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Clathrin-dependent APP endocytosis and Aβ secretion are highly sensitive to the level of plasma membrane cholesterol

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ABSTRACT

Several lines of evidence support a strong relationship between cholesterol and Alzheimer's disease pathogenesis. Membrane cholesterol is known to modulate amyloid precursor protein (APP) endocytosis and amyloid-β (Aβ) secretion. Here we show in a human cell line model of endocytosis (HEK293 cells) that cholesterol exerts these effects in a dose-dependent and linear manner, over a wide range of concentrations (-40% to +40% variations of plasma membrane cholesterol induced by methyl-beta-cyclodextrin (MBCD) and MBCD-cholesterol complex respectively). We found that the gradual effect of cholesterol is inhibited by small interference RNA-mediated downregulation of clathrin. Modulation of clathrin-mediated APP endocytosis by cholesterol was further demonstrated using mutants of proteins involved in the formation of early endosomes (dynamin2, Eps15 and Rab5). Importantly we show that membrane proteins other than APP are not affected by cholesterol to the same extent. Indeed clathrin-dependent endocytosis of transferrin and cannabinoid1 receptors as well as internalization of surface proteins labelled with a biotin derivative (sulfo-NHS-SS-biotin) were not sensitive to variations of plasma membrane cholesterol from -40% to 40%. In conclusion clathrin-dependent APP endocytosis appears to be very sensitive to the levels of membrane cholesterol. These results suggest that cholesterol increase in AD could be responsible for the enhanced internalization of clathrin-, dynamin2-, Eps15- and Rab5-dependent endocytosis of APP and the ensuing overproduction of Aβ.

1. Introduction

Several lines of evidence support a strong relationship between cholesterol- particularly high levels- and Alzheimer's disease (AD). Genetic association studies have incriminated the brain cholesterol transporter apolipoprotein E (APOE) ε4 allele as the major risk factor for late onset or sporadic AD [1]. Levels of total cholesterol are increased in the brain of AD patients (between 20% and 40%) and more specifically in the membrane fraction [2,3]. AD is characterized by the presence of neurofibrillary tangles and senile plaques. The core of the senile plaque is principally made of insoluble amyloid-β (Aβ) peptide in fibrillar form. In AD mouse models, a cholesterol-rich diet promotes Aβ deposition [4]. Aβ is derived from the amyloid-β precursor protein (APP) by sequential β- and γ-proteolytic cleavages. β-site APP cleavage occurs mainly in the endosomal compartment, where pH is optimal for β-secretase activity [5]. The presence of APP in this compartment depends on the tight regulation of APP endocytosis from the plasma membrane [6]. Inhibition or acceleration of the endocytic pathway reduces or increases Aβ production, respectively [7–11]. Methyl-β-cyclodextrin (MBCD)-based cholesterol depletion at the plasma membrane decreases the endocytosis and amyloidogenic processing of APP [7,10,12–14]. Recently Schneider *et al.* showed that APP is internalized through a specialized clathrin-dependent pathway involving the adaptor protein-2 but not epsin1 and requiring cholesterol and flotilin2. In their study they investigated a decrease of plasma membrane cholesterol using MBCD [10].

There are, to our knowledge, no reports in the literature on the relationship between the concentration of membrane cholesterol and the amplitude of the effect. In the present study we investigated the effects of both decreases and most importantly increases of plasma membrane cholesterol. We studied the dose-dependent effects of cholesterol on APP endocytosis and Aβ secretion in a human cell line model of endocytosis. We analyzed the effects of an 80% variation, from -40% to +40% of the normal level of membrane cholesterol obtained by MBCD and MBCD-cholesterol complex treatment respectively. Using GFP fusion constructs, confocal microscopy and flow

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cytometry, we show that the internalization of APP from the plasma membrane to the endosomal compartments is sensitive to a modest increase of cholesterol concentration at the plasma membrane. This effect is Rab5-, Eps15-, dynamin2- and clathrin-dependent. Additionally, increased membrane cholesterol was shown to be associated with enhanced secretion of Aβ peptide, an effect that was inhibited by siRNA against clathrin. APP appears particularly sensitive to small variations of cholesterol at the plasma membrane, since other membrane proteins are not similarly affected.

2. Experimental procedures

2.1. Antibodies, plasmids and reagents

C-terminal anti-APP antibody was purchased from Calbiochem and N-terminal anti-APP antibody from Chemicon. Anti-β-actin and biotin antibodies came from Abcam, anti-GFP and anti-Flag (clone M1) came from Sigma, anti-EEA1 antibody from Affinity Bioreagents and anti-clathrin heavy chain antibody from BD Bioscience. Alexa-labelled secondary antibodies came from Molecular Probes. HRP-conjugated anti-rabbit IgG and anti-mouse IgG were, respectively, from Jackson Immunoresearch and Molecular Probes. MBCD, MBCD-cholesterol complex and filipin were from Sigma. Rab5-GFP plasmids were generously provided by Dr. Robert Lodge and dynamin2-GFP by Dr. M. McNiven; these plasmids were validated in a previous study [15]. Wild-type and mutant eps15-eGFP plasmids were gifts from Dr. Alice Dautry-Varsat (Institut Pasteur, Paris, France) [16]. APP_{751} plasmid was a gift of Dr. Frederic Checler. The Swedish double mutations (APP_{751}sw: K651N and M652L) were obtained using the Quikchange mutagenesis kit (Stratagene). The siRNA target sequence for the clathrin heavy chain was UAAUCCAAUUCGAAGACCAAU [17].

