Effect of rosiglitazone on amyloid precursor protein processing and $\beta$-clearance in streptozotocin-induced rat model of Alzheimer’s disease

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ABSTRACT

Objective(s): Increasing evidence suggests that Alzheimer’s disease (AD) is associated with diabetes. Rosiglitazone, a peroxisome proliferator-activated receptor y (PPAR-y) agonist and anti-diabetic agent, may improve symptoms of AD. However, the underlying therapeutic potential of it has not been fully elucidated.

Materials and Methods: Rats were divided into four groups: control group, sham operated group, Streptozotocin (STZ) group, rosiglitazone (RGZ) group. Particularly, the STZ-induced rat model was established by intracerebroventricular injection (3 mg/kg) on the first and third day. The water maze behavioral test was performed to evaluate spatial reference learning and memory of the rats. $\beta$1-40 and $\beta$1-42 levels were measured by ELISA method. To determine APP-derived fragment, BACE1 and $\beta$-degrading enzymes levels, such as NEP and IDE, as well as $\beta$-transportation protein level, such as LRP1, RAGE, Abca1 and APOE, which were analyzed by Western blot. Immunohistochemistry was used to observe the change of $\beta$1-40 and $\beta$1-42 in hippocampus.

Results: Chronic treatment with RGZ could reduce the $\beta$ level and improved spatial memory performance in STZ-induced rat model. However, RGZ modified the expression of specific transport proteins monitoring Aβ clearance, such as ATP-binding cassette transporter 1 (ABCA1), lipoprotein receptor-related protein 1 (LRP1), and the advanced glycation end product-specific receptor (RAGE) rather than change levels of $\beta$-degrading enzymes, such as IDE and NEP, nor affect APP processing.

Conclusion: As a potential therapeutic strategy, rosiglitazone might exert anti-AD effect not by alteration of APP processing pathway and Aβ degradation directly, but through promotion of Aβ clearance indeed.

Introduction

It has been argued in recent years that an altered balance between the production and clearance of $\beta$-amyloid peptide (Aβ), the important pathologic hallmark of Alzheimer’s disease (AD), might be a critical and initial step for the neurodegenerative disease. It had been found that Aβ peptides are proteolytically degraded in the brain, mainly by neprilysin (NEP) and insulin degrading enzyme (IDE) (1). It has also been recently stated that apolipoprotein E (ApoE) might facilitate the proteolytic clearance of soluble Aβ from the brain, both within microglia by NEP and extracellularly by IDE, depending on the ApoE isoform subtype and its lipidation level. Furthermore, ATP-binding cassette transporter A1 (ABCA1), and the nuclear liver X receptors (LXR) have similarly played a major role in the clearance of soluble Aβ (2). Besides the enzymatic removal of Aβ, efflux to the blood across the blood-brain barrier (BBB) is the alternative main pathway in maintaining an appropriate brain Aβ level. The low-density lipoprotein receptor-related proteins (LRP1 and 2) and ATP binding cassette (ABC) are the two main transporters that mediate Aβ efflux (3-5). In addition, it has been demonstrated that an increase in the plasma levels of Aβ could induce Aβ influx into the brain through a specific transporter in the BBB, glycation end product-specific receptor (RAGE) (6-9), as well.

Interestingly, epidemiological evidence has implied that type 2 diabetes is associated with an increased incidence of AD (10). Intracerebroventricular administration of streptozotocin (STZ) in rodents is used to create an animal model of dementia. Single or double intracerebroventricular (ICV) STZ injections chronically

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decrease cerebral glucose uptake and produce multiple other effects that resemble molecular, pathological, and behavioral features of Alzheimer’s disease (AD)(11). So ICV STZ injections are exploited by some investigators as a non-transgenic model of this disease and used for preclinical testing of pharmacological therapies for AD. Rosiglitazone, a PPAR-γ agonist used in the treatment of type 2 diabetes, is currently undergoing Phase III clinical trials in individuals with AD (12-14). Mounting evidence has suggested that rosiglitazone could improve cognition and ameliorate Aβ pathology and inflammation in vivo and in vitro (15, 16). However, the potential therapeutic mechanism of action of PPAR-γ agonists in sporadic AD has not yet been elucidated. This study aimed to investigate the effect of rosiglitazone in a STZ-induced rat model of early pathological changes in AD.

