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Modulation of Secreted β-Amyloid Precursor Protein and Amyloid β-Peptide in Brain by Cholesterol*

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The effects of dietary cholesterol on brain amyloid precursor protein (APP) processing were examined using an APP gene-targeted mouse, genetically humanized in the amyloid β-peptide (Aβ) domain and expressing the Swedish familial Alzheimer's disease mutations. These mice express endogenous levels of APP holoprotein and abundant human Aβ. Increased dietary cholesterol led to significant reductions in brain levels of secreted APP derivatives, including sAPPα, sAPPβ, Aβ1–40, and Aβ1–42, while having little to no effect on cell-associated species, including full-length APP and the COOH-terminal APP processing derivatives. The changes in levels of sAPP and Aβ in brain all were negatively correlated with serum cholesterol levels and levels of serum and brain apoE. These results demonstrate that secreted APP processing derivatives and Aβ can be modulated in the brain of an animal by diet and provide evidence that cholesterol plays a role in the modulation of APP processing *in vivo*. APP gene-targeted mice lacking apoE, also have high serum cholesterol levels but do not show alterations in APP processing, suggesting that effects of cholesterol on APP processing require the presence of apoE.

Alzheimer's disease (AD)[1] pathology includes extracellular amyloid deposits, intracellular neurofibrillary tangles, synaptic loss, and neuronal death (for a review, see Ref. 1). Alterations in the production or processing of APP have been implicated in the etiology of at least some forms of AD (2, 3). Multiple pathways for APP processing have been described, including a nonamyloidogenic pathway in which a putative α-secretase cleaves within the Aβ domain (4, 5), resulting in the formation of a secreted NH_2-terminal fragment, sAPPα, and a cell-associated 9-kDa COOH-terminal derivative. Another fraction of APP is processed along an amyloidogenic pathway in which cleavage by a putative β-secretase at the NH_2 terminus of the Aβ domain results in the formation of a secreted NH_2-terminal fragment, sAPPβ (6), and a cell-associated 12-kDa COOH-terminal derivative that may be the immediate precursor of Aβ (7, 8). Cleavage of APP by both β-secretase and γ-secretase results in formation of Aβ, 40 or 42 amino acids in length (9, 10), that is found deposited in extracellular amyloid plaques in the AD brain (1, 11).

In order to elucidate mechanisms of APP processing and Aβ generation *in vivo*, an animal model was developed by gene targeting that converted the mouse Aβ sequence to human and incorporated the Swedish familial Alzheimer's disease mutations (12). Enhanced amyloidogenic APP processing by the Swedish mutations, resulting in higher level Aβ production has been well documented in cell culture systems (13–15) and in the APP gene-targeted mice (12). These mice are well suited for investigating modulation of APP processing *in vivo*, because brain Aβ levels are nearly 10-fold above normal endogenous levels, thereby reducing the stringency for assays to detect Aβ, particularly for the less abundant but more amyloidogenic 42-residue form. Furthermore, proper developmental and tissue-specific expression of APP and Aβ is maintained in the gene-targeted mice (12).

Here, we have examined the impact of dietary cholesterol and apoE on APP processing and Aβ levels using the APP gene-targeted mice. Cholesterol is an integral component of all eukaryotic cell membranes and is essential for normal cellular functions including caveolae formation (16, 17) and covalent modification of embryonic signaling proteins (18). Cholesterol also dramatically affects physical properties of cell membranes such as increasing ordering and rigidity and decreasing permeability and lateral diffusion (19). Alterations in membrane lipid and cholesterol content have been reported to modulate the activities of intrinsic membrane enzymes (20–24). Cellular cholesterol content arises either through intracellular synthesis or by uptake of cholesterol through the low density lipoprotein (LDL) receptor pathway (25). These pathways are tightly regulated by sterol regulatory element-binding proteins (SREBPs), membrane-bound transcription factors whose proteolytic cleavage and subsequent translocation to the nucleus regulate transcription of multiple cholesterol homeostatic genes (26). In the brain, apoE primarily directs the mobilization and redistribution of cholesterol during membrane remodeling associated with the plasticity of synapses (27–31). The apoE gene is a risk factor for sporadic and late onset AD (32–38). Alterations in cholesterol metabolism have been reported to occur with age (39–45) and have been implicated in the pathogenesis of AD (46–49). Directly pertinent to APP metabolism, cholesterol has been recently linked to decreased release of secreted APP in cultured cells (50, 51). However, neither of these studies presented data concerning the effects of cholesterol on Aβ production. We demonstrate that increased dietary cholesterol lowers levels of secreted APP derivatives

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[1] The abbreviations used are: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid β-peptide; LDL, low density lipoprotein; SREBP, sterol regulatory element-binding protein; KO, knockout; TC, total cholesterol; Ab, antibody; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ELISA, enzyme-linked immunosorbent assay; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

including sAPPα and sAPPβ in mouse brain. Additionally, cholesterol in the diet resulted in decreases in the levels of both major secreted Aβ forms, Aβ1–40 and Aβ1–42. The reported effects of cholesterol on APP processing derivatives in brain appeared to require the presence of apoE.

