ACAT1 Gene Ablation Increases 24(S)-Hydroxycholesterol Content in the Brain and Ameliorates Amyloid Pathology in Mice with AD

Elena Y. Bryleva
Dartmouth College

Maximillian A. Rogers
Dartmouth College

Catherine C. Y. Chang
Dartmouth College

Floyd Buen
Dartmouth College

Follow this and additional works at: https://digitalcommons.dartmouth.edu/facoa

Part of the Medical Genetics Commons, Medical Neurobiology Commons, and the Medical Pathology Commons

Recommended Citation
Bryleva, Elena Y.; Rogers, Maximillian A.; Chang, Catherine C. Y.; and Buen, Floyd, "ACAT1 Gene Ablation Increases 24(S)-Hydroxycholesterol Content in the Brain and Ameliorates Amyloid Pathology in Mice with AD" (2010). Open Dartmouth: Faculty Open Access Articles. 1489.
https://digitalcommons.dartmouth.edu/facoa/1489

This Article is brought to you for free and open access by Dartmouth Digital Commons. It has been accepted for inclusion in Open Dartmouth: Faculty Open Access Articles by an authorized administrator of Dartmouth Digital Commons. For more information, please contact dartmouthdigitalcommons@groups.dartmouth.edu.
ACAT1 gene ablation increases 24(S)-hydroxycholesterol content in the brain and ameliorates amyloid pathology in mice with AD

Elena Y. Blyleva a,1, Maximillian A. Rogers b,1, Catherine C.Y. Chang a, Floyd Buen a, Brent T. Harris c, Estelle Rousselet c, Nabil G. Seidah a, Salvatore Oddo d, Frank M. LaFerla e, Thomas A. Spencer f, William F. Hickey b, and Ta-Yuan Chang a,2

aDepartment of Biochemistry, Dartmouth Medical School, Hanover, NH 03755; bDepartment of Pathology, Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756; cLaboratory of Biochemical Neuroendocrinology, Institut de Recherches Cliniques de Montréal, Montreal, QC H2W 1R7, Canada; dDepartment of Physiology, University of Texas Health Science Center, San Antonio, TX 78229-3900; eDepartment of Neurobiology and Behavior, University of California, Irvine, CA 92697; and fDepartment of Chemistry, Dartmouth College, Hanover, NH 03755

Communicated by P. Roy Vagelos, Bedminster, NJ, December 9, 2009 (received for review September 30, 2009)

Cholesterol metabolism has been implicated in the pathogenesis of several neurodegenerative diseases, including the abnormal accumulation of amyloid-β, one of the pathological hallmarks of Alzheimer disease. Acyl-CoA:cholesterol acyltransferases (ACAT1 and ACAT2) are two enzymes that convert free cholesterol to cholesteryl esters. ACAT inhibitors have recently emerged as promising drug candidates for AD therapy. However, how ACAT inhibitors act in the brain has so far remained unclear. Here we show that ACAT1 is the major functional isoenzyme in the mouse brain. ACAT1 gene ablation (A1−) in triple transgenic (i.e., 3XTg-AD) mice leads to more than 60% reduction in full-length human APPsw as well as its proteolytic fragments, and ameliorates cognitive deficits. At 4 months of age, A1− causes a 32% content increase in 24-hydroxycholesterol (24SOH), the major oxysterol in the brain. It also causes a 65% protein content decrease in HMG-CoA reductase (HMGR) and a 28% decrease in sterol synthesis rate in AD mouse brains. In hippocampal neurons, A1− causes an increase in the 24SOH synthesis rate; treating hippocampal neuronal cells with 24SOH causes rapid declines in hAPP and in HMGR protein levels. A model is provided to explain our findings: in neurons, A1− causes increases in cholesteryl and 24SOH contents in the endoplasmic reticulum, which cause reductions in hAPP and HMGR protein contents and lead to amelioration of amyloid pathology. Our study supports the potential of ACAT1 as a therapeutic target for treating certain forms of AD.

