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Serum deprivation increases the expression of low density lipoprotein receptor-related protein in primary cultured rat astrocytes ☆

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Abstract

The low density lipoprotein receptor (LDLR)-related protein (LRP) is a multifunctional receptor which mediates the endocytic uptake of several ligands implicated in Alzheimer's disease pathophysiology. Although LRP, as a member of the LDLR family, is likely to be regulated in response to various cellular stresses, this regulation has not been fully understood yet. In the present study we studied the regulation of LRP expression in primary cultured rat astrocytes in response to serum deprivation as a general cellular stress. A significant increase in LRP expression was detected after serum deprivation and this increase was blocked by treatment of U0126, an inhibitor of MAP kinase. This serum deprivation action was partially reversed by either serum or D-glucose supplementation, but further augmented by glutamine. This result contrasted with a finding that glutamine suppressed gadd153 protein induced by serum deprivation. Taken together, the present data suggest that serum deprivation induces dramatically LRP expression in astrocytes partly by MAPK signaling pathways and by signaling pathways apparently distinct from gadd153 induction.

Keywords: Low density lipoprotein receptor-related protein; Astrocyte; Serum deprivation; Glutamine; Growth arrest and DNA damage-inducible; MAP kinase

The low density lipoprotein receptor-related protein (LRP) is a glycoprotein that belongs to the low density lipoprotein (LDL) receptor family [1]. It binds and endocytoses more than 10 structurally and functionally distinct ligands including apolipoprotein E, β-amyloid precursor protein, and activated α_2-macroglobulin [2–4] which are associated with pathogenesis of Alzheimer's disease (AD). LRP has an apparent molecular mass of 600 kDa, and during transport from the endoplasmic reticulum to the cell surface it undergoes physiological cleavage into two subunits of 515 kDa heavy chain and 85 kDa light chain [5]. The 515 kDa chain contains the ligand binding sites and is anchored to the cell surface membrane by non-covalent interaction with 85 kDa chain, which constitutes a transmembrane domain [6].

LRP is widely expressed by numerous cell types, especially highly in nerve cells, and astrocytes [1,6,7], and its expression seems to be tightly regulated in vivo and in vitro [8–10]. For example, LRP expression is known to be regulated by various growth factors, cytokines and hormones. Increased level of LRP was found in murine bone marrow macrophage treated with colony-stimulating factor-1 [11], HepG2 cells with dexamethasone [12], adipocytes with insulin [13, 14], and murine hepatocytes with interleukin-1 [15]. In addition, recent studies have suggested that LRP level (or function) may be significantly down-regulated in probable AD patients [16] and that LRP surface expression is preferentially up-regulated in response to amyloid peptide, a major cellular stress associated with AD.

In the present study, to test how gene expression of LRP is regulated by stresses, we studied the regulation

☆ *Abbreviations:* LRP, low density lipoprotein receptor-related protein; gadd, growth arrest and DNA damage-inducible protein; LDL, low density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PEA, *Pseudomonas* exotoxin A; HSP, heat shock protein; Erk, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase.

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of LRP expression by serum deprivation which we employed as a general cellular stress. Serum is composed of a variety of growth and nutritional factors and thus essential to cell growth [17]. Therefore, cells without it should be highly stressed. For this study we used primary cultured rat astrocytes where LRP is highly expressed [19]. Astrocytes, with highly dynamic physiology, constitute a major population of brain cells that respond to numerous brain damages and stresses [18]. Therefore, the abundant expression of LRP in astrocytes [19], along with the fact that LRP is structurally and functionally related to the LDL receptor whose expression is modulated by serum factor [20,21], suggests that the expression of LRP may be modulated by serum deprivation. In this paper, we report, for the first time, that LRP is markedly increased by serum deprivation and this increase is mediated partly by extracellular signal-regulated kinase (Erk) signaling pathways. These findings suggest that LRP is another newly identified serum deprivation-responsive protein.

