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Tomatidine inhibits iNOS and COX-2 through suppression of NF-κB and JNK pathways in LPS-stimulated mouse macrophages

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Abstract **We use the LPS-stimulated macrophage as a model of inflammation to investigate the anti-inflammatory effects of tomatidine and solasodine, whose structures resemble glucocorticoids. We found that tomatidine exhibited a more potent anti-inflammatory effect than solasodine. Tomatidine could decrease inducible nitric oxide synthase and cyclooxygenase-2 expression through suppression of I-κBα phosphorylation, NF-κB nuclear translocation and JNK activation, which in turn inhibits c-jun phosphorylation and Oct-2 expression. Here, we demonstrate that tomatidine acts as an anti-inflammatory agent by blocking NF-κB and JNK signaling, and may possibly be developed as a useful agent for the chemoprevention of cancer or inflammatory diseases.**

Keywords: Tomatidine; iNOS; COX-2; NF-κB; JNK; AP-1; Oct-2

1. Introduction

Inflammation is involved in numerous diseases, such as chronic inflammatory disease and the development of cancer [1–3]. Many stimuli can activate inflammatory leukocytes, such as macrophages, resulting in the induction and synthesis of proinflammatory proteins and enzymes. The release of large amounts of proinflammatory and cytotoxic nitric oxide (NO) and prostaglandins (PGs) has been associated with many inflammatory conditions, through the activity of their inducible enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [1–4]. The macrophage inflammatory response induced by the bacterial endotoxin lipopolysaccharide (LPS) activates several intracellular signaling pathways including the Iκ-B kinase (IKK)–NF-κB pathway and three mitogen-activated protein kinase (MAP kinase) pathways: extracellular signal-regulated kinase (ERK) 1 and 2, c-jun N-terminal kinase (JNK) and p38 [5]. In addition, octamer (Oct) factors are another class of transcription factors that play a central role in the immune system [6]. In a recent study, it was shown that the transcription factor Oct-2 binds to the octamer motif in the iNOS promoter [7].

Many plants in the Solanaceae family, such as tomatoes, potatoes and eggplant, possess steroidal alkaloids based on a C_{27} cholestane skeleton, such as tomatidine and solasodine. These compounds are essentially nitrogen analogues of steroid saponins such as diosgenin, which is a precursor of steroidal hormones and anti-inflammatory steroids [8]. Steroidal alkaloids and their glycosides are known to possess a variety of biological activities, including antifungal and antibacterial actions [9,10]. However, understanding of the anti-inflammatory effects of steroidal alkaloids is very limited. The structure of steroidal alkaloids is similar to that of glucocorticoids. In addition, corticoids mainly inhibit inflammatory responses requiring iNOS-mediated NO production and COX-2 expression. Here, we have investigated whether the steroidal alkaloids tomatidine and solasodine could also inhibit expression of the inflammatory enzymes iNOS and COX-2 in LPS-stimulated macrophages, and we have examined the underlying mechanism of action.

2. Materials and methods

2.1. Agents

Fig. 1 illustrates the chemical structures of tomatidine, solasodine and diosgenin, all of which were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Tomatidine was dissolved in DMSO, and the concentration was adjusted to 50 mmol/L. Solasodine and diosgenin were dissolved in 100% ethanol, and the concentration was adjusted to 25 mmol/L, as stock solutions.

2.2. Cell culture

RAW 264.7 cells, derived from murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA), penicillin (100 units/mL), and streptomycin (100 μg/mL; Gibco) in a 5% CO_2 atmosphere at 37 °C in a humidified incubator. For all assay, cell was plated at 2×10^5 cells/cm^2 in culture dishes or plates. Treatment with vehicle (0.1% DMSO or 0.1% ethanol), test compounds and/or LPS was carried out under serum-free conditions.