Alzheimer disease | cholesterol esterification | lipid metabolism | oxysterols

ACAT Expression in Mouse Brains. Whether the brain has active ACAT enzyme was previously unknown. To examine this issue, we prepared brain homogenates from WT, Acat1−/− (A1−) and Acat2−/− (A2−) mice and found that WT and A2− mouse brains contained comparable ACAT enzyme activity, whereas A1− mouse brains contained negligible activity (Fig. L4). Various regions prepared from WT mouse brains, but not A1− mouse brains, all contained active ACAT (Fig. IB). Mouse ACAT1 is a 46-kDa protein (22). Immunoblot analysis showed that, in homogenates prepared from mouse brain (but not from other mouse tissues), a non-ACAT1 protein band appeared in the 46-kDa region; the presence of this nonspecific band precluded us from using immunoblotting or histochemical staining to identify ACAT1 in the mouse brain. To unambiguously identify the ACAT1 protein, we first performed immunoprecipitation (IP) experiments with detergent-solubilized WT mouse brain extracts. The results showed that ACAT activity could be efficiently immunodepleted by ACAT1-specific antibodies (i.e., A1), but not by control antibodies (Fig. IC). We then performed immunoblotting on the immunoprecipitates; the result showed that, in homogenates from WT mouse brain regions, the ACAT1 antibody specifically identified a 46-kDa protein band; this band is absent in homogenates prepared from the adrenals and brains of A1− mice (Fig. 1D). These results indicate that mouse brains do express ACAT1 as the major ACAT isoenzyme.


The authors declare no conflict of interest.

1E.Y.B. and M.A.R. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: ta-yuan.chang@dartmouth.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0913828107/DCSupplemental.
To determine the ACAT1 mRNA distribution in mouse brains, we performed in situ hybridization experiments. Both hippocampus and cortex contain ACAT1 mRNA; hippocampus expresses a stronger signal (Fig. 1E). Other ACAT1-positive regions included choroid plexus, medial habenular nucleus, amygdala, and rostral extension of the olfactory peduncle. We next isolated hippocampus-rich regions and cortex-rich regions from WT mice and compared their ACAT1 mRNA levels by real-time PCR (primer sequences are listed in Table S1). The result showed that ACAT1 mRNA is approximately twofold higher in hippocampus than in cortex (Fig. 1F). A separate RT-PCR experiment using ACAT2-specific primers showed that only the thalamus-rich region, and no other brain regions, expresses low but detectable ACAT2 mRNA levels (Fig. S1A), confirming an early report by Anderson et al. (23), who showed in monkey brains that the ACAT2 mRNA level was nearly undetectable.

**Effect of A1− on Apβ Deposition/hAPPsw Processing and on hTau.** To investigate the effect of inactivating ACAT1 on amyloid and tau pathologies in the triple transgenic (3XTg)-AD mice (24), we produced Acat+/−/AD (A1−/−/AD) mice by crossing the Acat+/− mice with the 3XTg-AD mice. The breeding scheme is described in Fig. S2. To examine the effect of A1− on amyloid pathology, we first used the human specific anti-Aβ antibody 6E10 to perform intraneuronal immunostaining in the CA1 region of hippocampi of 4-month-old mice. Results showed that the staining was significantly diminished (by approximately 78%) in the A1+/−/AD mice (Fig. 2A). We next used ELISA to measure the total Aβ40 and Aβ42 levels in mouse brain homogenates at 17 months of age. Results showed that the Aβ42 levels were significantly decreased (by approximately 78%) in A1+/−/AD mice; the Aβ40 levels were also decreased, but the difference observed was not statistically significant. The brains of nontransgenic (NTG) mice did not contain measurable Aβ (Fig. 2B). We next used thiocyanate S to stain amyloid plaques in AD mouse brains at 17 months of age. The results showed that, in A1+/−/AD mice, the amyloid plaque load in the hippocampus was significantly reduced (by approximately 77%; Fig. 2C); in the cortex, the amyloid plaque load in these mice showed a trend toward decreasing (P = 0.17; Fig. S1B).