EXPERIMENTAL PROCEDURES

Animals and Diets—APP gene-targeted mice ($APP^{nNLh/nNLh}$), wild-type for apoE, containing a humanized Aβ domain and bearing the Swedish familial Alzheimer's disease KM to NL mutations have been described previously (12). Homozygous $APP^{nNLh/nNLh}$ mice, 210–408 days in age, were maintained for 8 weeks on a basal diet (number 5755C Purina Test Diet), containing trace amounts (0.005%) of cholesterol and 10% fat or a high cholesterol diet (number 5801C Purina Test Diet) containing 10% fat and 5% cholesterol. APP gene-targeted mice were bred with apoE knockout (KO) mice to generate double homozygous mice ($APP^{NLh/NLh}$, ApoE−/−). $APP^{NLh/NLh}$, ApoE−/− ($n = 4$) and APP gene-targeted mice, wild-type for apoE, ($APP^{NLh/NLh}$, ApoE+/+) ($n = 4$), each approximately 180 days old, fed a laboratory chow diet (Rodent 5001, Purina Mills Inc., St. Louis, MO) containing 0.027% cholesterol and 4.5% fat, were used to compare the effect of the apoE genotype on APP processing fragments in brain. Mice had access to the pelleted diets and water *ad libitum*. All animals were sacrificed prior to analysis by CO_2 asphyxiation. Brains were removed, washed in saline, and frozen at −70 °C prior to analysis.

Measurement of Serum and Tissue Total Cholesterol—Trunk blood was collected at sacrifice from mice in a nonfasted state in order to avoid potential complicating effects of food deprivation on APP metabolism. Serum total cholesterol (TC) was measured using an enzymatic-colorimetric assay (number 352, Sigma). To measure tissue total cholesterol levels, liver, mouse half-brain, or dissected frontal cortices were homogenized in B buffer (20 mM Tris-HCl, 2 mM EGTA, 1 mM EDTA, 1 mM benzamidine, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Lipids and cholesterol were extracted with 10 volumes of a chloroform/methanol mix (2:1, v/v) as described previously (52). Dried samples were resuspended in 400 μl of 2:1 (v/v) chloroform/methanol, and aliquots were mixed with 1 ml of a 2% Triton X-100/chloroform solution. Dried samples were resuspended in 0.5 ml of water, and total cholesterol was determined using the enzymatic-colorimetric assay.

Antibodies—Monoclonal Ab 6E10, specific for the human Aβ peptide 1–17 sequence, was obtained from Senetek (Maryland Heights, MO). Immunoprecipitating rabbit polyclonal Ab 1153 was raised against human Aβ1–28 (53), and Ab 97 was raised against the 30 COOH-terminal APP residues, APP741 to APP770 (APP770 coordinates) (12). Rabbit polyclonal Ab 9 was directed against APP724 to APP747 (54) and recognizes both the 12-kDa and 9-kDa COOH-terminal APP derivatives (12). Rabbit polyclonal Ab 54 is specific for the last five amino acids of Swedish sAPPβ (SEVNL) (12, 55). Rabbit polyclonal Ab specific for mouse apoE was obtained from BioDesign Inc. (Kennebunkport, ME; number K23100R). The monoclonal Ab C4 (Boehringer Mannheim) was used to detect mouse actin. An Aβ40-selective polyclonal Ab was used in the Aβ40 ELISA (Quality Control Biochemicals, Hopkinton, MA). All secondary antibodies (GAM-IgG and GAR-IgG) conjugated to horseradish peroxidase were obtained from Bio-Rad or from Southern Biotechnology Associates (Birmingham, AL).

Measurement of sAPP (sAPPα, Swedish sAPPβ) and ApoE in Brain—One-half brain or dissected frontal cortex was homogenized in 3 ml of B buffer and centrifuged at 100,000 × *g* for 1 h. Tris-soluble supernatant fractions were removed and assayed for total protein concentration by BCA assay (Pierce). Fifty μg of each extract was electrophoresed on 8% Tris-Glycine SDS-polyacrylamide gels (Novex, San Diego, CA) and subsequently transferred to nitrocellulose. For detection of sAPPα, blots were incubated with 1:2000 Ab 6E10 followed by 1:2000 GAM-IgG conjugated to horseradish peroxidase. For detection of Swedish sAPPβ, blots were incubated with 1:500 Ab 54 followed by 1:2000 GAR-IgG conjugated to horseradish peroxidase. Ab C4 was used at 1:2000 to detect actin in each brain extract for normalization. Enhanced chemiluminescence (ECL) was used as the detection reagent (Amersham Pharmacia Biotech). To detect mouse brain apoE, 50 μg of Tris-soluble extracts from half-brain or frontal cortex were electrophoresed on 10–20% Tris-Tricine SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. Membranes were incubated with 1:2000 anti-apoE Ab followed by 1:2000 GAR-IgG conjugated to horseradish peroxidase and ECL as the detection reagent.

Measurement of Cell-associated APP (Full-length APP (flAPP), 12- and 9-kDa Fragments)—Tris-insoluble pellets following 100,000 × *g* centrifugation of brains extracted with B buffer were washed with an additional 3 ml of B buffer followed by sonication in 1 × radioimmune precipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM benzamidine, 0.05 mM leupeptin, 0.02 mM pepstatin A). Supernatants from 100,000 × *g* spin were removed and assayed for total protein by BCA assay. Radioimmune precipitation-soluble protein containing cell-associated APP was immunoprecipitated with Ab 97 as described previously (12). Immunoprecipitates were split equally and electrophoresed either on 8% Tris-Glycine SDS-polyacrylamide or 16% Tris-Tricine SDS-polyacrylamide gels. For detection of flAPP, 8% gels were transferred to nitrocellulose and subsequently incubated with Ab 6E10 (1:2000) followed by 1:2000 GAM-IgG-conjugated to horseradish peroxidase and ECL detection. For detection of the 12- and 9-kDa COOH-terminal APP derivatives, 16% gels were transferred to polyvinylidene difluoride followed by subsequent incubation with Ab 9 (1:500) and 1:2000 GAR-IgG conjugated to horseradish peroxidase and ECL detection reagent. Ab C4 was used at 1:2000 to detect actin in each brain extract and was used for normalization.

