Human chorionic gonadotropin increases β-cleavage of amyloid precursor protein in SH-SY5Y cells

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Key words: Alzheimer's disease (AD), amyloid precursor protein (APP), amyloid-β peptide (Aβ), β-secretase, β-site APP cleaving enzyme (BACE), luteinizing hormone (LH), human chorionic gonadotropin (hCG); SH-SY5Y neuroblastoma

Abstract

Elevated levels of amyloid-β (Aβ) peptides, the main component of amyloid plaques in Alzheimer's disease (AD), are the result of excessive β-and γ-cleavage of the amyloid precursor protein (APP) and/or impaired Aβ clearance. It has been suggested that high concentrations of luteinizing hormone (LH) in women contributes to increased Aβ generation after menopause, but the mechanism for this is incompletely understood. We investigated the effect of human chorionic gonadotropin (hCG), an LH receptor agonist, on APP β-cleavage in the SH-SY5Y neuroblastoma cell line. Treatment of these cells with hCG induced elevated β-cleavage in a dose-dependent manner: administration of 30 mIU but not 10 mIU/ml of hCG significantly increased sAPPβ levels in the cell medium 1.7 fold as measured by Enzyme-linked Immunosorbent Assay (ELISA). These results support the notion that LH contributes to elevated Aβ levels at least in part by increasing β-cleavage of APP by BACE.

Introduction

Hallmarks of Alzheimer's disease (AD) include neurofibrillary tangles composed of intra-neuronal paired helical filaments of hyperphosphorylated tau protein and extracellular amyloid plaque deposits of aggregated amyloid-β (Aβ) peptides (Haass *et al*., 2012). Aβ peptides are derived from the trans-membrane amyloid precursor protein (APP) that is sequentially cleaved by proteolytic activities of β-secretase and γ-secretase (Haass *et al*., 2012). Activity of an additional secretase enzyme, α-secretase results in non-amyloidogenic processing of APP (Haass *et al*., 2012). APP is first cleaved in endosomes or at the plasma membrane by β-secretase or α-secretase enzymes releasing the large N-terminal APP ectodomains sAPPβ or sAPPα that are secreted from the cell. The membrane associated C-terminal fragment left after β-secretase activity is further processed by γ-secretase to generate Aβ. In AD, it is proposed that β-secretase activity followed by Aβ generation is favoured over α-secretase activity; hence, factors inducing β-secretase activity may contribute to the pathogenesis of AD (Haass *et al*., 2012).

Luteinizing hormone (LH) is a sex hormone produced in the pituitary of both genders and regulates secretion of estrogens and androgens via the hypothalamus-pituitary-gonad (HPG) axis. Age-related changes in the HPG axis, particularly declined levels of estrogens in menopausal women, reflects sustained high levels of LH. It has been suggested that LH may contribute to the pathogenesis of AD (Barron *et al.*, 2010; Short *et al*., 2001). In the brain, LH/hCG receptors are expressed in hippocampal and cortical structures affected by AD as well as other regions (Lei *et al.*, 1993). Studies *in vitro* and *in vivo* in animal models have suggested roles of LH in the generation of AD-like phenotypes. Studies in mice have shown that inhibition of LH release or suppression of LHR improved cognitive performance as measured in memory-based behavioral tests (Casadesus *et al.*, 2007). Other animal experiments demonstrated that LH or human chorionic gonadotropin (hCG),

that also acts on LHR, stimulated the production of APP (Porayette *et al,* 2007) and secreted Aβ peptide (Wahjoepramono *et al.,* 2011). Studies *in vitro* using the M17 neuroblastoma cell line showed that treatment with LH increased Aβ production however the mechanism for this remains unclear (Bowen *et al.,* 2004). Using a similar neuroblastoma cell model (SH-SY5Y), we sought to investigate specifically whether LH/hCG receptor activation triggers β-cleavage of APP. LH-mediated increases in the activity of the β-secretase BACE may be a mechanism whereby LH could stimulate Aβ generation.

