Research paper

Rolipram-induced improvement of cognitive function correlates with changes in hippocampal CREB phosphorylation, BDNF and Arc protein levels

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HIGHLIGHTS

• Cognitive function in rats with long-term diabetes was impaired.
• BDNF and Arc protein down-regulation were found in diabetic rats.
• CREB and phosphorylation of CREB were decreased in diabetic encephalopathy rats.
• Rolipram could improve cognitive impairment and the expression of BDNF, Arc CREB protein levels in diabetic rats.

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ABSTRACT

Diabetics suffer from a higher risk of cognitive decline. cAMP response element-binding protein (CREB) is a transcription factor associated with memory and synaptic plasticity. Here, we investigated the molecular changes in the hippocampus correlated with diabetes associated cognitive decline (DACD) from a CREB-centered perspective in a rat model of type 2 diabetes. Furthermore, we tested the therapeutic effect of rolipram on DACD. High-fat diet and low-dose streptozocin were adopted to induce diabetes in SD rats. Results show that supplementation with rolipram for 23 days (0.5 mg/kg, once a day) improved the performance of diabetic rats in Morris water navigation task with increased level of CREB, brain-derived neurotrophic factor (BDNF), and Arc protein in the hippocampus. Rolipram, acting as an inhibitor of PDE4, was found to repair the imbalance in the CREB/BDNF/Arc pathway. This study may provide important insights into the mechanisms underlying DACD and provide new therapeutic targets for clinical treatment.

1. Introduction

Recent studies have revealed that diabetic patients suffer from a higher risk of cognitive dysfunction or dementia, especially those related to Alzheimer’s disease. Basic research suggests that insulin accelerates AD-related pathology through its effects on the metabolism of amyloid beta (Aβ) and phosphorylation of tau [1,2]. Exact molecular mechanism underlying diabetes-associated cognitive decline (DACD) remains unclear. Molecular basis of learning and memory involves neuronal synapses modification in response to electrical activity, a process termed synaptic plasticity. While short-term memory involves modifications of existing proteins, long-term memory formation requires new gene transcription and protein production to maintain recent changes. cAMP-response element-binding protein (CREB), known as the “memory switch”, is a transcription factor binding to the promoter regions of many genes. CREB is the common downstream molecule of major signaling pathways controlling the process of memory and is associated with memory and synaptic plasticity [3,4].

It is well known that neurotrophins serve as important regulators of synaptic plasticity, the most extensively studied neurotrophin is brain-derived neurotrophic factor (BDNF). BDNF has been reported to be altered following CREB activation [5], and the CREB/BDNF pathway is thought to play an important role in long-term memory formation [6]. Furthermore, neurons are known to modify their synaptic strength through activation of specific genes, which brought our focus to one of the genes shown to
be involved in the BDNF-mediated modification of synapses and be vital for memory consolidation [7], namely activity-regulated cytoskeleton-associated gene/activity-Regulated gene 3.1 protein homolog (Arc/Arc3.1).

Rolipram, a selective inhibitor of phosphodiesterase4 (PDE4), works to prevent the hydrolysis of cAMP. The function of rolipram in improving memory has been revealed in many models of cognitive deficits, and is probably partly implemented through facilitation of long-term potentiation and phosphorylation of CREB in the hippocampus [8,9]. However, the effect of rolipram on DADC as well as relevant proteins in CREB/BDNF/Arc pathway remains to be explored. Based on the above, we designed the present study to explore the mechanism underlying the DADC. We aim to clarify whether rolipram is able to improve spatial working memory in rats with cognitive impairment induced by a high-fat diet (HFD) and low-dose streptozotocin (STZ) injection and whether these changes are correlated with the regulation of CREB and the rise of BDNF and Arc protein levels.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee at Shanghai Jiao Tong University and also complied with the NIH guide for the Care and Use of Laboratory Animals. Every possible effort was made to minimize the suffering of animals.

2.2. Animals

45 male 8-week-old Sprague-Dawley rats weighing 180–220 g were purchased from the experimental animal center of Shanghai Jiao Tong University. Animals were fed food and water ad libitum and were raised in the specific pathogen-free (SPF) animal experiment room at 24 ± 2 °C with 40–70% humidity on a 12 h light/dark cycle. After 1 week of acclimatization, rats were randomly divided into 3 groups: the diabetes group (DM), the diabetic rats treated with rolipram group (DM/ROL) and the control group (CON).

