Molecular mechanisms underlying the anti-obesity potential of prunetin, an O-methylated isoflavone

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ABSTRACT

Prunetin is an O-methylated isoflavone, which is a type of flavonoid. There are a limited number of reports detailing the biological activities of prunetin. Although an anti-inflammatory effect of prunetin has been reported in vitro, to our knowledge, there have been no reports on anti-adipogenic effects of prunetin in obese animals. The aims of this study were to determine whether prunetin suppresses high-fat diet (HFD)-induced adipogenesis in the liver and visceral adipose tissues of mice, and to explore the underlying mechanisms mediating the actions of prunetin. To this end, mice were fed a HFD for 10 weeks to induce obesity, and prunetin (10 μg/kg or 20 μg/kg) was administered in the last 3 weeks. Compared to saline-treated mice, mice treated with prunetin showed significantly reduced body weight gain, visceral fat pad weights, and plasma glucose levels. We found that prunetin significantly inhibited the HFD-induced upregulation of the expression of important adipogenic genes (PPARγ, C/EBPα, SREBP, Ap2, LPL adiponectin, and leptin), and suppressed HFD-mediated increase in expression of lipid metabolism-related genes (SREBP, PPARγ, LXR, and HMG-CoA) in the liver tissues. Furthermore, prunetin induced expression of adiponectin receptors 1 and 2 ( adipor1, adipor2 ), as well as that of AMP-activated protein kinase (AMPK) in the liver and adipose tissue. These results suggest that prunetin mediates anti-obesity/adipogenesis effects by suppressing obesity-related transcription through a feedback mechanism that regulates the expression of adiponectin, adipor1, adipor2, and AMPK.

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1. Introduction

An excessive increase in adipose tissue mass is defined as obesity [1]. Differentiation of the adipocytes, known as adipogenesis, is a complex process accompanied by changes in morphology, hormone sensitivity, and gene expression [2]. Adipogenesis is a differentiation process in which undifferentiated preadipocytes are converted to fully differentiated adipocytes. This process is regulated by a highly organized cascade of transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ), the CCAAT/enhancer-binding proteins α (C/EBPα), and thesterol regulatory element binding protein (SREBP) [3]. Furthermore, it is widely assumed that lipid metabolism, including lipogenesis, is primarily governed by a number of proteins including SREBP, liver × receptor (LXR), and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) [4,5].

Adiponectin is an adipokine that is secreted by adipocytes. Transcription of adiponectin is under the control of adipocyte transcription factors, including PPARγ and C/EBPα. It is known that secreted adiponectin circulates at relatively high concentrations in the bloodstream, and plays an important role in energy homeostasis and inflammation [6]. Some studies have reported that adiponectin gene expression and its circulating plasma levels are inversely correlated with adiposity [7,8]. Therefore, there have been significant efforts undertaken to identify the roles and the underlying mechanisms whereby adiponectin enhances energy metabolism.

Recently, 2 types of adiponectin receptor were identified. Adiponectin receptor 1 (adipoR1) is ubiquitously expressed, whereas adiponectin receptor 2 (adipoR2) is mainly expressed in the liver [9]. It has also been suggested that AMP-activated protein kinase (AMPK) may be activated by adiponectin and other downstream proteins involved in the adiponectin signaling
cascade [10,11]. Moreover, the activation of AMPK probably plays a critical role in mediating the stimulative effect of adiponectin on fatty acid oxidation [10,12].

A number of reports indicate that several flavonoids have anti-adipogenesis/anti-obesity effects [13–16]. Prunetin is an O-methylated isoflavone, which is a type of flavonoid. Therefore, we hypothesized that prunetin would also have the anti-adipogenesis/anti-obesity effects demonstrated by other flavonoids. Few studies have focused on the biological activities of prunetin except those on its anti-inflammatory effects, and there are no reports on the potential anti-adipogenic effects of prunetin in white adipose tissue (WAT) of obese animals. The present study was designed to investigate prunetin’s potential to suppress high-fat diet (HFD)-induced obesity, and to explore the possible transcriptional mechanisms involved in this attenuation.

