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PPARγ agonist pioglitazone improves scopolamine-induced memory impairment in mice

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Keywords
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Abbreviations
Aβ, amyloid peptide β; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; BBB, blood–brain barrier; ChAT, choline acetyltransferase; CNS, central nervous system; PPARγ peroxisome proliferator-activated receptor γ

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Abstract

Objectives This study was conducted to evaluate the effects of exposure to pioglitazone, a peroxisome proliferator-activated receptor agonist, on cognitive impairment induced by scopolamine, a muscarinic antagonist, in mice.

Methods Pioglitazone (9 mg/kg, 18 mg/kg) was orally administered for 9 days at 30 min before intraperitoneal injection with scopolamine (0.8 mg/kg, i.p.). Cognitive function was evaluated by the passive avoidance test and the Morris water maze test on the 10th day after treatment. Changes in cholinergic system reactivity were also examined by measuring the acetylcholine, acetylcholinesterase and choline acetyltransferase in the hippocampus and cortex.

Key findings Scopolamine injection induced impaired performance in the passive avoidance test and the water maze test and severe decrease of cholinergic system reactivity, as indicated by reduced acetylcholine levels, decreased choline acetyltransferase activity and increased acetylcholinesterase activity. Daily administration of pioglitazone significantly increased step-through latency in passive avoidance test, and significantly decreased the escape latency, and increased the time spent in the platform quadrant in the Morris water maze test. Pioglitazone also protected against scopolamine-induced cholinergic system deficit, including reduced acetylcholine levels, decreased choline acetyltransferase activity and increased acetylcholinesterase activity in the hippocampus or cortex.

Conclusions Pioglitazone demonstrates a significant neuroprotective effect against scopolamine-induced cholinergic system deficit and cognitive impairment.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease related to cognitive and behavioral impairments, which is shown by the most common form of dementia in the elderly. Although the primary cause of AD remains unclear, it may be considered that the β-amyloid and tau protein aggregation, reduced acetylcholine (ACh), and glutamatergic deficit are regarded as principal pathogenesis of AD.[1,2] Improvement of cholinergic neurotransmission is currently the strategy for developing drugs for the treatment of AD as a loss of cholinergic neurons and reduced acetylcholine activity in the cerebral cortex and hippocampus are consistent with findings in AD. The inhibitors of acetylcholinesterase (AChE) such as donepezil are commonly prescribed for treatment of AD.[3]

Peroxisome proliferator-activated receptor gamma (PPARγ) was recognized as a therapeutic target for AD about a decade ago because of not only its actions on inflammation, but also its effects on insulin sensitization and energy metabolism.[1,4] The synthetic thiazolidinediones (TZD), such as pioglitazone and rosiglitazone, which serve as PPARγ agonists, are widely prescribed for the treatment of type 2 diabetes mellitus, and have also been shown to be efficacious in a number of central nervous system (CNS) disease models.[5,6] However, the use of these drugs for CNS-targeted disease treatments is compromised due to their poor blood–brain barrier (BBB) penetrance. The permeability of pioglitazone across the BBB is poor, and rosiglitazone is even less permeable and subject to P-glycoprotein-mediated efflux from the brain.[7] A number of studies using AD mouse models have been carried out and demonstrated the utility of these synthetic agonists in AD pathogenesis.[8,9] Two trials

with pioglitazone demonstrated an improvement in cognitive function in patients with mild to moderate AD.[10,11] It is unfortunate that recent its phase III clinical trials have shown a negative outcome in the treatment of AD patients,[12] and there might be unidentified factors that influence pioglitazone therapy for AD.[13] In spite of this, the beneficial effects of pioglitazone have been demonstrated in improving cognition in the early stages of AD or prevention of AD.[13] However, whether pioglitazone affects the cholinergic system, which plays an important role in learning and memory, is unknown so far. In the present studies, we used a non-selective muscarinic receptor antagonist scopolamine to disrupt central cholinergic neurotransmission and investigated the effects of PPARγ agonist pioglitazone in improving learning and memory in mice exposed to repeated scopolamine-induced cholinergic activity reduction. To elucidate the neural mechanism underlying the memory-enhancing activity of pioglitazone, we also examined how these effects are related to the levels of cholinergic markers in the hippocampus and cortex.

