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Amyloid β -Protein Stimulates Trafficking of Cholesterol and Caveolin-1 from the Plasma Membrane to the Golgi Complex in Mouse Primary Astrocytes

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Abstract

The Golgi complex plays a key role in cholesterol trafficking in cells. Our earlier study demonstrated amyloid β -protein ($A\beta$) alters cholesterol distribution and abundance in the Golgi complex of astrocytes. We now test the hypothesis that the $A\beta$ -induced increase in Golgi complex cholesterol is due to retrograde movement of the cholesterol carrier protein caveolin-1 from the cell plasma membrane to the Golgi complex in astrocytes. Results with mouse primary astrocytes indicated that $A\beta_{1-42}$ -induced increase in cholesterol and caveolin abundance in the Golgi complex was accompanied by a reduction in cholesterol and caveolin levels in the plasma membrane. Transfection of DITNC1 astrocytes with siRNA directed at caveolin-1 mRNA inhibited the $A\beta_{1-42}$ -induced redistribution of both cholesterol and caveolin from the plasma membrane to the Golgi complex. In astrocytes not treated with $A\beta_{1-42}$, suppression of caveolin-1 expression also significantly reduced cholesterol abundance in the Golgi complex, further demonstrating the role for caveolin in retrograde transport of cholesterol from the plasma membrane to the Golgi complex. Perturbation of this process by $A\beta_{1-42}$ could have consequences on membrane structure and cellular functions requiring optimal levels of cholesterol.

Keywords

Caveolin; Cholesterol; Golgi Complex; Astrocytes; Alzheimer's Disease; Amyloid β -Protein

Introduction

Linkage between cholesterol and Alzheimer's disease (AD) has attracted considerable interest (Wolozin 2004; Blain and Poirier 2004; Hartmann 2006; Eckert et al. 2007). Several studies demonstrated cholesterol levels in modulating amyloid precursor protein (APP) synthesis and $A\beta$ production (Howlands et al. 1998; Bodovitz and Klein 1996; Simons et al. 1998; Runz et al. 2002). Lowering cholesterol levels, for example, reduces APP processing and $A\beta$ production and this interaction between cholesterol and $A\beta$ is reciprocal (Wood et al. 2003). $A\beta$ binds cholesterol with greater affinity as compared with phospholipids and fatty

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acids (Avdulov et al. 1997). A β alters cholesterol dynamics particularly cholesterol trafficking in astrocytes and neurons. Cholesterol efflux from rat hippocampal neurons to cyclodextrin is enhanced by A β ₁₋₄₀ (Liu et al. 1998). A β stimulates uptake of apoE-cholesterol complexes into astrocytes and neurons (Guillaume et al. 1996; Beffert et al. 1998). A β ₁₋₄₂ also increased cholesterol levels in the Golgi complex of DITNC1 astrocytes which was not due to a total change in cell cholesterol (Igbavboa et al. 2003).

The Golgi complex plays an integral role in lipid and protein trafficking (De Matteis et al. 2007). There is evidence that the cholesterol binding protein caveolin-1 cycles between the cell surface membrane and the Golgi complex (Smart et al. 1994; Conrad et al. 1995). Caveolin-1 is a 22-kDa protein associated with caveolae but this protein can also act independently of caveolae (Head and Insel 2007). Caveolin binds cholesterol and it is thought to be an important contributor to maintaining cholesterol homeostasis of cells (Murata et al. 1995; Smart et al. 1994; Conrad et al. 1995; Uittenbogaard and Smart 2000; Pol et al. 2001; Ito et al. 2002). Overexpression of caveolin-1 reduces cholesterol in the plasma membrane of Hs578T breast cancer cells, possibly due to internalization of caveolin-1 or caveolae (Chen et al. 2004). Movement of caveolin from the plasma membrane to the Golgi complex was observed when fibroblasts were treated with cholesterol oxidase (Smart et al. 1994). A subsequent study showed that caveolin-1 cycles constitutively between the plasma membrane and the Golgi complex and that this pathway may contribute to the trafficking of lipids including cholesterol (Conrad et al. 1995).