Materials and Methods

Animals and drug treatments

Sixty Sprague-Dawley male rats, aged between 12 and 16 weeks, weighted 300-350g were utilized in the study maintained temperature-controlled room (22 °C) with a standard diet. All animal experimental procedures were approved by the Animal Care and Ethical Committees of Harbin Medical University. The production license number of the second grade rats is scxk 2013-001. After selection, we excluded the 9 died rats during the stereotaxic operation, due to anesthesia accident and intracerebral hemorrhage, and the 3 rats because of unsuccessful ICV injection. So the final observed experimental rats were totaled 48, and there were 12 rats in each group. Rats were housed in separate cages and divided into four age matched groups: group 1, control rats (12 male rats) received vehicle injections (sodium citrate buffer); group 2, sham control rats (12 male rats) received sham operation only; group 3, STZ injection rats (12 male rats) received a low dose of STZ on the first and third day, after 3 weeks the STZ model was established; group 4, RGZ treated rats (12 male rats), three weeks following surgery, received 4 consecutive weeks daily gavage administration of rosiglitazone (17). In the STZ-treated groups, rats were injected bilaterally through ICV stereotactic injection (2 µl in each ventricle) with a streptozotocin solution, STZ (Sigma-Aldrich USA), dissolved in citrate buffer at 3 mg/kg on the first and third day. The solutions were freshly prepared just before the injection to avoid decomposition of the drug. Briefly, animals were anaesthetized with a mixture of ketamine (15 mg/kg) (Imalgene1000H, Merial) and xylazine (1.5 mg/kg) (RompunH 2%, Bayer) through intraperitoneal administration. Rats were then placed into a stereotactic apparatus and were injected through ICV either with the solvent or the STZ solution (stereotactic coordinates relative to bregma, dura mater and inter-hemispheric scissure: antero-posterior: -0.7mm; ventral: 23.6 mm, lateral: 61.5 mm)(18). After three weeks, STZ-treated rats were given an oral gavage of rosiglitazone maleate (Avandia, GSK, Brentford, UK) as a suspension in sterile water at a dose of 5 mg/kg-day\(^{-1}\) for 4 consecutive weeks. In all cases, animals were sacrificed 24 hr after the final administration of rosiglitazone. The brains were removed, hemisected along the midline, and immediately frozen on dry ice before dissection. One hemisphere was used for Western blotting and the other hemisphere for enzyme linked immunosorbent assay (ELISA) from the 6 rats in each group. Whereas the other 6 rats in each group, after transcardial perfusion with paraformaldehyde, was used for immunohistochemistry. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Harbin Medical University.

Behavioral procedures

To evaluate spatial reference learning and memory of the rats in response to rosiglitazone treatment, a water maze behavioral test was performed as previously described (19). Treatment animal groups underwent spatial reference learning in the Morris water maze (MWM) after after three weeks’ treatment in the STZ group and after 4 weeks’ treatment in the RGZ group. In brief, rats were trained on a visible platform (circular platform) for three consecutive days (eight trials per day), no visible cues were present during this phase. This was followed by four days of testing for learning and memory abilities with the platform submerged (hidden platform with all visible cues present). In both the visible-platform and hidden-platform versions, rats were randomly placed in selected locations, facing the wall of the pool to eliminate the potentially confounding contribution of extra-maze spatial cues. Each trial was terminated when the rats reached the platform within 90 sec, whichever came first. Rats failing to reach the platform were guided onto it. After each hidden platform trial, rats remained on the platform for 30 sec. On the fifth day, all rats were subjected to a probe trial in which they swam for 90 sec in the pool with no platform. Rats were monitored using a camera mounted in the ceiling directly above the pool. All trials were recorded using the HVS Image water maze software (HVS Image, Thornborough, UK) for the subsequent analysis of escape latencies, swimming speed, path length, and time spent in each quadrant of the pool during probe trials (WaterMaze 3 analysis program, Actimetrics, Evanston, IL, USA). All experimental analysis was performed blinded to treatment groups.
**Determination of Aβ1-40 and Aβ1-42 levels**

