Published in final edited form as:

Neuromolecular Med. 2017 September ; 19(2-3): 300–308. doi:10.1007/s12017-017-8446-x.

Low density lipoprotein receptor-related protein-1 (LRP1) C4408R mutant promotes amyloid precursor protein (APP) α -cleavage *in vitro*

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Abstract

Previous studies have demonstrated that the low-density lipoprotein (LDL) receptor-related protein-1 (LRP1) plays conflicting roles in Alzheimer's disease (AD) pathogenesis, clearing β -amyloid ($A\beta$) from the brain while also enhancing APP endocytosis and resultant amyloidogenic processing. We have recently discovered that coexpression of mutant LRP1 C-terminal domain (LRP1-CT C4408R) with Swedish mutant amyloid precursor protein (APP^{swe}) in Chinese hamster ovary (CHO) cells decreases $A\beta$ production, while also increasing sAPP α and APP α -C-terminal fragment (α -CTF), compared with CHO cells expressing APP^{swe} alone. Surprisingly, the location of this mutation on LRP1 corresponded with the α -secretase cleavage site of APP. Further experimentation confirmed that in CHO cells expressing APP^{swe} or wild-type APP (APP^w), coexpression of LRP1-CT C4408R decreases $A\beta$ and increases sAPP α and α -CTF compared with coexpression of wild-type LRP1-CT. In addition, LRP1-CT C4408R enhanced the unglycosylated form of LRP1-CT and reduced APP endocytosis as determined by flow cytometry. This finding identifies a point mutation in LRP1 which slows LRP1-CT-mediated APP endocytosis and amyloidogenic processing, while enhancing APP α -secretase cleavage, thus demonstrating a potential novel target for slowing AD pathogenesis.

Keywords

Low-density lipoprotein receptor-related protein-1 (LRP1); amyloid precursor protein (APP); secreted amyloid precursor protein- α (sAPP α); β -amyloid ($A\beta$); Alzheimer's disease (AD); Swedish mutant APP (APP^{swe})

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Competing financial interests

The authors declare no competing financial interests.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by plaques created by accumulation of amyloid-beta (A β) proteins and neurofibrillary tangles (NFT) composed of acetylated and phosphorylated tau proteins (Hardy and Selkoe, 2002; Cohen et al., 2011). A β is formed from the amyloid precursor protein (APP), which can be processed in two ways. In the non-amyloidogenic pathway, APP is cleaved by α -secretase to yield soluble amyloid precursor protein alpha (sAPP α) and APP intracellular domain (AICD) (Kimberly et al., 2003; Lefort et al., 2012). Alternatively, in the amyloidogenic pathway, APP is initially cleaved by β -secretase and then further cleaved by γ -secretase to create A β (Vassar et al., 1999). Although the physiological function of A β has yet to be clearly defined, the accumulation of A β plaques has been associated with memory loss characteristic of AD.

Because A β is a major component of the amyloid plaques associated with AD, much of the research on AD conducted to date has been concerned with ways to enhance A β clearance or reduce amyloidogenic APP processing. There has been speculation that lipoprotein receptors, specifically low-density lipoprotein (LDL) receptor-related protein-1 (LRP1), affect A β levels and consequently the onset or progression of AD. LRP1 is a part of the low-density lipoprotein receptor (LDLR) family with a plethora of functions, including scavenging, transporting and signaling (Dieckmann et al., 2010; Boucher and Herz, 2011). LRP1 is found on a variety of cell types and comes in two forms, cell surface bound and a truncated soluble form (sLRP1) (Moestrup et al., 1992). LRP1 via its extracellular domain and sLRP1 potentially interact with over 100 ligands (Lillis et al., 2008; Zlokovic et al., 2010; Boucher and Herz, 2011), including apolipoprotein E (apoE), α 2-macroglobulin (α 2M) (Hussain et al., 1999; Neels et al., 1999; Herz & Strickland, 2001), and A β (Deane et al., 2004; Sagare et al., 2007), lending to its versatility. In addition, the LRP1 intracellular domain potentially interacts with APP through FE65, an LRP1 adaptor protein, allowing it to promote APP endocytosis and consequent amyloidogenic processing (Kounnas et al., 1995; Pietrzik et al., 2004; Waldron et al., 2008).

