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Increases in β-amyloid protein in the hippocampus caused by diabetic metabolic disorder are blocked by minocycline through inhibition of NF-κB pathway activation

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Abstract:
Activation of the NF-κB pathway plays an important role in the pathophysiology of Alzheimer's disease (AD), and blocking NF-κB pathway activation has been shown to attenuate cognitive impairment. Diabetic metabolic disorder contributes to β-amyloid protein (Aβ) generation. The goal of this study was to determine the effect of minocycline on Aβ generation and the NF-κB pathway in the hippocampus of diabetic rats and to elucidate the neuroprotective mechanisms of minocycline for the treatment of diabetic metabolic disorder.
The diabetic rat model was established using a high-fat diet and an intraperitoneal injection of streptozocin (STZ). Behavioral tests showed that the capacity of learning and memory was significantly lower in diabetic rats. The levels of NF-κB, COX-2, iNOS, IL-1β and TNF-α after the STZ injection were significantly increased in the hippocampus. Significant increases in Aβ, BACE1, NF-κB, COX-2, iNOS, IL-1β and TNF-α were found in diabetic rats. The levels of Aβ, NF-κB, COX-2, iNOS, IL-1β and TNF-α were significantly decreased after minocycline administration; however, minocycline had no effect on BACE1 expression.
In sum, diabetes contributes to the activation of the NF-κB pathway and upregulates BACE1 and Aβ. Minocycline downregulates Aβ in the hippocampus by inhibiting NF-κB pathway activation.

Key words:
diabetes mellitus, minocycline, β-amyloid protein, NF-κB

Introduction

Diabetes mellitus (DM) has been closely linked to the pathogenesis of Alzheimer's disease (AD) [18, 34, 49, 56, 74], and it has been proposed that AD is type 3 diabetes [19, 34]. AD and DM have a similar pathogenesis; inflammation [53], oxidative stress [26], apoptosis [62] and vascular dysfunction [43, 55] are found in both diseases. Further evidence shows that Aβ generation and failure of Aβ clearance are symptoms of both AD and DM [6, 45].

NF-κB is a transcription factor that regulates the expression of the genes involved in the immune response [30], embryo or cell lineage development [2], cell apoptosis [13, 17], inflammation [39] and oxidative stress [69]. Tremendous attention has been focused on upstream signaling pathways leading to

NF-κB activation. Many of these signaling molecules represent potential pharmaceutical targets for the specific inhibition of NF-κB activation and the subsequent interference with disease processes [5, 8, 72].

AD is a neurodegenerative disorder characterized by progressive memory loss and a decline of cognitive functions. Its histopathological hallmarks include extracellular Aβ deposition in neuritic plaques, intracellular deposits of hyperphosphorylated tau protein (causing formation of neurofibrillary tangles) and neuronal death [20]. Aβ generation and deposition represents a key feature and is the triggering mechanism of AD [41]. Aβ peptides are generated from amyloid precursor protein (APP) by the sequential actions of two proteolytic enzymes, i.e., the b-site APP cleavage enzyme and γ-secretase. β-Amyloid precursor protein cleavage enzyme 1 (BACE1) is the β-secretase that processes APP, and β-secretase activity is dependent on protein levels of BACE1 [27, 73].

Minocycline, a tetracycline derivative, is a potential neuroprotective agent that blocks inflammation, oxidative stress and apoptosis [35, 66]. Previously, we demonstrated that minocycline can inhibit oxidative stress and inflammation in the hippocampus of rats with permanent bilateral occlusion of both common carotid arteries and improve behavioral deficits [11, 12]. Because BACE1 is the β-secretase that processes APP and induces Aβ generation in the pathogenesis of AD and because activation of the NF-κB pathway is present in the pathogenesis of both DM and AD, it is possible that minocycline inhibits the activation of the NF-κB pathway and Aβ generation by downregulating BACE1. To test this hypothesis, we analyzed the influence of minocycline on the expression of Aβ, BACE1 and upstream signal transduction molecules of the NF-κB pathway (such as COX-2, iNOS, IL-1β, TNF-α and NF-κB) in the hippocampus of diabetic rats with cognitive impairment induced by a high-fat diet and STZ injection [59, 63]. The aim of this study was to investigate the neuroprotective mechanisms of minocycline against diabetic brain injury.

