A Protective Role of the Low Density Lipoprotein Receptor-related Protein against Amyloid β-Protein Toxicity*

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Received for publication, February 10, 2000, and in revised form, July 7, 2000
Published, JBC Papers in Press, July 17, 2000, DOI 10.1074/jbc.M001151200

In order to delineate the neuroprotective role of the low density lipoprotein receptor-related protein (LRP) against amyloid β-protein toxicity, studies were performed in C6 cells challenged with amyloid β-protein in the presence or absence of activated α₂-macroglobulin. Toxicity was assessed via two cell viability assays. We found that this endocytic receptor conferred protection against amyloid β-protein toxicity in the presence of activated α₂-macroglobulin and its down-regulation via inhibition by receptor-associated protein or transfection of cells with presenilin 1, increased susceptibility to amyloid β-protein toxicity. Increased surface LRP immunoreactivity in response to amyloid β-protein challenge was associated with increased translocation of LRP from the endoplasmic reticulum to the surface, rather than from increased mRNA or protein expression. Furthermore, this translocation of LRP to the surface was mediated by a calcium/calmodulin protein kinase II-dependent signaling pathway. These studies provide evidence for a protective role of LRP against amyloid β-protein toxicity and may explain the aggressive nature of presenilin-1 mutation in familial Alzheimer’s disease.

Low density lipoprotein (LDL) receptor-related protein (LRP) is a multifunctional endocytic receptor that binds a variety of ligands including apolipoprotein E (apoE)-containing lipoproteins (1, 2), activated α₂-macroglobulin (*α₂m) complexes, and proteins containing Kunitz-type protease inhibitor domains such as secreted amyloid precursor protein (3). Recent studies have suggested that altered LRP regulation may be an integral component of Alzheimer’s disease (AD) pathogenesis (4, 5). Importantly, LRP and its ligands, APP, apoE, and *α₂m, have all been genetically linked to AD (6–9), and colocalize in senile plaques, a hallmark of AD (4, 10). In addition, patients with probable AD have increased serum levels of LRP ligands including apoE, *α₁-antichymotrypsin, plasmin, and urokinase (11–13), suggesting that LRP expression (or function) may be deficient in these patients. Furthermore, we have previously shown that overexpression of or mutations in the presenilin 1 (PS1) gene, which has been closely linked to the majority of early onset familial AD cases, results in significant down-regulation of LRP (5). Taken together, these data indicate that LRP plays a central role in AD pathogenesis.

Although the mechanisms through which LRP might be involved in AD remain unclear, a recent study has suggested a role for LRP in clearance of amyloid β-protein (Aβ) (14). Specifically, Aβ is internalized by LRP when bound to apoE or *α₂m (14–18). Whether this effect is toxic or protective is not completely known. Thus, the main objective of the present study was to determine if addition of an Aβ-binding LRP ligand prevents Aβ toxicity. The results suggest that LRP mediates protection against Aβ toxicity in the presence of *α₂m and that LRP surface expression is preferentially up-regulated in response to Aβ challenge via a calcium/calmodulin-dependent protein kinase II (CaM KII)-dependent mechanism. Furthermore, PS1-transfected C6 cells, which are more susceptible to Aβ toxicity relative to vector-transfected control cells, do not show this protective LRP up-regulation in response to Aβ challenge. These studies provide evidence for a protective role of LRP against Aβ toxicity and may explain the rapid progression of familial AD linked to mutations in the PS1 gene.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—Since glial activation is a pathological hallmark of AD and glia have been shown to undergo apoptosis in AD cases (19, 20), we used C6 glioma cells for our studies. Furthermore, these cells express high levels of LRP and can be easily transfected (5). Cells were maintained at 37 °C (5% CO₂ atmosphere) in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human wild type and mutant PS1 (M146L) stably transfected C6 cells were also used for these experiments as described previously (5). For the surface and viability assays, cells were seeded at a density of 1 × 10⁵/cm². Two days after seeding, cells were treated for 24 h with Aβ 1–40 (5–20 μM, Sigma) in the presence or absence of *α₂m. Aβ 1–40 was selected for these studies because this peptide has been shown to complex with *α₂m and to be cytotoxic (17, 21). *α₂m was activated by incubation with 0.4 M methionylamine for 2 h at room temperature. Control experiments were performed by incubation with *α₂m alone or the reverse peptide Aβ 40–1 (5–20 μM, Sigma). Excess methionylamine was removed by filtration on a PD-10 column (Amersham Pharmacia Biotech), and the activated *α₂m (*α₂m) was incubated with Aβ 1–40 in a 8:1 molar ratio in FBS for 24–72 h at 37 °C. Similar conditions have previously been shown to induce covalent modification of *α₂m with Aβ (17, 21). The Aβ-*α₂m solution was used at a working concentration of 10 μM (based on the starting free Aβ concentration). Receptor-associated protein (RAP)-glutathione S-transferase fusion protein (200 nm), an LRP ligand, and potent LRP antagonist was
used as described previously (25, 26). Fucoidan (Sigma), a scavenger receptor inhibitor was used at 200 ng/ml. All kinase inhibitors were purchased from Alexis Corp. (San Diego, CA) unless otherwise noted. KN93 (specific CaM KII inhibitor), KN92 (inactive control compound for KN93), KT 5720 (cAMP-dependent protein kinase A (PKA) inhibitor), and staurosporine (PKC inhibitor) were purchased from Alexis Corp. (San Diego, CA). For the mouse and rat LRP templates, a 225-base pair (bp) fragment from the narrow peak of cells with normal diploid DNA content. 

