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Successful abrogation by thymoquinone against induction of diabetes mellitus with streptozotocin via nitric oxide inhibitory mechanism

AbuBakr El-Mahmoudy[a,b], Yasutake Shimizu[b], Takahiko Shiina[b], Hayato Matsuyama[b], Mossad El-Sayed[a], Tadashi Takewaki[b,*]

[a]*Department of Pharmacology, Faculty of Veterinary Medicine, Zagazig University-Benha Branch, 13736 Moshtohor, Egypt*
[b]*Department of Basic Veterinary Science, United Graduate School, Gifu University, Yanagido 1-1, Gifu 501-1112, Japan*

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Abstract

Nitric oxide (NO) is involved in the destruction of β-cells during the development of type I diabetes mellitus (DM). We demonstrated the possibility of rescuing β-cells by intervention with thymoquinone (TQ) using streptozotocin (STZ) rat diabetic model. The hyperglycemic and hypoinsulinemic responses to STZ were significantly abrogated in rats cotreated with TQ, and this abrogating effect has persisted for 1 month after stopping of TQ treatment. Unlike observations recorded after diabetic chronicity of 1month, where there was a significant reduction of both serum and pancreatic nitrites, a significant increase in both nitrites was observed within the first 3 days in STZ rats, with or without lipopolysaccharide (LPS) stimulation, compared with controls and the TQ-cotreated. In vitro production of nitrite was significantly higher by 3-day-diabetic macrophages with or without stimulation compared to control or TQ-treated ones. However, 1-month-diabetic macrophages showed insignificant decrease of nitrite which turned significant upon stimulation. TQ has no effect on either I*k*B degradation or NF-*k*B activation; however, it significantly inhibited both p44/42 and p38 mitogen-activated protein kinases (MAPKs) which contribute to the transcriptional machinery of inducible nitric oxide synthase and NO production. These data emphasize the protective value of TQ against development of type I DM via NO inhibitory pathway.
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An understanding of cellular mechanisms involved in the etiology of type I diabetes mellitus (type I DM; insulin-dependent DM, IDDM) may lead to the development of therapeutic agents to intervene in the disease inflammatory process. The pancreatic β-cells are subjected to various internal and environmental toxins in addition to their programmed cell death. Among these toxic agents is nitric oxide (NO)

* Corresponding author. Tel.: +81 58 293 2938; fax: +81 58 293 2992.
E-mail address: tt@cc.gifu-u.ac.jp (T. Takewaki).

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which has been identified as a primary toxic effector molecule in the lysis of islet cells. This NO is evident to be produced by inflammatory macrophages [1] and the inflamed pancreatic β-cells themselves [2]. In addition, it was suggested by Tanaka et al. [3] that NO produced spontaneously from streptozotocin (STZ) may participate in pancreatic β-cell damage depending on that STZ has a nitrosourea group in its molecule. Furthermore, it has also been shown that peritoneal macrophages isolated from low-dose-STZ-treated mice are activated and produce effector molecules including NO which could participate in the destruction of pancreatic islets [4]. The islet toxic role of NO is evidenced by the fact that *N*-nitro-L-arginine-methylester (NAME), an inhibitor of endothelial and macrophage nitric oxide synthase, partially suppressed the development of diabetes mellitus in low-dose-STZ-induced DM model [5,6].

Thymoquinone (TQ), the active principle of *Nigella sativa* plant oil [7] has been shown to possess a hypoglycemic effect [8] and to suppress the in vitro production of NO and expression of inducible isoform of nitric oxide synthase (iNOS) by lipopolysaccharide (LPS)-activated peritoneal macrophages [9]. However, there is no any available information about the prevention by TQ of the pathogenetic inflammatory process of DM itself. On the basis of this background, therefore, the purpose of the present piece of research is to investigate the hypothesis of protection of β-cell and abrogation of type I DM by intervention with TQ depending on its in vitro NO inhibitory properties. Both in vivo and in vitro assessment of nitrites, as indicator for NO production, have been carried out in STZ model of DM. The site of action of TQ on iNOS transcriptional pathway has been also investigated.

1. Methods

1.1. Animals

Male Wistar rats, 10 weeks of age, specific pathogen free, purchased from Seven (Gifu, Japan) were used for the present investigation. The rats were housed at 22 °C, with a 12-h dark, 12-h light cycle and food and water freely available except during the time of experiments. Care and experimental procedures were approved by Gifu University, Animal Care and Use Committee and were in accordance with Japanese Department of Agriculture guidelines. Freshly prepared STZ (Sigma, St. Louis, MO, USA) was used to induce IDDM (type I diabetes mellitus) as mentioned in the following.

1.2. Experimental protocol

The effect of TQ (Aldrich, Milwaukee, WI, USA; purity 99%) was examined on diabetes development in Wistar rats. Animals were randomly allocated to four experimental groups. The first group, to be diabetic, was injected with small single dose of STZ (45 mg/kg, i.p.) dissolved immediately before administration in saline. The second group, to test the effect of TQ, was injected with TQ (3 mg/kg, i.p.) dissolved in 10% DMSO, then challenged with STZ 30 min later. Administration of TQ (3 mg/kg, i.p., once daily) was continued for further 2 days for total of 3 days. As a standard for NO assessment, the third group was administered L-N^6-(1-Iminoethyl) lysine hydrochloride (L-NIL; a specific iNOS inhibitor; Sigma), at the same dosage of TQ. The fourth group was left as control where it was administered 10% DMSO (the solvent of TQ) and then saline (the solvent of STZ) after 30 min. Ten hours before sacrificing, blood sampling and macrophage collection, some animals (5 out of 10) in each group were injected with LPS (5 mg/kg; Sigma) for activation of macrophages and induction of NO production; this procedure was carried out twice; once on the 3rd and the second on the 30th day of the experiment. The following assessments have been performed.