Materials and Methods

Cell culture and differentiation

SH-SY5Y neuroblastoma cells were maintained in high glucose DMEM supplemented with 10% FBS, penicillin, and streptomycin (Invitrogen). The cells were maintained at 37°C/5% CO2 and media was changed every 3 days. All-trans retinoic acid (ATRA) (Sigma-Aldrich) was diluted to1 µM in DMEM with supplements and added to cells for 7 days to induce cell differentiation. Human chorionic gonadotropin (hCG) was added to cells in Neurobasal medium supplemented with 1% FBS, 2% B27, 1% glutamax, penicillin, and streptomycin (Invitrogen). The hCG was prepared by dissolving 1 ampulla of hCG (Organon Pharmaceuticals, USA) in 1 ml of autoclaved water. Each ampulla contains 5000 International Unit (IU) of hCG powder; hence, the stock concentration was 5000 IU/mL. The stock hCG solution was serially diluted into the cell media to generate the required concentrations.

RNA extraction and PCR

Cells were plated into 6-well plates (Corning) at a density of 2.0×10^6cells per well. After 2 days, some cells were treated with ATRA for a further 7 days. Cells were washed with ice cold PBS and then lysed with Trizol reagent and centrifuged at 12,000 g for 15 minutes at 4°C to extract the cellular RNA which was sequentially precipitated by applying isopropyl alcohol. The RNA pellet was then washed twice with 75% ethanol and air-dried and dissolved in (Diethylpyrocarbonate) DEPC-treated water. The purity and concentration of RNA was measured using a NanoVue plus spectrophotometer. LHR primer was designed using the Primer-Blast program. The High Capacity RNA-to-cDNA Master Mix kit (Applied Biosystems) was employed to synthesize cDNA from the purified cellular mRNA. PCR was conducted employing a Platinum Taq Polymerase kit

(Invitrogen). The TaqMan®Gene Expression Master Mix kit(Applied Biosystems) was used to synthesize cDNA from cellular RNA and conduct real-time PCR.

hCG treatment and detection of total APP and secreted ectodomains

Cells were plated into 6-well plates at a density of 2.0×10^6 cells per well. The following day, DMEM medium was replaced with supplemented Neurobasal medium to which 0 or 10 or 30 mIU/ml of human chorionic gonadotropin (hCG) was added. At the end of the treatment period (day 8), the culture medium was collected and centrifuged at 13000 g for 15 minutes at 4°C. The supernatant was used to conduct ELISA and Western blot to compare the levels of sAPPβ and sAPPα, respectively. The cell pellets were lysed to extract cellular protein. RIPA Lysis buffer consisted of TRIS (50 mM, pH:7.4), NaCl (150 mM), Na Deoxycholate (10%), NP-40 (10%), SDS (0.1%), distilled water, and freshly added protease inhibitor cocktail (Roche). Protein concentrations in lysates were measured using the Micro BCA protein assay reagent kit (Pierce). An AlphaLisa kit (PerkinElmer) was used to measure the concentrations of sAPPβ in cell medium according to the manufacturer's protocol. To probe total APP, 6% acrylamide gels were prepared. Pre-cast 10% acrylamide gels (Invitrogen) were employed to probe sAPPα, and LHR. Mouse anti-APP antibody (Signet, 6E10) was applied at 1:1000 dilution to probe Western blots for APP and sAPPα in cell lysates and culture medium, respectively. To detect LHR protein, 0.2 µl/ml (1:5000) of goat polyclonal anti-LHR antibody (Novus Biologicals) was used. As a loading control to normalize the band densities, mouse anti-GAPDH antibody (Sigma) was applied at 1:2000 dilution. Image J (NIH) and excel softwares were employed to calculate normalized band densities which were afterwards analyzed with *Prism software* to determine statistical significance.

Statistical Analysis

The Mann-Whitney test was employed to measure the effect of retinoic acid administration on production and maturation of APP and the expression of LH-receptor. Bonferroni's Multiple Comparison test was used to study the effect of hCG administration on β-cleavage of APP.

