ALTERED CHOLESTEROL ESTER CYCLE IN *EX VIVO* SKIN FIBROBLASTS FROM ALZHEIMER PATIENTS.

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Nature Precedings : hdl:10101/npre.2008.1602.1 : Posted 18 Feb 2008

Recent studies in both animal and cell models of Alzheimer's disease (AD) indicated that sub-cellular cholesterol distribution seems to regulate amyloid-beta (Aβ) generation in the brain. In particular, cholesterol-esters (CE), rather than total cholesterol levels, appear directly correlated with Aβ production. Here we observed that, similarly to brain cells, skin fibroblasts obtained from AD patients produce and accumulate more CE than skin fibroblasts from age-matched healthy controls do. AD fibroblasts also exhibited a 2 fold increase in the expression of ACAT1, in addition to lower levels of SREBP2, nCEH, Caveolin-1 and ABCA1 mRNA levels, all of which are involved in the CE cycle. HMGCoA-reductase and LDL-receptor mRNAs levels did not show statistically significant changes in AD, compared to non-AD, cells. Furthermore, although APP mRNA did not significantly vary, neprilysin (NEP), the most important enzyme in the proteolysis of Aβ, was expressed at very low levels in skin fibroblasts of sporadic AD patients. Our results contribute to the concept that AD may be the consequence of a basic and systemic defect in the CE cycle. Moreover, our results identify new possible targets for the diagnosis, prevention, and cure or, at least, amelioration of the symptoms of AD.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory deficit, cognitive impairment, and personality changes accompanied by specific structural abnormalities in the brain[1]. Deposition of amyloid-beta (Aβ) peptide, generated by proteolytic cleavage of amyloid precursor protein (APP), is a consistent feature of the brains of patients affected by AD[2]. Normally, APP follows the constitutive non-amyloidogenic pathway via α-secretase releasing the ectodomain (soluble APP, sAPP) in the extracellular space, but it can also undergo an amyloidogenic processing via β-site cleaving enzyme (BACE1) leading to Aβ

formation[3]. The cellular mechanism that moves APP towards the pathological amyloidogenic pathway is still unclear. Recent studies in both animal and cellular models of AD indicated that sub-cellular cholesterol distribution seems to regulate Aβ generation in the brain[4, 5]. In particular, cholesterol-esters (CE), rather than total cholesterol levels, appear directly correlated with Aβ production[6, 7]. It has been suggested that acyl-coenzyme A: cholesterol acyltransferase (ACAT), the enzyme that catalyses the formation of CE, modulates the generation of Aβ through a tight control of the equilibrium between free cholesterol (FC) and CE pools. Inhibitors of ACAT, developed for the treatment of atherosclerosis, were also found to be potent modulators of Aβ generation in the brain[7, 8]. These studies indicate that CE metabolism plays an integral part in the pathogenesis of AD, and suggests that genes that regulate lipid metabolism could also be implicated.

Our previous studies in ex vivo cultures of peripheral blood mononuclear cells (PBMCs) and skin fibroblasts from AD subjects revealed an unbalance in the CE vs. FC pool, similarly to that found in AD brains[9]. This result, confirming the link between AD and CE, supports the view that AD should be considered a systemic disorder, and that fibroblasts and other peripheral cells may represent an easy source of biological material for use in investigations on the mechanisms leading to amyloid generation and deposition.

By utilizing skin fibroblast cultures from AD subjects and age-matched controls (non-AD subjects), herein we analyze the synthesis and content of CE, as well as the mRNA levels of the major proteins involved in intracellular cholesterol homeostasis; namely: i) low density lipoprotein receptor, LDLR (cholesterol uptake); ii) hydroxy-methyl-glutaryl coenzyme A reductase, HMGCoAR (cholesterol neosynthesis); iii) caveolin-1, Cav-1, and ATP binding cassette-A, ABCA1 (cholesterol trafficking); iii) ACAT1, and neutral cholesterol ester hydrolase, nCEH (cholesterol ester cycle); and iv) sterol regulatory element binding protein-2, SREBP2 (cholesterol homeostatic element). The gene expression of some proteins involved in Aβ production (i.e. APP and BACE1) and degradation (i.e. neprylisin) were also investigated. We found that AD fibroblasts displayed marked changes in their ability to control intracellular CE levels, and that this defect was associated with altered APP metabolism. We propose that variations in the intracellular CE pool might favour Aβ deposition and AD progression by triggering a permanent disturbance of the delicate equilibrium between amyloid formation and its catabolism.

RESULTS

Cholesterol esterification potential is higher in AD, compared to non-AD, skin fibroblasts.

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We initially determined the extent of cholesterol esterification by measuring the incorporation of [^{14}C]oleate into neutral lipids in AD and non-AD skin fibroblasts. The synthesis of CE was higher in AD than in non-AD cells at all time points considered (Fig. 1). This increased synthesis was not accompanied by a parallel rise in the incorporation of radioactivity into triacylglycerol (TG), thus indicating that cholesterol esterification was the process specifically affected (data not shown).

In AD cells, the greater amount of cholesterol ester synthesis was associated with an expanded cytoplasmic pool of neutral lipid droplets, as revealed by Oil red-O (ORO) staining (Fig. 2a). The fact that three different inhibitors of cholesterol esterification, 40-O-(2-hydroxyethyl) rapamycin, everolimus (EVE, 20 nM); progesterone (PG, 10 μM), and the acyl amide ACAT inhibitor (SaH, 4 μM), were able to reduce the accumulation of lipid droplets in these cells, implied that CE was the primary neutral lipid in the cells of AD subjects. Interestingly, non-AD cells were not only less sensitive to the inhibitory effects of the cholesterol esterification inhibitors (not shown), but they also displayed very low basal (time 0) levels of CE (Fig. 2b). This suggests, as originally proposed by Goldstein and Brown[10], that under physiologic conditions intracellular CE levels are maintained low by cyclic cholesterol ester hydrolysis and ester re-synthesis (CE cycle), and indicates that an impairment in this regulatory pathway may be responsible for abnormal CE accumulation in AD cells. The accrual of lipid droplets increased with time, in both AD and non-AD cells, following growth stimulation of the cultures with FCS (Fig. 2a and 2b). Although this behavior was expected as a response to proliferative induction[11, 12], the effect was significantly more pronounced in AD cells. These data support the belief that cholesterol regulatory mechanisms in AD patients are altered not only in the CNS, but also in peripheral cells. In addition, they suggest that these alterations may be, at least partially, due to changes in the CE cycle. Accordingly, we completed densitometric evaluations of the ACAT-1 bands generated by Western blot analyses (Fig. 2c) and showed a statistically significant change between skin fibroblasts from non-AD and AD subjects; the relative expression of ACAT1 was greater than 50% higher in the AD fibroblast samples (Fig. 2d).