TABLE I
Effect of diet on cholesterol and ApoE levels in APP gene-targeted mice

Data are mean ± S.E.; *n* values are shown in parentheses.

	Basal diet	Cholesterol diet
Liver weight (g)	1.6 ± 0.1 (9)	3.7 ± 0.1 (20)[a]
Serum TC (mg/dl)	109.9 ± 20.1 (9)	332.5 ± 62.4 (20)[b]
Liver TC (mg/g)	4.1 ± 0.3 (5)	70.5 ± 5.8 (10)[a]
Brain TC (mg/g)[c]	6.1 ± 0.6 (5)	7.7 ± 0.9 (10)
Frontal cortex TC (mg/g)	13.3 ± 0.3 (4)	16.0 ± 0.7 (4)[b]
Serum ApoE (%)[d]	100.0 ± 10.5 (11)	244.1 ± 20.9 (16)[a]
Brain ApoE (%)[d]	100.0 ± 2.8 (7)	112.9 ± 4.0 (13)[b]
Frontal cortex ApoE (%)[d]	100.0 ± 25.3 (4)	237.6 ± 25.8 (3)[b]

[a] $p < 0.0001$.
[b] $p < 0.05$.
[c] Values from brain were determined from half-brain samples.
[d] %, percentage of basal diet mean.

Measurement of Brain Aβ—A sandwich ELISA that specifically detects Aβ1–40 (56), but not Aβ17–40 or Aβ1–42, was used on brain samples. Frozen mouse half-brains were Dounce homogenized at a ratio of 150 mg of tissue/ml of 70% (v/v) formic acid, homogenates were centrifuged at 100,000 × *g* for 1 h, and the supernatants were recovered. Samples were diluted 1:20 in 1 M Tris-HCl, 0.5 M sodium phosphate dibasic, 0.05% sodium azide, and Aβ40 was detected using the ELISA as described previously (56).

To simultaneously measure Aβ1–40 and Aβ1–42 from brain samples, a bicine-urea gel system of immunoprecipitated Aβ was used. Mouse half-brain was homogenized in 3 ml of 6 M guanidine, 50 mM Tris-HCl, pH 7.4, at 4 °C. Human Aβ was immunoprecipitated with Ab 1153 as described previously (12), and the precipitates were eluted in bicine gel sample buffer (0.36 M Bistris, 0.16 M bicine, 1% (w/v) SDS, 15% (w/v) sucrose, 2.5% 2-mercaptoethanol) by heating for 10 min at 90 °C. Samples were resolved on 10% acrylamide, 5% bisacrylamide, 8 M urea gels (57, 58) and were blotted to polyvinylidene difluoride membrane. Human Aβ was detected using Ab 6E10 at 1:2000 followed by GAM-IgG conjugated to horseradish peroxidase (1:2000) and ECL substrate (12). Human Aβ1–40 and Aβ1–42 (Bachem) size standards were included on the gels.

Data Analysis—Densitometry of immunoblot data was performed using the Docugel V Scanalytic system (CSP Inc., Billerica, MA). All results are reported as mean ± S.E. Statistical significance was determined using student's *t* test analyses (StatView, Abacus Concepts Inc., Berkeley, CA). Correlations shown are reported as Pearson product moment.

RESULTS

Serum TC and ApoE Levels Increase in Response to High Cholesterol Diet—Serum TC (Table I) was significantly elevated in APP gene-targeted mice fed high dietary cholesterol when compared with basal diet controls. The range of serum TC in the high cholesterol diet group was 247–1152 mg/dl. Conversely, animals maintained on basal diets had levels typically ranging from 44 to 145 mg/dl. Occasional outliers on the basal diet were observed (*i.e.* animal number 061; TC = 249 mg/dl). In animals fed high dietary cholesterol, liver size and weight were increased compared with those of mice fed the