Materials and Methods

Animals and drugs

Eight-month-old male Wistar rats provided by the Experimental Animal Center of Chongqing Medical University, weighing 220–300 g, were housed in individual cages at a constant temperature (25°C) under a 12-h light-dark cycle. All rats were habituated to the cage for at least 5 days before the experiments. Animals were administered a high-fat and high-sugar diet for 2 months (food composition: 10% lard, 20% sucrose, 2.5% cholesterol, 1% bile salt and 6.5% conventional food) to induce insulin resistance, and the diabetes model was induced by a 55 mg/kg intraperitoneal injection of streptozocin (STZ) [14, 52].

To determine successful establishment of the DM model, blood glucose levels were measured weekly using blood glucose test strips (Biosynthesis Co., Beijing, China). Animals that had uric acid and insulin resistance and blood glucose levels ≥ 16 mmol/dl were classified as having DM [14, 58]. STZ-injected animals were continuously fed a high-fat and high-sugar diet. Diabetes developed spontaneously in Wistar rats at 63 ± 2 days. Animals were sacrificed at corresponding time points. The brain tissues from the hippocampal region were rapidly removed into ice-cold artificial cerebrospinal fluid and frozen at –80°C for analysis. When the animals were sacrificed, the glycated hemoglobin (Hb) and cholesterol levels were measured (Biosynthesis Co., China). Rats were sacrificed at 2, 4 or 8 weeks after STZ injection (average age was 8 weeks after STZ injection). Animals were randomly divided into a control group (C, intraperitoneal injection with buffer, n = 6), model groups (subdivided into M2, M4 and M8 for 2, 4 and 8 weeks after STZ injection, respectively, with 6 animals per group) and a minocycline administration group (subdivided into MT2, MT4 and MT8 for 2, 4 and 8 weeks after minocycline administration, respectively, with 6 animals per group). Minocycline was administered by gavage two days after STZ injection. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal experiments followed the ethical standards approved by the Research Ethics Committee of Chongqing Medical University, Chongqing, China.

Minocycline (Huishi Pharmaceutical Ltd. Co., China) was diluted to 0.5 mg/ml in PBS buffer. The minocycline-treated groups were given minocycline by gavage at 50 mg/kg/d. Diabetic rats were administered the same volume of buffer by douche *via* stomach. The minocycline dosage was determined based on previous studies [28, 61].

Enzyme-linked immunosorbent assay

Rat tissues were dissected and homogenized in T-PER buffer (Biosource International, Inc., USA) in the presence of protease inhibitors (Biosource International, Inc., USA). The concentrations of IL-1β, TNF-α and Aβ were measured using IL-1β, TNF-α and Aβ colorimetric ELISA kits (Biosynthesis Co., Beijing, China) according to the manufacturer's instructions and previous studies [50, 51].

Western blot

Western blots were carried out as described previously [54, 57]. Rat tissues were dissected and homogenized in T-PER buffer in the presence of protease inhibitors. Following homogenization, the lysates were centrifuged at 100,000 × g, and the supernatants were used for western blotting using Ciphergen (Biosource) protein chip arrays. Equal amounts of protein were subjected to SDS-PAGE (tris-glycine mini gel,