Expression levels of genes involved in cholesterol homeostasis in AD and non-AD fibroblasts.

The cycle of cholesterol esterification and hydrolysis represents the major short-term buffering of cholesterol levels in cells[13]. Although the above results unequivocally confirm that in AD patients this regulatory cycle is altered, the molecular mechanism/s and factor/s involved are yet to be determined. Therefore, we analyzed the mRNA levels of major proteins involved in intracellular cholesterol homeostasis; namely: i) LDLR (cholesterol uptake); ii) HMGCoAR (cholesterol

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neosynthesis); iii) Cav-1, and ABCA1 (cholesterol trafficking); iv) ACAT1 (cholesterol esterification); v) nCEH (cholesterol ester hydrolysis); and vi) SREBP2 (cholesterol homeostatic element). As expected, ACAT1 mRNA levels were higher in AD compared to non-AD fibroblasts (Fig. 3a and 3b). In contrast, nCEH and ABCA1 mRNA levels were significantly reduced in AD cells. Alzheimer's disease cells also exhibited lower levels of Cav1 mRNA, although not statistically significantly so (Fig. 3a and 3b). The expression levels of LDLR and HMCOAR mRNA were similar, but the expression of SREBP2 mRNA was decreased by 2.5-fold in AD, compared to non-AD, fibroblasts (Fig. 4a and 4b).

Expression levels of genes involved in Aβ production and catabolism in AD and non-AD fibroblasts.

Changes in proteins involved in the CE cycle were associated with parallel variations in proteins that regulate Aβ production and catabolism. Although mRNA levels of APP did not significantly change in the AD, compared to the non-AD, cells, lower neprylisin (NEP) mRNA levels were found in the AD fibroblasts. Unexpectedly, AD cells exhibited very low mRNA levels of BACE1 (Fig. 5a and 5b). Overall, these data establish that the accumulation of CE in AD cells likely depends on an increase in the cellular rate of cholesterol esterification coupled with a decrease in CE hydrolysis. In addition, they also suggest that increased amounts of CE may influence Aβ aggregation by down-regulating the levels of neprylisin that is the most important enzyme in the proteolysis of Aβ[14].

DISCUSSION

Alzheimer's disease is the most common form of age-related dementia. The social and economic consequences of this neurodegenerative disease are devastating, thus it is crucial that strategies to prevent or delay the onset of AD are developed. Brain deposition of Aβ aggregates into insoluble fibrils, considered the key event in AD pathogenesis, is a slow, continuous and gradual process. Many studies over the last decade have provided abundant information about structural and biochemical co-factors favoring Aβ aggregation in AD patients[15, 16]. Among these, changes in neuronal membrane cholesterol levels and/or in sub-cellular cholesterol distribution have received particular attention. However, no consensus arises on how defects in cholesterol homeostasis relate to AD[4, 15, 17]. Cellular cholesterol is present either as FC in the membrane or stored as CE in the form of cytoplasmic droplets. It is thought that CE in cytoplasmic droplets serves as storage and for synthesis-activating purposes. The importance of CE in the control of Aβ levels was first suggested by Puglielli and coworkers[6, 7] who, working with cells carrying mutations that either increased or decreased the formation of CE, showed that Aβ production correlated with the CE levels, rather

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than with total cholesterol. Consistent with this, they also found that compounds that inhibit ACAT, also inhibit the release of Aβ. Given that Aβ is generated in the membrane domains rich in FC (lipid rafts)[17], and that a consequence of ACAT inhibition is an additional FC enrichment of membranes[18], it has been proposed that CE may regulate Aβ production by modulating the amount of FC in nervous cell membranes. In an attempt to broaden the view of the relationship between CE and AD, we hypothesized that, similarly to other metabolic and biochemical alterations found in AD patients, changes in cholesterol metabolism, rather than being confined to the brain tissue, might be systemic. Here we observed that, like brain cells, skin fibroblasts obtained from AD patients[19, 20] produce and accumulate more lipid droplets than do skin fibroblasts from age-matched healthy controls (non-AD cells). Furthermore, we found that treating these cells with drugs targeting cholesterol esterification at various levels strongly inhibits the accumulation of lipid droplets, indicating that CE is the primary neutral lipid found in AD fibroblasts. Considering that a similar lipid pattern also characterizes AD-PBMCs[9], it is conceivable that the lipid droplet increase in peripheral cells of AD subjects may be indicative of a systemic alteration of intracellular cholesterol homeostasis. In the brain this alteration likely creates a cellular milieu favorable to the production and/or aggregation of Aβ peptides. However, the molecular mechanisms by which CE influences Aβ peptides to undergo extracellular aggregation remain to be established. It is well understood that changes in the organization of lipids can have profound effects on cellular functions such as signal transduction and membrane trafficking[21, 22]. Cholesterol is one of the most important regulators of lipid organization, and cells have developed a sophisticated and complex network of mechanisms to maintain cholesterol within controlled ranges[10] (see Fig. 6a); the cycle of cholesterol esterification and hydrolysis is considered the major short-term buffering of cell cholesterol. The proteolysis of APP yielding Aβ peptides is a normal process observed in multiple cell types, including fibroblasts[16]. It has been reported that Aβ peptides are produced at physiological levels if cells preserve their cholesterol homeostatic mechanisms, thus an altered balance of intracellular cholesterol pools (FC vs. CE), as indicated by the lipid droplet swelling in AD patients, may be responsible, at least in part, for the abnormal production of Aβ aggregates in these patients. Given this, an alteration in cholesterol levels, either an increase or decrease, would be sufficient to induce changes in membrane proteins' compartmentalization, thereby affecting the normal segregation of APP and its secretases[23]. We report herein that lipid droplet accumulation in AD fibroblast cultures was associated with changes in messenger RNA synthesis of proteins related to the regulation of cholesterol metabolism. In particular, very low levels of SREBP2 and nCEH were observed. Levels of ABCA1 and caveolin-1 mRNA were also reduced, whereas HMGCoAR and LDLR mRNAs remained similar in AD and non-AD cells. In light of these results, it seems likely that the