Our previous study demonstrated the ability for A β ₁₋₄₂ to increase cholesterol levels in the Golgi complex of DITNC1 astrocytes without altering levels of total cell cholesterol (Igbavboa et al. 2003). In this study, we test the hypothesis that the A β ₁₋₄₂-induced increase in Golgi complex cholesterol is due to retrograde movement of caveolin-1 from the cell plasma membrane to the Golgi complex in astrocytes. Caveolin-1 is abundant in astrocytes (Ito et al. 2002; Ge and Pachter 2004), and reduction of caveolin-1 protein expression can be achieved using specific siRNA. Results show that reduction of caveolin-1 protein expression by siRNA inhibited the A β ₁₋₄₂-induced redistribution of both cholesterol and caveolin-1 from the plasma membrane to the Golgi complex. These findings demonstrate the importance of caveolin-1 in regulating cholesterol distribution in the Golgi complex and plasma membrane of astrocytes. Perturbation of this process by A β could have consequences on membrane structure and cellular functions

Experimental procedures

Materials

NBD-cholesterol (22-(*N*-7-nitrobenz-2oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-chole-3- β -ol) and BODIPY TR-C₅-ceramide were obtained from Invitrogen-Molecular Probe (Carlsbad, CA). A β ₁₋₄₂ was purchased from American Peptide (Sunnyvale, CA).

All other chemicals unless specifically mentioned were purchased from Sigma.

Cell culture

Mouse cortical astrocytes were prepared from 1day-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) as we previously reported (Igbavboa et al. 2006). Briefly, cerebral cortices were dissected and meninges removed. Tissue was minced and then suspended in Tryple™ Express (Invitrogen) and incubated for 10 min at 37°C. The cell suspension in media was passed through a 20-gauge needle 5 times. Cells were filtered through 75 μ m nylon mesh, sedimented by centrifugation and then suspended in 10% (v/v) FBS in DMEM containing 100 IU/ml penicillin and 100 μ g/ml streptomycin, and transferred to culture flasks and maintained at 37°C, 5% CO₂ and 90% relative humidity. Medium was

changed after 24 h and then every second day. When cells reached confluence (around 1 week) flasks were gently shaken to remove microglia and oligodendrocytes. After shaking, cells were rinsed three times with phosphate-buffered saline (PBS), suspended in Tryple™ Express and treated in cell culture media as above and the cells were subcultured at 3×10^5 cells/75 mm flask. Using this method, we have shown that these cultures contain over 95% astrocytes, as determined by immunostaining for glial fibrillary acidic protein (Xu et al. 2003). Cells at 80–85% confluence were treated with medium containing 1% (v/v) lipoprotein-deficient serum replacing the 10% FBS and incubated for 16 h prior to experimentation. All experiments were done using confluent astrocytes.

Immortalized rat astrocytes (DITNC1) were purchased from American Type Culture Collection (ATCC, Manassas, VA). These cells were used primarily in the siRNA experiments. We have previously reported that A β_{1-42} increased cholesterol levels in the Golgi complex of DITNC1 rat astrocytes (Igbavboa et al. 2003).

Preparation of A β_{1-42}

In all experiments A β_{1-42} was prepared as described previously with small modification (Igbavboa et al. 2003). Briefly, 1 mg of the A β -protein was dissolved in 2.3 ml distilled water containing NH₄OH (1 μ L of 14.8 N Ammonium Hydroxide in 1 ml bidest water). The completely solubilized preparation was used immediately for treating cells without aging. In some experiments, oligomeric A β_{1-42} was prepared using the protocol as described (Dahlgren et al. 2002). For analysis, fresh A β_{1-42} and oligomeric A β_{1-42} were mixed with glycerol and separated with 15% non-denaturing gels using the Kaleidoscope protein standards (Bio-Rad). Bands were visualized using silver staining (Bio-Rad silver stain plus kit) and band density was quantified by densitometry using a Eagle Eye II video system and EagleSight software using procedures reported by our laboratory (Mason et al. 1999). Results of gel electrophoresis analysis indicated that the freshly solubilized A β without pretreatment had monomers and some lower molecular wt oligomers, mainly trimers, as compared to the higher molecular wt oligomers in the preparation as described (Dahlgren et al. 2002) (Fig. 1)

Localization of cholesterol in the Golgi complex of mouse primary astrocytes and DITNC1 rat astrocytes

The determination of cholesterol abundance in the Golgi complex of astrocytes was accomplished using confocal microscopy as reported previously (Igbavboa et al. 2003). Cells were grown on Lab-Tek™ chamber slides pretreated with Poly-D-Lysine. After incubating with NBD-cholesterol (8 μ M) for 1 h, and then washed three times with 1 ml of PBS, A β_{1-42} (1 μ M) was added to cells and incubated for 2 h. Following incubation with A β_{1-42} , cells were rinsed three times with PBS, and the Golgi marker BODIPY TR-C₅-ceramide (2 μ M) was added and incubated on ice for 30 min. The culture media was subsequently replaced with Hanks buffer (pH 7.4) and incubated at 37°C under relative humidity of 90% for 30 min. Cells were then washed with PBS, fixed with 4% (v/v) paraformaldehyde, and mounted for confocal microscopy using Gel/Mount (Biomedica Corp., Foster City, CA)