2.2. Cell transfections and cholesterol treatments

HEK293 cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Cells were transfected with plasmids using the DNA/Effectene mixture (Qiagen), except for siRNA, for which cells were incubated with 100 pmoles of siRNA and 4 µl of lipofectamine 2000 (Invitrogen) for 4 h at 37 °C. Stable HEK293 clones over-expressing APPsw-GFP or Flag-CB1-GFP were selected on G418 containing medium.

Levels of cholesterol were measured using the Amplex Red Cholesterol Assay kit (Molecular Probes) after extraction with chloroform-methanol solution (2:1) for 45 min. Treatment with 1.75 mM or 10 mM of MBCD induced a decrease of 15% and 40%, respectively, while 15% and 40% overloads were obtained after treatment with 0.5 mM or 1.4 mM of MBCD-cholesterol, respectively.

Filipin staining of HEK cells (0.05%, DMSO 2%) was performed for 30 min at room temperature after cholesterol depletion or enrichment to confirm the change of cholesterol, mainly at the plasma membrane (Supplementary Fig. 1)

For the $Aβ_{42}$ and $Aβ_{40}$ dosages, 50 µl of cell culture medium were collected. $Aβ_{42}$ and $Aβ_{40}$ levels were determined using the Biosource ELISA kit.

2.3. Image Quantification

Images (1024×1024 pixels) of individual cells were obtained on a Leica TCS NT confocal microscope using a 63× numerical aperture 1.4 oil-immersion objective and 6× zoom. Each image was obtained on the equatorial cross-section through the cell that maximized nuclear diameter. Co-detection of GFP and Alexa-Fluor® 568 fluorescence was done by excitation at 488 and 568 nm sequentially. Membrane/intracellular Fluorescence Ratios (MFR) were calculated for each cell analyzed, according to Leterrier et al. [15].

2.4. Antibody feeding experiments

HEK293 cells transfected with the APPsw plasmid were incubated for 30 min at 37 °C with the N-terminal anti-APP antibody directly coupled to Alexa-Fluor® 647 (Invitrogen). Treatment with trypsin (5 min, 0.05%) was used to remove all membrane-bound antibody. Cells were analyzed using the flow cytometry analyzer CyAn ADP LX 7 Color (Dako) (up to 5,000 cells for each condition). For studying the effects of cholesterol, HEK293 cells expressing either APPsw-GFP, FLAG-CB1-GFP or the endogenous transferrin receptor were incubated for 30 min with various concentrations of MBCD-cholesterol and an anti-APP N-terminal antibody, anti-Flag antibody (clone M1) or holo-transferrin, respectively, all directly coupled to Alexa-Fluor® 647. Non-internalized antibodies and holo-transferrin were stripped from the plasma membrane by trypsin (0.05%, 5 min), also serving to detach cells, and Alexa-Fluor® 647 fluorescence of individual cells was quantified.

To label all membrane proteins, HEK293 cells were incubated for 30 min with various concentrations of MBCD-cholesterol and the Sulfo-NHS-SS-Biotin (0.8 mM, Thermo scientific). Non-internalized Sulfo-NHS-SS-Biotin was stripped from the plasma membrane by trypsin treatment (0.05%, 5 min), also serving to detach cells. After permeabilization (4% PFA for 12 min), cells were incubated with a biotin antibody (Abcam) for 1 h, then with a secondary goat anti-rabbit Alexa-Fluor® 488 antibody for 45 min. Alexa-Fluor® 488 fluorescence of individual cells was quantified using the flow cytometer.

To characterize endosomes, cells transfected with the APPsw plasmid were incubated with an anti-APP N-terminal antibody for 12 min. The membrane-bound antibody was removed as described above and intracellular APP was detected using an Alexa-Fluor® 568 secondary antibody. Quantifications were performed using ImageJ (http://www.rsbweb.nih.gov/ij/).

2.5. Protein extraction and western blot

Cells were washed twice and extracted for 2 h in 0.05 M Tris buffer containing 0.1 M NaCl, 5 mM EDTA, 1% Nonidet P-40 and a protease inhibitor cocktail (Roche). Cell debris were removed by centrifugation at 13000 ×*g*. Protein concentration was determined using the Dc protein assay kit (BIO-RAD). For western blot analysis, 50 µg of total protein per lane were loaded on a 4-12% gradient Bis-Tris gel under reducing conditions. Nitrocellulose membranes (Whatman) were probed with anti-GFP, clathrin heavy chain or actin antibodies. After incubation with horseradish-conjugated secondary antibodies (Molecular Probes) the signal was revealed using Immobilon Western chemoluminescent HRP substrate (Millipore).

3. Results

3.1. Plasma membrane cholesterol modulates APP endocytosis

To study the effects of increased cholesterol at the plasma membrane on APP endocytosis, we used the antibody-feeding technique. HEK293 cells were stably transfected with APP_{751} carrying the Swedish mutation (APPsw) fused to green fluorescence protein (GFP) at the C-terminus. We first tested whether the GFP tag or the Swedish mutations altered the subcellular distribution of APP by measuring the membrane/intracellular GFP fluorescence ratio (MFR) by confocal microcopy in equatorial optical sections of individual cells. At steady-state, about 95% of both APP-GFP and APPsw-GFP was intracellular, while the remaining 5% was localized at or just beneath the plasma membrane (Supplementary Fig. 2C, D). Most of the GFP signal corresponded to full-length APP, since it overlapped with APP labelled with either N-terminal or C-terminal anti-APP antibodies in fixed and permeabilized cells (Supplementary Fig. 2A, B). In the

following experiments, we used APPsw-GFP because it produced higher levels of Aβ [18].

