PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2019,46(6):578~586
www.pibb.ac.cn

Nobiletin Rescues Cognitive Impairment in Naturally Aging Mice*

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Abstract With the aging of the world's population, the threat of age-related cognitive impairment is increasing. It is of great significance to study the mechanism of age-related cognitive impairment and find effective strategies to rescue it. Our previous studies have demonstrated that S-nitrosoglutathione reductase(GSNOR) was significantly increased in the hippocampus of aging mice and neuronal-specific GSNOR transgenic mice showed cognitive impairment in behavior tests. However, the mechanisms underlying this process remain unclear. Here, we found that CREB signaling was significantly decreased by GSNOR in cultured mice hippocampus neurons and GSNOR transgenic mice. Up regulation of the CREB signaling pathway by nobiletin rescued the cognitive impairment in GSNOR transgenic mice in Y-maze test. Nobiletin also showed protective effects on memory impairment in aging mice model. These results provide a new mechanism for the cognitive impairment in GSNOR transgenic mice and provide a new potential strategy to improve the cognitive function by nobiletin. GSNOR transgenic mice may be used as a screen model as age-related cognitive impairment.

Key words S-nitrosoglutathione reductase (GSNOR), nitric oxide (NO), CREB phosphorylation, nobiletin, age-related cognitive impairment
DOI: 10.16476/j.pibb.2019.0108

Population aging is one of the major global social problems today. The number of people suffering from cognitive impairment with increasing age is rising dramatically[1]. Age-related cognitive impairment seriously reduces the quality of life for elderly people and increases the economic burdens of care for families. There are as yet no effective drugs for age-associated cognitive impairment[2]. A better understanding of the underlying mechanisms and discovery of efficient enhancers for age-associated cognitive impairment is urgent[3].

S-nitrosoglutathione reductase (GSNOR) is a key protein S-nitrosation metabolic enzyme that is widely expressed and highly conserved from bacteria to humans[4]. Our laboratory studies have shown that GSNOR plays an important role in many neuronal physiological functions in *Drosophila*, including neurite growth[5], neuronal differentiation[6] and visual pattern memory[7]. In particular, our previous work demonstrated GSNOR is significantly increased in the hippocampus of aging mice and neuronal-specific GSNOR overexpression mice show cognitive impairment in behavior tests. Knockout of GSNOR rescues age-related cognitive impairment[8]. However, detailed knowledge of how GSNOR regulates age-related cognitive impairment is still lacking. Discovering this mechanism can provide new insight

* This work was supported by grants from The National Key Research and Development Program of China (2017YFA0504000) and The National Natural Science Foundation of China (31570857, 91849203, 31800969).
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Received: May 14, 2019 Accepted: May 28, 2019

on how to solve age-related cognitive impairment problems.

It is known that CREB (cyclic AMP-dependent response element-binding protein) signaling linked to CREB-mediated transcription is crucial for a wide range of biologic processes[9]. CREB is a multifunctional transcription factor expressed in different regions of the brain and plays an essential role in cognitive function, including in synaptic efficacy and long-term potentiation. CREB regulates specific memory genes and is activated in the nucleus by phosphorylation at Ser133 by various protein kinases *via* cAMP and/or Ca^{2+}. Alterations in CREB signaling lead to cognitive impairment as observed in neurodegenerative diseases and normal aging[10]. *c-fos* is one of immediate early genes which is stimulated by CREB signaling in neurons. Induction of c-Fos in long-term neuronal plasticity plays an important role in learning and memory[11-12]. Nobiletin, a natural polyethoxylated flavone compound from *Citrus depressa*, is reported to increase the CREB-dependent signaling pathway in the mouse hippocampus and in cultured hippocampal neurons[13-14].

