The effect of rosiglitazone on LRP1 expression and amyloid β uptake in human brain microvascular endothelial cells: a possible role of a low-dose thiazolidinedione for dementia treatment

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Abstract
Thiazolidinediones, such as rosiglitazone or pioglitazone, are anti-diabetic agents that have been expected to show a beneficial effect in Alzheimer’s disease (AD) because of their anti-inflammatory effect. However, these agents have failed to show a significant beneficial effect on AD in recent clinical trials. Here, we suggest that low-dose rosiglitazone treatment, and not the conventional doses, has an amyloid β (Aβ)-clearing effect by increasing LRP1, an Aβ outward transporter in the blood–brain barrier. Rosiglitazone up-regulated LRP1 mRNA and protein expression and LRP1 promoter activity in human brain microvascular endothelial cells (HBMECs). Aβ uptake through LRP1 in HBMECs was also increased by rosiglitazone. This increase in LRP1 and Aβ uptake was observed in up to 10 nM rosiglitazone concentration. At concentrations above 20 nM rosiglitazone, the LRP1 expression and Aβ uptake in HBMECs were not altered. The possible mechanism of this unusual dose response is discussed. This study suggests a new therapeutic application of thiazolidinediones for AD at a much lower dose than the doses used for diabetes treatment.

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Introduction
Alzheimer’s disease (AD), the most frequent form of senile dementia, is characterized by extracellular senile plaques and neurofibrillary tangles, as well as progressive neurodegeneration (Ballard et al. 2011; Wirths et al. 2004). Amyloid β (Aβ), a 38–43 kDa peptide, is derived from the proteolytic cleavage of the amyloid precursor protein and forms the core of the senile plaque (Bates et al. 2009). The amyloid hypothesis as a pathogenesis of AD states that overproduction and/or impaired clearance of Aβ peptide in the human brain result in pathological deposition of Aβ in AD brain, serial neuronal dysfunction and loss, and atrophy in involved brain regions, and finally, clinical symptoms (Ballard et al. 2011; Bates et al. 2009; Wirths et al. 2004). Low-density lipoprotein receptor-related protein 1 (LRP1) is a member of the low-density lipoprotein (LDL) receptor gene family and this cell surface glycoprotein binds and internalizes diverse ligands (Herz & Strickland, 2001). Several studies have revealed that LRP1 in brain capillaries mediates outward transport of Aβ from the brain across the blood–brain barrier (BBB) and plays a key role in clearing Aβ to the blood circulation (Goto & Tanzi, 2002; Shibata et al. 2000; Ueno et al. 2010; Zlokovic et al. 2010).

As the epidemiological association between AD and diabetes has been reported, anti-diabetic agents

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have attracted attention as a new therapeutic option for AD (Akter et al. 2011; Riederer et al. 2011). Thiazolidinediones, such as rosiglitazone or pioglitazone are oral hypoglycaemic agents that improve insulin resistance through activation of the peroxisome proliferator-activated receptor-γ (PPARγ). These agents have been expected to show a beneficial effect in AD because of its anti-inflammatory effect (Geldmacher et al. 2011), but the clinical evidence is still controversial (Geldmacher et al. 2011; Gold et al. 2010; Landreth et al. 2008). In one previous in-vitro study, rosiglitazone was reported to increase LRP1 expression in adipocytes via PPARγ activation (Gauthier et al. 2003), and we reaffirmed rosiglitazone-induced LRP1 up-regulation in hepatocytes (J. H. Moon et al., unpublished data). On the basis of these data, we hypothesized that rosiglitazone would increase LRP1 expression and Aβ efflux in the BBB. In this study, we elucidated the effect of rosiglitazone on the expression and function of LRP1 in endothelial cells derived from human brain microvessels. This study will provide important clues on a possible new application of thiazolidinediones in the treatment of AD.

Methods

Cell culture and preparation

Primary human brain microvascular endothelial cells (HBMECs; Sciencell, USA) were plated on fibronectin-coated dishes and cultured in endothelial cell medium (ECM) supplemented with 5% fetal bovine serum and endothelial cell growth supplement (ECGS) according to the manufacturer’s instructions. Rosiglitazone was provided by GlaxoSmithKline (UK) and prepared in DMSO. HBMECs were incubated with various concentration of rosiglitazone for 48 h by adding the stock solution to the culture media. The final concentration of DMSO in the culture media was adjusted to 0.5% (v/v).