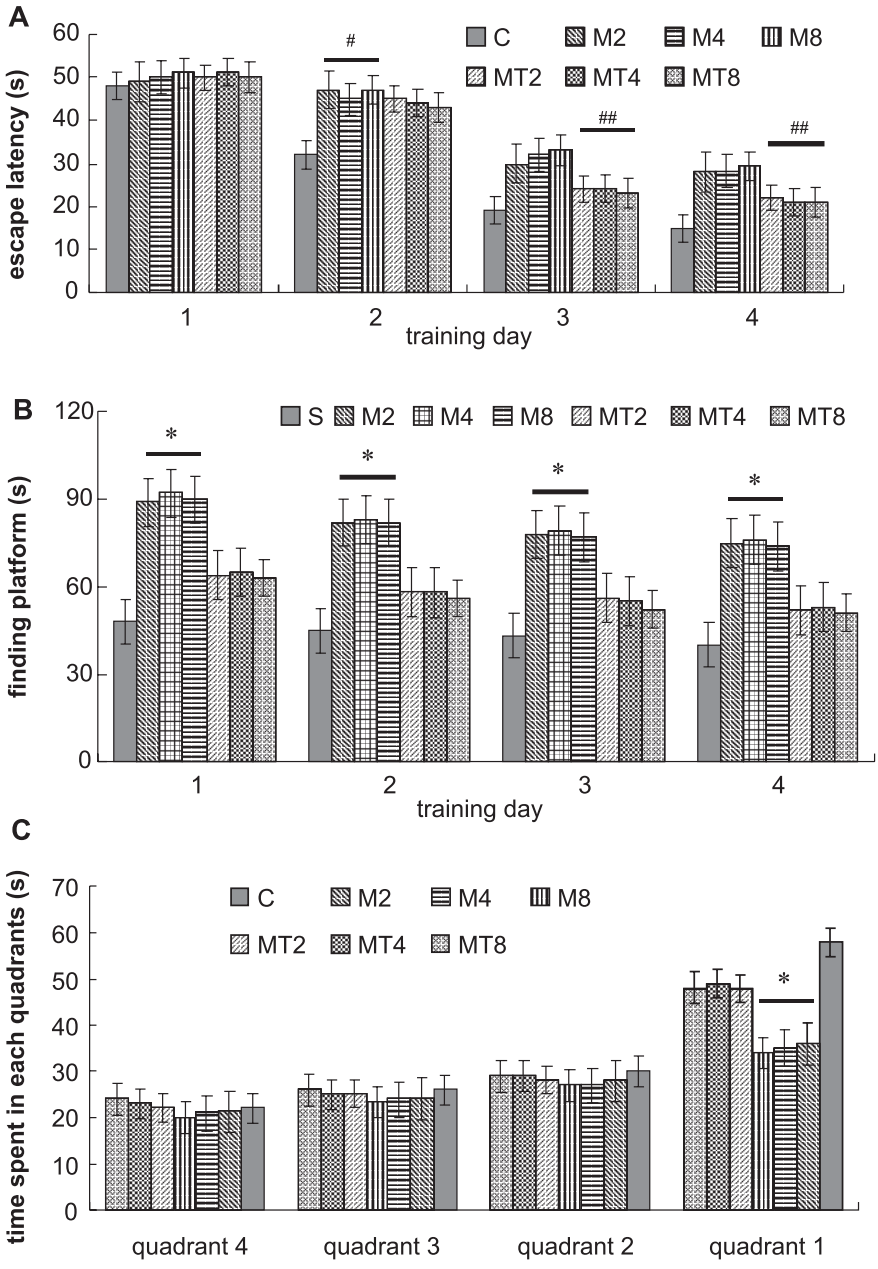

Fig. 1. Morris water maze performance. (**A** and **B**) Escape latency increased in DM rats on testing days 2, 3 and 4 ($^{\#}$ $p < 0.01$). Minocycline treatment decreased escape latency compared to DM animals on days 3 and 4 ($^{\#\#}$ $p < 0.01$). The escape latency was longer in STZ-injected rats that than in control rats (* $p < 0.01$), whereas the latency to find the platform was shorter in STZ-injected rats after minocycline administration (* $p < 0.01$). (**C**) The time spent in quadrant 1 during the last probe trial was longer in control rats than in STZ-injected rats (* $p < 0.01$), while minocycline treatment increased the time spent in quadrant 1 during the last probe trial in STZ-injected rats (* $p < 0.01$)

Biosource). The BACE1 antibody was purchased from Biosource International Inc., USA, and the NF-κB, COX-2, iNOS, IL-1β and TNF-α antibodies were obtained from the Biosynthesis Co., China. The optical densities of the specific bands were scanned and measured by image analysis software (HPIAS 2000, Tongji Qianping Company, Wuhan, China).

Statistical analysis

Quantitative data were expressed as the mean ± standard deviation ($\bar{x}$ ± SD). SPSS software for Windows 2000 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. For statistical evaluation, one-way analyses of variance (ANOVA) were employed.

Results

Minocycline improved behavioral deficits caused by diabetes

Following STZ injection, rats were subjected to the Morris water maze as described in the Materials and Methods section. Escape times decreased significantly after one day of training. On days 3 and 4 of training, the latency to find the platform in STZ injected groups was considerably longer than in the control group ($p < 0.01$); however, minocycline administration considerably reduced the latency to find the platform ($p < 0.01$) (Fig. 1A and B). In the probe trials, the time spent in quadrant 1 during the last probe trial for the control group was significantly longer than in STZ-injected rats ($p < 0.01$). Control rats acquired significantly higher preferences for the platform location compared to diabetic rats. The time spent in quadrant 1 during the last probe trial was significantly longer in minocycline-treated animals than in diabetic rats ($p < 0.01$) (Fig. 1C). These results suggest that minocycline improved behavior deficits caused by diabetic metabolism disorder.