Here, we investigated if CREB signaling might be involved in GSNOR induced cognitive impairment and if nobiletin can recover the impairment. Using cultured primary hippocampal neuron, GSNOR neuronal-specific transgenic mice (GSNOR TG) and naturally aged mice as models, we detected the regulation of CREB signaling by GSNOR and the effect of nobiletin in cognitive function. Our findings show that CREB signaling is involved in GSNOR-induced cognitive impairment and that nobiletin can rescue the cognitive impairment both in GSNOR TG mice and naturally aged mice.

1 Materials and methods

1.1 Animals and treatment

GSNOR TG mice were generated as described[8] and compared with age-matched littermates. 18-month old naturally aged female CD-1 mice, acclimatized in an SPF barrier facility at the Institute of Biophysics, Chinese Academy of Science, were used in experiments. All experimental procedures involving animals were approved by the Institute of Biophysics Animal Center. Mice were randomly divided into two groups (6 per group), a vehicle group and a nobiletin group, nobiletin (30 mg/kg, i.p) or vehicle (DMSO) being administrated daily for at least seven days until all behavior tests were completed. The dose of nobiletin and administration procedures used were based on descriptions in the published literature[15].

1.2 Behavioral tests

All behavioral tests were performed at approximately the same time of day (lights on from 9:00 am to 6:00 pm). Mice were habituated with a standard 12 h light/12 h dark cycle. Data are presented as means ± SEM.

1.3 Open field test

Mice was gently placed in a clear open chamber (40 cm×40 cm×40 cm), and their locomotor activity was monitored automatically for 20 min. Traveled in the open field was analyzed.

1.4 Y−maze test

The maze consisted of three equally-sized arms arranged at 120 angels from each other. Each mouse was gently placed at one fixed arm and allowed to freely explore the maze for 8 min, the sequence and the number of entries into each arm being recorded, one consecutive sequence being defined as an alternation. The percentage of alternation was defined as follows: % = [(number of alternations)/ (total arm enteries−2)]×100%. The total number of arm entries was determined as locomotor activity.

1.5 Contextual fear−conditioning test

During the training session, mice were placed in a chamber with a metal floor, and were allowed to explore freely for the first 2 min. An electric shock (0.5 mA, 100 V, 2 s) was then delivered to their feet 5 times at interval of 15 s. The mice were then allowed to move freely again in the chamber for an additional 1 min. The fear-conditioning memory was performed after 24 h. The freezing response was measured by placing the mice back into the conditioned chamber without stimulus for 5 min.

1.6 Cell cultures and transfection

Primary hippocampal neurons were prepared as described[16]. Briefly, hippocampal neurons were isolated from embryos of 18-day pregnant mice and quickly dissociated with 0.25% trypsin. Hippocampus neurons were collected according to standard methods and plated on dishes coated with poly-L-lysine (Sigma). Neuronal cells were cultured in Neurobasal Medium (Invitrogen, 21103-049)

containing B-27 supplement (Invitrogen, 17504-044) and 2 mmol/L L-glutamine (Invitrogen, 35050-061). Cells were transfected with Lipofectamine 2000 (Life Technologies) or lent virus according to the manufacturers' instructions.

1.7 Western blotting

Hippocampal neurons were collected and tissues were homogenized in ice-cold lysis buffer to extract proteins. Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore). Membranes were blocked with 5% skimmed milk in TBS containing 0.05% Tween-20 for 2 h, and incubated with the indicated antibody for 2 h at room temperature or overnight 4℃, followed by incubation with peroxidase-conjugated anti-rabbit or mouse IgG (Santa Cruz) for 2 h. Protein bands were analyzed quantitatively using imaging software (BioRad).

1.8 Antibodies and regents

The antibodies used were as follows: β-Actin from Santa Cruz Biotechnology; PSD95, GluR1 from Cell Signaling Technology. GSNOR inhibitor C3 was purchased from ChemDiv[17]. All chemical reagents were obtained from Sigma-Aldrich unless otherwise indicated.