Hippocampal Aβ1-40 and Aβ1-42 levels were measured by using a sensitive sandwich ELISA kit (Biosource, Camarillo, CA, USA). In brief, tissue was weighed and homogenized in eight volumes of ice-cold guanidine buffer (5 M guanidine HCl/50 mM Tris-HCl pH 8.0) and diluted 1:20. The homogenates were mixed overnight at room temperature of about 25° C and were diluted 1:50 in Dulbecco’s phosphate-buffered saline containing 5% bovine serum albumin and 0.03% Tween-20, followed by centrifugation at 16,000 g for 20 min at 4° C. The supernatant was diluted with standard diluent buffer supplemented with a protease inhibitor cocktail and 1 mM PMSF. A total of 100 µl of each homogenate was loaded onto ELISA plates in duplicate, and the manufacturer’s instructions were followed. Aβ standards were prepared in a buffer with the same composition as the tissue samples.

**Western blot analysis**

For the determination of APP-derived fragments and BACE1 levels, hippocampal tissue was homogenized in a buffer containing 2% SDS in Tris-HCl (10 mM, pH 7.4) and protease inhibitors (1 mM PMSF and Complete Protease Inhibitor Cocktail, Roche Diagnostics). The homogenates were sonicated for 2 min and centrifuged at 100,000 g for 1 hr. Aliquots of the supernatant were frozen at -80° C and protein concentrations were determined using the Bradford method using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Protein extracted from hippocampal tissues was subjected to SDS-polyacrylamide electrophoresis and protein bands were transferred to a PVDF membrane with 0.2 m removal rating (Hybond LFP, Amersham Biosciences, Little Chalfont, UK). The membranes were blocked with 5% Tween-20 in Tris-buffered saline (TBS) followed by overnight incubation with the following primary antibodies: monoclonal mouse anti-BACE1, Cat No MAB5308, (1:500, Chemicon, Temecula, CA), rabbit polyclonal anti-APP C-terminal (amino acids 676-695), Cat No A8717, (1:2000, Sigma, St Louis, MO, USA), goat polyclonal anti-LRP1, Cat No ab62829, (1:2000, Abcam, USA), rabbit monoclonal anti-neprilysin, Cat No ab126593, (1:1000, Abcam, USA), rabbit polyclonal anti-RAGE, Cat No. ab3611, (1:1000, Abcam, USA), mouse monoclonal anti-LRP1, Cat No ab92544 (1:20000, Abcam, USA), rabbit polyclonal anti-APOE, Cat. No. ab20874 (1:500, Abcam, USA), mouse monoclonal anti-Abca1, Cat No ab18180, (1:200, Abcam, USA), and mouse monoclonal anti-β-actin, Cat No AS5441, (1:10000, Sigma, USA). After two washes in TBS/Tween20 and one wash in TBS alone, immunolabeled protein bands were detected using HRP-conjugated anti-rabbit or anti-mouse antibody (1:5000, Dako, Denmark) with an enhanced chemiluminescence system (ECL, Amersham Biosciences), and autoradiographic exposure to Hyperfilm ECL (Amersham Biosciences). Signal quantification was carried out using Quantity One v.4.6.3 software (Bio-Rad) with β-actin as a loading control.