Results

Studies *in vitro* using the M17 neuroblastoma cell line showed that treatment with LH increased Aβ production (Bowen *et al.,* 2004). This study inferred an increase in β-cleavage of APP based on elevated levels of intracellular C99, which is the membrane bound product left behind after cleavage of APP by BACE. C99 is further processed by γ-secretase to generate Aβ or it is degraded by the proteasome (Watanabe *et al*., 2012). We sought to investigate this pathway further by measuring β-cleavage in neuroblastoma SH-SY5Y cells treated with hCG as the LHR agonist. Direct measurement of β-cleavage was achieved by using ELISA to detect the ectodomain sAPPβ released in to the cell media.

As we did not find any previous literature demonstrating that SH-SY5Y cells express LHR, we first conducted RT-PCR (Fig. 1A, left panel) and Western blot (Fig. 1A, right panel) to investigate LHR gene expression and protein synthesis in SH-SY5Y cells and the effect of cell differentiation on APP expression and maturation. For this purpose, SH-SY5Y cells were treated with 1 μM all trans-retinoic acid (ATRA) for 7 days. At the end of this period, significant morphological changes including rounding of perikarya and the development of thin neurite-like processes were observed which indicated cellular differentiation (Figure 1B, images). The results of Western blots were analyzed and showed that 1 μM ATRA administration significantly increased total intracellular APP levels ($p < 0.05$; Figure 1B, lower panels). Furthermore, an increase in slower-running bands representing the mature glycosylated forms of APP indicated that SH-SY5Y cell differentiation correlated with the maturation of APP through the secretory pathway (Fig.1B, arrows). By contrast, LHR levels were sometimes reduced after ATRA treatment. As some differentiated cells appeared to express reduced levels of LHR, undifferentiated SH-SY5Y cells were used for the following

experiments. Cells were treated for 8 days with 0, 10 or 30 mIU/ml of hCG. The media was replaced every other day. On the last day, we compared the levels of total APP, sAPPα and sAPPβ in the 3 groups. Total APP levels were measured by Western blot of protein lysates (Fig. 1C, upper panel). Quantification of the band densities showed that administration of neither 10 nor 30 mIU/ml of hCG significantly altered total APP synthesis although a trend to increased APP was observed (Fig. 1D, top) (p >0.05). The sAPPα levels were measured by applying Western blot of the culture medium (Fig. 1C, lower panel). This demonstrated that hCG administration caused a trend of decline in sAPPα production (Figure 1D, bottom). Although this reduction was not statistically significant, it did exhibit a dose-dependent pattern - p values for 10 and 30 mIU/mL were 0.5887 and 0.0586, respectively. Finally, we conducted ELISA to measure the concentration of sAPPβ in the same culture medium (Fig. 1E and Supplementary Information). The mean concentration of sAPPβ was 343 pg/mL in the absence of hCG administration. When cells were treated with 10 mIU/mL or 30 mIU/mL of hCG, the mean sAPPβ concentration increased to 429 pg/ml or 591 pg/ml, respectively corresponding to a 1.3 or 1.7 fold elevation in sAPPβ secretion. The 1.7 fold increase at 30 mIU/mL of hCG was statistically significant (p < 0.005; Bonferroni's Multiple Comparison test) (Fig. 1E).

Discussion

We have shown that 30 mIU/mL hCG can significantly increase β-cleavage of APP and secretion of sAPPβ from SH-SY5Y neuroblastoma cells. This result is consistent with a previous study that showed intracellular C99 (the intracellular C-terminal membrane bound APP fragment left behind after β-cleavage by BACE) levels are increased in neuroblastoma cells treated with LH (Bowen *et al.,* 2004). The apparent elevated BACE activity may be due to different scenarios – increased trafficking of BACE and/or APP to subcellular domains where cleavage takes place, increased expression of BACE or increases in BACE activity via other mechanisms. It has been shown that BACE protein and mRNA levels are elevated in AD, and that BACE activity but not necessarily protein levels are elevated during ageing (Holsinger *et al.,* 2006; Fukumoto *et al.,* 2004). This suggests that an age-related increase in BACE trafficking and activity and/or BACE expression levels could contribute to predisposition to AD. Age-related changes in hormonal signalling through LH could be one triggering mechanism for elevated APP β-cleavage in neurons. This is a reasonable hypothesis because brain regions affected by altered APP metabolism, amyloid accumulation and tau pathology early in AD, such as the entorhinal cortex and hippocampus, have been shown to express LHR in adults (Lei *et al.*, 1993). The localization of LHR in these brain regions suggests potential diverse functional roles for LH-mediated signalling that have so far not been investigated (Apaja *et al.,* 2004).

