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Research Paper

Luteolin prevents uric acid-induced pancreatic β-cell dysfunction

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Abstract

Elevated uric acid causes direct injury to pancreatic β-cells. In this study, we examined the effects of luteolin, an important antioxidant, on uric acid-induced β-cell dysfunction. We first evaluated the effect of luteolin on nitric oxide (NO) formation in uric acid-stimulated Min6 cells using the Griess method. Next, we performed transient transfection and reporter assays to measure transcriptional activity of nuclear factor (NF)-κB. Western blotting assays were also performed to assess the effect of luteolin on the expression of MafA and inducible NO synthase (iNOS) in uric acid-treated cells. Finally, we evaluated the effect of luteolin on uric acid-induced inhibition of glucose-stimulated insulin secretion (GSIS) in Min6 cells and freshly isolated mouse pancreatic islets. We found that luteolin significantly inhibited uric acid-induced NO production, which was well correlated with reduced expression of iNOS mRNA and protein. Furthermore, decreased activity of NF-κB was implicated in inhibition by luteolin of increased iNOS expression induced by uric acid. Besides, luteolin significantly increased MafA expression in Min6 cells exposed to uric acid, which was reversed by overexpression of iNOS. Moreover, luteolin prevented uric acid-induced inhibition of GSIS in both Min6 cells and mouse islets. In conclusion, luteolin protects pancreatic β-cells from uric acid-induced dysfunction and may confer benefit on the protection of pancreatic β-cells in hyperuricemia-associated diabetes.

Keywords: luteolin, uric acid, nitric oxide, nuclear factor (NF)-κB, MafA

INTRODUCTION

The association between elevated serum uric acid level and type 2 diabetes mellitus (T2D) has been established for years[1,2]. Several large epidemiological studies also provide evidence that hyperuricemic patients are susceptible to T2D[3]. In hyperuricemia, elevated uric acid not only induces insulin resistance in peripheral tissues but also exerts a strongly negative effect on pancreatic β-cell survival and insulin secretion[4,5]. The underlying mechanism of uric acid-induced dysfunction of pancreatic β-cells has not been well elucidated. Recently, we found that uric acid activates the NF-κB signaling pathway through IκBα phosphorylation, resulting in upregulated inducible nitric oxide synthase (iNOS) expression and excessive

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nitric oxide (NO) production. The expression of MafA is also inhibited by elevated NO production, which leads to pancreatic β-cell dysfunction[6]. We speculated that suppression of the activation of the NF-κB signaling pathway may confer benefit on the prevention of uric acid-induced dysfunction of pancreatic β-cells.

Luteolin, a common flavonoid present in many types of plants, has been proved to be effective in the treatment of various diseases like hypertension, inflammatory disorders and cancer[7]. As an antioxidant, luteolin significantly attenuates increased reactive oxygen species (ROS) production and prevents decreases in the number of mitochondria, catalase and glutathione activities in ROS-insulted primary neurons[8]. Moreover, luteolin was found to improve insulin sensitivity in peripheral tissues as well as increase insulin secretion in pancreatic β-cells[9,10]. Kim et al. reported that luteolin suppressed cytotoxicity in pancreatic β-cells and attenuated cytokine-induced decrease in glucose-stimulated insulin secretion (GSIS) in islets through inhibition of NF-κB activity[11]. However, the effect of luteolin on uric acid-induced dysfunction in pancreatic β-cells has not been studied yet. In this study, we evaluated the effects of luteolin on uric acid-stimulated pancreatic β-cells usign an insulin-secreting cell line Min6 and primary cultured mouse islets. We found that luteolin decreased iNOS expression and NO production by inhibiting NF-κB activity, and led to up-regulation of MafA expression in uric acid-stimulated pancreatic β-cells, which reversed uric acid-induced decrease of insulin secretion.

MATERIALS AND METHODS

Reagents and cell culture

All chemical reagents were purchased from Sigma (St. Louis, MO, US) unless otherwise stated. Min6 cells were maintained in 25 mmol/L glucose Dulbecco′s Modified Eagle′s Medium (DMEM, Hyclone, Logan, UT, USA), and supplemented with 10% fetal bovine serum (FBS), 50 μmol/L β-mercaptoethanol, 100 U/mL penicillin and 0.1 mg/mL streptomycin in 5% CO_2 at 37°C. For cell treatments, uric acid solution was prepared as previously described[12]. Antibodies to iNOS and MafA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The detergent compatible (DC) protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Recombinant human uric acid was purchased from R&D System (Minneapolis, MN, USA).

