A (H2O, 0.1% TFA). A linear gradient of 100% solvent A to 100% ethanoll in 40–60 min, followed by 100% ethanol for 40–60 min, at a flow rate of 0.3 ml/min. Absorbance was monitored at 210 nm and 0.3-min fractions (except as noted) were collected in glass scintillation vials. Two milliliters of Biofluor (Research Products International) was added, and the radioactivity was counted in a Beckman LS 3801 liquid scintillation counter.

**Preparation of ApoE-Sepharose 4B—**Apolipoprotein E (100 μg, Calbiochem) was dialyzed for 72 h at 4 °C against 10 mM NaHCO3, pH 8.0. Cyanogen bromide-activated Sepharose 4B (0.25 g, Amersham Biosciences) was then suspended in 1 mM HCl, shaken gently at room temperature for 15 min to rehydrate it, then collected by centrifugation. The apoE was diluted to 5 μl in coupling buffer (0.1 mM NaHCO3 plus 0.5 mM NaCl), added to the rehydrated CNBr-Sepharose, and reacted at room temperature with gentle shaking for 4 h. Unreacted binding sites were then blocked by addition of Tris-HCl (pH 8) to 0.1M, and the mixture was removed, and the cells were washed twice with phosphate-buffered saline, removed by gentle scraping, and collected by centrifugation at 800 g for 5 min.

**Purification of APP—**Two frozen rat brains or two frozen guinea pig brains were sonicated in 20 ml of 50 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, and 2 mM benzamidine. The supernatant was centrifuged at 5,000 × g for 20 min at 4 °C, and the cytosolic supernatant was saved. The pellet was re-sonicated in 10 ml of 10% Triton X-100 and re-centrifuged, and the supernatant containing the solubilized extract was saved. The cytosol and the solubilized extract were then added to separate tubes containing apoE-Sepharose and incubated for 1 h at room temperature with gentle shaking. The Sepharose was then collected by centrifugation and transferred to two separate columns at room temperature and washed extensively with 10 ml Tris-HCl, pH 7.4, followed by 20 ml of 10 mM Tris-HCl, pH 7.4, containing 100 mM NaCl. The APP was then eluted by sequentially applying 2.5 ml of 5 mM NaCl plus 10 mM Tris-HCl, pH 7.4, 2 M NaCl, and sodium acetate (pH 4.5), and 2.5 ml of 1 M Tris-HCl, pH 8.0, plus 1 M NaCl. The elutes were collected directly into Centricon-30 ultrafiltration devices. The Centricon devices were then centrifuged at 5000 × g overnight. Centrifugation was repeated with two additions of 10 mM Tris-HCl, pH 7.4. The fractions containing purified APP were identified by Western blotting, and the desalted protein was stored at −20 °C.

**Two-dimensional Western Blot—**Samples of purified APP were applied to an In vitro gen immobilized pH gradient, pH 3–10, polyacrylamide isoelectric focusing strip that had been rehydrated with isoelectric focusing sample buffer (8.5% urea, 2 M thiourea, 0.4% CHAPS, 0.5% immobilized pH gradient buffer (Amersham Pharmacia Biotech), and 0.01% bromphenol blue) and subjected to isoelectric focusing on an LKB flatbed Multiphor apparatus (27). The focusing strip was then equilibrated for 15 min with SDS equilibration buffer (0.05 M Tris-HCl, pH 6.8, 1.2 M urea, 6% glycerol, 0.2% SDS, and 0.1% bromphenol blue), transferred to a Bio-Tris minigel (Invitrogen, Zoom Gel), and subjected to SDS electrophoresis, nitrocellulose blotting, and antibody staining using anti-APP (Santa Cruz Biotechnology) and alkaline phosphatase conjugated secondary antibody as described previously.

**H2O2 Measurement—**Samples containing unlabeled cholesterol (170 μM), CuCl2, or Cu(Glycine) (50 mM, dissolved in 50% Me2SO), Tris-HCl (0.1 M, pH 7.4), Triton X-100 (0.66%), and EDTA (0.1 μM) were incubated under air in 1.5-ml conical polypropylene centrifuge tubes for 2 h at 37 °C. Hydrogen peroxide was then measured fluorometrically using horseradish peroxidase assays based on horseradish peroxidase labeled on 10-kDa small, homovanillic acid, horseradish peroxidase, and Tris-HCl buffer, pH 7.4, were added to final concentrations of 83 μM, 0.05 unit/μl, and 0.01 μl, respectively. Samples were incubated for 10 min at room temperature, diluted to 3 ml, and fluorescence was measured using a Spex Fluorolog-2 spectrofluorometer using excitation and emission wavelengths of 321 and 421 nm, respectively.

