PPAR gamma agonist reduces amyloid pathology in aged senescence mice model: mechanisms involving LRP1 expression

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ABSTRACT

PPAR gamma agonist reduces amyloid pathology in aged senescence mice model: mechanisms involving LRP1 expression

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Objective. The role of peroxisome proliferator-activated receptor-γ (PPAR-γ) activation in Alzheimer's disease (AD) is still controversial. Low-density lipoprotein receptor-related protein 1 (LRP1) is known to play an important role in secretion of amyloid-β (Aβ) in AD. The current study is carried out to investigate the effect of pioglitazone, PPAR-γ agonist, on Aβ deposition, LRP1 expression, and memory impairment in a senescence mouse model.

Methods. Pioglitazone was orally administrated to 9-month-old SAMP8 mice in variable doses (0.5 mg/kg/day, 2 mg/kg/day and 5 mg/kg/day) for 7 weeks. Memory function was assessed using Morris water maze test. Aβ deposition and LRP1 expression in mice brain were investigated.

Results. Pioglitazone treatment showed improvement in memory impairment. Pioglitazone-treated SAMP8 mice showed reduced Aβ deposition than vehicle-treated mice. In addition, increased LRP1 expression in microvessel was observed in pioglitazone-treated SAMP8 mice.

Conclusion. These results suggest that pioglitazone may provide a protective effect against memory impairment through increasing LRP1 expression in

blood-brain barrier and reducing Aβ deposition. Pioglitazone could have a therapeutic potential for the treatment of AD.

Key words : Alzheimer's disease, amyloid-β, PPAR-γ agonist, LRP1

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I. INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia. The main pathogenesis of AD is deposition of amyloid-β (Aβ) in the brain parenchyma. Pathologic Aβ deposits result in senile plaques, neurofibrillary tangles, and serial neuronal loss[1]. Therefore, reducing production of Aβ and increasing clearance of Aβ from the brain parenchyma represent an important target for treatment of AD.

Peroxisome proliferator-activated receptor gamma (PPAR-γ) is a ligand-activated nuclear receptor that is expressed in various tissues. PPAR-γ activation is associated with improved insulin resistance, decreased inflammation, and metabolic homeostasis[2]. Therefore, PPAR-γ agonists such as rosiglitazone and pioglitazone are used in the treatment of type 2 diabetes. Recent studies have shown that PPAR-γ agonists improve memory dysfunction and reduce Aβ accumulation in animal models of AD[3,4]. However, the effects of

PPAR-γ agonists on AD remain controversial, and the underlying mechanisms are still unknown.

Low-density lipoprotein receptor-related protein 1 (LRP1) is a member of the low-density lipoprotein (LDL) receptor family and is a cell surface glycoprotein that binds and internalizes a diverse array of ligands[5]. Several studies have reported that LRP1 present in brain capillaries mediates secretion of Aβ from the brain across the blood-brain barrier (BBB) and thus plays a key role in clearing Aβ into blood circulation[6-8].

Several recent *in vitro* studies have reported that PPAR-γ agonists increase expression of LRP1 in various cells, including adipocytes, hepatocytes, and microvascular endothelial cells[9-11]. Based on these findings, we evaluated the effect of pioglitazone on LRP1 expression and Aβ accumulation in the brain using the senescence-accelerated prone mouse 8 (SAMP8), a model of age-related cognitive decline[12]. SAMP8 mice have a natural mutation leading to age-related increases in amyloid-β protein precursor (AβPP) and Aβ deposition in the brain, and exhibit impaired learning and memory[13-15].

The aim of this study was to investigate whether pioglitazone could modulate LPR1 expression in the BBB and decrease pathologic Aβ deposition in SAMP8 mice resulting in memory deficit improvement. The results of this study may be of potential therapeutic interest for the use of PPAR-γ agonists in the treatment of AD.

II. MATERIALS AND METHODS

1. Animals and drug treatments

9-month-old male SAMP8 mice were purchased (Japan SLC, Inc., Shizuoka, Japan). Animals were housed individually with free access to food and water, and maintained under controlled conditions (12 hr light/dark cycle; 25 ± 2°C). Age-matched senescence-accelerated resistant mouse (SAMR1) mice were used

as a normal aging control and were treated with vehicle. SAMP8 mice were treated with vehicle or pioglitazone hydrochloride (AD-4833, Takeda Pharmaceuticals, Kanagawa, Japan) as a suspension in sterile water by oral gavage at doses of 0.5, 2, and 5 mg/kg/day for 7 weeks (n = 6-10 animals per group). In all cases, animals were killed 24 hr after the last administration. The brains were then removed and immediately frozen on dry ice before dissection. All procedures were approved by the Animal Research Committee of the Yonsei University College of Medicine.