Islet isolation

Animal experiments were approved by the Experimental Animal Ethics Committee at the authors′ affiliated institution. Collagenase digestion was used to isolate islets from 6-8-week-old male C57BL mice (Slac, Shanghai Experimental Animal Center, Chinese Academy of Science, Shanghai, China) as previously described[13]. Isolated islets were then cultured in a complete culture medium RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Hyclone), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 11.1 mmol/L glucose in 5% CO_2 at 37°C overnight. The animal study was carried out according to the instittutional and state guidelines on the experimental use of animals.

Glucose-stimulated insulin secretion assay (GSIS)

Min6 insulin secreting cell line or primary cultured mouse islets (10 islets per well) were planted in 24-well plates and pretreated with or without luteolin for 2 hours, and then incubated with uric acid for 24 hours. After treatment, the cells/islets were incubated for 1 hour in glucose-free Krebs-Ringer bicarbonate (KRB) buffer (115 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 20 mmol/L $NaHCO_3$, 16 mmol/L HEPES, 2.56 mmol/L $CaCl_2$ and 0.2% bovine serum albumin (BSA)), and then the cells were treated for 1 hour in KRB buffer with low (3.3 mmol/L) or high (16.7 mmol/L) concentrations of glucose[14]. Supernatants were collected and insulin concentrations were determined using radioimmunoassay as previously described[15].

RT-PCR

After treatment, total RNA was extracted from Min6 cells using total RNA extraction reagent (Vazyme Biotech, Nanjing, China) following the manufacturer′s instructions. cDNA was synthesized from total RNA (1 μg) using PrimeScript® RT reagent kit (Takara, Dalian, China) according to the manufacturer′s protocol. RT-PCR was performed using the SYBR® Premix Ex Taq™ system (Takara, Dalian, China). The *iNOS* mRNA expression was determined using RT-PCR analysis. Primers used to identify *iNOS* were: forward, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-AGC-3′ and reverse, 5′-GGCTGTCAGAGCCTC-GTGGCTTTGG-3′. β-actin was used as an input control: forward, 5′-GCAAGTGCTTCTAGGCGGAC-3′ and reverse, 5′- AAGAAAGGGTGTAAAACGC-AGC -3′.

Western blotting assays

After treatment, Min6 cells were washed twice in prechilled phosphate buffed saline (PBS), and then lysed in RIPA buffer (Vazyme Biotech, Nanjing, China). Western blotting assays were performed as previously described[6]. Immunoreactivity was detected by ECL reagents (MultiSciences Biotech, Hangzhou, China) and observed by a Bio-Rad ChemiDoc™ XRS+ Universal Hood II machine.

Nitrite assays

Min6 cells were placed in 48-well dishes for 24 hours, and pretreated with or without luteolin for 2 hours, and then incubated with uric acid for 24 hours. The medium was sampled for NO determination using a Griess assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Each experiment was performed in triplicate and repeated 3 times independently.

Construction of expression plasmids

To construct the iNOS expression plasmid, coding sequences (CDS) for these factors were PCR-amplified from the mouse full-length cDNA based on the sequences from GeneBank. The following primers (including the sites of restriction enzymes) were synthesized: forward, 5′-AAGGATCCATGGCTTGCCCCTGGAAGTTTCTCTTC -3, reverse, 5′-AAGAGCTCTCAGAGCCTCGTGGCTTTGGGCTCCTC-3′. The PCR was at 94°C for 5 minutes (1 cycle), 95°C for 10 seconds, 58°C for 15 seconds, and 72°C for 2 minutes (30 cycles) with a final extension of 10 minutes at 72°C. The PCR products were resolved on agarose gel, and the correct sized fragments were recovered using a DNA extraction kit. The recovered fragments and pcDNA3.1 vector were digested with the corresponding restriction enzymes to construct the iNOS expression plasmids (pcDNA3.1-iNOS). All sequences were confirmed by automated DNA sequencing.

Transient transfection and luciferase reporter assays

To investigate the activity of NF-κB, the NF-κB luciferase reporter construct was performed in Min6 cells. A plasmid containing the *β-galactosidase* gene driven by the cytomegalovirus (CMV) promoter (Clontech Laboratories, Palo Alto, CA, USA) was used as an internal control. Min6 cells were transfected with plasmids containing the NF-κB luciferase reporter construct and β-galactosidase by Lipofectamine plus transfection kit (Invitrogen) according to the manufacturer's instructions. 24 hours after transfection, the cells were pretreated with or without luteolin for 2 hours, followed by incubation of uric acid for another 24 hours. After the cells were lysed using lysis buffer, luciferase activity was determined as previously described[16].

