RETRACTED ARTICLE: Tetramethylpyrazine relieves LPS-induced pancreatic β-cell Min6 injury via regulation of miR-101/MKP-1

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ABSTRACT
Tetramethylpyrazine (TMP) is a traditional Chinese medicine with anti-inflammation and immunomodulatory effects. In this context, our purpose was to investigate the associated regulatory mechanisms of TMP against lipopolysaccharide (LPS)-caused pancreatic β cell Min6 injury. The injury of Min6 cells was induced by 10 μg/mL of LPS. Viability of Min6 cells was detected through CCK-8 assay, apoptosis process through flow cytometry, and the proteins involved in apoptosis through western blot. Insulin secretion was valued through the glucose-stimulated insulin secretion (GSIS) assay. microRNA-101 (miR-101) was measured through qRT-PCR. Mitogen-activated protein kinase phosphatase 1 (MKP-1) and signaling regulators was measured through western blot. We found that, TMP treatment effectively attenuated LPS-induced injury in Min6 cells by suppressing cell apoptosis and promoting insulin secretion. Further investigation revealed that TMP exerted protective effect through down-regulating miR-101, and MKP-1 was demonstrated as a target of miR-101. Moreover, TMP-attenuated LPS-triggered inflammation by inactivating the JNK1/2 and NF-κB through the down-regulation of miR-101. In conclusion, our present study revealed that TMP alleviated LPS-induced injury in pancreatic β-cell Min6 injury via regulation of miR-101/MKP-1 with the bluntness of JNK1/2 and NF-κB pathways.

Introduction
In the last few years, diabetes mellitus (DM) is accepted as a chronic endocrine disease with serious complications and it is also reported as the third biggest killer of human health besides cancer and heart disease [1]. DM is divided into type 1 diabetes mellitus (T1DM) and T2DM [2]. Among them, T1DM often occurs in children or adolescents with other complications, which may lead to early childhood death [3]. The aetiology of T1DM is very complex, including genetic factors, environmental factors and so on [4]. At present, T1DM is considered to be an autoimmune deficiency disease, mainly due to the specific autoimmune destruction of islet β cells, which is characterized by β cells loss [3]. Lifelong subcutaneous insulin injection is still the main method of treatment, but this method brings great pain to the children, because of the less function of the remaining islet beta cells, the greater dependence on insulin, the more unstable blood sugar, the earlier the occurrence of acute and chronic complications, and the worse the prognosis of the children [5]. Therefore, searching for drugs that can protect β-cell against the inflammatory damage is of great significance for the treatment of T1DM.

Tetramethylpyrazine (TMP) is an alkaloid compound, possessing numerous activities, for example, antioxidant, anti-inflammation and immunomodulatory properties, and it is generally extracted from the roots of Ligusticum chuanxiong Hort (a traditional Chinese herbal medicine) [6,7]. Recently, researches were set out to investigate the protective activities of TMP against lipopolysaccharide (LPS)-evoked injury of cells and the protection mechanisms. For example, Huang et al. reported that TMP represses LPS-induced damages through promoting the production of 14-3-3y [8]. In addition, it has been found that TMP treatment can significantly improve blood glucose and renal function in the streptozotocin-induced diabetic nephropathy rat model compared to the untreated diabetic rats [9]. However, the specific protective mechanisms have not been thoroughly explored. As a consequence, in-depth study of the role of TMP and its mechanism is of great significance. In our present study, the effect of TMP and its associated modulatory mechanisms was investigated in LPS-treated Min6 cells.

microRNAs (miRNAs) is a single-stranded, endogenous nucleotide with a length of about 18–22 nucleotides. miRNAs bind with 3’-untranslated region (3’-UTR) of specific target genes through complete complementation or incomplete complementation, and thus regulate gene expression and exert its biological effects [10,11]. In recent years, several reports implied that miRNAs can mediate the synthesis and secretion of insulin, and miRNAs have been identified as a regulator of blood sugar balance. For example, Poy et al. reported that glucose-induced insulin secretion is repressed
by miR-375 while enhanced by miR-375 silence [12]. Besides, Grieco et al. found that there are a set of miRNAs involved in the apoptotic process of pancreatic β cell, and these miRNAs are down-regulated in T1DM and they act on B lymphoma-2 gene to promote apoptosis of pancreatic β cells [13]. Thus, we further confirmed the participation of miRNA in the protective process of TMP against LPS-caused damages.