**Assessment of Surface LRP Levels**—Surface LRP levels were determined using immunocytochemical detection on a fluorescence-activated cell scanner (FACScan, Becton Dickinson, Franklin Lakes, NJ). Briefly, cells were trypsinized and washed with FACS buffer (1.5% FBS in PBS). Cell pellets were resuspended in 100 μl of the monoclonal anti-LRP primary antibody (5A6, 1:1500, kindly provided by Dr. D. Strickland) diluted in FACS buffer and incubated at 4 °C for 45 min. Parallel samples were incubated with anti-IgG1 isotype control (for nonspecific staining), mouse monoclonal anti-transferrin receptor (1:1000, Chemicon International, Temecula, CA), or mouse monoclonal anti-LDL-R (1:1000, Calbiochem, La Jolla, CA) (control for surface receptor expression). Cells were then incubated for 45 min at 4 °C with 1:50 diluted secondary antibody (goat anti-mouse FITC for LRP, transferrin, and LDL-R) (Vector Laboratories, Burlingame, CA). Cells were pelleted and resuspended in FACS buffer containing 1 μg/ml propidium iodide. Ten thousand cells per condition were collected on a FACs flow cytometer using a 488-nm argon laser at empirically determined instrument settings. Median FL1 (FITC) fluorescence values of live cells were determined for anti-LRP-stained and control cells, and dead cells were excluded from analysis on the basis of their propidium iodine fluorescence on FL3.

Additional analysis of LRP localization was performed by double-immunolabeling and laser scanning confocal microscopy. Briefly, as described previously (5), cells were plated on poly-l-lysine-coated glass coverslips, treated in the presence or absence of Aβ1–40, as described above and fixed for 20 min with 2% paraformaldehyde. Cells were then incubated overnight at 4 °C with the rabbit polyclonal antibody against RAP (1:40,000), followed by detection with the Tyramide Signal Amplification (TSA)-Direct (Red) system (NEL Life Science Products), followed by an overnight incubation with the rabbit polyclonal anti-LRP456 (1:100) and FITC-conjugated anti-rabbit IgG secondary antibody (1:75, Vector). This approach allows the identification of two different antigens using primary antibodies generated in the same species. In addition, cells were also incubated overnight at 4 °C with a mixture of anti-LRP456 (1:100, Vector) and the mouse monoclonal anti-protein disulfide isomerase (PDI, StressGen Biotechnologies, Victoria, Canada) a marker of the endoplasmic reticulum (ER). Next day coverslips were incubated with the FITC-conjugated goat anti-rabbit and Texas Red-conjugated horse anti-mouse secondary antibodies (Vector). Immunolabeled coverslips were then air-dried, placed cell-side up on SuperFrost slides (Fisher Scientific, Taunton), coverslipped with anti-fading media (Vector) and imaged using a scanning confocal microscope (MRC600, Bio-Rad). The signal corresponding to LRP immunoreactivity was visualized in the FITC channel, while the one corresponding to the ER was visualized in the Texas Red channel.