Living HEK293 cells stably transfected with APPsw-GFP were incubated for either 5 or 20 min with the anti-APP N-terminal antibody. Cells were fixed and permeabilized, and internalized anti-APP N-terminal antibody was visualized with a secondary antibody coupled to Alexa568. In contrast to the 5 min incubation, after which anti-APP N-terminal antibody was mainly localized at the plasma membrane, after 20 min a significant amount of immunoreactivity was also visible in GFP-positive intracellular vesicles (Supplementary Fig. 3). We repeated the anti-APP N-terminal antibody-feeding experiments while simultaneously increasing plasma membrane cholesterol. HEK293 cells stably expressing APPsw-GFP, and incubated with 1.4 mM MBCD-cholesterol and the anti-APP N-terminal antibody, demonstrated a 40% increase in cholesterol. Non-internalized antibodies were stripped from the plasma membrane with trypsin and APP was visualized as above. Cells with increased plasma membrane cholesterol demonstrated elevated levels of N-terminal anti-APP antibody internalization compared to controls, suggesting increased cholesterol levels promoted APPsw endocytosis (Fig. 1A). The labelled intracellular structures were identified as early endosomes, due to co-localization with an antibody against EEA1, the early endosome associated protein 1 (Fig. 1B). In cholesterol-treated cells, the size and the number of EEA1 positive structures were significantly increased (Fig. 1C; size: +59.3%, $p=0.003$; number: +35%, $p=0.0359$; Student's t-test), as demonstrated earlier in HEK293 cells and in cultured neurons [19].

To precisely quantify the effects of cholesterol overload on APP endocytosis, cells were analyzed using a flow cytometer. HEK293 cells were incubated with various concentrations of MBCD-cholesterol and the anti-APP N-terminal antibody directly coupled to Alexa647. Treatment with 0.5 mM and 1.4 mM MBCD-cholesterol complex produced a 15% and a 40% elevation of cholesterol at the plasma membrane, respectively. Alexa647 fluorescence of individual cells was quantified in a flow cytometry analyzer. Noticeably, a 40% overload of plasma membrane cholesterol significantly increased APPsw endocytosis, as compared to untreated cells (Fig. 1D; $p=0.022$, one-way ANOVA).

We then investigated whether this effect of cholesterol was selective for APP by analyzing two different transmembrane receptors: the transferrin receptor and the type 1 cannabinoid (CB1) receptor, a constitutively active seven transmembrane receptor. Both the transferrin and the CB1 receptors are internalized through the clathrin-dependent endocytic pathway [15]. HEK293 cells expressing either APPsw-GFP or FLAG-CB1-GFP or the endogenous transferrin receptors were incubated with various concentrations of MBCD-cholesterol and either an anti-APP N-terminal antibody or anti-Flag antibody (clone M1) or holo-transferrin, all directly coupled to Alexa647. The Alexa647