2. Behavioral assessments

Animals underwent spatial reference learning using the Morris water maze test after 7 weeks of treatment. The Morris water maze consists of a large circular pool (100 cm in diameter, 35 cm in height), filled to a depth of 30 cm with water at 20 ± 3°C. The water was made opaque with a non-toxic black colored dye. The pool was divided arbitrarily into four equal quadrants. A submerged platform (15 cm in diameter) was centered in one of the target quadrants of the pool and submerged 1 cm below the water surface. The position of the platform was unaltered throughout the training trial sessions. The training trials were performed twice daily for five consecutive days. At the start of each training trial, the mouse was placed on the platform for 15 s and then randomly placed in the water pool. Each trial was terminated when the mouse reached the platform or after 60 s, whichever came first. After each trial, mice remained on the platform for 15 s. The time taken to find the hidden platform (escape latency) was recorded in each trial. After finishing the last training trial session, mice were subjected to a probe-trial session in which the platform was removed from the pool. Probe-trials were performed with a cut-off time of 60 s and examined whether mice could find the previous platform site. Mice were monitored by a camera mounted in the ceiling directly above the pool, and all trials were recorded using a water maze program.

3. Amyloid-β ELISA

Cortical and hippocampalAβ40 levels were measured with a sensitive sandwich ELISA kit (Biosource International, Camarillo, CA, USA). Briefly, tissue was weighed and homogenized in 100μl of PBS followed by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was diluted with standard diluent buffer supplemented with Halt® protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). A total of 600 pg/ml was loaded onto ELISA plates in duplicate, and the manufacturer's instructions were followed.

4. Western blot analysis

Proteins were extracted from homogenized frozen brain tissue with TPER® (Thermo Scientific) mixed with Halt® protease inhibitor cocktail (Thermo Scientific). Protein concentrations were determined with a BCA Protein Assay kit (Thermo Scientific) and appropriate amounts of protein were mixed with SDS sample buffer (62.5 mM Tris-HCl pH6.8, 25% (v/v) glycerol, 2% SDS (w/v), 0.01% (w/v) Bromophenol Blue, 5% (v/v) β-mercaptoethanol). Lysates (20 μg for each sample) were separated by 10% (w/v) SDS-PAGE, and the resolved proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, USA). Blots were blocked with 5% w/v non-fat milk in TBST (Tris-buffered saline containing 0.1% v/v Tween 20) for 1 hour and incubated with an anti-LRP1 antibody (1:5,000, Abcam®, Cambridge, MA, USA) overnight at 4°C. After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000, Thermo Scientific) for 1 hour at room temperature, signals were detected using SuperSignal® West Pico Chemiluminescent Substrate reagent (Thermo Scientific). Images were captured and analyzed with a LAS-4000 luminescent image analyzer (Fujifilm Life Science, Stamford, CT, USA)

5. Immunohistochemistry

Brain tissues from mice were fixed in 10% formaldehyde and embedded in paraffin. The paraffin sections were cut and deparaffinized using xylene and ethanol. After inactivation of endogenous peroxidase with 3% H_2O_2 for 10 min at room temperature, samples were blocked with 5% BSA for 1 hr. Next, tissues were incubated overnight with a mouse anti-amyloid β 1-42 antibody (AnaSpec Inc, Fremont, CA, USA) at 4°C. After washing, tissues were incubated with a probe labeled with anti-mouse IgG antibodies and peroxidase for 20 min at room temperature. Staining for mouse primary antibodies was performed using an EnVision+ System-HRP Labeled Polymer Anti-Mouse kit (K4001, Dako, Glostrup, Denmark). Coverslips were washed and incubated with DAB (K3468, Dako) for 5 min and counterstained with hematoxylin prior to examination under a light microscope (Olympus BX40, Olympus Optical Co. Ltd., Tokyo, Japan). ImageJ v.1.47d software (National Institutes of Health, Bethesda, MD, USA) was used for quantitative analysis of plaque burden. Selection of areas for measurement was performed by manual threshold adjustment. Quantifications were performed blindly (five fields per section, three sections per animal, spaced 500 μm apart) at 40 x magnification.