**Mass Spectrometry—**Mass spectrometry was carried out by M-Scan, Inc. (Westchester, PA) using a VG analytical ZAB-2SE high field mass spectrometer. The source temperature was maintained at 200 °C, and the spectra were acquired with an electron voltage of 70 eV. The sample was transferred to a solids probe using dichloromethane, inserted into the instrument via the solids probe, and heated until an appropriate spectrum was obtained.

**Two-dimensional Gel Analysis—**Image quantitation and molecular weight estimation were done using the Unix-based image analysis program tnimage (available at www.braneurosci.org/tnimage.html). Enzyme kinetic parameters were obtained by nonlinear fitting using the Marquard-Levenberg algorithm.

**Cell Culture—**Rat hippocampal H19–7/IGF-IR cells (ATCC) were cultured on poly-L-lysine-coated plates and grown at 35 °C in Dulbecco’s modified Eagle’s medium/10% fetal calf serum for several days until ~50% coverage was obtained. The cells were then induced to differentiate into a neuronal phenotype by replacing the medium with 5 ml of N2 medium containing 10 ng/ml basic fibroblast growth factor and grown in T-25 flask at 39 °C. Varying concentrations of the cholesterol metabolite were then added in 10 μl of ethanol. After 48 h the medium was removed, and the cells were washed twice with phosphate-buffered saline, removed by gentle scraping, and collected by centrifugation at 500 rpm (30 × g) for 5 min.

**sAPP Measurement—**Cell culture medium was centrifuged at 5000 × g for 5 min and filtered through a Pasteur pipette containing a cotton plug to remove any detached or dead cells. A 1-ml aliquot of the filtrate was then precipitated by adding 0.1 ml of 100% (w/v) trichloroacetic acid and centrifuging (15,000 × g, 4 °C, 10 min). The pellet was redissolved in 100 μl of H2O by directing NH4 vapor into the test tube and mixed with SDS sample buffer, and the proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blots were incubated with anti-β-amyloid antibody (Santa Cruz sc-5399) and developed with an alkaline phosphatase-linked secondary antibody. The bands were detected with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. The images were photographed using a Fujifilm LAS-1000 Plus charge-coupled device gel documentation system, and 16-bit images were quantitated by densitometry.

**TACE Assay—**TACE (TNF-α-converting enzyme, ADAM-17) activity was measured using a fluorometric fluorescence resonance energy transfer assay (29). Cultured cells or rat brain homogenate were incubated with 10 μM TACE substrate IV (Abz-LAQAVRSSR-DPa, Calbiochem) and substrate concentrations for TACE were determined by thin-layer chromatography using an HPLC—Mass spectrometry—Two-dimensional Western Blot—Two-dimensional Gel Analysis—Mass spectrometry—Cell Culture—Cell Culture—sAPP Measurement—sAPP Measurement—TACE Assay—TACE (TNF-α-converting enzyme, ADAM-17) activity was measured using a fluorometric fluorescence resonance energy transfer assay (29). Cultured cells or rat brain homogenate were incubated with 10 μM TACE substrate IV (Abz-LAQAVRSSR-DPa, Calbiochem).
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chem) in 50 mM Tris-HCl (pH 7.4), 25 mM NaCl, and 4% glycerol at room temperature for 40 min. The reaction was stopped by diluting to 3 ml, and the fluorescence was measured in a Spex Fluorolog-2 spectrofluorometer using excitation and emission wavelengths of 320 and 420 nm, respectively.

BACE1—BACE1 activity was measured using a fluorometric fluorescence resonance energy transfer assay using a kit based on the BACE1 substrate 7-methoxyxocoumarin-4-acetyl[Asn670,Leu671]-Amyloid β/4 protein precursor 770 fragment 667–676(2,4-dinitrophenyl)-Lys-Lys-Lys-Arg amide trifluoroacetate (Sigma). Samples were incubated with 100 µM substrate in a total volume of 100 µl for 2 h at 37 °C, and fluorescence was measured in a Spex Fluorolog-2 using excitation and emission wavelengths of 320 and 405 nm, respectively.

Protein Kinase C Assay—Recombinant PKC (1.1, 2.4, 2.0, 1.8, 2.5, and 1.1 ng of α, βII, γ, δ, ε, ζ, and η isoforms, respectively) was incubated for 15 min at 37 °C in the presence of 10 µM histones, 4.89 mM CuCl2, 1.2 µg/ml phosphatidylserine, 0.18 µg/ml 1,2,6-octanoyl-sn-glycerol, 10 mM MgCl2, 20 mM HEPES (pH 7.4), 0.8 mM EDTA, 4 mM EGTA, 4% glycerol, 8 µg/ml aprotinin, 6.6% Tween 20, and 2 mM benzamidine. 0.5 µCi of [32P]ATP was added, and [32P]phosphoprotein formation was measured by adsorption onto phosphocellulose as described previously (30).