2.3. Nitrite assay

The nitrite concentration in the RAW 264.7 cell culture medium was measured as an indicator of NO production, according to the Griess reaction [11]. Briefly, the cells were treated with the test compounds for 1 h before the addition of LPS, and the cells were further incubated for 24 h. The isolated supernatants were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min; absorbance of the mixture at 550 nm was determined with a Dynatech MR-7000 enzyme-linked immunosorbant assay plate reader (Dynatech Labs, Chantilly, VA).

2.4. PGE_2 assay

The medium from cultured RAW 264.7 cells was collected for the determination of prostaglandin E_2 (PGE_2) concentrations by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. Briefly, the

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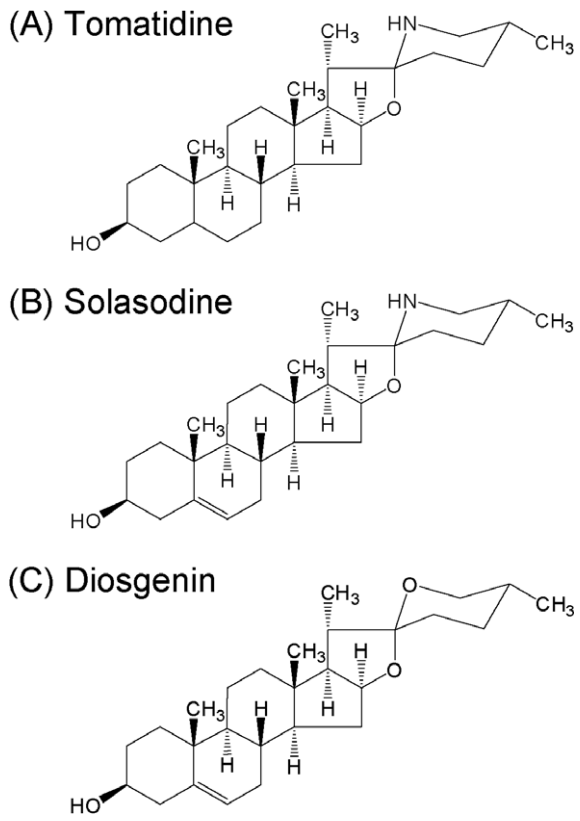

Fig. 1. The chemical structures of tomatidine, solasodine and diosgenin.

cells were treated with the test compounds for 1 h before the addition of LPS, and the cells were further incubated for 16 h. Cultured medium was incubated in goat anti-mouse microplate coated with a goat anti-mouse PGE_2 with PGE_2-conjugated to horseradish peroxidase (HRP) with red dye for 2 h at room temperature. The plate was emptied and rinsed five times with wash buffer contained in the kit. And then, 200 μL of substrate reagent was added to each well and incubated for 30 min at room temperature. The developed plate was read at 450 nm and the PGE_2 concentration of each sample was determined according to the standard curve.

2.5. Western blot analysis

Details of the procedure for immunoblotting have been described previously [12]. Immunoreactive protein bands were visualized using a chemiluminescent substrate and quantitated using the Bio-Rad (Hercules, CA, USA) PDQuest Image software and normalized against bands for either β-actin or α-tubulin (Sigma Chemical Co.). The following antibodies were used: anti-iNOS, anti-NF-κB and anti-Oct-2 (Santa Cruz Biotechnology, CA, USA), anti-COX-2 (BD Transduction Laboratories, San Jose, CA, USA). Anti-I-κBα and anti-phospho-I-κBα, and anti-MAPKs and anti-phospho-MAPKs antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

2.6. Analysis of p65/DNA binding

Cells were serum-starved for 2 h, followed by incubation without and with the indicated concentration of Td for 1 h and then LPS for an additional 30 min. After treatment, nuclear extracts were prepared [13] and DNA binding activity was quantified using the ELISA-based Trans-AM™ NF-κB p65 Kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's protocol. Briefly, nuclear extracts (5 μg of protein per well) were incubated in 96-well plates coated with immobilized oligonucleotide, containing a NF-κB consensus binding site. NF-κB binding to target oligonucleotides was detected by incubation of samples with primary antibodies against the p65 subunit provided with the kit. For quantification of activity, optical densities were measured at 450 nm with a microplate reader.