We next studied the effect of A1− on human APP processing in 4-month-old AD mice. We used the antibody 6E10 to detect full-length APP [human APP harboring the Swedish mutation (hAPPsw)] and its proteolytic fragments sAPPα (soluble APP fragment produced by α-secretase cleavage [hAPPα]) and CTFβ (C-terminal APP fragment produced by β-secretase cleavage [hCTFβ]). The results showed that, in A1+/−/AD mice, hAPPα and hCTFβ levels were decreased (by approximately 67% and 37%, respectively; Fig. 3A, C, and D). To our surprise, the hAPP level was also significantly reduced (by approximately 62%; Fig. 3A and B). In contrast to the hAPP protein levels, there was no difference in hAPP mRNA levels between the A1+/−/AD mice and the A1+/−AD mice (Fig. 3E) (primer sequences are listed in Table S1). hAPP is synthesized in the ER in its immature form (with a molecular weight of approximately 105 kDa); the immature form moves from the ER to the Golgi via the secretory pathway (25) and becomes highly glycosylated (mature form has a molecular weight of approximately 115 kDa) (26, 27). We examined the effects of A1− on the levels of immature and mature forms of hAPP in young AD mice (at 25 d of age). The results showed that A1− led to decrease in both forms to approximately the same extent (by approximately 52%–54%; Fig. 3F–I), suggesting that the effect(s) of A1− act on newly synthesized hAPP. The AD mice express both hAPP and endogenous (mouse) APP (mAPP). To test the possibility that A1− may affect both the...
hAPP and the mAPP levels, we used a different antibody (antiserum 369), which recognizes the C-terminal fragments of both hAPP and mAPP (28) to investigate the total APP levels in AD mice. The results showed that there was no detectable difference in the total APP levels among NTG, A1+/AD, and A1−/AD mice (Fig. 3I), indicating that, in our AD mouse strain, the hAPP is not overexpressed compared with the endogenous mouse APP protein level. We also examined the mAPP processing in mice that do not contain the hAPP gene. In these mice, A1− also did not affect the levels of mAPP and its homologue APLP2 (29), or any of the proteolytic fragments derived from mAPP (Fig. 3K). These results led us to conclude that A1− leads to reduction in only the hAPP level, not the mAPP level. Subtle sequence differences exist between hAPP and mAPP, and these differences may play important roles in causing differential fates of hAPP and mAPP (30, 31). We investigated the effect of A1− on mutant human tau (htau) in 3XTg-AD mice. The results showed that, at 17 months of age, no significant change was observed in the number of hippocampal neurofilibrillary tangles between the A1+/AD and the A1−/AD mice, suggesting that A1− may not lead to tau pathology attenuation in AD mice.

A1− Ameliorates Cognitive Deficits of AD Mice. (SI Results, Fig. S3). Effects of A1− on sterol metabolism in AD mouse brains. A1− is involved in cellular cholesterol homeostasis. We hypothesized that A1− may cause a decrease in hAPP content by affecting sterol metabolism in AD mouse brains. To test this possibility, we isolated the sterol fractions from A1+/AD and A1−/AD mouse brains and analyzed them by GC/MS. The results showed that, at 4 months of age, lack of A1 caused an approximate 13% decrease in cholesterol content (Fig. 4A; P = 0.04) and an approximate 32% increase in 24SOH content per wet weight tissue (Fig. 4B; P = 0.007). The decrease in cholesterol content of the A1+/AD mouse brains was confirmed when a colorimetric enzyme assay kit (Wako) was employed to determine free cholesterol. The lanosterol or desmosterol contents per milligram tissue in the A1+/AD and A1−/AD mouse brains did not change significantly (Fig. 4B). Additional results showed that a lack of A1 caused an approximately 10% decrease in cholesterol content and an approximate 23% increase in 24SOH content in the 2-month-old AD mouse brains. We next compared the relative sterol synthesis and fatty acid synthesis rates in the brains of these mice in vivo. The results showed that A1− caused an approximate 28% decrease in the sterol synthesis rate (Fig. 4C; P = 0.04) without significantly changing the fatty acid synthesis rate (Fig. 4D). In mouse brains, CE contents are reported to be very low (32). We attempted to measure CE in A1+ mouse brains by separating the CE fraction from the free cholesterol fraction using column chromatography.
and determining the cholesterol content in CE by GC/MS after CE is saponified. The result suggested that CE might be present at no more than 1% of the total cholesterol mass in mouse brains. The low level of CE prevented us from reliably measuring a value. We used a similar procedure to determine the 24SOH ester content, and estimated that no more than 1% of total 24SOH is esterified in the brain. These results are consistent with the finding that ACAT prefers to use cholesterol to various oxysterols as its enzymatic substrate (33).

To test the functionality of ACAT1 in the intact mouse brain, we developed a procedure to measure CE synthesis in vivo by injecting [3H]-labeled cholesterol as a cyclodextrin complex into intact mouse brains. We monitored the [3H]-CE produced in A1+ and A1− mice 3 h after injection. The result showed that, in A1+/AD mice, a small percentage of [3H] cholesterol was converted to [3H] CE (0.56% in 3 h); in contrast, such conversion was not detectable in the A1−/AD mouse brains (Fig. 4E). This result demonstrates that ACAT1 in intact mouse brains does biosynthesize CE, although at a low rate.