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accumulation of lipid droplets in AD cells primarily depends on an altered cholesterol ester cycle, rather than on increased *de novo* cholesterol synthesis or uptake. Changes in cholesterol ester homeostasis that result in reduced transport of FC to the membrane may alter the structure and functional integrity of cholesterol–rich membrane micro-domains (rafts) and, consequently, the activity of the raft-resident proteins, including those involved in APP processing. Accordingly, even if APP mRNA did not significantly vary, mRNA neprilysin, an enzyme critical to the proteolysis of Aβ, was found to be lower in AD than in non-AD cells. BACE1 was expressed at very low levels in skin fibroblasts of AD patients. This finding, although seemingly paradoxical, is not surprising because BACE-1 mRNA has never been found to be elevated in the brains of AD patients[24]. To explain this discrepancy, it has been proposed that BACE-1 expression is controlled at the translational level. However, it is important to remember that AD is a progressive disorder, and that cerebral deposition of Aβ precedes symptoms of dementia by years, or even decades. Thus, the possibility that, year after year, aberrant accumulation of Aβ could decrease the rate of enzyme synthesis at the level of gene expression cannot be excluded. In this respect, it is worth noting that the fibroblasts used in this study were derived from subjects with a probable diagnosis of sporadic AD since at least five years. A schematic model of the molecular mechanisms by which lipid droplet accumulation may promote AD generation and progression is presented in Fig. 6b.

To our knowledge, these findings are the first *ex vivo* demonstration that peculiar alterations in CE cycle and APP metabolism characterize peripheral tissues of AD patients as well as those of the SNC. The emerging picture is that systemic chronic alterations of cholesterol ester metabolism, by reducing FC transport to plasma-membranes, may lead to modifications in distribution and/or function of the major pool of raft-resident proteins, beside AD-related proteins, including: glycosylphosphatidylinositol (GPI)-linked proteins (i.e. prion), fatty-acylated proteins, receptor and cytoskeleton proteins, as well as many proteins known to be important for signal transduction[17, 21, 22]. Of recent interest, the Cu/Zn superoxide dismutase (SOD1) mutations, which are related to the familial form of amyotrophic lateral sclerosis (ALS), have been found in lipid rafts[25]. Consequently, it is likely that altered cholesterol esterification might trigger molecular events leading to pathologic processes related to each other, but not correlated. In summary, our results lend credence to the belief that AD may be the consequence of a basic and systemic defect of the CE cycle, even though not conclusively demonstrated. In addition, these findings introduce the possibilities of new molecular targets for the diagnosis, prevention, cure, or at least amelioration of, the symptoms of many human diseases that are characterized by changes in the properties or composition of raft resident molecules. Interestingly, promising results have recently been obtained with an inhibitor of ACAT that markedly reduced amyloid deposition in a mouse AD model[8]. An important challenge

for the future will be to understand how even subtle alterations in cholesterol transport and metabolism, perhaps over the span of decades, contribute to the pathogenesis of chronic diseases.

MATERIALS AND METHODS

Participants.

In this study, we took advantage of a pre-existing bank of skin fibroblasts collected as part of a project of "Ricerca Finalizzata Alzheimer", funded by the Ministero della Salute through Regione Toscana and entitled "Identification and validation of peripheral markers of altered proteolysis in AD patients". All individuals included in this study were enrolled at the Azienda USL 4, Geriatric Unit of Prato Hospital (Italy). The dermal biopsies were obtained, following informed written consent, from the upper forearm of the subjects by a 2-mm punch after local anesthesia with 2% xylocaine, according to the guidelines established and approved by the local Ethical Committee of Azienda USL 4, Prato (Italy). We analyzed fibroblasts from six non-AD controls (six females, mean age 67.0 ± 6.1 years) and six AD patients (five females, two males, mean age 73.8 ± 9.4 years).

Routine clinical and laboratory evaluation, including magnetic resonance imaging, was performed to exclude other causes of cognitive impairment. The Reisberg Global Deterioration Scale (GDS) was used to indicate the severity of the cognitive impairment in AD patients. Abnormal GDS levels start from level 3 and maximal deterioration grade corresponds to level 7. Patient evaluation included medical history, physical and neurological examinations, laboratory blood tests to rule out metabolic causes of dementia (thyroid hormones, Vitamin B12, and erythrocyte sedimentation rate), and a neuro-imaging (computed tomography and/or magnetic resonance) of the brain. In addition, all patients received neuropsychological tests, including the Mini-Mental State Examination (MMSE) (mean ±S.E. = 18.9±0.5). Patients with neoplastic or hematological disorders, recent infections or surgery, severe hepatic or renal failure, myocardial infarction or cranial trauma in the previous 6 months, or who had received statins, antineoplastic, corticosteroid, or immunosuppressive drug treatments were not included in the present study.

Cell cultures.

Skin fibroblasts were isolated by dermal biopsies obtained from the upper forearm by a 2-mm punch after local anesthesia with 2% xylocaine. Biopsies were washed with sterile PBS supplemented with penicillin and streptomycin, minced to pieces, transferred to culture dishes and overlaid with a coverslip. Cultures were incubated at 37°C in 5% CO_2 humidified atmosphere with

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DMEM containing 4.5 g/l glucose and 10% FCS for 18-21 days to obtain primary cultures of human diploid fibroblasts. These were then subcultured, seeded onto 60-mm plastic Petri dishes (28 cm^2) in 10% FCS–DMEM and grown to confluence All experiments were completed using fibroblasts between passages two and three of culture. For *in vitro* kinetic experiments, cells were plated at a density of 10.000 cell/cm^2 in 6 well plates and then incubated for 48 h in DMEM with 0.2% FCS to synchronize cells at a quiescent state. Quiescent fibroblasts were then diluted in complete growth medium with 10% FCS, either in the presence or in the absence of 20 nM of 40-O-(2-hydroxyethyl) rapamycin (EVE) (supplied by Novartis Pharma AG, Basel, Switzerland). In addition, 10 μM of Progesterone (PG) (Sigma-Aldrich, St. Louis, MO) and 4 μM of the acyl amide ACAT inhibitor SaH (Novartis Pharma AG, Basel) was added to some cultures as broad-spectrum regulators of cholesterol ester cycle. Preliminary experiments were carried out to find the minimal effective dosages with the least effect on cell viability. Cells were harvested after treatments at indicated time points. Cell proliferation was assessed by direct cell counting with a hemocytometer. The trypan blue exclusion test was used to assess cell viability.

Cholesterol esterification.

Cholesterol esterification was evaluated by incubating cells for 6 hours in medium containing [1-^{14}C]oleic acid (Dupont, NEN 55 mCi/mmol), bound to bovine serum albumin (BSA) at a final concentration of 2 μCi/ml. After incubation, cells were washed with PBS and lipids extracted with acetone. Lipid subclasses were separated by thin layer chromatography (TLC) and incorporation of [^{14}C]oleate into cholesterol esters was measured.