The biomedical literature contains seemingly conflicting reports describing LRP1's role in AD onset and progression. LRP1 mediates the clearance of A β from the brain (Mawuenyega et al., 2010), functioning as its main clearance receptor across the blood-brain barrier (BBB, Sagare et al., 2012), thereby potentially slowing AD progression. It has been shown that AD patients have lowered LRP1 expression in the BBB and in cerebral arterial vascular smooth muscle cells (VSMC) (Shibata et al., 2000; Bading et al., 2002; Deane et al., 2004; Donahue et al., 2006; Herring et al., 2008; Bell et al., 2009; Silverberg et al., 2010). In contrast, LRP1 can also mediate neuronal uptake and retention of A β in the brain through its apoE and α 2M ligands (Narita et al., 1997; Qiu et al., 1999; DeMattos et al., 2004; Zerbinatti et al., 2004; Zerbinatti & Bu, 2005; Deane et al., 2008). Moreover, LRP1 mediates APP endocytosis, a key step in APP amyloidogenic processing. Less LRP1 expression results in less APP endocytosis (Pietrzik et al., 2002; Waldron et al., 2008), which leads to not only less A β production (Pietrzik et al., 2002; Ulery et al., 2000) but also greater levels of sAPP α (Parvathy et al., 1999). Other studies have demonstrated that LRP1-deficient cells co-transfected with APP and LRP1 leads to a significant increase in A β compared to cells

transfected with APP alone (Ulery et al., 2000). The rapid endocytosis rate of LRP1 has been shown to be responsible for both increased APP internalization and processing to A β (Pietrzik et al., 2002; Zerbinatti et al., 2004; Cam et al., 2005).

The present study was undertaken to further examine the effects of the LRP1 intracellular domain on APP processing and A β production. A CHO cell line expressing both APP^{swe} and truncated LRP1 C-terminal domain (LRP1-CT) constructs was created to examine the effect of truncated LRP1-CT on APP processing. However, while creating the cell line, a new LRP1 mutation was discovered that resulted in lower A β levels. The following analysis was performed to further examine how this mutation impacts APP processing.

Materials and Methods

Antibodies

The antibodies for immunofluorescence staining and Western blot (WB) were used as follows: a mouse anti-N-terminal A β monoclonal antibody (6E10, Covance, Emeryville, CA, USA), rabbit anti-C-terminal APP polyclonal antibody (pAb751/770, Millipore, Billerica, MA, USA), mouse anti-human intracellular domain LRP1 monoclonal antibody (5A6, Millipore), rabbit anti-human intracellular domain LRP1 antibody (gift from Dr. G Bu, Mayo Clinic, Jacksonville, FL, USA), anti-LRP1 C-terminal antibody (Sigma, St. Louis, MO), and a β -actin antibody (Sigma-Aldrich). Secondary antibodies employed include: Alexa Fluor 488-conjugated donkey anti-rabbit IgG, and Alexa Fluor 594-conjugated donkey anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) used for immunofluorescence staining; HRP conjugated horse anti-mouse IgG, and HRP conjugated goat anti-rabbit IgG (Cell Signaling, Danvers, MA, USA) used for WB.

Cell lines and cell culture

Chinese hamster ovary (CHO) cell lines with stable expression of human APP bearing the Swedish mutation (APP^{swe}) and human LRP1-CT within PLHCX vector construct were kindly provided by Dr. Stefanie Hahn and Dr. Sascha Weggen. (University of Heinrich Heine, Dusseldorf, Germany). As described in our recent report (Li et al., 2015), these cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1 mM sodium pyruvate and 100 U/mL of penicillin/streptomycin. For subclone selection, these cells were counted and diluted to one single cell/well in 96 well plate. After one week of culture, the cells from each well were detached by trypsin-EDTA and some of the cells were plated on an 8 well chamber for immunocytochemical staining. The remaining cells were further cultured in 24 well plates for analysis by WB, ELISA and flow cytometry.