Materials and Methods

Animals

The study was conducted in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China. All procedures and the care of the mice were in accordance with institutional guidelines for animal use in research. ICR male mice, aging 8–10 weeks old and weighing 20–25 g, were purchased from Yangzhou University Medical Center (Yangzhou, China). They were maintained at a constant humidity (c. 60%) and temperature (c. 23°C) with a light/dark cycle of 12 h.

Experimental groups

Seventy mice were randomly divided into seven groups: saline plus vehicle (Sal+Veh), scopolamine plus vehicle (Sco+Veh), scopolamine plus Aricept (Sco+Ari) (2 mg/kg), scopolamine plus pioglitazone (Sco+Pio) (18 mg/kg), scopolamine plus pioglitazone (Sco+Pio) (9 mg/kg), saline plus pioglitazone (Sal+Pio) (18 mg/kg) and saline plus pioglitazone (Sal+Pio) (9 mg/kg). Pioglitazone and aricept were dissolved in 0.5% sodium carboxymethyl cellulose solution (0.5%CMC-Na) and stored at 4°C. Scopolamine was diluted by saline and stored at normal temperature. The mice were orally administered with pioglitazone (9 mg/kg, 18 mg/kg; 0.3 ml/10 g body weight) 30 min before intraperitoneal injection with scopolamine (0.8 mg/kg; 0.2 ml/10 g body weight) or saline for 9 consecutive days. The mice in the vehicle group received 0.5% CMC-Na. Aricept (2 mg/kg; 0.3 ml/10 g body weight) was administered as the positive control. From the tenth day onwards, the passive avoidance test and subsequent Morris water maze test were carried out 30 min after scopolamine injection. To investigate the effect of pioglitazone on the memory of unimpaired animals, pioglitazone alone was administered 1 h before the test without scopolamine treatment. The mice were killed under ether anesthesia and the brain hemispheres were quickly removed to an ice plate. After being weighed, the brain hemispheres were homogenized in ice-cold phosphate-buffered saline (PBS, pH 7.6) and centrifuged at 3000 × g for 15 min at 4°C. The supernatant was decanted and diluted fivefold with enzyme immunoassay buffer containing 1% bovine serum albumin and 0.05% Tween-20 in PBS. The amounts of ACh, AChE and choline acetyltransferase (ChAT) in mouse brain were determined as described in the manufacturer's instructions. The fasting blood glucose (FBG) was determined by the glucose oxidase method.

Passive avoidance test

The passive avoidance test is a widely accepted simple and rapid means of memory testing. The passive avoidance response was determined using a 'step-through' apparatus (Huaibei Zhenghua Biologic Equipment Co., Ltd, China), which consisted of illuminated and dark compartments (each 32.5 × 14 × 36 cm) adjoining each other through a guillotine door. Floors were constructed of 3.175 mm stainless steel rods set 8 mm apart. The mice underwent two separate trials, a training trial and 24 h later a test trial. For the training trial, mice were initially placed in the illuminated chamber. When mice entered the dark compartment, the door closed and an electrical foot shock (0.4 mA) of 2 s duration was delivered through the stainless steel rods. One hour before the training trial, mice were administered pioglitazone (18 or 9 mg/kg, orally). After 30 min, memory impairment was induced by administering scopolamine (0.8 mg/kg, i.p.). Control animals received 0.5% CMC-Na rather than pioglitazone. Twenty-four hours after the training trial, mice were placed in the illuminated compartment for the test trial. The time taken for a mouse to enter the dark compartment after door opening was defined as latency for both training and test trials. Latency to enter the dark compartment was recorded up to 300 s. To avoid a ceiling effect in unimpaired animals, pioglitazone alone was administered 1 h before the training trial without treatment of scopolamine.