Confocal fluorescence imaging was performed on an Olympus Fluoview laser scanning confocal imaging system (Olympus America Inc., New York). Several images from each slide were captured using multiple photomultiplier tubes regulated by Fluoview 5.0 software (Olympus). Excitation of the fluorescent probes was accomplished using a 15-milliwatt krypton-argon lasers with 5-milliwatt output. An Olympus BX 50 fluorescence microscope was used to capture the images using an oil immersion objective. NBD-cholesterol was excited at 488 nm, and emission was recorded at 540 nm. BODIPY TR-C₅-ceramide was

excited at 568 nm, and emission was recorded at 598 nm. The captured images for the red and green channels were merged and appeared yellow, which is indicative of colocalization. Quantitative analysis of the colocalization of NBD-cholesterol and BODIPY TR-C₅-ceramide was determined by image processing using MetaMorph imaging system V4.3 from Universal Imaging Corp. (Downingtown, PA) and expressed as percent colocalization (Atshaves et al. 2000; Zhang et al. 1998; Igbavboa et al. 2003).

Localization of caveolin-1 in the Golgi complex of mouse primary astrocytes and DITNC1 rat astrocytes

Astrocytes (5×10^3 cells) were seeded on to an 8-well chamber slide (Nalge Nunc International, Naperville, IL) which was pretreated with Poly-D-Lysine. After incubation with A β_{1-42} for 2 h, cells were rinsed three times with PBS, and the Golgi marker BODIPY TR-C₅-ceramide (2 μ M) was added and incubated on ice for 30 min. The solution was replaced with Hanks' media and the slide placed in an incubator at 37°C for 30 min. Cells were then washed with PBS, fixed with 4% (v/v) paraformaldehyde then permeabilized with 0.1% (v/v) Triton X-100 at room temperature for 7 min. This was followed by blocking with 0.5% (w/v) BSA at room temperature for 1 h and then rinsed gently with PBS and treated with anti-caveolin-1 (Abcam Inc., Cambridge, MA) for 1.5 h. Cells were rinsed gently 3X with PBS then treated with (20 μ g/well) FITC-goat anti-mouse antibody IgG in PBS containing 0.1% (v/v) goat serum for 2.0 h and then rinsed gently 3X and mounted for confocal microscopy using Gel/Mount (Biomedica Corp., Foster City, CA). Imaging and quantification of colocalization were similar as described for cholesterol except FITC was excited at 495 nm and emission was recorded at 520 nm.

Caveolin-1 protein determination using Western blot analysis

Isolated cells were treated with RIPA buffer (Pierce Biotechnology Inc., Rockford, IL) and protein concentration was determined using the Bradford method. The suspension was treated with Electrophoresis Sample buffer 2X (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and proteins separated by 11.5% (w/v) SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and blocked with 2% (w/v) BSA and 3% (v/v) milk for 1 h before treating with polyclonal primary rabbit anti-mouse caveolin-1 antibodies for 2 h. The membrane was gently rinsed (4X) and then treated with HRP-conjugated secondary goat anti-rabbit IgG antibody (Pierce, Rockford, IL) for 2 h. The membrane was washed 4X and the immunoreactivity was visualized with Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The membrane was then placed on Kodak film and developed using Agfa Developer CP1000. Band density was quantified by densitometry using Eagle Eye II video system and EagleSight software.

Caveolin-1 knockdown by siRNA transfection

DITNC1 cells were harvested, counted and then treated with predesigned caveolin-1 siRNA (Ambion Inc., Austin, TX) with the following sequences (5'→3') CCAUCAAUUUGGAGACUAUtt (sense) and AUAGUCUCCAAAUUGAUGGtg (antisense). Ambion procedures were used for transfection with modification, i.e., 40 μ l of 100 μ M siRNA solution was added to 160 μ l of Opti-MEM-1 and 80 μ l Siport™ NeoFX™ was added to another 320 μ l Opti-MEM1, mixed separately and let stand at room temperature for 10 min. An equal volume of each solution was then mixed together and left at room temperature for 20 min. Cell suspension (1 million per 19.6 ml media) was mixed with 400 μ l of the above mixture and cultured in 25 cm³ flasks (0.2×10^6 cells $\approx$ 4 ml) or slide chambers (160 μ l $\approx$ 8×10^3 cells) for 2 days. Control samples were treated only with Siport™ NeoFX™ as above and incubated at 37°C, 90% relative humidity and 5% CO₂. Incubation time for transfection reagents was 24 h, at which time the medium was replaced with DMEM.