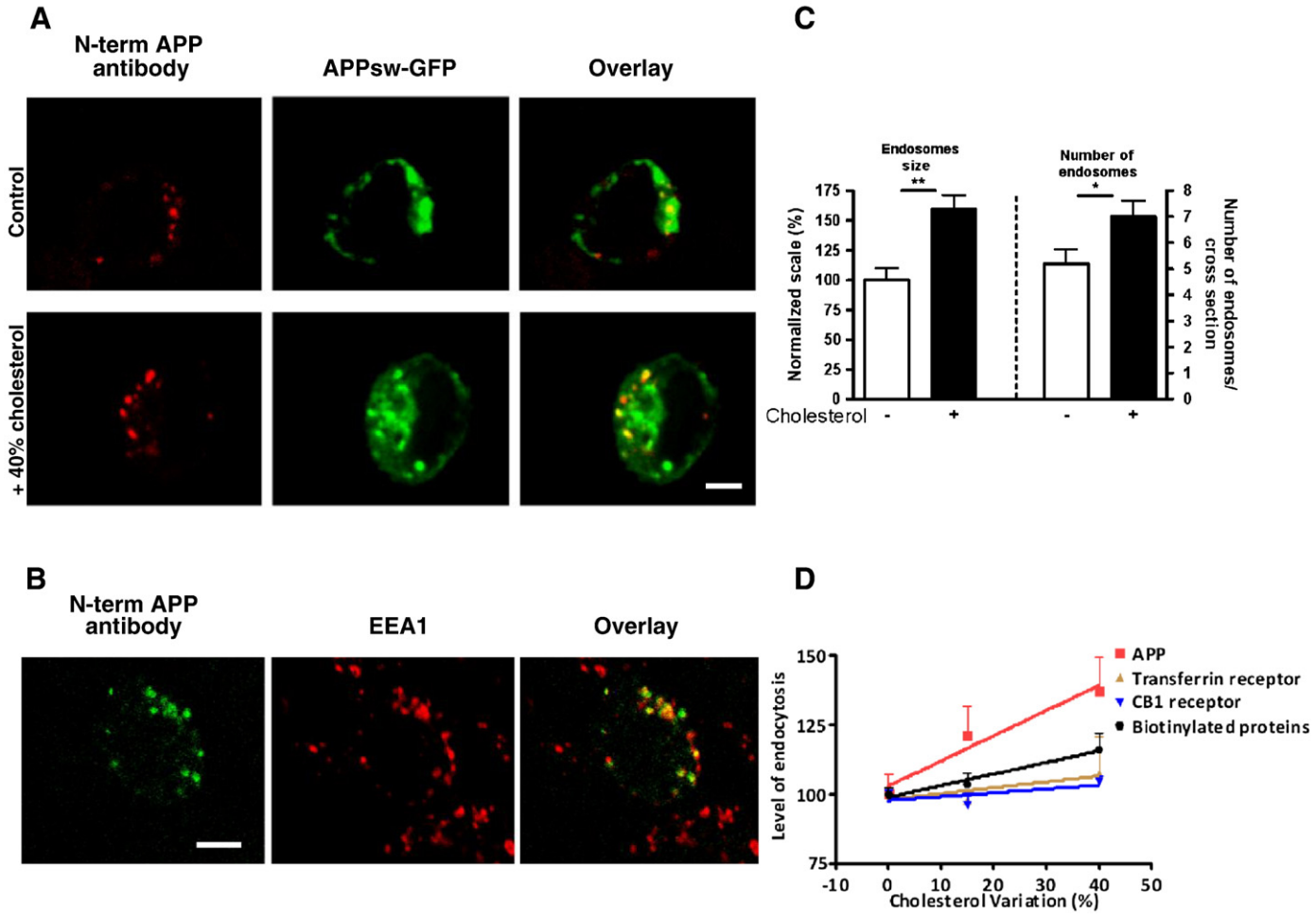

Fig. 1. Increase of APP endocytosis in HEK293 cells after MBCD-cholesterol treatment. (A) Confocal images of HEK293 cells transfected with APPsw-GFP and treated with an anti-APP N-terminal antibody with or without MBCD-cholesterol complex (40% cholesterol and control respectively). (B) Colocaliztion of APP-positive endocytosed vesicles with anti-EEA1 antibody. After 12 min treatment of HEK293 cells with an anti-APP N-terminal antibody, cells were incubated with an anti-early endosome antigen protein 1 (EEA1) antibody. Anti-APP N-terminal antibody and anti-EEA1 antibody were detected with two different secondary antibodies. The overlay shows an example of cells transfected with APPsw in which the majority of APP-positive vesicles correspond to early endosomes. Scale bar, 4 μm (C) Quantification of the number ($p=0.0359$, Student's t-test) and size ($p=0.003$, Student's t-test) of APP-positive structures in 22 untreated and in 22 cholesterol-treated cells, detected with the N-terminal anti-APP antibody in 2 independent experiments. (D) Effect of cholesterol on the level of endocytosis of APP ($p=0.022$, one-way ANOVA), transferrin receptor, cannabinoid receptor and biotinylated cell surface proteins as measured by flow cytometry from 3 independent experiments. Scale bar: 3 μm.

fluorescence level of individual cells was quantified as before. A 15% to 40% increase in membrane cholesterol did not alter the level of endocytosis for either transferrin or CB1 receptors (Fig. 1D).

To investigate the effect of an excess of cholesterol on the global internalization of cell surface proteins, we used a biotin derivative (sulfo-NHS-SS-biotin) that makes covalent bonds with the extracellular domain of plasma membrane proteins. After incubation of this biotin derivative with various concentrations of MBCD-cholesterol, cells were stripped, permeabilized and incubated with a biotin antibody detected by an Alexa-488 secondary antibody. Flow cytometry revealed that a 15% to 40% increase in membrane cholesterol did not alter the level of internalization (and, therefore, endocytosis) of plasma membrane proteins (Fig. 1D). As a positive control, cells were treated with 10 mM of cholesterol-MBCD complex, producing a 200% elevation of cholesterol at the plasma membrane. In this particular condition, an increase of intracellular fluorescence was detected for the cell surface proteins as well as for the transferrin receptor, the CB1 receptor and APP, indicating a non-specific effect on endocytosis (data not shown).

These results demonstrate that APP endocytosis is more sensitive to increases in membrane cholesterol than other transmembrane proteins.

3.2. Cholesterol modulates APP endocytosis through the dynamin2-, Eps15- and Rab5-dependent pathway

We next studied the effects of a wide range of cholesterol changes (increase or decrease) on APP endocytosis in HEK293 cells transfected