Materials and methods

Materials. Fetal bovine serum (FBS), MEM, trypsin–EDTA, and streptomycin–penicillin were obtained from Gibco-BRL (Grand Island, NY). The monoclonal antibody against 85 kDa subunit of human LRP was collected in supernatants from hybridoma cultures (11H4) obtained from the American Tissue Culture Collection (Rockville, MD). The polyclonal antibodies against gadd153 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and those against p42/44 MAPK (Erk), phospho-specific Erk, phospho-MEK1/2, and phospho-Raf were obtained from Cell Signaling (Beverly, MA). *Pseudomonas* exotoxin A (PEA) was purchased from Boehringer–Mannheim (Indianapolis, IN) and U0126 was from Calbiochem (La Jolla, CA). L-Glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and mouse anti-glial fibrillary acidic protein (GFAP) antibody were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were of the purest analytical grade.

Cell culture and serum deprivation. Rat cortical astrocyte cultures were prepared from forebrain of 4–5-day-old Sprague–Dawley rats according to the method of Chiu and Goldman [22] with minor modifications. In brief, forebrains were dissected free of meninges, minced, and incubated in phosphate-buffered saline containing 0.25% trypsin, 0.02% EDTA for 10 min at 37 °C. After MEM containing 10% FBS was added, the cells were dissociated by several passages through a pipette and filtered through a 125 μm nylon mesh. Cells were then centrifuged at 200*g* for 10 min, resuspended in the culture medium (MEM containing 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin), and allowed to grow in the culture dishes for 7–10 days. Non-astroglial cells were removed by shaking the cultures on an orbital shaker for 15–18 h at 37 °C as described by McCarthy and de Vellis [23]. Isolated astrocytes were approximately 95% pure as determined by immunostaining with mouse anti-GFAP (1:400 dilution) antibody and maintained in the culture media containing 10% FBS. For serum deprivation assay, cells were rinsed with MEM and treated with the culture medium containing reduced concentrations (0.5% or 5%) of FBS for the indicated times.

Measurement of cell viability. The viability of astrocytes after serum deprivation was measured by the MTT reduction method. Briefly, at the indicated time after serum deprivation, the cells were rinsed with phosphate-buffered saline, pH 7.2, and incubated with 0.5 mg/ml MTT reagent for 3 h. The reagent was reduced by living cells to form insoluble blue formazon product. The cells were washed, solubilized with DMSO, and quantified as the absorbance (*A*) at 570 nm.

Western blot analysis. To compare relative density of LRP and gadd153 protein expressed in rat astrocytes, cells were scraped and homogenized in 10 mM Tris buffer (pH 7.4) containing 250 mM sucrose, 2 mM EDTA, 100 μM PMSF, 5 μg/ml leupeptin, and 20 μg/ml aprotinin. Crude homogenates were subjected to a centrifugation at 200*g* for 10 min to prepare post-nuclear supernatants. Equal quantities of protein (20 μg) of post-nuclear supernatants were directly solubilized in a solubilization buffer and separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by electrophoretic transfer onto nitrocellulose membranes. The blots were then probed with anti-LRP (1:250 or 1:500 dilution), anti-gadd153 protein (1:1000 dilution), anti-Erk (1:1000), anti-phospho-specific Erk (1:2000), anti-phospho-specific MEK1/2 (1:2000), or anti-phospho-specific Raf (1:2000) followed by corresponding secondary antibodies, and developed using enhanced chemiluminescence reagents (ECL, Amersham, DE).

Northern blot analysis. Total RNA was isolated from cells using Trizol reagents (Gibco-BRL, Gaithersburg, MD), quantified by UV spectrophotometry, and stored at −80 °C prior to use. Total RNA (20 μg) from control and serum-deprived (for 24 h) astrocytes was electrophoresed on 1% (w/v) agarose gels and electroblotted to Zeta probe nylon membrane (Bio-Rad Laboratories, Richmond, CA). The cDNA fragment (619 bp) specific for rat LRP was prepared by PCR using forward (5′-GGCTGCTGATGGCTCCCGAC-3′) and backward (5′-TCTCGTCCGTGCTGGCCAGG-3′) primers, and separated on low-melting Seaplague agarose (FMC BioProducts, Rockland, ME). The probe (25 ng) was labeled with [^{32}P]dCTP (50 μCi) using a Random-primed DNA Labeling Kit (Gibco-BRL). Electroblotted RNA on nylon membranes was hybridized with the labeled cDNA fragment at 42 °C for 24 h. In a control experiment, membrane was also hybridized with labeled actin cDNA probe. Hybridized bands were quantitated by autoradiography using a bio-imaging analyzer (FUJIFILM BAS-1500, Fuji Photo Film, Japan).

