Profile of cholesterol-related sterols in aged amyloid precursor protein transgenic mouse brain

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Abstract Cholesterol is implicated to play a role in Alzheimer disease pathology. Therefore, the concentrations of cholesterol, its precursors, and its degradation products in brain homogenates of aging wild-type and β-amyloid precursor protein transgenic mice carrying the Swedish mutation (APP23) were analyzed. Among the sterols measured, lanosterol is the first common intermediate of two different pathways, which use either desmosterol or lathosterol as the predominant precursors for de novo synthesis of brain cholesterol. In young mice, cholesterol is mainly synthesized via the desmosterol pathway, while in aged mice, lathosterol is the major precursor. 24*S*-hydroxycholesterol (cerebrosterol), which plays a key role in the removal of cholesterol from the brain, modestly increased during aging. No differences in the levels of cholesterol, its precursors, or its metabolites were found between wild-type and APP23 transgenic mice. Moreover, the levels of the exogenous plant sterols campesterol and sitosterol were significantly elevated in the brains of APP23 animals at age 12 and 18 months. This time point coincides with abundant plaque formation.— Lütjohann, D., A. Brzezinka, E. Barth, D. Abramowski, M. Staufenbiel, K. von Bergmann, K. Beyreuther, G. Multhaup, and T. A. Bayer. **Profile of cholesterol-related sterols in aged amyloid precursor protein transgenic mouse brain.** *J. Lipid Res.* 2002. **43:** 1078–1085.

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In the human brain, cholesterol is entirely produced in situ. Currently, there is little evidence for the transfer of cholesterol or its precursors from the plasma via the blood-brain barrier (BBB) of fetus, newborn, or adult (1). Brain cholesterol metabolism is well balanced by a low rate of synthesis, elimination of excessive cholesterol after conversion into 24*S*-hydroxycholesterol (24*S*-OH-Chol), and release into the systemic circulation (2–4). Neurons are continuously supplied with free cholesterol, either by de novo synthesis or by uptake using ligands and specific membrane transport systems.

Epidemiological, biochemical, and genetic evidence link cholesterol metabolism with Alzheimer disease (AD), one of the most frequent age-dependent neurodegenerative diseases of the human brain (5). So far, little is known about the levels of cholesterol and its precursors (such as lanosterol, lathosterol, or desmosterol) in the brain during aging. The apolipoprotein E (apoE) $\varepsilon 4$ allele is the major genetic risk factor for AD (6). Additionally, it is associated with increased plasma cholesterol concentrations. Cholesterol has been demonstrated to modulate β-amyloid precursor protein (APP) processing. APP and its proteolytic product Aβ play an important role in the etiology of AD. The amyloid deposits in AD brains are aggregates of the 40- to 42-residue Aβ peptides (7, 8), originating from the large precursor protein APP (9, 10). High cholesterol concentrations in the medium of cultured cells inhibit secretion of α-cleaved soluble APP (11–13). Low cholesterol concentrations stimulate the non-amyloidogenic pathway by its effect on ADAM-10, the major α-secretase (14). APP transfected hippocampal neurons exhibit lower Aβ secretion after cellular cholesterol depletion (15). High dietary cholesterol accelerates plaque formation in APP transgenic mice (16). Inhibition of the enzyme converting 7-dehydrocholesterol to cholesterol by the synthetic drug BM 15.766 is associated with reduced

Aβ accumulation (17). Simvastatin, an inhibitor of cholesterol synthesis, reduces Aβ levels in vivo and in vitro (18). Moreover, a significantly reduced incidence of AD and dementia was reported in patients treated with statins (19, 20). Simvastatin treatment of hypercholesterolemic patients affected the ratio of 24S-OH-Chol to cholesterol in plasma (21). Increased plasma and cerebrospinal fluid levels of 24S-OH-Chol, the major degradation product of brain cholesterol (2, 22, 23), have been observed in early-onset AD and vascular demented patients (24).

The lack of information on cholesterol sterol levels in the aging brain prompted us to study the brain sterol metabolism in APP23 transgenic mice, which develop typical AD-related pathological changes (25). Concentrations of brain cholesterol, cholesterol precursors, the brain-specific cholesterol metabolite 24S-OH-Chol, and the plant-derived sterols campesterol and sitosterol were analyzed.