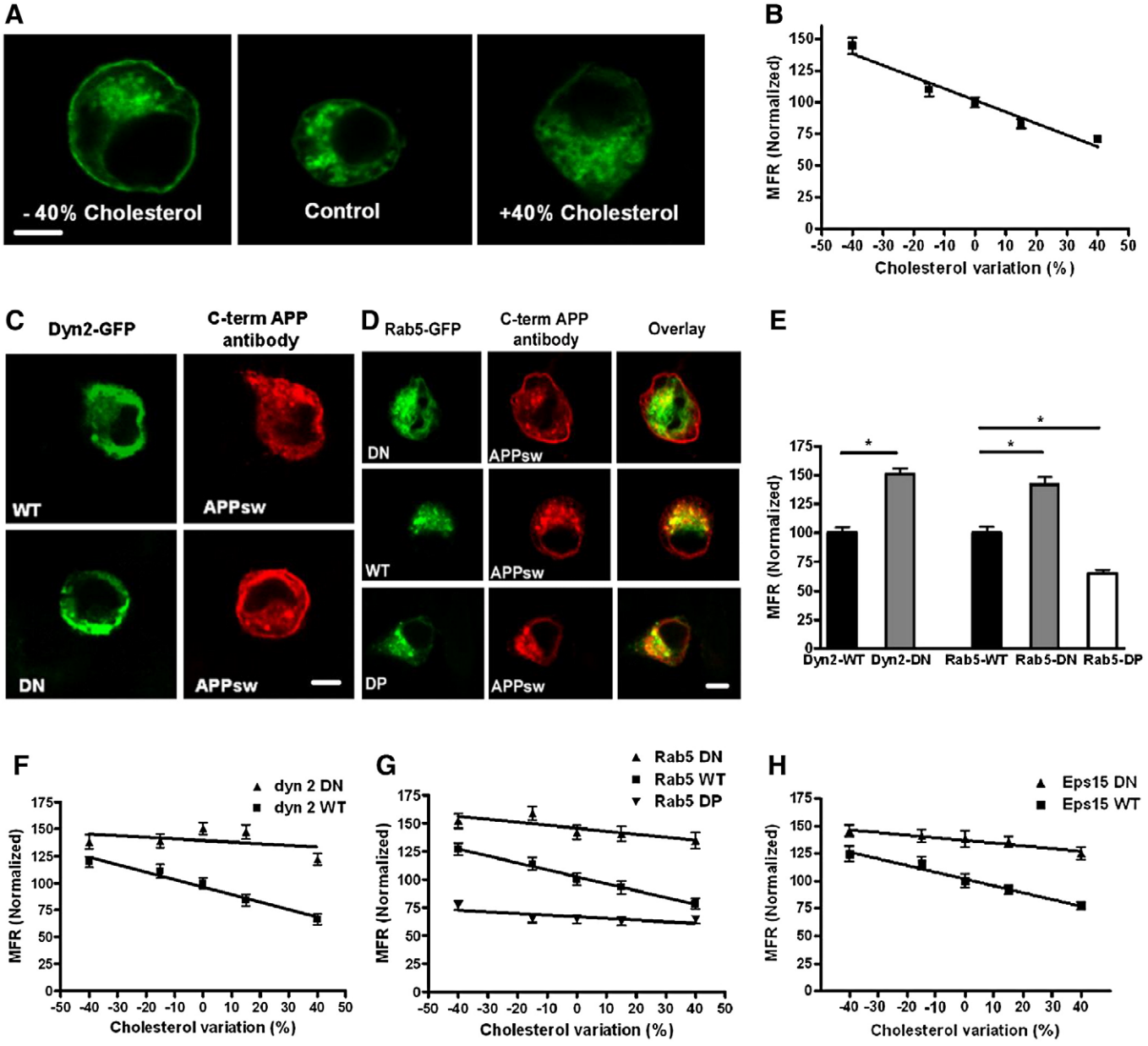

Fig. 2. Effects of plasma membrane cholesterol variations on APP endocytosis. (A) Confocal images of HEK293 cells transfected with APPsw-GFP and treated with MBCD or MBCD-cholesterol for 30 minutes. Scale bar: 3 µm. (B) Dose-dependent effect of cholesterol on the membrane/intracellular fluorescence ratio (MFR). For HEK293 cells, 84 cells in 3 independent experiments were analyzed for cholesterol concentration ($p = 0.0015$, one-way ANOVA). (C) HEK293 cells were co-transfected with the APPsw plasmid and wild type (WT) or dominant negative (DN) mutant of Dyn2-GFP. APP was detected using a C-terminal polyclonal anti-APP antibody and an anti-rabbit secondary antibody labelled with Alexa568. Scale bar: 3 µm. (D) Quantification of the MFR after transfection of Dyn2-GFP (30 cells analyzed in 2 independent experiments) or Rab5-GFP (75 cells analyzed in 3 independent experiments). * corresponds to p values <0.05 in one-way ANOVA. (E) Confocal images of HEK293 cells co-transfected with the APPsw plasmid and WT, DN or dominant positive (DP) mutants of Rab5-GFP. Scale bar: 3 µm. (F) Quantification of the MFR of APP in Dyn2 WT and DN transfected cells treated with various concentration of cholesterol modulating drugs (30 cells analyzed in 2 independent experiments). (G) MFR of APP in HEK293 cells transfected with WT or mutants of Rab5 (40 cells analyzed in 2 independent experiments). (H) MFR of APP in HEK293 cells transfected with WT or DN mutant of Eps15 (50 cells analyzed in 2 independent experiments).

with APPsw-GFP. Cells were treated with increasing concentrations of drugs inducing either acute depletion (MBCD) or enrichment (MBCD-cholesterol complex) of plasma membrane cholesterol. Treatment with 1.75 mM or 10 mM of MBCD induced a decrease of 15% and 40%, respectively (Fig. 2A). The subcellular distribution of APP (membrane/intracellular GFP fluorescence ratio: MFR) was measured by confocal microcopy in equatorial optical sections of individual cells.