Blood analysis

Rats showed elevated blood glucose and glycated hemoglobin levels after 2, 4 or 8 weeks following STZ injection ($p < 0.001$). Cholesterol levels were also elevated in diabetic rats ($p < 0.001$). Elevated blood glucose levels in STZ-injected rats were slightly decreased following minocycline treatment, whereas glycated hemoglobin levels, weights and cholesterol levels did not change after minocycline administration (Tab. 1).

Minocycline decreased expression of diabetes-induced Aβ

Aβ expression in the hippocampal tissues of diabetic rats was measured using a colorimetric ELISA method to clarify the neuroprotective mechanisms of minocycline against diabetic brain injury. Aβ40 levels were significantly elevated from 34.13 ± 6.76 pg/mg

Tab. 1. Body weight, plasma glucose, plasma insulin, glycated hemoglobin and cholesterol levels in control and DM model rats

Group	Weight (g)	Plasma glucose (mmol/dl)	Glycated Hb (%)	Cholesterol (mg/dl)	Plasma insulin (pmol/l)
C group rats (n = 6)	482.2 ± 16.5	6.23 ± 0.62	3.44 ± 0.26	121.6 ± 26.2	365.3 ± 26.5
M2 group rats (n = 6)	392.1 ± 13.4*	23.25 ± 4.02**	12.32 ± 0.76**	290.5 ± 26.3**	405.5 ± 25.2*
M4 group rats (n = 6)	391.5 ± 15.2*	23.21 ± 3.46**	12.41 ± 0.82**	291.1 ± 27.5**	406.2 ± 24.5*
M8 group rats (n = 6)	381.2 ± 18.3*	22.89 ± 3.61**	12.56 ± 0.67**	290.3 ± 24.2**	403.4 ± 28.7*
MT2 group rats (n = 6)	373.5 ± 15.9*	21.82 ± 3.58**	11.42 ± 0.56**	288.6 ± 20.7**	396.7 ± 23.1*
MT4 group rats (n = 6)	376.3 ± 16.7*	21.89 ± 3.70**	11.52 ± 0.61**	287.3 ± 21.1**	398.4 ± 22.5*
MT8 group rats (n = 6)	372.4 ± 19.1*	21.82 ± 4.53**	11.31 ± 0.59**	285.2 ± 22.6**	397.2 ± 28.1*

Data are expressed as the mean ± SD. * $p < 0.05$, ** $p < 0.001$ *vs.* control group. C – control group; M2 – rats studied 2 weeks after STZ injection; M4 – rats studied 4 weeks after STZ injection; M8 – rats studied 8 weeks after STZ injection; MT2 – rats studied 2 weeks after STZ injection and minocycline treatment; MT4 – rats studied 4 weeks after STZ injection and minocycline treatment; MT8 – rats studied 8 weeks after STZ injection and minocycline treatment

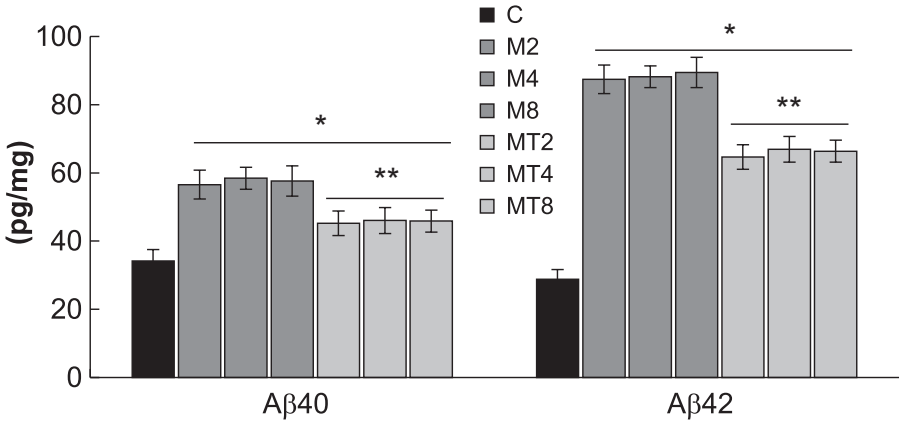

Fig. 2. Aβ40/42 ELISA assay. Aβ42 and Aβ40 levels were elevated in DM rats (* $p < 0.01$). Aβ42 and Aβ40 levels in minocycline-treated rats were lower than in diabetic rats (** $p < 0.01$). Data are expressed as the mean ± SD