MATERIALS AND METHODS

Animals

We analyzed material from transgenic mice, which express human APP751 with the Swedish mutation under the control of the murine Thy1 promoter (APP23), and also from wild-type littermate control mice (25). The animals received a standard chow (pellets) containing plant sterols (0.08% of all sterols) and cholesterol (10% of all sterols). Analysis of sterol content in brain homogenates was performed in groups of five wild-type and five APP transgenic mice at 3, 6, 9, and 12 months, respectively, and in 10 wild-type and 11 APP transgenic mice at 18 months. The animal experiments were in accordance with the German animal protection laws.

Western blot

For immunoblotting, snap-frozen hemispheres were homogenized in lysis buffer (1% Triton X-100, 50 mM Tris pH 8.0, 120 mM NaCl) including complete protease inhibitor cocktail (Roche), and mixed with an equivalent volume of a 2-fold sample loading buffer. Electrophoresis was performed on 16% Tricine sodium dodecylsulfate-polyacrylamide gels (Novex, San Diego, CA) and the proteins were electroblotted to Hybond® nitrocellulose membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Western blots were probed with the monoclonal antibody W0-2 (1 μg/ml). For Aβ detection, the blotted membrane was heated in boiling phosphate buffered saline for 5 min to enhance the signal. It was then blocked with 10% non-fat dry milk in TBS for 2 h at room temperature. After washing the membrane the primary antibody was added and incubated overnight at 4°C. The immunoreactivity of bound antibodies was detected by horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody (Amersham) followed by chemiluminescense detection (Amersham) according to the manufacturer's instructions.

Preparation of brain homogenates for sterol extraction

Brain hemispheres were carefully rinsed with 0.9% NaCl solution and snap-frozen in liquid nitrogen. The frozen brain aliquots were cut into small slices and kept frozen at −80°C until homogenization. Aliquots (ranging from 4–13 mg) of the frozen slices were homogenized in 1.2 ml Tris buffer solution. Fifty microliters of butylated hydroxytoluene in methanol (5 mg/ml) was added to protect the sterols from autoxidation.

Sterol analysis

Fifty micrograms of 5α-cholestane (Serva) (50 μl from a stock solution of 5α-cholestane in cyclohexane; 1 mg/ml), 1 μg epicoprostanol (Sigma) (10 μl from a stock solution epicoprostanol in cyclohexane; 100 μg/ml), and 200 ng racemic [23,23,24,25-^{2}H$_4$]24-OH-Chol (Medical Isotopes Inc., Pelham) (50 μl from a stock solution 24-OH-Chol in toluene; 4 μg/ml) were added as internal standards to an aliquot (500 μl) of brain homogenate. Sterols and oxysterols were extracted by cyclohexane after saponification and neutralization. The solvents were evaporated and the residual sterols and oxysterols were derivatized to trimethylsilyl (TMSi)-ethers by adding 1 ml TMSi-reagent (pyridine-hexamethyldisilazane-trimethylcholorsilane; 9:3:1, v/v/v; all reagents were supplied by Merck) and incubated for 1 h at 64°C. The solvents were evaporated under nitrogen at 65°C. The pellet was dissolved in 160 μl n-decane. Eighty microliters of the solution was transferred into micro-vials for gas-liquid chromatographic-mass spectrometric (GC-MS) analysis of cholesterol precursors, metabolites, and plant sterols. The residual 80 μl was diluted with 400 μl n-decane for analysis of cholesterol by gas chromatography-flame ionization detection. 5α-cholestane and cholesterol/TMSi ethers were separated on a cross-linked methyl silicone DB-XLB 122-1232 fused silica capillary column (J&W) (30m × 0.25 mm id × 0.25 μm film thickness) in a Hewlett Packard (HP6890) gas-chromatograph. The oven temperature was initially kept at 150°C for 3 min, then increased by 30°C/min to a final temperature of 290°C. At 280°C, an aliquot of 1 μl was injected in a splitless mode by an automated sampler and injector (HP 7683). Hydrogen was used as the carrier gas with an inlet pressure of 9.9 psi, resulting in a total gas-flow of 1.1 ml/min. Flame ionization detection was performed at 280°C. The concentration of cholesterol was calculated by one-point calibration using 5α-cholestane as internal standard. The peak area of cholesterol is divided by the peak area of 5α-cholestane and multiplied by the amount of 5α-cholestane added to the sample (50 μg). This calculation method was validated against standard curves for cholesterol and showed a high validity. The identity of cholesterol was proven by comparison with the mass spectrum of authentic cholesterol as a standard (Sigma).