Pseudomonas exotoxin A (PEA) sensitivity assay. Astrocytes grown for 24 h with the culture medium containing either 10% or 0.5% FBS were treated with PEA (500 ng/ml) for 10 min and then washed three times with the culture medium. The viability of cells was analyzed by phase contrast microscopy and by the MTT reduction method.

Statistical analysis. Results were expressed as means ± SD. Statistical significance of the data was determined by Student's unpaired *t* test where a value of $p < 0.05$ was considered statistically significant.

Results

Serum deprivation up-regulates the expression of LRP in rat astrocytes

Isolated astrocytes from neonatal rat brain expressed abundant LRP and interestingly, its expression was largely limited to the intracellular region with a little expression at the cell surface (data not shown). To elucidate if serum deprivation affects LRP expression, astrocytes maintained in the 10% FBS-containing culture media were rinsed with MEM and treated for 24 h with the culture medium containing various concentrations (0.5%, 5%, and 10%) of FBS. Both LRP protein and its mRNA were significantly up-regulated with decreasing concentrations of FBS (1.9- and 3.1-fold at 5% and 0.5% FBS, respectively; Fig. 1A). Similar increases in LRP

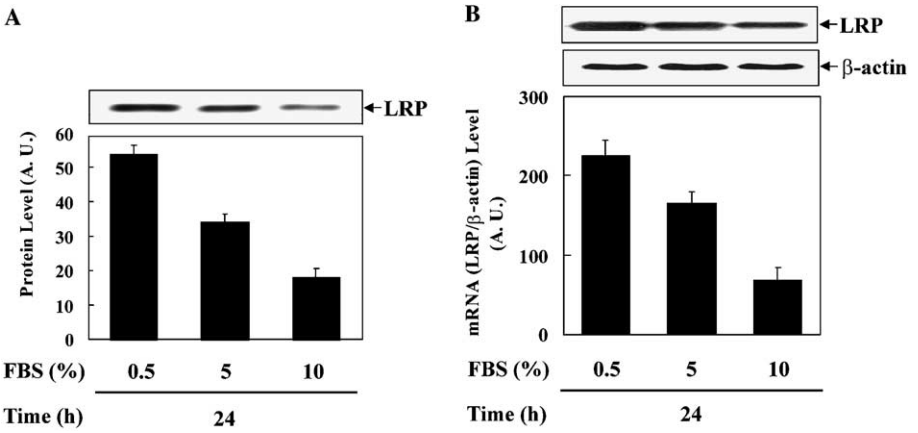

Fig. 1. Concentration-dependent expression of LRP and its mRNA in primary cultured rat astrocytes. Astrocytes maintained in the culture media containing 10% FBS were rinsed with MEM, incubated with different concentrations of FBS for 24 h, and harvested. (A) Western blot analysis of 85 kDa subunit of LRP. Cellular protein (20 μg) was electrophoresed by SDS–PAGE. The electroblotted nitrocellulose was then reacted for 1 h at room temperature with monoclonal anti-LRP antibody (11H4) followed by anti-mouse peroxidase-conjugate. The final detection was carried out using enhanced chemiluminescence reagents. (B) Northern blot analysis of LRP mRNA. Total RNA (20 μg) isolated from astrocytes was electrophoresed on 1% (w/v) agarose gels and electroblotted to Zeta probe nylon membrane. The blot was probed with [^{32}P]dCTP-labeled LRP and β-actin cDNAs. The level of β-actin expression was used as the internal control for the expression of LRP mRNA. Data are means ± SD of triplicate experiments. Significant differences in LRP protein and mRNA levels relative to control (10% FBS) are shown ($p < 0.001$).