The known cellular functions of LHR activation involve steroid hormone production and associated cholesterol transport and metabolism. However, little is known about LHR signalling in the brain or more specifically in neurons. Cholesterol is known to modulate APP metabolism in lipid rafts (Hicks *et al.,* 2012). Cholesterol- and sphingolipid-rich membrane microdomains are involved in

regulating trafficking and processing of APP and active BACE resides in lipid rafts (Hicks *et al.*, 2012). It is possible then that LHR activation could exert its effect on BACE activity by modulating cholesterol transport and metabolism and the extent of BACE and APP localization in lipid rafts. Addressing these questions and dissecting the intracellular signalling cascade involved would open a new area of future work.

Some epidemiologic studies have suggested that AD is more prevalent among women at old age (Miech *et al.*, 2002). This hypothesis is supported by some limited studies that have shown that LH levels are higher in AD patients than age-matched controls (Short *et al*., 2001). Aging is a major risk factor for AD and postmenopausal women in particular have elevated levels of LH (Miech *et al.*, 2002). Although some studies have suggested that hormone replacement therapy (HRT) may have protective effects against AD, this remains controversial as the period of HRT administration could be critical, encompassing the onset of menopause and the subsequent following early first years (Zandi *et al*., 2002). Considering that LH levels are maximal during this critical period and HRT suppresses LH production, it is conceivable that any beneficial effects of estrogen administration on reducing AD risk could be mediated in-part through reducing the level of circulating LH. Furthermore, people with Down's syndrome have a high prevalence of AD, develop the disease at younger ages than the general population and also have relatively high levels of LH starting at puberty indicating abnormalities in primary gonadal function that correlate with earlier-onset AD (Schupf *et al.*, 1998).

I(n summary, the demonstrated dose-dependent increase in β-secretase activity upon treatment of SH-SY5Y cells with the LHR agonist hCG suggests that reported LH/LHR-mediated increases in Aβ levels are due to activation of β-secretase. Elevated sAPPβ and Aβ production *in vivo* may be

generated in-part through LH binding to LHR-expressing neurons in the regions of the brain affected by AD that are potentially exposed to increased levels of LH during ageing and menopause.

Conflict of interest

The authors declare that they have no conflicts of interest.