Combined GC-MS analysis for quantification of lathosterol, desmosterol, lanosterol, campesterol, sitosterol, and 24S-OH-Chol was performed on a cross-linked methyl silicone DB-XLB 122-1232 fused silica capillary column (J&W) (30 m × 0.25 mm id × 0.25 μm film thickness) using an HP5890 Series II plus gas-chromatograph combined with an HP5971 mass selective detector. An aliquot of 1 μl was injected by automated injection (HP 7673 autosampler and automatic injector) in a splitless mode at an injection temperature of 280°C. The initial oven temperature was kept at 150°C for 1 min, thereafter it was increased at a rate of 30°C/min to a final temperature of 290°C. The temperature of the transfer line was kept at 280°C. Multiplier voltage was set to 2700 eV. Electron impact ionization was employed at 70 eV ionization energy. Selected-ion monitoring (SIM) was performed by cycling the quadrupole mass filter between the chosen m/z values at a rate of 2.0 cycles s^{-1}. TMSi-ether of epicoprostanol was measured at m/z 370 (M$^+$-OTMSi), lathosterol at m/z 458 (M$^+$), desmosterol at m/z 441 (M$^+$-CH$_3$), lanosterol at m/z 393 (M$^+$-OTMSi-CH$_3$), campesterol at m/z 472 (M$^+$), sitosterol at m/z 486 (M$^+$), 24-OH-Chol at m/z 413 [M$^+$-OTMSi-CH(CH$_3$)$_2$], and deuterated 24-OH-Chol at m/z 416 [M$^+$-OTMSi-CD(CH$_3$)$_2$]. The concentrations of lathosterol, desmosterol, lanosterol, campesterol, and sitosterol were calculated from standard curves using epicoprostanol as internal standard. The concentrations of 24S-OH-Chol were calculated from standard curves using racemic 24R,S-OH-Chol in increasing concentrations and deuterated ra-

cemic 24R,S-OH-Chol as internal standard (isotope dilution method). Identification of all sterols was proven by comparison with the full-scan mass spectra of authentic compounds. Additional qualifier (characteristic fragment ions) ions were used for structural identification.

Statistics

Data are expressed as mean ± SD. Statistics for differences in wild-type and transgenic mice and for age differences were performed using two-tailed Student's t-test. Differences between months of age in transgenic and wild-type mice were evaluated by ANOVA and by Bonferroni adjusted post-hoc tests. Correlation analysis was performed with a Pearson's correlation coefficient. The level of significance used was 5%. All statistical calculations were performed using SPSS software (SPSS Inc.).

RESULTS

Western blotting of brain lysates

As expected, APP23 mice expressed abundant human full-length APP, as well as β-cleaved C-terminal-stubs (C-99 fragment). The W0-2 monoclonal antibody recognizes the amino acid epitope 1–8 of Aβ. Detectable Aβ was found as early as 3 months of age and it increased in an age-dependent manner with significantly elevated concentrations at 9, 12, and 18 months of age (**Fig. 1**).

Cholesterol metabolites in aging wild-type and APP transgenic mice

Bonferroni adjusted post-hoc test of variance showed no differences in the level of cholesterol or its precursors between wild-type and APP23 mice.

The relationship of different precursors and the 24S-OH-Chol metabolite to cholesterol was expressed as the ratio of the different non-cholesterol sterols to cholesterol. The ratio of 24S-OH-Chol to cholesterol varied from 2.1 to 3.0 ng/μg in APP23 mice, and from 1.9 to 2.9 ng/μg in wild-type mice. ANOVA revealed a modest age-related effect in the ratio of 24S-OH-Chol to cholesterol ($P = 0.05$). Ratios of 24S-OH-Chol to cholesterol increased from 3 months to 9 months (2.16 ± 0.04 ng/μg, 3

months, to 2.36 ± 0.07 ng/μg, 6 months, to 2.52 ± 0.07 ng/μg, 9 months in APP23 mice; and 2.17 ± 0.18 ng/μg, 3 months, to 2.43 ± 0.17 ng/μg, 6 months, to 2.53 ± 0.05 ng/μg, 9 months in wild-type mice). The ratio of 24S-OH-Chol to cholesterol showed highly significant positive correlations with the corresponding ratios of lathosterol ($R = 0.637$, $P < 0.0001$) and lanosterol ($R = 0.360$, $P = 0.005$) as well as a weak negative correlation with that of desmosterol ($R = -0.323$, $P < 0.02$) (**Fig. 2**).