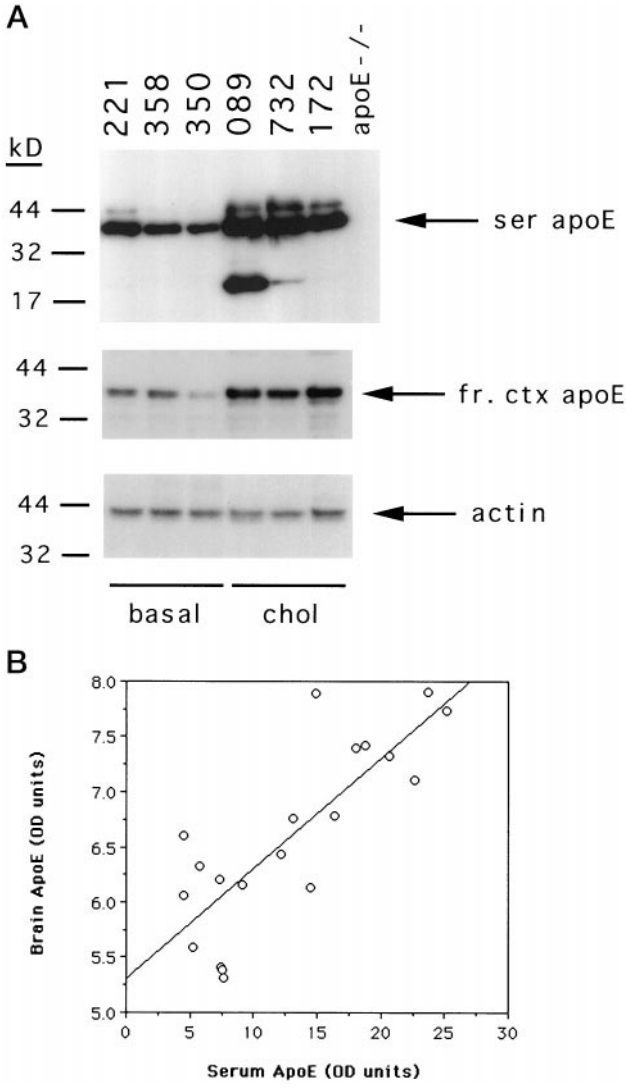

FIG. 1. **Mouse serum and brain apoE levels increase in response to high dietary cholesterol.** *A* (*top*), immunoblot of mouse serum (*ser*) apoE from APP gene-targeted mice fed either basal or high cholesterol (*chol*) diets. Serum from an APP gene-targeted mouse also harboring the apoE KO locus (labeled as *apoE*−/−) is devoid of apoE immunoreactivity. *Middle*, immunoblot of apoE extracted from frontal cortex (*fr. ctx.*) from animals fed either basal or high cholesterol (*chol*) diets. *Bottom*, actin signal from frontal cortex samples. *B*, correlation of mouse serum apoE levels to total brain apoE levels from APP gene-targeted mice on basal or high cholesterol diets, combined ($r = 0.81$; $p < 0.0001$).

basal diet (Table I). Mean liver weight increased more than 2-fold in gene-targeted mice as a result of the high cholesterol diet. Liver-associated TC was dramatically increased more than 17-fold in mice fed the high cholesterol diet (Table I).

Elevations in serum apoE in response to increases in dietary cholesterol have been shown to be the result of transcriptional up-regulation of the apoE gene (59, 60). Serum protein from gene-targeted mice fed either basal or high dietary cholesterol was immunoblotted using a mouse apoE-specific antibody. This antibody recognizes a 34-kDa polypeptide that is clearly absent in serum from APP gene-targeted mice that also harbor the apoE-KO (61) locus ($APP^{NLh/NLh}$, ApoE−/−) (Fig. 1*A*). A 2.4-fold increase in mean serum apoE was observed in APP gene-targeted mice fed the high cholesterol diet (Fig. 1*A* and Table I). A strong correlation between serum apoE and TC levels was found in these mice ($r = 0.86$, $p < 0.0001$).

TC and ApoE Levels Increase in Brain in Response to High Dietary Cholesterol—TC was extracted from half-brains of APP gene-targeted mice fed either basal or high cholesterol diets. A trend toward increased brain TC in mice fed the high cholesterol diet compared with the basal diet was evident (Table I), although these results did not reach statistical significance. However, extraction and measurement of TC from frontal cortices of APP gene-targeted mice fed high cholesterol, as compared with mice on basal diet, revealed a more robust and statistically significant increase in TC (Table I). Levels of apoE extracted from half-brains of APP gene-targeted mice fed either basal or high cholesterol diet were also compared. A small but statistically significant increase in the levels of brain apoE in mice fed high dietary cholesterol relative to basal diet controls was observed (Table I). Levels of brain apoE positively correlated with TC levels ($r = 0.79$, $p < 0.0001$) and with serum apoE levels (Fig. 1*B*). A 2.4-fold increase in apoE levels was observed in the frontal cortex of APP gene-targeted mice fed the high cholesterol diet relative to those on the basal diet (Fig. 1*A* and Table I). The differences in the magnitude of the increased TC and apoE when comparing half-brain *versus* frontal cortex samples (see Table I) most likely reflected the lower amount of white matter present in the dissected frontal cortex. It is likely that white matter-associated lipid dilutes the magnitude of the measured increases in both TC and apoE observed from half-brain preparations. Also, increased serum cholesterol and apoE originating from brain blood would not be expected to significantly contribute to the measured changes of TC and apoE in brain tissue from mice on the high cholesterol diet, since blood comprises less than 2% of total brain wet weight. The increases in brain TC in response to the cholesterol diet reported here are similar in magnitude to increases observed in brains from both rat (20) and rabbit (62) following similar high cholesterol diet regimens. Rabbits also show increased apoE immunoreactivity in brain following the high cholesterol diet (63).

No changes in measured phospholipid, total glycerol, and free fatty acids were observed in the serum or brain of mice fed basal *versus* high cholesterol diets (data not shown).