Taken together, we found that TMP treatment effectively attenuated LPS-induced injury in pancreatic β Min6 cells by regulation of microRNA-101 (miR-101)-mitogen-activated protein kinase phosphatase 1 (MKP-1) via inactivating JNK1/2 and NF-κB, shedding a new insight in the treatment of T1MD.

Materials and methods

Cell culture and treatment

Min6 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco’s modified Eagle medium (HyClone, Logan, UT, USA) in addition with 5 mM glucose, 10% foetal bovine serum (Gibco, Gaithersburg, MD, USA), 50 mmol/L b-mercaptoethanol (Sigma, St. Louis, MO, USA), 100 U/mL penicillin (Sigma, St. Louis, MO, USA) and 0.1 mg/mL streptomycin (Sigma, St. Louis, MO, USA) in 5% CO2 at 37 °C. TMP was purchased from Solarbio (Beijing, China, purity ≥98%). TMP was dissolved in water and cells were incubated with TMP at indicated concentration for 24 h before LPS treatment. LPS was bought from the Sigma-Aldrich (St. Louis, MO, USA). U6 SnRNA was used as the internal reference. Relative level was quantified by the 2−ΔΔCt method. The primers used for RT-PCR were as following, miR-101 forward, 5′-GCAAAGUCUUACAGUCAUAGC-3′ and 5′-TTGTGTCGTTGAGATCG-3′; U6 forward 5′-AACGCTTCGAGCACA-3′, reverse, 5′-AACGGCTTCAGAATTTGCGCT-3′.

CCK-8 assay

Min6 cells were seeded in 96-well plate (5 × 10³/well) and its viability was valued by a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan). After administration, the cells were cultured with the CCK-8 solution for 1 h at 37°C. Microplate Reader (Bio-Rad, Hercules, CA, USA) was exploited to detect absorbance of the culture at 450 nm.

Apoptosis analysis

Min6 cells (5 × 10⁵/well) were plated in six-well plates. The cells were washed twice with cold phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA), suspended in binding buffer and stained by Annexin V-propidium iodide (PI) (Beijing Biosea Biotechnology, Beijing, China). FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was applied to observe the apoptotic cells.

Glucose-stimulated insulin secretion (GSIS) assay

Min6 cells was plated in 24-well plates and treated with or without TMP, and then were stimulated with LPS. Continually, the cells were cultured with glucose-free Krebs-Ringer bicarbonate (KRB) buffer (115 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 20 mmol/L NaHCO₃, 16 mmol/L HEPES, 2.56 mmol/L CaCl₂ and 0.2% bovine serum albumin (BSA)) for 1 h, and then were treated in KRB buffer with low (3.3 mmol/L) or high (16.7 mmol/L) concentrations of glucose for 1 h. Cell culture supernatants were collected for determining insulin concentrations by radioimmunoassay.

qRT-PCR

TRizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA. Reverse transcription proceeded by MultiScribe kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed by SYBR-green PCR Master Mix on a Fast Real-time PCR 7500 System (Applied Biosystems, Foster City, CA, USA). U6 SnRNA was used as the internal reference. Relative level was quantified by the 2−ΔΔCt method. The primers used for RT-PCR were as following, miR-101 forward, 5′-ACACCTCACCTGGGTACAGTACTGTGATA-3′, reverse, 5′-TGTTGTCGTTGGAGATCG-3′; U6 forward 5′-CTCGGTTCCGGCAGCACA-3′, reverse, 5′-AACGCTTCAGAATTTGCGCT-3′.

miRNAs transfection

miR-101 mimic (mimic sense 5′-UCAGUAUACACAGUCUGAUG-3′; mimic antisense 5′-UACAGCACUGUGAUAACUGAUU-3′), NC mimic (mimic sense 5′-CAGAAUAUUAAUUCGCAUGUGA-3′; mimic antisense 5′-UAACAUAGUUAGCAGAGUAGCCG-3′), si-NC (5′-GCAAGGCUCCUAGACCAUAGC-3′) and si-MKP-1 (5′-GUGUUUGACUGAUACGCUUCAGUUAC-3′) were provided by Ribobio (Shanghai, China) and transfected into Min6 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Dual luciferase activity assay

The wild-type (wt) and mutant (mut) 3′-UTR of MKP-1 reporter plasmids were provided by Genechem (Shanghai, China). Min6 cells were grown in a 24-well plates and co-transfected with miR-101 mimics or NC mimic (0.5 μg) in the presence of Lipofectamine 2000 reagent. After 48 h, the cells were collected. Dual-luciferase assay system (Promega, Madison, WI, USA) was used to evaluate the luciferase activities.