2.7. Statistics

Statistical analysis was based on the standard error of the mean, and *P* values determined using an independent sample, two-tailed Student's *t*-test assuming equal variances.

3. Results

3.1. Effect of tomatidine, solasodine and diosgenin on nitric oxide production and iNOS protein expression in LPS-stimulated RAW 264.7 macrophage cells

To investigate the effect of tomatidine, solasodine and diosgenin on NO production, we measured the accumulation of nitrite, the stable metabolite of NO, in culture media using Griess reagent. We examined the inhibition of NO production in treatment of RAW 264.7 cells with test compounds either prior to stimulation with LPS (pretreatment) or with LPS at the same time (co-treatment). The preliminary results indicated that the pretreatment protocol was more effective on inhibiting NO production. Therefore, in this study we used the pretreatment of the cells with test compounds and then challenged with LPS in all experiments. As shown in Fig. 2A, LPS evoked an eight-fold increase of nitrite production compared with the naïve control, and this induction was inhibited in a dose-dependent manner by treatment with the test compounds. In similar treatments, tomatidine, solasodine and diosgenin (40 μmol/L) showed 66%, 22% and 41% inhibition of nitrite production, respectively. The iNOS protein was barely detectable in unstimulated cells but markedly increased after LPS treatment, and tomatidine caused dose-dependent inhibition of LPS-induced iNOS expression (Fig. 2B). These results indicated that tomatidine have a more potent inhibitory effect than diosgenin and solasodine.

3.2. Effect of tomatidine on PGE_2 production and COX-2 protein expression in LPS-stimulated RAW 264.7 cells

To characterize further the role of tomatidine in anti-inflammatory events, we studied the expression of another important proinflammatory enzyme, COX-2. PGE_2 is a major product of the metabolism of arachidonic acid via the COX pathway in macrophages exposed to stimuli that elicit immediate responses [14]. As shown in Fig. 3A and B, COX-2 and PGE_2 were barely detectable in unstimulated cells, but markedly increased after LPS treatment. Tomatidine suppressed the COX-2 protein level in a dose-dependent manner (Fig. 3A), and PGE_2 production was decreased by tomatidine (Fig. 3B). These results indicated that tomatidine could modulate inflammatory effects through inhibition of the COX pathway.

3.3. Effect of tomatidine on LPS-induced degradation of I-κBα and activation of NF-κB

To clarify the mechanism of action of tomatidine in the inhibition of the LPS-induced expression of iNOS and COX-2, the effects of tomatidine on LPS-induced degradation of I-κBα and activation of NF-κB were examined. Treatment of cells with tomatidine for 1 h before the addition of LPS inhibited the LPS-induced NF-κB nuclear translocation (Fig. 4A). Because p65 is the major component of NF-κB in LPS-stimulated macrophages, we determined the effect of tomatidine on p65 DNA-binding activity. In the presence of tomatidine at 10–40 μmol/L, the binding activity of NF-κB was suppressed in a dose-dependent manner (Fig. 4B).