The ratios of lathosterol to cholesterol and lanosterol to cholesterol showed similar profiles. They increased during the time from three to nine months and slightly decreased in the time up to 18 months. In contrast, the profile of desmosterol to cholesterol reached a maximum at 6 months and significantly decreased thereafter. The ratio at 18 months was less than a third of the ratio observed at the age of 3 months. ANOVA revealed highly significant differences between the ratios of all three cholesterol precursors with aging ($P < 0.001$), except for the ratio of lanosterol to cholesterol in the transgenic mice with only a moderate change ($P = 0.05$). Bonferroni adjusted post-hoc test showed highly significant lower ratios of lathosterol to cholesterol when comparing the ratios for all wild-type mice between 3 and 6 months with 9, 12, and 18 months at a level of $P < 0.001$.

Concentrations of campesterol and sitosterol in the brain are normally kept at a very low level. In transgenic animals, levels of campesterol as well as sitosterol to cholesterol were significantly elevated during aging (ANOVA; $P < 0.001$). The ratio of campesterol to cholesterol was 2.3- to 4.1-fold higher than the ratio of sitosterol to cholesterol, with the most differences in the younger animals. Ratios of campesterol and sitosterol to cholesterol were significantly higher in the APP23 compared with wild-type mice at 12 months (1.09 ± 0.06 ng/μg vs. 0.83 ± 0.08 ng/μg and 0.44 ± 0.02 ng/μg vs. 0.26 ± 0.02 ng/μg, respectively; $P < 0.001$ for both) and 18 months of age (1.33 ± 0.10 ng/μg vs. 0.81 ± 0.05 ng/μg, and 0.59 ± 0.06 ng/μg vs. 0.29 ± 0.03 ng/μg, respectively; $P < 0.001$ for both). Bonferroni adjusted post-hoc test revealed significant differences in the ratios of sitosterol to cholesterol in wild-type mice between 3 months and 12 and 18 months ($P = 0.014$ and $P < 0.001$, respectively), between 6 months and 12 and 18 months ($P = 0.022$ and $P < 0.001$, respectively), and between 9 and 18 months ($P < 0.001$). Significantly lower ratios of campesterol and sitosterol to cholesterol in transgenic mice were found between 3, 6, and 9 months and 12 and 18 months ($P < 0.001$ for all) and between 12 months and 18 months of age (**Fig. 3**).

DISCUSSION

Cholesterol and its precursors

ApoE is the main carrier of free cholesterol in the brain. The ε4 allele of apoE is the major known genetic risk factor for sporadic AD (6). The ε4 isoform correlates positively with amyloid plaque formation and Aβ deposition. Growing evidence indicates a potentially im-