Caveolin-1 mRNA expression

Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine caveolin-1 mRNA expression levels in samples treated with or without siRNA. Briefly, total RNA from each sample was isolated using magnetic ChargeSwitch® (Invitrogen Corp., Carlsbad, CA) as per the manufacturer's instructions. Total RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) and the manufacturer's instructions. For RT-PCR, 0.15 µg total RNA was used with the Invitrogen SuperScript™ III One-Step RT-PCR System, with platinum® *Taq* DNA polymerase and the PTC-100 Peltier Thermal Cycler (Bio-Rad). The primer sequences for caveolin-1 were 5'-AGGGAAACCTCCTCAGAGC-3' (sense) and 5'-GTGCGCGTCATACACTTGC-3' (antisense). QuantumRNA™ 18S (Ambion Inc., Austin, TX) was used as an internal control. The incubation parameters were as follows: preheat-1 Cycle-54.4°C for 30 min; denaturing period-1 cycle at 94°C for 2 min; amplification period-35 cycles (94°C for 15 s to denature, 54.4°C for 30 s for annealing and 68°C for 1.5 min to extend). The PCR cycle was terminated by extension at 60°C for 5 min and product resolved on a 2% (w/v) agarose gel with 8% (w/v) ethidium bromide. Bands were visualized with UV light and densitometric readout using a Strategene Eagle Eye II densitometer.

Astrocyte plasma membrane isolation

Astrocyte plasma membranes were isolated and characterized using procedures previously reported by our laboratory (Igbavboa et al. 2003; Arelin et al. 2002; Wood et al. 1995). Briefly, cells were suspended in 5mM Tris Buffer Solution (pH 8.5) together with protease inhibitors and transferred into a Potter-type homogenizer and homogenized with approximately 20 strokes. The suspension was then kept on ice and vortexed every 15 min for 20 sec for a period of 1.25 h. The suspension was then centrifuged at 35000 × g for 20 min. The pellet was resuspended in water and underlayered with 0.75 M sucrose buffer containing 2.5 mM Tris, 3 mM HEPES and 0.25 mM EDTA (pH 7.4). The gradient was centrifuged at 42000 × g for 30 min in a Beckman LC Ultra Centrifuge rotor using a SW 55Ti rotor. The membrane layer appearing between the water and sucrose phase was removed and centrifuged at 35000 × g for 20 min using a Sorvall RC-5C centrifuge. The pellet was suspended in 50 mM Tris buffer (pH 7.4) and protein concentrations were determined using the Bradford assay. The cholesterol content in these samples was determined enzymatically in a microassay using a diagnostic kit from Roche Molecular Biochemicals (Palo Alto, CA) as reported earlier (Igbavboa et al. 2003). Also, the Ca²⁺/Mg²⁺ and the Na⁺/K⁺ ATPase activities of the plasma membrane were determined. (Wood et al. 1995)

Gel electrophoresis of Aβ₁₋₄₂

Solution of fresh Aβ₁₋₄₂ and oligomeric Aβ₁₋₄₂ were mixed with glycerol respectively and separated with 15% nondenaturing gels. The following protein reference standards were used: insulin, aprotinin, lysozyme, soybean trypsin inhibitor, carbonic anhydrase. Bands were visualized using silver staining (Bio-Rad silver stain plus kit) and band density was quantified by densitometry using a Eagle Eye II video system and EagleSight software using procedures reported by our laboratory (Mason et al. 1999).