Neutral lipid staining.

To visualize the degree of cytoplasmic neutral lipid accumulation, cultured fibroblasts were washed three times with PBS and fixed by soaking in 10% formalin. Cells were then treated with isopropyl alcohol (60%), washed, stained with Oil red-O (ORO) (a lipid-soluble dye that stains cholesterol esters but not free cholesterol) and counterstained with Mayer's hematoxylin. Stained cells were examined by light microscopy and digital images were recorded. For quantification of mean neutral lipid content in skin fibroblasts, red color intensity was measured in single cells using the software Scion Image (Scion Corp., Fredrick, Maryland, USA). Values were expressed as the mean of red color intensity in each cell, determined in at least 30 single cells in six random microscopic fields.

RT-PCR and Southern blotting.

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The expression levels of mRNAs were evaluated in skin fibroblasts by reverse transcription polymerase chain reaction (RT-PCR). The mRNA levels for the housekeeping gene β-actin were used to normalize the amount of RNA inputs in the RT-PCR. Total RNA was extracted from 10^6 cells using TRIZOL reagent (Invitrogen Corporation). Equal amounts of total RNA (1 μg) were reverse transcribed into cDNA using the random hexamer method and amplified by PCR in the presence of specific primers, according to the manufacturer's instructions (GeneAmp RNA PCR Kit, Perkin-Elmer Cetus).

Amplicons were labeled during PCR with Digoxigenin-11-dUTP (DIG; Roche Applied Science), immuno-detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche Applied Science) and visualized with the chemiluminescent substrate CSPD®. The intensity of the autoradiographic bands was measured after exposure to X-ray film with the Kodak Digital Science Band Scanner Image Analysis System containing HP ScanJet ID Image Analysis Software. The overall procedure was normalized by expressing the amount of PCR products for each target mRNA relative to the amount of PCR products obtained for the housekeeping gene β-actin.

Western blotting.

Proteins were extracted from fibroblast monolayers with the RIPA buffer (0.05 mL per mg tissue or cell pellets from 10^6 fibroblasts). Protein concentration was evaluated by the Bicinchoninic Acid Protein determination kit (Sigma). Aliquots of protein extracts were separated by 10% SDS polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Millipore, Bedford MA). Membranes were subjected to immunoblotting with a 1:5000 dilution of the anti-ACAT1 antibody (H-125, Santa Cruz Biotechnology, Santa Cruz, CA) as indicated by the manufacturer. Blots were then treated with the appropriate dilution of a HRP-conjugated secondary antibody (1:6500 for ACAT-1). Specific bands were detected after addition of a chemiluminescent substrate (Amersham, Freiburg Germany), and analyzed by the NIH Image 1.63 Analysis Software program (Scion Image).

Statistical analysis.

Data are reported as mean ± standard error (SE). Statistical calculations were performed using the statistical analysis software of Origin 7.0 version (Microcal, Inc, Northampton, MA, USA). A value of $p<0.05$ was considered to be statistically significant.

Funding source.

Nature Precedings : hdl:10101/npre.2008.1602.1 : Posted 18 Feb 2008

This study was supported by grants of Regione Autonoma della Sardegna and of Monte dei Paschi di Siena.

AKNLOWLEDGEMENTS

The Authors wish to thank Anna Saba for excellent technical assistance and Marina Julian for revision of theEnglish.

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LEGENDS TO FIGURES

Figure 1. **Cholesterol esterification in AD and non-AD skin fibroblasts**.

Fibroblasts between passages two and three of culture were plated at a density of 10.000 cell/cm^2 in 6 well plates, and then incubated for 48 h in DMEM with 0.2% FCS to synchronize cells at a quiescent state. Quiescent fibroblasts were then diluted in complete growth medium with 10% FCS, and harvested at the indicated time points. Cholesterol esterification was evaluated by incubating cells for 6 hours in medium containing [1-^{14}C]oleic acid (Dupont, NEN 55 mCi/mmol), bound to bovine serum albumin (BSA) at a final concentration of 2 μCi/ml. Following incubation, cells were washed with PBS and lipids extracted with acetone. Lipid subclasses were separated by thin layer chromatography (TLC), and incorporation of [^{14}C]oleate into cholesterol esters was measured. Data shown are the mean ± SEM of six AD and six non-AD cell cultures. *$p<0.05$.

Figure 2. **Accumulation of lipid droplets and increased expression of ACAT1 protein in AD, compared to non-AD, fibroblasts.**

Representative microscopic visualization of lipid droplet accumulation in 1 non-AD and 1 AD subject (a). Quiescent fibroblasts were diluted in complete growth medium with 10% FCS and harvested at the indicated time points. 40-O-(2-hydroxyethyl) rapamycin, everolimus (EVE, 20 nM), progesterone (PG, 10 μM), and one of the acyl amide ACAT inhibitors (SaH, 4 μM) were added to cultures as broad-spectrum regulators of the cholesterol ester cycle. After washing three times with PBS, cultured fibroblasts were fixed by soaking in 10% formalin. a) Cells were then treated with isopropyl alcohol (60%), washed, stained with ORO and counterstained with Mayer's hematoxylin. Stained cells were examined by light microscopy and digital images recorded. (**b**) Quantitative analysis of ORO-stains performed on six different non-AD and AD fibroblast cell cultures. Bars indicate the mean ± SE of red color intensity/cell as determined in each culture in at least 30 individual cells in six random microscopic fields. Red color intensity was measured in single cells using the software Image J. (Scion Corp., Fredrick, Maryland, USA. **c**) Immunoblots of ACAT1 protein levels in skin fibroblasts. ACAT1 protein was extracted from 10^6 fibroblast monolayers with the RIPA buffer. Aliquots of protein extracts were separated by 10% SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were subjected to immunoblotting with a 1:5000 dilution of the anti-ACAT1 antibody. Blots were then treated with a 1:6500 dilution of an HRP-conjugated secondary antibody. Specific bands were

detected after addition of a chemiluminescent substrate, and analyzed by the NIH Image 1.63 program (Scion Image). **d**) Densitometric analysis of ACAT1 protein immunoreactivity relative to β-actin in 6 AD patients and 6 age-matched non AD subjects. Data values are represented as mean ±S.E. $^{*}p < 0.05$.