6. Immunofluorescence

Brain tissues were sectioned with a cryostat (5 μm) and fixed with 3% H_2O_2. Next, brain sections were washed and blocked for 1h at room temperature with 5% BSA. The slides were then incubated with a rabbit anti-LRP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a mouse anti-PECAM-1 antibody (Santa Cruz Biotechnology) overnight at 4°C. After three 5 min washes in TBS, slides were incubated with an Alexa 488 conjugated chicken anti-rabbit IgG antibody (A21441; 1:100, Invitrogen, Carlsbad, CA, USA) and PerCP-Cy5.5 conjugated goat anti-mouse IgG antibody (sc-45013, 1:100, Santa Cruz Biotechnology) in blocking buffer for 30 min at room temperature. The sections were then washed and coverslipped with fluorescent mounting media

(Dako). Fluorescent images were captured using a Zeiss Laser Scanning Confocal Microscope (LSM 700, Carl Zeiss, Oberkochen, Germany).

7. Statistical analysis

Data analysis was carried out using SPSS package for Windows (version18.0; SPSS, Chicago, IL, USA). Results are expressed as the mean ± S.E. For statistical analysis, normally distributed data were analyzed by one-way analysis of variance (ANOVA) with *post hoc* tests performed using the Tukey test. Non-normally distributed data were analyzed by the Kruskal-Wallis test. Values of $P < 0.05$ were taken as statistically significant.

III. RESULTS

1. Assessment of spatial learning and memory deficits following pioglitazone treatment

A previous study reported that SAMP8 mice exhibit significant learning and memory dysfunction between 8 to 12 months of age compared to SAMR1 mice[16]. In this study, SAMP8 mice showed spatial learning and memory impairment at 11 months of age in the water maze test. In training trials, the escape latency (time taken to find the hidden platform) was improved in SAMR1 mice, but not in SAMP8 mice (Fig. 1A).This increased latency was improved by pioglitazone (2 mg/kg/day) treatment on the 5th day.

In probe trials, SAMP8 mice showed significantly decreased escape latency and increased searching time (as measured by passing the previous platform site) compared with SAMR1 mice. No significant difference of escape latency or passing count was found between pioglitazone-treated SAMP8 mice and vehicle-treated SAMP8 mice (Fig. 1B-C).