**Immunohistochemistry**

For immunohistochemistry, six free-floating tissue sections from six animals per group were processed. Brain sections were washed (3-10 min) with 0.1 M phosphate buffered saline (PBS) (pH 7.4) and incubated in blocking solution (PBS containing 0.3% Triton X-100, 0.1% BSA, and 2% normal goat serum) for 2 hr at room temperature. Primary and secondary antibodies were diluted in blocking solution. Sections were incubated with the primary antibody (mouse monoclonal anti-Aβ1-40, 1:1000, Cat. No. AS-25230; and mouse monoclonal anti-Aβ1-42, 1:200, Cat. No. AS-55922, AnaSpec, Eurogentecent, Belgium) overnight at 4° C, washed with PBS, and subsequently incubated with a biotin-labeled secondary antibody for 30 min at room temperature and washed three times with PBS. Diluted streptavidin biotin complex was incubated with the sections for 30 min at room temperature, and washed three times with PBS; 100 µl 3,3’-diaminobenzidine (DAB) was added as a color reagent solution to the sections, and the reaction was observed under a microscope and terminated with tap water. The sections were re-stained with hematoxylin and treated with hydrochloric acid alcohol. Sections were dehydrated with gradient alcohol, transparentized with xylene, and sealed with neutral gum. Brown DAB reaction products appearing inside the neuronal cells were identified as positive staining at higher magnification.

**Statistical analysis**

Values represent the mean±standard deviation from three separate experiments. The data were normally distributed. One-way ANOVA followed by the Scheffe F-test was used to determine the statistical significance of the differences. P<0.05 was considered statistically significant.

**Results**

**Rosiglitazone treatment ameliorates memory deficits**

In order to investigate spatial reference learning and memory in response to treatment with rosiglitazone in the STZ-induced rat model, a water maze behavioral test was performed. In the hidden platform swimming test in the MWM, the STZ group spent more time searching for the hidden platform compared with the control and RGZ groups (Figure 1a). After 4 weeks’ treatment, rosiglitazone ameliorated the STZ-induced impairment in spatial memory. The escape latencies in the hidden platform test were significantly improved in the RGZ group (Figure 1a). The average path length in the four consecutive days spent
Rosiglitazone decreases Aβ levels in hippocampal regions

Hippocampal levels of Aβ40 and Aβ42 were determined in the STZ-injected rats using ELISA after 4 weeks of rosiglitazone treatment. Aβ40 and Aβ42 levels were significantly increased in the STZ group compared with the control and sham groups (Figure 2b). Rosiglitazone treatment significantly decreased Aβ42 levels by 41% (P<0.01), and Aβ40 levels by 54% (P<0.01) compared with the STZ group (Figure 2a, b). Moreover, immunohistochemical staining using anti-Aβ40 and Aβ42 antibodies, which recognize intracellular Aβ deposits in the brain, revealed that STZ-injected rats had greater intracellular Aβ40 and Aβ42 deposits in hippocampal areas compared with the control and sham groups (Figure 2a, b). In contrast, in rosiglitazone-treated rats the intracellular deposition of Aβ40 and Aβ42 was almost little observed in hippocampal regions (Figure 3a, b). These results suggested that the drug may promote clearance of soluble intracellular Aβ deposits (Figure 3a, b).

Effects of rosiglitazone on APP processing and degradation

To determine whether rosiglitazone decreased Aβ levels by changing APP processing, we analyzed the levels of APP-derived fragments and level of the APP cleaving enzyme, BACE1, using western blot analysis with anti-BACE1 and anti-APP C-terminal antibodies. BACE1 protein levels increased after STZ-treatment suggesting an up-regulation of BACE1 (P<0.05) (Figure 4a). The increase in BACE1 protein level was not significantly reduced by rosiglitazone treatment (P>0.05) (Figure 4a). Additionally, the expression of the two main Aβ degrading enzymes, NEP and IDE, were analyzed. The protein expression level for both enzymes did not change in the rosiglitazone-treated group compared with the STZ group (P>0.05) (Figure 4a). Furthermore, the levels of full-length APP and

Figure 1. Effect of daily treatment with rosiglitazone (5 mg/kg) for four weeks on performance of STZ-treated rats in the MWM
(a) STZ treated rats spent more time searching for the hidden platform than control and rosiglitazone-treated rats; (b) The average path length of the consecutive four days in searching for the hidden platform in the STZ group was significantly prolonged compared with the control and rosiglitazone group;
(c) The percent time spent in the target quadrant of the former platform position in the STZ group was significantly lower compared with the control and rosiglitazone-treated group;
(d) The average number of hidden platform crossings of the STZ group was significantly lower compared with control and rosiglitazone-treated groups. Values are means±SD (n =12 rats per group). *P<0.05, **P<0.01 vs. control group, and *P<0.05, **P<0.01 vs. STZ group
◆◆: control group; ■: STZ group; ▲: RGZ group
searching for the hidden platform was significantly prolonged in the STZ group compared with the control and RGZ groups (Figure 1b). In the probe trial test to evaluate memory function in the MWM, the percentage of time spent in the target quadrant, i.e., the quadrant where the platform was previously found, was significantly lower in the STZ group compared with control and RGZ groups (Figure 1c). The average number of hidden platform crossings in the STZ group was significantly lower compared with the control and RGZ groups (Figure 1d). These results indicated that rosiglitazone treatment caused improved learning and memory performance in the STZ-induced rat model.