The data in Fig. 4A−C suggest that, in AD mouse brains, A1− leads to an increased 24SOH level, which in turn leads to a down-regulation of the sterol synthesis rate. Studies in cell culture have suggested that 24SOH may down-regulate sterol synthesis by two mechanisms: (i) blocking transcriptional activations of SREBP2 target genes and/or (ii) increasing the degradation rate of HMGR protein (38). To test the first possibility, we compared the mRNA levels of various SREBP2 target genes in the A1+/AD and A1−/AD mouse brains, but we failed to detect statistically significant alterations in the expression levels of these genes (Fig. 4F) (primer sequences are listed in Table S2). Additional results showed that no statistically significant alterations in the mRNA levels of various LXR target genes occurred in the brains of mice with or without A1 (Fig. 4G) (primer sequences are listed in Table S2). To test the second possibility, we performed immunoblot analysis in brain homogenates prepared from the AD mice with or without A1. The result showed that the HMGR protein content is decreased by approximately 65% in A1−/AD mouse brains (Fig. 4F and G; P = 0.0009), whereas the HMGR mRNA in A1− mouse brains was not changed (Fig. 4H). Additional results showed that, in AD mice at 25 d of age, A1− caused an approximate 62% decrease in HMGR protein content, demonstrating that the effect of A1− on HMGR content occurs in mice at a young age.

**Biosynthesis of 24SOH in Hippocampal Neuronal Cell Cultures.** The results described here show that A1−/AD mouse brains exhibit elevated 24SOH levels, suggesting that, in mouse neurons, A1− may cause an increase in the biosynthesis of 24SOH. Cultured neurons isolated from human and mouse brains synthesize and secrete 24SOH (13, 34). Based on these reports, we established a hippocampal-rich neuronal cell culture system from A1+/AD and A1−/AD mice to test this possibility. We first monitored CE biosynthesis in these neurons by incubating them with labeled [3H] oleic acid. Upon entering cells, [3H] oleic acid is rapidly converted to [3H] CE by ACAT. Both the A1+ cells and the A1− cells biosynthesize CE; however, A1− cells synthesize [3H] CE at much reduced capacity than A1+ cells (Fig. 5A). We next examined the effect of A1− on 24SOH biosynthesis by feeding neurons with the sterol precursor [3H] acetate for 3 h, then isolated and analyzed the labeled sterols present in the cells and in the media. The results showed that A1− cells exhibited a reduced trend in cholesterol synthesis rate; the difference observed between A1+ cells and A1− cells approached but did not reach statistical significance (P = 0.05; Fig. 5B Right). The 24SOH synthesis rate in A1− cells was significantly increased (by approximately 27%; Fig. 5C Right). We also analyzed the [3H] sterols in the media of A1+ and A1− cells. The result showed that the [3H] cholesterol contents were not significantly different (Fig. 5B Left); in contrast, the [3H] 24SOH content in the media of A1− cells was significantly (approximately 56%) higher than in A1+ cells (Fig. 5C Left). We calculated the percentage of total [3H] sterols secreted into the media, and found that neurons secreted only approximately 2% of total [3H] cholesterol (Fig. 5D Left), but secreted 13% to 15% of total [3H] 24SOH into the media (Fig. 5D Right).

The results described earlier (Fig. 5C) demonstrate that A1− causes an increased 24SOH biosynthesis rate in cultured neurons. Mouse neurons maintained in culture express CYP46A1 as a single 53-kDa protein, which can be identified by immunoblotting (13). It is possible that the increased synthesis of 24SOH observed in A1− neurons may be a result of an increase in CYP46A1 protein content in these neurons. To test this possibility, we examined CYP46A1 protein content in A1+ and A1− neurons by immunoblotting. The results showed that the intensities of the single 53-kDa protein band were comparable between these two cell types (Fig. 5E). This result suggests that, in hippocampal neurons, the mechanism(s) involved in A1−-dependent increase in 24SOH synthesis may not require an increase in CYP46A1 protein content.

**24SOH Provided to AD Mouse Neurons Decreases hAPP Protein Content.** The observations made in intact A1−/AD mouse brains [i.e., an increase in 24SOH content (Fig. 4B) and a decrease in hAPP content (Fig. 3A, B, and F)] suggest that 24SOH may decrease hAPP content in neurons. To test this possibility, we treated hippocampal neuronal culture from A1+/AD mice with 24SOH, and monitored the hAPP protein content and the HMGR protein content in parallel. We found that 1 μM 24SOH rapidly decreased the protein contents of both hAPP and HMGR (within 3 h; Fig. 5F). A separate experiment showed that 1 to 5 μM 24SOH causes a rapid decrease in hAPP protein content (Fig. 5G) without affecting its mRNA level (Fig. 5H) (primer sequences are listed in Table S1). This result
supports the interpretation that accumulation of 24SOH in neurons may down-regulate hAPP protein content in vivo.