Figure 3**. RT-PCR analysis of human ACAT1, nCEH, CAV1 and ABCA1 mRNAs from non-AD and AD skin fibroblasts.**

Total mRNA was extracted from quiescent cells and mRNA levels for ACAT1, nCEH, CAV1 and ABCA1 were determined by reverse transcriptase-polymerase chain reaction using the appropriate primer sets. **a**) representative autoradiograms of RT-PCR of the target mRNAs' expression. **b**) densitometric analyses of the bands normalized for the endogenous β-actin mRNA content that was used as an internal control. Data represent mean values ± SE of triplicate determinations of six non-AD and six AD samples.* $p<0.05$.

Figure 4**. RT-PCR analysis of human HMGCoAR, LDLR and SREBP2 mRNAs from non-AD and AD skin fibroblasts.**

Total mRNA was extracted from quiescent cells and mRNA levels for HMGCoAR, LDLR and SREBP2 were determined by reverse transcriptase-polymerase chain reaction using appropriate primer sets. **a**) representative autoradiograms of RT-PCR of the target mRNAs expression. **b)** densitometric analyses of the bands normalized for the endogenous β-actin mRNA content that was used as an internal control. Data represent mean values ± SE of triplicate determinations of six non-AD and six AD samples.* $p<0.05$.

Figure 5**. RT-PCR analysis of human BACE1, APP and neprylisin mRNAs from non-AD and AD skin fibroblasts.**

Total mRNA was extracted from quiescent cells, and mRNA levels for BACE1, APP and neprylisin were determined by reverse transcriptase-polymerase chain reaction using the appropriate primer sets. **a**) representative autoradiograms of RT-PCR of the target mRNAs' expression. **b**) densitometric analyses of the bands normalized for the endogenous β-actin mRNA content that was used as an internal control. Data represent mean values ± SE of triplicate determinations of six non-AD and six AD samples.*$p<0.05$.

Figure 6. **a)** In a controlled situation, FC derived from the hydrolysis of LDL-CEs in late endosomes/lysosomes, and/or from *de novo* synthesis in the ER, is directly transferred to the plasma membrane where it may be used for lipid membrane organization. If FC in the ER exceeds a

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critical threshold value, its excess is rapidly converted to CE by ACAT and stored as lipid droplets. These stores are continuously mobilized by the action of a neutral cholesterol ester hydrolase (nCEH), re-producing FC that is either used to replenish membranes or secreted to extracellular acceptors, namely high-density lipoproteins (HDL), via the ATP-binding-cassette-sub-family A-member 1 (ABCA-1) transporter (cholesterol ester cycle). High FC levels in the ER block the function of the SREBP-cleavage-activating-protein (SCAP), which is needed for the proteolytic activation of the sterol-regulatory-element-binding-protein-2 (SREBP2), a transcriptional factor that promotes the expression of HMGCoA-R and LDL-R. In this case the major pool of BACE1 is in rafts, while its substrate APP is in non-raft membranes. This segregation keeps the production of Aβ at physiological levels. In addition, the activation of the amyloid degrading enzyme neprylisin contributes to the degradation of the peptide and to the maintenance of non deleterious steady state levels of Aβ necessary for multiple physiological functions. **b)** During the years, as a result of genetic, environmental and ageing effects, cholesterol homeostatic mechanisms become altered, e.g.: increased CE synthesis (higher levels of ACAT1 activity and protein) and decreased CE catabolism (lower levels of nCEH, cav-1, and ABCA1). This leads to excessive accumulation of CE, present as cytoplasmic lipid droplets, and consequently to a reduced transport of FC to the plasma membrane. Low membrane cholesterol, by causing raft domain disorganization, may foster Aβ production by favoring the interaction of BACE1 and APP in the non-raft membrane domains. Due to raft disorganization, the activation of neprylisin is impaired. This renders inefficient the Aβ clearance, thus contributing to its aggregation.

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Figure 1. **Cholesterol esterification in AD and non-AD skin fibroblasts.**