MTT assays

Cell viability was determined using MTT (Sigma) assays. Briefly, Min6 cells were seeded in 96-well plates at a density of 1×10^4 cells per well. The cells were pretreated with or without luteolin for 2 hours followed by incubation with uric acid for 24 hours. Then, each well was supplemented with 0.5 mg/mL MTT and incubated for 4 hours at 37°C. The medium was then removed, and 150 μL dimethysulfoxide was added to solubilize MTT formazan. Optical density was read at 490 nm to calculate cell viability.

Statistical analysis

All statistical analysis was performed by SPSS 11.0 software (USA). Student's *t* test between 2 groups, or ANOVA in multiple groups followed by Tukey's multiple-comparison posttest was used to compare significant difference. Results were presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

RESULTS

Luteolin attenuated uric acid-stimulated iNOS expression and NO formation in pancreatic β-cells

Our previous study showed that uric acid-mediated destruction of β-cells was related to an increase of NO. Min6 cells in normal state released 0.91 ± 0.48 μmol/L of NO, whereas cells treated with uric acid markedly increased NO production[6]. We found that 10 μmol/L luteolin did not affect NO production in normal cultured Min6 cells. However, this compound significantly inhibited uric acid-induced NO production (***Fig. 1A***).

iNOS plays a critical role in NO production[17]. To determine whether luteolin inhibited NO production via suppression of iNOS expression, we investigated the mRNA and protein expression levels of iNOS by real-time PCR and Western blotting assays, respectively. As shown in ***Fig. 1B***, *iNOS* mRNA expression was significantly increased in uric acid-treated Min6 cells; however, luteolin attenuated uric acid-induced alteration of *iNOS* mRNA. In accordance with the results of mRNA determination, luteolin significantly inhibited uric acid-induced iNOS protein expression

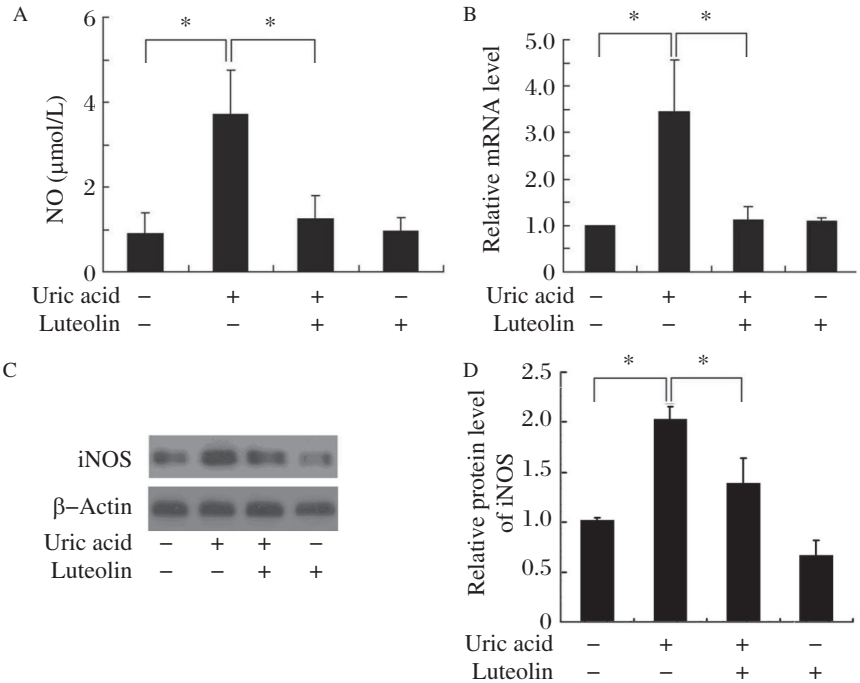

Fig. 1 **Luteolin inhibits uric acid-stimulated NO formation and iNOS expression.** A: After Min6 cells were treated with or without 10 μmol/L luteolin in the presence/absence of 5 mg/dL uric acid for 24 hours, NO formation in medium was measured. $n = 4$. B: After Min6 cells were treated as described above, the expression of *iNOS* mRNA of cells was determined by real time PCR. $n = 4$. C: The expression level of iNOS protein of differently treated Min6 cells were measured by Western blotting assays. D: All blots were repeated for 3 times. The densitometric analysis of protein bands were performed with Quality One software. * $P < 0.05$ indicates significant difference compared to the uric acid-treated alone group (A, $P = 0.008$ and 0.011, respectively; B, $P = 0.003$ and 0.003; D, $P < 0.001$ and $= 0.012$, respectively). NO: nitric oxide; iNOS: inducible NO synthase