mRNA were observed (2.3- and 3.3-fold at 5% and 0.5% FBS, respectively; Fig. 1B). In time-course experiments, a significant increase (about 2.0-fold) of LRP protein was seen as early as 12 h and this augmentation was time-dependent up to 24 h (Fig. 2A). At longer incubation (for 48 h) of cells in 0.5% FBS, however, no further increase was observed (3.2-fold for 24 h incubation and 3.4-fold for 48 h incubation, $p = 0.1115$). Similar increasing pattern was also observed in the induction of LRP mRNA up to 24 h (3.9-fold for 24 h; Fig. 2B). Accordingly, all subsequent experiments referred to as 'serum-deprived' were performed using 0.5% FBS and for 24 h incubation. However, the viability of astrocytes in the absence and presence of serum deprivation for 24 h was not significantly different as that measured in MTT reduction (10% FBS, 0.166 ± 0.008 in $A_{(570–630)\,nm}$; 0.5% FBS, 0.162 ± 0.004 in $A_{(570–630)\,nm}$, respectively, $p = 0.1223$).

The addition of either serum or D-glucose blunts the induction of LRP by serum deprivation

To confirm that LRP induction observed in this study was indeed mediated by serum, serum-deprived astrocytes were again treated with fresh culture medium containing 10% FBS and incubated further for 24 h (total 48 h: serum deprivation for 24 h and serum replenishment for 24 h). As shown in Fig. 3, serum

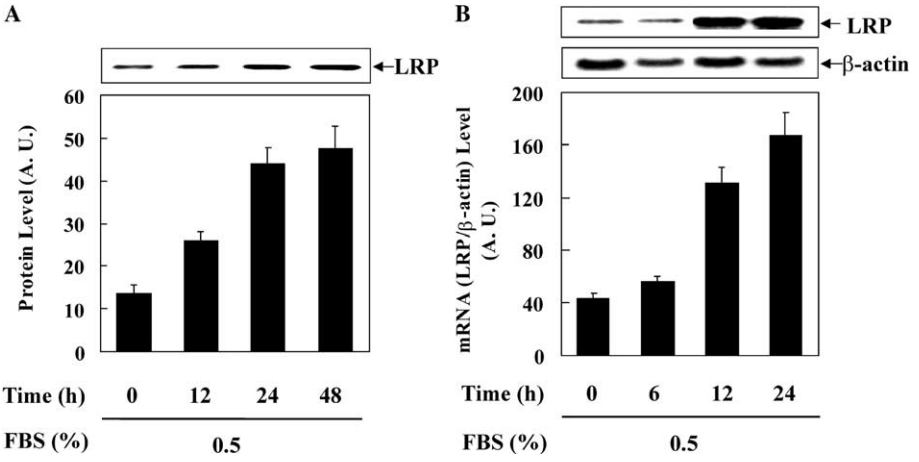

Fig. 2. Time-dependent expressions of LRP and its mRNA in cultured rat astrocytes. Cells were incubated with the culture medium containing 0.5% FBS for different times and harvested. LRP protein (A) and mRNA (B) expressions were measured by Western and Northern blot analysis, respectively, as described in Fig. 1. Data are means ± SD of triplicate experiments. Significant differences in LRP protein and mRNA levels relative to control (0 time) are shown ($p < 0.001$), except for the difference in LRP level between 24 and 48 h incubation as shown in (A) ($p = 0.1115$).