1.9 Data analysis

Two-tailed Student's *t* tests were used for two-group comparisons. ANOVA and appropriate post hoc analyses were used for comparisons of more than two groups, $P<0.05$ was considered statistically significant.

2 Results

2.1 CREB signaling is down-regulated by GSNOR

We detected p-CREB and CREB expression in the hippocampus of GSNOR neuronal specific transgenic mice by Western blotting. Results showed that levels of p-CREB were lower in GSNOR TG mice compared to WT mice (Figure 1a, b). When we measured *c-fos* mRNA levels (downstream of CREB signaling), by real-time PCR in the hippocampus of GSNOR TG mice, we found that *c-fos* mRNA levels indeed decreased in GSNOR TG mice compared to WT mice (Figure 1c). In order to verify the regulation of GSNOR at the c-Fos level, we examined the effect of induction of nitric oxide (NO) with L-arginine treatment (NO synthesis substrate) on c-Fos protein levels in primary cultured hippocampal neurons. Results showed that overexpression of GSNOR inhibited the c-Fos level up-regulation induced by L-arginine (Figure 1d, e). These results show that GSNOR down-regulates hippocampal CREB signaling in mice and primary cultured neurons.

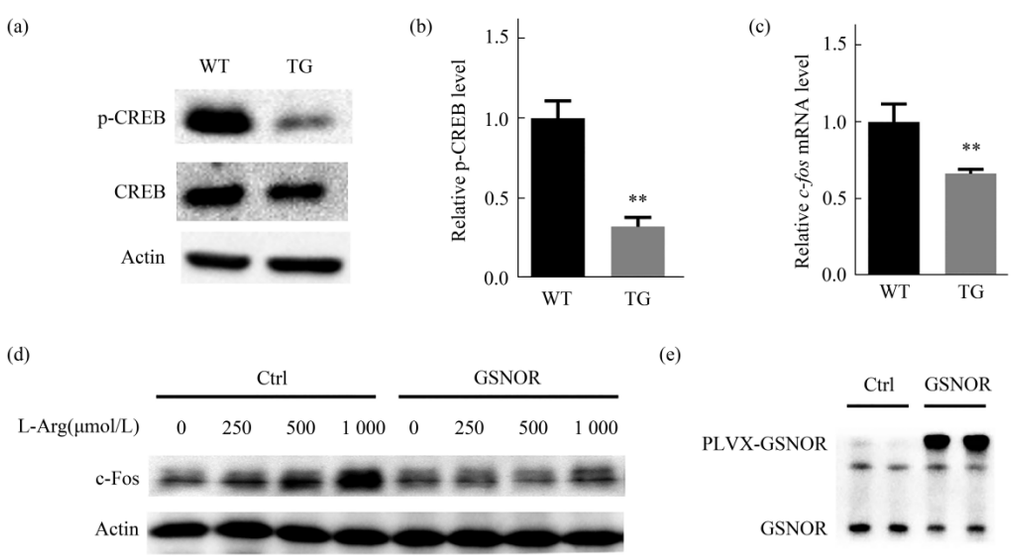

Fig. 1　CREB signaling in neuronal specific GSNOR transgenic mice and primary cultured hippocampal neuron

(a) p-CREB (S133) level in the hippocampus of GSNOR TG mice. (b) Quantitation of p-CREB level in GSNOR TG mice hippocampus (n=3). (c) Relative *c-fos* mRNA level in GSNOR TG mice hippocampus. (d) c-Fos expression level in primary hippocampal neuron transfected by PLVX or PLVX-GSNOR, and then treated by different concentration L-arginine for 15 min. (e) GSNOR expression level in primary hippocampal neuron transfected by PLVX or PLVX-GSNOR to indicated GSNOR overexpression efficiency.