2. Materials and methods

2.1. Reagents

Dimethyl sulfoxide (DMSO) was obtained from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Prunetin and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless otherwise specified in the text. Prunetin was dissolved in DMSO as a stock solution (70.4 μM) and stored at 4°C. For intraperitoneal (i.p.) injection, the prunetin stock solution was diluted in distilled water up to 3.52 μM or 7.04 μM.

2.2. Animals

Three-week-old male C57BL/6J mice (15–17 g) were purchased from Daehan Biolink (Daejeon, Republic of Korea). Animals were maintained under conditions that were in accordance with the guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the Institutional Animal Care Committee, Sangji University (Reg. No. 2013-3). Mice were adapted to the modified conditions in 4 weeks prior to the start of the experiment. Thereafter, animals were given free access to food and water for 10 weeks and were housed under a 12-h light/12-h dark cycle at a constant temperature of 22 ± 2°C and relative humidity of 55 ± 7% throughout the experiment. Mice were randomly distributed into 4 groups of 10 mice each: the normal diet (CON) group, high-fat diet (HF) group, and 2 additional treatment groups that were fed a high-fat diet (HFD) along with daily injections of either 10 μg/kg or 20 μg/kg i.p. prunetin (P10 and P20, respectively). Normal diet and HFD was obtained from Research Diets, Inc. (New Brunswick, NJ, USA). The caloric density and ingredient composition of each diet is shown in Table 1. Body weight and food intake were recorded every week. During the last 3 weeks, prunetin was administrated to the P10 and P20 groups, and PBS was administrated to the CON and HF groups. At the end of the 10-week period, all animals were fasted for 12 h. The following day, the mice were anesthetized with Zoletil (Virbac, France), and blood samples were collected by cardiac puncture. The liver tissue and visceral fat pads were excised, rinsed, weighed, and stored at −70°C pending further analysis.

2.3. Serum lipid and toxicity markers analysis

Serum concentrations of glucose and total cholesterol (TC) were determined by enzymatic methods using a commercially available assay kit (BioVision Research Products, Inc., CA). Serum concentrations of ALT and CPK were determined with the help of commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA). Serum concentration of BUN was quantified using commercial kit from Bioo Scientific Corp. (Austin, TX, USA).

2.4. Histological analysis

The liver tissue and visceral fat pads from representative mice in each group were fixed in 4% buffered formalin and embedded in paraffin, and sections of a thickness of 8 μm or 4 μm were cut. The sections were stained with hematoxylin and eosin (H&E) for the histological examination of fat droplets. Images were acquired using an SZX10 microscope (Olympus, Tokyo, Japan).

2.5. Quantitative Real-time PCR analysis

The liver and adipose tissues from each animal were homogenized, and total RNA was isolated using Easy-Blue® Reagent (Intron Biotechnology Inc., Gyeonggi-do, Republic of Korea) according to the manufacturer’s instructions. Total RNA was quantified using an Epoch® micro-volume spectrophotometer system (BioTek Instruments, Inc. Winooski, VT). Total RNA

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<tr>
<th>Table 1</th>
<th>Caloric content and ingredient composition of each diet.</th>
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<td>Normal diet</td>
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<td>Adipor2</td>
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<tr>
<td>AMPK</td>
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Experimental group. 