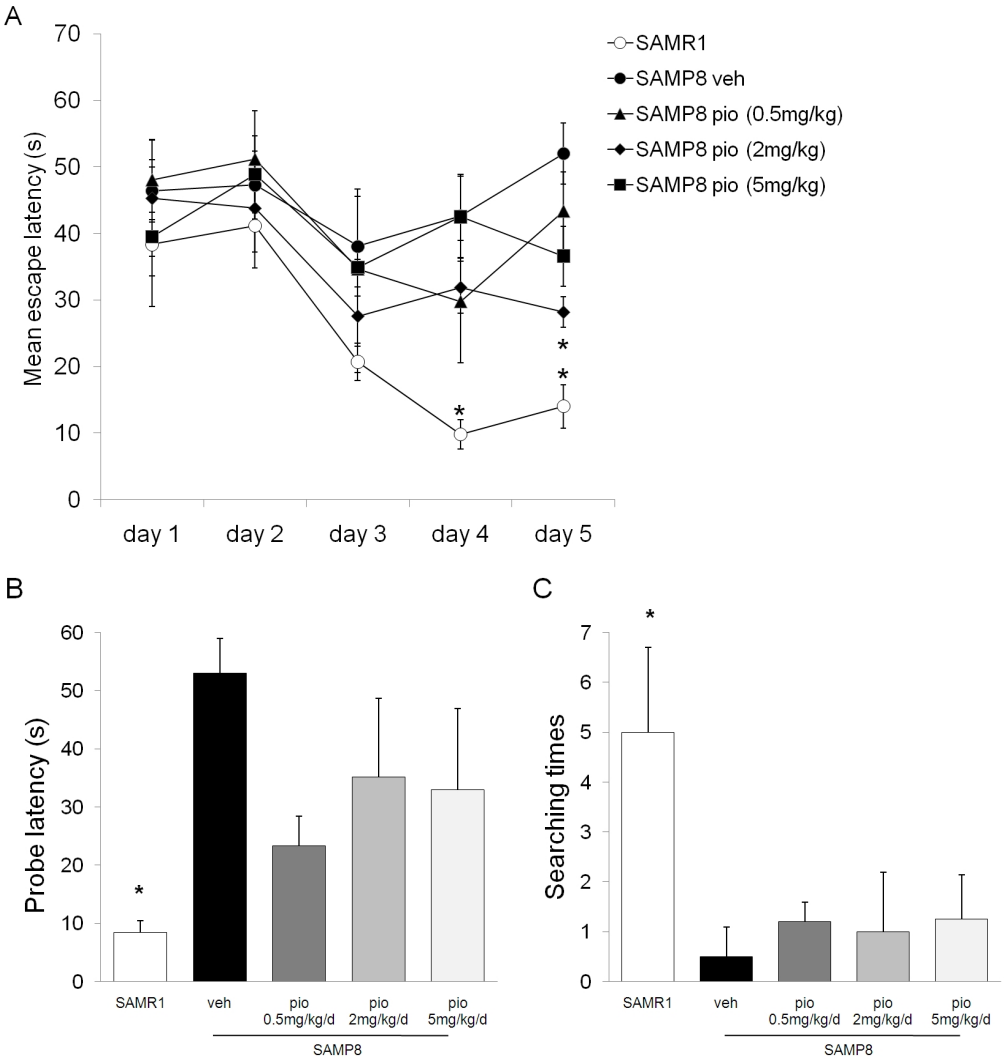

Figure 1. Effect of pioglitazone on escape latency in training trial sessions (A) and probe trial sessions (B,C) of the Morris water maze test. (A) During the training trial, the escape latency was significantly improved in SAMR1 mice, but not in SAMP8 mice. Pioglitazone treated SAMP8 mice (2 mg/kg/day) showed improved latency on the 5th day. In the probe trial session, escape latency (B) and searching times (C) were significantly improved in SAMR1 mice but not in SAMP8 mice. * p <0.05 compared to vehicle-treated SAMP8 mice.

2. Effects of pioglitazone treatment on Aβ levels and Aβ plaque depositions

SAMP8 mice had increased Aβ deposits in the cortex and hippocampus compared to SAMR1 mice (Fig. 2). In pioglitazone-treated SAMP8 mice, Aβ deposits were reduced in both the cortex and hippocampus compared with vehicle-treated SAMP8 mice. Plaque burdens were significantly reduced in pioglitazone-treated SAMP8 mice compared with vehicle-treated SAMP8 mice.