Age [Mo] 3 6 9 12 18

APP ▶

β-stub ▶

◀ Aβ dimer
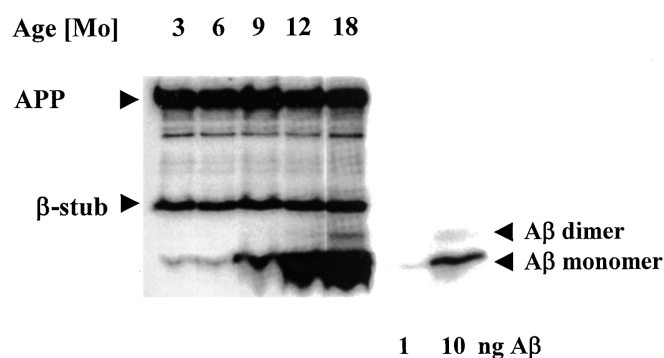◀ Aβ monomer

1   10 ng Aβ

Fig. 1. Western blot analysis of brain lysates (20 μg per lane) of aged APP23 transgenic mice using Aβ antibody W0-2. Abbreviations: Mo, month; APP, β-amyloid precursor protein; Aβ, Abeta; β-stub, C-99 APP fragment).
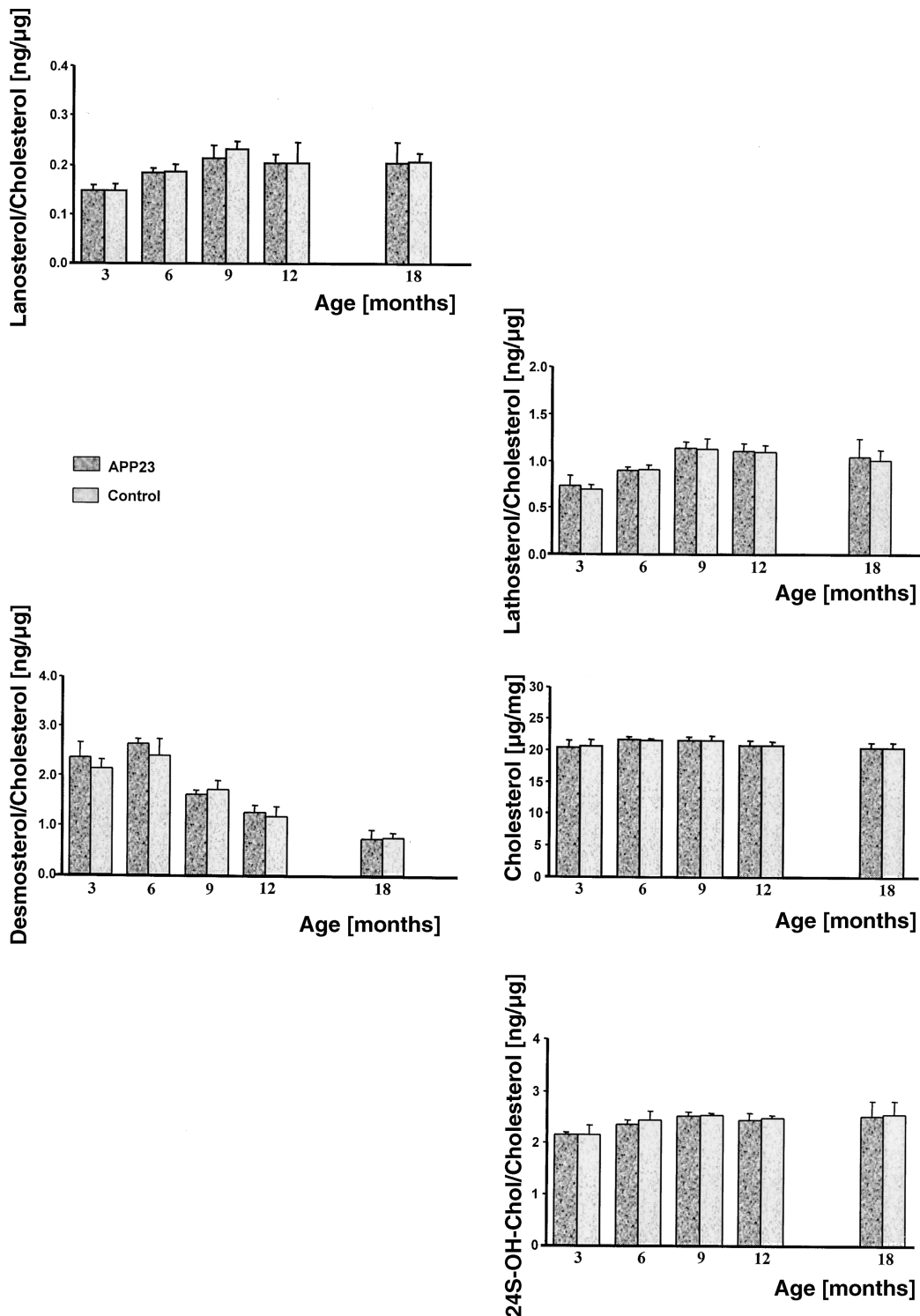
Fig. 2. Cholesterol metabolism in the aging APP23 transgenic (dark bars) and wild-type mouse (light bars) brain, with ratios of lathosterol, lanosterol, and desmosterol to cholesterol, cholesterol/mg wet weight, and ratio of 24S-OH-Chol to cholesterol.

