The Relationship between Cholesterol and Tau Protein Expression in Human Neuroblastoma SH-SY5Y Cells

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ABSTRACT

Microtubule associated Tau protein is one of pathogenic factors of Alzheimer's disease (AD) since phosphorylated Tau protein accumulation is found inside neurons in AD brains. Recently, some reports revealed the direct linkage between apolipoprotein E cholesterol and Tau protein phosphorylation and polymerization in vivo. This work aimed to study the relationship between cholesterol and Tau protein expression in neurons. The human neuroblastoma SH-SY5Y cells were grown in a cholesterol-containing medium for either two or four days. Total proteins were extracted and immunoblotted with an anti-Tau antibody. Cholesterol had not altered the Tau expression in the two-day treatment, but diminished Tau expression occurred with increased cholesterol concentration in the four-day treatment. This finding may contribute to basic knowledge about pathogenesis caused by cholesterol in Alzheimer's disease

Keywords: Cholesterol, Tau protein, Neuroblastoma SH-SY5Y cells

INTRODUCTION

Alzheimer's disease (AD), the most common dementia in aging, has been proposed to be attributed to the gradual degeneration of neurons progressing to disability in memory, learning and cognition. Characteristic patterns of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) accumulation as well as the disappearance of neurons and synaptic regions [Sun et al, 2015].

Presently, there are several significant pathogenic causes and progression of the neurodegenerative process underlining AD. One of the noticeable factors is Tau protein, as the prominent occurrence of NFTs of hyperphosphorylated Tau proteins accumulate inside the neurons of the AD brain. Tau is a microtubule associating protein, which is heat resistant and was discovered more than thirty years ago. Normally, Tau protein can facilitate the assembly of microtubules [Weingarten et al, 1975]. In humans, the Tau gene is composed of 16 exons which are 100 kb long at the 17q21 region of chromosome 17 [Neve et al, 1986]. In the brain, there are six isoforms of Tau proteins consisting of 352 to 441 amino acid residues with

molecular weight ranging from 45 to 65 kDa [Lee et al, 1989]. Tau proteins also assist in microtubule stability in neurons [Weingarten et al, 1975]. In a pathogenic condition, Tau proteins are also expressed in other non-neural cells such as glial cells [Chin and Goldman, 1996]. Some reports have demonstrated that Tau protein is one of pivotal factors contributing to AD. Those studies reveal the relationship between the progression of NFT numbers and positions and the decline of cognitive function and the increase of severity of AD [Arriagada et al, 1992].

Cholesterol occurs in the highest proportion in the brain, (containing 25% of all cholesterol in the body) even though brain weight is only 2% of body weight. Apolipoprotein E (APOE), constructed of 299 amino acids, acts as a cholesterol transporter, not only in the blood circulation system, but also in the central nervous system (CNS) [Lund-Katz and Phillips, 2010]. Some studies have demonstrated that the amount of APOE is significantly directly related to Tau and phosphorylated Tau levels in AD patients [Leoni et al, 2010; Shafaati et al, 2007]. Other studies have reported that a sufficient amount of APOE leads to the significant enhancement of Tau phosphorylation [Glockner et al, 2011] and a lack of cholesterol due to an obstruction of cholesterol synthesis in cultured neuronal cells. This is attributed to Tau hyperphosphorylation and microtubule depolymerization synchronously, which in turn leads to axon degeneration [Fan et al, 2001].

However, the causative effect of cholesterol in AD pathogenesis remains uncertain. This work studied the relationship between cholesterol and Tau protein expression in cultured human neuroblastoma SH-SY5Y cells in case this knowledge could form a basic undertsanding of AD pathogenesis.

METHODOLOGY

Cell culture

Human neuroblastoma SH-SY5Y cells (ATCC; CRL-2266) were brought up in a 75 cm^2 flask containing DMEM/F12 medium (Gibco) with 10% fetal bovine serum (Capricon Scientific) and incubated in one atmosphere of pressure at 37 °C. The cells were refreshed with new medium every 2 to 3 days and were subcultured when the cell density reached 2 - 4 x 10^6 of viable cells/ mL (at 80% confluence). When the cells reached 100% confluence, the cultures were differentiated by exposure to 20 µM retinoic acid for 4 days. The cells were then shifted to mediums containing 0, 150, 250, or 300 µM of cholesterol (Sigma Aldrich) and cultured further for either 2 or 4 days.