RESULTS

To determine whether β-amyloid possessed oxidative activity toward cholesterol, we incubated 50 nM fresh human amyloid β-protein fragment 1–42 with [1,3-3H]cholesterol in the presence of 2 µM CuCl2, 0.1 mM EDTA, and 0.66% Tween 20 or Triton X-100 for 2 h at 37 °C. The lipid fraction was extracted into chloroform, evaporated, dissolved in 15 µl of EtOH, and injected onto a C18 HPLC column. The cholesterol metabolites were separated and detected by monitoring absorbance at 210 nm. Fractions were collected every 0.2 min, mixed with scintillation counting fluid, and counted in a scintillation counter.

As shown in Fig. 1 and Table I, incubation of radiolabeled cholesterol with β-amyloid in the presence of 2 µM divalent copper produced a new product (t½ ~ 48 min) that was absent in samples incubated with β-amyloid or Cu2+ alone. The retention time of this peak coincided with that of 5-cholesten-3,7-diol (7-hydroxycholesterol). The retention time of this peak did not coincide with any of a number of other neurotoxins or cholesterol metabolites tested.

The radioactive cholesterol metabolite produced by β-amyloid was isolated by reversed-phase HPLC and chromato-graphed on silica gel TLC using toluene/ethanol (2:1). The Rp of the metabolite was identical to that of cholesterol, 3,6-diol and 5-cholesten-3,7-diol and different from the Rp values of the other cholesterol derivatives tested.

To further identify the cholesterol metabolite, we incubated 1 µM β-amyloid 1–42 with 1 mg of cholesterol, 0.66% Tween 20 or Triton X-100, 3 µM CuCl2, 150 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA at 37 °C for 3 h, extracted the lipids into CHCl3, and purified the cholesterol metabolite by repeated injections onto a C18 reversed-phase HPLC column. The metabolite was subjected to electron impact mass spectrometry (Fig. 2A), yielding a M+ ion of m/z 402 (predicted mass = 402.73) and a major fragment of m/z 385 (M-18), corresponding to M-H2O (predicted mass = 384.715), or 1 mass unit less than cholesterol, indicating the presence of at least 2 OH groups. Consistent with the identification of the metabolite as 7-hydroxycholesterol is the low abundance of the M-88 ion (m/z = 317), which results from scission of the 5,6-double bond in cholesterol (32) (m/z = 301 in cholesterol), and the absence of the M-111 and M-113 ions (m/z 275 and 273 in cholesterol), which are produced by fragmentation of the B ring by a mechanism that involves C-7 (32).

7-Hydroxycholesterol is thermally unstable and undergoes dehydration to cholesta-5,7-dien-3-ol, which may account for the imperfect alignment of some of the peaks in Fig. 2A. Similar

![Fig. 1. HPLC detection of radiolabeled cholesterol metabolite produced by β-amyloid.](image)

**FIG. 1.** HPLC detection of radiolabeled cholesterol metabolite produced by β-amyloid. β-Amyloid 1–42 peptide (50 nM) was incubated with 2 µM CuCl2, 0.1 mM EDTA, 0.66% Tween 20, and 100 mM Tris-HCl for 2 h at 37 °C. Lipids were extracted into chloroform and applied to a reversed-phase Macrosphere RP-300 C18 5 µm HPLC column. The cholesterol metabolites were eluted using a linear gradient of 36% methanol plus 0.07% trifluoroacetic acid in water, changing to 100% methanol from 0 to 40 min, followed by 100% ethanol for an additional 20 min.

| **Table I** Production of cholesterol metabolite by β-amyloid

| Sample | nmol/min 1 mg⁻¹ | mol product/mmol peptide |
|--------|-----------------|--------------------------|
| Cholesterol only | 0.31 ± 0.10 | 0.17 ± 0.09 |
| Cholesterol plus β-amyloid | 1.07 ± 0.87 | 0.58 ± 0.58 |
| Cholesterol plus Cu²⁺ | 1.72 ± 1.18 | 0.94 ± 2.03 |
| Cholesterol plus β-amyloid plus Cu²⁺ | 21.48 ± 3.84 | 11.64 ± 1.81 |
| Cholesterol plus H₂O₂ | 0.18 ± 0.69 | 0.29 ± 1.74 |
| Cholesterol plus β-amyloid plus Cu²⁺ plus KI | 22.53 ± 3.76 | 12.20 ± 2.04 |