Total RNA and cDNA preparation

Total RNA was isolated from HBMECs using Trizol reagent (Invitrogen, USA) and quantified using NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA). Following RNA extraction, 4 μg RNA was treated with 1 U RNase-free DNase I to remove all contaminating genomic DNA. After removing DNase I, DNase-treated RNA was subsequently used for cDNA synthesis using MMLV reverse transcriptase (Promega, USA) according to the manufacturer’s protocol. The synthesized cDNA was stored at −20°C for later use.

Quantitative real-time PCR (RT-PCR)

Quantitative RT-PCR analysis was performed using Taqman assay kits for LRP1 (Hs00233856_m1) with ABI 7500 instrument (Applied Biosystems, USA). The β-actin (Hs99999903_m1) gene was used as an invariant control. PCR reactions were performed in triplicate reactions in a final volume of 20 μl according to the manufacturer’s protocol. For each assay, a standard curve was obtained by analysing a series of dilutions of pooled cDNA samples for the relevant gene. Data were analysed with Sequence Detector 1.7 software (Applied Biosystems).

Immunoblot analysis

Cell lysates were prepared using MPER® (Thermo Scientific, USA) and aliquots of cell lysates were denatured under reducing conditions (1.75% SDS, 15 mM 2-mercaptoethanol) for 5 min at 100°C. Total protein amount in each cell lysate was determined by Bradford assay (Sigma-Aldrich, USA). Cell lysates including 10 μg protein were loaded to SDS–PAGE for immunoblot analysis. For LRP1, nitrocellulose membranes were incubated with anti-LRP1 antibody (Epitomics, USA) at 1:1500 dilution overnight at 4°C, then subsequently with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, USA) at 1:5000 dilution overnight at 4°C, then detected with the ECL Western Blotting Analysis System (Thermo Scientific). β-actin immunoreactivity, detected with monoclonal anti-β-actin antibody (Sigma-Aldrich) at a 1:5000 dilution and horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) at a 1:5000 dilution, were used as loading control.

Small interfering (si)RNA transfections

siRNA targeting human LRP1 (siLRP1) and non-targeting negative siRNA (siCTRL) were purchased from Thermo Scientific. Each siRNA was transfected into HBMECs using Lipofectamine 2000 (Invitrogen, USA). We determined siRNA silencing efficiency by RT-PCR of LRP1 mRNA in siLRP1- and siCTRL-transfected HBMECs.

Aβ uptake analysis

The Aβ40 (HiLyte Fluor 488-labelled) and Aβ42 (HiLyte Fluor 555-labelled) synthetic peptides were purchased from Anaspec (USA) and reconstituted in 1% NH4OH. After treatment with the indicated concentration of rosiglitazone for 48 h, HBMECs were treated with Aβ40 or Aβ42 (1 μM, respectively) and
incubated at 37 °C for 1 h in complete medium. If needed, siLRP1 was transfected to HBMECs for LRP1 gene silencing prior to rosiglitazone treatment. Incubated cells were washed with PBS and lysed with 0.2% SDS in water. The fluorescence in cell lysate was measured with a Berthold Fluorometer (Berthold, Germany), and the values in cell lysates were standardized with protein amounts.

To obtain fluorescence microscopic images of Aβ uptake, HBMECs were plated on fibronectin-coated chambered glass slides (BD Bioscience, USA) 1 d prior to the experiment. Cells were incubated with Aβ at 37 °C for 1 h before fixation in 2% paraformaldehyde for 5 min. Slides were mounted using ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) visualizing the nuclei. Images were captured using a IX 71 inverted fluorescence microscope equipped with DP71 digital camera (Olympus, Japan).

Construction of the LRP1 promoter-reporter vector

The LRP1 promoter-reporter was constructed according to the method reported by Gauthier et al. (2003). The LRP1 promoter region containing a peroxisome proliferator response element (PPRE) was amplified by PCR using the LRP1-BAC construct as a template (Source BioScience imagenes, Germany) using the following primers: 5'-GCAACGAGCTCCG TAAAAGGGGAAG-3’ and 5'-GCAGCAGATCTTT CCCCGACTGAAG-3’. The amplified fragment was subcloned into the ScaI and BglII sites of the firefly luciferase reporter vector, pGL3-Basic (Promega). This construct was designated as pGL3-PPRE. The integrity of reporter plasmid sequences was confirmed by DNA sequencing.