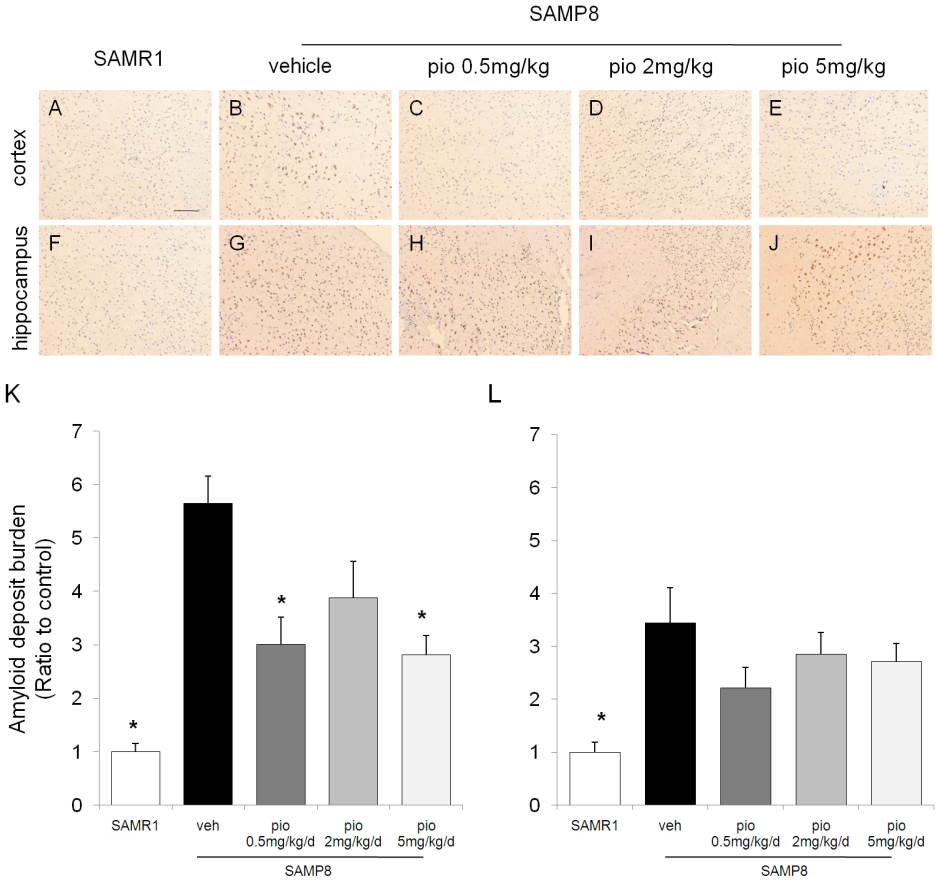

Figure 2. Representative immunohistochemistry images of brain regions for Aβ deposits. Compared to cortex and hippocampus of SAMR1 mice (A,F), multiple deposits of Aβ were detected in SAMP8 mice (B,G). Pioglitazone treatment reduced staining of Aβ deposits in both the cortex (C,D,E) and hippocampus

(H,I,J). Plaque burden was also reduced after pioglitazone treatment in cortex (K) and hippocampus (L). The scale bar represents 100μm.* p <0.05 compared to vehicle-treated SAMP8 mice.

Cortical and hippocampal levels of Aβ were determined by ELISA. As shown in Fig. 3, the levels of Aβ in SAMP8 mice were significantly higher than those in SAMR1 mice. Likewise, cortical and hippocampal levels of Aβ40 exhibited a decreasing trend in SAMP8 mice treated with pioglitazone compared with vehicle-treated SAMP8 mice.

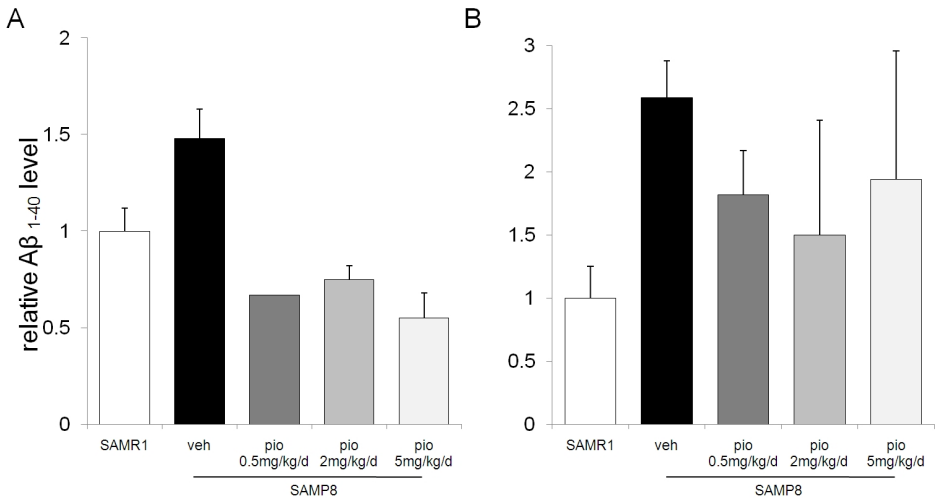

Figure 3. Effect of pioglitazone on levels of Aβ in brain regions of SAMP8 mice. Levels of Aβ40in the cortex (A) and hippocampus (B) were decreased after pioglitazone treatment as determined by ELISA. Values are the mean± SEM. * p <0.05 compared to vehicle-treated SAMP8 mice.

3. Effects of pioglitazone treatment on LRP1 expression in microvessels

LRP1 expression in the cortex and hippocampus was evaluated in conjunction with PECAM-1, an endothelial marker, because LRP1 in the blood brain barrier has been shown to be an important exporter of Aβ. LRP1 expression merged

with PECAM-1 in SAMP8 mice was decreased compared with SAMR1 mice (Fig. 4), whereas LRP1 expression merged with PECAM-1 was improved in SAMP8 mice upon treatment with pioglitazone.