RESULTS

Freshly prepared Aβ₁₋₄₂ induced increase localization of cholesterol in the Golgi complex of mouse primary astrocytes

Experiments in this study tested the hypothesis that Aβ₁₋₄₂ increases cholesterol levels in the Golgi complex due to the retrograde movement of the cholesterol carrier protein,

caveolin-1, from the plasma membrane to the Golgi complex. In the first experiment, we compared cholesterol localization in Golgi of mouse primary astrocytes by taking confocal images of NBD-cholesterol and the Golgi marker BODIPY TR-C₅-ceramide. As shown in Fig 2A, incubation of astrocytes with freshly prepared A β ₁₋₄₂ showed higher cholesterol in the Golgi complex as compared to that with the oligomeric preparation. Analysis of colocalization data using the MetaMorph software and expressed as percent colocalization shows that fresh A β ₁₋₄₂ significantly ($p \leq 0.001$) increased NBD-cholesterol colocalization in the Golgi complex as compared with control astrocytes and oligomeric A β ₁₋₄₂ (Fig. 2B). The stimulatory effect of fresh A β ₁₋₄₂ on cholesterol localization in the Golgi complex of mouse primary astrocytes was similar to what we reported earlier in DITNC1 rat astrocytes and it was also observed that scrambled protein A β ₄₂₋₁ had no effect on colocalization (Igbavboa et al. 2003). Oligomeric A β ₁₋₄₂ did not alter colocalization of cholesterol and the Golgi marker and therefore subsequent experiments were performed using only fresh A β ₁₋₄₂.

A β ₁₋₄₂ reduces cholesterol abundance in the plasma membrane of mouse primary astrocytes

The A β ₁₋₄₂-induced increase in cholesterol levels in the Golgi complex could be due to an increase in cholesterol synthesis, redistribution among intracellular organelles or uptake into the cell. It can be seen in Figure 3 that cholesterol levels were similar in the cell lysates of control and A β ₁₋₄₂ treated cells as determined by an enzymatic assay. This finding does not support the effect of A β ₁₋₄₂ to enhance cholesterol synthesis or uptake. The plasma membrane contains the majority of cellular cholesterol and there is evidence that perturbation of the membrane can induce movement of cholesterol from the cell surface to intracellular cholesterol pools (Slotte and Bierman 1988; Slotte et al. 1989). Data in Figure 3 show that incubation of primary astrocytes with A β ₁₋₄₂ significantly ($p \leq 0.05$) reduced cholesterol levels in the plasma membrane as compared with control cells, thus suggesting a redistribution of cholesterol in astrocytes.

Fresh A β ₁₋₄₂ enhanced localization of caveolin-1 in the Golgi complex of mouse primary astrocytes

A retrograde pathway from the plasma membrane to the Golgi complex has been proposed previously involving the cholesterol-carrier protein caveolin-1 (Smart et al. 1994; Conrad et al. 1995). In this study, confocal images were used to test whether treatment of astrocytes with A β ₁₋₄₂ alter colocalization of caveolin-1 in the Golgi. As shown in Fig 4A and B, there was significant ($p \leq 0.01$) increase in colocalization of caveolin-1 fluorescent antibodies with the Golgi marker BODIPY TR-C₅-ceramide in cells incubated with A β ₁₋₄₂ as compared with control cells. Furthermore, the increase in caveolin-1 localization in the Golgi complex of A β ₁₋₄₂-treated cells was offset by a significant ($p \leq 0.01$) time-dependent reduction in caveolin-1 protein abundance in the plasma membrane as determined by Western analysis (Figure 5).

Suppression of caveolin-1 mRNA and protein levels by siRNA in DITNC1 rat astrocytes

In order to test the role of caveolin-1 in transport of cholesterol, we used the siRNA protocol to down-regulate this protein in the immortalized rat astrocytes (DITNC1). In an earlier study (Ge and Pachter 2004), caveolin-1 protein levels in astrocytes were reported to be reduced by approximately 90% after 48 h following caveolin-1 siRNA transfection. In the present study, astrocytes incubated with caveolin-1 siRNA for 48 h showed a 74% reduction of mRNA as determined by RT-PCR and 80% of protein as determined by Western analysis (Fig. 6B).

Reduction of caveolin-1 protein expression inhibited the A β ₁₋₄₂-induced increase in cholesterol in the Golgi complex

Similar to the data in Figure 2A with primary astrocytes, it can be seen that A β ₁₋₄₂ significantly ($p \leq 0.002$) increased cholesterol localization in the Golgi complex of DITNC astrocytes from 13% to 26% (Figure 7A, panels f versus c and 7B). However, cells treated with siRNA to lower caveolin-1 protein showed lower percentage of cholesterol in the Golgi (5% as compared to 13%)(Figure 7B). Furthermore, siRNA transfected cells showed little or no respond to A β ₁₋₄₂ in mediating increase in cholesterol localization in Golgi (Figure 7B). These findings indicate that caveolin-1 is a contributor to maintaining cholesterol in the Golgi complex.