Figure 2. Daily treatment with rosiglitazone for four weeks markedly reduced β-amyloid (Aβ) levels in STZ-treated rats
(a) Hippocampal Aβ42 levels determined by ELISA were decreased by 41% after chronic treatment with the drug in the STZ group; (b) Hippocampal Aβ40 levels were decreased by 54% after chronic treatment with the drug in the STZ group. Values are means±SD (n =12 rats per group). **P<0.01 vs. control group, and ##P<0.01 vs. STZ group

Figure 3. Representative images of immunohistochemical staining for Aβ40 and Aβ42 in the hippocampal region in levels in STZ-induced AD rats
Rosiglitazone almost nearly reduced staining of internal Aβ deposits in hippocampal areas in the RGZ group compared with the control and STZ-treated groups (scale bar=10 um, representative sections of n = 12 animals per group)
carboxy-terminal fragments, C83 and C99, were significantly increased in STZ-treated rats compared with the control group (Figure 4b), while in rosiglitazone-treated rats, the levels of total APP and C83 were significantly lower compared with STZ-treated rats (P<0.01) (Figure 4b). In addition, the levels of C99, the precursor of Aβ peptide, was reduced but not to a significant extent (P>0.05), suggesting that rosiglitazone most likely did not reduce Aβ production by affecting the APP amyloidogenic pathway.

Effects of rosiglitazone on Aβ transportation

In order to investigate if rosiglitazone affected Aβ levels via a transport mechanism, the protein expression levels of LRP1, RAGE, and ABCA1 were measured using Western blot analysis. LRP1 and ABCA1 protein expression levels were decreased in STZ-treated rats compared with the control group (P<0.01) (Figure 4c), while after rosiglitazone treatment the expression levels of both proteins significantly increased compared with the STZ-treated group (P<0.05) (Figure 4c). The expression of RAGE was increased in STZ-treated rats compared with the control group (P<0.01) (Figure 4c). However, after rosiglitazone treatment, its expression was significantly decreased (P<0.01) (Figure 4c). In contrast, ApoE protein expression levels remained unchanged (P>0.05) (Figure 4c). These results suggest that rosiglitazone may affect Aβ levels via regulating transportation and may increase the lipiddation of ApoE through an overexpression of ABCA1 rather than affect ApoE expression levels in the STZ-treated rat model.

Discussion

In the present study we found that rosiglitazone, a high-affinity PPAR-γ agonist, decreased Aβ burden and eliminated the abundant amyloid plaques found in the hippocampus of an STZ-treated rat model. The drug could modify the expression of specific transport proteins, such as ABCA1, LRP1, and RAGE, all of which are considered to alter the Aβ clearance rate. Moreover, rosiglitazone rescued impaired learning and spatial memory in STZ-treated rats.