Dietary Cholesterol Reduces Levels of sAPP, but Not Cell-associated APP, in Brain—Immunoblot and ELISA-based methods were used to quantify the effects of dietary cholesterol on APP and its processing derivatives in brain. Tris-soluble brain homogenates were analyzed on immunoblots using antibodies 6E10 and 54, which recognize secreted sAPPα and Swedish sAPPβ, respectively. Both full-length cell-associated and secreted APP expressed in the APP gene-targeted mouse brain are primarily APP695, as previously demonstrated (56). Mice fed high dietary cholesterol exhibited significant decreases in both brain sAPPα (Figs. 2*A* and 4) and brain sAPPβ (Figs. 2*B* and 4) relative to mice on the basal diet. Some variability was observed among individual mice in the magnitude of decreases in the sAPPα and sAPPβ (Fig. 2). Differences were attributable to the levels of serum TC attained in mice within both diet groups. For instance, several mice fed a high cholesterol diet that showed only subtle increases in serum TC had only modest decreases in sAPP (*i.e.* number 784, Fig. 2*A*). Conversely, some mice on a basal diet with unexpectedly high levels of serum TC exhibited more substantial decreases in sAPP (*i.e.* number 061, Fig. 2*A*). A strong negative correlation was found between levels of serum TC attained and levels of both brain sAPPα ($r = -0.63$; $p = 0.01$) and sAPPβ ($r = -0.74$; $p = 0.002$) when data from both diet groups were combined.

To determine if dietary cholesterol resulted in changes in cell-associated APP, Tris-insoluble fractions were analyzed for flAPP and the 12- and 9-kDa COOH-terminal APP cleavage products that result from cleavage by the β- and α-secretases,

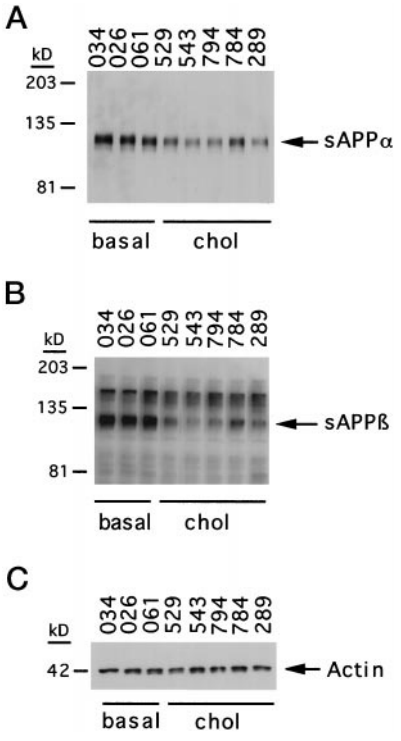

FIG. 2. **Brain sAPPα and Swedish sAPPβ decrease in response to dietary cholesterol.** *A*, detection of brain sAPPα by antibody 6E10 from APP gene-targeted mice fed either basal or high cholesterol (*chol*) diets. *B*, detection of brain Swedish sAPPβ by antibody 54 from APP gene-targeted mice on the different diets. *C*, actin immunoreactivity from samples depicted in *A* and *B*.

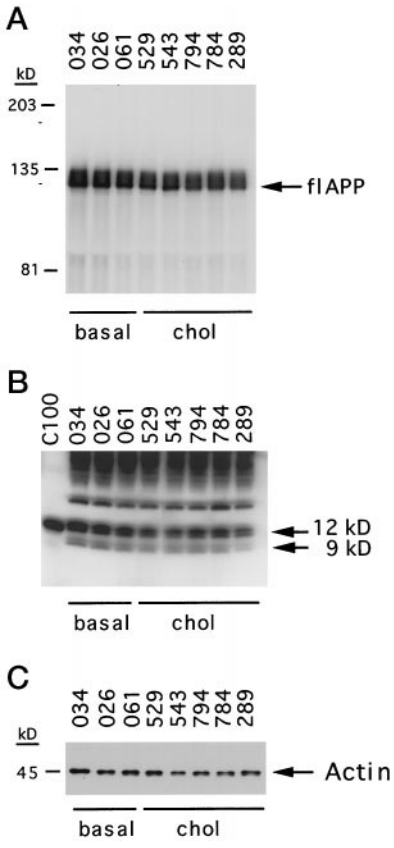

FIG. 3. **Full-length APP and COOH-terminal APP processing derivatives are not significantly altered in brain in response to dietary cholesterol.** *A*, immunoblot of brain flAPP from APP gene-targeted mice fed either basal or high cholesterol (*chol*) diets. *B*, immunoblot of the 12- and 9-kDa COOH-terminal APP processing derivatives. Recombinant C100 (12 kDa) (53) was included on the blot as a size comparison. *C*, actin immunoreactivity from samples depicted in *A* and *B*.

respectively (12). No significant differences in cell-associated flAPP in brain resulted from high dietary cholesterol (Figs. 3*A* and 4). A trend toward decreased levels of the 12- and 9-kDa COOH-terminal derivatives due to high cholesterol diet was noted; however, these effects did not reach statistical significance ($0.05 < p < 0.15$) (Figs. 3*B* and 4).

Brain Aβ1–40 and Aβ1–42 Decrease in Response to High Dietary Cholesterol—Brain Aβ from APP gene-targeted mice, fed either basal or high dietary cholesterol, was analyzed by two independent methods. Using a sandwich ELISA that specifically detected Aβ1–40 (56), but not Aβ17–40 or Aβ1–42, a significant reduction in brain Aβ40 was measured in mice fed a high cholesterol diet (Table II). To determine the effect of the cholesterol diet on Aβ peptide ending at residue 42, a bicine-urea analysis of immunoprecipitated brain Aβ was performed under conditions previously shown to resolve Aβ1–40 and Aβ1–42 (57, 58). As shown in Fig. 5*A*, both brain Aβ1–40 and Aβ1–42 decreased in response to high dietary cholesterol. Quantitation of these effects revealed highly significant reductions in both Aβ1–40 and Aβ1–42 from brains of mice fed high dietary cholesterol compared with mice on a basal diet (Table II).