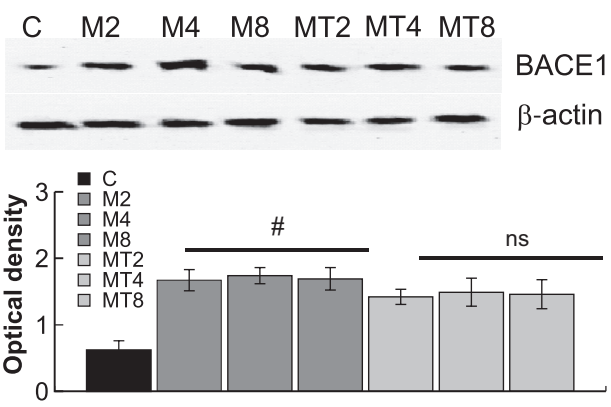

Fig. 3. Western blot analysis of BACE1 expression. Relative amounts of BACE1 expression expressed as the densitometry ratio of BACE1 to β-actin (mean ± SD). DM model groups had a significantly higher *OD* value for BACE1 than the C group (# $p < 0.01$), whereas BACE1 levels in the minocycline-treated groups were not significantly different from DM model rats (not significant (ns) $p > 0.05$)

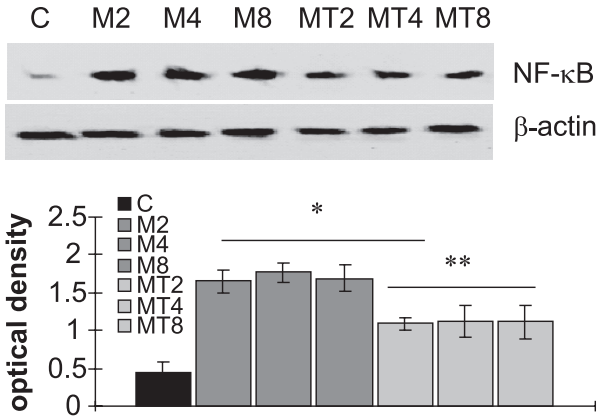

Fig. 4. Western blot analysis of NF-κB expression. Relative amounts of NF-κB expression expressed as the densitometry ratio of NF-κB to β-actin (mean ± SD). DM model groups had a higher *OD* value for NF-κB than the C group (* $p < 0.01$), and expression of NF-κB in minocycline-treated groups had a lower *OD* value than DM model groups (** $p < 0.01$)

in the control animals to 58.43 ± 7.03 pg/mg in the STZ-injected animals ($p < 0.01$), and Aβ42 levels increased from 67.43 ± 5.12 pg/mg in control animals to 89.45 ± 5.28 pg/mg diabetic rat tissue lysates ($p < 0.01$). The Aβ concentration in the minocycline-treated group was significantly lower than in the diabetic animals; Aβ40 levels decreased from 58.43 ± 7.03 pg/mg in the diabetic animals to 46.03 ± 8.13 pg/mg ($p < 0.01$) in the minocycline-treated rats, and Aβ42 levels decreased from 89.45 ± 5.28 pg/mg to 79.04 ± 8.03 pg/mg, respectively ($p < 0.01$) (Fig. 2). These results indicate that minocycline may improve diabetes-induced behavior deficits by downregulating Aβ levels.

Minocycline had no influence on diabetes-induced BACE1 expression

Aβ generation has been shown to be closely linked to cognitive impairment [22, 45, 48]. Previously, we demonstrated that minocycline improved behavior deficits caused by diabetic metabolic disorder by blocking Aβ generation. To further explore the mechanism by which minocycline improved behavior deficits, we measured the levels of the β-amyloid precursor protein cleavage enzyme BACE1. BACE1 levels in diabetic rats were higher than those of the control group ($p < 0.001$), whereas BACE1 levels in minocycline-treated groups

were not significantly different from the diabetic groups ($p > 0.05$) (Fig. 3). Inhibition of Aβ levels by minocycline treatment had no effect on BACE1 downregulation, although elevation of BACE1 in DM contributes to the pathogenesis of AD.