Effects (body weight) of prunetin vs. HF (A), visceral vs. period weight of group 1TBW gain (B), changes for values in each group. ### shown. T.-G. Ahn et al. Biochemical Pharmacology 85 (2013) 1525–1533

Fig. 1. Effects of prunetin injection on weight gain, FER, and fat-pad weights of experimental mice. (A), (B) Changes in total body weight, body weight gain (g), (C) FER, and (D) visceral fat-pad weight in each group are shown. The arrows indicate initiation of prunetin injection. Values are expressed as mean ± SEM of 10 mice per group. *p < 0.05 vs. CON group, **p < 0.001 vs. CON group, *p < 0.05 vs. HF group, ***p < 0.001 vs. HF group. 1TBW (total body weight). 2FER (food efficiency ratio) = (body weight gain for the experimental period (g)/food intake for the experimental period (g)) × 1000.

from the liver and visceral adipose tissues was each converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Reverse transcription was carried out using a thermocycler (Gene Amp<sup>®</sup> PCR system 9700, Applied Biosystems), where the program was set for initiation at 25 °C for 10 min, followed by incubation at 50 °C for 90 min, and a final step of 85 °C for an additional 5 min. The synthetic cDNA products were stored at −20 °C pending further analysis. Primers were obtained from Bioneer (Daejeon, Republic of Korea). All primer sequences and annealing temperatures are shown in Table 2. Real-time PCR analysis was carried out on a StepOnePlus<sup>®</sup> Real-time PCR system (Applied Biosystems, Foster City, CA) using SYBR<sup>®</sup> green master mix (Applied Biosystems, Foster City, CA). For the liver tissue, the reaction commenced with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 45 s. The final melting curve was 95 °C for 15 s, 55 °C for 1 min, and 95 °C for 15 s. For visceral adipose tissue, the reaction consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 45 s, and a final melting curve of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Gene expression was calculated according to the comparative threshold cycle (Ct) method (Applied Biosystems). Each value was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.6. Western blot analysis

The liver tissue and adipose tissue were homogenized in a commercial lysis buffer PRO-PREP<sup>®</sup> (Intron Biotechnology Inc., Gyeongi-do, Republic of Korea) and incubated for 25 min on ice to induce cell lysis. Tissue extracts were centrifuged at 13,000 rpm (4 °C) for 5 min, and supernatant transferred to a clean 1.5-mL
tube. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instruction (Bio-Rad, Hercules, CA). Aliquots of each protein sample (15 µg) were separated on a 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane. Membranes were blocked with 5% skim milk at 4 °C for 1 h. The membranes were incubated overnight with primary antibodies, which included the anti-PPARγ, anti-C/EBPα, anti-SREBP, or anti-β-actin (Santa Cruz, CA) antibody. Blots were washed 3 times with Tween-20/Tris-buffered saline (TTBS) followed by incubation with the corresponding secondary antibody (Santa Cruz, CA) for 1 h at room temperature. Blots were again washed 3 times with TTBS, and immunoreactive protein bands visualized using enhanced chemiluminescence (ECL) and X-ray film (Amersham Life Science, Buckinghamshire, UK).

2.7. Statistical analysis

All reported values are expressed as the mean ± SEM for 10 mice. Data were analyzed using one-way analysis of variance ANOVA, with Duncan’s multiple range tests or Dunnett’s test. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) statistical analysis software (Version 19.0).

3. Results

3.1. Prunetin reduces body weight gain, total fat mass, and liver weight in mice with HFD-induced obesity

Total body weight of mice in the HF group increased compared with that of mice in the CON group (11.12%, p < 0.01). Body weight gain in the HF group also increased compared with that in the CON group (17%, p < 0.05). In contrast to the HF group, prunetin injection suppressed body weight gain by 9.44% and 11.09% in the
P10 and P20 groups, respectively (Fig. 1A and B). During the experimental period, there were no significant differences in food and water intake in the HF group compared to the other treatment groups (data not shown). Importantly, the food efficiency ratio (FER) in the P10 and P20 groups was lower than that in the HF group (Fig. 1C). In the P10 group, the total fat weight (−42.28%) and weight of the epididymal (−49.72%) fat pads were all significantly lower than those in the HF group. Similarly, the epididymal (−50.31%) and total (−58.21%) fat pad weights in the P20 group were significantly lower than those in the HF group (Fig. 1D).