1.2.1. Blood glucose

Blood glucose levels were measured by glucose test meter (GT 1640, KDK, Kyoto, Japan) using the glucose oxidase method.

1.2.2. Serum insulin

Blood samples were obtained fresh, allowed to clot at room temperature and sera immediately separated by centrifugation at 3000 rpm and stored at −30 °C. Serum insulin levels were determined using a commercially available ELISA insulin kit (Morinaga, Tokyo, Japan) in accordance with the manufacturer's instructions.

1.2.3. Serum nitrite

NO as a free radical is relatively unstable in oxygenated solutions where it is rapidly and spontaneously reacts with molecular oxygen to yield a variety of nitrogen oxides. It was demonstrated that the only stable products formed by spontaneous decomposition of NO in oxygenated solutions are nitrites and nitrates, thus they were measured as indicators for NO production. Serum nitrite content was measured after reduction of nitrates to nitrites with *Aspergillus* nitrate reductase (Sigma) [10]. Nitrite was then measured spectrophotometrically (540 nm, MPR.A4i Microplate Reader, Tosoh, Japan) after mixing equal amounts (100 μl) of serum samples and Griess reagent (solution of 1 part of 1.32% sulfanilamide in 60% acetic acid and 1 part of 0.1% naphthyl-ethylene-diamine HCl) in 96-well plate and incubation for 10 min at room temperature in the dark [10]. Nitrite concentrations were then interpolated from sodium nitrite (Sigma) standard curve (10–250 nM).

1.2.4. Pancreatic nitrite

Equal weights from pancreatic tissues were firstly homogenized and deproteinized. Nitrates in the supernatants were then reduced to nitrites using nitrate reductase; and total nitrites were measured by Griess reaction as described in case of serum.

1.2.5. Macrophage isolation and culturing and in vitro assessment of nitrite

Peritoneal macrophages were isolated from the sacrificed animals as described previously [9]. Cells were washed, re-suspended in fresh Dulbecco's modified Eagle's medium (DMEM), and seeded at 2×10^5 cells in 200 μl per well of 96-well flat-bottom tissue culture plates. Cells were incubated for 2 h at 37 °C, 5% CO_2, in a humidified chamber to allow macrophages to adhere and spread. Non-adherent cells were removed by washing of the wells three times by DMEM and the remaining adherent cells (>97% macrophages as assessed by morphologic examination) were used. The macrophage cells prepared for experimentation from control, STZ, L-NIL+STZ, and TQ+STZ rats were either unstimulated or stimulated with LPS either in vivo (5 mg/kg) or in vitro (1 μg/ml) and incubated for 24 h. Culture-conditioned medium was collected at the appropriate time in fresh plates and stored at −30 °C for assessment of nitrite.

The viability of the cells was examined by using 3-[4,5-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma), which is based on the ability of mitochondrial enzyme, succinate dehydrogenase to cleave MTT to the blue compound formazan as previously described [11]. Insignificant difference in viability was observed among cells of all groups.

1.2.6. Assessment of nitrite in culture supernatants

NO production in culture supernatants was assessed by measuring nitrite using Griess reaction as described above. The medium supernatants were centrifuged before assessment.

1.3. Detection of NF-kappaB p65 activation

Peritoneal macrophage cells were seeded in six-well plates at a density of 5×10^5 cells/ml and pre-treated with TQ (1, 3, and 10 μM) for 30 min, then stimulated with LPS (100 ng/ml) for further 30 min. Supernatants were then removed, and cells were lysed in a lysis buffer containing 20 mM HEPES (pH 7.5), 350 mM NaCl, 20% glycerol, 1% Igepal-CA630, 1 mM $MgCl_2$, 0.5 mM EDTA, and 0.1 mM EGTA. Activation of the p65 subunit of the NFkappaB (NF-*k*B p65) complex was detected by using TransAM NF-*k*B p65 ELISA kit (ActiveMotif, Rixensart, Belgium; *n*=3).

1.4. Western Blotting Analysis for IkappaB and p38 and p44/42 mitogen-activated protein kinases (MAPKs)

Cellular levels of macrophage IkappaBα (I*k*Bα) and MAPKs, namely, p38, phosphorylated p38 (pp38), p44/42, and phosphorylated p44/42 (pp44/42) as well as control α-tubulin proteins were estimated by immunoblotting. Peritoneal macrophage cells were seeded in six-well plates at a density of 5×10^5 cells/ml and exposed to LPS (100 ng/ml) for 15 min (kinases) or 30 min (I*k*B) after pre- or non-treatment with TQ (1, 3, and 10 μM) for 30 min. After removal of the supernatant, the cells were washed with cold phosphate-buffered saline (PBS) three times, then scrapped off, centrifuged (3000 rpm, 5 min, 4 °C) and the pellets were lysed in lysis buffer without dithiothritol (DTT) or bromophenol blue (BPB) but containing 100 μM sodium orthovanadate as phosphatase inhibitor. The protein concentration in cell lysates was determined