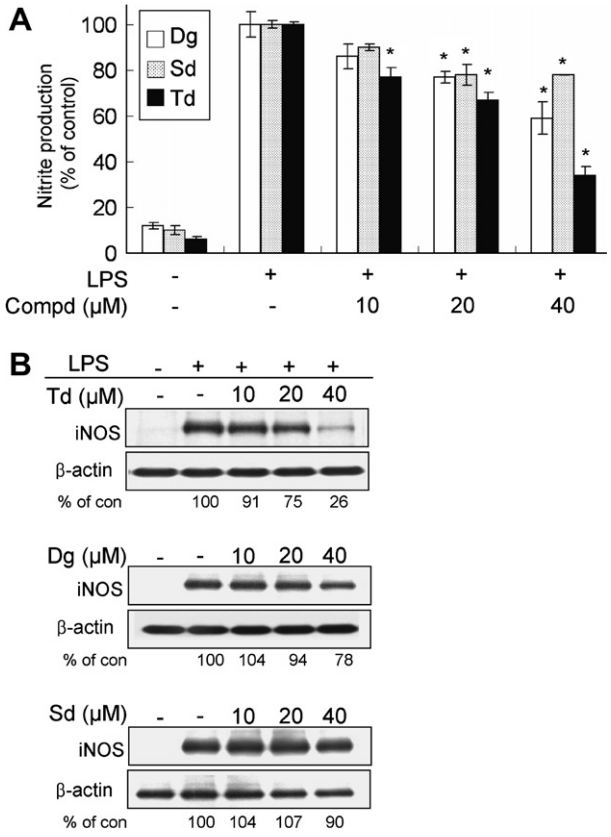

Fig. 2. Effects of tomatidine, solasodine and diosgenin on LPS-induced nitrite production and iNOS expression in RAW 264.7 cells. (A) Inhibition of LPS-induced NO production by tomatidine (Td), solasodine (Sd) and diosgenin (Dg) in RAW 264.7 cells. The cells were treated with the indicated concentrations of the test compounds for 1 h before the addition of 100 ng/mL LPS, and the cells were further incubated for 24 h. The concentration of nitrite in the culture medium was monitored, as described in Section 2. Each bar represents the means ± S.D. from four separate experiments. $^{*}P < 0.05$ compared with LPS alone. (B) Inhibition of LPS-induced iNOS expression by the test compounds tomatidine (Td), solasodine (Sd) and diosgenin (Dg). The cells were treated with the indicated concentrations of compounds for 1 h before the addition of 100 ng/mL LPS, and the cells were further incubated for 16 h. The levels of iNOS protein were monitored, as described in Section 2. This experiment was repeated four times, with similar observations in each experiment.

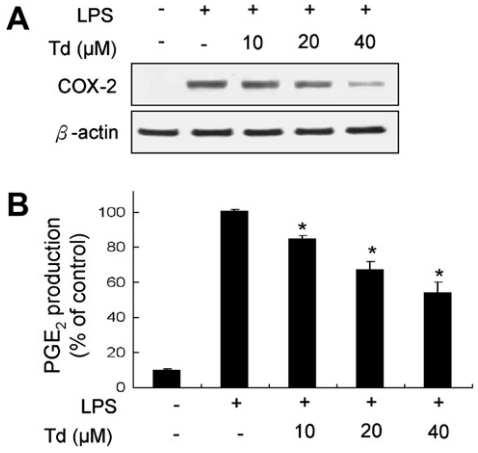

Fig. 3. Effects of tomatidine on LPS-induced PGE_2 production and COX-2 expression in RAW 264.7 cells. The cells were treated with the indicated concentrations of tomatidine (Td) for 1 h before the addition of 100 ng/mL LPS, and the cells were further incubated for 16 h. (A) Inhibition of LPS-induced COX-2 expression by tomatidine. The levels of COX-2 protein were monitored, as described in Section 2. (B) Inhibition of LPS-induced PGE_2 production by tomatidine in RAW 264.7 macrophages. The concentrations of PGE_2 in culture medium were monitored, as described in Section 2. Each bar represents the means ± S.D. from three separate experiments. $^{*}P < 0.05$ compared with LPS alone. This experiment was repeated four times with similar observations in each experiment.