Morris water maze test

Cognitive function was evaluated by the Morris water maze. The apparatus and test procedure have been previously described.[14] The test included 6 days of learning and memory training and a probe trial on day 6. Mice were individually trained in a circular pool (110 cm diameter, 60 cm height) filled to a depth of 40 cm with water maintained at

25°C. The maze was located in a lit room with visual cues. An escape platform (10 cm diameter) was placed in the center of one quadrant of the pool. The platform's position was fixed and the animal's starting positions, facing the pool wall, were pseudo-randomized for each trial. Each mouse was separately tested on both visible-platform and hidden-platform versions of the water maze. The hidden-platform version evaluates spatial learning and was used to determine the retention of memory to find the platform. During the hidden-platform training trials, the escape platform was placed 1 cm below the surface of the water. On each day, the animal was subjected to three trials with a 20 min interval between trials. Each trial lasted for 90 s unless the animal reached the platform before that time elapsed. The time taken for the mouse to reach the platform (a successful escape) was noted. If an animal failed to find the platform within 90 s, the test was ended and the animal was gently navigated to the platform by hand. Whether a mouse found or failed to find the platform within 90 s, it was maintained on the platform for 30 s. On the last day (day 6), the platform was removed from its previous location and the animals were given a probe trial in which they had 90 s to search for the platform. The time taken to reach the missing platform and the number of times the animals crossed the platform location were recorded. All animals underwent non-spatial pre-training during the first two training days, which prepared them for the spatial learning test. During the visible trials of the water maze, mice were trained to find the platform, made visible to them by a small flag (5 cm tall). Data for the escape latency, the distances traveled and the number of platform location crossings were collected by video equipment and processed by a computer equipped with an analysis-management system (Viewer2 Tracking Software, BIOBSERVE, Fort Lee, NJ, USA). Pioglitazone (18 and 9 mg/kg) was orally administered before intraperitoneal injection with scopolamine (0.8 mg/kg) or saline, and 30 min later the test trial began.

ELISA assay for ACh

The ACh in the brain homogenate was assayed using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems of Shanghai, China). In short, 50 μl of sample was added into the precoated plate and was incubated for 30 min at 37°C in the dark. After washing each well of the precoated plate with washing buffer, 50 μl of labeled antibody solution was added and the mixture was incubated for 30 min at 37°C in the dark. After washing, chromogen was added and the mixture was incubated for 15 min at 37°C in the dark. After the addition of stop solution, the resulting color was recorded at 450 nm using a microplate absorbance reader. The protein content was measured using Coomassie blue-based assay reagent and bovine serum albumin as standard. Results were expressed as millimole of ACh per milligram of protein.

AChE activity assay

AChE activity was measured by the method of Ellman[15] with slight modification.[16] Acetylthiocholine iodide solution (25 μl, 75 mM) and 100 μl of buffered Ellman's reagent (10 mM 5,5′-dithio-bis [2-nitrobenzoic acid] and 15 mM sodium bicarbonate) were used and reacted at room temperature for 10 min. Absorbance was measured at 412 nm immediately after adding the enzyme source to the reaction mixtures using a UN spectrometer (UV/V-16/18 MAPADA Co. Ltd, China). The assay procedure followed the manufacturer's instructions. The protein content was measured using Coomassie blue-based assay reagent, and the results were expressed by U of AChE per milligram of protein.

Assay of ChAT activity

The ChAT activity was measured using the amount of acetyl-coenzyme A (acetyl-CoA) formed from coenzyme A (CoA) and ACh[17] and with slight modification. Tissue homogenate (5%) was boiled for 60 s and then mixed well on a vortex mixer. The activity of ChAT in the sample was deactivated. The samples were vortexed and placed at room temperature for 15 min. The absorbance of Ach at 324 nm in a quartz cuvette was measured (lightpath 1 cm; inside diameter 2 mm; volume 600 μl; measurement against deionized water). One unit of ChAT catalytic activity is defined as the amount of enzyme that will catalyse the transfer of 1.0 nanomol of acetate from acetyl CoA to choline per minute at pH 7.2 at 37°C in 1.0 g tissue.

Statistical analysis

The experimental results were expressed as the mean ± SEM. The behavioral data were calculated and analysed by repeated measures analysis of variance using SPSS 14.0 (IBM). The statistical significance of the differences among groups was further analysed using Tukey's post-hoc test. Other data were also analysed by one-way analysis of variance followed by Tukey's post-hoc test. In all analyses, $P \leq 0.05$ was considered significant.