A depletion of plasma membrane cholesterol favored APPsw localization at the plasma membrane, while an increase of plasma membrane cholesterol significantly enhanced the intracellular pool of APPsw, demonstrated by a decrease of membrane/intracellular GFP fluorescence (Fig. 2A, B). The slope of the curve (MFR vs cholesterol variations) was significantly different from 0 ($p = 0.0035$). Similar results were obtained with WT APP fused to GFP (Supplementary Fig. 2D). These results indicate that cholesterol level at the plasma membrane is an important regulator of the endosomal pool of APP.

We hypothesized that APP endocytosis could be modulated by targeting proteins involved in internalization (dynamin2, the epidermal growth factor receptor substrate 15 Eps15 and the small GTPase Rab5). Dominant-negative (DN) mutants of these proteins are known to induce a deficit in endocytosis, while dominant-positive (DP) Rab5 increases endocytosis [20,21]. HEK293 cells were transfected with APPsw (detected with an anti-APP C-terminal antibody) and wild type (WT) or mutant dynamin2, Eps15 or Rab5 fused to GFP. DN mutants of dynamin2 or Rab5 induced an accumulation of APPsw at the plasma membrane (Fig. 2C, D). MFR increased by 50% and 42% for dynamin2 and Rab5 DN mutants, respectively (Fig 2E $p < 0.05$ one-way ANOVA). A 38% increase of MFR was also observed with Eps15 DN mutant (data not shown). In contrast, over-expression of DP Rab5 increased APPsw endocytosis and resulted in the formation of unusually large vesicles, leading to a significant MFR decrease (Fig. 2D, E). These results suggest that the steady-state distribution of APP is regulated by dynamin2-, Eps15- and Rab5-dependent constitutive endocytosis.

To determine whether the regulation of APP endocytosis by cholesterol also occurs through the upregulation of dynamin2-, Eps15- and Rab5-dependent APP internalization, we compared the effects of acute cholesterol loading between cells transfected with WT or DN mutants of dynamin2, Eps15 or Rab5. Over-expression of WT dynamin2, Eps15 or Rab5 did not modify the concentration-dependent effects of cholesterol on APP endocytosis (Fig. 2F, G, H versus 2B). The slopes of the curves were significantly different from 0 ($p = 0.0025$ for Dyn2 WT, $p = 0.0098$ for Eps15 and $p = 0.0003$ for Rab5 WT). However, DN mutants blocked the effects of cholesterol (Fig. 2F, G, H). The slopes of the curves (MFR vs. cholesterol variations) obtained with DN mutants were not significantly different from 0 ($p = 0.5093$ for Dyn2 DN, $p = 0.1521$ for Eps15 DN, $p = 0.082$ for Rab5 DN) ($p > 0.08$; Fig. 2F, G, H), indicating that in these conditions APP internalization became insensitive to cholesterol changes at the plasma membrane. In contrast, over-expression of WT and DP Rab5 induced the formation of characteristic large vesicles containing APPsw, leading to lower MFRs (Fig. 2D). Interestingly, cholesterol addition did not further decrease the MFR, suggesting that endocytosis was maximal when DP Rab5 was over-expressed (Fig. 2G). These results indicate that plasma membrane cholesterol regulates the steady-state distribution of APP in a dynamin2-, Eps15- and Rab5-dependent manner.

3.3. Cholesterol modulates APP endocytosis through the clathrin-dependent pathway

Next, to confirm the role of clathrin in upregulation of APP endocytosis by plasma membrane cholesterol overload, we directly targeted endogenous clathrin using siRNA against the clathrin heavy chain [17]. HEK293 cells co-transfected with APPsw and anti-clathrin siRNA, but not with the control anti-luciferase siRNA, expressed the clathrin protein at a very low level (control, Fig. 3A). Knock-down of clathrin increased APPsw at the plasma membrane, as visualized with an anti-APP C-terminal antibody (Fig. 3B) and demonstrated by elevated MFR (Fig. 3C). Increased cholesterol at the plasma membrane decreased the MFR (Fig. 3C, $p = 0.034$, one-way ANOVA), but did not change the inhibition of APP endocytosis by clathrin siRNA (Fig. 3C, $p = 0.71$, one-way ANOVA).

Taken together, we concluded that the cholesterol content of the plasma membrane controls APP internalization by tight regulation of clathrin-, dynamin2-, Eps15 and Rab5-dependent endocytosis of APP.