**Assessment of LRP Expression Levels**—In order to assess changes in LRP transcriptional levels, we utilized the ribonuclease protection assay. Riboprobe templates were amplified by PCR from human and mouse cDNA. For the PS1 template, a 305-base pair fragment corresponding to bases 1133–1437 of human PS1 (GenBank accession no. L76517) was obtained and subcloned into pCR II (Invitrogen Corp., Carlsbad, CA). For the mouse and rat LRP templates, a 225-base pair fragment corresponding to bases 2358–2601 of mouse LRP (GenBank accession no. X67469) was similarly isolated and subcloned. An actin ribonucleoprotein was used to normalize variations in loading or mRNA concentration (GenBank accession no. M18194). RPAs were carried out with 32P-labeled antisense riboprobes, and signals were quantified with a PhosphorImager (Molecular Dynamics) to assess levels of immunoreactive specific mRNA.

**DNA Fragmentation Assay and FACS Analysis**—Genomic DNA undergoes extensive DNA fragmentation into oligonucleosomal subunits late in apoptotic cell death. These DNA fragments are divided into apoptotic bodies, while both the nuclear and plasma membranes of these bodies remain intact. A flow cytometric method of Nicoletti et al. (28) was used to assess the percentage of fragmented nuclei. This assay is highly correlated (R2 = 0.82) with percentage of cells undergoing apoptosis as determined via annexin-V staining (data not shown). Briefly, cell nuclei were stained with a hypotonic lysis buffer containing 50 μg/ml propidium iodide to stain DNA at 4°C for at least 2 h. Samples were then run on a FACScan (Becton Dickinson) to quantitate DNA fragmentation on the FL3 channel (propidium iodide). Debris was excluded from collection of 10,000 nuclei by empirical setting of forward scatter channel and side scatter channel threshold levels. Apoptotic nuclear bodies appeared as a broad hypodiploid peak easily discernible from the narrow peak of cells with normal diploid DNA content.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT) Assay—The MTT assay used to assess cell viability is based on the ability of viable cells to convert MTT (a tetrazolium salt) into a blue formazan product. Following a 24-h treatment with various reagents as per the experimental design, 10 μl of MTT solution (2.5 mg/ml) was added to each well and cells were incubated overnight at 37 °C. Next, 100 μl of solubilization solution (50% dimethylformamide and 20% sodium dodecyl sulfate, pH 4.7) was added to the wells and cells were incubated overnight. Plates were then read at 570 nm using a Thermomax microplate reader (Molecular Devices).

**Statistical Analysis**—All experiments were done at least in triplicate and blind-coded. After results were obtained, the code was broken and averaged according to the experimental group and design. All values were expressed as mean ± S.E. Statistical analysis was done using the StatView program (Abacus Concepts, Cary, NC) by one-way ANOVA with post hoc Scheffe.

**RESULTS**

**LRP Is Protective against Aβ Toxicity**—Although previous studies have shown that LRP mediates clearance of Aβ when bound to β-mim (14), it has not been determined if this internalization is protective. To this end, C6 cells were incubated with Aβ 1–40 in the presence or absence of β-mim, and toxicity was assessed via FACS analysis of DNA fragmentation, and the MTT assay. We found that in cells treated with free Aβ (10 μm) approximately 50% of the nuclear bodies were apoptotic (Fig. 1A). In the presence of β-mim, Aβ-induced apoptosis was significantly decreased to 31.2 ± 2.4% (Fig. 1A). Treatment with RAP (200 nM), a potent LRP antagonist, ameliorated this protective effect and significantly increased the percentage of apoptotic bodies to 52.4 ± 2.8% (Fig. 1A). Concomitant RAP treatment did not alter observed apoptosis in cells treated with Aβ alone (Fig. 1A). Similar effects on cell viability were observed by the MTT assay (Fig. 1B). Specifically, in cells challenged with Aβ, concomitant β-mim significantly increased viability and this effect was ameliorated by addition of RAP (Fig. 1B). Since it is possible that, in addition to LRP, the observed protective effects may be mediated through other Aβ receptors, cells were also treated with fucoidan (200 ng/ml), a scavenger receptor inhibitor. This compound had no effect on toxicity in the presence of the mouse receptor inhibitor. This further supports the contention that the observed effects are mediated by LRP.