in Min6 cells (***Fig. 1C*** and ***1D***). The results suggested that luteolin mediated a significant inhibition of iNOS expression in Min6 cells exposed to uric acid.

Luteolin inhibited uric acid-induced activation of the NF-κB pathway

Following exposure to deleterious factors, including inflammatory cytokines, high glucose and elevated level of free fatty acid, activation of the NF-κB signaling pathway has been reported as a key event promoting death and dysfunction of pancreatic islet cells[18]. Therefore, we investigated whether luteolin could reverse the activation of NF-κB induced by uric acid. When Min6 cells were stimulated with uric acid (5 mg/dL), the transcriptional activity of NF-κB was significantly increased. However, as shown in ***Fig. 2***, luteolin inhibited uric acid-activated NF-κB in Min6 cells significantly.

Luteolin improved GSIS of uric acid-treated mouse islet

After exposure to uric acid for 24 hours, insulin secretion was significantly decreased in response to stimulation of 16.7 mmol/L glucose. Pretreatment with luteolin (10 μmol/L) suppressed this detrimental effect and restored islet insulin secretion to near normal level in Min6 cells (***Fig. 3A***). The effect of luteolin on GSIS in primary cultured mouse islets was also evaluated. The results were in accordance with those of Min6 cell

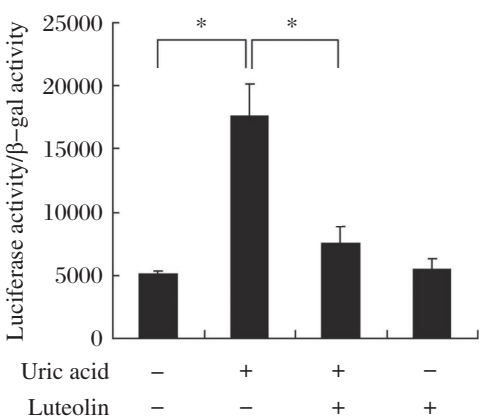

Fig. 2 **Luteolin inhibits urid acid-induced activation of NF-κB.** After Min6 cells were treated with or without 10 μmol/L luteolin in the presence/absence of 5 mg/dL uric acid for 24 hours, the transcriptional activation of NF-κB was determined by luciferase assay in different groups. $n = 4$. * $P < 0.001$ indicates significant difference in comparison with the uric acid-treated alone group.