Antibodies

Anti-Tau monoclonal antibody (Clone Tau-5, MAB361, Millipore) recognized phosphorylated and non-phosphorylated isoforms of microtubule-associated protein Tau. Anti-β-tubulin monoclonal antibody (Clone KMX-1, MAB3408, Millipore) reacted specifically with β-tubulin. These are primary antibodies applicable for immunoblotting techniques and all have species reactivity for humans. By the same method, goat anti-mouse IgG peroxidase-conjugated affinity-purified antibody (AP181P, Millipore) was used as a secondary antibody for following detection.

Immunoblotting

The cells from each flask were harvested and the cell suspension was removed to a sterilized 15 mL centrifuge tube. Then the cell suspension was centrifuged at 180-225 xg for 5 min. The supernatant was then decanted and the cell pellets were collected. The pellets were lysed with ice-cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP=40, 1% sodium deoxycholate, 0.5% SDS, 1X protease inhibitor cocktail (VMR, Amresco) and 1X phosphatase inhibitor (Bio Basic Canada TNC) by at least 5×10^6 cells / ml RIPA buffer, and were incubated on ice for 30 min. The cell suspension was centrifuged at 16,000 xg at 4 ºC for 15 min for collection of the protein part of the supernatant. The total protein concentrations were determined using Quick StartTMBradford 1X Dye Reagent (Bio-Rad) by measuring absorbance at 595 nm. After that, an equal amount of total protein (4 µg of total protein) was resolved on 8% SDS-polyacrylamide gel and then transferred onto a polyvinylidenedifluoride membrane. This membrane was incubated in a blocking buffer containing 10 mM Tris pH 7.5, 100 mM NaCl, 0.05% (v/v) Tween 20 (TBST) plus 5% drymilk (w/v) overnight at 4 ºC. Then the primary antibodies: anti-Tau (1:500) or anti-β-tunbulin (1:500) were incubated for 3 hours at 4 ºC. The membranes were then washed three times in TBST (5 min/each). Subsequently, the membranes were further incubated in goat anti-mouse IgG peroxidase-conjugated secondary antibody (1:2500) for 1 hour at room temperature. The Western Blots were analyzed using LuminataTMForte Western HRP substrate (Millipore) following the manufacturer's instructions and then the protein bands of antigen expression were detected by Image QuantTMLAS 500 (GE Healthcare). Quantitative assay of antigen expression was based on density measurement of protein bands, using the Image J program. Tau immunoreactivity was derived from normalizing the amount of Tau expression by the amount of corresponding β-tubulin expression. All of the data was analyzed by a statistical one-way ANOVA with $\alpha = 0.05$.