The following sterols were tested: 3β-hydroxy-5α-cholestan-3α,6β-diol (7β-hydroxycholesterol), 5α-cholesten-3β,7β-diol (7β-hydroxycholesterol), 5α-cholesten-3β,6β-diol (6α-hydroxycholesterol), 5α-cholestan-3β,6β-diol (6β-hydroxycholesterol), cholestan-3β,5α-diol (5α-hydroxycholestanol), 5α-cholestan-3β,4α-diol (4α-hydroxycholesterol), 5-cholesten-3β,20α-diol (20α-hydroxycholesterol), 5-cholesten-3β,22R-diol (22R-hydroxycholesterol), 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, cholestan-5α,6α-epoxy-3β-ol (cholesterol-5,6α-epoxide), cholestan-5β,6α-epoxy-3β-ol (cholesterol-5,6β-epoxide), 4-cholesten-3-one, 5-cholesten-3-one, 5-androsten-3β,17β-dione (dehydroandrosterone), pregnenolone, pregnenolone sulfate, 5α-pregn-3-ol-20-one, corticosterone, deoxycorticosterone, progesterone, estrone, 4-androstene-3,17-dione, cholesterol-5α-hydroperoxide (26), 3β-hydroxycholesterol-4-ene-6α-hydroperoxide (26), 5α-cholestan-3β-ol-6-one (6-ketocholesterol), 5α-cholestan-3β-ol-7β-one (7-ketocholesterol), 5α-cholestan-3β-ol-7-one (7-ketocholesterol), cholesterol, and cholesterol acetate.

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2 The following sterols were tested: 3β-hydroxy-5α-cholestan-3α,6β-diol (7β-hydroxycholesterol), 5α-cholesten-3β,7β-diol (7β-hydroxycholesterol), 5α-cholesten-3β,6β-diol (6α-hydroxycholesterol), 5α-cholestan-3β,6β-diol (6β-hydroxycholesterol), cholestan-3β,5α-diol (5α-hydroxycholestanol), 5α-cholestan-3β,4α-diol (4α-hydroxycholesterol), 5-cholesten-3β,20α-diol (20α-hydroxycholesterol), 5-cholesten-3β,22R-diol (22R-hydroxycholesterol), 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, cholestan-5α,6α-epoxy-3β-ol (cholesterol-5,6α-epoxide), cholestan-5β,6β-epoxy-3β-ol (cholesterol-5,6β-epoxide), 4-cholesten-3-one, 5-cholesten-3-one, 5-androsten-3β,17β-dione (dehydroandrosterone), pregnenolone, pregnenolone sulfate, 5α-pregn-3-ol-20-one, corticosterone, deoxycorticosterone, progesterone, estrone, 4-androstene-3,17-dione, cholesterol-5α-hydroperoxide (26), 3β-hydroxycholesterol-4-ene-6α-hydroperoxide (26), 5α-cholestan-3β-ol-6-one (6-ketocholesterol), 5α-cholestan-3β-ol-7β-one (7-ketocholesterol), 5α-cholestan-3β-ol-7-one (7-ketocholesterol), cholesterol, and cholesterol acetate.
mass spectra for 7-hydroxycholesterol, showing a predominant ion at $m/z = 385$, have been seen by other researchers (33, 34).

Identification of the metabolite as 7-hydroxycholesterol (Fig. 3A) was also supported by its position on C18 HPLC. 7-Hydroxycholesterol eluted slightly before cholesterol-5,6-epoxide, consistent with its slightly greater polarity. The absence of $n \to \pi$ transitions in the metabolite's UV spectrum ($\lambda_{\text{max}}$(in ethanol) = 200 nm) (Fig. 3B) indicated the absence of ketones, dienes, or conjugated double bonds.

To determine the stereochemistry at the 7-position, the me-
tabolite produced from $[^3]$Hcholesterol by $\beta$-amyloid was isolated by HPLC then rechromatographed on silica Gel TLC plates using hexane/ethyl acetate (2:1), and 1-mm sections were scraped and counted in a scintillation counter. The ratio of the $^7\beta/\gamma$ isomer was $\sim$9:1, suggesting that the $\beta$-isomer was the predominant product.