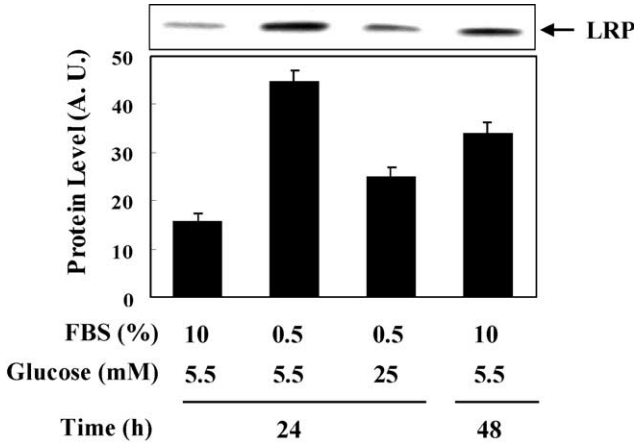

Fig. 3. The effect of glucose and serum on LRP induction by serum deprivation. Cultured rat astrocytes were incubated for 24 h with the culture medium containing either 10% or 0.5% FBS. For glucose supplementation, cells were incubated with the culture medium containing 0.5% FBS supplemented with 25 mM (final) D-glucose at the beginning of experiment. For serum supplementation, the cells incubated for 24 h with the culture medium containing 0.5% FBS were again supplied with fresh culture medium containing 10% FBS and incubated further for 24 h (total 48 h). The LRP expression at each condition was measured by Western blot analysis. Data are means ± SD of triplicate experiments.

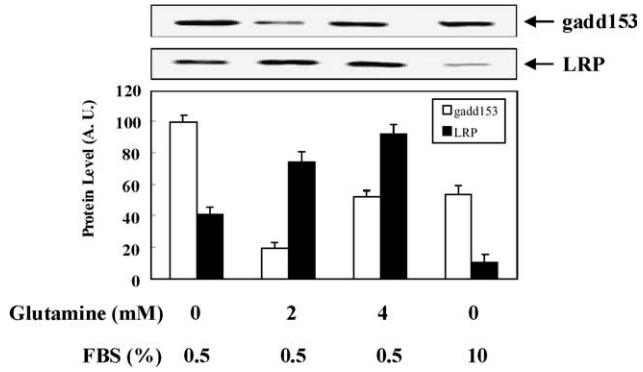

Fig. 4. The effect of glutamine on the induction of gadd153 protein and LRP by serum deprivation. Cultured rat astrocytes were incubated for 24 h with the culture medium containing either 10% FBS or 0.5% FBS. In the selected experiments, serum-deprived cells were supplemented with either 2 or 4 mM glutamine at the beginning of experiment. The expressions of gadd153 protein and LRP at each condition were measured by Western blot analysis using polyclonal anti-gadd153 protein antibody and anti-rabbit peroxidase conjugate as a secondary antibody. Data are means ± SD of triplicate experiments.

deprivation induced LRP expression approximately 2.7-fold, but the addition of serum partially reversed LRP induction (about 37%) caused by serum deprivation, suggesting that our result is not artifact. In this study, the level of LRP protein in astrocytes deprived of serum for 24 h was used as the control for LRP level in cells with serum supplementation (data not shown) since the former was not significantly different from the level of LRP in cells deprived of serum for 48 h (Fig. 2A). Next, we tested to define serum component(s) responsible for this action. Serum is known to play an important role in supporting survival and growth of many mammalian cells [17]. Under limited concentration of serum, therefore, cells are likely to be under significant stress. Since it is known that energy supply allows cells to resist and survive many different types of stresses, we first tested if glucose, a universal energy source to the cells, can reverse the increase of LRP induced by serum deprivation. As shown in Fig. 3, the addition of 19.5 mM D-glucose (25 mM final) to the culture medium containing 0.5% FBS significantly restored LRP expression level by 68%. In contrast, no alteration of LRP induction by serum deprivation was found when D-glucose was replaced by L-glucose (data not shown).