3.2. Effects of prunetin on serum lipid and toxicity markers

Plasma levels of glucose and TC in the HF group were significantly higher than those in any other group. Plasma concentrations of glucose were −17.21% in the P10 group and −19.14% in the P20 group compared to those in the HF group (Fig. 2A). The plasma levels of glucose decreased significantly in prunetin treated groups, but the plasma levels of TC do not. The levels in prunetin treated groups have a tendency to decrease. The TC concentrations decreased by 0.79% in the P10 group and 3.84% in the P20 group.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Fig. 4.** Effects of prunetin on the mRNA levels of lipid metabolism and adipogenesis in liver and adipose tissue each. (A) The expression of genes (SREBP, PPARγ, LXR, and HMG-CoA) involved in lipid metabolism in liver tissues, (B) expression of genes (PPARγ, C/EBPα, SREBP, LXR, LPL, adiponectin, aP2, and leptin) related to adipogenesis in epididymal adipose tissues were analyzed by quantitative Real-time PCR (A, B). Data were normalized to the GAPDH mRNA levels and then compared to CON group measurements, which were assigned a value of 1.0. Each bar represents the mean ± SEM of 3 independent experiments of the mRNA from 10 mice per each group. (C) The protein levels of SREBP and PPARγ in liver tissues were analyzed by Western blot. (D) The protein levels of PPARγ, C/EBPα, and SREBP in adipose tissues were analyzed by Western blot. All Western blot lanes were each two of CON, HF, P10 and P20 sequentially from left to right. *p < 0.05 vs. CON group, †p < 0.05 vs. HF group, ‡p < 0.01 vs. HF group, ***p < 0.001 vs. HF group.
in the P20 group compared to those in the HF group (Fig. 2B). In addition, concentrations of BUN, ALT, CK in prunetin-treated group were within normal range (Table 3).

3.3. Prunetin suppresses lipid droplet accumulation in the liver and visceral adipose tissue

Fig. 3 shows H&E staining of the liver and visceral adipose tissues. Lipid droplets in the representative liver tissue of the HF group appeared as small vacuoles within the liver cells (Fig. 3A). Enlargement of adipocytes in the visceral adipose tissue of the mice in the HF group was more pronounced than that in the visceral adipose tissue of the mice in the P10 and P20 groups. Fig. 3B and D clearly revealed that lipid accumulation in the liver and visceral adipose tissue was considerable in the HF group compared with the P10 and P20 groups. In the prunetin-treated groups, lipid accumulation decreased in a dose-dependent manner.

3.4. Prunetin suppresses expression of adipogenesis-related genes

To investigate whether the reduced adiposity in prunetin-treated mice was associated with molecular signaling by the genes involved in lipid metabolism and adipogenesis, we examined the expression levels of several genes that are key transcription factors for lipid metabolism and adipogenesis in the liver and visceral adipose tissues. In the liver tissue, the expression of SREBP, PPARγ, LXR, and HMG-CoA mRNAs significantly decreased in a dose-dependent manner in the prunetin-treated groups compared to the HF group (Fig. 4A). The expression of several genes related to adipogenesis in visceral adipose tissue is shown in Fig. 4B. The mRNA levels of the adipogenesis/obesity-related transcription factors (PPARγ, C/EBPα, SREBP, and LXR), and their target genes (LPL, aP2, and leptin) were significantly lower, while the mRNA level of adiponectin was higher than that observed in the HF group. Importantly, the decrease in the expression of PPARγ, C/EBPα, SREBP, LXR, LPL, aP2, and leptin in visceral adipose tissue and increase in adiponectin expression was dependent on the dose of prunetin. Furthermore, results from Western blot analysis confirmed that protein levels of PPARγ, C/EBPα, and SREBP were down-regulated in prunetin-treated mice. These proteins were significantly suppressed in the P20 group (Fig. 4D and E).

3.5. Prunetin induces expression of adiponR1, adiponR2, and AMPK

Prunetin stimulated the expression of adiponR1 in the liver tissue and adipose tissues. We checked another adiponectin receptor, adiponR2, but it was not changed in liver tissues. Furthermore, prunetin induced AMP-activated protein kinase (AMPK) expression in the liver and adipose tissues (Fig. 5). The relative adiponectin expression ratio (RAER) was also increased significantly in a dose-dependent manner by prunetin.