Production of the cholesterol metabolite was strictly dependent on the presence of both copper and $\beta$-amyloid (Table I). More than 11 molecules of product were produced per molecule of $\beta$-amyloid, indicating that the reaction was not a simple chemical reaction between cholesterol and one of the functional groups on the peptide, but that $\beta$-amyloid was acting, at least in part, as a catalyst. Addition of potassium iodide (1 mM) after the incubation to reduce any hydrogen peroxide or cholesterol hydroperoxides had no effect on the reaction, indicating that the product is not a hydroperoxide. Also, incubation of cholesterol with 1 mM H$_2$O$_2$ did not produce any product, indicating that the metabolite is not formed by the action of H$_2$O$_2$ on cholesterol (35, 36) or arachidonate (37) in the presence of oxygen to produce hydroperoxide derivatives. To avoid this problem, we reacted cholesterol (0.1 g) with 10 mM CuCl$_2$ under 100% oxygen in tetrahydrofuran at 60 °C overnight. This converted up to 70% of the cholesterol to a highly polar derivative with properties indicating a stenodiol or triol (38), most likely cholest-5-ene-3$\alpha$,5,7$\alpha$-triol (38). However, the retention time of this product was markedly different from the product produced by incubation with $\beta$-amyloid ($t_R = 30$ min versus 48 min for the $\beta$-amyloid product) (not shown).

To determine whether the metabolite could be produced in cells under more physiological conditions, cultured rat hippocampal H19–7/IGF-IR neurons were preincubated for 10 min with $[^3]$Hcholesterol. Then 2 $\mu$M Cu$^{2+}$ and 100 nM $\beta$-amyloid were added, and the cells were grown for an additional 2 h in a total volume of 100 $\mu$L. The lipids were extracted into CHCl$_3$, fractionated by HPLC, and the radioactivity was counted as before (Fig. 4). The rate of product formation was 28.7 nmol min$^{-1}$ mg$^{-1}$ $\beta$-amyloid, comparable to that produced in vitro, although the overall counts/min were lower because of the large excess of unlabeled cholesterol. The amount of product formed corresponds to a concentration of metabolite of 155 nM. This experiment also demonstrates that Me$_3$SO, Tween, Triton, and Tris are not involved in the reaction.

If $\beta$-amyloid, which is a proteolysis product of APP, possesses oxidative activity, it is possible that the parent protein may also act as an enzyme. To avoid any potential problems with cloned proteins, which may lack a post-translational modification that may be essential for activity, we devised a purification method for APP from rat and guinea pig brain based on its affinity for apolipoprotein E (39).

Although rat $\beta$-amyloid differs at three amino acid residues from human $\beta$-amyloid, which may account for the scarcity of amyloid deposits in the brains of aged rats (40), it is likely that rat APP fulfills the same biological function in rats as in humans. Any differences in activity would be an interesting subject of further investigations, because these would be highly significant in tracing the pathogenesis of AD. Therefore, we began by isolating native APP from rat and guinea pig brain.

To purify APP from rat brain, we homogenized two rat brains in 10 mM Tris-HCl containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 2 mM benzamidine) and centrifuged the homogenate at 100,000 × g. The pellet was then re-extracted by sonication with 0.66% Triton X-100, and the two soluble fractions (cytosol and membrane extract) were incubated with apoE-Sepharose for 1 h at room temperature. The mixtures were then transferred to small columns and washed with increasing concentrations of NaCl in buffer containing 1 mM phenylmethylsulfonyl fluoride. This produced small amounts of highly purified APP, estimated to be $\sim$96% pure by densitometric analysis of a Coomassie-stained gel (Fig. 5, left). All preparations showed two protein bands in approximately a 1:1 ratio. A two-dimensional Western blot (Fig. 5, right) showed that these same two bands reacted with anti-APP antibody, indicating that both bands were APP, and that no other forms of APP were detectable.

To test whether APP could also metabolize cholesterol, we incubated 2 $\mu$M purified rat brain APP with [1,3-$^3$H]cholesterol in the presence of 2 $\mu$M CuCl$_2$ as described above, and analyzed the samples by HPLC as described in Fig. 1. APP produced a metabolite with the same retention time as the metabolite produced by $\beta$-amyloid (Fig. 7). The reactions with $\beta$-amyloid and APP both followed saturable Michaelis-Menten kinetics, with a V$_{max}$ of 0.149 ± 0.046 and 0.030 ± 0.003 pmol min$^{-1}$ and a K$_{m}$ for cholesterol of 4.04 ± 2.36 and 1.61 ± 0.27 $\mu$M, respectively (Fig. 6). The rate of reaction from APP, for equal molar concentrations of protein, was about 200 times lower than the rate of reaction with $\beta$-amyloid. Apolipoproteins E2, E3, and E4 had no effect on either reaction. The reaction with APP was strongly inhibited by an unidentified contaminant that accumulated in the cholesterol solution. To avoid this, source vials of [3H]cholesterol were stored at $-80$ °C and used within 2 weeks of receipt. The rate from guinea pig APP, which has a

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**Fig. 4. Production of cholesterol metabolite in cultured cells.** Cultured rat hippocampal H19–7/IGF-IR neurons were preincubated for 10 min with 5 $\mu$Ci of [3H]cholesterol. Then 2 $\mu$M Cu$^{2+}$ 100 nM $\beta$-amyloid was added, and the cells were grown for an additional 2 h in a total volume of 100 $\mu$L. The lipids were extracted into CHCl$_3$, fractionated by HPLC, 0.5-min fractions were collected, and the radioactivity was counted in a scintillation counter. The rate of product formation shown here represents 28.7 nmol min$^{-1}$ mg$^{-1}$. 
primary sequence similar to human APP, was similar to that of rat APP (not shown).