2.2 Nobiletin rescues GSNOR TG mouse cognitive impairment by increasing CREB signaling

In order to explore whether CREB signaling is the mechanism of GSNOR-induced cognitive impairment, we used nobiletin (chemical structure in Figure 2a), a CREB signaling activator, to rescue GSNOR TG mouse cognitive impairment[14]. In the Y-maze test, intraperitoneal injection of nobiletin into mice did not affect the total arm entry number (Figure 2b). Consistent with previous results[8], the alternation percentage of GSNOR TG mice injected with DMSO solvent was significantly lower than WT mice. However, after injection with nobiletin for 7 days, the alternation percentage of GSNOR TG mice was not significantly different from WT mice (Figure 2c), indicating that up-regulation of CREB signaling by nobiletin rescued GSNOR TG mouse cognitive impairment. We thus concluded that CREB signaling deficit is the underlying molecular mechanism of GSNOR-induced cognitive impairment.

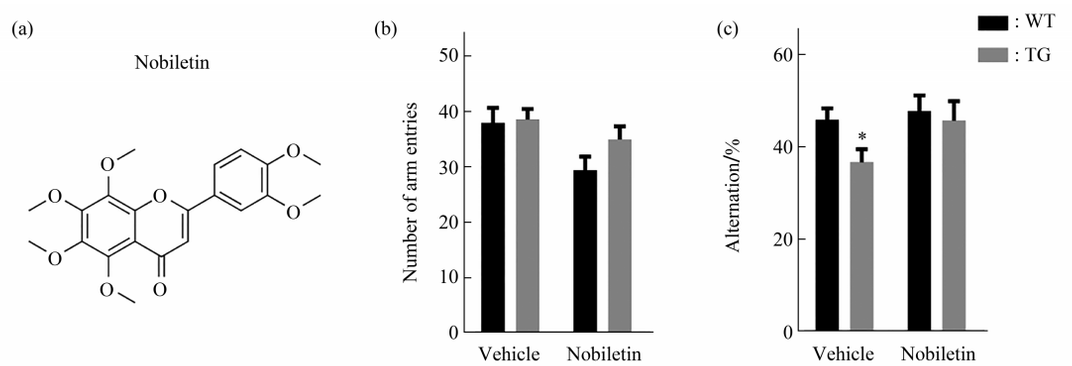

Fig. 2 Nobiletin rescued GSNOR TG mice cognitive impairment in behavior tests

（a）Nobiletin chemical structure.（b）Number of arm entries that mice explored the Y maze during 8 min. Nobiletin is intraperitoneal injected for seven days.（c）Percent alternations among Y maze arms during 8 min exploration. Nobiletin is intraperitoneal injected for seven days. TG （n=15），WT （n=16），TG nobiletin （n=13），WT nobiletin （n=14）.

2.3 Nobiletin rescues cognitive impairment in naturally aging mice

To test whether nobiletin can rescue cognitive impairment in naturally aging mice, we measured cognitive function in naturally aging mice (18 months) treated with nobiletin. We first compared naturally aging mice treated with nobiletin with mice injected with vehicle in an open field test. The nobiletin treatment group showed the same amount of exploratory and locomotor activity (as measured by the total distance traveled and percentage time in the center area) as the vehicle group in the open field (Figure 3a, b). We then subjected these two groups to two cognitive functional tasks, the Y-maze and fear conditioning test. In the Y-maze test, naturally aging mice treated with nobiletin showed a similar entry to the arms (Figure 3c), but more alternations than controls (Figure 3d), indicating that working memory was enhanced in nobiletin treated mice. In the fear-conditioning test, naturally aging mice treated with nobiletin showed higher percentage of freezing time in the contextual box than controls after 24 h retention (Figure 3e, f), but had a similar freezing percentage in the exploration and pre-end stage. When the freezing percentage was analyzed every minute, the freezing percentage of the nobiletin group at 2−3 min was significantly higher than that of the vehicle group. Our results thus indicate that nobiletin can rescue cognitive impairment in naturally aging mice.