Results

Pioglitazone improves scopolamine-induced memory impairment in mice

In the passive avoidance test, at the learning trial (day 0), mice of all groups entered the dark compartment and there were no significant differences among the mice. In the testing trial (day 1), the mice which received intraperitoneal injection of

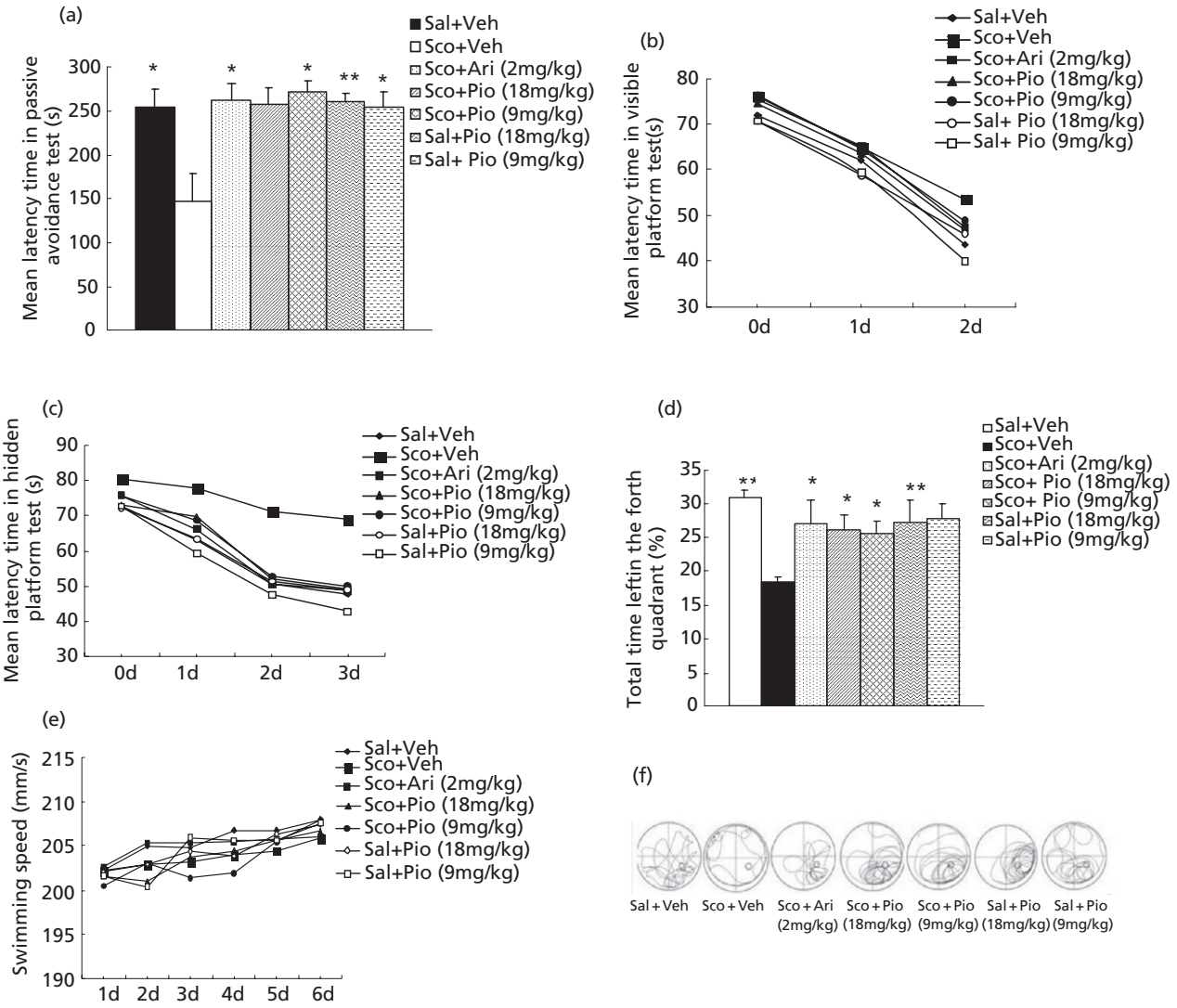

Figure 1 Effects of pioglitazone on scopolamine-induced memory impairment evaluated by the passive avoidance test (a) and the Morris water maze in mice (b–f). Mean latency time (b) during the visible platform test, mean latency time (c) during the hidden platform test, the percentage of total time left in the fourth quadrant (d), swimming speed (e) and representative swim paths (f) during spatial probe test are shown. Aricept (2 mg/kg), pioglitazone (18 mg/kg) or pioglitazone (9 mg/kg) was orally administered 30 min before intraperitoneal injection with scopolamine (0.8 mg/kg) or saline. Vehicle refers to 0.5%CMC-Na oral administration. Sal+Veh, saline plus vehicle; Sco+Veh, scopolamine plus vehicle; Sco+Ari (2 mg/kg), scopolamine plus aricept; Sco+Pio (18 mg/kg), scopolamine plus pioglitazone; Sco+Pio (9 mg/kg), scopolamine plus pioglitazone; Sal+Pio (18 mg/kg), saline plus pioglitazone; Sal+Pio (9 mg/kg), saline plus pioglitazone. Values are expressed as the mean ± SEM ($n = 10$). *$P < 0.05$, **$P < 0.01$ vs Sco+Veh.

scopolamine showed a significant reduced step-through latency (148.10 ± 30.39 s, $P < 0.05$) compared with those injected with saline (255.70 ± 19.45 s) (Figure 1a). The escape latency of mice treated with scopolamine plus pioglitazone (18 or 9 mg/kg) for 9 consecutive days showed a significant protective effect on memory impairment (257.70 ± 14.77 s, $P < 0.05$ or 272.33 ± 11.80 s, $P < 0.05$) compared with that of animals in the group of scopolamine plus vehicle, which indicated that pioglitazone had a similar effect to aricept (263.40 ± 18.28 s) (Figure 1a). To further confirm the memory-enhancing activity of pioglitazone, we determined its improvement of the spatial memory function using the Morris water maze test. Figure 1b and 1c show that