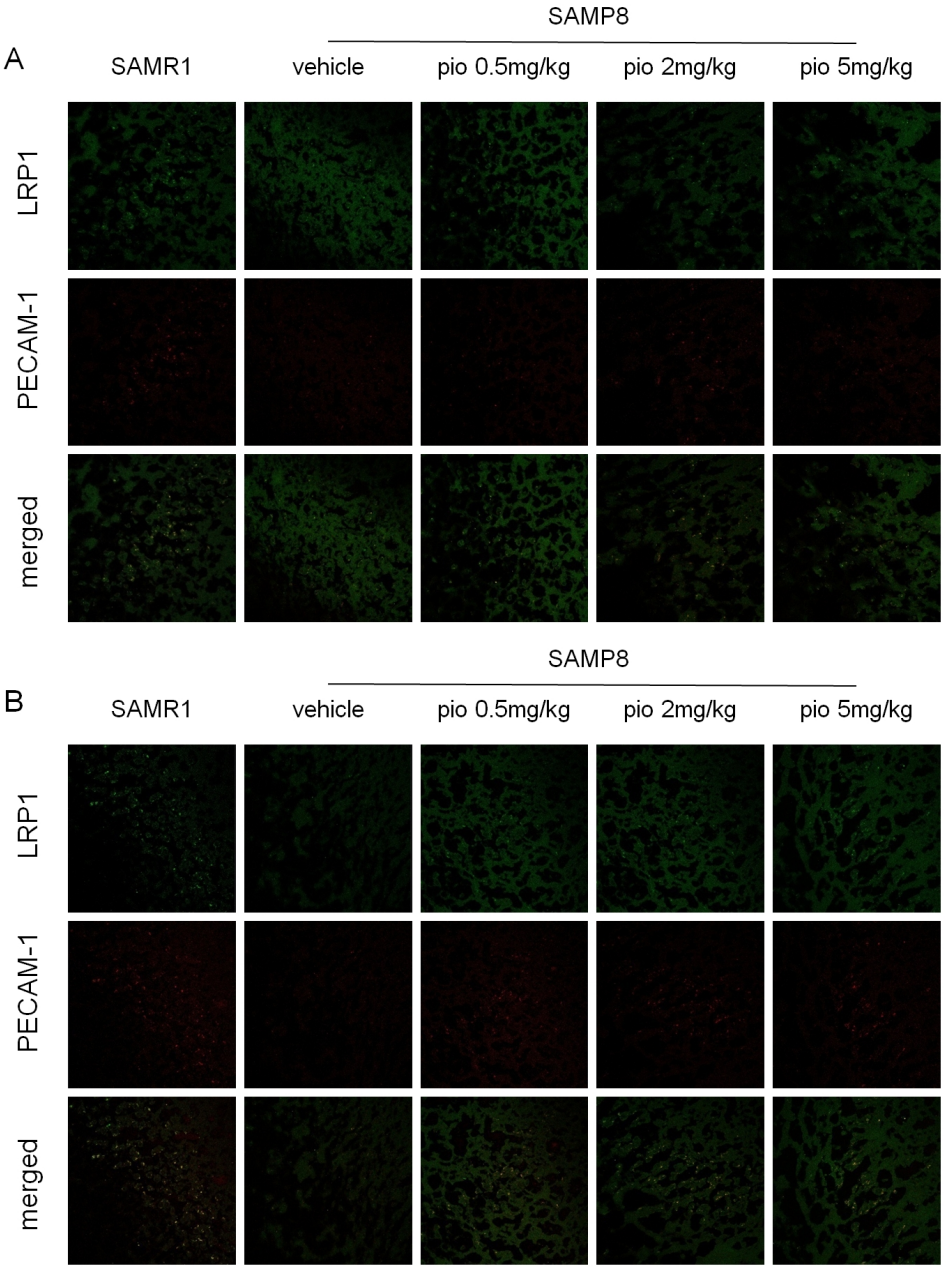

Figure 4. Representative immunofluorescent staining images of brain regions for LRP1 and the endothelial marker PECAM-1. Staining with LRP1 (green) expression merged with PECAM-1 (red) was decreased in the cortex (A) and hippocampus (B) of SAMP8 mice, which was ameliorated by pioglitazone treatment.

The levels of LRP1 were also analyzed in the cortex and hippocampus by Western blot. The levels of LRP1 were not significantly different between SAMR1 mice and SAMP8 mice (Fig. 5). Similarly, levels of LRP1 in pioglitazone-treated SAMP8 mice were not statistically different compared to vehicle-treated SAMP8 mice.