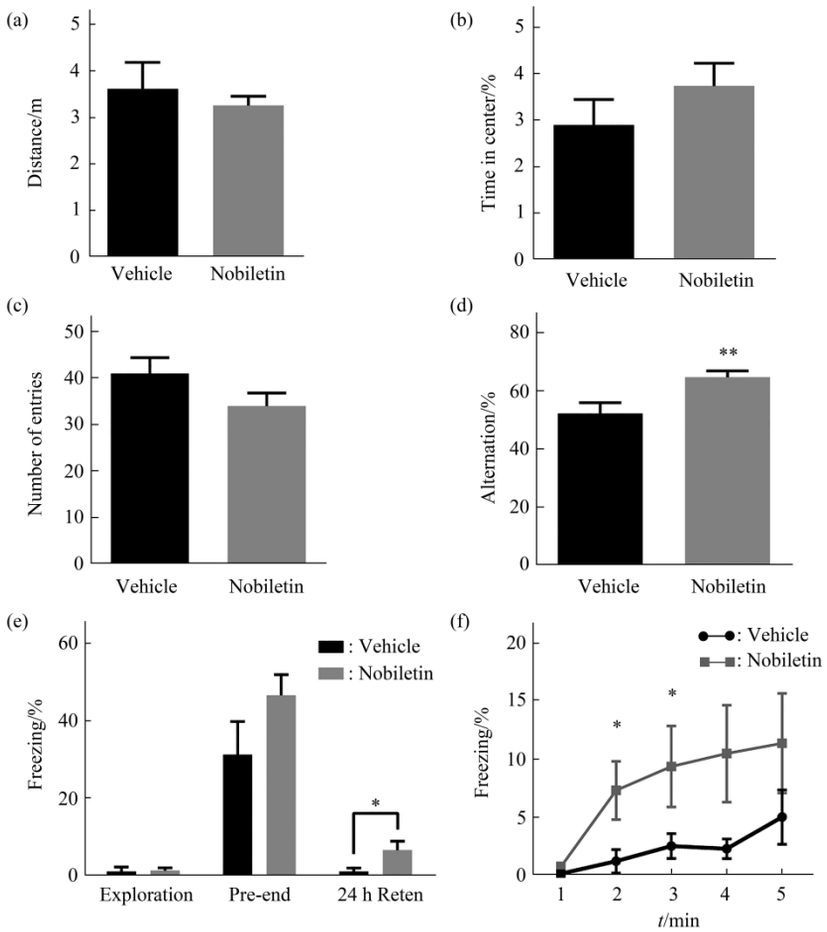

Fig. 3　Nobiletin rescued cognitive impairment in natural aging mice

（a，b）Distance and time percentage in center in the open field test of aging mice （16 – 18 month） treated with nobiletin and vehicle. （c） Number of arm entries that mice explored the Y-maze. （d） Percent alternations among Y-maze arms in exploration. （e） Percentage time of mice spent freezing at exploration， pre-end and 24 h after context training in a fear-conditioning task. （f） The freezing time percentage of every minute in the 24 h retention test.

2.4　CREB signaling was up−regulated by nobiletin in the hippocampus of naturally aging mice

To explore the mechanism by which nobiletin promoted recovery in naturally aging mice, we examined CREB signaling in the hippocampus. Western blottig showed that p-CREB expression levels were significantly increased in naturally aging mice treated with nobiletin (Figure 4a, b). In addition, we also detected other synaptic proteins that play an important role in synaptic plasticity and the stabilization of synaptic changes during long-term potentiation, including PSD95 (postsynaptic density protein 95) and GluR1 (AMPA receptor subunit). We found PSD95 and GluR1 expression levels were also significantly increased in naturally aging mice treated with nobiletin. Expression levels of c-Fos, a downstream indicator of p-CREB signaling, were also significantly increased in the nobiletin treatment group (Figure 4c, d). These experiments demonstrated that nobiletin rescues age-related cognitive impairment at least in part by up-regulating CREB signaling.