Daily treatment with rosiglitazone for four weeks markedly decreased Aβ40 and Aβ42 levels, and at the same time, impairments in spatial learning and memory were rescued in STZ-treated rats. Thus, the decreased amyloid pathology may account for the rescue of impaired recognition and spatial memory in the AD rat model. It is interesting to note that amyloid plaques were eliminated prominently in the hippocampus after chronic rosiglitazone treatment. The amelioration of amyloid pathology found in this study may be due to a lower production of Aβ. However, rosiglitazone neither modified the levels of the Aβ peptide precursor, C99, nor changed the levels of BACE1, the key enzyme cleaving APP. These results suggest that in an STZ-induced rat model of AD, a reduction of Aβ levels may not be caused by a decrease of APP processing but due to an increase of the brain’s Aβ clearance ability. In a previous study, Pedersen et al (20) reported that rosiglitazone attenuated the reduction of IDE mRNA observed in Tg2576 mice in the hippocampus but not in the frontal cortex, and at the same time decreased Aβ42, rather than Aβ40, levels. While Vekrellis et al (21) reported that IDE degrades Aβ42 and Aβ40 to the same extent. On the other hand, Meilandt et al (22) doubted that increased NEP levels may be beneficial to amyloid pathology, as this enzyme neither degrades Aβ oligomers nor restores memory function in an AD mouse model. Our research revealed that rosiglitazone treatment did not change IDE and NEP protein levels in STZ-treated rats. These findings indicated that the decrease of Aβ levels was not due to the degradation effect of IDE and NEP.

ApoE is a major ligand for LRP1 and interferes with AD by affecting Aβ aggregation, cellular uptake, and degradation. While decreased ApoE levels reduce Aβ deposition, Jiang et al (23) reported that
lipidated ApoE facilitates the proteolytic degradation of Aβ by NEP and IDE. Furthermore, Wahrlie et al (24) proposed that genetic loss of the lipid transporter ABCA1 impairs ApoE lipidation and promotes amyloid deposition in AD mouse models. In our experiments, we found that rosiglitazone did not modify ApoE levels in STZ-treated rats, but significantly increased the expression of ABCA1. The increased ABCA1 could lead to a more marked lipidation of ApoE in rosiglitazone-treated rats, which in turn facilitates Aβ clearance. Therefore, the increase in ABCA1 expression, which results in increased lipidation of ApoE-containing lipoproteins, facilitating ApoE-Aβ interaction in the perivascular space, making Aβ more accessible to transport by LRP1 is a novel potential mechanism of action for PPARγ agonists in the STZ-treated rat model.

Several previous studies have investigated the effects of altered LRP1 expression on Aβ metabolism. Shinohara et al. (25) showed that the hydroxy methylglutaryl-CoA reductase inhibitor, fluvastatin, increases LRP1 in mouse cerebral vessels, resulting in reduced brain Aβ levels. In our study, we found an up-regulation of LRP1 and down-regulation of RAGE protein levels after rosiglitazone treatment in STZ-treated rat brains, which may enhance Aβ efflux from the brain through the BBB.

Although the link between the Aβ and AD has been known for decades, the importance of Aβ balance mechanisms, involving Aβ production, clearance mechanisms, and efflux, has only been addressed recently. To date, only a few studies have ever focused on disease-related expression variations of BBB transporters, and even less is known regarding disease-induced modification of the transporters through the BBB (26). Based on our findings, we propose that PPAR-γ activation, through natural or synthetic ligands, leading to the increased expression of specific transporters which maintain BBB integrity and functionality to enhance Aβ clearance.

The findings of the present research indicate that rosiglitazone reduces AD pathology and restores hippocampal function leading to a rescue of memory impairment observed in STZ-treated rats. Of particular interest was that rosiglitazone led to the virtual elimination of the abundant amyloid plaques found in the hippocampus of the AD rat model. We hypothesize that PPAR-γ agonists mediate the activation of specific transporters monitoring Aβ clearance via the BBB, such as ApoE, LRP1, and RAGE. In the STZ-induced animal model, the PPAR-γ agonist did not influence the expression of BACE1, APP processing, nor intervene with the expression of Aβ-degrading enzymes, such as IDE and NEP. These novel findings provide an underlying linked mechanism between rosiglitazone and amyloid clearance and suggest the potential therapeutic employment of PPAR-γ agonists against AD.

**Conclusion**

Rosiglitazone improved impaired spatial memory by decreasing amyloid pathology in STZ-treated rats. But the drug did not appear to reduce Aβ production by merely modifying amyloid precursor protein (APP) processing, whereas intervening the facilitation of Aβ clearance. Therefore, it might be prospected that rosiglitazone will be a potential exploited orientation for Aβ clearance in sporadic AD patients.

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