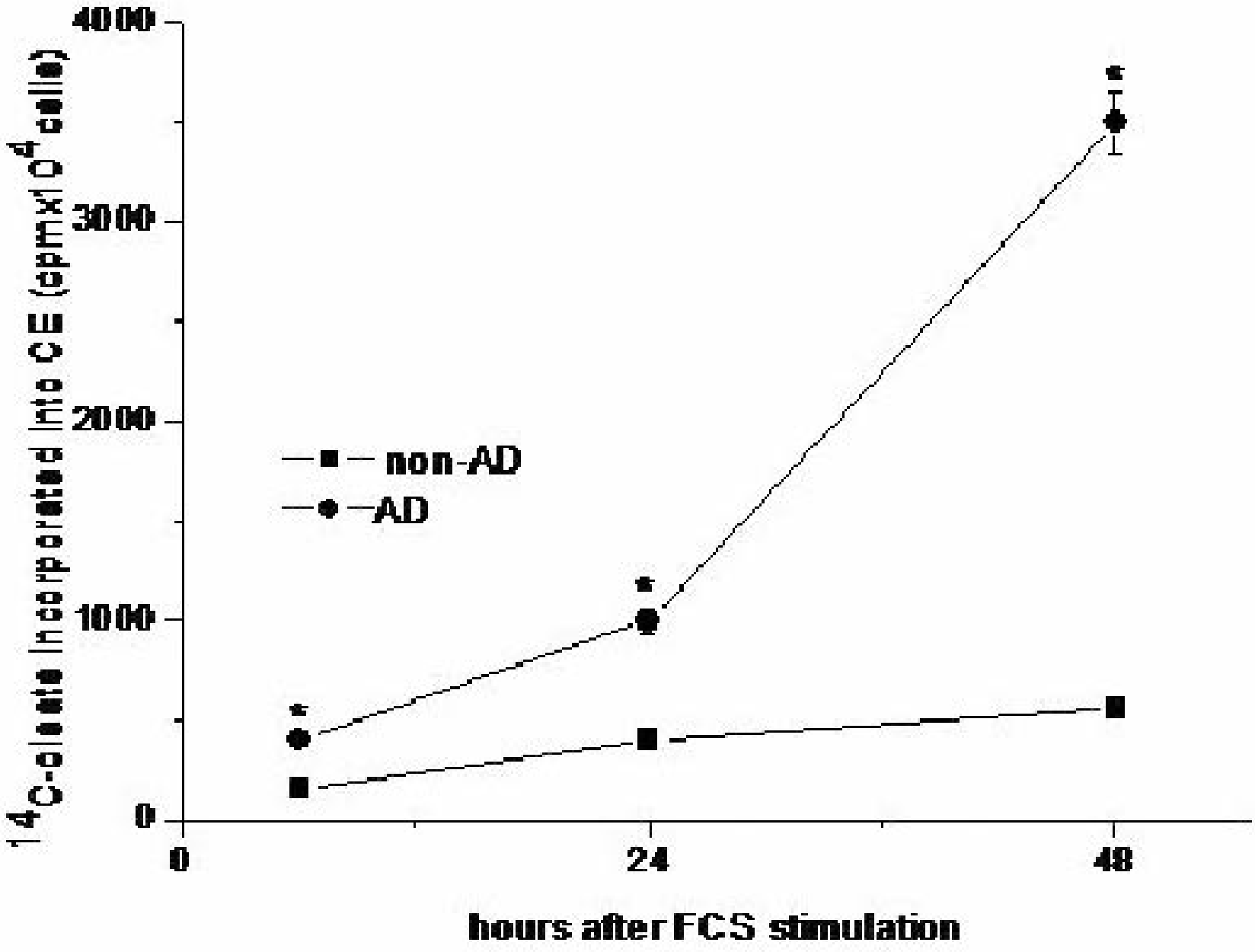

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Figure 2. **Accumulation of lipid droplets and increased expression of ACAT1 protein in AD, compared to non-AD, fibroblasts.**

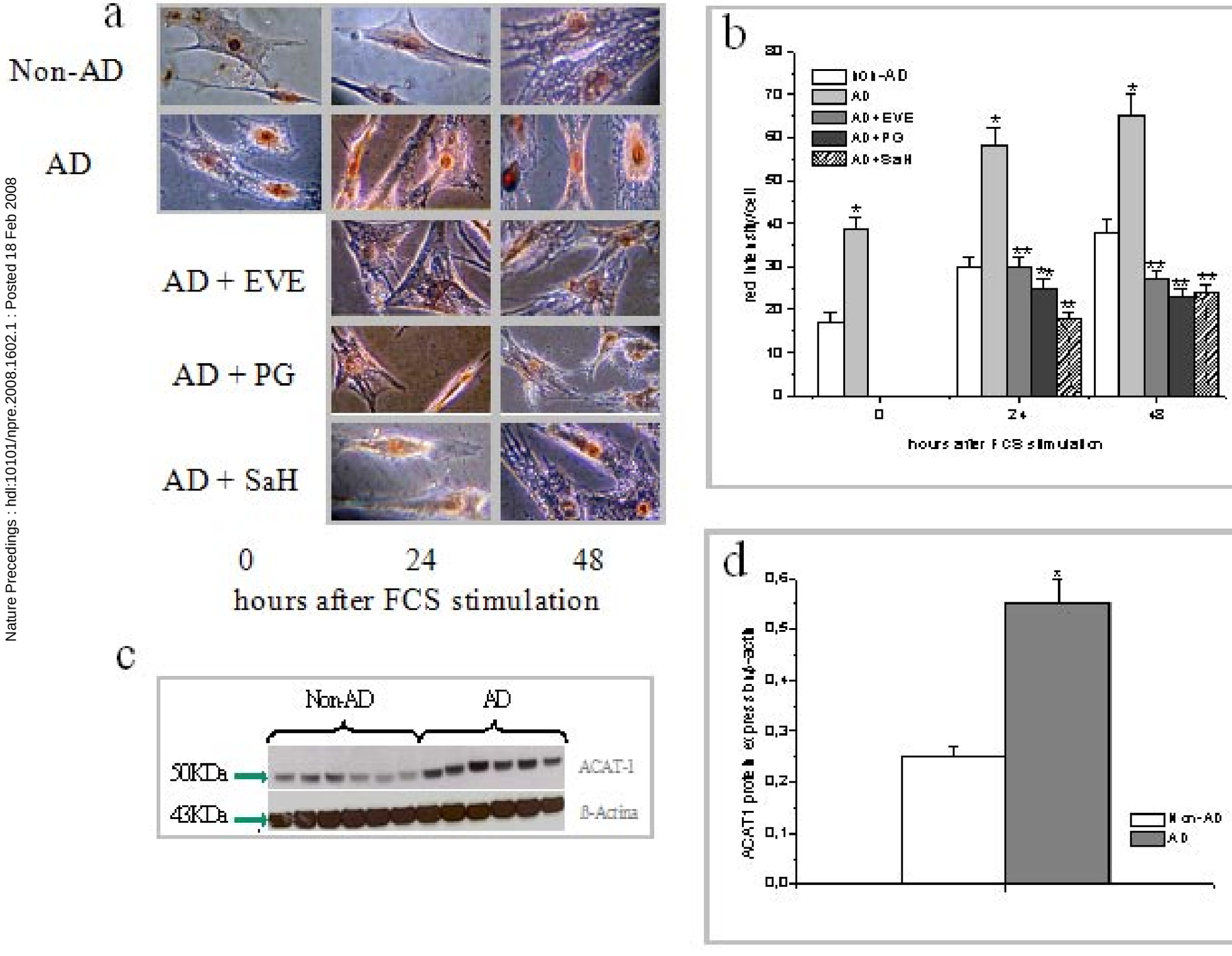

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Figure 3. **RT-PCR analysis of human ACAT1, nCEH, CAV1 and ABCA1 mRNAs from non-AD and AD skin fibroblasts.**