Western blot examination

RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) was applied to isolate proteins. The concentration was measured using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Equal amount (50 μg) of protein was separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis using a Bio-Rad Bis-Tris Gel system according to the manufacturer’s instructions (Solarbio, Beijing, China) and was transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocked by non-fat milk (5%), the proteins were probed by primary antibodies detecting Bax (ab32503) (Abcam, Cambridge, UK), caspase-3 (14220) (Cell
Signaling Technology, CST, Danvers, MA, USA), caspase-9 (3409-100) (BioVision, Milpitas, CA, USA), MKP-1 (ab61201) (Abcam, Cambridge, UK), β-actin (4967) (CST, Danvers, MA), p65 (8242) (CST, Danvers, MA, USA), phospho-Ser276 p65 (MBS001656) (MyBioSource, San Diego, CA, USA), IκBx (4812) (CST, Danvers, MA, USA) and phospho-Ser32 IκBx (2859) (CST, Danvers, MA, USA), overnight at 4°C. After washed with PBS supplemented with 0.6% Triton-X 100 at least three times, the target proteins were detected with secondary antibody marked by horseradish peroxidase (7074) (CST, Danvers, MA, USA) for 1 h at room temperature. At last, the signals were captured using chemiluminescence (Pierce, Appleton, WI, USA) and Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

**Statistical analysis**

All data were depicted as the mean ± standard deviation from the results of multiple experiments (n = 3). SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was applied to perform statistical analysis. The p values were calculated using a one-way analysis of variance. The significant difference was considered when p values were less than .05.

**Results**

**LPS facilitated apoptosis and insulin secretion in Min6 cells**

First, cell injury induced by LPS was examined. As shown in Figure 1(A), viability was suppressed by LPS. At the same time, LPS induced apoptosis process (Figure 1(B)). Furthermore, the apoptosis was accompanied by the accumulation of Bax and the cleavage of caspases as shown in the results from western blot assay (Figure 1(C)). We could see that cell apoptosis rate and the production of proteins associated with apoptosis were both elevated by LPS. Besides, glucose at a concentration of 16.7 mM induced the high level of insulin secretion (Figure 1(D)). However, LPS stimulation suppressed the secretion of insulin. Thus, these above results showed that LPS induced apoptosis and inhibited insulin secretion by Min6 cells.

**TMP moderated LPS-caused injury of Min6 cells**

The results of the CCK8 assay in the Figure 2(A) showed that the treatment of TMP (10–50 μM) had no obvious cytotoxicity on Min6 cells. Then, the results in Figure 2(B) exhibited that TMP treatment effectively rescued LPS induced cell viability inhibition and the protective effect of 30 μM TMP was the most obvious among the selected concentration. Thus, 30 μM TMP was selected for our following experiments. The result of the flow cytometry showed that elevated cell apoptosis rate induced by LPS was then suppressed by TMP treatment (Figure 2(C)). The increase of Bax, cleaved-caspase-3 and cleaved-caspase-9 was also repressed by TMP treatment (Figure 2(D)). As expected, we found that the TMP treatment stimulated insulin secretion suppressed by LPS (Figure 2(E)). Thus, these above results showed that TMP alleviated LPS induced Min6 injury and recovered insulin secretion.

![Figure 1](image1.png)

**Figure 1.** LPS induced apoptosis and repressed insulin secretion in Min6 cells. (A) CCK-8 was conducted to examine the effect of LPS on cell viability. (B) Cell apoptosis rate was detected through flow cytometry. (C) The expression of cell apoptosis related proteins (Bax, caspase-3 and caspase-9) was valued through western blot. (D) Glucose-stimulated insulin secretion (GSIS) assay was conducted to value the effect of LPS on insulin secretion. n = 3. **p < .01, ***p < .001.
In order to explore whether miR-101 was associated with the protective capacity of TMP against LPS-triggered damages, miR-101 in TMP-treated cells was detected through qRT-PCR. The result in Figure 3 showed that miR-101 was greatly elevated by LPS. However, TMP treatment down-regulated the production of miR-101 induced by LPS. TMP alleviated LPS induced-injury and inhibition of insulin secretion by down-regulating miR-101

Specific miR-101 mimic was transduced into Min6 cells to exogenously up-regulate miR-101 (Figure 4(A)). The protective roles of TMP against LPS-elicited viability inhibition were largely weakened by miR-101 mimic (Figure 4(B)). Similarly, the inhibitory effect of TMP on LPS induced cell apoptosis detected through flow cytometry (Figure 4(C)) and western blot (Figure 4(D)) was also reversed by the addition of miR-101 mimic. Moreover, the insulin secretion ability of Min6 cells was rescued by TMP treatment but was then largely weakened by the combination of miR-101 mimic (Figure 4(E)). Thus, the above results revealed that TMP alleviated LPS-caused damages of Min6 cells by down-regulating miR-101. Besides, miR-101 mimic abolished the protective function of TMP against LPS-evoked apoptosis and suppression of insulin secretion.