mice treated with scopolamine plus pioglitazone exhibit progressively decreased escape latency during training (four times/day) compared with the mice treated with scopolamine plus vehicle in place navigation. In the probe trial, a putative measure of spatial learning and memory retention, only mice exposed to scopolamine failed to favor the target platform location. Administration of pioglitazone at 18 or 9 mg/kg

significantly improved overall target quadrant preference during the final probe trial (26.19 ± 2.03, $P \leq 0.05$, 25.61 ± 1.82, $P \leq 0.05$, respectively) compared with that of animals in the scopolamine plus vehicle group (18.35 ± 0.72) (Figure 1d). In addition, there were no differences in swimming speed among groups in the Morris water maze test (Figure 1e). Furthermore, 18 and 9 mg/kg of pioglitazone had no effect on learning and memory in unimpaired mice.

Effects of pioglitazone on blood glucose levels

Table 1 shows that there were no differences among groups, which suggests that pioglitazone at 18 or 9 mg/kg showed no significant effects on blood glucose levels in mice.

Effects of pioglitazone on the ACh content of the hippocampus and cortex in mice

The ACh content significantly decreased in the hippocampus by 74% and in the cortex by 51% in mice injected with scopolamine compared mice injected with saline. Pioglizatone at 18 or 9 mg /kg increased ACh content in the hippocampus by 1.9 and 1.7 times and in the cortex by 1.5 and 1.3 times compared with the scopolamine plus vehicle group. Furthermore, 18 and 9 mg/kg of pioglitazone had no effects on the ACh content of hippocampus and cortex in unimpaired mice (Table 1).

Effects of pioglitazone on the AChE activity of the hipppocampus and cortex in mice

Table 2 shows that scopolamine increased the AChE activity of the hippocampus, and pioglizatone (18, 9 mg/kg) inhibited the increase in AChE activity caused by scopolamine in the hippocampus. There was no statistical significance, although pioglizatone also inhibited the AChE activity in the cortex. Furthermore, pioglitazone had no effect on the AChE activity of the hippocampus and cortex in unimpaired mice.