using the Bradford protein assay using bovine serum albumin (BSA) as a standard [12]. Before electrophoresis, BPB and DTT (10 mM) were added to the samples which were then denaturized by boiling for 5 min. Equal amounts of proteins (50 μg) from corresponding samples were used for Western Blotting Analysis, where they were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12% running gels with the Laemmli buffer system [13]. Proteins were then transferred to 0.45 μm nitrocellulose membranes (Amersham) by semidry blotting and then blots were blocked by gentle shaking in 5% non-fat dried milk solution for 1 h. Blots were rinsed twice for 5 min Tris-buffered saline (TBS) containing 0.05% Tween-20 and incubated with the primary antibody (rabbit anti-p38 MAPK, SantaCruz, Heidelberg, Germany, 1:500; mouse anti-pp38 MAPK, SantaCruz, 1:100; rabbit anti-p44/42 MAPK, SantaCruz, 1:500; goat anti-pp44/42, SantaCruz, 1:100; rabbit anti-I*k*Bα, SantaCruz, 1:100; and mouse anti-α-tubulin, Sigma, 1:500) diluted in TBS containing 0.1% Tween-20 and 1% BSA for 2 h at 37 °C. Membranes then washed extensively five times (5 min each) using TBS containing Tween-20. Target proteins were detected by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse and anti-goat IgGs (secondary antibody; 1:2000 dilutions, Amersham) for 1 h at 37 °C. Again, blots were washed extensively as described after the 1st antibody. Immunoreactive bands were detected by exposure for at least 3 min to enhanced chemiluminescence (ECL) detection system and developed with Hyperfilm-ECL according to the procedure recommended by the maker (Amersham). Quantification of kinases, I*k*Bα and α-tubulin proteins was carried out by densitometric analysis using Scicon Image beta 4.02 software (Scicon, Maryland, USA). All blots were repeated at least three times from three different experiments.

1.5. Statistical analysis

Data were expressed as means±S.E. Statistical analysis was performed by the Student's *t*-test to express the difference between two groups. Multiple comparisons were carried out using one-way analysis of variance (ANOVA) followed by Dunnett's test. Immunoblotting experiments were done at least three times with similar results. Results in which the *P* value is <0.05 were considered significantly different.

2. Results

2.1. Characteristics of experimental animals

Before administration of indicated agents, all groups of rats presented practically the same degree

Table 1
Ages, body weights, fluid intakes, food intakes, and urine volumes of control, streptozotocin-diabetic (STZ) rats; those cotreated with L-N^6-(1-Iminoethyl) lysine hydrochloride and STZ (L-NIL+STZ); and those cotreated with thymoquinone and STZ (TQ+STZ) rats

	Control		STZ		L-NIL+STZ		TQ+STZ	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Age (week)	10	14	10	14	10	14	10	14
Body weight (g)	308.75±4.89	368.25±6.75	303.50±5.81	195.00±10.94**	310.00±4.98	350.75±4.46	305.±7.81	358.50±7.76
Water intake (ml/24 h)	32.14±2.14	36.28±3.93	97.14±5.23**	152.14±7.78**	33.65±5.54	51.24±5.44*	35.38±4.97	48.98±3.88*
Food intake (g/24 h)	25.32±3.65	28.76±3.55	29.15±4.67*	42.94±5.68**	26.47±3.66	29.57±3.75	26.38±3.78	29.13±2.99
Urine volume (ml/24 h)	12.56±2.12	14.51±2.23	35.50±6.13**	46.32±5.12**	13.37±2.03	20.24±4.07*	16.84±2.09	18.35±3.23*

Initial: Results recorded 3 days after start of experiment.
Final: Results recorded 30 days after start of experiment.
Values are means±S.E.M. of 10 observations in each group.
* Significantly different from corresponding control ($P<0.05$).
** Significantly different from corresponding control ($P<0.01$).

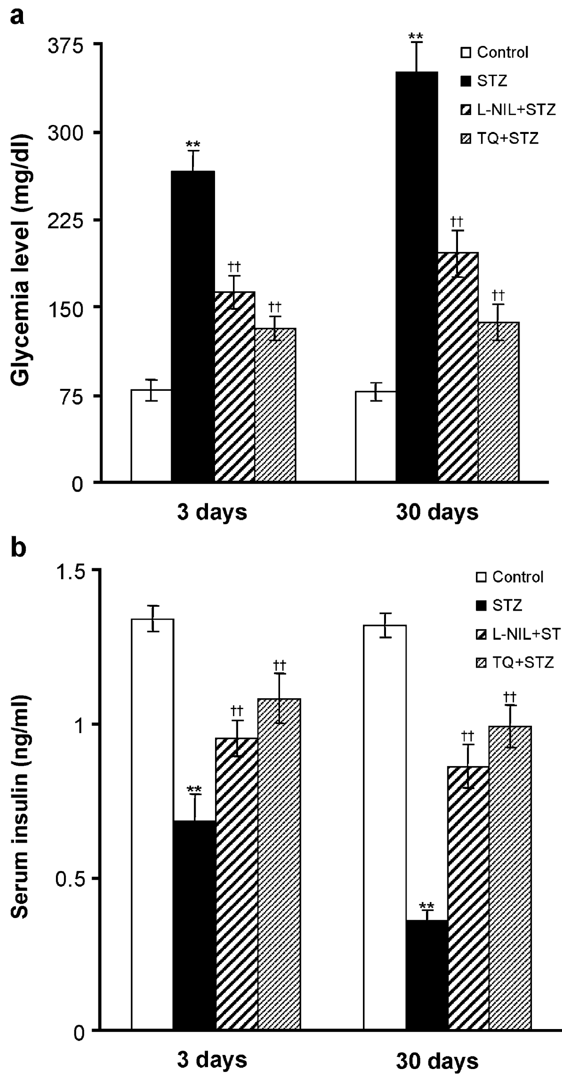

Fig. 1. (a) Glycemia levels of control and streptozotocin (STZ; 45 mg/kg, i.p. single dose on the day 1) diabetic rats, injected or not with the iNOS inhibitor, L-NIL (3 mg/kg on days 1, 2, and 3) or thymoquinone (TQ; 3 mg/kg on days 1, 2, and 3). Blood samples were taken after 3 and 30 days from start of the experiment and glucose levels were directly measured using a glucometer. **$P<0.01$ vs. control; ††$P<0.01$ vs. STZ. (b) Serum insulin levels of control and streptozotocin (STZ; 45 mg/kg, i.p. single dose on the day 1) diabetic rats, injected or not with the iNOS inhibitor, L-NIL, (3 mg/kg on days 1, 2, and 3) or thymoquinone (TQ; 3 mg/kg on days 1, 2, and 3). Serum samples were taken after 3 and 30 days from start of the experiment and insulin levels were measured by ELISA. **$P<0.01$ vs. control; ††$P<0.01$ vs. STZ.