Immunocytochemistry staining

The cells were cultured in 8 well chambers, fixed in 4% paraformaldehyde at RT for 30 minutes, permeabilized with 0.05% triton X-100 for 5 minutes, blocked with 5% horse serum for 1 hour and incubated with primary antibody at 4°C overnight. The cells were then incubated with appropriate secondary antibody, mounted with DAPI medium (Vector Laboratories, Burlingame, CA) and visualized with an Olympus FV1000 confocal microscope (Tokyo, Japan).

WB analysis

WB analysis was performed as previously described (Deng et al., 2015). Briefly, the proteins in cell-free suspensions and cell lysates were electrophoretically separated using 10% bicine/tris gel (8 M urea) for proteins less than 5 kD or 10% tris/SDS gels for larger proteins. Electrophoresed proteins were transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA), washed and blocked for 2 hours at room temperature in tris-buffered saline containing 5% (w/v) nonfat dry milk (TBS/NFDM). After blocking, membranes were hybridized for 2 hours with various primary antibodies, washed, incubated for 1 hour with the appropriate HRP-conjugated secondary antibody and developed using the luminol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Blot densitometric analysis was performed as described previously (Deng et al., 2015; Rezai-Zadeh et al., 2005), using a Fluor-S MultiImager with Quantity One software (Bio-Rad).

ELISA

Total A β _{1–40, 42} levels in cell conditioned media were detected by A β _{1–40, 42} ELISA kits (Invitrogen) according to the manufacturer's instructions. The sAPP α level in cell conditioned media was determined using an sAPP α ELISA kit (IBL-America, Minneapolis, MN, USA).

PCR and sequencing

DNA from CHO subclone #0, which only overexpressed LRP1-CT but not APP, were extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), amplified, purified (QIAquick Gel Extraction Kit, Qiagen) and sent to Moffitt Molecular Genomics Core Facility (Tampa, FL) for DNA sequencing. PLHCX forward (AGCTCGTTTAGTGAACCGTCAGATC) and reverse primers (ACCTACAGGTGGGGTCTTTCATTCCC) and LRP primer (GTGGGGATGTCAC CT) were used for amplification and sequencing.

Flow cytometry analysis

CHO/APPwt, CHO/APPswe and CHO/APPswe/ LRP1-CT C4408R stable cells were stained with Alexa Fluor 488-conjugated 6E10 (Covance, Emeryville, CA) in the dark at RT for 30 min, washed with Flow buffer, and analyzed with an Accuri C6 Flow Cytometer (Rochester, MN, USA).

Statistical analysis

Data are expressed as mean $\pm$ S.D. of 3–4 independent experiments. Comparison between two groups was performed by Student's *t*-test. *P* < 0.05 was considered statistically significant.

Results

A novel discovery made after subcloning CHO/APPswe/LRP1-CT cells

LRP1 C-terminal domain has been shown to mediate A β generation through enhancement of APP endocytosis and amyloidogenic processing (Pietrzik et al., 2002). In order to further