The induction of LRP by serum deprivation may be mediated by signaling pathways apparently distinct from gadd153 protein induction

Next, we tested if the intracellular molecular mechanism responsible for LRP induction shares that for gadd induction. There are ample evidences to suggest that gadd expression was readily induced under stress conditions such as serum deprivation and malnutrition [24–27]. Furthermore, the replenishment of L-glutamine has been shown to suppress gadd153 mRNA induction by nutrient deprivation [28]. In this study, we also found that serum deprivation enhanced the gadd153 protein level (1.8-fold) in rat astrocytes (Fig. 4) and that this induction was greatly suppressed by the addition of glutamine. A maximal reduction of gadd153 protein occurred at 2 mM, and this effect subsequently decreased at the higher concentration (4 mM) showing the restoration to the control level. In contrast, the addition of glutamine in the culture medium containing 0.5% FBS failed to overcome LRP induction and rather further increased LRP expression in a concentration-dependent manner (Fig. 4). A maximal induction of LRP expression by glutamine (about 8.6-fold) was observed at 4 mM. This result suggests that the induction of LRP and gadd153 protein by serum deprivation may be modulated by different molecular mechanisms in rat astrocytes.

Serum deprivation also increased the LRP level on the cell membrane

PEA is selectively internalized by cell surface LRP and is thus toxic primarily for LRP-expressing cells [29–31]. Therefore, using PEA toxicity assay it is possible to examine indirectly the presence of LRP on the cell surface membrane and if it is functional. As shown in Fig. 5, approximately half of astrocytes (about 45.1%) grown with 0.5% FBS died and were detached from the culture plates under toxin treatment, while most of control cells grown with 10% serum survived and appeared morphologically intact when examined by phase-contrast microscopy.

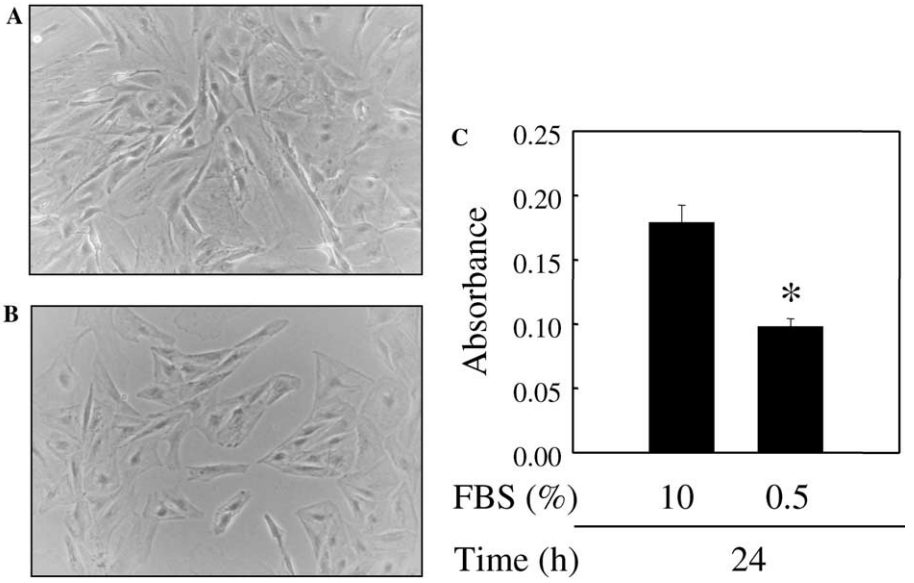

Fig. 5. Toxicity of serum-deprived cells to the cytotoxin *Pseudomonas* exotoxin A (PEA). Cultured rat astrocytes incubated for 24 h with the culture medium containing either 10% FBS or 0.5% FBS were treated with 500 ng/ml PEA for 10 min at 37 °C. Phase-contrast micrographs of cells grown in 10% (A) or 0.5% (B) are shown. The viability of cells was determined by MTT reduction assay (C). Data are means ± SD of triplicate experiments. Significant reduction in the number of viable cells was found in the cultures grown with 0.5% serum compared with control (*$p < 0.001$).