portant link between cholesterol, β-amyloid, and AD. Changing cholesterol levels affect APP metabolism in vitro (11, 14, 15, 26) and in vivo (16, 18). The mechanism for these observations is largely unknown. As there is an obvious lack of information about cholesterol metabolism in the aging brain, the levels of cholesterol, its

precursors, or degradation products were analyzed in mouse brain. In agreement with previous studies (see below), two different cholesterol synthesizing pathways were found. Desmosterol levels decreased dramatically with age, whereas lathosterol and lanosterol levels increased, indicating that the aged brain utilizes the la-
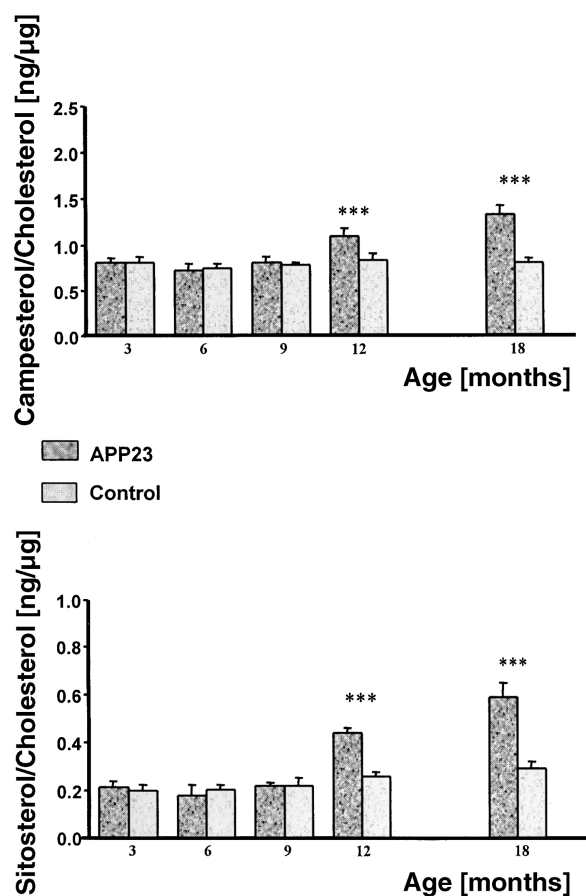
Fig. 3. Ratios of campesterol and sitosterol to cholesterol in APP23 (dark bars) and wild-type mouse brain (light bars). The levels were significantly elevated (***$P < 0.001$) in 12 and 18 months old APP23 transgenic mice.

thosterol rather than the desmosterol pathway as a precursor route.

There are three main known functional differences between the central nervous system (CNS) and the peripheral nervous system (PNS): 1) While axons in the PNS can regenerate after injury, those in the CNS cannot. 2) Degeneration of axons and the myelin sheath following injury occurs much more rapidly in the PNS than in the CNS; and 3) in rodents, PNS myelination commences before CNS myelination (27). Bourre et al. described how Schwann cells in the PNS use the 7-dehydrocholesterol pathway, while oligodendrocytes in the CNS may prefer the desmosterol pathway (28). Cholesterol biosynthesis in mammals is a very complex biochemical process that includes a great number of enzymatical reactions in early-step (29) and late-step cholesterol synthesis (**Fig. 4**). In late-step synthesis, discrete oxidoreductive and/or demethylation reactions occur, which start with the common precursor lanosterol. Desmosterol (24-dehydrocholesterol) has been implicated with myelination processes. While high desmosterol levels could be detected in the brain of young rat and mouse (30, 31) and in fetal human brain (32), no desmosterol was found in the brain of adult rats (33) or humans (32). The same holds true for the

brain of newborn guinea pigs, which is fully myelinated at birth (33). The brain of Sprague-Dawley rats exhibited 4-fold decreasing desmosterol contents between 4 and 21 days, and almost undetectable levels at 6 months of age. In the present study, concentrations of desmosterol decreased significantly after 6 months of age. This discrepancy could be explained by the sensitivity of the methods used to quantify cholesterol related sterols. The continuous increase of lathosterol and lanosterol further indicated that cholesterol synthesis is dominated by the lathosterol pathway during aging. The late-step cholesterol precursors are involved in a variety of CNS and peripheral processes. For example, 1) elevated desmosterol concentrations in an infant with lethal multiple malformations (such as macroencephaly) have been described. GC-MS demonstrated an abnormal accumulation of desmosterol in kidney, liver and brain tissue (34). Abnormally elevated levels of precursors of the lathosterol pathway is a biochemical consequence in cerebrotendinous xanthomatosis, a disorder which is characterized by neurological and psychiatric symptoms (35).