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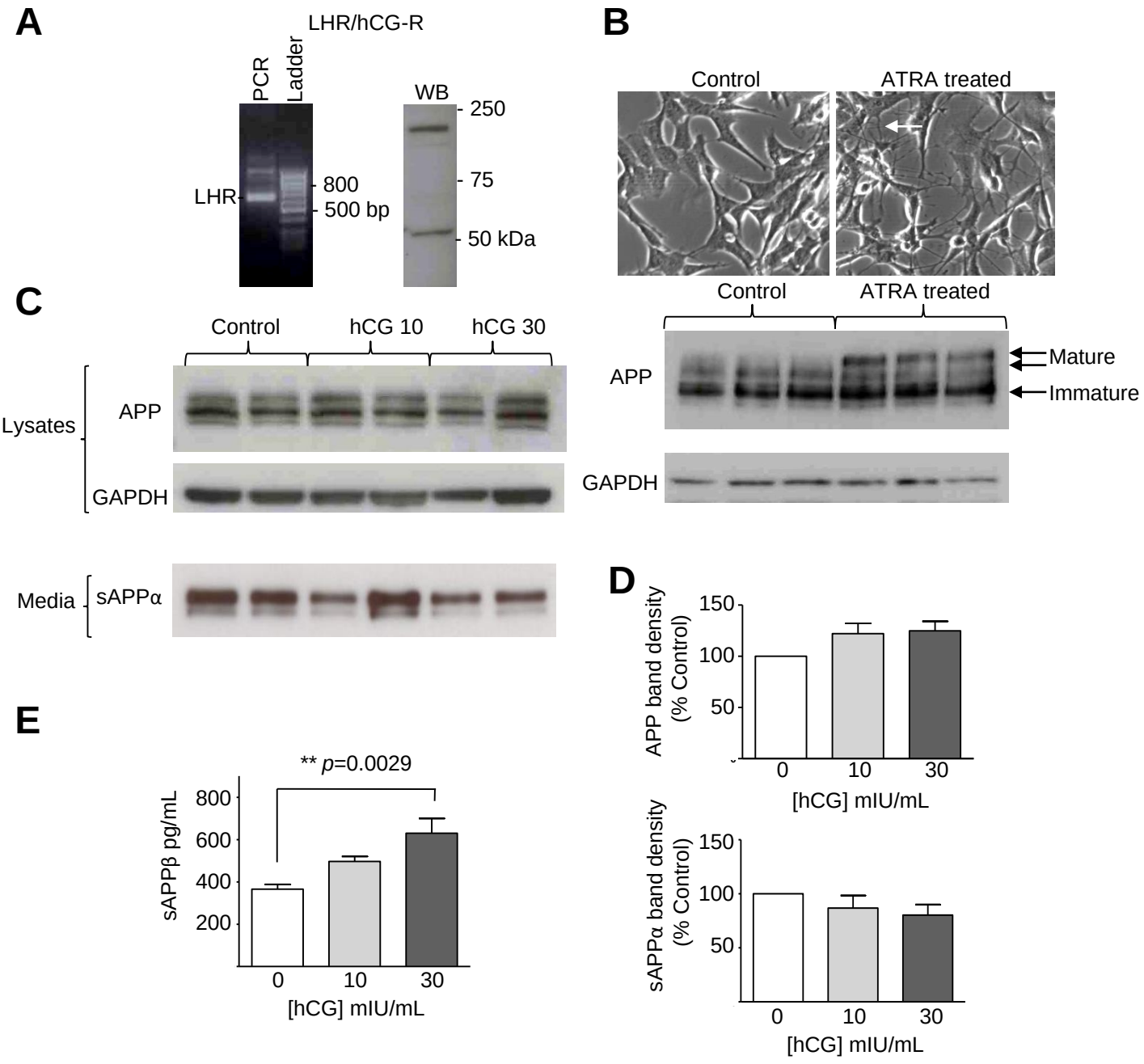
Figure Legend

Figure 1

(A) Indication that LHR mRNA and protein is expressed in SH-SY5Y cells. A 655 bp fragment was amplified using LHR-specific primers (left panel). A 59 kDa protein band was observed on Western Blot consistent with LHR protein expression (right panel; probed with 0.2 µl/mL, 1:5000 anti-LHR goat antibody**). (B)** ATRA administration induces cell differentiation and stimulates APP production and maturation in SH-SY5Y cells. Lanes 1-3 and 4-6 are triplicate lysates from untreated and ATRA treated cell cultures respectively. **(C)** Treatment of SH-SY5Y cells with hCG followed by Western blot for total intracellular APP levels (upper panel) and sAPPα in cell media (lower panel). Representative examples of results from duplicate cultures treated with 0, 10 or 30 mIU/ml hCG are shown from one of 3 experiments. **(D)** Quantification of the Western blot band densities in (C) and two other experiments (n= 6 samples for each condition). Total APP measured in cell lysates showed a trend to increase after treatment with hCG, but this was not statistically significant (top bar graph). By contrast, sAPPα measured in the cell medium showed a trend to decline after treatment with hCG but this was also not statistically significant (lower bar graph) (p>0.05). Western blots of SH-SY5Y cell lysates and cell media for detection of APP and sAPPα, respectively were probed using the mouse monoclonal 6E10 anti-APP antibody at 1:1000 dilution. (E) The concentrations of sAPPβ in the same sets of cell medium were measured by ELISA (AlphaLisa kit, Perkin Elmer). A significant increase in sAPPβ was observed with 30 mIU/ml (p = 0.0029) but not 10 mIU/ml (p >0.05) hCG, demonstrating a dose dependent effect. Statistical significance was considered for p values <0.05.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