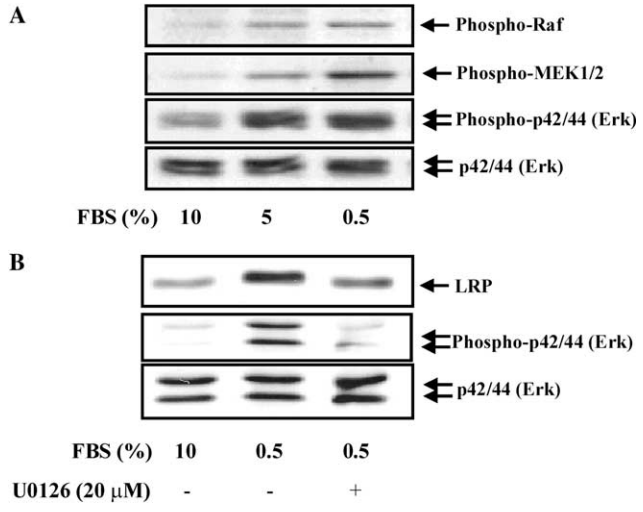

Fig. 6. MAP kinase (Erk) pathways are involved in serum deprivation-induced LRP expression. (A) Cultured rat astrocytes were incubated for 24 h with culture medium containing either 10%, 5%, or 0.5% FBS and harvested. Phosphorylation of Raf, MEK1/2, and Erk was assayed by Western blot analyses by using phospho-specific antibodies (see Materials and methods). Serum deprivation (5% or 0.5% FBS) caused phosphorylation of Raf, MEK1/2, and Erk while the level of total Erk protein was unchanged. (B) Cells were treated with U0126 (20 μM) 30 min before serum deprivation and further incubated for 24 h with culture medium containing 0.5% FBS; control cells were incubated in the presence of 10% FBS. The levels of LRP protein and phosphorylation of Erk were measured by Western blot analyses and all data shown were obtained from the same blot. U0126 significantly blocked serum deprivation-induced LRP increase and phosphorylation of Erk.

Increased LRP by serum deprivation is mediated partly by Erk signaling pathways

It is known that serum deprivation induces variety of cell responses, such as cell survival, cell death, and gene expression through MAP kinase signaling pathways [32,33]. Therefore, we tested whether LRP induction by serum deprivation is mediated by activation of Erk signaling pathways. As shown in Fig. 6A serum deprivation (0.5% or 5% FBS) of astrocytes for 24 h induced phosphorylation of Raf, MEK-1/2, and p42/44 Erk as detected by Western blot analyses by using their anti-phospho antibodies. Moreover, both Erk phosphorylation and LRP induction by serum deprivation were blocked in the presence of a specific MEK/Erk inhibitor, U0126 (20 μM, 24 h; Fig. 6B), suggesting that Erk signaling pathways are involved in an increase of LRP induced by serum deprivation. SB203580 (20 μM), a specific p38 MAP kinase inhibitor, slightly but not as efficiently as U0126 blocked LRP induction by serum deprivation (data not shown).

Discussion

There are numerous reports showing that the expression of LRP in brain cells including astrocytes is up-regulated in response to various cellular stimuli and trophic factors, in addition to changes in its cellular distribution [9,34,35]. Although specific role of LRP in brain cells is not well characterized, both LRP and some of LRP ligands such as apolipoprotein E, amyloid pre-

cursor protein, α_2-macroglobulin, and tissue-plasminogen activator have been implicated in neurodegeneration and brain injury, which are associated with various cellular stresses. In this regard, previous studies have shown that high levels of LRP were found in brains from Alzheimer's disease (AD) patients [3,34,36] at senile plaques, tangles, and dystrophic neurites. Brain cells are likely to be frequently challenged by various stresses [18,37], particularly, during the periods of their development and progressive neurodegeneration. In support of above tissue-level findings, cell surface LRP level is found to increase significantly in response to amyloid β-peptide (Aβ) [35] which is known to exert brain cell toxicity via, in part, oxidative stress [38,39]. Serum deprivation is another common type of cellular stress and challenges astrocytes in certain situations such as ischemia. In addition, LRP is structurally and functionally related to LDL receptor whose expression is modulated by serum factors [20,21]. Thus, although expression of LRP was expected to increase under in vitro cellular stress, serum deprivation, this study demonstrates, for the first time, that expression of LRP was markedly augmented under serum deprivation.