2.3. Induction of type 2 diabetes model and design of experiment

The control group was fed with a normal pelleted diet (NPD) while rats in the diabetes group and rolipram group were given a high-fat diet (HFD, 40% energy supplied from fat; SLAC laboratory animal, Shanghai, China) until the end of experiment. STZ (30 mg/kg, freshly dissolved in citrate buffer, PH4.4, 0.1 mol/L, Sigma–Aldrich, USA) was injected intraperitoneally to induce diabetes in HFD-fed rats after 12-hour fasting at the end of the 4th week. The NPD-fed rats received an equal volume of citrate buffer. Blood samples were collected 72 h after administration to measure random blood glucose (RBG) through a fast blood glucose meter (OneTouch Surestep, Johnson & Johnson, USA). The diabetes model was considered to be successfully established if the RBG of rat was more than 16.7 mmol/L [10].

At the end of the 3rd week (7 days before STZ injection) as well as at the end of the whole experiment, blood samples were collected from the caudal vein to analyze fasting blood glucose (FBG) and fasting serum insulin (FSI) using an enzyme linked immunosorbent assay (ELISA) kit (BlueGene Biotech, Shanghai, China). An oral glucose tolerance test (OGTT), insulin tolerance test (ITT) were carried out in the 6th and 7th weeks to evaluate glucose tolerance and insulin sensitivity of rats (50% glucose, 2 g/kg, given orally; regular human insulin 0.75 U/kg, injected subcutaneously, Lilly, Suzhou, China). From the 13th week (after STZ had been injected for 8 weeks), animals in the DM + ROL group were administrated with rolipram for 23 days (0.5 mg/kg, qd, i.p, Sigma–Aldrich, USA). Rolipram was dissolved in 0.9% saline containing 10% dimethyl sulfoxide, and injected at a volume of 1 ml/kg. The control group and diabetes group were given the same volume of saline. From the 15th week, water maze tests began 1 h after the administration of rolipram and saline. The body weight and blood glucose of rats were monitored once a week (Fig. 1).

2.4. Morris water maze test

Rats were tested in a Morris water maze apparatus to assess spatial learning and memory as described previously [11]. After 2 weeks of rolipram treatment, the 5-day hidden platform training began. Before the training, each rat was given 2 min to swim without platform to adapt to the new environment. Four plastic pallets in different shapes and colors were hung on the curtains for rats to identify direction. The platform was submerged 2 cm beneath the water surface, and the water was dyed black with edible pigment. Each rat was placed into the maze facing the pool wall at one randomly designated entry point. The rats were allowed to swim freely for 90 s to find the hidden platform, and after mounting the platform they were allowed to stay there for 30 s. If a rat failed to locate the platform within 90 s, it was gently guided to the platform and allowed to stay on it for 30 s. The animals were dropped into the maze randomly at the four different entry points each day. The escape latency (time spent in searching and climbing onto the platform), swimming speed and escape routes were recorded by the video tracking-analysis system. The average of the four escape latencies for that day were calculated for each rat. After being dried with a towel and placed in a warm cage, rats were given a 10-minute rest between trials.

The probe trial test was conducted without a platform the day after the acquisition training. Each rat was placed into the tank facing the wall in a position farthest away from the target quadrant and allowed to swim freely for 90 s. The time spent in the target quadrant, swimming speed and search path were recorded. Twenty-four hours after the probe trial test, a visible platform test was carried out on the subsequent three consecutive days. The platform with a 4 cm² red flag on it was 1 cm above water surface, and each rat was placed into the maze facing the pool wall at the closest entry point. Rats were allowed to search and climb onto the platform for 90 s. The escape latency was recorded. Rats were guided to the platform if they failed to find it in 90 s. The location of the platform changed every day. ELISA assay for Aβ1-40 and Aβ1-42.