RESULTS AND DISCUSSION

In this work, protein bands of Tau expression were detected at a size of approximately 48 kDa. After two-day cholesterol exposure, the Tau expression altered in an uncertain pattern when cholesterol concentrations were increased as shown in Figure 1A, while Tau immunoreactivity at each cholesterol concentration did not differ, as shown in Figure 1B. After four-day cholesterol exposure, Tau expression tended to have a declining pattern with increasing cholesterol concentrations as shown in Figure 2A, but Tau immunoreactivity at each cholesterol concentration was not statistically different as shown in Figure 2B.
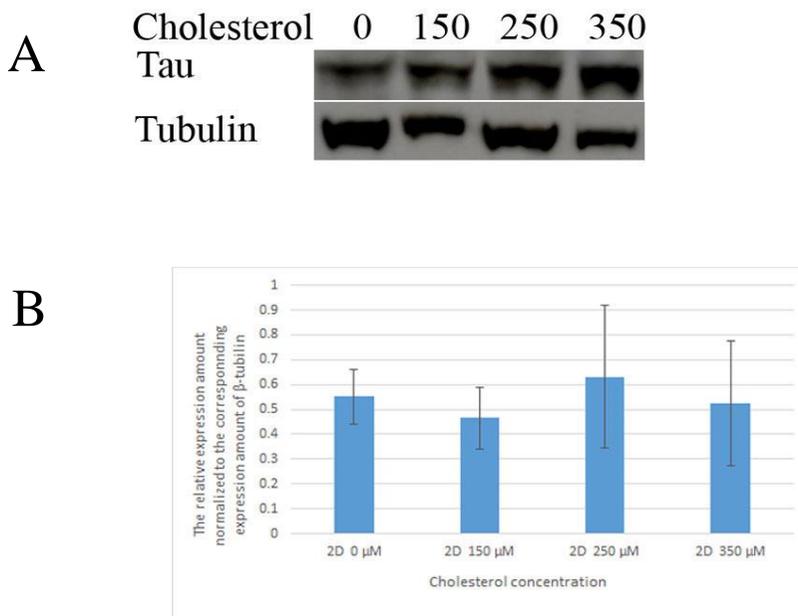

Figure 1. The effect of cholesterol on Tau expression in the two-day experiment. (A) The immunoblot of Tau expression at various cholesterol concentrations. (B) The Tau immunoreactivity at various cholesterol concentrations. Each group corresponds to the mean + SEM of seven independent experiments. Analysis was done by One-way ANOVA with $P > 0.05$.
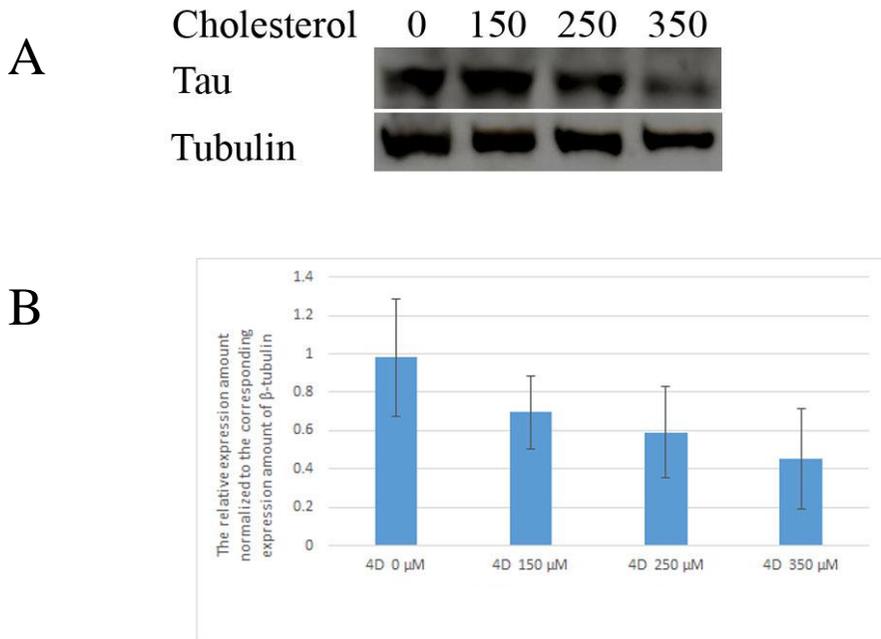
A

B

Figure 2. The effect of cholesterol on Tau expression in the four-day experiment. (A) The immunoblot of Tau expression at various cholesterol concentrations. (B) The Tau immunoreactivity at various cholesterol concentrations. Each group corresponds to the mean + SEM of seven independent experiments. Analysis was done by One-way ANOVA with $P > 0.05$.