Strong negative correlations of brain Aβ levels in gene-targeted mice to several parameters including levels of serum TC, serum apoE, and brain apoE were evident. Each of these correlations is depicted in Fig. 6 using the Aβ40 values obtained by ELISA (Table II).

Cholesterol Effects on APP Processing Require the Presence of ApoE—Mice homozygous for a KO apoE locus have been shown to have elevated levels of serum cholesterol and develop atherosclerotic lesions in blood vessels (61, 64, 91). Mice homozygous for both the targeted APP and the apoE KO loci ($APP^{NLh/NLh}$, ApoE−/−) were used to determine the effect of elevated serum TC, in the absence of apoE, on APP processing in the

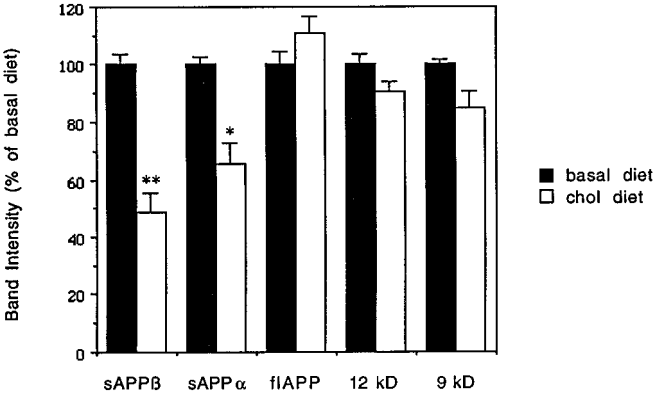

FIG. 4. **Quantitation of APP and processing fragments from APP gene-targeted mouse brain in response to dietary cholesterol.** Densitometric analyses of bands for sAPPα, sAPPβ, flAPP, and the 12- and 9-kDa COOH-terminal derivatives are represented as a percentage of the mean signal from basal diet samples. This allows comparison across multiple immunoblots. The number (n) of animals analyzed for each APP fragment was 8 in the basal diet group (■) and 19 in the cholesterol diet group (□). The *asterisks* denote statistical significance relative to basal diet groups at $p = 0.007$ (sAPPα) and $p < 0.0001$ (sAPPβ). Other nonsignificant effects had p values greater than 0.1.

mouse brain. $APP^{NLh/NLh}$, ApoE−/− mice exhibited significantly ($p < 0.0001$) increased serum TC (mean 542 mg/dl) relative to APP gene-targeted mice harboring the wild-type apoE locus ($APP^{NLh/NLh}$, ApoE+/+) (mean 145 mg/dl). This

TABLE II
Effect of diet on Aβ levels in APP gene-targeted mouse brain
n values are shown in parentheses.

	Aβ40[a]	Aβ1–40[b]	Aβ1–42[b]
	ng/mg protein	*% of basal diet*	*% of basal diet*
Basal diet	1.8 ± 0.1 (5)	100.0 ± 6.2 (9)	100.0 ± 12.5 (9)
Cholesterol diet	1.3 ± 0.1 (10)[c]	50.0 ± 4.3 (7)[d]	30.0 ± 7.0 (7)[d]

[a] Aβ40-specific ELISA.
[b] Bicine-urea gel analysis.
[c] $p = 0.02$.
[d] $p < 0.001$.

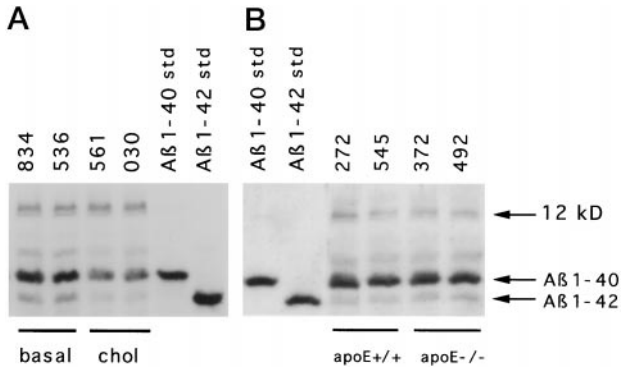

FIG. 5. **Analysis of brain Aβ1–40 and Aβ1–42 in response to dietary cholesterol and apoE genotype.** *A*, brains from APP gene-targeted mice (wild-type for apoE) fed either basal or high cholesterol (*chol*) diets were extracted in 6 M guanidine, and Aβ was immunoprecipitated from extracts as described under "Experimental Procedures." Samples were run on bicine-urea gels and immunoblotted with Ab 6E10 to afford resolution and detection of Aβ1–40 and Aβ1–42. Human Aβ1–40 and 1–42 standards (Bachem) were included on the gels for comparison. Levels of the 12-kDa signal shown for each sample do not change in response to dietary cholesterol. The band just above Aβ1–40 in each sample comigrates with the Aβ1–38 standard (data not shown) and also decreases in response to cholesterol diet. *B*, Aβ1–40 and Aβ1–42 from brains of APP gene-targeted mice, either wild-type for apoE (apoE+/+) or KO for apoE (apoE−/−).

elevation in serum TC was even greater than that generally observed from APP gene-targeted mice harboring the wild-type apoE gene when fed high dietary cholesterol (see Table I). Despite increases in serum TC in APP gene-targeted mice devoid of apoE, relative to gene-targeted mice wild-type for apoE, no significant differences in levels of sAPPβ ($p = 0.47$) or sAPPα ($p = 0.58$) between the two groups were observed (data not shown). Similarly, no differences in levels of Aβ1–40 ($p = 0.88$) and Aβ1–42 ($p = 0.82$) could be attributed to the apoE genotype (Fig. 5*B*).