Similar to primary astrocytes, A β ₁₋₄₂ induced increase in colocalization of caveolin-1 in the Golgi in DITNC cells as compared with control cells (Figure 8A, c versus f, and B). Treatment with caveolin-1 siRNA significantly reduced ($p \leq 0.002$) the levels of caveolin-1 in the Golgi and the stimulatory effects of A β ₁₋₄₂ on caveolin-1 colocalization with the Golgi (Fig. 8A, l versus f, and B). It has been previously proposed that caveolin-1 may cycle between the Golgi complex and the plasma membrane (Smart et al. 1994; Conrad et al. 1995) and the reduced colocalization of caveolin-1 and the Golgi marker observed in the present study is consistent with such a pathway.

Discussion

There are several different lines of evidence demonstrating an interaction between A β and cholesterol. Much of that work has focused on the effects of altering cholesterol levels both *in vivo* and *in vitro* and the subsequent production of A β (Wolozin 2004; Blain and Poirier 2004; Hartmann 2006; Eckert et al. 2007). This interaction between A β and cholesterol however is reciprocal and there is evidence that cholesterol dynamics such as cholesterol trafficking are affected by A β . Our study here confirms previous results that the Golgi complex plays a key role in lipid and protein trafficking and that A β ₁₋₄₂ increases cholesterol levels in the Golgi complex of astrocytes (Igbavboa et al. 2003). There is evidence that the cholesterol carrier protein caveolin may cycle between the plasma membrane and the Golgi complex and it has been suggested that such a pathway may contribute to lipid trafficking (Smart et al. 1994; Conrad et al. 1995). In the present study, we provided information linking this cholesterol trafficking in astrocytes with caveolin-1.

Data in this present study indicate that freshly prepared A β ₁₋₄₂ increases colocalization of NBD-cholesterol and a fluorescent marker of the Golgi complex in mouse primary astrocytes and these data are in agreement with an earlier study using immortalized rat DITNC1 astrocytes (Igbavboa et al. 2003). It is intriguing that although the fresh A β indicated presence of oligomers, results are different as compared with the conventional oligomeric preparation as described (Dahlgren et al. 2002). A possibility for the difference in results is that the freshly prepared A β ₁₋₄₂ contained mainly trimers whereas the oligomeric preparation has more tetramers and higher molecular polymer species.

Our results demonstrated that A β ₁₋₄₂ caused a net transfer of cholesterol from the plasma membrane to the Golgi complex which is mediated by the cholesterol carrier protein caveolin-1. Clearly, suppression of caveolin-1 protein expression by siRNA significantly inhibited the A β ₁₋₄₂ stimulated increases in both cholesterol and caveolin-1 levels in the Golgi complex. Caveolin-1 is a 22-KDa protein associated with caveolae and this protein binds cholesterol and is thought to be a key contributor to cholesterol homeostasis (Murata et al. 1995; Smart et al. 1994; Conrad et al. 1995; Uittenbogaard and Smart 2000; Pol et al. 2001; Ito et al. 2002). A pathway from the plasma membrane to the Golgi complex for transporting sphingolipids has been previously proposed and it was suggested that this

pathway may also regulate cholesterol distribution in cells. We now show that cholesterol abundance is lower in the Golgi complex of caveolin-1 siRNA treated astrocytes, consistent with a role for caveolin-1 in the trafficking of cholesterol. Caveolin-1 has been reported to cycle between the cell surface membrane and the Golgi complex (Smart et al. 1994; Conrad et al. 1995) and this cycling may regulate the distribution of cholesterol between the plasma membrane and the Golgi complex (Fielding and Fielding 1997).

Although beyond the focus of the present study, it is reasonable to search the answer to how A β ₁₋₄₂ stimulates movement of caveolin-1 out of the plasma membrane. A potential explanation may involve A β increasing membrane fluidity and oxidative stress. We and others have reported that A β increases synaptic plasma membrane fluidity although there are some studies finding opposite effects (Wood et al. 2003). Movement of caveolin from the plasma membrane to the Golgi complex was observed when fibroblasts were treated with cholesterol oxidase (Smart et al. 1994) and incubation of synaptic plasma membranes with cholesterol oxidase increases membrane fluidity (Wood et al. 1995). A β increases lipid and protein oxidation which would be consistent with the effects of oxidation on caveolin trafficking (Butterfield et al. 2007). Our earlier study indicated that A β ₁₋₄₂ alter cholesterol homeostasis in the Golgi complex; cholesterol levels were reduced in the *cis*-medial regions but increased in the *trans*-region of the astrocyte Golgi complex (Igbavboa et al. 2003). There is evidence that protein transport within the Golgi complex is dependent on an optimal level of cholesterol (Stüven et al. 2003). In addition, it is well-established that cholesterol plays a key role in membrane fluidity, lipid domains and modulates activity of certain proteins (Wood et al. 2007). A reasonable conclusion is that A β -induced changes in cholesterol homeostasis in the Golgi complex and plasma membrane would have detrimental effects on cellular functions.