Transient transfection assays

HBMECs were seeded at a density of 1.5 × 10⁶ cells/well in six-well plates 48 h prior to transfection. When the cell density reached a confluency of 90%, cells were co-transfected with 4 μg pGL3-PPRE and 0.25 μg pRL-CMV, the Renilla luciferase reporter vector using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s protocol. For negative control, pGL3-Basic vector was used. Four hours after transfection, the cells were treated with indicated concentrations of rosiglitazone for 48 h. The cell lysates were prepared with 250 μl reporter lysis buffer (Promega).

Luciferase activities derived from both firefly (pGL3-PPRE) and Renilla (pRL-CMV) proteins were measured using the dual luciferase reporter assay system (Promega) using a luminometer (Berthold, Germany). The firefly luciferase activity was normalized with Renilla luciferase activity to minimize experimental variability caused by differences in cell viability or transfection efficiency.

Statistical analysis

All statistical analyses were conducted using SPSS software version 18.0 (SPSS, USA). Values are expressed as the mean ± s.e. Statistical comparisons between groups were performed using Student’s t test. Data with a p value < 0.05 were considered significant.

Results

Rosiglitazone increased LRP1 expression in a dose-dependent manner in HBMECs

LRP1 mRNA and protein expressions were increased by 0.5 and 10 nm rosiglitazone (Fig. 1a, b). However, at concentrations above 20 nm rosiglitazone, LRP1 mRNA and protein expression were not changed compared to those in non-treated cells (Fig. 1a, b). We confirmed that rosiglitazone increased the amount of LRP1 protein in a dose-dependent manner up to 10 nm in HBMECs (Fig. 1c).

Rosiglitazone induced transcriptional activation of the LRP1 promoter

The transcription activity of the LRP1 promoter was assayed using the promoter-reporter construct in HBMECs. The promoter-reporter construct contains the LRP1 promoter region where the conserved PPRE resides at positions −1185 to −1173 (Gauthier et al. 2003). The reporter assay in HBMECs showed that rosiglitazone increased the transcriptional activity of the LRP1 promoter in a dose-dependent manner up to 10 nm (Fig. 1d). However, the LRP1 promoter activity remained unchanged at concentrations above 20 nm rosiglitazone in HBMECs (Fig. 1d).

Rosiglitazone increased Aβ uptake through LRP1 in HBMECs

We performed Aβ uptake analysis in HBMECs to investigate the function of LRP1 increased by rosiglitazone. The uptake of both Aβ40 and Aβ42 in HBMECs was increased by rosiglitazone treatment in a dose-dependent manner up to 5 nm rosiglitazone, and plateaued at 10 nm (Fig. 2a-c). Concentrations above 20 nm rosiglitazone did not alter Aβ uptake of HBMECs (Fig. 2a, b). The increase in Aβ uptake by rosiglitazone was not shown in HBMECs transfected with siLRP1 (Fig. 2d). The transfection efficiency determined by RT-PCR of LRP1 was 84.3 ± 5.1%.
Discussion

Recently, Mawuenyega et al. (2010) reported that Aβ clearance rate from brain parenchyma to blood was decreased in AD patients compared to the control group, while their production rate was not different between AD and control. These results suggested that an impairment in Aβ clearance in the central nervous system (CNS) is the main pathogenetic mechanism of AD. LRP1 has been known to mediate the efflux of Aβ in the BBB (Goto & Tanzi, 2002; Shibata et al. 2000; Ueno et al. 2010; Zlokovic et al. 2010). Furthermore, a specific mutation of Aβ associated with hereditary cerebrovascular amyloidosis reduces its binding affinity for LRP1 and results in CNS accumulation of Aβ (Monro et al. 2002), and a direct LRP1/Aβ protein–protein interaction regulates Aβ clearance across the BBB (Deane et al. 2004). In view of this, the regulation of LRP1 expression in the BBB is considered to be an important target of AD treatment. In addition, a recent study reported that a soluble form of LRP1 reduced Aβ-related pathology and dysfunction by sequestering plasma Aβ (Sagare et al. 2007).

In this study, we employed HBMECs to evaluate the effect of rosiglitazone on LRP1 expression in the BBB (Shinohara et al. 2010). Gauthier et al. (2003) reported