Discussion

Earlier work showed that when the ACAT inhibitor CP113818 or CI-1011 was administered to mice with AD, it significantly reduced amyloid plaques and rescued cognitive deficits, suggesting that inhibiting ACAT may prevent and/or slow the progression of AD (20, 35). The present work supports this hypothesis. However, close comparison revealed that several important differences exist between the effects of the ACAT inhibitors and the effects of A1–

CP113818 inhibited the processing of both human APP and mouse APP; CI-1011 decreased the mature/immature ratio of hAPP. In contrast, A1– causes a decrease in only the full-length human APP protein content; it does not affect the mouse APP at any level, and it does not alter the mature/immature ratio of hAPP. In addition, unlike the effect of A1–, CP113818 did not cause a reduction in the full-length hAPP content (20). The differences in results raise questions about the specificity of the ACAT inhibitors employed. ACAT is a member of the membrane bound O-acyltransferase enzyme family (36), which comprises 16 enzymes with similar substrate specificity and catalytic mechanisms, but with diverse biological functions. In addition, many ACAT inhibitors are hydrophobic, membrane-active molecules (37). When administered to cells, they may partition into membranes at high concentration and perturb membrane properties nonspecifically. Although CP113818 and CI-1011 are designated as ACAT inhibitors, they may also inhibit other enzymes in the membrane bound O-acyltransferase enzyme family and/or interfere with other biological processes. Our present work shows that inactivating the ACAT1 gene alone is sufficient to ameliorate amyloid pathology, at least in the 3XTg-AD mouse model. In this mouse model, A1– acts to reduce Aβ load mainly by reducing the hAPP protein content. The action of A1– is similar to that of cerebrolysin, which might lead to an 24SOH content decrease (38, 39). To explain how A1– leads to hAPP content reduction, we show that the brains of A1–/AD mice contain a significantly greater amount of 24SOH. We then demonstrate that, in neuron-rich cultures, 24SOH added to the medium leads to rapid decrease in hAPP protein content. How 24SOH acts on hAPP is currently unknown. APP may be a sterol-sensing protein (40); APP contains three CRAC motifs, a consensus motif known to bind cholesterol (41). It is possible that cholesterol and/or oxysterol may directly interact with the hAPP protein to accelerate its rate of degradation. Other possibilities cannot be excluded. The AD mice used in our current study express a mutant form of hAPP. Further investigations are required to determine whether A1– also leads to decreases in nonmutated hAPP. We also show that, in mouse brains, A1– causes a decrease in HMGR protein and a decrease in cholesterol biosynthesis. Earlier, Tabas et al. (42) and Sheck et al. (43), showed that inhibiting ACAT in macrophages or in CHO cells increases the ER “regulatory sterol pool” that mediates down-regulation of HMGR levels and SREBP processing. The “regulatory sterol” could be cholesterol itself and/or an oxysterol derived from cholesterol; however, whether oxysterol(s) play(s) important roles in regulating sterol biosynthesis in the brain in vivo is currently debated, as reviewed by Björkhem et al. (44).

Materials and Methods

Generation of Acat1−/−/AD (A1−/−/AD) and Acat2−/−/AD (A2−/−/AD) Mice. The Acat1−/− and Acat2−/− mice (53, 54) in C57BL/6 background were received from Sergio Fazio (Nashville, TN) and Shailesh Patel (Charleston, SC), respectively. The 3XTg-AD mice (AD mice) in hybrid 129C57BL/6 background contain two mutant human transgenes, hAPP harboring Swedish mutation (hAAPPsw), and mutant htau (htauP301L), and contain the knock-in mutant presenilin 1 (PS1M146V) (55). Breeding strategy is described in SI Text.

Statistical Analysis. Statistical comparisons were made using two-tailed, unpaired Student t test. The difference was considered significant when the P value was less than 0.05.

Acknowledgments. We thank Drs. Robert Leaton, Yasuomi Urano, Gregory Holmes, Lorenzo Sampere, Scott Howell, Yohei Shibuya, and Paul Huang at Dartmouth University; Barbara Tate and Jim Harwood at Pfizer (New York, NY); Gregory Brewer at Southern Illinois University; Chunjiang Yu at University of Illinois Medical Center; and Irene Cheng at National Yang-Ming University (Taiwan, ROC) for advice during the course of this work. We also thank Stephanie Murphy for careful editing of the manuscript. The laboratory of T.Y.C. is supported by National Institutes of Health grant HL060306. The laboratory of N.G.S. is funded by Canadian Institutes of Health Research grants MOP 44363 and MOP 36496.