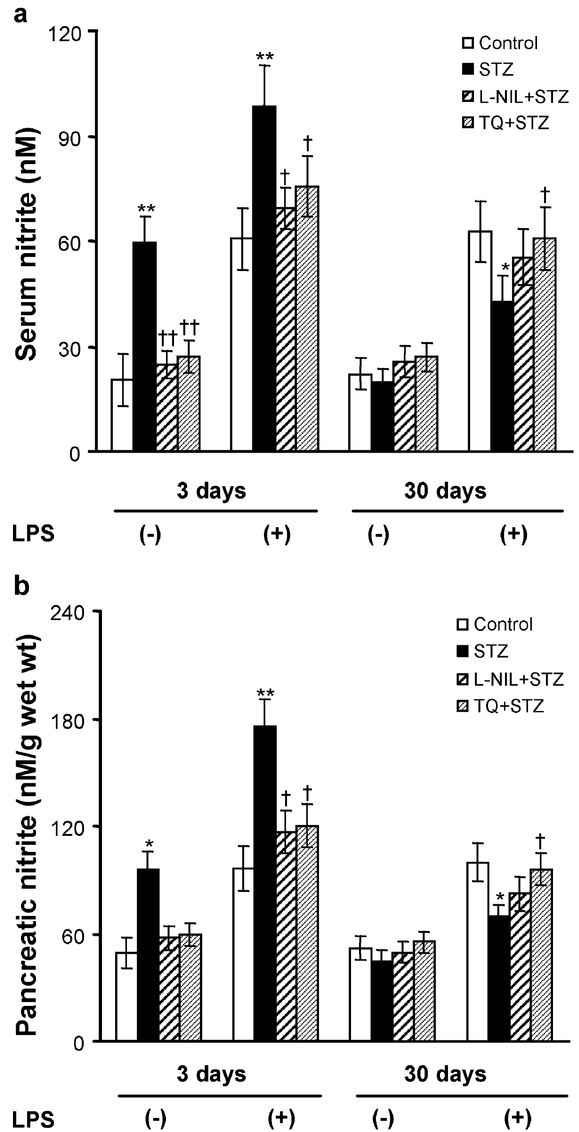

Fig. 2. (a) Serum nitrite levels of control and streptozotocin (STZ; 45 mg/kg, i.p. single dose on the day 1) diabetic rats, injected or not with the iNOS inhibitor, L-NIL (3 mg/kg on days 1, 2, and 3) or thymoquinone (TQ; 3 mg/kg on days 1, 2, and 3). Serum samples were taken after 3 and 30 days from start of the experiment and nitrite levels were measured by Griess reaction. *$P<0.05$ and **$P<0.01$ vs. control; †$P<0.05$ and ††$P<0.01$ vs. STZ. (b) Pancreatic nitrite levels of control and streptozotocin (STZ; 45 mg/kg, i.p. single dose on the day 1) diabetic rats, injected or not with the iNOS inhibitor, L-NIL, (3 mg/kg on days 1, 2, and 3) or thymoquinone (TQ; 3 mg/kg on days 1, 2, and 3). Equal weights from pancreata were taken after 3 and 30 days from start of the experiment, then homogenized, deproteinized and nitrite concentrations were measured in the homogenates using Griess reaction. *$P<0.05$ and **$P<0.01$ vs. control; †$P<0.05$ vs. STZ.

of physico-metabolic symptoms. However, it can be seen from Table 1, at the end of the 30 days, the body weight of the untreated diabetic rats was found to be significantly decreased compared with control rats. Untreated diabetic rats also had elevated food and fluid intakes as well as urine volume over those of control ones. Cotreatment of STZ-injected rats with TQ or L-NIL resulted in insignificant alterations in weight gain and food intake from controls. However, at the end of the experiment, this cotreatment could not prevent slight significant increases in both water intake and urine volume.

2.2. Glycemia levels

Fig. 1a shows the results of cotreatment effects of TQ on STZ-induced hyperglycemia in rats. Overnight fasting blood glucose levels of the control group were within normal range, whereas in the group administered STZ, the blood glucose levels were markedly elevated on the 3rd day and were gradually increasing by time along the 1-month experimental course. TQ caused highly significant inhibition of STZ-induced hyperglycemia which persisted even after stopping of TQ treatment. Interestingly, the inhibitory effect of TQ (50.6%) was greater than that (38.9%) of the used standard NO inhibitor, L-NIL. The administration of either L-NIL or TQ only did not influence normoglycemia.

2.3. Serum insulin levels

Fig. 1b illustrates the profile of serum insulin concentration of control rats, those injected with STZ alone and rats treated with TQ/L-NIL and challenged with STZ. There was no difference in the basal serum insulin concentration before any treatment among all groups. As expected, intraperitoneal injection of STZ was associated with time-dependent inhibition of serum insulin level. Intervention with TQ or L-NIL resulted in prevention of such inhibition of serum insulin with more significant preventive effect in case of the former.