TMP restored MKP-1 expression dependent on miR-101

The results in Figure 5(A) showed that MKP-1 was diminished by LPS and was then increased by the treatment of TMP. However, miR-101 mimic further decreased MKP-1 level. The results from luciferase reporter assay proved that miR-101 mimic repressed the activity of luciferase in Min6 cells transfected with MKP-1 WT compared with the control group, identifying that there existed target sites of miR-101 on MKP-1 3’-UTR, rather than MKP MUT (Figure 5(B)). Thus, the above results implied that MKP-1 might be a target of miR-101.

TMP inactivated the NF-κB and JNK1/2 signal pathways through up-regulating MKP-1

The associated signal pathways were further investigated in our following experiments. Specific si-MKP-1 was transfected into Min6 cells for inducing MKP-1 silence as shown in Figure 6(A). Then, we found that the NF-κB was over-activated by LPS and was then inactivated by TMP treatment. However, the NF-κB was activated again by si-MKP-1 (Figure 6(B)). Similarly, the inhibiting effect of TMP on JNK1/2 signal pathway was also abolished by the combination of si-MKP-1 (Figure 6(C)). Thus, we concluded that TMP inactivated the NF-κB and JNK1/2 signal pathways through up-regulating MKP-1.
Discussion

T1DM is categorized as a type of T cell-mediated autoimmune disease and is evoked by multiple factors, such as oxygen free radicals, cytokines, T lymphocyte, immune response disorder, etc. [14,15]. T1DM is characterized by the selective destruction of islet β cells, absolute insufficient secretion of insulin, and it results in metabolic disorders of sugar, fat and protein [16,17]. The death of a large number of islet β cells is the causative factor of the disease, and apoptosis of β cells may be the main form of death and play a leading role in T1DM process [18]. Thus, trying to find appropriate ways to cope with the destruction of autoimmune β-cell is a prospective treatment direction for T1DM.

Islet β cells are sensitive to diabetes-associated inflammatory regulators, for instance, interleukin (IL)-β and tumour necrosis factor (TNF)-α, and impaired islet β cells in turn activate signaling pathways involved in the dysfunction and apoptosis of β cell and infiltrate of immune cells [19,20]. LPS is a cell membrane endotoxin of gram-negative bacteria which is able to produce inflammatory reactions after bacterial infection, thus LPS is widely used as an inducer for the conduction of disease model, such as obesity, insulin resistance and diabetes [21,22]. In our present study, the pancreatic β cell Min6 cells injury model was established by using LPS stimulation. We found that viability was suppressed and apoptosis was induced by LPS. At the same time, insulin

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**Figure 3.** TMP down-regulated the level of miR-101. Relative expression of miR-101 in TMP treated cells and the control cells was detected through qRT-PCR. \( n = 3 \). \(* p < .05, **p < .01.\)

**Figure 4.** TMP alleviated LPS induced Min6 injury by down-regulating miR-101. Specific miR-101 mimic was transfected into Min6 cells to exogenously up-regulate the expression of miR-101 and the NC mimic was used as a control. (A) Relative expression of miR-101 was detected through qRT-PCR. (B) Cell viability was detected through CCK-8 assay. (C) The effect of TMP on LPS induced cell apoptosis was detected through flow cytometry. (D) The expression of Bax, caspase-3 and caspase-9 was detected through western blot. (E) The insulin secretion ability of Min6 cells was examined through glucose-stimulated insulin secretion (GSIS) assay. \( n = 3. \) \(* p < .05, **p < .01, ***p < .001.\)
secretion was also largely inhibited by LPS, identifying the successful establishment of cell injury model.

Natural products have been widely used in various diseases due to its anti-inflammatory effect, safety and rich sources [23]. TMP has been widely applied in the cardiovascular and inflammatory diseases treatment. Just as Bai et al. reported, TMP effectively ameliorates experimental autoimmune encephalomyelitis by maintaining the balance between pro-inflammation and anti-inflammatory responses [24]. Besides, Li et al. reported that TMP suppresses the level of IL-8 induced by LPS thorough modulating signaling transduction pathways [25]. In addition, TMP also remarkably reduced the degree of lipoperoxidation for the treatment of DM complications with oxidative stress [26]. However, specific protection mechanisms of TMP in DM have not been explored. In our present study, we found that TMP treatment exerted protective effect on LPS-evoked apoptosis of Min6 cells by elevating viability, suppressing apoptosis and restoring insulin secretion. So, associated protection mechanisms of TMP were further investigated.