Effects of pioglitazone on the ChAT activity of the hipppocampus and cortex in mice

Table 2 shows that the ChAT activity of hippocampus and cortex significantly decreased in mice treated with scopolamine, and pioglizatone at 18 or 9 mg/kg significantly inhibited scopolamine-induced decreases of ChAT activity in either the hippocampus or cortex of mice. Pioglitazone had no effect on the ChAT activity of the hippocampus and cortex in unimpaired mice.

Table 1 Effects of pioglitazone on levels of blood glucose and intracerebral ACh in scopolamine-treated mice (mean ± SEM, n = 10, 5)

		Ach content (mmol/mg prot)	
Groups	Blood glucose (mmol/l)	Hippocampus	Cortex
Sal+Veh	6.88 ± 0.38	0.0405 ± 0.0027**	0.0218 ± 0.0011*
Sco+Veh	6.68 ± 0.38	0.0102 ± 0.0012	0.0104 ± 0.0007
Sco+Ari (2 mg/kg)	6.50 ± 0.34	0.0245 ± 0.0017**	0.0163 ± 0.0014*
Sco+Pio (18 mg/kg)	6.87 ± 0.31	0.0198 ± 0.0022**	0.0158 ± 0.0017*
Sco+Pio (9 mg/kg)	6.51 ± 0.54	0.0175 ± 0.0013*	0.0131 ± 0.0018*
Sal+Pio (18 mg/kg)	6.81 ± 0.27	0.0358 ± 0.0026*	0.0202 ± 0.0006*
Sal+Pio (9 mg/kg)	6.49 ± 0.29	0.0359 ± 0.0019*	0.0195 ± 0.0012*

*P < 0.05, **P < 0.01 vs Sco+Veh.

Table 2 Effects of pioglitazone on the AchE and ChAT activity of the hippocampus and cortex in scopolamine-treated mice (mean ± SEM, n = 5~6)

	AchE activity (U/mg prot)		ChAT activity (U/mg prot)	
Groups	Hippocampus	Cortex	Hippocampus	Cortex
Sal+Veh	1.60 ± 0.15*	1.25 ± 0.06*	235.16 ± 11.90**	130.89 ± 11.81**
Sco+Veh	2.30 ± 0.17	1.76 ± 0.13	68.71 ± 8.42	64.35 ± 6.79
Sco+Ari (2 mg/kg)	1.67 ± 0.12*	1.34 ± 0.03*	160.33 ± 7.41**	116.05 ± 8.90**
Sco+Pio (18 mg/kg)	1.69 ± 0.14*	1.45 ± 0.05	148.11 ± 6.87**	113.22 ± 9.68**
Sco+Pio (9 mg/kg)	1.74 ± 0.05*	1.63 ± 0.08	144.52 ± 5.20**	111.25 ± 14.79**
Sal+Pio (18 mg/kg)	1.64 ± 0.16*	1.29 ± 0.06*	228.61 ± 12.23**	127.61 ± 4.16**
Sal+Pio (9 mg/kg)	1.65 ± 0.08*	1.33 ± 0.05*	232.98 ± 10.37**	126.25 ± 7.90**

*P < 0.05, **P < 0.01 vs Sco+Veh.

Discussion

The present study demonstrates that repeated scopolamine-induced dementia produces severe deficits in the performance of cognitive function tests, with corresponding signs of cholinergic system deficit in the brain, including depleted ACh, increased AChE activity and decreased ChAT activity in the hippocampus. Our results show that treatment with the PPARγ agonist pioglitazone improved learning and memory retention in the Morris water maze test and increased step-through latencies in the passive avoidance test. It also increased the ACh levels that resulted from the elevation of ChAT activity and the reduction of the AChE in the hippocampus or cortex of scopolamine-induced dementia mice. No effects of pioglitazone on memory in normal mice were observed. This suggests that pioglitazone alleviated the memory impairment induced by repeated scopolamine injection.