2.4. Serum nitrite levels

Mean serum nitrite concentrations in control, STZ, L-NIL+STZ, and TQ+STZ administered rats

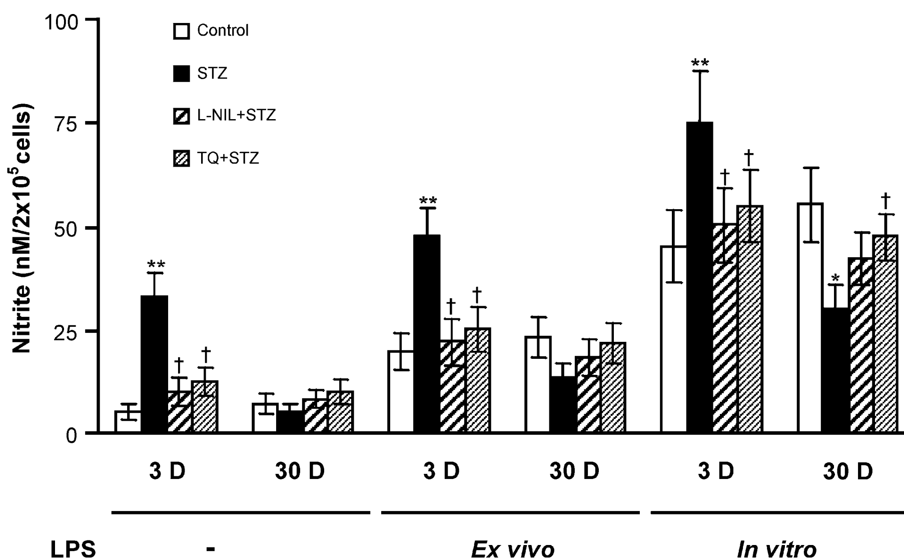

Fig. 3. In vitro NO release from peritoneal macrophages in control and streptozotocin (STZ; 45 mg/kg, i.p. single dose on the day 1) diabetic rats, injected or not with the iNOS inhibitor, L-NIL (3 mg/kg on days 1, 2, and 3) or thymoquinone (TQ; 3 mg/kg on days 1, 2, and 3). Peritoneal macrophage cells were collected 3 and 30 days from the start of the experiment, cultured, and nitrite was measured by Griess reaction in the cell culture supernatants after 24 h of incubation in the absence or presence of ex vivo or in vitro lipopolysaccharide (LPS) stimulation (5 mg/kg and 1 μg/ml, respectively). *$P<0.05$ and **$P<0.01$ vs. control; †$P<0.05$ vs. STZ.

are presented in Fig. 2a. In unstimulated condition, serum nitrite concentration was significantly increased in diabetic rats within the first 3 days after STZ injection. However, there was a tendency to decrement of serum nitrite with the chronicity of diabetic course. After 1 month, serum nitrite was insignificantly lower than that of corresponding control, while L-NIL- and TQ-cotreated rats showed insignificant differences in comparison with the control. Induction of NO production by intraperitoneal injection of LPS resulted in elevation of serum nitrite in all groups of rats within the first 3 days of the experiment. When the LPS-stimulated nitrite levels were evaluated together, a highly significant increase in diabetic rats was found, while there were insignificant differences between control and cotreated groups. One month data, in contrast, showed significant decrease in serum nitrite level of diabetic rats in comparison with corresponding control. Again, cotreated rats showed insignificant differences from control.

2.5. Pancreatic nitrite levels

For more evaluation of NO production and TQ action, we have measured, in addition, the generation of nitrites in pancreatic tissue from control, STZ, L-NIL+STZ, and TQ+STZ rats. Fig. 2b shows that nitrites were significantly higher in the pancreatic homogenates from diabetic than control rats within the first 3 days of the experiment even without LPS stimulation. L-NIL and TQ were able to prevent the increase due to the diabetic condition. After chronicity of 1 month, pancreatic nitrites become insignificantly lower in comparison with control and cotreated animals. Pancreatic nitrite levels from LPS-stimulated rats showed parallel results, yet, the decrease in nitrite from diabetic pancreata become significant.

2.6. In vitro assay of macrophage-derived nitric oxide

As shown in Fig. 3, unstimulated rat peritoneal macrophages released negligible amounts of nitrite. Ex vivo or in vitro stimulation of macrophage cells with LPS (5 mg/kg and 1 μg/ml, respectively) resulted in release of relatively large amounts of nitrite. The levels of nitrite in culture supernatants were significantly higher in 3-day-diabetic macrophages either in unstimulated or stimulated conditions. After 30 days, there was no significant difference between nitrite levels in culture supernatants of unstimulated diabetic macrophage and those of the corresponding control cells. However, ex vivo and in vitro stimulation of cells showed insignificant and significant decreases, respectively, in NO release when compared with the corresponding stimulated control cells. Macrophages derived from TQ-treated rats showed insignificant changes compared with control cells. However, in comparison with cells isolated from STZ-rats, it could be stated that TQ was able to prevent the enhanced release of NO by macrophages due to the diabetic condition. Thirty-day data showed that there were