Twenty-four hours after the completion of Morris water maze test, rats were sacrificed under deep anesthesia 1 h after the last rolipram injection. Hippocampi were quickly dissected on ice, immediately frozen in liquid nitrogen and stored at −80 °C. The level of Aβ in each hippocampus was measured using rat Aβ1-40 and Aβ1-42 ELISA kits (BlueGene Biotech, Shanghai, China). Hippocampi were cut into small pieces and homogenized in PBS on ice. The homogenates were then centrifuged for 15 min at 5000 rpm at 4 °C. The supernatant was assayed in strict accordance with the instructions in the ELISA kits. Tissue preparation and western blotting Hippocampal tissues was homogenized in ice-cold T-PER tissue protein extraction reagent ( Pierce Biotechnology, Waltham, Massachusetts, USA) and Halt phosphatase inhibitor cocktail ( Pierce Biotechnology, Waltham, Massachusetts, USA), and assessed for expression of pCREB, CREB, BDNF and Arc/Arg3.1 proteins. The homogenates were centrifuged at 12,000 × g for 10 min at 4 °C. The protein concentration was determined with a BCA Protein Assay Kit ( Pierce Biotechnology, Waltham, Massachusetts, USA). Samples (60 μg each) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF membranes ( Millipore, Boston, Massachusetts, USA), then incubated in blocking buffer [5% BSA in Tris-buffered.
saline (TBS) with 0.1% Tween 20 for 1 h, followed by an overnight incubation at 4°C with rabbit anti-pCREB (Ser133, 43 kDa), anti-
CREB (43 kDa: 1:1,000; Cell Signaling Technology, USA), anti-
BDNF (14 kDa, 1:5000; Abcam, USA), anti-Arc/Arg3.1 (55 kDa: 1:5000; Abcam, USA), and anti-β-actin (42 kDa, 1:5000; BioTNT, Shanghai, China). Membranes were then incubated for 1 h with goat anti-rabbit antibody (1:5000; BioTNT, Shanghai, China). Specific bands were developed using enhanced chemiluminescence reagent (Pierce Biotechnology, USA), visualized on the ImageQuant LAS4000 mini (GE Healthcare, USA), and quantified using Image-Pro software (www.mediacy.com).

2.5. Statistical analysis

Statistical analysis was performed using SPSS 13.0 (SPSS, Chicago, IL, USA). Quantitative data was expressed as mean ± SD. All the data were analysed using one-way ANOVA followed by Newman–Keuls tests for post-hoc comparisons between groups, with the exception of the data of the water-maze acquisition trials, which were analysed by two-way repeated-measures ANOVA. Statistical significance was considered when p < 0.05.
3. Results

3.1. Changes in body weight, blood glucose and FSI

At the end of the experiment, each group contained 13 rats. Two rats were excluded from the Con group because of fighting, one died and another became blind. Two rats in DM group and two in DM + ROL group died 2 weeks after the establishment of the model. Body weights of rats were matched between groups at the beginning of the experiment. After 4 weeks, HFD-fed rats weighed more than NPD-fed rats. However after injection of STZ, the weight gain of HFD-fed animals were slower than the NPD-fed rats. Diabetic rats treated with rolipram showed no significant difference in weight compared with diabetic rats without drug intervention. There was no significant difference in FBG between NPD-fed rats and HFD-fed rats before STZ injection. The levels of blood glucose began to rise after STZ injection in HFD-fed rats and remained at a high level until the end. Rolipram did not change the hyperglycemia of HFD-fed rats. Glucose tolerance and insulin tolerance in diabetic rats appeared to be impaired. In addition, insulin sensitivity decreased in OGTT and ITT. Levels of FSI in diabetic rats remained unchanged after they received rolipram (Supplementary data 1 and Supplementary data 2).

3.2. Results of the Morris water maze test

During the 5-day acquisition trials in the water-maze test, all the rats displayed progressive decreases in escape latency to reach the hidden platform. Comparisons by two-way repeated-measures ANOVA revealed significant changes in drug effect $F(2,144) = 35.68$, $p < 0.001$ and time effect $F(4,144) = 3008.48$, $p < 0.001$ as well as the interaction of drug and time effect $F(8,144) = 4.76$, $p < 0.001$. Post hoc Newman–Keuls comparisons indicated that the escape latency in untreated diabetic rats was longer than the control group (all $p < 0.05$); these were reversed by rolipram (0.5 mg/kg for 14–19 days, all $p < 0.05$) from the day 3 in the acquisition trial (Fig. 2A). In the probe trial test, time spent in the target quadrant in untreated diabetic rats was less than in the control and rolipram-treated groups ($p < 0.01$ vs. control group; $p < 0.001$ vs. rolipram-treated group, Fig. 2C). The searching swimming paths in target quadrant were different in different groups (Fig. 2D). There was no significant difference in swimming speed among rats in three groups. [CON vs DM vs DM + ROL (25.59 ± 2.89 m/s) vs (25.29 ± 2.82 m/s) vs (24.12 ± 2.45 m/s)], $F(2,36) = 1.060$, $p = 0.357$. The visible platform test was performed after the probe trial test in order to evaluate the rats' vision and movement abilities, and we saw no significant changes among animals in different groups (Fig. 2B).