There is a body of data indicating that certain cholesterol carrier proteins are associated with Alzheimer's disease. The most recognized protein in that regard is apolipoprotein E4, a protein that binds and transports cholesterol and other lipids, and apolipoprotein E4 has been identified as a risk factor for familial and sporadic Alzheimer's disease (Strittmatter et al. 1993; Strittmatter and Roses 1996). A β ₁₋₄₂ increases apoE protein levels in astrocytes (LaDu et al. 2000; Igbavboa et al. 2003; Igbavboa et al. 2006) which could affect cholesterol trafficking. Other data also suggest that ABCA1, a protein of the ATP-binding cassette family of transporters which transports cholesterol and phospholipids may play a role in Alzheimer's disease (Hirsch-Reinshagen and Wellington 2007). The sterol carrier protein SCP-x/Pro-SCP-2 gene was shown to have transcriptional activity which contributed to the regulation of γ -secretase activity required for the generation of A β (Ko and Puglielli 2007). A recent study found that caveolin-1 mRNA and protein levels were increased in brains of Alzheimer's disease patients as compared with control brains (Gaudreault et al. 2004) and it was suggested that such an increase could have a dysfunctional effect on cholesterol homeostasis in the plasma membrane. The present report demonstrates that A β ₁₋₄₂ reduces caveolin-1 levels in the plasma membrane and increases its abundance in the Golgi complex. These results add to the growing body of data demonstrating that cholesterol carrier proteins are targets of A β and Alzheimer's disease which may affect cellular cholesterol homeostasis and play a role in the pathophysiology of Alzheimer's disease.

Abbreviations

Aβ	amyloid beta-protein
AD	Alzheimer's disease
APP	Amyloid precursor protein

RT-PCR	reverse transcription polymerase chain reaction
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum

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Figure 1. Fresh and oligomeric Aβ₁₋₄₂

Fresh Aβ₁₋₄₂ was prepared as indicated in “Experimental procedures” and oligomeric Aβ₁₋₄₂ was prepared using procedures reported by our lab and others (Johnson-Anuna et al. 2007; Dahlgren et al. 2002; Stine et al. 2003). The samples were examined by electrophoresis on 15% nondenaturing gels as described under “Experimental procedures.” Lane 1 is the standards insulin, aprotinin, lysozyme, soybean trypsin inhibitor, carbonic anhydrase. Lane 2 is fresh Aβ₁₋₄₂ and Lane 3 is oligomeric Aβ₁₋₄₂.

A.

B.

Figure 2. Localization of cholesterol in the Golgi complex of mouse primary astrocytes incubated with Aβ₁₋₄₂

A: Shown are confocal images of astrocytes labeled with NBD-cholesterol (a,d,g, green) and BODIPY TR-C₅-ceramide (b,e,h, red) and treated without or with fresh Aβ₁₋₄₂ or oligomeric Aβ₁₋₄₂ for 2 h. Colocalization of NBD-cholesterol and BODIPY TR-C₅-ceramide is shown in panels c,f, and i. **B:** Quantitative measurements of colocalization of NBD-cholesterol and BODIPY TR-C₅-ceramide were performed using MetaMorph software and data were expressed as percent colocalization. Data are means ± S.E. (n = 3 independent experiments). *, p ≤ 0.001 as compared with control astrocytes and oligomeric Aβ₁₋₄₂.

Figure 3. Cholesterol levels in cell lysate and plasma membranes from mouse primary astrocytes incubated with Aβ₁₋₄₂

Cholesterol levels were determined in a microassay kit (Roche Molecular Biochemicals) according to the manufacturer's instructions and procedures reported by our laboratory (Igbavboa et al. 2003; Igbavboa et al. 2006). Data are means ± S.E. (n = 3–4 independent experiments). *, p ≤ 0.05 as compared with control plasma membranes.

A.

B.