investigate the effect of LRP1-C-terminal domain on APP amyloidogenic processing, a Chinese hamster ovary (CHO) cell line co-overexpressing both Swedish mutant human APP (APP^{swe}) and truncated LRP1 (LRP1-CT) constructs (CHO/APP^{swe}/LRP1-CT) was obtained from Drs. Stephanie Hahn and Sascha Weggen (University Heinrich Heine, Düsseldorf, Germany), cultured and analyzed. As determined by immunofluorescence staining and confocal microscopy, a small portion of the cells stained positive for both APP and LRP1 (Fig. 1a, top). In order to isolate these cells, they were subcloned by separating them into single cell cultures and then expanding them in 96-well plates. We obtained several subclones expressing only APP^{swe}, such as #15, and several subclones expressing both APP^{swe} and LRP1-CT, such as #39, 41 and 67 (Fig. 1a), which were further expanded for biochemical analysis. Surprisingly, subclones #39, 41 and 67 produced more sAPP α and less A β than subclone #15 as determined by WB and ELISA (Fig. 1b, d & e). In addition, whereas all subclones produced full length APP (holo APP) as well as APP α -CTF and β -CTF, only subclones #39, 41 and 67 consistently increased α -CTF as determined by WB analysis of cell lysates using pAb751/770. WB analysis using an anti-LRP1 C-terminal antibody (pAbLRP1) also confirmed the presence of LRP1-CT only in subclones #39, 41 and 67 (Fig. 1c, middle). Therefore, co-overexpressing LRP1-CT reduces APP amyloidogenic processing in CHO/APP^{swe} cells. This finding led us to analyze the truncated LRP1-CT DNA sequences transfected in these cells.

LRP1-CT C4408R mutation discovered in CHO/APP^{swe}/LRP1-CT cells

Based on the construct map of the truncated LRP1-CT provided by Dr. Sascha Weggen, we designed two primers to amplify and sequence this construct. Sequence analysis revealed a point mutation at position 757 of LRP-CT (T to C), resulting in an amino acid change from cysteine (TGT) to arginine (CGT) at position 4408 or LRP1 (Fig. 2a & b). Most interestingly, this location corresponds exactly to the APP695 α -secretase cleavage site.

LRP1-CT C4408R promotes APP α -cleavage processing

In order to confirm and further investigate the role of the C4408R mutant LRP1 C-terminal domain in modulation of APP processing, we generated CHO/APP^{wt}/LRP1-CT^{wt}, CHO/APP^{wt}/LRP1-CT C4408R, CHO/APP^{swe}/LRP1-CT^{wt} and CHO/APP^{swe}/LRP1-CT C4408R stable cell lines. Preconditioned media and cell lysates prepared from cultures of these cell lines were then analyzed for LRP1 and APP processing by WB. WB analysis using a rabbit anti-human intracellular domain LRP1 antibody (provided by Dr. G Bu) showed the presence of three LRP1 C-terminal fragments, α -CTF, β -CTF (very light), and γ -CTF, in the cell lysates from each cell line (Fig. 3a). These results were confirmed using the mouse anti-LRP1 monoclonal antibody (5A6). In addition, WB analysis using pAb751/770 and anti-N-terminal A β antibody (6E10) shows three bands corresponding to α -CTF and β -CTF of APP as well as sAPP α in all cell lines. LRP1-CT C4408R expression in CHO/APP^{wt} or CHO/APP^{swe} cells markedly promoted LRP1 α CTF, APP α -CTF and sAPP α production, indicating enhanced α -cleavage of both LRP1-CT and APP.

We further characterized LRP1-CT glycosylation and LRP1 and APP processing in CHO/APP^{swe}/LRP1-CT^{wt} and CHO/APP^{swe}/LRP1-CT C4408R cells by WB analysis. WB analysis with long exposure confirmed that LRP1-CT C4408R markedly increased LRP1 α -

CTF (Fig. 3b, top). WB analysis with short exposure reveals LRP1 C4408R markedly increased unglycosylated LRP1 C-terminal domain (unglyco LRP1-CT) (Fig. 3b, bottom). LRP1 C4408R also markedly promoted APP nonamyloidogenic processing as evidenced by increased levels of APP α -CTF and sAPP α , without altering holo APP (Fig. 3c, d and e).