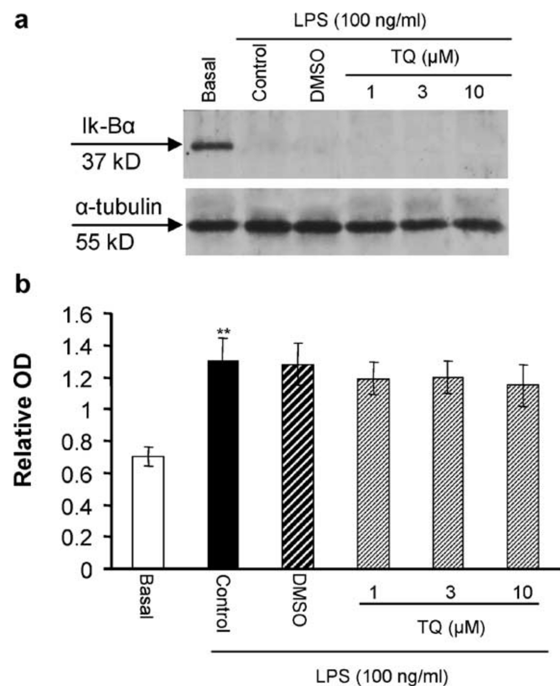

Fig. 4. Effects of thymoquinone (TQ) on LPS-induced activation of p65 NF-*k*B and degradation of I*k*B in peritoneal macrophages. Cells were either pretreated with TQ (1, 3, and 10 μM) for 30 min or not and then incubated with LPS (100 ng/ml) for further 30 min. The protein samples prepared from the cells were then probed for p65 NF-*k*B activation using ELISA or I*k*B degradation using Western Blotting Analysis as described in the Methods. TQ was found not to inhibit either LPS-induced I*k*B degradation (a) or p65 NF-*k*B activation (b).

insignificant changes between TQ+STZ- and STZ-only-treated rats regarding nitrite accumulation in culture supernatants of unstimulated macrophages. However, after in vitro stimulation with LPS, the significant decrease of nitrite performed by STZ-rats was not evident in TQ-cotreated group. As a standard, L-NIL inhibited nitrite production which showed insignificant changes from control in all concerned groups.

2.7. Effect of thymoquinone on NF-kappaB activation and IkappaB degradation

The possibility that the inhibitory effect of TQ on NO/iNOS synthesis is due to the inhibition of the I*k*B degradation induced by LPS has been investigated. The macrophage cells were pretreated with TQ (1, 3, or 10 μM) for 30 min, before adding LPS (100 ng/ml) and incubation for further 30 min. As expected, LPS

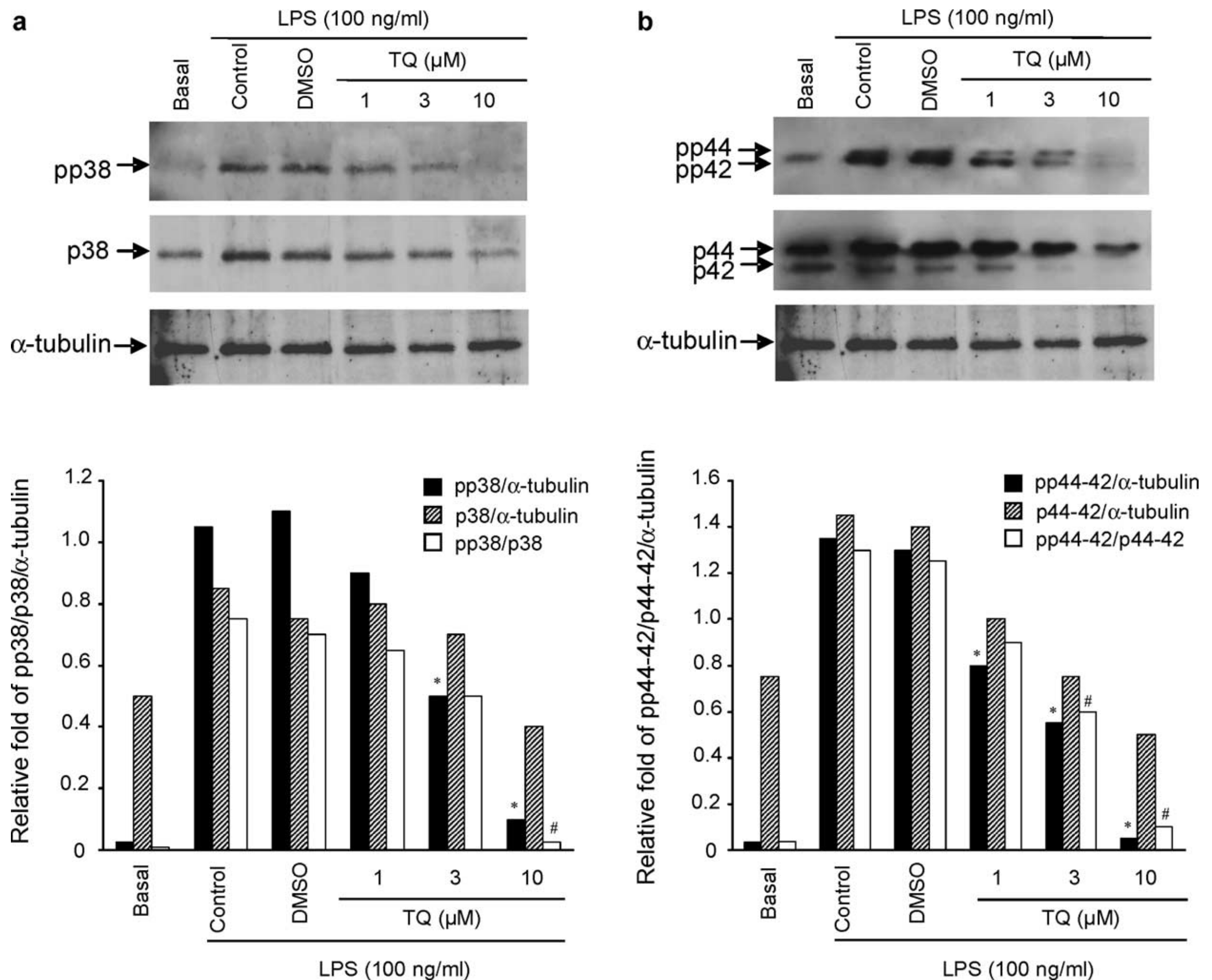

Fig. 5. Immunoblot analyses of cytoplasmic extracts of macrophages left untreated or treated with lipopolysaccharide (LPS; 100 ng/ml) in the absence or presence of thymoquinone (TQ; 1, 3 or 10 μM) for 15 min. Cell lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes. Phosphorylation of p38 MAPK and p44/42 MAPK was analyzed using anti-phospho-p38 MAPK (pp38) and anti-phospho-p44/42 MAPK (pp44/42) antibodies. The same samples were probed for unphosphorylated forms of p38 MAPK (p38) and p44/42 MAPK (p44/42) as well as for α-tubulin expressions. The lower panels show band intensities quantified by densitometer. Total and LPS-phosphorylated p38 (a) and those of p44/42 (b) were inhibited by TQ in a dose-dependent manner. *$P<0.05$ vs. α-tubulin; #$P<0.05$ vs. unphosphorylated forms. Intensities of p44 and p42 were summated and compared as one unit.

induced significant degradation of I*k*B which has not been prevented by TQ (Fig. 4a).