Figure 4. Localization of caveolin-1 in the Golgi complex of mouse primary astrocytes incubated with A β_{1-42}

A: Shown are confocal images of astrocytes labeled with anti-cav-1 primary antibody FITC-anti-IgG secondary antibody (a,d green) and BODIPY TR-C₅-ceramide (b,e red) and treated without or with A β_{1-42} for 2 h. Colocalization of caveolin-1 and BODIPY TR-C₅-ceramide is shown in panels (c,f, yellow). Quantitative measurements of colocalization of caveolin-1 and BODIPY TR-C₅-ceramide were performed using MetaMorph software and data are expressed as percent colocalization. Data are means $\pm$ S.E. (n = 3 independent experiments).

*, p $\leq$ 0.01 as compared with control astrocytes.

Figure 5. Aβ₁₋₄₂ reduces caveolin-1 protein abundance in the plasma membrane of mouse primary astrocytes

Astrocytes were treated as described under “Experimental procedures”. Levels of caveolin-1 protein were determined by Western analysis and densitometry. Data of cells treated with Aβ₁₋₄₂ are the levels of caveolin-1 relative to control cells and are the means ± S.E. from three to four independent experiments. *, $p \leq 0.01$, **, $p \leq 0.001$ as compared with control astrocytes; + $p \leq 0.01$ as compared with the earlier time points for Aβ₁₋₄₂-treated astrocytes. The inset shows a Western blot of a representative experiment

Figure 6. Caveolin-1 mRNA expression and protein levels are reduced by caveolin-1 siRNA in DITNC1 astrocytes

Astrocytes were treated as described under “Experimental procedures.” **Panel A.** Relative caveolin-1 mRNA levels (18S mRNA used as an internal control) were measured using RT-PCR and densitometry was used to determine changes in relative caveolin-1 mRNA levels following caveolin-1 siRNA transfection. **Panel B.** Relative caveolin-1 protein levels in cell lysate were determined by Western analysis and densitometry. The insets show a RT-PCR gel (panel A) and a Western blot (panel B) from representative experiments. Data are the means $\pm$ S.E. from three to four independent experiments. * $p \leq 0.01$ as compared to the control astrocytes.

Figure 7. Caveolin-1 siRNA reduces the effect of A β_{1-42} on localization of cholesterol in the Golgi complex of DITNC1 astrocytes

Astrocytes were transfected with caveolin-1 siRNA or transfection reagent alone for 48 h after which time cells were incubated with A β_{1-42} for 2 h as described under “Experimental procedures.” **A:** Shown are confocal images of astrocytes labeled with NBD-cholesterol (a,d,g,j, green) and BODIPY TR-C₅-ceramide (b,e,h,k, red) and treated without or with A β_{1-42} . Colocalization of NBD-cholesterol and BODIPY TR-C₅-ceramide is shown in panels c,f,i,l. **B:** Quantitative measurements of colocalization of NBD-cholesterol and BODIPY TR-C₅-ceramide were performed using MetaMorph software and data are expressed as percent colocalization. Data are means $\pm$ S.E. (n = 3 independent experiments).

*, $p \leq 0.002$ as compared with control astrocytes and caveolin-1 siRNA and $A\beta_{1-42}$ treated astrocytes; +, $p \leq 0.005$ as compared with control astrocytes.

Figure 8. Caveolin-1 siRNA reduces effects of Aβ₁₋₄₂ on localization of caveolin-1 in the Golgi complex of DITNC1 astrocytes

Astrocytes were transfected with caveolin-1 siRNA or transfection reagent alone for 48 h after which time cells were incubated with Aβ₁₋₄₂ for 2 h as described under “Experimental procedures.” **A:** Shown are confocal images of astrocytes incubated with anti-cav-1 primary antibody and FITC-anti-IgG secondary antibody (a,d,g,j, green) and BODIPY TR-C5-ceramide (b,e,h,k, red) and treated without or with Aβ₁₋₄₂. Colocalization of caveolin-1 and BODIPY TR-C5-ceramide is shown in panels c,f,i,l. **B:** Quantitative measurements of colocalization of caveolin-1 and BODIPY TR-C5-ceramide were performed using MetaMorph software and data are expressed as percent colocalization. Data are means ± S.E. (n = 3 independent experiments). *, p ≤ 0.02 as compared with control astrocytes; **, p ≤ 0.002 as compared with Aβ₁₋₄₂-treated astrocytes; +, p ≤ 0.005 as compared with control astrocytes.