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Research Article
Protective Effect of Theaflavin-3-digallate on Lipopolysaccharide-induced Inflammation Injury in Macrophage Cells

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Abstract

Background and Objective: Inflammation is a defensive response against a multitude of harmful stimuli and stress conditions such as tissue injury and is one of the most common pathological processes of human diseases. Tumor Necrosis Factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) play important roles during the inflammatory state. The aim of this study was to investigate the suppressive effects of theaflavin-3-digallate on lipopolysaccharide (LPS)-induced inflammation in U937 and RAW 264.7 cells. **Materials and Methods:** The U937 and RAW 264.7 cells were treated with 100 ng mL^{-1} LPS in the presence of theaflavin-3-digallate (6.25-50 μM). The qRT-PCR assay was used to test the levels of TNF-α, IL-1β and IL-6 in LPS-treated Phorbol Myristate Acetate (PMA)-primed U937 and murine RAW 264.7 cells and Western blot assays were used to identify potential mechanism of anti- inflammatory potential of the theaflavin-3-digallate. Significance between groups data were analyzed by Graph Pad Prism 5.0, using Student's t-test, One-way ANOVA. **Results:** The present study has clearly shown that theaflavin-3-digallate markedly decreased the mRNA expression of TNF-α, IL-1β and IL-6 in a dose-dependent manner. Moreover, western blot analyses demonstrated that theaflavin-3-digallate also blocked phosphorylation of IκB from cytosolic fraction and reduced LPS-induced nuclear accumulation of transcription factor nuclear factor-kappa B (NF-κB) p65. **Conclusion:** These results demonstrated that theaflavin-3-digallate might abolish inflammation in RAW 264.7 macrophages and PMA-primed U937 cells via NF-κB pathway.

Key words: Theaflavin-3-digallate, inflammation, lipopolysaccharide, tumor necrosis factor, interleukin-1 β, interleukin 6

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Theaflavin-3-gallate (TF-2a) is one kind of theaflavin in black tea. The TF-2a has been applied for antioxidant, anti-tumor, anti-virus in cell cultures[1-3]. Nevertheless, the underlying mechanisms of the suppressive effects of TF-2 on LPS-induced inflammation remain unknown.

Numbers of human diseases even cancer are associated with inflammation. Inflammation is a defensive biological reaction involving various cells and cytokines. During the process of inflammation, many immune cells especially macrophages, hyper-secrete pro-inflammatory cytokines, which further accelerate inflammation reaction. Immune cells play crucial roles in eliminating exogenous pathogens and release various chemokine and pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF)-α, interleukin (IL)-1β and IL-6[4]. As one of the key downstream mediators in inflammation, the pro-inflammatory cytokine TNF-α controls inflammatory cells as well as influencing the processes of inflammation[5]. There have few IL-1β in cells of healthy persons, however, the level of IL-1β in monocytes from patients of inflammatory diseases is elevated significantly[6-11]. Furthermore, IL-1β requires a series of other intracellular cytokines, such as TNF-α, IL-1α, IL-6 and IL-18 before it trigger inflammation. Therefore, once the release of IL-1β increased rapidly, there is more serious inflammation[12]. Worse still, TNF-α and IL-1β also activate other transcription factors to further induce the secretion of IL-6. As reported by previous study, TNF-α, IL-1β and IL-6 play a key role in activating the host response through inducing an acute inflammatory response and alerting immune system to perform its function[4].

TNF-α, IL-1β and IL-6 are also related to generic Mitogen-Activated Protein Kinase (MAPK) and NF-κB signaling pathways. The MAPK pathway is associated with cell life cycle, which can be activated by lipopolysaccharide (LPS)[13]. The signaling pathway includes extracellular signal-related kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK), p38 and ERK5[14]. These MAPK signaling pathways are vital for LPS-induced production of TNF-α, IL-1β and IL-6 in activated macrophages. Suppression of any of these MAPK pathways is enough to block the LPS-induced increasing secretion of TNF-α[15]. Furthermore, MAPKs are also related to the activation of nuclear factor-kappa B (NF-κB)[16]. The MAPKs and NF-κB can work synergistically to cause the increasing expression of pro-inflammatory genes and the secretion of cytokines[17]. During inflammatory response, NF-κB is phosphorylated and translocate into the nucleus, then it induces the expression of genes relevant to inflammatory responses[18,19]. Therefore, MAPK and NF-κB signaling pathway have been recognized as key target for treatment of inflammation and inflammatory diseases.