DISCUSSION

Cholesterol and alterations in its homeostasis have been implicated by several studies to be risk factors in the development of AD (46, 48, 49, 62, 65). However, the specific role that cholesterol might play in AD has not been defined. A potential link between AD and hypercholesterolemia, a defined risk factor for coronary artery disease (62, 66, 67), has been proposed. Other studies have linked decreases in brain cholesterol content to AD. Brain membranes isolated from AD subjects show significant decreases in membrane cholesterol content (41, 47). Furthermore, it has been proposed that inefficient cholesterol transport resulting from low levels of apoE in the brain may lead to the loss of synaptic integrity observed in AD (68, 69). In support of this, apoE levels in brain, cerebrospinal fluid, and plasma (59, 70–73) of individuals carrying one or two copies of the apoE4 allele are lower than those with other apoE genotypes.

Several studies have provided evidence that serum cholesterol is transported across the blood-brain barrier. Measurable increases in brain cholesterol have been observed in rodents fed high cholesterol diets (20, 62). Furthermore, transport of LDL across the blood brain barrier has been shown to be mediated by the LDL receptor and has been proposed to be a critical mechanism by which essential lipids, including cholesterol, are delivered to brain cells (74). The ability to experimentally modulate cholesterol levels in both serum and brain of an animal, taken together with evidence that alterations in cholesterol metabolism may be involved in AD, has led us to pursue a potential relationship of cholesterol to APP metabolism using the APP gene-targeted mouse. Our results demonstrating an increase in brain cholesterol as a result of high dietary cholesterol are not only consistent with previous reports (20, 62) but are also very similar in magnitude. We found that secreted APP fragments and Aβ1–40 and Aβ1–42 are modulated in the brain of the APP gene-targeted mice as a function of cholesterol levels. Not surprisingly, mice fed either basal or high cholesterol diets showed some variability in their responsiveness. A likely explanation for these differences is the efficiency with which dietary cholesterol is absorbed (75). We demonstrated that cholesterol and apoE levels, measured in combined data from animals fed basal and high cholesterol diets, showed significant negative correlations to levels of brain sAPP and Aβ. Levels of these secreted APP processing fragments were highly predictive based on measurable changes of both serum cholesterol and apoE from mice in both the basal and high cholesterol diet groups. These data support the hypothesis that the observed changes in sAPP and Aβ1–40 and Aβ1–42 are a direct result from cholesterol in the diet as opposed to other minor differences that may exist between the diet formulations. This is further supported by a lack of change in measured phospholipid, total glycerol, and free fatty acid in the serum and brains of mice fed basal *versus* high cholesterol diets.

The effects of dietary cholesterol on APP derivatives were most robust on the secretory products of the APP processing pathway, since no significant changes were observed in cell-associated full-length APP or in the COOH-terminal derivatives generated by α- and β-secretase processing. There are several possibilities to explain the observed effects of cholesterol on secreted APP derivatives in the brain. Increased cellular cholesterol could act to increase membrane rigidity (19) and thereby decrease accessibility of secretases (α-, β-, and possibly γ-secretase) to APP substrate or to any cofactors associated with proteolytic activity. Decreases in sAPP secretion, presumably sAPPα, have been reported in 293 cells when cultured in the presence of increasing concentrations of free cholesterol, and it was suggested that this resulted from reduced cleavage by the α-secretase due to reduced lateral mobility of secretase and APP substrate in the membrane (50). Increased cellular cholesterol has also been demonstrated to inhibit, either directly or indirectly, proteolytic cleavage of the SREBP-1 precursor protein, a mechanism to prevent the soluble form of SREBP-1 from entering the nucleus to up-regulate genes involved in cholesterol synthesis and uptake (26, 76). Further understanding of the SREBP-1 pathway may provide insight into the mechanism of the cholesterol-mediated effects on APP processing.

Alternatively, changes in cellular cholesterol could act to alter the trafficking of vesicles containing APP to sites where secretase cleavage occurs or possibly by altering the efficiency at which secreted APP derivatives or Aβ are released from cells. Cholesterol within the cell is not uniformly distributed. Plasma membrane contains the highest levels of cholesterol, while ER and mitochondria contain very little (42, 77). The Golgi also contains substantial levels of cholesterol, and there