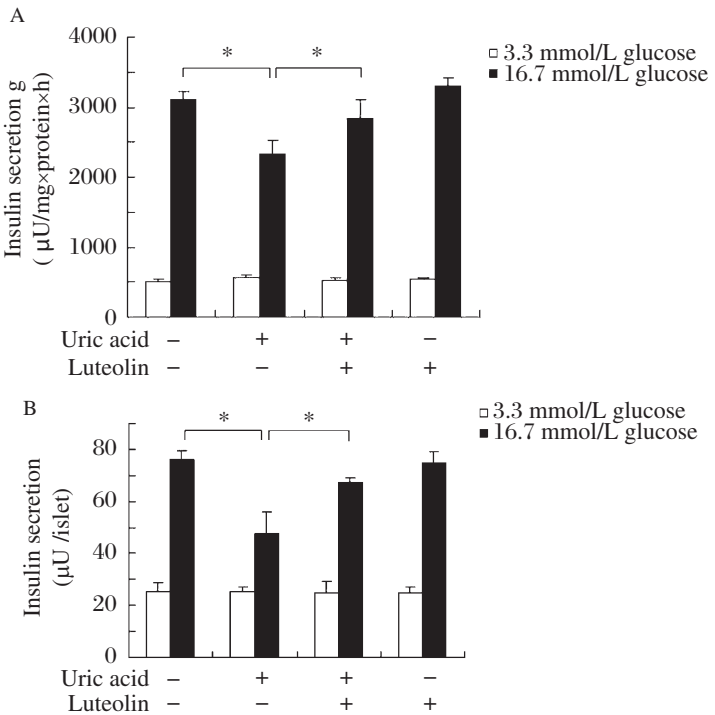

Fig. 3 **Luteolin improves GSIS in uric acid-treated Min6 cells and cultured mouse islets**. A: After Min6 cells were treated with or without 10 μmol/L luteolin in the presence/absence of 5 mg/dL uric acid for 24 hours, glucose stimulated insulin secretion was measured. White bar and black bar show secreted insulin levels modified by protein concentration of Min6 cells induced by 3.3 and 16.7 mmol/L glucose, respectively. N = 4. B: After primary cultured islets were treated with or without 10 μmol/L luteolin in the presence/absence of 5 mg/dL uric acid for 24 hours, glucose stimulated insulin secretion was measured. White bar and black bar show secreted insulin levels modified by the number of islets induced by 3.3 and 16.7 mmol/L glucose, respectively. $n = 4$. * $P < 0.05$ indicates significant difference compared to the uric acid-treated alone group (A, $P = 0.002$ and 0.04, respectively; B, $P = 0.002$ and 0.004, respectively).

lines (***Fig. 3B***). Meanwhile, treatment of luteolin alone had no effect on insulin release.

Luteolin and uric acid had no effect on pancreatic β-cells viability

NO production has been implicated in uric acid-induced pancreatic β-cells dysfunction and disruption[20]. In the present study, the finding that luteolin inhibited uric acid-induced NO production prompted us to evaluate the potential effects of luteolin and uric acid on pancreatic β-cell viability using MTT assays. As shown in ***Fig. 4***, treatment with uric acid (5 mg/dL) or luteolin (10 μmol/L) alone or in combination for 24 hours did not significantly inhibit the viability of Min6 cells. It suggested that luteolin restored uric acid-induced dysfunction of pancreatic β-cells without affecting cell survival.

Luteolin increased uric acid-induced decrease of MafA expression

Insulin biosynthesis and secretion by β-cells were finely regulated by various essential transcription factors, such as MafA, PDX-1 and NeuroD. The suppression of MafA led to a significant reduction in insulin production and secretion[19]. To examine whether luteolin affected MafA expression, we measured the expression level of MafA in Min6 cells. As shown in ***Fig. 5A*** and ***5B***, by pretreatment with luteolin, MafA protein

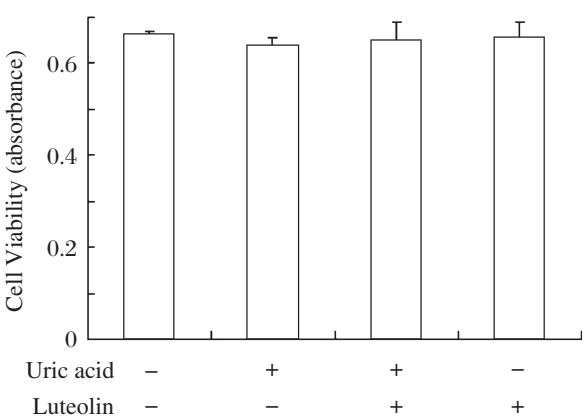

Fig. 4 **Luteolin and uric acid have no effect on Min6 cell viability**. The cell viability was measured after Min6 cells were treated with or without 10 μmol/L luteolin in the presence/absence of 5 mg/dL uric acid for 24 hours. There was no significant difference in cell viability between each treated group. $n = 4$.