From the data in Fig. 7, the $k_{cat}$ of reaction for β-amyloid was calculated as 0.211 min$^{-1}$. This rate is comparable to the rates observed for known cholesterol-synthesizing and -metabolizing enzymes. For example, the $k_{cat}$ values for hydroxylation of cholesterol and 7α-hydroxycholesterol by recombinant mitochondrial sterol 27-hydroxylase are 0.09 and 0.018 min$^{-1}$, respectively (41). The $k_{cat}$ values for recombinant cholesterol
7α-hydroxylase, with cholesterol and 4β-hydroxycholesterol as substrates, were reported as 0.184 and 0.087 min⁻¹, respectively (42). The value of 0.211 min⁻¹ is also comparable to the value of 0.17 min⁻¹ calculated from the data of Opazo et al. (24) for production of H₂O₂ from β-amyloid in the presence of dopamine.

Previous researchers have shown that β-amyloid 1–42 can bind copper with high affinity (15, 16). If the reaction between APP or β-amyloid and cholesterol is a transition-metal enzyme catalyzed oxidation-reduction reaction, it should produce hydrogen peroxide or some other form of active oxygen as a byproduct. To determine whether this is the case, we incubated β-amyloid 1–42 (0.19 μM) with 10 μM cholesterol and 2 μM Cu²⁺, either free in solution or chelated with glycine (16), for 1 h at 37 °C and then measured the H₂O₂ fluorometrically by reaction with horseradish peroxidase and homovanillic acid (28). As shown in Fig. 8A, H₂O₂ was produced in the presence of β-amyloid at the rate of 6.6 ± 2.0 pmol/min, or 52 ± 15 nmol-min⁻¹-mg⁻¹ (kcat = 0.23 min⁻¹). This was comparable to the rate of production of the cholesterol metabolite (Fig. 7, legend), indicating a 1:1 stoichiometry. The small differences in rate may be caused by instability of the cholesterol metabolite.

H₂O₂ has also been found as a product of a catalytic reaction between β-amyloid and cholesterol in the presence of Cu²⁺ by other researchers (24, 43). These researchers reported that other peptides, including apotransferrin and insulin, did not produce H₂O₂. They also presented evidence for a free-radical reaction and ruled out the possibility of a superoxide intermediate. Our results confirm these findings and indicate that the same reaction occurs with APP.

Elevated temperatures (60 °C) or addition of higher levels of Cu²⁺ (≥30 μM) greatly increased the rate of non-enzymatic production of H₂O₂ from the reaction between Cu²⁺ and oxygen (Fig. 8B). This suggests that one possible mechanism of action of β-amyloid may be to lower the energy of activation by increasing the availability of copper to the cholesterol. Similar results were obtained whether H₂O₂ was measured using the horseradish peroxidase/homovanillic acid method (28), the horseradish peroxidase-Amplex Red method (44) or with horseradish peroxidase and 2′,7′-dichlorofluorescein diacetate (45, 46) (not shown).

When applied to cultured rat hippocampal H19–7/IGF-IR cells, the metabolite caused cell death (Fig. 9), consistent with reports of apoptosis reported by other investigators using synthetic 7β-hydroxycholesterol (47–51). Approximately half the cells were killed by 100 nM 7β-hydroxycholesterol metabolite within 96 h (Fig. 10). This implies that rat hippocampal neurons were significantly more sensitive than the macrophages and fibroblasts used by previous researchers. For example, 24-h viability was 50–60% after addition of 30 μM 7β-hydroxycholesterol to U937 monocytes (47).

At concentrations above 50 nM, 7β-hydroxycholesterol also inhibited secretion of sAPP into the culture media (Fig. 11). The same concentrations also inhibited TACE α-secretase activity in rat brain cytosol but had no effect on BACE1 activity (Fig. 12). However, no inhibition was observed with commercial His-Tag recombinant TACE, indicating significant differences between the native and cloned proteins. These differences could be explained by post-translational modification or the presence of a necessary cofactor in rat brain. To distinguish between these possibilities, recombinant TACE was measured with the presence of a small amount of rat brain cytosol. No inhibition was observed (not shown), indicating that the differences are not the result of an easily replaceable soluble cofactor.