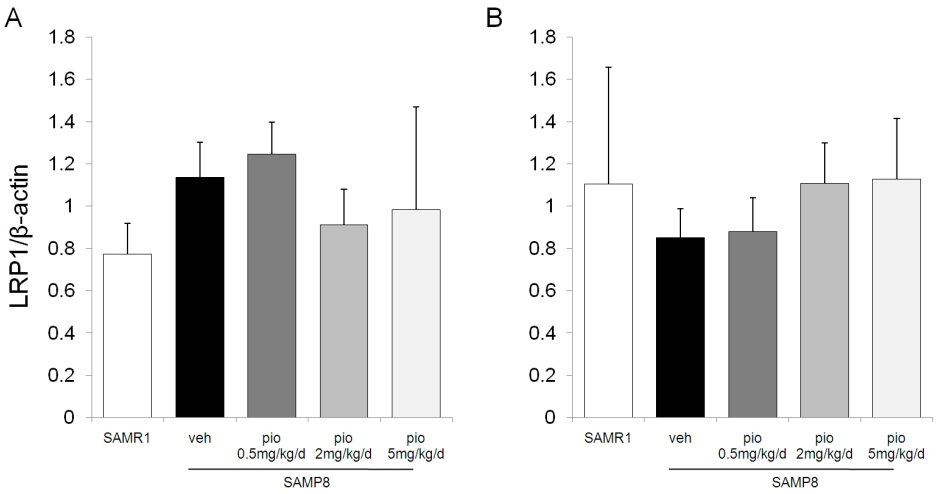

Figure 5. Effect of pioglitazone on the expression of LRP1 in the brains of SAMP8 mice. Levels of LRP1 in the cortex (A) and hippocampus (B) were not different between SAMR1 and SAMP8 mice as determined by Western blot. There was no significant difference in levels of LRP1 between pioglitazone-treated mice and vehicle-treated SAMP8 mice.

IV. DISCUSSION

It has been suggested that an age-related inability to clear Aβ from the brain may be a cause of Aβ accumulation in AD patients[6-8]. In this study, pioglitazone showed effects on reduction of Aβ depositions in SAMP8 mice. Treatment with pioglitazone also increased expression of LRP1, which plays a key role in clearance of Aβ in brain microvessels.

Escape latency in probe-trials was improved in SAMP8 mice treated with 2 mg/kg/day pioglitazone, but not by the other dosages. The effects of PPAR-γ agonists on behavioral tests of AD mice model continue to be controversial. Several studies have reported conflicting results with respect to PPAR-γ agonists in memory and cognitive dysfunctions. One study reported that APP transgenic mice treated with pioglitazone (20 mg/kg/day, for 6-8 weeks) do not exhibit improved spatial memory in the Morris water maze test[17]. In contrast, a separate study reported that pioglitazone treatment (9 mg/kg and 18 mg/kg for 9 days) improves spatial memory in the scopolamine-induced memory impairment mice model[18]. It was also reported that rosiglitazone treatment (5 mg/kg/day for 4 weeks) in APP mice improves recognition memory but not spatial memory[3]. Another study showed that chronic treatment with rosiglitazone (30 mg/kg of food for 7months) rescues impaired spatial memory[19]. It has also been suggested that spatial memory requires more complete hippocampal function than does recognition memory[20]. Thus, differences in the sites of improvement being evaluated after treatment may have contributed to these controversial findings.

In this study, a significant reduction of the Aβ burden was observed after pioglitazone treatment, whereas levels of Aβ40 were not affected. There is conflicting data in the literature with respect to the effects of PPAR-γ agonists on Aβ burden in AD mouse models. In a previous study, chronic treatment of rosiglitazone in Tg2576 mice was shown to reduceAβ42 levels by ~25%, but did not affect levels of Aβ40 or abundance of amyloid plaques[19]. Another study

reported that rosiglitazone treatment (5mg/kg/day, 4 weeks) decreases both Aβ40 and Aβ42 levels by ~72% and ~57%, respectively[3]. In contrast, a separate study performed with Tg2576 mice showed that pioglitazone treatment (20 mg/kg/day, for 16 weeks) reduces Aβ40 levels by 19% without affecting Aβ42 levels or amyloid plaques[21]. These differing results may be due to the dose of PPAR-γ agonists, treatment duration, and/or differences in the ages and specific mouse models utilized.