With the development of molecular biotechnology, the discovery of miRNA opens a new door for the study of complications of DM. In recent years, more and more studies have shown that miRNA is implicated in the pathogenesis of DM and its complications [27]. The destruction, apoptosis and dedifferentiation of islet β cells contribute to cell loss, the decrease of insulin secretion, and the disorder of blood sugar, which ultimately leads to DM and its complications. Bao et al. reported that overexpression of miR-185 enhances insulin secretion of pancreatic β-cells, promotes cell proliferation and protects cells from apoptosis [28]. Similarly, in our study, we found that the treatment of TMP down-regulated miR-101. miR-101 largely weakened the protective activities of TMP, indicating that TMP exerted protective effect by suppressing miR-101. Insulin is a protein hormone secreted by stimulated pancreatic β cell and exerts an essential function in the balance of human blood sugar [29]. Insufficient secretion of insulin will cause complications of hyperglycaemia and diabetes [30]. In recent years, related studies have found that miRNAs can regulate the synthesis and secretion of insulin, and miRNA has become a regulator of human blood sugar balance. For example, Zhao et al. reported that miR-30d protects β cells against the suppression of insulin transcription and secretion induced by TNF-α through repressing mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) [31]. In our study, we also found that the insulin secretion was increased by TMP and was then suppressed by the adding of miR-101 mimic compared with the individual

![Figure 5](image1.png)  **Figure 5.** MKP-1 was a target of miR-101. (A) The expression of MKP-1 was examined through western blot. (B) Luciferase reporter assay was conducted to further demonstrate the targeting relationship between miR-101 and MKP-1. n = 3. *p < .05, **p < .01.

![Figure 6](image2.png)  **Figure 6.** TMP inactivated the NF-κB and JNK1/2 signal pathways through up-regulating MKP-1. (A) Specific si-MKP-1 was transfected into Min6 cells to knockdown the expression of MKP-1. The expression of MKP-1 was examined through western blot. (B) The expression of NF-κB signal pathway related proteins was examined through western blot. (C) The expression of JNK1/2 signal pathway related proteins was examined through western blot. n = 3. *p < .01, **p < .01.
TMP, identifying that TMP promoted insulin secretion in LPS induced Min6 cells by suppressing the expression of miR-101. MKP-1 inactivates the MAPK signal through de-phosphorylation, which is essential for regulating the activity of the MAPK pathway [32]. It is a negative regulator of the MAPK pathway and has very important physiological and pathological significance. Up to now, at least 10 kinds of MKP have been identified in mammalian cells, of which MKP-1 is the prototype of MKP family [33]. Jacob et al. reported that MKP-1 decreases the phosphorylation of MAPK, abates migration, and then restores insulin’s ability to inhibit vascular smooth muscle cell migration in Goto-Kakizaki diabetic [34]. We observed that TMP treatment induced MKP-1, and we proved MKP-1 was a target of miR-101. In addition, associated signal pathway was also investigated. NF-kB and JNK1/2 signal pathways are recognized immune-related pathways and Li et al. has demonstrated that glucagon-like peptide-2 suppresses LPS-evoked over-inflammatory response in BV-2 cells dependent on the suppression of ERK1/2, JNK1/2 and NF-κB [35]. We also found JNK1/2 and NF-κB were activated by LPS and were then suppressed by TMP treatment. However, the addition of specific si-MKP-1 abolished the inhibiting effect of TMP on the JNK1/2 and NF-κB signaling pathways, indicating that TMP attenuated LPS-caused inflammation by inactivating the JNK1/2 and NF-κB by down-regulating miR-101.

Taken together, we found that TMP treatment effectively attenuated LPS-induced injury in pancreatic β Min6 cells by suppressing cell apoptosis and promoting insulin secretion. Further investigation revealed that TMP exerted protective effect through down-regulating miR-101 and MKP-1 was demonstrated as a target by miR-101. Moreover, TMP attenuated inflammation induced by LPS by inactivating the JNK1/2 and NF-κB through the inhibition of miR-101. Our present study more clearly revealed the protective mechanism of TMP on islet β cells, demonstrating new targets and methods for the treatment of T1MD.

Disclosure statement
Authors declare that there is no conflict of interest.

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