Because α-secretase is regulated by protein kinase C (53–56), we also examined the effect of the metabolite on PKC activity.

7β-Hydroxycholesterol was found to be a potent inhibitor of α and γ-PKC, which are abundant forms of PKC in neurons, but had little effect on the minor isoforms, including β and η (Fig. 13). For α-PKC, 50% inhibition was observed at ~0.2 nM (Fig. 13). Similar large differences in substrate specificity are observed with many PKC inhibitors, such as bisindolylmaleimide (57, 58). Application of the metabolite to cultured neurons also increased cellular APP levels to about 70% above normal, possibly due to reduced cleavage by α-secretases (Fig. 14).

**DISCUSSION**

Our results show that both APP and β-amyloid behave as catalysts that convert cholesterol to 7β-hydroxycholesterol and that this oxysterol possesses an exceptional level of toxicity to neuronal cells. Cholesterol plus hydrogen peroxide alone or in the presence of copper did not produce this metabolite. The
affinity for cholesterol (1–4 μM) would appear to be more than adequate for maximal activation by brain cholesterol levels (~2–2.5% by weight, or 518 mM). However, in the brain, whereas most of the cholesterol is free, much of the free cholesterol is found in myelin membranes (2). Elsewhere, most cholesterol is not free; in blood, for example, 68% of the cholesterol is esterified, resulting in a free cholesterol concentration of 1.6 mM (59). Significantly, 66% of the total cholesterol in Alzheimer's fibroblasts is unesterified, compared with only 23% in controls (60). Thus, Alzheimer's cells would have higher levels of both cholesterol substrate and β-amyloid peptide necessary to produce oxysterols.

At high concentrations (~20 μM), copper alone is capable of oxidizing cholesterol non-enzymatically to oxysterols, including 7-ketocholesterol, 7-hydroxycholesterol, and 5,6-epoxycholesterol (61). Under these conditions, as shown in Fig. 8B, large amounts of H₂O₂ are also produced. Our results show that at physiologically relevant concentrations, H₂O₂ and cholesterol are produced in a 1:1 stoichiometric ratio. 

β-Amyloid peptides contain a hydrophobic region or “patch” (LVFFA), containing two phenylalanine residues and three other hydrophobic residues. This hydrophobic region may participate in binding of cholesterol, facilitating contact with Cu²⁺. Increased solvation of this region has been suggested as a possible factor in the
pathogenic differences between wild type and the E22Q ("Dutch") mutation (62). Point substitution of phenylalanine 19 with threonine also affects the folding and plaque competence of \(H9252\)-amyloid (63). Thus, one possible mechanism of action of \(H9252\)-amyloid may be to lower the energy of activation by increas-
ing the availability of copper to cholesterol.

Zhang et al. (64) recently reported evidence for oxidation of cholesterol by ozone in human brain. However, the reaction products of ozone, including 5,6-secosterol and the ketoaldehyde \(3\beta\)-hydroxy-5-oxo-5,6-secocholestan-6-al (64) are readily distinguished from the 5- and 7-hydroperoxy derivatives that are the principal products of singlet oxygen reactions and metal ion-catalyzed oxidation by ground state oxygen \(^{3}\text{O}_2\), respectively (65). In our hands, reacting ozone with cholesterol produced only trace amounts of 7-hydroxycholesterol (not shown). Thus, it is unlikely that ozone is an intermediate in the formation of 7-hydroxycholesterol. 7-Hydroxycholesterol can also be produced \textit{in vitro} by reaction of cholesterol with hypochlorite ion (66).

7-Hydroxycholesterol, its oxidation product, 7-ketochole-
teron, and its unstable precursor, 7-hydroperoxycholesterol, are among the most cytotoxic oxysterols and induce apoptosis in non-neuronal cells at micromolar concentrations (47–50) accompanied by condensed and fragmented nuclei and DNA, by a
mechanism that involves generation of oxidative stress (51). 10 μM 7α-hydroxycholesterol caused 50% mortality in human neuroblastoma cells (67). Changes in other oxysterols, including 24(S)-hydroxycholesterol and 27-hydroxycholesterol, have been observed in brains from AD patients (68). Micromolar concentrations of the oxysterol 25-hydroxycholesterol induce apoptosis (69). Levels of oxysterols in atherosclerotic plaques are greatly increased compared with control arteries (70) suggesting that cholesterol metabolism is also markedly altered in vascular disease. 7-Hydroxycholesterol was also reported to stimulate accumulation of cholesterol and cholesteryl esters in macrophages (71).