In the present study, immunofluorescence staining of LRP1 merged with the endothelial marker PECAM-1 showed increased LRP1 expression in pioglitazone-treated SAMP8 mice. However, no statistical difference in cortical and hippocampal levels of LRP1 was found between SAMR1 mice and SAMP8 mice, nor between SAMP8 mice treated with pioglitazone or vehicle. LRP1 is considered to play an important role in the clearance pathway of Aβ. Evidence suggests that LRP1 in the BBB may play a key role in Aβ efflux from the brain parenchyma to the blood[8]. A recent study reported that LRP1 expression is increased in the microvasculature compared to neurons in aged non-AD individuals, and interestingly, this pattern of expression is reversed in AD. These findings suggest that LRP1 expression is predominant in neurons, but becomes down-regulated in the brain microvasculature in AD[22]. Another recent study showed that neuronal LRP1 expression is increased in old SAMP8 mice[23]. Based on their findings, the authors of that study suggested that LRP1 can bind certain domains of AβPP, the interaction of which may increase production of Aβ. These results also suggest that the role of LRP1 expression may differ between the microvasculature and parenchyma. Unfortunately, LRP1 levels of microvasculature and other parenchyma were not measured separately in this study. Thus, follow-up studies should be performed to clarify the role of LRP1 in AD as well as the effects of PPAR-γ agonists on LRP1 expression in microvessels and other tissues.

Recent *in vitro* studies have shown that rosiglitazone increases LRP1

expression and Aβ uptake in microvascular endothelial cells at concentration approximately 100- to 200-fold lower than clinically used doses, where the highest plasma concentration is approximately 300 ng/ml (840 nM) after a single dose administration of 4 mg rosiglitazone)[11]. Thus, in the present study, a relatively low dose of pioglitazone was used (0.5 mg/kg/day, 2 mg/kg/day and 5 mg/kg/day). These doses of pioglitazone did not affect inflammations of brain tissues (data not shown). However, consistent with the results of previous *in vitro* studies, the low dose of pioglitazone had a partial effect on Aβ burden and LRP1 expression. And as a result, low-dose pioglitazone showed beneficial effects in improvement of memory dysfunction. These findings suggest that low doses of pioglitazone may be used for the treatment of AD to achieve a decreased hypoglycemic effect in non-diabetic patients.

V. CONCLUSION

In conclusion, our data demonstrated that pioglitazone reduces AD pathology and restores memory impairment in SAMP8 mice. We hypothesize that PPAR-γ agonists may facilitate a clearance mechanism of Aβ, possibly through activation of LRP-1 in the BBB. The results of this study provide mechanistic support for the therapeutic potential of pioglitazone in the treatment of AD.

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ABSTRACT (IN KOREAN)

PPAR gamma 작용제가 고령의 노화 동물 모델에서 LRP1의 발현 및 amyloid beta 단백의 침적에 미치는 영향

<지도교수 차 봉 수>

연세대학교 대학원 의학과

석 한 나

배 경: 치매의 주된 원인으로 알려진 알츠하이머 병에서 PPAR gamma 작용제의 역할은 아직 확실하지 않다. Low-density lipoprotein receptor-related protein 1 (LRP1)은 amyloid beta 단백의 제거에 관여하는 수송체 단백으로 알츠하이머 병에서 중요한 역할을 하는 것으로 알려져 있다. 본 연구에서는 PPAR gamma 작용제인 pioglitazone 이 amyloid beta 단백의 침적과 LRP1의 발현 및 뇌기능장애에 미치는 영향에 대해 노화 동물 모델을 이용하여 확인하고자 한다.

방 법: 9개월 령의 SAMP8 쥐를 군을 나눈 후 pioglitazone 을 각각 다른 농도 (0.5 mg/kg/일, 2 mg/kg/일, 5 mg/kg/일)로 7주간 경구 투여하였다. Morris water maze test를 통해 기억 기능을 확인하였으며, 쥐의 뇌를 추출하여 amyloid beta 단백의 침적과 LRP1의 발현을 확인하였다.

결 과: Pioglitazone 을 투여한 SAMP8 쥐 군에서는 기억 장애의 호전을 보였다. Pioglitazone을 투여한 경우 뇌 실질에서 amyloid beta 단백의 침적이 감소되어 있었으며, 뇌혈관에서 LRP1의 발현이 증가되어 있었다.
결 론: 본 연구는 pioglitazone이 뇌혈관 장벽에서 LRP1의 증가 및 amyloid beta 단백의 침적을 감소시키고, 결과적으로 기억 장애의 호전을 가져올 수 있음을 보여주고 있다. 따라서 본 연구는 pioglitazone의 알츠하이머 병의 치료제로서의 가능성을 제시한다고 하겠다.

핵심되는 말: 알츠하이머 병, amyloid beta 단백, PPAR gamma 작용제, LRP1