The nuclear translocation of NF-κB is preceded by the phosphorylation and degradation of I-κBα [15]. To determine whether the inhibition of nuclear translocation of NF-κB by tomatidine is because of an effect on I-κBα degradation, the cytoplasmic levels of I-κBα were determined. Incubation of RAW 264.7 cells with LPS for 30 min could induce I-κBα phosphorylation and degradation; and this induction was inhibited by tomatidine in a dose-dependent manner (Fig. 4C). These findings suggest that tomatidine suppresses the action of NF-κB at least partly through the inhibition of I-κBα degradation.

3.4. Effect of tomatidine on LPS-induced phosphorylation of JNK and c-jun

As MAP kinases have been shown to be required for iNOS induction mediated by LPS in RAW 264.7 macrophages, we investigated the effect of tomatidine on the activation of MAP kinases in LPS-stimulated RAW 264.7 cells. After incubation with LPS, the phosphorylation of c-jun was significantly increased, and this activation was blocked by tomatidine (Fig. 5A and B).

To further determine if inhibition of MAP kinases contributes to the inhibitory action of tomatidine, cells were pretreated with the MEK1/2, p38 and JNK inhibitors—PD98059, SB203580 and SP600125, respectively—before the addition of LPS. The LPS-induced increases in iNOS and COX-2 levels were unaffected by PD98059 and SB203580 (data not shown) but were inhibited by the JNK inhibitor SP600125 (Fig. 5C). Based on this information, it seems that JNK signaling is of primary importance in controlling iNOS and COX-2 expression.

3.5. Effect of tomatidine on LPS-induced Oct-2 expression

The Oct-2 transcription factor had been shown to bind to the octamer motif in the iNOS promoter [7,16], and Oct-2 is required for iNOS activation by LPS [17]. To determine whether tomatidine treatment decreases the level of LPS-induced Oct-2 expression, RAW 264.7 cells were exposed to LPS with and without tomatidine, and examined using Western immunoblot analysis. Incubation of RAW 264.7 cells in the presence of LPS could induce expression of Oct-2 and this induction was inhibited by tomatidine in a concentration-dependent manner (Fig. 6A). To examine further the signaling cascade triggering the increase in Oct-2 expression by LPS, specific pharmacological antagonists were used. The LPS-induced increase in Oct-2 levels was unaffected by PD98059 and SB203580 (data not shown) but was blocked by SP600125 (Fig. 6B). These findings indicate that inhibition of LPS-induced iNOS expression by tomatidine may proceed through down-regulation of Oct-2 by the JNK signaling cascade.