Scopolamine in humans may be used to investigate the role of acetylcholine in cognition. Liem-Moolenaar *et al*. reported that 7-day administration of scopolamine caused impairments in cognitive and psychomotor function in healthy subjects.[18] Scopolamine, when administered to animals, is capable of transiently producing some of the deficits in the processes of learning acquisition and short-term memory that are considered to be characteristic of AD.[19–21] However, the duration of scopolamine administration differs greatly in the literatures, which refers to 3 to approximately 30 consecutive days.[22–26] Nine-day consecutive administration of scopolamine, a regular regime in our laboratory, exhibited accurate and reproducible cognitive impairment in mice, in which the changes of ACh, ChAT and AChE were in agreement with the findings of previous studies.[23,24] Theoretically, long-term administration of a receptor antagonist such as scopolamine can induce an increase in the number of receptors in the brain (upregulation). If scopolamine is withdrawn suddenly, the cells become more sensitive to ACh (supersensitivity). The consecutive administration of a higher dose of scopolamine prevailed over this limited upregulation of the receptor, which is conformed by the behavior tests.

Two higher dosages of pioglitazone, 9 mg/kg (equivalent to the maximum dosage for patients, 0.75 mg/kg) and 18 mg/kg, were used in the studies because of its poor BBB permeability, so the level of intracerebral pioglitazone was elevated based on its pharmacokinetics.[27] Both dosages are effective in inhibiting repeated scopolamine-induced memory impairment in the Morris water maze test and in the passive avoidance test. However, the development of a new formulation of brain-targeted pioglitazone is required to improve its permeability across the BBB since higher dosages would increase the risk of adverse drug reactions. ACh is an important regulator of memory and neural plasticity. Some studies have shown a significant reduction in the levels of brain acetylcholine following experimental dementia.[22] The disruption of basal forebrain cholinergic projections to the hippocampus and other limbic structures impairs the functions of learning and memory.[28,29] Thus, the results of the present and other studies suggest that the reduced cholinergic transmission might, at least in part, be responsible for the cognitive deficits in repeated scopolamine-induced dementia in mice. Because the central cholinergic system is important in the regulation of cognitive function, decreased ACh levels may contribute to the observed impairment of learning and memory during chronic dementia. ChAT and AChE belong to a family of enzymatic proteins that are expressed in cholinergic neurons. ChAT is responsible for the biosynthesis of ACh and is required for cholinergic neurotransmission in the central and peripheral nervous systems. Because ACh is rapidly hydrolysed by AChE, the duration of ACh action in the synaptic cleft is dependent upon AChE activity.[2,30] We found that repeated scopolamine-induced dementia caused a reduction in ChAT activity and an increase in AChE activity in the hippocampus or cortex, and treatment with pioglitazone produced significant changes in cholinergic markers, such as ChAT increase and AChE decrease in the hippocampus, compared with the scopolamine-induced group. How pioglitazone affects AChE and ChAT is unknown. There two possibilities: direct action of the drug itself on the enzymes or gene expression of enzymes regulated by its stimulation of PPARγ. This remains to be further investigated. It is reasonable that the observed improvement in learning and memory in the Morris water maze test and the passive avoidance test was associated with the elevation of hippocampal ACh in pioglitazone plus scopolamine-treated animals. Recent other studies have shown that acute administration of pioglitazone improved the acquisition and retrieval of spatial recognition memory impairment by scopolamine in mice, and the enhancing effect of pioglitazone on acquisition was dependant on the NO pathway.[31] It is well known that long-term administration of pioglitazone may improve insulin-mediated glucose disposal via activation of PPARγ in liver and peripheral tissues in type 2 diabetic patients.[5] However, we did not find any effect of pioglitazone for 16 consecutive days on blood glucose in the mice of each group in present study. This study has provided the first evidence that the stimulation of PPARγ results in the enhancement of the cholinergic nerve and subsequent improvement of learning and memory, implying there might be some connection between PPARγ and the cholinergic nerve in the CNS.

Conclusions

In this study we found that scopolamine can induce memory and learning deficits associated with cholinergic degeneration in mice in the Morris water maze test and the passive avoidance test. Pioglitazone attenuated scopolamine-induced memory

deficits by the enhancement of the cholinergic nerve system, indicated by the elevation of ACh levels, which result from the decreased AChE activity and increased ChAT activity in hippocampus. We illustrated for the first time that there is some association between PPARγ and the cholinergic nerve in the CNS.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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