The cholesterol brain content increases dramatically during postnatal development between 5 and 21 days, to about 12–13 mg/g at the age of 3 months, but only very slightly during adulthood and aging (14 mg/g at 12 months of age) (28). However, an increase of the ratio of cholesterol to protein in mouse of about 14% between 1 month and 12 months of age has also been reported (36). The authors performed quantification of cholesterol by a modified enzymatic assay using unspecific sterol oxidases, which may be not very sensitive. This method is suitable and validated for determination of total cholesterol in serum or plasma in healthy humans where the content of cholesterol is 10^4–10^6-fold higher than normal levels of other sterols and oxysterols (37). We strongly believe that it is important to quantify sterols with highly sensitive and specific HPLC, GC, and/or GC-MS methods. In the present study, we observed no elevation of cholesterol during aging.

Importance of 24*S*-OH-Chol

Peripheral cholesterol is eliminated and converted into bile acids by the liver through oxidation and degradation of the cholesterol side-chain (38). In contrast, no evidence for such metabolism is found in the brain (39). A new mechanism for elimination of cholesterol from the brain was recently described by Lütjohann et al. and Björkhem et al. (2, 4, 22); the conversion of cholesterol into 24*S*-OH-Chol and a flux of this oxysterol through the blood-brain barrier into the circulation. The enzyme involved in this reaction was recently identified as a cytochrome P-450 species denoted CYP46 (40). This enzyme is almost exclusively expressed in brain and evidence was presented that more than 90% of the 24*S*-OH-Chol in the human circulation originates from this organ (22, 41), while in mouse and rat, only 50% and 70% originate from the brain, respectively. 24*S*-OH-Chol levels increased only mildly with age in both APP23 and wild-type mice. This observation is remarkable despite a massive pathology
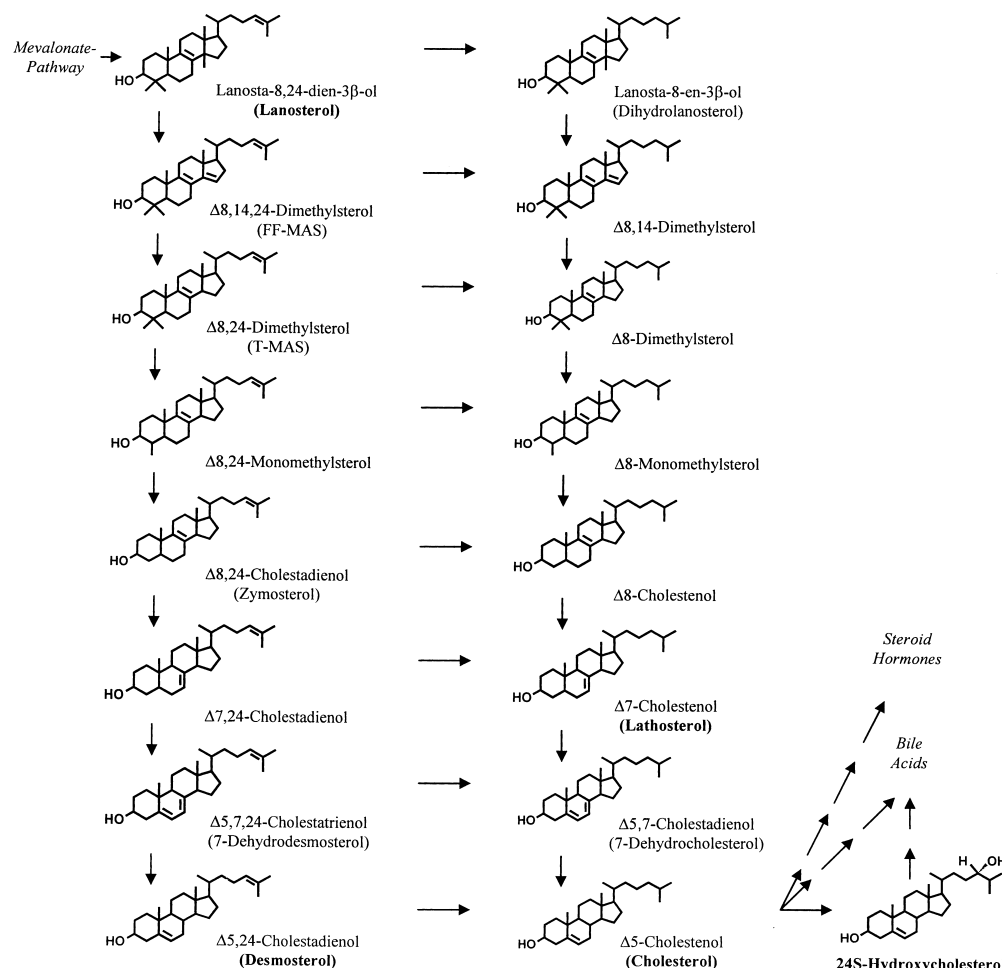
Fig. 4. The steroid pathway from lanosterol to cholesterol and the conversion of cholesterol to 24*S*-hydroxy-cholesterol (24*S*-OH-Chol).