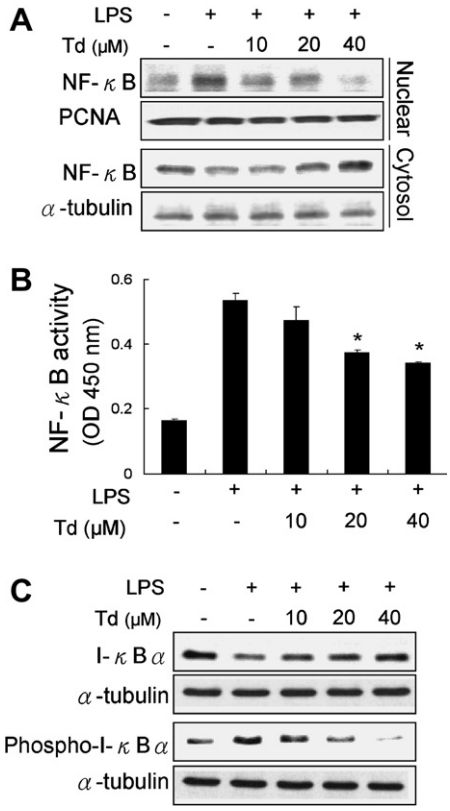

Fig. 4. Effects of tomatidine on LPS-induced NF-κB activation and I-κBα degradation and phosphorylation in RAW 264.7 cells. The cells were treated with the indicated concentrations of tomatidine (Td) for 1 h before the addition of 100 ng/ml LPS, and the cells were further incubated for 30 min. After the isolation of nuclear and cytoplasm extracts, NF-κB p65 translocation (A) was measured by Western blot, and NF-κB p65 binding activity (B) was determined, as described in Section 2. Each bar represents the means ± S.D. from three separate experiments. $^{*}P < 0.05$ compared with LPS alone; the proliferating cell nuclear antigen (PCNA) and α-tubulin as the nuclear and cytosol fractions marker, respectively. (C) Inhibition of LPS-induced I-κBα phosphorylation and degradation by tomatidine. RAW 264.7 cells were treated with the indicated concentrations of tomatidine (Td) for 1 h before the addition of 100 ng/mL LPS, and the cells were further incubated for 30 min. Western blot analysis was carried out, as described in Section 2. These experiments were repeated four times with similar observations in each experiment.

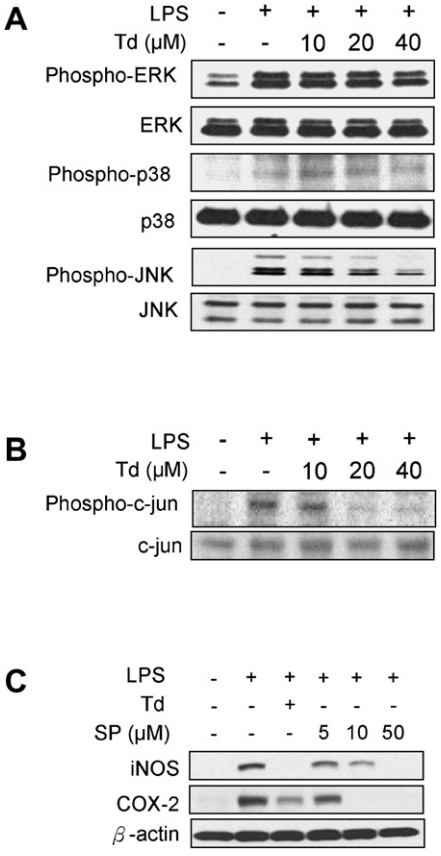

Fig. 5. Effects of tomatidine on LPS-induced phosphorylation of MAPKs in RAW 264.7 cells. The cells were stimulated with 100 ng/mL LPS alone or LPS plus tomatidine (Td; 10, 20 and 40 μmol/L) for 30 min, and whole-cell lysates were analyzed by immunoblot analysis using various antibodies against activated MAPKs (A) and phospho-c-jun (B). (C) Cells were pretreated with tomatidine (Td; 40 μmol/L) and SP600125 (SP; 5, 10 or 50 μmol/L) for 1 h before LPS (100 ng/mL) treatment for 16 h. Whole-cell lysates were analyzed by immunoblot analysis using various antibodies against iNOS and COX-2. The results presented are representative of three independent experiments.

4. Discussion

Tomatidine is the aglycone of tomatine, which is a major tomato glycoalkaloid. Tomatidine has been associated with a variety of effects on human health, including lowering cholesterol, enhancing immune responses when used as a cancer chemotherapy agent, and protecting against pathogenic fungi and other microorganisms [18]. Tomatidine may benefit cancer chemotherapy by inhibiting multidrug resistance in human cancer cells [19] and by inhibiting the growth of colon and liver cancer cells [20]. Tomatidine was developed into a pregnane derivative possessing neuritogenic and NGF-enhancing activities [21]. In addition, tomatidine suppresses the oxidative burst and is able to suppress induced plant defense responses [22,23]. However, the effects of tomatidine on inflammatory processes are still unclear. Our present results provide, for the first time, evidence for the anti-inflammatory effects of tomatidine.