3.3. Changes in expression of CREB and pCREB levels in hippocampus

Rats treated with HFD and STZ that impaired memory in the Morris Water-maze test, with or without rolipram, displayed alterations of CREB $F(2,30) = 201.84$, $p < 0.001$ and pCREB in the hippocampus $F(2,30) = 19.81$, $p < 0.001$ in a pattern similar to that of memory changes. Post hoc Newman–Kuels tests indicated that CREB and pCREB levels decreased in the hippocampus of HFD-STZ treated diabetic rats compared with control group (both $p < 0.05$); this was reversed by repeated treatment with rolipram for 23 days (both $p < 0.05$) (Fig. 3).

3.4. Regulation of BDNF and Arc protein

One-way ANOVA revealed significant changes in the levels of BDNF $F(2,30) = 115.92$, $p < 0.001$ and Arc protein $F(2,30) = 2291.71$, $p < 0.001$ in the hippocampus following the drug treatment. Post hoc Newman–Keuls tests indicates that BDNF were significantly decreased in the hippocampus of rats in DM group compared with the CON group ($p < 0.05$); this was reversed by repeated treatment with rolipram for 23 days ($p < 0.05$). Arc levels were significantly reduced in diabetic rats relative to the rats in the control group ($p < 0.05$), but were elevated in rats treated with rolipram compared with diabetic rats without drug intervention ($p < 0.05$) (Fig. 3).

4. Discussion

Physicians around the world have witnessed a growing number of older patients consulting for diabetes who also exhibit or complain about cognitive decline. Diabetes itself has been recognized as an independent risk factor for the development of cognitive impairment, which has been confirmed in several large prospective studies based on large populations with follow-up durations of up to 18 years [12,13]. In the present study, we successfully built the Type 2 Diabetes Mellitus rat model through high-fat diet and low-dose STZ administration, with the metabolic characteristics of early insulin resistance and later hyperglycemia, which is consistent with previous studies [14]. Our study found increased Aβ deposition in the hippocampus of HFD-STZ treated diabetic rats compared with the control group. We also found the learning and memory abilities, as indicated by Morris water maze tests, were decreased in diabetic rats compared with control rats. However, the exact relationship between diabetes and cognitive decline remains to be explored. Researchers emphasize the important role of the hippocampus in the consolidation of information from short-term memory to long-term memory and spatial navigation. Hippocampal damage will...
further underpin the progression and severity of brain disorders resulting in cognitive and behavioral impairment. Therefore, we chose to focus on the hippocampus in our current research.

Researchers have found that rolipram, working as a PDE4 inhibitor, could enhance memory and reverse memory deficits due to a variety of reasons from pharmacological to genetic [15,16]. In the present study, we administered a repeated dose of 0.5 mg/kg rather than a high dose of 3 mg/kg acute injection to avoid side effect of sedation, and gave animals one hour time to rest before starting the water maze tests as suggested in previous studies [17]. In our study, diabetic rats showed impaired cognitive ability compared with control group. After rolipram administration, memory and cognitive performance of diabetic rats were improved while levels of FBG and FSI as well as Aβ remained unchanged. Therefore, rolipram might not exert its effect through ameliorating the metabolism of diabetes or Aβ deposition. Rolipram has been shown to reverse learning and memory deficits in AD models at least in part via the CREB signaling [18]. Improvements in long-term memory require synthesis of mRNAs and proteins and CREB, working as a transcription factor, is of great importance in the switch of short-term memory to long-term memory [19]. We observed increased levels of CREB and phosphorylated CREB (pCREB) expression in hippocampus of rolipram-treated rats. The P-CREB/CREB ratio is a known index of CREB activation [8]. Our results indicate that the level of CREB activation is higher in diabetes than the other two groups. We suggest two reasonable explanations. Firstly, our results show significantly decreased expression of total CREB levels, pCREB probably increased due to negative feedback. Memory processing was impaired in diabetic rats, and it needs more phosphorylation of CREB to sustain this, however it is still inadequate. Meanwhile we found the phosphorylation of CREB in rolipram treatment group was slightly higher than the control group, although it showed no significant difference. This trend could be a supplementary explanation that the increased phosphorylation level of CREB in diabetic rats might due to a compensatory reaction or negative feedback, the expression of CREB increased in rolipram treated diabetic rats, thus high phosphorylation was not needed so much as diabetic rats. Secondly, we detected the pCREB proteins after the Morris water maze test, which is a learning process. The correlation between learning and activation of hippocampal CREB has been demonstrated [9]. However, as CREB and pCREB separately decreased in the diabetes group, and rolipram treatment can reverse this trend, we can still conclude that the imbalance of CREB/pCREB contributes to the pathological process of DACD.