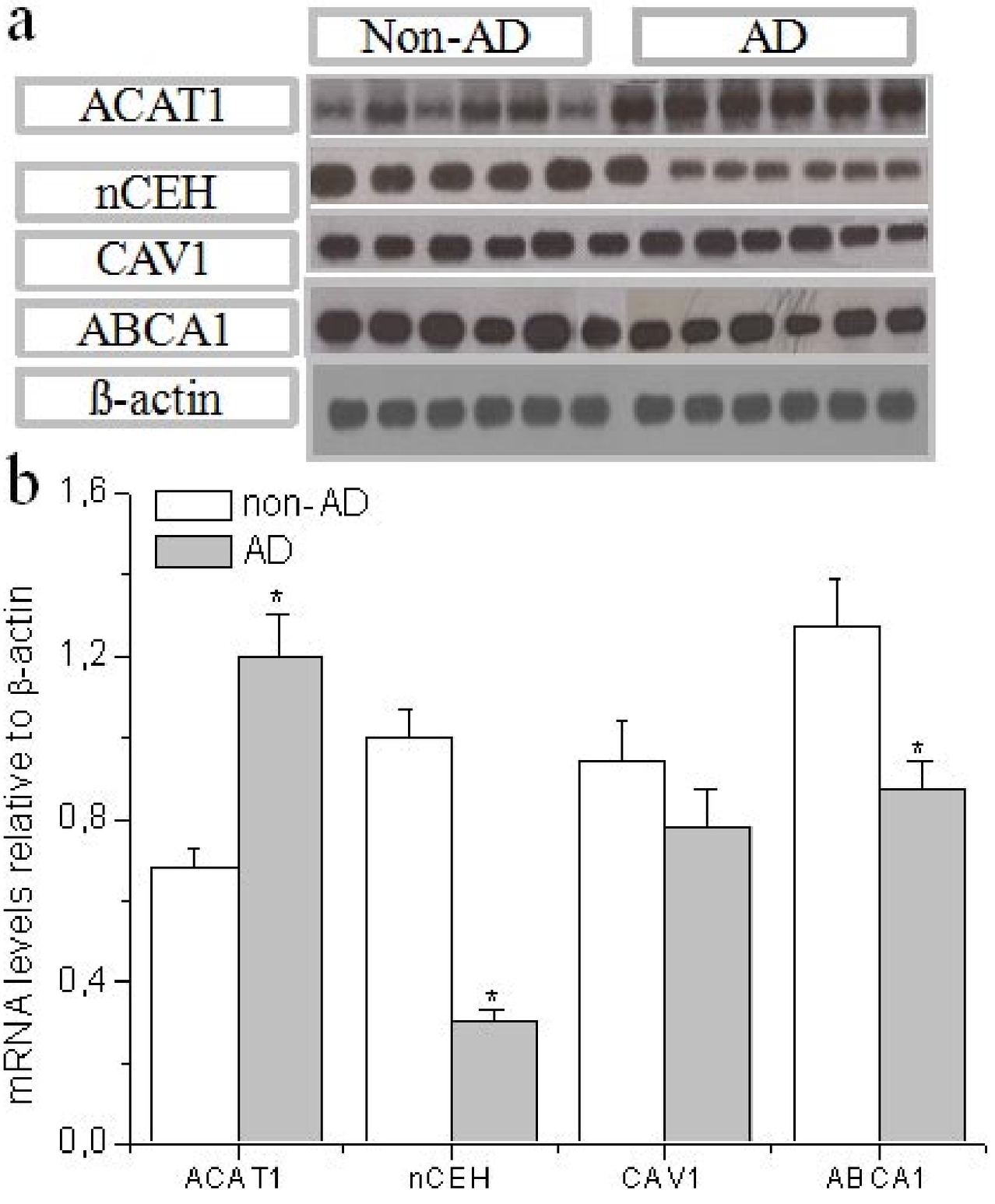

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Figure 4. **RT-PCR analysis of human HMGCoAR, LDLR and SREBP2 mRNAs from non-AD and AD skin fibroblasts.**

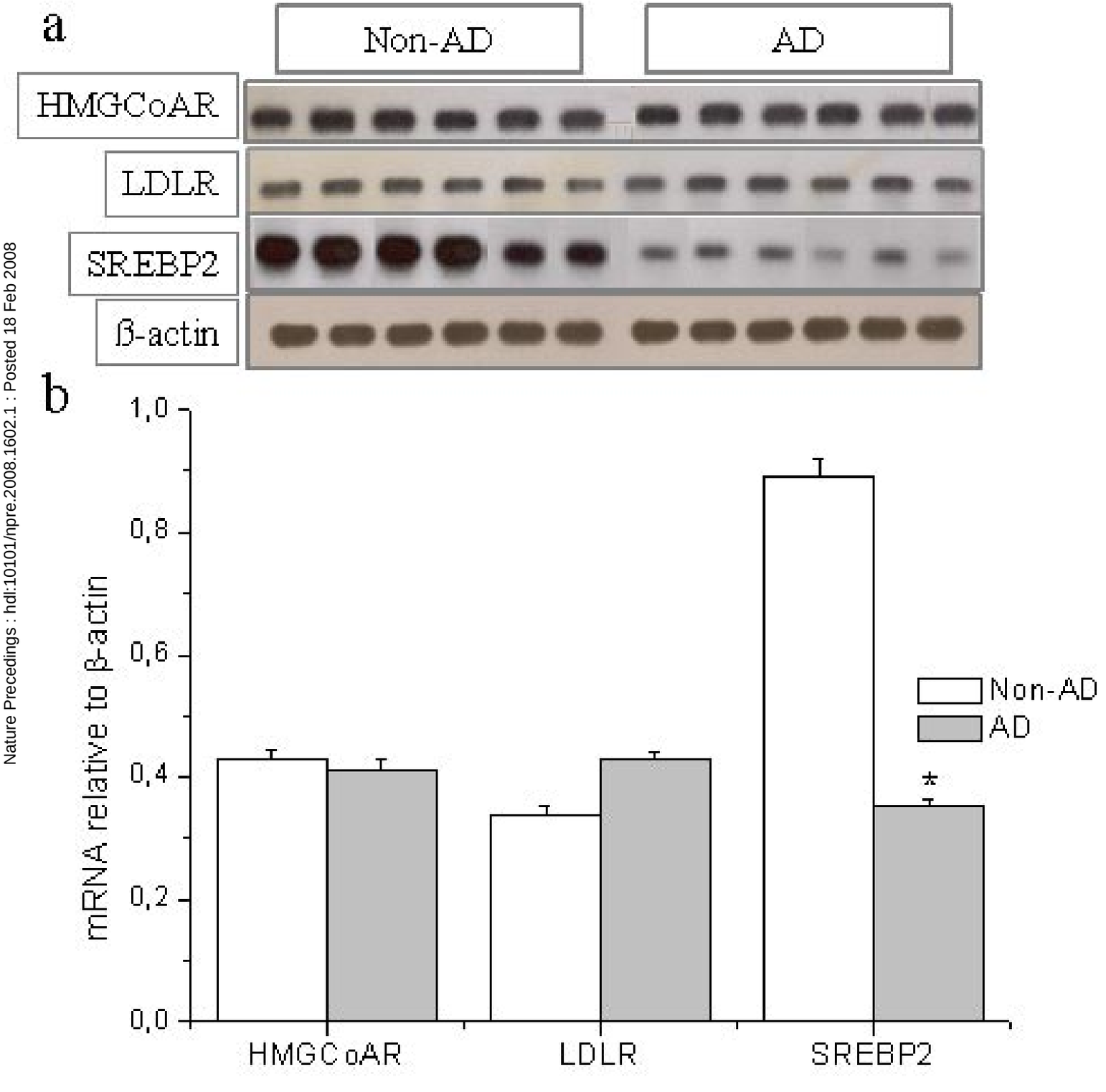

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Figure 5. **RT-PCR analysis of human BACE1, APP and neprylisin mRNAs from non-AD and AD skin fibroblasts.**