The role of increased LRP (Figs. 1 and 2) in response to serum deprivation remains unknown at the present time. There are considerable evidences in the literature showing that serum deprivation leads to induction of many genes; these include a set of stress genes such as gadd [24,26,27] and heat shock proteins (HSPs) [40,41]. Their up-regulations are often speculated to support maintain cell viability under adverse conditions. In this regard, recent study showed that, in human saphenous veins, serum deprivation stimulated the expression of HSP70 coincidental with the disappearance of apoptotic markers, suggesting a protective effect of HSP70 on tissue damage [41]. In addition, the presence of increased cell surface LRP after serum deprivation (Fig. 5), thereby exerting itself to be functionally active as a receptor, may support the possibility of a protective role as suggested in case of increased cell surface LRP in response to Aβ insult [35]. In this study, we also raised a question as to if up-regulation of astrocytic LRP induced by serum deprivation plays a role in protecting astrocytes against cellular stress. Serum constitutes a variety of nutritions, hormones, and growth factors [17], and therefore its deprivation significantly limits cells to either singular or combined above-mentioned factor(s). Since the increased LRP expression by serum deprivation was markedly suppressed by D-glucose supplementation (Fig. 3), it appears to be associated with energy metabolism in astrocytes. A variety of cells deprived of energy are likely to obtain energy primarily from nutritional intake for survival at their early times. The important role of LRP in the regulation of lipid and lipoprotein metabolism in a variety of cells suggests that the increased level of LRP may be the consequence of the higher requirements of lipid and lipoprotein of serum-deprived astrocytes in relation to their energy production. However, further experiments such as overexpression of LRP in astrocytes are needed to clarify if LRP indeed plays a role in protecting astrocytes under serum deprivation.

Although a protective role of LRP is speculated to share that of other stress-activated proteins, our data showed that the molecular mechanism of LRP induction by serum deprivation is different from that of gadd153 induction (Fig. 4). Previously, in renal proximal tubular epithelial cells (LLC-PK1), gadd153 mRNA was reported to be induced by nutrient deprivation but its induction was significantly suppressed by glutamine supplementation [28]. Our results of the biphasic glutamine response, such as maximal suppression of gadd153 protein expression at a lower concentration of glutamine (2 mM) and then attenuation, were consistent with those shown in the previous study. In contrast with the regulation of gadd153 protein, however, glutamine failed to suppress serum deprivation-induced LRP expression and rather increased in a concentration-dependent manner (Fig. 4), suggesting that the mechanism underlying LRP induction by serum deprivation was apparently distinct from that of gadd153 in astrocytes. In this regard, it is interesting to compare the signaling pathways involved in serum deprivation-induced gadd153 and LRP expression. Our data showed that induction of LRP by serum deprivation involves Raf/MEK/Erk signaling pathways (Fig. 6). Although it is not clear how serum deprivation-induced gadd153 increase is regulated, literatures [42,43] showed that stresses, such as low level glucose and bile acid, induce activation of gadd153 by p38 MAP kinase and Erk, respectively. Thus, induction of LRP and gadd153 by serum deprivation could require activation of different MAP kinases. Alternatively, if they share Erk activation, other signaling molecules at downstream of Erk may play a role in differential effects by glutamine on serum deprivation-induced gene expression.

Although our data were focused on energy deprivation as one of the major regulators responsible for LRP induction, there is room for considering other serum factors such as hormonal and growth factors. Nonetheless, this study clearly demonstrated that serum deprivation induced LRP expression in brain cells. It is yet to be known, however, if the increased gene expression of LRP is one of the common cellular consequences in response to many other stresses. Recent studies showing that LRP expression was induced by hypoxia [44], a well-known cellular stress in brain, by hydrogen peroxide (data not shown) or at amyloid plaques of AD brain indicate a possible existence of common cellular responses in response to stresses. Further studies on the molecular mechanism of LRP induction by serum deprivation will enhance our understanding of specific role of LRP in astrocytes and perhaps general mechanism of stress-induced gene expression.

Acknowledgments

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