Minocycline inhibited expression of NF-κB

Minocycline improved behavioral deficits mediated by diabetes by downregulating Aβ, although it had no effects on BACE1, which is the primary contributor to Aβ generation [33, 48]. However, diabetes-induced NF-κB activation enhances both Aβ generation and Aβ clearance [3, 36]. Therefore, activation of the NF-κB pathway was investigated to further elucidate the influence of minocycline on Aβ metabolism. The results from western blot experiments showed that minocycline downregulated NF-κB in the hippocampus of diabetic rats. NF-κB levels in the hippocampus were decreased 1.3-fold in the minocycline-treated rats compared to the diabetic rats ($p < 0.001$), whereas NF-κB levels in the diabetic model rats was increased 3.6-fold compared to control rats ($p < 0.001$) (Fig. 4).

Minocycline inhibited oxidative stress triggered by diabetes

Oxidative stress is a hallmark of pathophysiological responses resulting from alterations in cellular redox homeostasis due to either an over-production of reactive oxygen species (ROS) or a deficiency in the buffering or scavenging systems for ROS [55]. ROS might mediate the activation of NF-κB in response to a broad range of stimuli. COX-2 and iNOS, markers of upstream signaling molecules of the NF-κB pathway and oxidative stress [9, 24], play an important role in Aβ metabolism [23, 40]. Therefore, COX-2 and iNOS levels were tested to explore the effect of minocycline on decreasing the oxidative stress associated with increases in Aβ levels. COX-2 levels in diabetic rats increased 3.5-fold compared to control rats ($p < 0.001$), but decreased 1.1-fold following minocycline administration ($p < 0.001$) (Fig. 5A). In agreement with these changes in COX-2 expression, iNOS levels in the hippocampus of diabetic rats increased 3.4-fold compared to control rats ($p < 0.001$), but decreased 1.2-fold following minocycline administration ($p < 0.001$) (Fig. 5B). Clearly, minocycline downregulated oxidative molecules upstream of NF-κB in the hippocampus of diabetic rats.

Minocycline inhibited neuroinflammation in diabetic rats

The upstream and proximal kinases of the NF-κB signal pathway, such as TNF-α, IL-1β, Toll and CD28, lead to the activation of NF-κB [4]. Accordingly, TNF-α and IL-1β levels, which are markers of inflammation [37], were used to explore the effect of minocycline on inflammation. In agreement with findings suggesting that minocycline promotes anti-neuroinflammation in cerebral ischemia and neurodegenerative diseases [10, 38], minocycline decreased TNF-α and IL-1β levels in the hippocampus of diabetic rats. IL-1β levels were elevated from 18.19 ± 5.06 pg/mg in the control rats to 36.32 ± 6.02 pg/mg in diabetic rats ($p < 0.001$), whereas minocycline treatment decreased IL-1β levels from 36.32 ± 6.02 pg/mg in the diabetic rats to 25.48 ± 6.35 pg/mg in diabetic rats treated with mino-

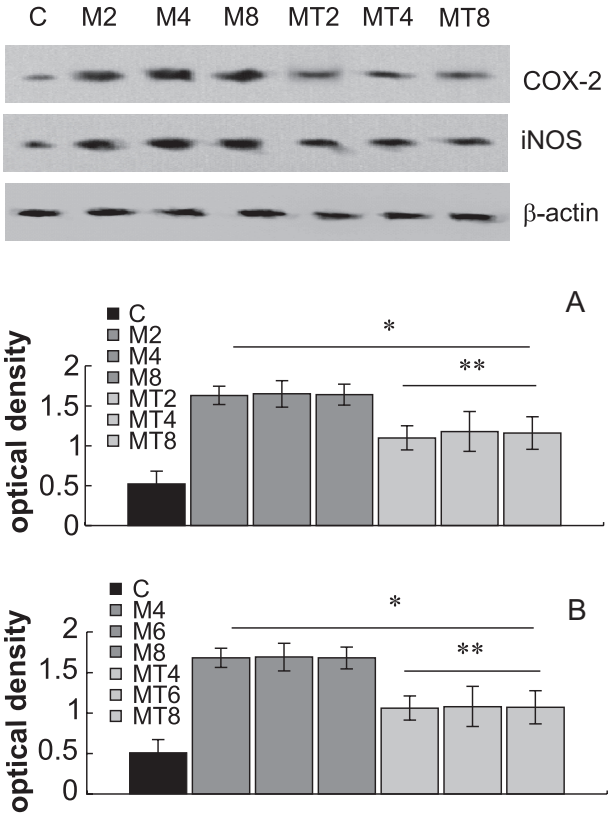

Fig. 5. Analysis of COX-2 (**A**) and (**B**) iNOS expression. Western blot analysis of the protein levels of COX-2 and iNOS, expressed as the densitometry ratio of COX-2 and iNOS to β-actin (mean ± SD). Model rats had higher levels of COX-2 and iNOS than the C group (* $p < 0.01$). COX-2 and iNOS levels in the minocycline-treated groups were significantly lower than in the model groups (** $p < 0.01$). ** $p < 0.01$ *vs.* M and S group