LRP1-CT C4408R reduces APP endocytosis

Koo and Squazzo (1994) reported that APP endocytosis is required for A β production. In addition they reported that full length APP accumulated on the cell membrane when APP endocytotic processing is inhibited and that more full length APP accumulated on the cell membrane of CHO/APPwt cells compared to CHO/APPswe cells. In as much as LRP1 can mediate APP endocytosis (Bu et al., 2006), we compared the effect of LRP1-CT C4408R with LRP1-CTwt on APP endocytic processing in CHO/APPswe cells. Using the flow cytometry method recently established in our laboratory and anti-N-terminal human A β ₁₋₁₇ antibody (6E10), we measured full length APP (holo APP) accumulation on cell membranes of CHO/APPswe/LRP1-CT C4408R subclone 4 (blue), CHO/APPswe subclone 5 (red) and CHO/APPwt subclone cells (black). CHO/APPswe/LRP1-CT C4408R subclone #4 (blue) accumulated more cell membrane associated holo APP compared to CHO/APPswe subclone #5 (red), suggesting that LRP1 C4408R overexpression reduces APP endocytosis (Fig. 4a). Cellular membrane associated holo APP, presented as median FL-1-A, increased by 47% in CHO/APPswe/LRP1-CT C4408R subclone #4 and by 116% in CHO/APPwt cells compared to CHO/APPswe subclone #5 (Fig 4b). Therefore, LRP1-CT C4408R overexpression reduces APP endocytosis, thereby potentially reducing A β production.

Discussion

Mechanisms of APP processing and A β production are being studied in hopes of finding a method to prevent or slow AD. LRP1 is a receptor that has shown promise. LRP1 has been shown to be involved in cell signaling and macromolecule transporting; binding to many ligands including A β . In addition, LRP1 can mediate APP amyloidogenic processing through its internalization as well as interaction of its intracellular domain with FE65.

In CHO cells, overexpression of LRP1-CT C4408R with either APPswe or APPwt resulted in less A β and more APP α -CTF and sAPP α production, without altering the level of holo APP, compared to endogenous LRP1 expressing (Fig. 1) or wild type LRP1-CT overexpressing cells (Fig. 3). Therefore, the C4408R mutation of LRP1-CT enhances APP nonamyloidogenic processing. Surprisingly, this point mutation of cysteine to arginine is at the same corresponding location as the APP695 α -secretase cleavage site, suggesting that this mutation may directly modify α -secretase activity (Fig 2). Since the LRP1 C4408R mutation also increased the production of LRP1 α -CTF, one might speculate that this mutation increases both LRP1 and APP α -processing by a single mechanism. This provides the potential that this mutation may slow AD progression by reducing APP amyloidogenic processing and increasing sAPP α production. In addition, an increase in sAPP α has been shown to reduce GSK3 β activity and tau phosphorylation both *in vitro* and *in vivo* (Deng et al., 2015). This consequently can lead to a decrease in NFT and hence further slow AD progression.

Whether the C4408R mutation of LRP1 directly inhibits APP β -secretase processing or indirectly inhibits this process through increasing APP α -secretase processing could be a point of further investigation. We have previously shown that promoting APP α -secretase processing and sAPP α production reduces β -secretase activity (Deng et al., 2015). However, if there is a direct pathway that links LRP1 to β -secretase, this could play a role in AD pathogenesis as well. Whether the modulation is direct or indirect, this proves to be a very exciting finding, as this provides another possibility to reduce the amount of A β produced in the body. Furthermore, LRP1 C4408R increased the unglycosylated form of APP (Fig 3b) and thereby potentially reduced APP endocytosis (Fig. 4). Waldron et al. (2008) recently showed a role for LRP1 glycosylation in APP trafficking along the ER and golgi. When APP is internalized, it undergoes amyloidogenic processing by β - and γ -secretase (Zhang and Song, 2013). However, since the C4408R mutation decreases APP endocytosis, APP stays on the cell membrane and this APP is primarily cleaved by α -secretase to yield sAPP α . The frequency or existence of the C4408R mutation has not been reported in the literature to date. However we have screened several hundred samples from non-cognitively impaired elderly adults and did not detect this mutation. It is possible that reduced endocytosis and amyloidogenic processing occurs because the mutation reduces LRP1 association with lipid rafts through FE65, thus pulling APP away from the rafts. Further research will need to be conducted to confirm this hypothesis.