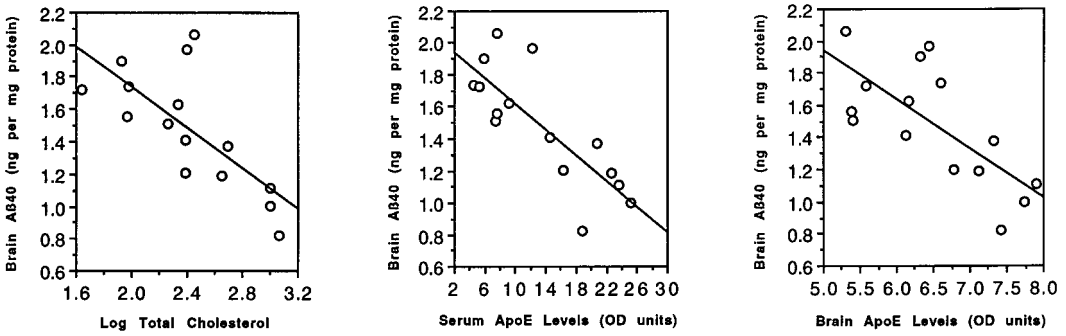

FIG. 6. **Negative relationship of brain Aβ40 to levels of serum total cholesterol, serum apoE, and brain apoE in the APP gene-targeted mice.** The raw Aβ40 data used to generate values shown in Table II were correlated with serum total cholesterol, serum apoE, and brain apoE levels measured from the APP gene-targeted mice fed basal ($n = 5$) or high cholesterol ($n = 10$) diets. Brain Aβ40 *versus* log serum total cholesterol ($r = -0.717$; $p = 0.003$), *versus* serum apoE ($r = -0.799$; $p = 0.0004$), and *versus* brain apoE ($r = -0.719$; $p = 0.003$) were all highly significant correlations.

is evidence of an increasing gradient in the cis to trans direction (77, 78). Cholesterol gradients may function in the sorting of membrane-associated proteins to specific subcellular compartments (77). It is therefore possible that alterations in cholesterol content in subcellular membranes could affect the efficiency at which APP is trafficked along the secretory pathway, thereby altering cleavage and secretion of APP derivatives.

In considering a hypothesis where cellular cholesterol regulates APP cleavage or trafficking and secretion, one must reconcile the lack of significant changes in cell-associated full-length APP and the carboxyl-terminal derivatives. Only a minority of cell-associated APP is processed for secretion and subsequently released (79, 80). The majority of cell-associated APP is trafficked into alternative intracellular pathways, presumably for ultimate degradation. If the majority of cell-associated APP residing in intracellular pathways is largely unaffected by increased cellular cholesterol, then measurable decreases of cell-associated APP fragments produced in the secretory pathway would be diluted. Indeed, a trend toward decreases in the 9- and 12-kDa COOH-terminal derivatives in brain as a result of elevated cholesterol was observed in APP gene-targeted mice. Whatever the mechanism, the data reported here indicate that the reciprocal relationship between sAPPα and Aβ described in numerous cell culture systems (*i.e.* see Refs. 81–83) does not appear to extend to the cholesterol effects on these APP processing derivatives *in vivo*.

Another possible mechanism to explain the cholesterol effects on secreted APP and Aβ that needs to be considered is increased clearance of these fragments from the brain. ApoE-lipoprotein complexes are taken up by both astrocytes and neurons as a means of delivering cholesterol to cells (25, 84, 85). This uptake is mediated by the LDL, LDL receptor-related protein, and very low density lipoprotein receptors in the brain (74, 86, 87). It has also been shown that apoE-enriched lipoproteins bind Aβ and that the LDL receptor mediates uptake of these complexes into primary neurons and astrocytes (88). Similarly, the LDL receptor-related protein receptor mediates uptake and degradation of secreted APP from the extracellular space (86). Based on these observations, it is possible that reduced brain levels of sAPPα, sAPPβ, and Aβ, all secretory products of APP processing, may result at least in part from increased clearance triggered by increased brain apoE and/or cholesterol levels. However, this mechanism may be less likely, given that the cholesterol-mediated effects on sAPPα have been reported in cells cultured in serum-free conditions (50), where clearance systems are presumably not present.

A cholesterol-cell membrane effect on APP processing or trafficking and secretion is supported by examining secreted APP derivatives in APP gene-targeted mice also harboring the apoE knockout locus. Despite a genetic predisposition to elevated serum cholesterol in APP gene-targeted mice lacking apoE, no alterations in levels of sAPPα, sAPPβ, or Aβ1–40 and Aβ1–42 were found compared with APP gene-targeted mice, wild-type for apoE. Similarly, although absence of apoE has been shown to dramatically reduce amyloid deposition in transgenic PDAPP mice, no differences in APP processing to Aβ could be attributed to absence of apoE (89). Although we cannot discount the possibility that changes in lipid metabolism that may occur in the apoE KO mice, other than elevated serum cholesterol, could impact APP processing in the brain, these results support the hypothesis that apoE and the cellular uptake of the apoE-cholesterol complex are required to elicit the observed changes in the modulation of secreted APP derivatives in brain. An important distinction in this hypothesis to be emphasized is that despite very high levels of serum cholesterol attained in the apoE KO mice, an effect on cellular processing of APP would not be expected. The absence of apoE- lipoprotein complexes in brain would be predicted to severely impact the ability of brain cells to take up exogenous sources of cholesterol.

Elucidating the mechanism of changes in cholesterol to APP metabolism, amyloid deposition, and other pathological features observed in the AD brain will be important. Our results demonstrate a negative relationship between cholesterol levels and brain apoE levels to secreted APP processing fragments including Aβ1–40 and Aβ1–42. It is therefore conceivable that low steady-state apoE levels that have been documented in cerebrospinal fluid, plasma, and brains of individuals with at least one apoE4 allele (59, 70–73) may result in elevations in Aβ1–40 and Aβ1–42. To our knowledge, no study has been reported documenting the effects of apoE genotype on brain Aβ levels in the undiseased brain. Transgenic mice that express exclusively apoE2, -E3, or -E4 (90) provide mouse model systems to address the relationship of both cholesterol and apoE genotype to Aβ levels and potentially the progression of AD-like pathologies in the brain.

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