cycline ($p < 0.001$). TNF-α was upregulated from 19.59 ± 6.16 pg/mg in the control rats to 42.43 ± 6.62 pg/mg in the diabetic rats ($p < 0.001$), whereas minocycline treatment decreased TNF-α levels from 42.43 ± 6.62 pg/mg in diabetic rats to 30.44 ± 6.52 pg/mg in diabetic rats treated with minocycline ($p < 0.001$) (Fig. 6A). Through western blot analysis, the TNF-α levels were found to be increased 3.5-fold in diabetic rats compared to controls ($p < 0.001$), but decreased in the hippocampus of diabetic rats by 1.4-fold following minocycline administration ($p < 0.001$) (Fig. 6B). Additionally, western blot analysis showed that IL-1β levels in diabetic rats decreased 1.3-fold following minocycline administration ($p < 0.001$) (Fig. 6B). These results demonstrate that minocycline blocked upstream molecules of the NF-κB signaling pathway to inhibit inflammation in the hippocampus of diabetic rats.

Discussion

STZ, a powerful alkylating agent, can interfere with glucose transporters and glucokinase function and induce double-strand DNA breaks. To determine successful establishment of the DM animal model, a fasting blood glucose test and glucose tolerance test were conducted [68]. Animals that presented with hyperglycemia and uric acid and insulin resistance were classified as successful DM models. In this study, Morris water maze performance showed that cognitive impairment occurred in diabetic rats. The levels of BACE1 and Aβ proteins were increased in the hippocampus of diabetic rats, and oxidative stress and inflammation were present concurrently in DM animals. Minocycline not only decreased expression of β-amyloid protein expression and improved behavioral deficits of diabetic rats but also inhibited NF-κB activation and upstream signal transduction molecules of the NF-κB pathway (mediators of inflammation and oxidative stress) in the hippocampus. Therefore, increases in β-amyloid protein in the hippocampus caused by diabetic metabolic disorder are blocked by minocycline through inhibition of NF-κB pathway activation.

Sustained oxidative stress produced during chronic or acute inflammatory responses to environmental toxicant exposure is cytotoxic and enhances NF-κB activation [25, 29]. Chronic or acute inflammation induced by diabetic metabolism disorder can exacerbate oxidative stress and increase NF-κB activation [16]. There is increasing evidence implicating dysregulation of the NF-κB signaling pathways in the pathology of various diseases, including autoimmune diseases, neurodegenerative diseases, inflammation and cancer [47]. Moreover, several human diseases caused by inherited mutations in the genes encoding NF-κB signaling molecules have been recently described, especially in oxidative stress and inflammation [21, 44]. The signal transduction pathways of NF-κB activation therefore represent potential targets for therapeutic intervention [42]. In this study, minocycline decreased COX-2 and iNOS levels in the hippocampus of diabetic rats. Furthermore, as shown in studies demonstrating an anti-inflammatory effect of minocycline in cerebral ischemia and neurodegenerative diseases, minocycline decreased TNF-α and IL-1β levels in the hippocampus of diabetic rats. Therefore, minocycline represents a possible anti-oxidant and anti-inflammatory agent in the treatment of diabetic metabolism disorder.

Minocycline is a semi-synthetic tetracycline antibiotic that effectively crosses the blood-brain barrier. Minocycline has been reported to have significant neuroprotective effects in cerebral ischemia [71], amyotrophic lateral sclerosis [32], Alzheimer's disease [7, 15], Huntington's disease [65] and Parkinson's disease [1]. Minocycline inhibits brain inflammation, astrocyte reactivation [11], microglial activation [31, 59], oxidative stress, apoptosis and extracellular matrix degradation [67, 70]. One common pathophysiological mechanism of diabetic brain damage is the production of reactive oxygen species, reactive nitrogen, oxidative stress and inflammation. Minocycline downregulated NF-κB, TNF-α, COX-2, iNOS and IL-1β and improved cognitive impairment in diabetic rats. In addition, minocycline downregulated β-amyloid protein. Therefore, minocycline improves cognitive impairment and downregulates β-amyloid protein by inhibiting neuroinflammation and oxidative stress caused by abnormal glucose metabolism in diabetic rats.