4. Discussion

In the present study, we first induced obesity by feeding a HF to mice for 7 weeks, followed by administration of prunetin for 3 weeks to obese mice maintained on the HFD. Compared to the mice in the HF group, the prunetin-treated mice had significantly reduced body weight gains and reduced total fat pads weight (Fig. 1A and B). In agreement with these results, the size of adipocytes significantly reduced in the prunetin-treated groups compared with that in the HF group (Fig. 3D and C). Importantly, these beneficial effects of prunetin on body and fat weights did not result from decreased food intake, as there was no difference in the amount of food ingested per mouse between the prunetin-treated group and the HF group.

However, when we analyzed the results, the body weight of P10 and P20 groups in the third week was suddenly decreased. The reason is not clear, but food intake amount in this week found to be sharply decreased compared with other weeks. The sudden decrease of body weight in third week might probably be influenced by the less food intake. There are some reports that the decline of food intake and decrease of body weight may be caused by some metabolic changes which are able to happen in animal experiments [17,18].

FER means how much efficiently HFD induce weight gain. Therefore, if experiments were proceeded on the same conditions only except the type of diet, HFD-fed group should be the higher levels of FER than any other group and CON group should be lower levels of FER compared with HF group. Because HFD has higher caloric density than normal diet has. The most important thing is
Fig. 6. The possible mechanism of prunetin action. Injection of prunetin significantly reverse the expression of adipOR1, AMPK, adiponectin, PPARγ, C/EBPα, SREBP, and their target genes in white adipose tissue. Prunetin also recovered expression of adipOR1 and AMPK and suppress SREBP, LXR, and HMG-CoA in liver tissues. It seems like that there would be feedback loop action which attenuates obesity/adipogenesis-related genes expressions among adiponectin, adipOR1, and AMPK.

This: if prunetin have suppressing effect on weight gain, a level of FER in prunetin-treated group should be lower than that in HFD group. In our study, the FER was lower in P10 and P20 groups than that in the HF group (Fig. 1D). It means injections of prunetin suppressed body weight gain induced by HFD. In addition, the FER level of P20 was lower than that of P10 group. Therefore, it is thought that injecting high concentration of prunetin has more favorable effects on weight gain than low concentration of prunetin has.

In this study, we used DMSO as a solvent of prunetin. The concentration of DMSO used in working solution had no effect on experimental animals and experimental results, which is consistent with previous reports [19,20]. Furthermore, toxicity makers such as ALT, BUN, and CK (markers of liver, kidney, and cardio-toxicity) were within normal range and we couldn’t find any significant increases of toxicity markers in prunetin-treated group according our blood serum analysis (Table 3).

Compared with the HF group, prunetin decreased lipid accumulations in the liver tissue (Fig. 3A and C) and transcription factors for lipid metabolism and adipogenesis in the liver tissue. These data suggest that prunetin may be useful for treatment of non-alcoholic fatty liver diseases [21,22].

Anti-obesity and anti-adipogenic effects of prunetin were further confirmed by the significant reversal of expression of various adipogenesis obesity-related genes (Fig. 4B). PPARγ and C/EBPα are widely accepted as few of the most critical transcription factors in adipogenesis and obesity [23–25]. The role of SREBP and LXR in the regulation of obesity and adipogenesis has been established in previously published studies [5,26,27]. A dramatic increase in the expression of PPARγ induces C/EBPα expression, promoting the differentiation of preadipocytes to mature and expanded adipocytes. While the induction of expression of C/EBPα depends on the expression of PPARγ, the increase in C/EBPα expression also provides positive feedback with regard to expression of not only PPARγ but also various other genes required for adipogenesis and obesity. This interaction between PPARγ and C/EBPα expression is assumed to be essential for the progression of adipogenesis [2]. Interestingly, while PPARγ is known to be specifically up-regulated by HFD, other genes that are involved in fatty acid oxidation and fatty acid synthesis failed to be regulated by HFD [28]. Earlier studies reported that PPARγ expression was closely associated with fatty liver or liver steatosis [29–31]. LXR, another marker for adipogenesis, stimulates adipocyte differentiation through induction of the expression of several adipogenic genes, especially PPARγ [5].