Acknowledgments

This work was supported by the NIH/NIA (R01AG050253, R01AG032432) and the Silver Endowment to J Tan. We would like to thank Dr. David Kang, Dr. Demian Obregon and Dr. Doug Shytle for their helpful discussion, and Mr. Yang Gao for his technical support in confocal image analysis. We also appreciated Dr. Stefanie Hahn and Dr. Sascha Weggen for kindly providing us with CHO cells overexpressing human APP bearing the Swedish mutation (APP^{Swe}) and human LRP-CT.

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Fig. 1. Subcloning CHO cells co-transfected with APPswe and LRP1-CT constructs

Double immunofluorescence staining with rabbit anti-human C-terminal APP polyclonal antibody (pAb751/770) and mouse anti-human intracellular domain LRP1 monoclonal antibody (5A6) reveal that a small portion of the cells are double positive (**panel a, top**). CHO/APPswe (#15) and CHO/APPswe/LRP1-CT subclones (#41, 39 and 67) were obtained as indicated. Subclones #39, 41 and 67 markedly increased sAPP α and decreased A β production compared to subclone #15 as determined by Western blot analysis (**b**) and ELISA (**d & e**, *** $P < 0.001$). WB analysis using pAb751/770 shows three bands corresponding to full length APP (holo APP) as well as α -CTF and β -CTF of APP in all four subclones (**c**). However, WB analysis using a polyclonal anti-LRP1 C-terminal antibody

(pAbLRP1) only shows LRP1 C-terminal fragments in subclones #41, 39 and 67. These results are representative of three independent experiments with each condition triplicated.

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Fig. 2. LRP1-CT construct sequencing
DNA prepared from the subclone #0 cells, which only overexpressed LRP1-CT but not APP, was amplified utilizing two primers designed for the LRP1-CT construct and sequenced (a). One point mutation was identified in LRP1-CT (T – C), resulting in an amino acid change from cysteine (TGT) to arginine (CGT) at position 4408 of LRP1-CT. The location of this mutation exactly corresponds with the APP α-secretase cleavage site.

Fig. 3. LRP1-CT C4408R effects on APP and LRP processing

LRP1 and APP processing in CHO cells overexpressing human wild-type APP (CHO/APPwt) or Swedish mutant APP (CHO/APPswe) and co-expressing wild-type LRP1-CT or LRP1-CT C4408R were examined by WB analysis. WB by C-terminal LRP1 and N- and C-terminal APP antibodies, 6E10 and pAb751/770, respectively, shows three bands corresponding to α-CTF, β-CTF (very light) and γ-CTF of LRP1-CT, sAPPα as well as α-CTF and β-CTF of APP, respectively (a). Further WB analysis reveals co-expression of LRP1-CT C4408R with APPswe markedly enhanced LRP1 and nonamyloidogenic APP α-

secretase processing as evidenced by markedly increased LRP1 α -CTF (long exposure), unglycosylated LRP1 CTF (short exposure, **b**), APP α -CTF (**c**) and sAPP α (**d**), without altering holo APP. For WB quantitative analysis, the band density ratio of sAPP α to holo APP was analyzed and presented as mean $\pm$ SD (**e**, $*P < 0.05$). These WB results are representative of three to four independent experiments with each condition duplicated.

Fig. 4. Effect of overexpressing LRP1-CT C4408R on APP endocytosis in CHO/APPswe cells
Flow cytometry analysis shows CHO/APPswe/LRP1-CT C4408R subclone #4 (blue) accumulated more cell membrane associated full length APP (holo APP) compared to CHO/APPswe subclone #5 (a, red). Cellular membrane associated holo APP was presented as median FL-1-A (b). As indicated, median FL-1-A was increased by 46% in CHO/APPswe/LRP1-CT C4408R subclone #4 and by 110% in CHO/APPwt cells compared to CHO/APPswe subclone #5 cells. Data are representative of three independent experiments with each condition triplicated (* $P < 0.05$).