**Surface LRP Levels Are Up-regulated upon Aβ Challenge**—Since previous studies have shown that LRP is up-regulated in response to neuronal injury (29) and it has been implicated in Aβ clearance (14), it is possible that LRP expression and/or subcellular compartmentalization might be modified in response to Aβ challenge. To this end, LRP levels were measured via FACS analysis, ribonuclease protection assay, and Western blot. Additional qualitative analysis was done by immunocyto-
FIG. 1. LRP protects C6 cells against Aβ toxicity. A, FACS analysis of propidium-stained cells showed a significant increase in the number of apoptotic bodies in Aβ-treated compared with untreated cells. Percentage of apoptotic bodies was significantly decreased by preincubation of Aβ with activated αm (Aβ-αm) or by addition of RAP (an LRP antagonist). B, analysis of cell viability by the MTT assay further confirmed that addition of Aβ resulted in significant damage in C6 cells. In contrast, preincubation of Aβ with activated αm (Aβ-αm) or by addition of RAP protected cells against Aβ toxicity. *, p < 0.05 by one-way ANOVA with post hoc Scheffe.

FIG. 2. Colocalization of LRP and the ER marker PDI in C6 glioma cells. Cells were double-immunolabeled with the antibodies against LRP456 (green) and PDI (red) and imaged with the laser scanning confocal microscope. Colocalization between the two markers is shown in yellow. A, in untreated cells, most of the LRP immunoreactivity was seen in the ER and, to a lesser extent, in the cell surface. B, cells treated with αm alone showed that LRP is more abundant in the ER than in the cell surface. C, cells treated with the reverse peptide (Aβ 40–1) showed that LRP is present in the ER more than on the cell surface. Cells treated with Aβ 1–40 alone (D) or in the presence of αm (E) showed increased LRP immunoreactivity in the cell surface (arrow). F, elimination of primary antibody shows no immunostaining. Scale bar = 15 μm.

chemistry/laser scanning confocal microscopy. Consistent with previous studies (5), LRP immunoreactivity was preferentially observed both at the cell surface and in the cytoplasm where it was colocalized with the ER marker PDI (Fig. 2A). Treatment with αm alone (Fig. 2B) or reverse Aβ peptide (Fig. 2C) had no effect. Upon challenge with Aβ 1–40 and αm, LRP immunoreactivity was observed predominantly on the cell surface and, to a lesser extent, in the ER (Fig. 2, D and E). Similar results were in double-labeling experiments with the chaperone protein RAP, which is primarily localized in the ER (data not shown). Consistent with this, FACS analysis showed that surface LRP levels increased 2-fold after 24 h of treatment (Fig. 3, A and B). Free Aβ 1–40 increased surface LRP levels by 45.0 ± 17.2% (Figs. 2C and 3B). Control experiments where cells were immunolabeled with IgG isotype, anti-transferrin receptor, or anti-LDL-R did not show concomitant up-regulation upon Aβ challenge (data not shown). Additionally, treatment with other LRP ligands including αm (Figs. 2C and 3B), RAP (data not shown), and apoE (data not shown), or the reverse peptide, Aβ 40–1 (Fig. 2E), did not result in LRP up-regulation. In order to determine whether the observed LRP up-regulation occurred at the transcriptional or post-transcriptional levels, total protein and RNA were measured by Western blot and ribonuclease protection assay. No evidence of increase in protein or message was observed after 24 h of treatment (data not shown), suggesting that the observed increase in surface LRP immunoreactivity was due to translocation of LRP from reserve stores.