It is well known that the liver plays a central role in lipid metabolism. SREBP, LXR, and HMG-CoA all play critical roles in lipid metabolism, mediating lipogenic activities, such as accumulation of lipid droplets and cholesterol homeostasis. SREBP, LXR, and HMG-CoA also support anti-adipogenic effects by suppressing obesity-related genes such as PPARγ and C/EBPα [32,33].

The expression pattern of adiponectin in visceral adipocytes appeared distinct from the other genes that we investigated. As a result of our observations, we predicted that prunetin would stimulate adiponectin expression, and promote additional cellular interactions that would support the recovery of adiponectin expression. To explore possible mechanisms involving the anti-obesity effects of prunetin, we examined the expression of adipOR1, adipOR2, and AMPK, genes known to be closely involved in the regulation of adiponectin expression and function. AMPK has been described as a master regulator of lipid metabolism and glucose metabolism. It is generally assumed that AMPK can switch over from anabolic pathways to catabolic pathways by suppressing the expression of genes involved in adipogenesis, such as PPARγ. In short, AMPK acts like a metabolic sensor to regulate lipid and glucose metabolism. Activation of AMPK eventually results in diverse metabolic changes [34–36]. Adiponectin is primarily synthesized by and secreted from white adipocytes, acting as an endocrine hormone [1,37,38]. It is also known that decreased expression and plasma levels of adiponectin are paradoxically and inversely correlated with a number of metabolic disorders such as obesity, insulin-resistant type 2 diabetes, and adipogenesis. For example, the expression levels of adiponectin have been found to decrease in obesity and type 2 diabetes [7,39,40]. Recently, 2 types of adiponectin receptor, called adipOR1 and adipOR2, have been identified. The receptor adipOR1 is widely expressed in mouse, whereas adipOR2 is expressed primarily in the liver. These 2 receptors serve as signals transducers for diverse types of
adiponectin. Following activation by adiponectin, adiponR1 and adiponR2 mediate fatty acid oxidation, glucose uptake, and increase in AMPK [9,41,42]. Some investigators have also reported that adiponectin stimulated AMPK [10,43]. Our results from gene expression studies indicated that the expression of adiponR1 and AMPK genes in the liver and visceral tissue was significantly upregulated in the prunetin-treated animals compared with that in the HF group, and that effects of prunetin were dose-dependent (Fig. 5). It may also be possible that adiponectin was more efficiently expressed in the prunetin-treated group than in the HF group because RAER was significantly higher in the HF group (Fig. 5C). Taking into account all of these observations, we hypothesized that prunetin may have a suppressive effect on obesity through some kind of feedback loop (Fig. 6), and that adiponectin resistance resulted from a decrease in adiponectin receptor expression. Alternatively, decreased adiponectin expression itself may cause not only functional failure of adiponectin but also insulin resistance. These physiological circumstances could be critical mediators of obesity.

To summarize, prunetin efficiently suppressed adiponectin and obesity in HFD-induced obese mice. The mechanisms underlying the anti-obesity effect of prunetin appear to involve reinstatement of the expression of adiponectin through induction of adiponR1 and AMPK expression, together with suppression of several adipogenic transcription factors such as PPARγ, C/EBPα, and SREBP. In conclusion, our study clearly demonstrated an anti-obesity effect of prunetin, and suggested a possible mechanism involving transcriptional regulation. However, the precise mechanisms and molecules mediating the effects of prunetin need to be investigated further and more precisely.

Conflict of interest

There were no other relationships/conditions/circumstances that present a potential conflict of interest.

Acknowledgement

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References