This study aimed is to investigate the effects of TF-2a on the cytokine levels in LPS-treated Phorbol Myristate Acetate (PMA)-primed U937 and murine RAW 264.7 cells, to elucidate its potential mechanism of action.

MATERIALS AND METHODS

Chemicals and reagents: The TF-2a (>98% purity) was purchased from Pi and Pi Technology Inc. (Guangzhou, China), then solubilized in dimethyl sulfoxide (DMSO) and diluted with medium as needed. The final DMSO concentration was 0.1% (v/v). Dexamethasone (DEX) was obtained from Guangzhou Overseas Chinese Hospital. The 3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) and LPS (Escherichia coli 055:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle medium (DMEM) and penicillin-streptomycin mixture were bought from Invitrogen (Carlsbad, CA, USA). All antibodies used were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). All other reagents were of pharmaceutical grade.

Cell culture: The U937 human leukemia and RAW 264.7 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured either in RPMI-1640 or in DMEM with 10% FBS and penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) in a suitable humidified incubator with 5% CO_2 atmosphere at 37°C.

Cytotoxicity assays: The MTT assays were conducted to assess the cytotoxicity of TF-2a towards U937 human leukemia and RAW 264.7. Cells were cultured overnight in 96-well plates in 100 μL of growth media (RPMI-1640 or DMEM with 10% FBS) and exposed to a series concentrations of TF-2a for 48 h. Then, 10 μL of 5 mg mL^{-1} MTT solution was added to each well and the plate was incubated in the dark for 4 h. The MTT solution was then discarded and 100 μL of DMSO was added to each well. The plates were gently shaken for 15 min at room temperature, before the optical density at 570 and 630 nm was recorded with an enzyme immunoassay reader (Bio-Rad, Hercules, CA, USA). The normalized cell viability at each TF-2a concentration was determined by comparison to TF-2a-free cells.

LPS-induced inflammation assays: *In vitro* models of inflammation, such as LPS-treated, PMA-primed U937 human leukemia and murine RAW 264.7 cells have been widely used to investigate anti-inflammatory effects of small-molecule compounds[20-23]. Therefore, these two models were used in this study to assess the anti-inflammatory activity of TF-2a. To examine the inhibitory effects of TF-2a on LPS-induced TNF-α, IL-1β and IL-6 expression in LPS-induced cells, two approaches were used in this study. The following experiments were performed to study LPS-induced inflammation in U937 and RAW 264.7 cells: (1) U937 cells were primed with PMA (10 ng mL^{-1}) for 48 h. The medium was discarded and cells were washed three times with PBS. A range of TF-2a concentrations with LPS induction medium (100 ng mL^{-1}) in serum-free RPMI-1640 medium was added and cells were cultured for 24 h before total RNA isolation, (2) U937 cells were primed with PMA (10 ng mL^{-1}) for 48 h. The medium was discarded and cells were washed three times with PBS. A range of TF-2a concentrations in complete RPMI-1640 medium was added for 4 h. The medium was then discarded and cells were washed three times with PBS. The serum-free medium was then replaced with the LPS induction medium (100 ng mL^{-1}). The cells were cultured for 2 h before total RNA isolation. (3) RAW 264.7 cells were cultured in 12-well plates to 70~80% confluence. The medium was discarded and cells were washed three times with PBS. A range of TF-2a concentrations with LPS medium (100 ng mL^{-1}) in serum-free DMEM was added and cells were cultured for 6 h before total RNA isolation. (4) RAW 264.7 cells were cultured in 12-well plates to 70~80% confluence. The medium was discarded and cells were washed three times with PBS. A range of TF-2a concentrations in complete DMEM was added for 6 h. The medium was then discarded and cells were washed three times with PBS. The serum-free medium was then replaced with LPS induction medium (100 ng mL^{-1}). The cells were cultured for 6 h before total RNA isolation.