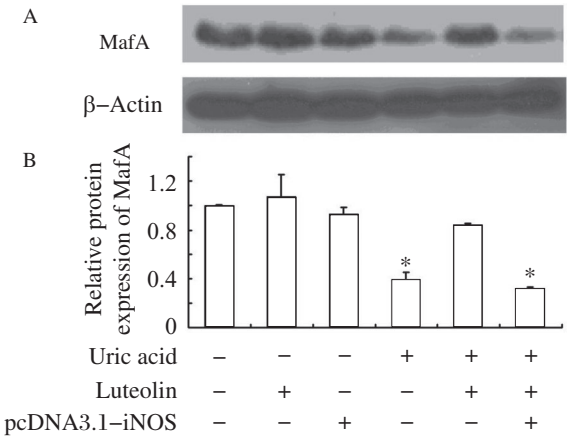

***Fig. 5* Luteolin increases MafA expression in Min6 cells treated by uric acid via regulation of iNOS.** A: After Min6 cells were treated with or without 10 μmol/L luteolin in the presence/absence of 5 mg/dL uric acid for 24 hours, the expression level of MafA protein was measured in the different groups. To confirm the effect of iNOS on this process, pcDNA3.1-iNOS was pre-incubated in luteolin and uric acid co-treated Min6 cells, and the pcDNA3.1-iNOS treated alone group was used for control. B: The blotting assay was repeated for 3 times. The densitometric analysis of protein bands was performed with Quality One software. * $P < 0.05$ indicates significant difference compared to the uric acid-treated alone group ($P = 0.002$ and < 0.001, respectively).

expression was significantly increased in uric acid-treated Min6 cells compared to that in the uric acid-treated alone group. Furthermore, the improvement of MafA expression by luteolin was significantly reversed by transfection with pcDNA3.1-iNOS plasmid, suggesting that iNOS expression was strongly involved in this process.

DISCUSSION

Hyperuricemia has been characterized by an increased level of serum uric acid, which has frequently been related to metabolic syndrome[21]. In recent years, since metabolic syndrome has been regarded as a risk factor for T2D, the potential association of hyperuricemia and future risk of T2D has been explored by multiple large clinical studies. Our previous study provided evidence that uric acid impacted on pancreatic β-cells through the NF-κB-iNOS-NO pathway[6]. Therefore, inhibition of this process may benefit prevention of uric acid-induced dysfunction in pancreatic β-cells. Several anti-inflammatory natural products have been reported[22,23]. Luteolin has been found to possess strong antioxidative and several pharmacological activities like anti-inflammatory effects[22]. Chen et al. had reported that luteolin could suppress the NF-κB pathway and inhibit some pro-inflammatory substances[23]. In this study, we also confirmed that luteolin attenuated uric acid-induced NF-κB activation in pancreatic β-cells.

It has been proved that NO generation was directly related to NF-κB-associated pathway[6]. Moreover, uric acid-induced dysfunction of pancreatic β-cells is mainly due to NO generation[4]. Interestingly, some reports showed that the protective effect of luteolin could be associated with reduced release of NO in macrophages and tumor cell lines[24–25]. Thus, we hypothesized that luteolin might generate a similar effect in pancreatic β-cells. Our results provided evidence that luteolin could reduce uric acid-induced NO formation in Min6 cells. The activity of iNOS contributes greatly to NO generation, and we also confirmed that luteolin could significantly inhibit uric acid-activated iNOS expression in mRNA and protein levels, indicating that luteolin inhibits NO production could be associated with the regulation of iNOS expression.

We reported that insulin secretion could be significantly impaired under stimulation of uric acid in pancreatic β-cells[6]. In the current study, we found that luteolin suppressed uric acid-induced dysfunction of GSIS in Min6 cells and primary cultured islets. It is known that the transcription factor of MafA is one of the key regulators of insulin secretion[19]. Furthermore, our previous results demonstrated that the expression of MafA was significantly decreased by uric acid[6]. The effect of luteolin on the expression of MafA under stimulation of uric acid in pancreatic β-cells was studied in this study. Our data showed that luteolin restored uric acid-induced decrease in the expression level of MafA in Min6 cells. Moreover, overexpression of iNOS reversed the protective effect of luteolin on MafA expression. Meanwhile, luteolin improved insulin secretion in uric acid-damaged pancreatic β-cells by suppressing the decrease of MafA mainly through the NF-κB-iNOS-NO signaling pathway.

It should be mentioned that 24 hours of 5 mg/dL uric acid significantly inhibited GSIS in Min6 cells and primary cultured mouse islets without affecting β cell viability. Besides, luteolin alone (10 μmol/L) did not affect Min6 viability, but it could significantly improve uric acid-induced dysfunction of GSIS in Min6 and cultured islets.

In conclusion, we found that the protective effect of luteolin on uric acid-induced dysfunction of β-cells was through the NF-κB-iNOS-NO signaling pathway via regulation of MafA expression. It should be mentioned that luteolin did not affect cell viability at its effective concentration in Min6 cells, suggesting that luteolin could provide benefit for protection of pancreatic β-cells without causing cytotoxicity. Therefore, this

study provided a better understanding of the molecular mechanism underlying the protective effect of luteolin on T2D and suggested that the compound may be of benefit for the treatment of T2D in hyperuricemic patients.

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