Down-regulation of LRP via PS1 Increases Aβ Toxicity—We have previously shown that overexpression of wild-type or mutant PS1 (M146L) down-regulates LRP total protein and RNA levels (5). Thus, we hypothesized that wild-type and mutant PS1-transfected cells would be more susceptible to Aβ toxicity relative to vector-transfected controls. Both wild-type and mutant PS1-transfected cells were significantly more susceptible to Aβ toxicity both in the presence or absence of αm (Fig. 4A). Apoptosis increased almost 2-fold in the PS1-transfected cells compared with vector controls when challenged with Aβ (Fig. 4A). In addition, Aβ-challenged PS1 transfectants showed corelative decreases in cell viability as measured by MTT absorbance (data not shown).

Although a protective effect of αm was observed in vector-transfected controls, in wild-type or mutant PS1-transfected cells no change in toxicity was observed either with addition of RAP (data not shown) or αm. Thus, we hypothesized that functional LRP was significantly decreased in these PS1 transfectants. FACS analysis of LRP surface expression showed that levels in wild type and mutant PS1 stably transfected cells were significantly decreased by approximately 30% and 40%, respectively (Fig. 4B). Furthermore, wild-type and mutant PS1-transfected cells did not show an up-regulation of surface LRP levels in response to Aβ challenge, whereas vector-transfected C6 cells responded with a 2-fold increase comparable to non-transfected C6 wild type controls (Fig. 4C). These results indicate that susceptibility to Aβ in PS1-transfected cells may be due not only to decreased LRP levels but also to the inability of these cells to up-regulate LRP expression to a level necessary for protection.
Translocation of LRP Is CaM KII-dependent—In order to determine which signaling cascade is important for LRP translocation, cells were treated with various kinase inhibitors upon Aβ challenge in the presence of *α2m. Surface LRP up-regulation was inhibited by the CaM KII inhibitor, KN93 (1 μM) (Fig. 5A). Staurosporine (1 μM), a broad spectrum protein kinase inhibitor (known to inhibit PKA, PKC, and CaM KII) also ameliorated the Aβ-induced up-regulation (Fig. 5A). The specific PKC inhibitor, bisindolylmaleimide IX methansulfonate (1 μM), the specific cAMP-dependent kinase (PKA) inhibitor, KT5720 (1 μM), or the control peptide KN92 (1 μM) did not affect LRP translocation (Fig. 5A). Similar effects of inhibition of LRP up-regulation upon KN93 or staurosporine treatment were observed with Aβ 1–40 alone (data not shown). This inhibition increased cell death only when cells were challenged with Aβ in the presence of *α2m (Fig. 5B).

Levels of CaM KII activity were determined via measurement of phosphorylation of autocamtide 3, a CaM KII-specific substrate peptide. A significant increase in CaM KII activity in response to Aβ:*α2m challenge was observed (Fig. 6). Addition of KN93, a CaM KII inhibitor, significantly decreased activity by approximately 30% below baseline (i.e. activity in untreated cells). Similar results were observed with Aβ peptide alone (Fig. 6). These results indicate that increased translocation of LRP from the ER to the cell surface upon Aβ challenge involves activation of the CaM KII pathway.

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FIG. 3. Surface LRP levels increase in response to Aβ challenge. A, a representative FL1 FACS histogram showed the shift in fluorescence values in untreated versus Aβ:*α2m-treated cells. B, Aβ 1–40 alone increased surface LRP immunoreactivity in C6 cells, while preincubation of Aβ with activated α2m (Aβ:*α2m) resulted in over a 2-fold increase in surface LRP expression. *, p < 0.05 by one-way ANOVA with post hoc Scheffe.

FIG. 4. FACS analysis of apoptosis and surface LRP immunoreactivity in PS1-transfected cells. A, wild-type and mutant (M146L) PS1-transfected cells showed an increased susceptibility to Aβ treatment as measured by percentage of apoptotic bodies. B, surface LRP immunoreactivity was significantly decreased in untreated wild-type and mutant PS1-transfected cells compared with vector-transfected cells. C, compared with vector-transfected and non-transfected cells, wild-type and mutant PS1-transfected cells did not show up-regulation of surface LRP levels in response to Aβ challenge.