Real-time PCR: Total RNA was extracted using the TRIzol® universal reagent (TIANGEN, Beijing, China) and RNA concentrations were measured using a Nanophotometer™ P-Class P330 (Implen, Munich, Germany). The extracted RNA (1,000 ng) was reverse-transcribed using a PrimeScript™ RT master mix kit (Takara Bio, Kusatsu, Japan). Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assays were performed using SsoFast™ Eva Green® supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The sequences of the primers used were as follows: Mouse TNF-α (F: 5'-CAAAATTCGAGTGAC AAGCCTG-3' and R: 5'-GAG ATCCATGCCGTTGGC-3'), mouse IL-1β (F: 5'-ATGCCACCTTTTGACAGTGAT-3' and R: 5'-TGC GAGATTTGAAGCTGGAT-3'), mouse IL-6 (F: 5'-TCCATCCAGTTG CCTTCTTG-3' and R: 5'-GAA GTCTCCTCTCCGGACTT-3') and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (F: 5'-TGTGTCCGTCGTGGATCT GA-3' and R: 5'-CCTGCTTCA CCACCTTCTTGA-3'), human TNF-α (F: 5'-GGGCCTGTACCTCAT CTA CT-3' and R: 5'-TGACCTTGGTCTGGTAGGAG-3'), human IL-1β (F: 5'-TGATGGCTTATTACAGTGGCAATG-3' and R: 5'-GTA GTGGTGGTCGGAGATTCG-3'), human IL-6 (F: 5'-CCACTCACC TCTTCAGAACG-3' and R: 5'-CATGTCTCCTTTCTCAGGGC-3') and human GAPDH (F:5'-AGCCTCAAGATCATCAGCAAT G-3' and R: 5'-CACGATACCAAAGTTGTCATGGAT-3'). The relative expression was determined by the Ct method and normalized to the GAPDH expression.

Western blot assays: PMA- primed U937 cells and RAW 264.7 cells were treated with LPS (100 ng mL^{-1}) alone or in combination with various concentrations of TF-2a. An hour later, the cells were lysed in a 1% Sodium Dodecyl Sulfate (SDS) solution containing 2 mM proteinase inhibitor, phenylmethanesulfonyl fluoride (Invitrogen, Carlsbad, CA, USA). The lysate was heated for 10 min at 100°C, then centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was collected. The protein concentration was determined using a bicinchoninic acid assay kit (Beyotime, Jiangsu, China). The protein samples were resolved by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline-Tween 20 (TBS-T) [150 mM NaCl, 50 mM Tris-HCl, pH 7.6 and 0.1% (v/v) Tween-20] for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. The membranes were then incubated with secondary antibodies diluted in 5% dried milk in TBS-T for 1 h. The immunoblots were visualized using the enhanced chemiluminescence reagent (Beyotime). The Image J software was used to quantify the band intensity. The signal intensity of each protein was normalized to that of GAPDH.

Statistical analysis: Results were presented as Mean±SEM and analyzed using Graph Pad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Significance between groups data were analyzed by Graph Pad Prism 5.0, using Student's t-test, one-way ANOVA. A significant difference was indicated by p-values less than 0.05, 0.01, or 0.001[24].

RESULTS

Cytotoxicity of TF-2a towards U937 and RAW 264.7 cells: Results of TF-2a cytotoxicity tests shown in Fig. 1b-c indicated