There are lots of genes with cAMP-response element (CRE) sequences in their promoter regions [20]. Particular concern has been given to the regulation of BDNF, which has long been associated with synaptic plasticity and long-term memory [21]. BDNF belongs to the neurotrophin family and has important effects on maintaining the development, regeneration, existence and function of neurons. Previous studies have verified that strengthening synaptic transmission or neuronal excitability induces the modification of synaptic function mediated by BDNF [22]. A recent study found lower serum BDNF levels in patients with type 2 diabetes compared with the control group [23]. In our study, we observed that decreased hippocampal BDNF levels in diabetic rats. The observed reduction in BDNF in diabetic rats may be linked to the reduced performance of this group of rats in spatial working memory tasks. This was further confirmed by the increases in BDNF in rolipram-treated animals.

Arc/Arg3.1 is a unique immediate early gene (IEG). Previous studies have found that Arc is involved in the BDNF-mediated synapse modification and is induced by BDNF in both dose and time-dependent manners [24]. The behavioral phenotype in animals without the Arc gene is irregular, with the ability to form short-term memories but not long-term memories. Injection of antisense-oligodeoxynucleotide in the hippocampus blocks Arc and interferes with synaptic plasticity as well as hippocampus-dependent learning and memory capacity [25]. Researchers explored the relationship between Arc and Alzheimer disease (AD). Ginsberg found a significant decrease in the levels of Arc/Arg3.1 mRNA in the CA1 region of AD patients compared with controls [26]. However, autopsy studies in the brains of AD patients show a great increase in the expression of Arc protein [27]. The same contradictions also appeared on the animal models. An AD rat model expressing synthetic Aβ, Wang [28] and Chen [29] showed decreased Arc expression in cultured primary cortical neurons while Lacor [30] found an increase in Arc expression in cultured primary hippocampal neurons. Interestingly, Palop [31] observed the rise and fall of Arc expression in the hippocampus of mice aged 4–7 months. Although different experiments presented inconsistent results of Arc expression, we suggest that altered Arc expression interferes with the nervous system responses and physiological consolidation of behavioral experience and then participates in AD progression [32].

The pathogenesis of diabetes associated cognitive decline is complex, but recent studies have found that with AD similar synaptic damage and Aβ deposition [33–35], it may also have something to do with the expression of Arc/Arg3.1 protein. Our findings suggest that rolipram supplementation of diabetic rats results in a significant increase in hippocampal Arc/Arg3.1 protein levels as well as memory and cognitive ability, an event which appears to be linked to increased BDNF and CREB activity in the hippocampus. In agreement with this, previous studies have indicated that subchronic Rolipram delivery increased the basal rat hippocampal expression and phosphorylation of CREB, as well as the expression of Arc protein [9,22]. An exogenous BDNF-induced LTP model increased CREB activation and Arc mRNA and protein expression was simultaneously observed [36].

As discussed above, the goal of this research is to provide evidence in support of correlations between some known proteins and DACD centered on CREB from a new perspective of the pharmacological mechanism of rolipram. At present, we are unable to elaborate the exact mechanism underlying DACD. However, the goal was partly achieved because we confirmed that rolipram played a positive role in improving cognitive function in neurodegenerative disease such as DACD. This improvement was reached by increased levels of CREB, pCREB, BDNF and Arc/Arg3.1, indicating the vital function of the CREB/BDNF/Arc pathway in the development and progression of DACD and providing a theoretical basis for improving cognition in the development of clinical therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2015.09.023.

References