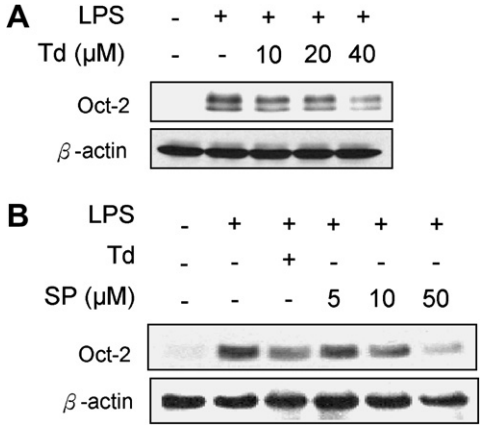

Fig. 6. Effect of tomatidine on LPS-induced Oct-2 expression in RAW 264.7 cells. (A) RAW 264.7 cells were pretreated with tomatidine (Td; 10, 20 or 40 μmol/L) for 1 h before LPS (100 ng/mL) treatment for 12 h. (B) Cells were pretreated with tomatidine (Td; 40 μmol/L) and SP600125 (SP; 5, 10 and 50 μmol/L) for 1 h, then LPS (100 ng/mL) was added for 16 h. Whole-cell lysates were analyzed by immunoblot analysis using various antibodies against Oct-2. These experiments were repeated three times with similar observations in each experiment.

Comparison of the chemical structures of tomatidine and solasodine shows that the major difference between them is that tomatidine lacks the double bond in the 5,6 position of the B-ring. We found that tomatidine was a more potent anti-inflammatory than solasodine. In other words, the absence of a 5,6 double bond in the B-ring of tomatidine results in a much stronger anti-inflammatory effect compared with the otherwise structurally similar solasodine (which contains such a double bond). In addition, the results of our cell viability assay indicated that tomatidine is much less toxic than solasodine and diosgenin; with similar concentration treatment (40 μmol/L) showed 5.5%, 13.5%, and 26.8% growth inhibition of tomatidine, solasodine, and diosgenin, respectively (data not shown). Recent studies also indicated that tomatidine is a much less toxic molecule in both pregnant and nonpregnant mice when compared with the structurally similar solasodine [24], and that it does not affect the body and liver weights of mice [25]. The other difference between them is that the 25-methyl group of tomatidine in the nitrogenous F-ring is epimeric. Therefore, the relationships between chemical structure and the anti-inflammatory characteristics of tomatidine need to further investigated. These results show that tomatidine has the best anti-inflammatory effect and least toxicity of the pair.

The NOS and COX systems are often present together, share a number of similarities, and play fundamental roles in similar pathophysiological conditions, such as inflammation [26] and cancer [27]. The transcription factor NF-κB is implicated in the regulation of many genes that code for mediators of the immune, acute-phase and inflammatory responses, e.g. iNOS and COX-2 [28,29]. The p50/p65 heterodimer is the most common dimer found in the NF-κB signaling pathway [30]. Activation of the NF-κB signaling cascade results in the complete degradation of I-κB via phosphorylation and ubiquitination. In our study, tomatidine inhibited the phosphorylation of I-κB, blocked the I-κB production, and furthermore suppressed p65 NF-κB translocation to the nucleus and modulated binding activity. The role of the MAP kinases in the regulation of iNOS and COX-2 expression has been investigated intensively. In this study, we found that incubation of RAW 264.7 cells with LPS brings about activation of MAP kinases, and that treatment with tomatidine inhibits LPS-induced phosphorylation of JNK but not p38 MAPK and ERK. These results suggest that NF-κB and JNK pathways could modulated the suppression of LPS-induced iNOS and COX-2 expression by tomatidine (Fig. 7).