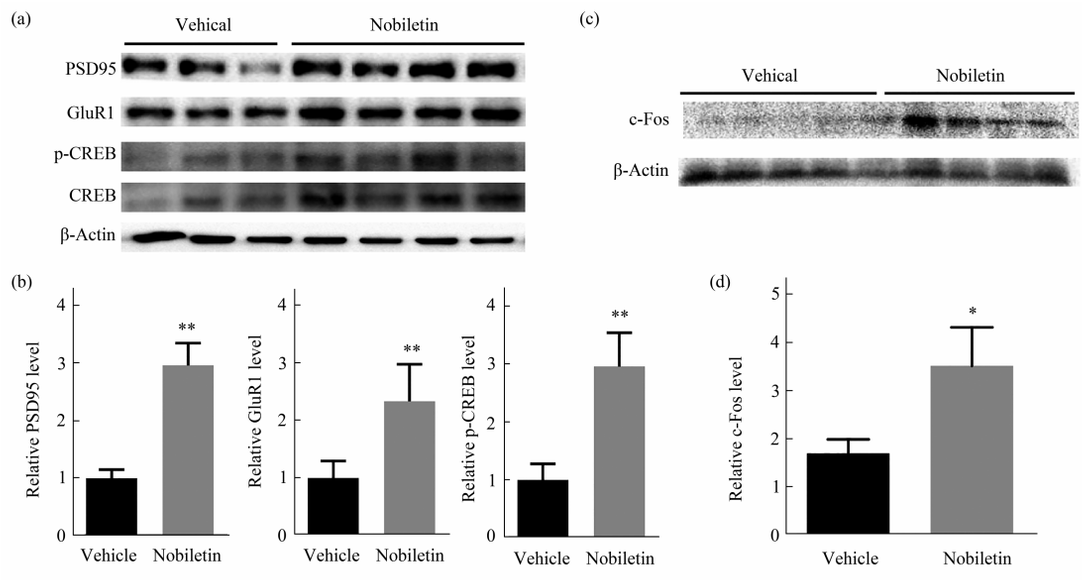

Fig. 4 Nobiletin up−regulated CREB signaling in natural aging mice

（a）Protein level of PSD95，GluR1，p-CREB （S133） and CREB in aging mice treated with vehicle and nobiletin. （b）Quantitation of PSD95，GluR1，and p-CREB level. （c）Protein level of c-Fos in aging mice treated with vehicle and nobiletin. （d）Quantitation of c-Fos level.

3 Discussion

Our research demonstrates that CREB signaling is down-regulated in the hippocampus and primary hippocampal neurons of GSNOR TG mice and that nobiletin increases CREB signaling and can rescue cognitive impairment. In addition, a further study demonstrated that nobiletin can also rescue cognitive impairment in naturally aging mice and confirmed that CREB signaling is also up-regulated in the hippocampus of naturally aging mice treated with nobiletin.

Our study is the first to report that CREB signaling is down-regulated by GSNOR, while nobiletin, as a CREB signaling activator, rescues cognitive impairment in GSNOR TG mice. These results indicate that down-regulation of CREB signaling is a new mechanism of GSNOR-induced cognitive impairment during aging. Nitric oxide is reported to up-regulated CREB signaling *via* S-nitrosylation of nuclear proteins that associate with CREB target genes [18]. Modification of protein S-nitrosylation is a crucial mechanism of nitric oxide, and dysregulation of protein S-nitrosylation contributes to many human pathologies[19]. GSNOR is a major enzyme involved in protein de-nitrosylation, metabolizing of GSNO, the main storage form of nitric oxide, which is widely expressed in eukaryotic organisms[20]. GSNOR and NOS double regulate the NO homeostasis of endogenous GSNO and S-nitrosylation[21]. Consistent with these reports, our results showed increased levels of NO can up-regulate c-Fos expression level and GSNOR rescued this effect (Figure 1d).