FIG. 5. FACS analysis of surface LRP immunoreactivity and apoptosis in the presence of CaM KII blockers. A, cells treated with Aβ:*α2m were unable to up-regulate levels of LRP upon treatment with CaM KII inhibitors KN93 or staurosporine. B, inhibition of CaM KII activity in Aβ:*α2m-treated cells significantly increased apoptosis compared with cells treated with Aβ:*α2m alone. *, p < 0.05 by one-way ANOVA with post hoc Scheffe.
The present study showed that LRP mediates protection against Aβ toxicity in the presence of α2m and that this effect might be due to complexation of Aβ with activated α2m with subsequent clearance via LRP. In support of this possibility, inhibition of LRP via RAP or expression of PS1 (5) significantly increased Aβ toxicity only in the presence of α2m.

Further supporting a role for LRP in protection against Aβ toxicity, we found that LRP surface expression increased in response to Aβ challenge. This effect was specific to Aβ (free or complexed) although it was more robust with complexed Aβ. The LDL-R and transferrin receptor did not show concomitant up-regulation in response to Aβ challenge. The mechanisms by which increased translocation of LRP from the ER to the surface decreased Aβ toxicity are not completely clear. One possibility is that surface LRP might increase Aβ clearance, which otherwise if remaining free on the cell surface may be toxic to the cells via generation of free radicals (30). This is in accordance with a recent report by Qiu et al. (14) in which α2m was found to enhance clearance of endogenous soluble Aβ via LRP. However, since protection is associated with Aβ complexing with α2m, then an alternative possibility is that α2m may decrease formation of fibrillar Aβ (21, 31). Supporting this, previous studies have shown that preincubation of Aβ with α2m resulted in decreased Aβ toxicity (14). However, if this is the mechanism operating in our system, one would not expect to see the observed increase in toxicity by addition of LRP inhibitors (such as RAP), which by itself most likely does not alter the kinetics of fibrillar Aβ formation. Then, although α2m might change the conformation of Aβ, our results suggest that LRP plays a more central role in protection against toxicity.

Another finding of the present study was that translocation of LRP is mediated via a CaM KII mechanism since the specific inhibitor of this kinase, KN-93, blocked surface up-regulation of LRP and CaM KII activity increased in response to Aβ challenge. These effects were observed at doses as low as the high nanomolar range of Aβ (data not shown); thereby, a physiological role for the reported phenomenon could not be excluded. Miguel, however, previous studies have also suggested that CaM KII, which is particularly abundant in the hippocampus, where it is believed to be involved in the modulation of neurotransmitter release and long term potentiation (32–37), is implicated in transcytosis. Furthermore, calmodulin, a CaM KII-binding protein, is required for the recycling of endocytosed receptors including transferrin and LDL, possibly through regulation of endosome fusion (38–41). Taken together, these results suggest that CaM KII signaling might regulate LRP translocation from the ER to the surface.

Although the effects of other Aβ receptors cannot be excluded, in addition to RLP RAP also blocks other receptors (42), there are reasons to believe that LRP is an important mediator of protection against Aβ toxicity. Specifically, of the three known Aβ receptors, the receptor for advanced glycation end products is believed to mediate the cytotoxic effects of Aβ and has limited expression in the adult central nervous system, while the scavenger receptor (SR) does not affect Aβ accumulation (3, 43–46). In this regard, the present study showed that addition of the SR inhibitor fucoidan did not affect Aβ toxicity. Furthermore, in vivo studies in APP transgenic mice crossed with SR-knockout mice did not show either increased susceptibility to Aβ toxicity or increased plaque formation (10). In addition, a recent study showed that LRP plays an important role in regulating the generation of Aβ (47). Thus, these results support the contention that Aβ clearance is the central nervous system might occur primarily via LRP.

In conclusion, these studies provide evidence for a protective role of LRP against Aβ toxicity via the complex formation with α2m and may explain the rapid progression of familial AD linked to mutations in the PS1 gene.

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FIG. 6. CaM KII activity in Aβ-treated cells. Cells challenged with Aβ in the presence or absence of α2m showed increased CaM KII activity. This effect was blocked by the CaM KII inhibitor KN93.
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