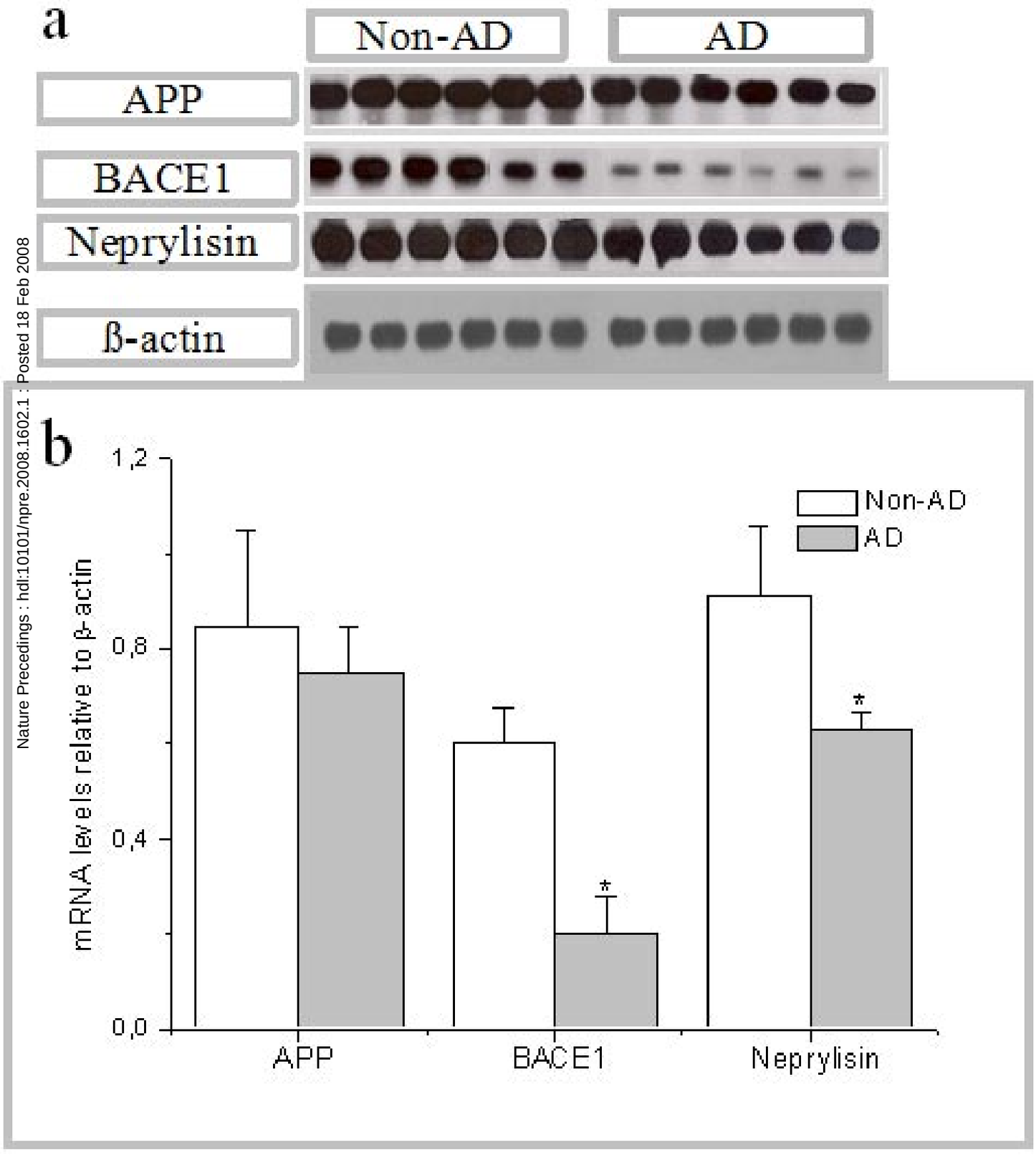

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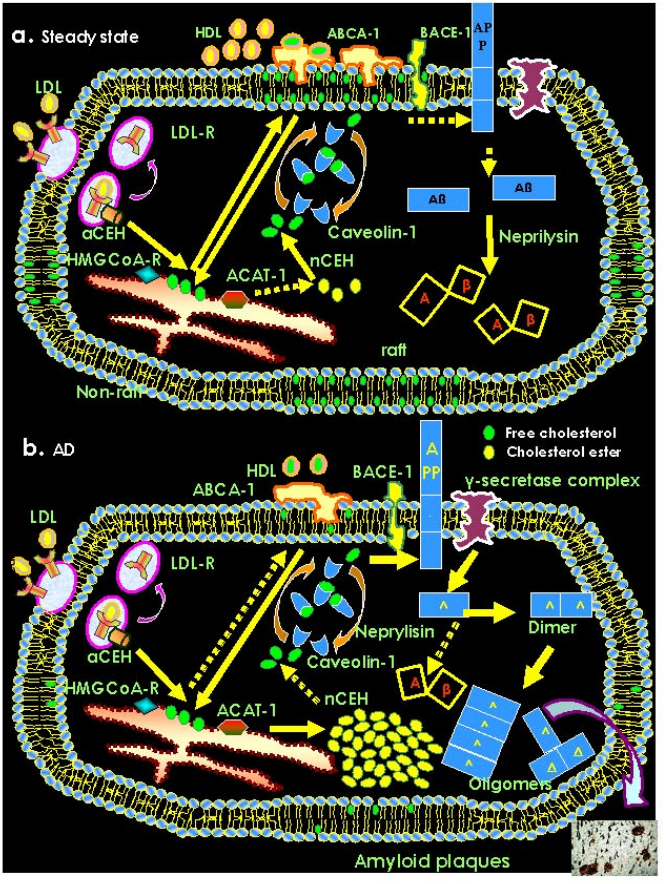
Figure 6.


a. Steady state
HDL
ABCA-1
BACE-1
APP
LDL
LDL-R
aCEH
HMGCoA-R
ACAT-1
nCEH
Caveolin-1
Aß
Aß
Neprilysin
A
β
A
β
raft
Non-raft
b. AD
Free cholesterol
Cholesterol ester
HDL
ABCA-1
BACE-1
APP
γ-secretase complex
LDL
LDL-R
aCEH
HMGCoA-R
ACAT-1
nCEH
Caveolin-1
Neprilysin
Dimer
Oligomers
Amyloid plaques