Here, we described the first evidence that nobiletin rescues cognitive impairment in naturally aging mice. In addition to phosphorylation of CREB, important synaptic proteins including PSD95 and GluR1 are regulated by nobiletin in the hippocampus of naturally aging mice. Previous research has shown that nobiletin exhibits memory-improving effects in various animal models of dementia including APP TG mice, MK-801-treated mice, ischemic mice and SAMP8 mice[22]. Our study has proved memory-improving effects of nobiletin in naturally aging mice. Nobiletin has very good bioavailability, important for drug to be effective in the nervous system. It can penetrate the Blood Brain Barrier (BBB) after peripheral administration[23] and 4'-demethylnobiletin, a major metabolite of nobiletin can also cross the BBB and have enhancer effects in cognitive impairment[24]. A previous report demonstrated that

one year oral-administration of nobiletin-rich *Citrus reticulata* peels extract prevented progression of cognitive impairment in donepezil pre-administered AD patients with no side effects[25]. Nobiletin maybe a potential safe and effective compound for drug development for the treatment of age-related memory impairment.

Our study is also the first to report that nobiletin can rescue cognitive impairment in both GSNOR TG mice and naturally aging mice. This suggests that we can perform initial screens of natural products using GSNOR TG mice as a cognitive impairment model and then verify effects in the naturally aging model. Using naturally aging mice as a model to screen for efficient enhancers of age-associated cognitive impairment requires a long time (at least 18 months) and much effort. Results presented here, along with those from a previous report[8], suggest that the neuronal-specific GSNOR TG mice constructed in our laboratory may be a good model for age-associated cognitive impairment.

In conclusion, our research demonstrates that increasing CREB signaling by nobiletin treatment rescues GSNOR induced cognitive impairment during aging. Neuronal specific GSNOR overexpression mice can be used as a screening model for age-related cognitive impairment and nobiletin is a compound that may have potential for drug development for age-related memory impairment. Further research on more signal pathways is required, along with natural product screening in GSNOR TG mice to find more ways to alleviate age-related cognitive impairment.

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川陈皮素改善衰老认知功能损伤的作用与机制*

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摘要　随着世界人口的老龄化，与年龄相关认知功能障碍的威胁越来越大.研究年龄相关认知功能损伤的发病机制及寻找有效的防治策略具有重要意义.我们之前的研究表明，衰老小鼠海马中S-亚硝基谷胱甘肽还原酶（S-nitrosoglutathione reductase，GSNOR）显著升高，神经元特异性高表达GSNOR转基因小鼠在行为学检测中表现出认知功能障碍.然而，其分子机制仍不清楚.在本研究中发现，CREB信号通路在GSNOR高表达转基因小鼠及原代培养小鼠海马神经元中均被GSNOR下调.在Y迷宫中检测表明，连续7 d腹腔注射CREB激活剂川陈皮素，能改善GSNOR过表达小鼠的认知损伤.进一步通过恐惧箱实验及Y迷宫测试研究川陈皮素对自然衰老小鼠认知功能的作用，发现川陈皮素能显著提高自然衰老小鼠在Y迷宫测试中的正确选择率以及在恐惧箱中的冻结时间，表明川陈皮素能显著改善衰老相关的认知功能.同样，川陈皮素上调了CREB磷酸化以及PSD95和GluR1的水平，表明CREB信号上调在改善自然衰老认知功能损伤中发挥了重要作用.本研究为衰老认知功能损伤机制及改善方法提供了新的依据，GSNOR转基因小鼠也可能成为一种新的认知功能损伤模型.

关键词　亚硝基谷胱甘肽还原酶，一氧化氮，磷酸化CREB，川陈皮素，衰老相关的认知损伤

中图分类号　Q2　　**DOI**：10.16476/j.pibb.2019.0108

* 国家重点研究研发项目（2017YFA0504000）和国家自然科学基金（31570857，91849203，31800969）资助项目.

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收稿日期：2019-05-14，接受日期：2019-05-28