The p65 subunit is a part of the NF-*k*B complex activated by LPS [14]. To investigate whether TQ prevents NF-*k*B activation by binding to the p65 subunit of NF-*k*B complex, peritoneal macrophages have been treated with TQ (1, 3, and 10 μM) for 30 min before stimulation with LPS (100 ng/ml) for further 30 min. Cells stimulated with LPS have shown significant increase in activated NF-*k*B p65 levels when compared with the resting ones. Unexpectedly, TQ have been found not to inhibit such activation induced by LPS (Fig. 4b).

2.8. Effect of thymoquinone on p38 and p44/42 mitogen-activated protein kinases

MAP kinases have been shown to be a part of the signal transduction pathways leading to iNOS expression [15]. Therefore, in the following experiment, we investigated whether TQ-induced inhibition of NO/iNOS synthesis is due to an interference with a MAP kinase(s). Macrophage cells have been pretreated with TQ (1, 3, and 10 μM) for 30 min before adding LPS for further 15 min as phosphorylation of MAP kinases appeared to peak after 10–15 min and then starts to decline. LPS resulted in phosphorylation of both kinases as well as up-regulation of their constitutive un-phosphorylated forms. These activating responses on p38 (Fig. 5a) and p44/42 (Fig. 5b) MAP kinases were not affected by DMSO, the vehicle TQ. However, the latter exhibited significant inhibitory effect on the activating response mediated by LPS on them. The inhibition of MAP kinases activation by TQ seems to be not only due to inhibition of LPS-induced phosphorylation but also due to inhibition of MAPK expression and de novo synthesis as the total p38 and p44/42 MAP kinases were also reduced in lanes of TQ-treated cells.

3. Discussion

NO and proinflammatory cytokines as interleukin-1β and tumor necrosis factor-α have been implicated as immunological effector molecules that mediate β-cell dysfunction and lysis associated with IDDM. NO that is constitutively released at low levels acts as signaling molecule that mediates several physiological responses, including vasodilatation, inhibition of platelet aggregation [16] and, interestingly, glucose-mediated insulin secretion [17]. In contrast, much large quantities that are induced by cytokines and endotoxins mediate cytostatic and cytotoxic effects including those associated with β-cell destruction and pathogenesis of IDDM [2]. More than one source for NO release can be considered to contribute to destruction of pancreatic islets: β-cell itself and resident and infiltrating macrophages; and, if the diabetic condition is experimentally induced by injection of STZ, the latter could be also an additional spontaneous releasing source for NO [3] either by itself or via activation of macrophage cells [4]. More than one mechanism can underline β-cell cytotoxicity mediated by NO. Studies of Drapier et al. [18] and Stadler et al. [19] indicated that NO causes destruction of iron–sulfur centers of iron-containing enzymes and results in an impairment of mitochondrial function and DNA synthesis. Stassi et al. [20] suggested that NO mediates upregulation of Fas, an apoptosis-inducing surface receptor involved in controlling tissue homeostasis and function, which further contributes to pancreatic β-cell damage in IDDM. Undoubtedly, understanding such cellular mechanisms involved in the etiology and pathogenesis of IDDM will be of great value in designing safe therapies that target pro-inflammatory pathways without inhibiting endogenous regulation. In spite of the extensive literature on and extensive use of *N. sativa* oil in treatment of DM [21], there is no report about the possible protective effect of the oil or its major component, TQ, against the process of pathogenesis of DM itself. Classically, investigations on DM in all stages were mostly confined to glucose–insulin circle; however, the establishment of the macrophage and other immune cells' contribution to β-cell dysfunction; and what is recently reported by El-Dakhakhny et al. [8] that the hypoglycemic effect of *N. sativa* oil, TQ, and nigellone may be mediated by yet-unidentified extrapancreatic actions, as well as the finding out the in vitro macrophage-derived NO inhibitory potential of TQ [9], has shifted the light focus in the present study toward the NO pathway intervention by TQ as a trial for controlling DM. Data of the present study showed clearly that STZ-induced diabetes in rats can be abrogated by cotreatment with TQ where

there was a reduction of the hyperglycemic response with preserved serum insulin levels. The response has persisted for 1 month even after stopping the administration of TQ on the 3rd day, indicating that it is mediated not only due to the hypoglycemic effect of the agent, but also, and more importantly, due to blocking the pathogenic effect of injected STZ and consequent inflammatory process toward insulin secreting cells. The anti-diabetic response of TQ was associated with decrement of the elevated production of NO induced in the early diabetic stage either in the absence or presence of LPS stimulation both in vivo and in vitro. NO assessments have been carried out in serum, pancreatic tissue and macrophage cultures. It was found that there were elevations in all nitrite levels in the early stage of diabetes that were almost normalized by TQ. These data indicate that the protection of β-cells by TQ could be mediated, at least partly, via decreasing release of NO from macrophage cells as a major factory for NO and probably from other cells including islet cell themselves.