Naturally, Tau proteins promote the assembly of microtubules and the stability of the neurons. Several isoforms of Tau proteins were synthesized from a single gene by alternative mRNA splicing. This leads to a different isoform expression in each cell type and at each stage. The Tau primary structure expected by molecular cloning has 31 or 32 amino acids with 3 or 4 repeating units at the carboxyl-end half of the molecule. These repeating units contain microtubule binding sites [Aizawa et al, 1989]. Additionally, each isoform contains an insertion of 0 (0N), 29 (1N), or 58 (2N) amino acids. Combining the amino acid insertion (0N, 1N, or 2N) with repeating units (3R or 4R) gives human Tau proteins six isoforms [Goedert et al, 1989]. The shortest human Tau isoform has 357 amino acids and contains only three repeating units (0N3R), while the longest human Tau isoform has 441 amino acids and contains both four repeating units and 58 amino acid insertions (2N4R). By analyzing human brains protected with RNase, mRNAs encoding three-repeat containing isoforms are found in both fetal and adult brains, but mRNAs encoding four-repeat containing isoforms as well as amino-terminal insertion (1N or 2N) isoforms are found only in the adult brain and not in the fetal brain. A set of six Tau isoforms on SDS-PAGE gel can be viewed at a molecular weight of 48-67 kDa [Goedert et al, 1990]. In this study, approximately 48 kDa of Tau protein was detected from human neuroblastoma SH-SY5Y cells, which may belong to the shortest 0N3R isoform of Tau proteins detectable on SDS-PAGE gel.

In a recent previous study, the viability of neuroblastoma SH-SY5Y cells had a tendency to increase when grown in 150 µM cholesterol for two days, while they had no change or were slightly decreased when raised on 250 µM or 300 µM cholesterol respectively in the same time period (unpublished data). In this work, enlarging the cholesterol concentration had no impact on Tau expression in two-day-treated cells. This result might be relevant to the study of Gratuze M., et al. [Gratuze et al, 2016], in which no significant Tau change was displayed in mice fed with high-fat, high-cholesterol and high sugar diets. After four days of treatment, 150-300 µM cholesterol diminished the cell viability of neuroblatoma SH-SY5Y cells [Ternchoocheep et al, 2017]. In this study the declining effect of enlarging cholesterol concentration on Tau expression in neuroblastoma SH-SY5Y cells may be a repercussion of the cell death process. There are some reports which demonstrate the causative effect of cholesterol in promoting cell death both by apoptotic and autophagic processes. Cholesterol can enhance Bax and caspase-3 expression in cholesterol-treated HepG2 cells compared to those in a normal group or a steatosic group [Zhu et al, 2014]. Additionally, cholesterol can extend gastric cancer cell death both by apoptosis and by autophagy via caspase-3 cleavage, nuclear fragmentation, ATG accumulation or LC3 cleavage [Lim et al, 2014]. Also, Tau protein has been involved in degradation processes both by apoptotic and autophagic mechanisms. Tau proteolysis parallels in the same time course with caspase-3 activation in sorbitol-induced apoptotic neuroblastoma SH-SY5Y cells, while the specific caspase-3 inhibitor (Z-DEVD-fmk) completely obstructs this

proteolysis both by reducing the Tau level and by causing the appearance of proteolytic fragments [Oliva-Santa Catalina et al, 2016]. Additionally, this sorbitol-induced apoptotic Tau proteolysis is controlled by JNK signaling though the regulation of caspase-3 activation in neuroblastoma SH-SY5Y cells [Oliva-Santa Catalina et al, 2017]. Recently, a report revealed that lysosomes can contribute to the degradation of full-length (441 amino acid residue) human Tau (2N4R-Tau), in a large part through chaperone-mediated autophagy (CMA), as LAMP-2a (CMA receptor) knock down almost completely abolished lysosomal degradation of 2N4R-Tau and lead to its accumulation. This isoform of Tau proteins accumulates in endosomal microautophagy (e-MI)-defective cells. However, once the N-terminal insert is lost (in 0N3R-Tau), a pronounced reduction in Tau uptake is present. The N-terminal inserts are crucial for Tau degradation via CMA as well as e-MI to a lesser extent as the internalization steps of CMA and e-MI are interrupted by the missing N-terminal inserts. In this work, Tau proteins may have undergone proteolysis during exposure to enlarged cholesterol for four days when the cells were under the death process, so the Tau protein expression pattern tended to diminish.

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