In conclusion, minocycline decreases the expression of β-amyloid protein to maintain neural function and improve behavioral deficits due to its inhibition of NF-κB and NF-κB-related molecules, including cytokines and markers of neuroinflammatory damage and oxidative stress. From a clinical standpoint, the ability of minocycline to modulate inflammatory reactions and inhibit oxidative stress responses may be of great impor-

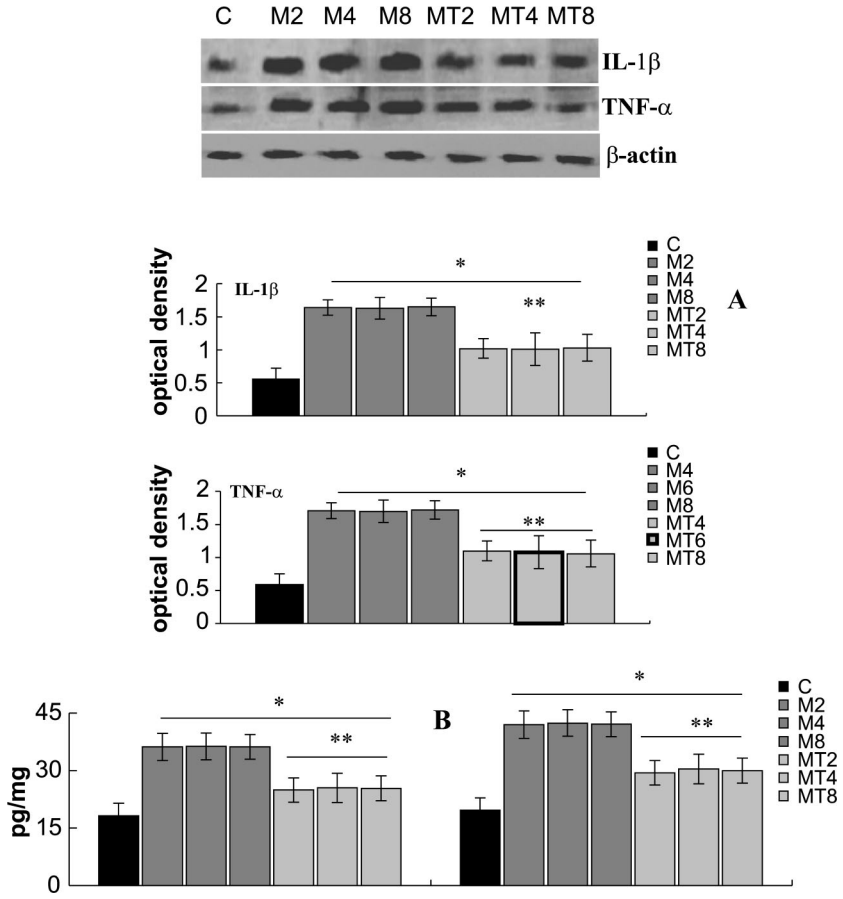

Fig. 6. Analysis of IL-1β and TNF-α expression. (**A**) Western blot analysis of the protein levels of IL-1β and TNF-α in the brains of rats with DM. Relative amounts of IL-1β and TNF-α are expressed as the densitometry ratio of IL-1β and TNF-α to β-actin (mean ± SD). The M group had higher levels of IL-1β and TNF-α expression than the C group (* $p < 0.001$). Additionally, IL-1β and TNF-α levels in the minocycline-treated groups were significantly lower than in the DM model groups. ** $p < 0.01$ *vs.* M and C group. (**B**) Analysis of protein levels of IL-1β and TNF-α by ELISA in rats with DM. Protein levels of IL-1β and TNF-α in DM groups were increased compared to the control group (mean ± SD) (* $p < 0.001$), whereas expression of IL-1β and TNF-α in the minocycline-treated groups were decreased compared to DM model groups (** $p < 0.001$). C – control group; M2 – rats studied 2 weeks after STZ injection; M4 – rats studied 4 weeks after STZ injection; M8 – rats studied 8 weeks after STZ injection; MT2 – rats studied 2 weeks after STZ injection and minocycline treatment; MT4 – rats studied 4 weeks after STZ injection and minocycline treatment; MT8 – rats studied 8 weeks after STZ injection and minocycline treatment

tance in the selection of neuroprotective agents to treat neurodegenerative diseases, especially in the treatment of chronic diseases such as Alzheimer's disease.

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