The octamer transcription factors are member of the Pit-Oct-Unc domain family, exerting their effects through binding to sequences related to the octamer motif (ATGCAAAT) [6]. Oct-1 and Oct-2 are the two most prominent octamer factors. Oct-1 was identified as a ubiquitous protein, whereas Oct-2 expression is restricted largely to B lymphocytes [31], but is also present in macrophages [17,32]. Recent studies have indicated that Oct-2 binds to the octamer motif in the iNOS promoter [7] and is required for iNOS activation by LPS [33]. Our results are consistent with previous studies, and we confirm that Oct-2 is required for iNOS activation by LPS. Furthermore, we demonstrated that LPS-induced Oct-2 expression was abolished by blocking the MAP kinase pathway with the JNK inhibitor SP600125 (Fig. 6B), but no effect was seen with PD980559 and SB203580 (data not shown). Here, for the first time, we demonstrated that JNK pathway could be modulated the LPS-induced Oct-2 expression. These results suggest that tomatidine's suppression of LPS-induced Oct-2 expression through the JNK pathway in turn modulates iNOS expression. Besides, this study indicates that NF-κB is required for transcriptional activation of the Oct-2 gene in transformed pre-B lymphocytes [34]. Therefore, more studies are needed to determine whether tomatidine inhibits inflammatory-mediator expression only through the MAPK and NF-κB pathways, or by cross-interactive regulation.

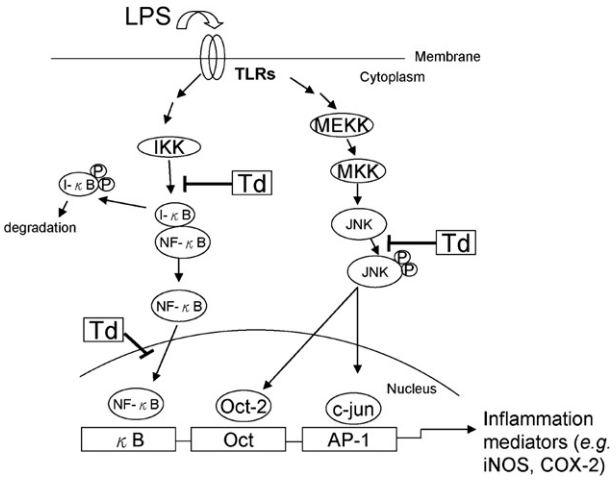

Fig. 7. Possible anti-inflammatory mechanisms of tomatidine (Td). LPS-induced activation of macrophages is mainly mediated through TLR 4 and results in the production of proinflammatory mediators including iNOS and COX-2. The promoter regions of proinflammatory mediators contain several binding site for transcriptional factors, such as κB, AP-1, and Oct. The anti-inflammatory effects of tomatidine are due to repression of NF-κB, c-jun, and Oct-2 acting on different regions of the genes promoter through NF-κB and JNK pathways. TLR, Toll-like receptor; IKK, inhibitor-κB Kinase; I-κB, inhibitor-κB; MEKK, mitogen-activated protein kinase kinase kinase; MKK, mitogen-activated protein kinase kinase; JNK, c-jun N-terminal kinase; NF-κB, nuclear factor-κB; AP-1, activating protein-1; Oct, octamer.

LPS-induced activation of macrophages is mainly mediated through the transmembrane signaling receptor toll-like receptor (TLR) 4 [35]. LPS stimulation of macrophages activates several intracellular signaling pathways that include the IKK–NF-κB pathway and three mitogen-activated protein kinase (MAPK) pathways: ERKs 1 and 2, JNK and p38. These signaling pathways in turn activate a variety of transcription factors that include NF-κB (p50/p65) [5,36] and activator protein-1, AP-1 (c-Fos/c-Jun) [37], as well as Octamer (Oct-2) [7,16], which coordinate the induction of many genes encoding inflammatory mediators. In conclusion, these experiments demonstrate that tomatidine inhibits LPS-induced expression of the iNOS and COX-2 genes through suppressing the phosphorylation of I-κBα and the activation of NF-κB, and by inhibiting the JNK pathway, which in turns inhibits c-jun phosphorylation and Oct-2 expression in RAW 264.7 cells (Fig. 7). Tomatidine shows great potential as an anti-inflammatory agent and may be used in the future as a novel agent for the chemoprevention of cancer or inflammatory diseases.

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