Fig. 1. The effect of rosiglitazone on LRP1 expression and the LRP1 promoter in human brain microvascular endothelial cells (HBMECs). HBMECs were treated with indicated concentrations of rosiglitazone for 48 h. (a) Real-time PCR quantification of LRP1 mRNA in HBMECs (n = 6/group). Results were normalized by β-actin mRNA level. (b) Western blot analysis of LRP1 (β-chain) in HBMECs (n = 6/group). Results were normalized by β-actin protein level. (c) Western blot analysis of LRP1 (β-chain) in HBMECs. (d) Reporter assay using the LRP1 promoter-reporter construct in HBMECs. The promoter region of human LRP1 was cloned into pGL3-Basic. This construct was transiently co-transfected into HBMECs along with the Renilla luciferase reporter vector, pRL-CMV. For negative control, pGL3-Basic vector was used. Firefly and Renilla luciferase activities were determined using the dual luciferase assay system (n = 3/group, each in triplicate reactions). Results are expressed as ratio relative to non-treated cells. Data are mean ± s.e. *p < 0.05 vs. 0 nM rosiglitazone; †p < 0.05 vs. 0.5 nM rosiglitazone.
that rosiglitzone increases LRP1 expression in primary human adipocytes and human liposarcoma cell lines (SW872). There is no other published report showing LRP1 up-regulation via thiazolidinediones in any tissue. They affirmed that 50 nM rosiglitazone in culture media up-regulated the transcription of LRP1 and that up-regulation was maintained in up to 1 μM rosiglitazone. In our previous study, we obtained similar, consistent data in HepG2 cells, a human hepatoma-derived cell line (data not shown). The increase in LRP1 mRNA transcription and protein expression was induced by 100 nM rosiglitazone and was maintained by up to 5 μM rosiglitazone in HepG2 cells. However, in HBMECs, the minimal concentration of rosiglitazone that increased LRP1 expression and Aβ uptake was 0.5 nM, and this increase was maintained by up to 10 nM rosiglitazone. These results clearly demonstrate that up-regulation of LRP1 expression and Aβ uptake in HBMECs is achieved by rosiglitazone at a concentration approximately 100- to 200-fold lower than that required in adipocytes or hepatocytes. In addition, the pharmacokinetics of rosiglitazone in the human body reveals that the plasma concentration of rosiglitazone reaches its highest – approximately 300 ng/ml (840 nM) – after a single dose administration of 4 mg (Cox et al. 2000; Niemi et al. 2003). In this regard, our

Fig. 2. The effect of rosiglitazone on Aβ uptake in human brain microvascular endothelial cells (HBMECs). HBMECs were treated with indicated concentrations of rosiglitazone for 48 h and subsequently, cells were incubated with fluorescence-tagged Aβ40 or Aβ42 for 1 h. For quantification, the fluorescence in cell lysate was measured with a fluorometer and the values in cell lysates were standardized to protein amount. (a) The uptake assay of Aβ40 into HBMECs (n = 6/group). (b) The uptake assay of Aβ42 into HBMECs (n = 6/group). (c) Fluorescence microscopic view of uptake of Aβ40 (green) and Aβ42 (red) in HBMECs (×100). (d) The cellular uptake of Aβ42 and Western blot analysis of LRP1 (β-chain) in HBMECs transfected with negative siRNA (siCTRL) or siRNA targeting LRP1 (siLRP1) (n = 6/group). HBMECs were transfected with siCTRL or siLRP1 prior to rosiglitazone treatment. Results are expressed as ratio relative to non-treated cells. Data are mean ± S.E. * p < 0.05 vs. 0 nM rosiglitazone; † p < 0.05 vs. 0.5 nM rosiglitazone; ‡ p < 0.05.
results suggest that a much lower dose of rosiglitazone than the doses for diabetes treatment could increase LRP1 expression and $\beta\beta$ clearance in the BBB.

Gauthier et al. (2003) reported a conserved PPRE in the promoter region of LRP1 gene and showed that up-regulation of LRP1 by rosiglitazone is mediated by PPAR$\gamma$ and PPRE in the LRP1 promoter at a transcriptional level in human adipocytes. We demonstrated that rosiglitazone also activates LRP1 promoter in HBMECs. Interestingly, the concentration of rosiglitazone showing the maximum LRP1 promoter activity was 10 nM in HBMECs, while it was 500 nM in human adipocytes (Gauthier et al. 2003). This result is consistent with other data of this study showing that a very low concentration of rosiglitazone up-regulates LRP1 expression and function in HBMECs. Although the underlying mechanism of this difference according to the cell type remains to be elucidated, we assume that the affinity of PPAR$\gamma$ with its ligand would be much higher in HBMECs than in adipocytes or hepatocytes.