such as abundant neuritic plaques with sprouting neurites, accompanying gliosis and a mild neuron loss in the brain of aged APP23 mice (42). Nevertheless, a differential distribution of cholesterol between subcellular membrane components during aging is most likely. Cholesterol is thought to condense the packing of sphingolipids in rafts (43). Both lipids form a concentration gradient along the secretory pathway with enriched compartments toward the cell surface (44, 45). Lipid rafts play an essential role in exocytic transport routes and endocytic uptake (46). We assume that misregulated intraneuronal Aβ/APP anterograde, retrograde, and transcytotic transport is a key feature of AD (47). A recent study reported that intracellular Aβ accumulates in late endosomes of Niemann-Pick Type C (NPC1) mutant cells linking Aβ with cholesterol transport (48).

Cholesterol and Aβ reduction

Recently, Refolo et al. (17) reported that treatment of presenilin-1 and APP double-transgenic mice with BM15.766, a 7-dehydrocholesterol reductase inhibitor in late-step cholesterol synthesis, decreased the amount of Aβ produced. A strong positive correlation between the amount of plasma cholesterol and Aβ was found in both untreated and BM15.766 cholesterol lowered animals.

It is well established that most of the cholesterol in the adult CNS is present in its free form (49–51), with only trace amounts of esterified cholesterol. Very recently, Puglielli et al. (52) analyzed the influence of esterification of cholesterol on Aβ production in different cell lines. The authors used two different ACAT inhibitors, a fatty-acid anilide derivative that mimics acyl-CoA, and a derivative of urea. They found that esterified cholesterol modulated the generation of Aβ. Similar to other in vitro studies, cholesterol turned out to be the effective compound influencing APP processing. However, the influence of cholesterol precursors was largely neglected.

Phytosterols in APP transgenic animals

Cerebral amyloid angiopathy (CAA) is one of the pathological hallmarks in AD brain and in APP transgenic mice (42, 53–55). It is generally assumed that the BBB integrity can be interrupted by aggregation of Aβ peptides causing CAA at later stages. APP transgenic mice have proven to be very useful for the study of the cascade of pathological alterations occurring early in the brain of AD patients.

The ratios of campesterol and sitosterol to cholesterol, both dietary plants sterols, were significantly higher in transgenic compared with wild-type mice at age 12 and 18 months. Our observation indicates either *i*) that the BBB is affected as early as 12 months of age in APP23 mice, or *ii*) that in aged APP23 mice there is an altered phytosterol transport into the brain.

Recently, Poduslo et al. compared BBB permeability of different proteins, such as insulin, albumin, and Aβ1-40 in an AD double transgenic mouse model expressing APP and presenilin-1 (56). The authors concluded that alterations in the BBB may be possible but of lower importance. We observed that the phytosterols campesterol and sitosterol are significantly higher at 12 months of age compared with littermate non-transgenic controls. At this at a time point, all transgenic APP mice exhibit plaques (25).

In conclusion, the aging brain favors the lathosterol rather than the desmosterol pathway for late-step cholesterol synthesis. Increased levels of plant sterols were identified only in the brain of APP23 transgenic mice, which may point to altered transport through or leakage of the blood brain barrier due to Aβ deposition. 🔒

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