Trials have been performed to locate the site of action of TQ as NO inhibitory agent. In a previous report [9], we have proven TQ ability to suppress iNOS protein synthesis and iNOS mRNA expression in rat LPS-stimulated peritoneal macrophage cells. However, its effects on the intracellular molecules leading to this final step remained to be investigated. The transcription factor NF-*k*B has been reported to be involved in iNOS production [22,23]. It was strongly activated by cytokines and endotoxins like LPS. Different mechanisms of interference with the NF-*k*B pathway have been suggested to be exhibited by anti-inflammatory agents such as binding and inhibition of I*k*B kinase [24,25] or direct binding to cysteine 38 of p65 protein of the NF-*k*B complex as shown by Gracia-Pineres et al. [26]. The present data show that TQ inhibits iNOS expression and NO release most likely neither by inhibiting I*k*B degradation as described by Kwok et al. [24] nor by preventing LPS-induced NF-*k*B (p65) activation as described by Gracia-Pineres et al. [26].

Feng et al. [27] reported that p44/42 and p38 MAP kinases are key molecules in the LPS induction of iNOS expression. He found that PD98059 (p44/42 MAPK antagonist) and SB203580 (p38 MAPK antagonist) have partially and completely blocked LPS induction of iNOS, respectively indicating the contributing roles of both kinases for NO production. The present findings revealed that TQ has the ability to not only reduce the LPS-induced phosphorylation of such kinases but also to reduce their expressions since the total MAP kinases also have been shown to be reduced by pretreatment with TQ. This result may be consistent with what has been reported by Chakravarty [28] that inhibition of the induced-histamine release from peritoneal mast cells by nigellone (the polymer of TQ) may be attributed partly to an inhibition of protein kinase C (PKC). He stated this conclusion depending on the fact that the induction of histamine release by PKC inducers, TPA, and DAG was partially inhibited by nigellone. PKC is an activator for p44/42 which is involved in the NO/iNOS production.

Although it was suggested that NF-*k*B is the upstream of p38 and p44/42 MAPKs activation, its dependence on the activations of MAP kinases for iNOS induction is controversial. Some reports indicated that NF-*k*B and p38 MAPK seem to lie on two distinct pathways where inhibition of p38 does not interfere with the activation of NF-*k*B [27,29,30]. However, NF-*k*B may represent a second independent pathway for iNOS production as it has been reported to be important for iNOS transcription, since the pretreatment of rat alveolar macrophages and glomerular mesengial cells with pyrrolidine dithiocarbamate, an inhibitor of NF-*k*B activation, completely blocked iNOS transcription [31,32]. Moreover, the anti-inflammatory actions of some medicinal plants have been attributed to inhibition of NF-*k*B/I*k*B activation pathways in several pieces of research work including the inhibition of iNOS in monocytic cells [33,34]. It could be stated that the responses of the MAP kinases and NF-*k*B are fast and could both induce independently one or two separate genes, yet to be defined, whose products may lead to induced iNOS transcription. The present results suggest that TQ has no effect on activation of the NF-*k*B, but its inhibitory effect on NO/iNOS system may be mediated, at least partly, through an inhibition of p38 and p44/42 MAPK activation which is a novel finding to be reported about TQ. It may be speculated that TQ may interfere with other transcription factors like AP-1 which is the upstream of p44/42 MAP kinase.

From the other aspect of view, relatively chronic diabetic animals showed decreases in all nitrites which could be explained on the basis of macrophage dysfunction that occurs as a complication due to the long exposure of macrophage cells to the hyperglycemic state with consequent formation of advanced glycation end products (AGEs) resulting from conjugation of persistently high glucose with proteins; macrophages as scavenger cells possess a number of receptor species capable of binding to and internalizing such products which are resistant to degradation [35]. Another opinion was reported by Doxy et al. [36] that hyperlipidemia may be more important than hyperglycemia relative to the development of many diabetic complications including macrophage dysfunction. Lipids may interact directly with the macrophage cell membrane, interfering with membrane bound receptors and enzyme systems altering macrophage gene expression for cytokines and essential polypeptide growth factors. Such decreases were not observed in TQ+STZ group; may be logically due to blocking of the causative diabetic condition through β-cell protection. Although the dramatic β-cell protective effect exhibited by TQ is not that simple to be definitely explained on one basis, it could be simplified collectively as follows; injected STZ has been reported to accumulate in the pancreatic islets [37] where it may cause damage of pancreatic islets through production of NO and other reactive OH and ROO radicals [38,39]. However, complete damage of all islets is not the case after STZ injection, especially when the injected dose of STZ is relatively small. We hypothesize that the partially damaged cells release intracellular proteins which act as autoantigens that initiate mononuclear cellular infiltration and the cascade of immunological inflammatory process in the islets (insulitis) with final result of more development of DM. Data listed in the present study together with other investigators' data indicate that TQ may produce its protective effects at both stages of the development of DM, where it could reduce the severity of the toxic stage through its antioxidant and free radical scavenging properties [40,41]. In addition, the immunological inflammatory stage can be calmed by TQ both indirectly as a consequence to the reduced severity of the toxic phase and reduction of the amount of autoantigens released; and directly through TQ modulatory effect on macrophage cells and inhibition of activated iNOS/NO system that occur during the pre- and early diabetic condition and thus limiting the disease at a mild state with consequent maintenance of macrophage function.

In conclusion, the results of the present study suggest that TQ may be effective for β-cell protection and prevention of IDDM initiated after injection of a small dose of STZ, apparently by downregulation of immunological inflammatory activity towards β-cells mediated by NO. Although the clinical application of TQ in control and therapy of IDDM needs detailed clinical studies, the beneficial effects documented with TQ in the present study emphasize the value of TQ and/or future drugs with TQ-like activity.

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