7-Hydroxycholesterol and its oxidation product, 7-ketocholesterol, are also believed to be responsible for the cytotoxicity of oxidized LDL to aortic smooth muscle cells (50). Oxysterols such as 7-ketocholesterol accumulate in the core of fibrotic plaques (72, 73), leading to the suggestion that oxysterols may be involved in atherogenesis (73) and chronic inflammation (74). Atherosclerosis and Alzheimer’s disease share many convergent elements (75), including hypercholesterolemia, oxidative stress, and inflammation.

The results reported here, in which a 50% reduction in cell number was produced by 100 nM 7-hydroxycholesterol, however, suggest that neurons are significantly more sensitive to oxysterols than other cell types. Lethal concentrations of 7β-hydroxycholesterol were produced in less than 2 h with 100 nM 7-hydroxycholesterol, how-

FIG. 14. Effect of 7β-hydroxycholesterol on cellular APP levels. Cultured rat hippocampal H19–7GF-IR neurons were incubated for 48 h after addition of varying concentrations of 7β-hydroxycholesterol in 10 μl of ethanol. Cells were collected and sonicated in SDS sample buffer. APP was measured by densitometry of Western blots. The y-axis represents densitometric signal expressed as a percentage of control.

amylodiogenic and non-amylodiogenic APP processing in Alzheimer’s disease patients.

Oxidative stress may be central to the apoptotic process since many factors that induce apoptosis also produce oxidative stress (77). Unlike hydrogen peroxide, which is rapidly detoxified by catalase, lipophilic substances such as oxysterols would accumulate in cell membranes and rapidly reach concentrations high enough to induce apoptosis (Fig. 4, legend). In contrast to H2O2, which induces acute cytotoxic effects, oxysterols would accumulate over longer time periods more consistent with the gradual course of AD. Thus, 7-hydroxycholesterol could be a significant contributor to the toxic effects of β-amyloid, and might also be useful as a biomarker for high-risk AD patients.

On the other hand, the finding that intact APP produces low levels of 7-hydroxycholesterol suggests that low levels of 7-hydroxycholesterol, its likely precursor, 7-hydroperoxysterol, or 7-ketocholesterol could also act as signaling molecules, analogous to the thromboxanes produced by peroxidation of arachidonic acid, or may serve some other beneficial function to the neuron. Normal functioning of APP is essential for synaptic function. Knock-out mice deficient in APP develop weight loss, cognitive defects, reactive gliosis, and low levels of presynaptic marker proteins (78). APP is rapidly transported to growing tips of nerve fibers where it participates in synaptogenesis (79) or cell adhesion (80). Conversely, down-regulation of APP inhibits neurite outgrowth (81) and injection of anti-APP antibodies blocked memory formation in chicks (82).

In addition to binding copper (15, 16), β-amyloid also binds to cholesterol at the α-secretase cleavage site (83). β-Amyloid 1–42 inhibits the binding of cholesterol to apoE or LDL (83). The cholesterol metabolite 22(R)-hydroxycholesterol has been found to protect against β-amyloid-induced cytotoxicity by binding to β-amyloid peptide (84) and was found at lower levels in AD patients compared with controls (84).

Serum amyloid A precursor, which is similar to APP, is known to bind cholesterol in the amyloid A fibril forming region with high affinity (1.7 × 10^7 M) (85). There is also considerable evidence for involvement of cholesterol in APP processing. The N-terminal ectodomain of APP fractionates with cholesterol-rich membrane rafts (86). Altering the subcellular distribution of cholesterol by inhibiting intracellular cholesterol transport reduces the cleavage of APP by β-secretases (87). The interaction of 125I-labeled β-amyloid 1–40 and AβPP is inhibited by the cholesterol-transport proteins apoE and apoE4 (88). The toxicity of various oligomeric β-amyloid peptides correlates with their ability to bind cholesterol (52).

However, there are still many questions left unanswered. The question of whether 7-hydroxycholesterol is formed via a hydroperoxide intermediate or by some other mechanism has not been studied. It is still unknown to what extent this reaction occurs in Alzheimer’s patients. Most importantly, the biological role and long term pathological effects of 7-hydroxycholesterol are unknown.

Recently, 24(S)-hydroxycholesterol and 27-hydroxycholesterol, in which the side chain is oxidized, were also found to be potent inhibitors of β-amyloid secretion (31) indicating that these oxysterols may have a therapeutic potential. Because of the selective expression of cholesterol 24-hydroxylase around neuritic plaques, it is possible that 24(S)- and 27-hydroxycholesterol may influence hydroxycholesterol synthesis by APP as well.

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