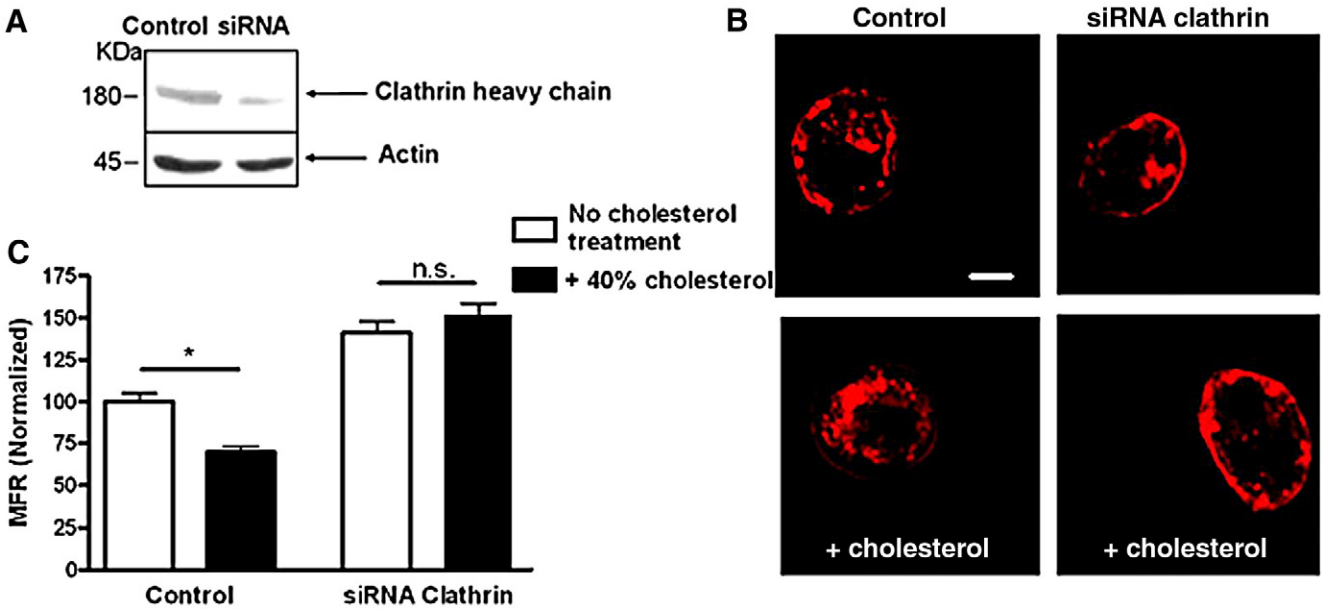

Fig. 3. Plasma membrane cholesterol promotes APP endocytosis through the clathrin-dependent pathway. (A) Measurement of clathrin expression in cells treated with siRNA against luciferase (control) or clathrin heavy chain (siRNA) using western blot. (B) Confocal images of cells co-transfected with APPsw and clathrin siRNA, treated or untreated with cholesterol. Scale bar: 3 µm. (C) Quantification of the membrane/intracellular fluorescence ratio (MFR) of HEK293 cells. MFR were compared using one-way ANOVA for 48 cells analyzed in 3 independent experiments. * $p < 0.05$.

3.4. Plasma membrane cholesterol controls Aβ secretion through direct regulation of clathrin-dependent endocytosis of APP

APP endocytosis is a key step in Aβ production [22]. Therefore, we investigated how the observed direct regulation of APP endocytosis by plasma membrane cholesterol was coupled to Aβ secretion. HEK293 cells stably overexpressing APPsw-GFP were acutely loaded with cholesterol for 30 min, and secretion of $A\beta_{40}$ and $A\beta_{42}$ was measured by ELISA eight hours after changing culture medium. Although a significant fraction of APPsw is processed through the secretory pathway [23], we observed a striking dose-dependent effect of cholesterol levels at the plasma membrane on both $A\beta_{40}$ and $A\beta_{42}$ secretion (Fig. 4A). To confirm that elevated Aβ secretion after cholesterol loading was indeed due to the increase of APP endocytosis, we tested the effects of clathrin siRNA on $A\beta_{42}$ secretion in HEK293 cells overexpressing APPsw-GFP. A 45% decrease of $A\beta_{42}$ secretion by clathrin siRNA was observed under normal cholesterol conditions, indicating that the steady-state production of $A\beta_{42}$ is critically dependent on the constitutive clathrin-dependent endocytosis of APP. Importantly, the elevated $A\beta_{42}$ secretion response to increased cholesterol at the plasma membrane was impaired in clathrin siRNA-treated cells, but not in luciferase siRNA-treated control cells (Fig. 4B). The difference in $A\beta_{42}$ secretion between cells transfected with clathrin siRNA treated or not with cholesterol may be due to the incomplete knockdown of clathrin and to the APP processing activity at the plasma membrane [7].