We observed no effect of rosiglitazone on LRP1 expression and $\beta\beta$ uptake in HBMECs at concentrations exceeding 20 nM. In primary human adipocyte, the induction of LRP1 expression by rosiglitazone diminished at a concentration of 2 $\mu$m, and concentrations of $\geq$750 nM did not alter the LRP1 mRNA abundance or the LRP1 promoter activity in SW872 cells (Gauthier et al. 2003). It was reported that the activation of PPAR$\gamma$ at the AF2 domain by its ligands (PPAR$\gamma$ agonists) increased its transcriptional function, and the same process enhanced subsequent proteosomal degradation of PPAR$\gamma$ (Hauser et al. 2000). This would explain the reduced efficacy of rosiglitazone at higher concentrations (Gauthier et al. 2003). In this regard, our assumption of high affinity of PPAR$\gamma$ with its ligand in HBMECs may explain our observations; LRP1 expression and $\beta\beta$ uptake in HBMECs were up-regulated by much lower concentrations of rosiglitazone than in adipocytes or hepatocytes, and the efficacy of rosiglitazone in HBMECs was reduced at a much lower concentration than in adipocytes or hepatocytes. Taken together, our results suggest that only low-dose rosiglitazone treatment, and not the doses for treatment of diabetes, would be effective in inducing the LRP1 expression in human brain microvessels and the $\beta\beta$ efflux through the BBB.

Our results include some important clinical implications. Recently, growing concerns regarding the adverse effects of rosiglitazone have reduced the use of this agent, particularly since the association of rosiglitazone with an increased risk of adverse cardiovascular events has been reported. Our data in HBMECs suggest the possibility that low-dose rosiglitazone treatment could be effective for AD without causing safety-associated problems, such as cardiovascular problems, and other reported side-effects, including weight gain, fluid retention or osteoporosis. A phase III clinical trial of rosiglitazone monotherapy in AD failed to show the efficacy of this agent in cognition or global function in 693 mild-to-moderate AD patients (Gold et al. 2010), and a pilot clinical trial to evaluate the safety of pioglitazone treatment in AD also failed to show a significant beneficial effect (Geldmacher et al. 2011). These clinical trials used doses more appropriate for the treatment of diabetes; 2 or 8 mg/d for rosiglitazone and 45 mg/d for pioglitazone. If our in-vitro data were applied to these clinical trials, the doses used in these clinical studies would be too high to show the effects on increasing CNS clearance of $\beta\beta$ through the BBB, although the background of the use of thiazolidinediones for AD in these studies was its anti-inflammatory effect in the CNS (Geldmacher et al. 2011).

In this study we only demonstrated the cellular uptake of $\beta\beta$ in HBMECs, not the transendothelial transport of $\beta\beta$. To demonstrate $\beta\beta$ transcytosis in HBMECs in vitro, a uni-directional monolayer of cells should be maintained and the transport of $\beta\beta$ from the basolateral side to the apical side should be demonstrated. In a review, Zlokovic et al. (2010) mentioned their unpublished preliminary data using a human BBB in-vitro model with primary brain endothelial cells and pericyte-conditioned media to direct LRP1 distribution. This BBB in-vitro model should be considered for future studies. In addition, they mentioned another preliminary data showing the distribution of LRP1 in brain endothelial cells. Although the exact distribution of LRP1 between the luminal side of the BBB, the cytoplasmic endothelial pool and the abluminal side of the BBB is presently unknown, their work using high-resolution confocal microscopy analysis demonstrated that LRP1 is mainly confined to the abluminal side of the BBB (Zlokovic et al. 2010). In this regard, cellular uptake of $\beta\beta$ in HBMECs may partially reflect the transcytosis of $\beta\beta$. Another limitation of this study is the lack of in-vivo data. Further study with an AD animal model investigating the effect of low-dose PPAR$\gamma$ agonist treatment on LRP1 expression in the brain microvessels, $\beta\beta$ efflux through the BBB, and cognitive or memory function will be required. However, for in-vitro study, a completely new drug delivery system which can maintain 10 nM of serum rosiglitazone concentration should be developed. Although we investigated the effect of rosiglitazone in this study, we assumed that other
thiazolidinedione class drugs, including pioglitazone, would show the same effect on LRP1 in HBMECs because we obtained data showing that pioglitazone increased LRP1 expression in HBMECs in a similar manner as rosiglitazone (Supplementary Fig. S1, available online).

In conclusion, this study suggests a theoretical background for a new application of a low-dose thiazolidinedione for AD treatment. Moreover, this ‘low-dose thiazolidinedione treatment’ is expected to reverse the key pathogenesis of AD by up-regulating LRP1 expression in brain microvessels as well as enhancing CNS clearance of Aβ through the BBB.

Note
Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org/pjn).

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Statement of Interest
None.

References