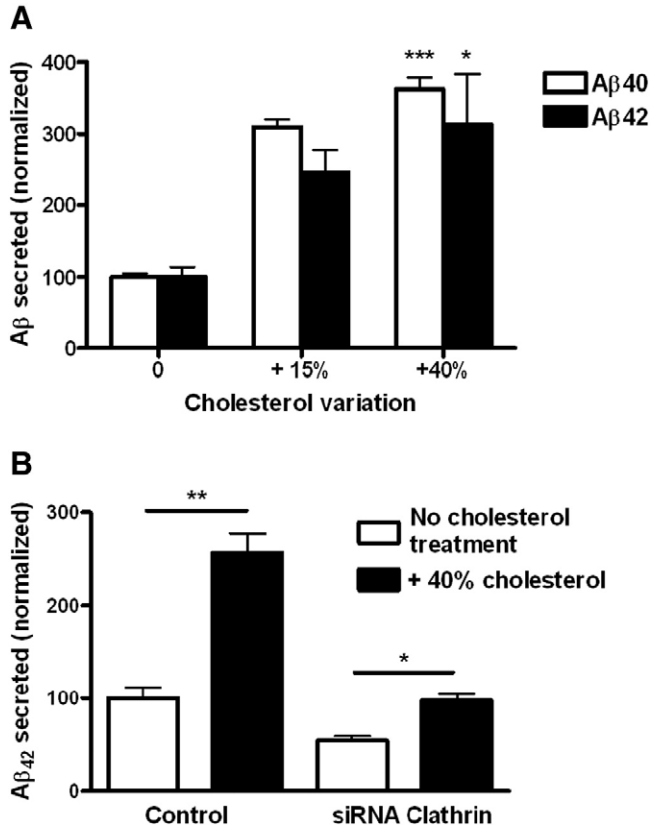

Fig. 4. Effect of plasma membrane cholesterol variations on Aβ secretion and APP cleavage. HEK293 cells stably transfected with APPsw-GFP were treated with MBCD-cholesterol (30 min at 37 °C). $A\beta_{42}$ and $A\beta_{40}$ secretions were quantified from the cell culture medium. (A) $A\beta_{42}$ and $A\beta_{40}$ secretions after 8 hours following treatment with cholesterol modulating drugs (n = 3 for HEK293 cells; 3 replicates in each experiment). $A\beta_{42}$ and $A\beta_{40}$ secretions were compared between different conditions using one-way ANOVA ($p<0.001$ for $A\beta_{40}$, $p<0.05$ for $A\beta_{42}$). (B) $A\beta_{42}$ and $A\beta_{40}$ secretions after 12 hours following MBCD-cholesterol treatment in HEK293 cells transfected with siRNA against luciferase (control) or clathrin heavy chain (siRNA) in 2 independent experiments; * $p<0.05$.

Since cholesterol is also enriched in caveolas containing caveolins and because caveolin-1 was shown to be expressed in the brain of elderly individuals [24], we wondered whether the effects of cholesterol on APP endocytosis were dependent on caveolin-1. We found that in HEK293 cells transfected with APPsw, siRNA against caveolin-1 did not significantly inhibit the effects of cholesterol on APP endocytosis and Aβ production (data not shown).

Thus, cholesterol content of the plasma membrane appears to tightly control $A\beta_{42}$ secretion through direct regulation of clathrin-dependent endocytosis of APP.

4. Discussion

Most studies related to APP processing have explored the effects of cholesterol extraction from the plasma membrane using MBCD in cell cultures. MBCD treatment results in a decrease of Aβ production [7,10,12–14]. In the present work, we explored the effects of a wide range of acute cholesterol changes (over 30 min) at the plasma membrane: from -40% to +40% as measured by colorimetric assay and labelling with the fluorescent dye filipin.

We showed that plasma membrane cholesterol regulates APP endocytosis and Aβ secretion in cell lines. These effects are dose-dependent and mediated through the clathrin-dependent and caveolin-independent endocytosis pathway. Additionally, we showed that cholesterol-dependent modulation of APP endocytosis is impaired by dynamin2, Eps15 and Rab5 mutants that inhibit clathrin-dependent endocytosis. Since the majority of Aβ secreted into the brain interstitial fluid is generated through endocytosis of APP [11], enhancement of clathrin-dependent APP endocytosis by cholesterol is likely the direct cause of the increased $A\beta_{42}$ secretion observed in our study.

We suggest that several clathrin-dependent endocytosis pathways exist, but one is more sensitive to cholesterol, thereby regulating APP endocytosis. Recently, the adaptor protein 2 was shown to regulate APP, but not transferrin receptor, endocytosis [10]. Further, endocytosis motifs are variable; the motif for APP differs from the one for the transferrin receptor [25,26].

The localization of membrane proteins within or outside cholesterol-enriched microdomains (lipid rafts) could also play an important role in endocytosis. Indeed, evidence indicates APP is partially localized in rafts, while the transferrin receptor is exclusively present outside the rafts [27]. Finally, recent papers proposed a strong interaction between APP and cholesterol: they either interact directly on residues located close to the transmembrane domain of APP [28,29], or indirectly through flotillin2, facilitating APP clustering to raft microdomains [10]. We suggest that lipid raft-associated APP internalizes through clathrin-mediated endocytosis, a process that is accelerated by an excess of cholesterol at the plasma membrane.

Increased cholesterol levels have been demonstrated in vulnerable brain regions of AD patients [2,3]. Further, cholesterol staining overlapped with β-secretase and Aβ, suggesting that cholesterol increases could be responsible for elevated Aβ deposition [3]. Cholesterol was also shown to contribute to increased Aβ production and accumulation in nerve terminals from AD brains [30]. More recently, AD mouse models overexpressing CYP46A1, the neuronal cholesterol 24-hydroxylase that allows the excretion of cholesterol through the blood brain barrier, had significantly reduced Aβ deposits [31]. The *in vivo* mechanisms underlying Aβ accumulation in sporadic AD are not well understood; we propose a scenario whereby increased cholesterol in AD brains could be responsible for increased APP endocytosis and overproduction of Aβ.

We showed here and confirmed in another study [19] that cholesterol induced the formation of enlarged early endosomes resembling those described in affected neurons from AD brains [32,33]. This effect could be directly related to the excess of cholesterol in AD brains. Alternatively, the formation of enlarged endosomes could result from the overproduction of the APP fragment

generated by β-secretase due to increased APP endocytosis after cholesterol treatment. Indeed, it was recently shown that βCTF induces the formation of enlarged early endosomes [34].

In summary, our results confirm a link between cholesterol levels and APP processing. These studies suggest a potential mechanism for the regulation of clathrin-mediated APP endocytosis by increased levels of cholesterol at the plasma membrane. Further, the observed enlarged early endosomes suggest cholesterol as a potential therapeutic target for AD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbalip.2010.05.010.

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