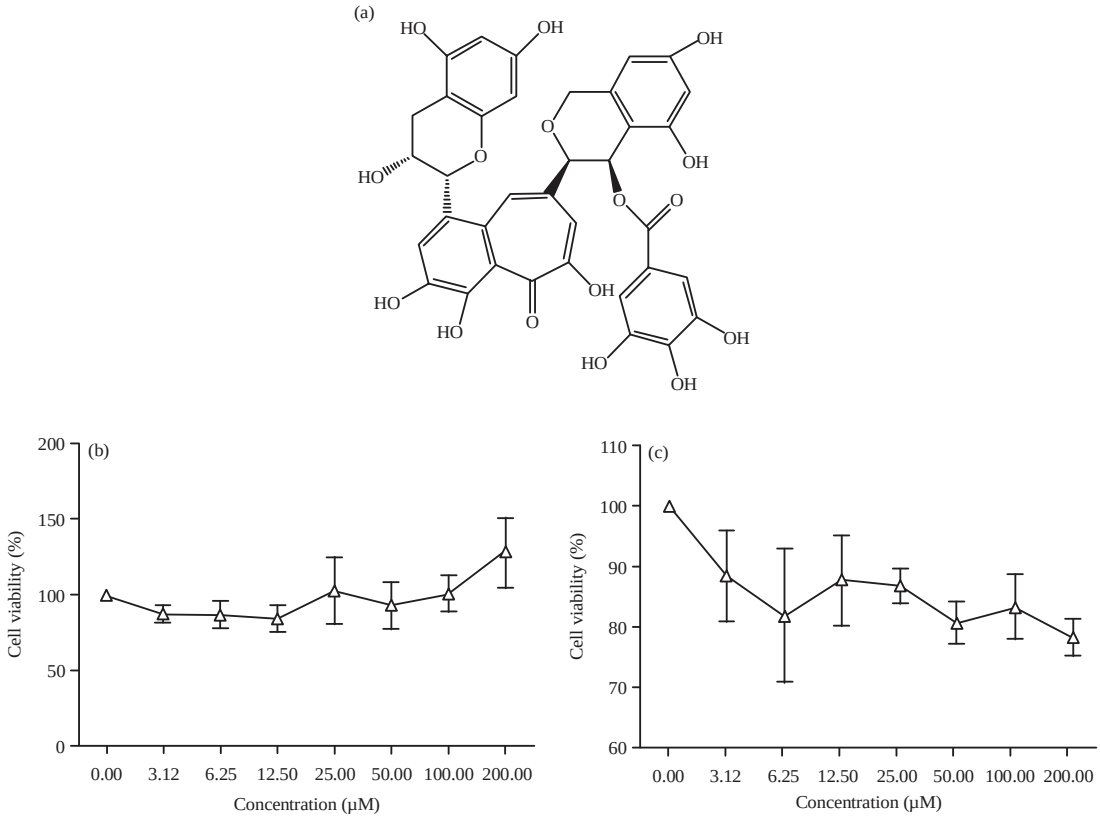

Fig. 1(a-c): (a) Chemical structure of theaflavin-3-digallate (TF-2a), Cytotoxic effect of TF-2a on (b) PMA-primed U937 and (c) RAW 264.7

Data are presented as the Mean±SEM of at least three independent experiments

that the half-lethal concentrations of TF-2a towards U937 and RAW 264.7 cells were all over 200 μM, suggesting that TF-2a (3.12~50 μM) was fairly non-toxic to cells.

TF-2a inhibited mRNA expression of TNF-α, IL-1β and IL-6 in PMA-primed U937 cells: As shown in Fig. 2a-b, compared with those of control group, TNF-α, IL-1β and IL-6 levels were increased obviously ($^{\#}p<0.05$, $^{\#\#}p<0.01$ or $^{\#\#\#}p<0.001$) after LPS stimulus. Interestingly, levels of TNF-α, IL-1β and IL-6 in PMA-primed U937 cells were all significantly inhibited (*p<0.05, **p<0.01 or ***p<0.001) by TF-2a in a dose-dependent manner when co-administered with LPS in PMA-primed U937 cells (Fig. 2a). However, compared with the LPS-treated control, the mRNA expressions of TNF-α, IL-1β and IL-6 almost were not inhibited by TF-2a, when pretreated of four hours (Fig. 2b). These results suggested that co-treated with TF-2a and LPS, TF-2a might exhibit inhibitory effect on LPS-induced increased release of inflammatory cytokines in PMA-primed U937 cells.

TF-2a inhibited mRNA expression of TNF-α, IL-1β and IL-6 in LPS stimulated RAW 264.7 macrophages: Following the observation that TNF-α, IL-1β and IL-6 production was inhibited by TF-2a in PMA-primed U937 cells, qRT-PCR analysis were carried out to determine whether the inhibition of TNF-α, IL-1β and IL-6 production by TF-2a in the LPS-stimulated RAW 264.7 cells. As mentioned methodology, two approaches were also used in this study. In response to LPS treatment, mRNA expressions of TNF-α, IL-1β and IL-6 were significantly induced ($^{\#}p<0.05$ or $^{\#\#\#}p<0.001$); However, co-treatment with TF-2a inhibited this up-regulation in a dose-dependent manner (***p<0.001) (Fig. 3a). In addition, pretreatment with TF-2a markedly suppressed the levels of TNF-α, IL-1β and IL-6(*p<0.05, **p<0.01 or ***p<0.001), respectively (Fig. 3b). The results indicated that TF-2a can down-regulate LPS-induced increased production of TNF-α, IL-1β and IL-6 in RAW 264.7 cells in different treatment ways.

TF-2a didn't suppress the activation of MAPK signaling pathway *in vitro:* The MAPK pathways are also involved in inflammation response. Suppression of any of three MAPK pathways is enough to block the LPS-induced increasing secretion of TNF-α, IL-1β and IL-6 by LPS[15]. Thus, to clarify the anti-inflammatory mechanism of TF-2a, Western blot analysis

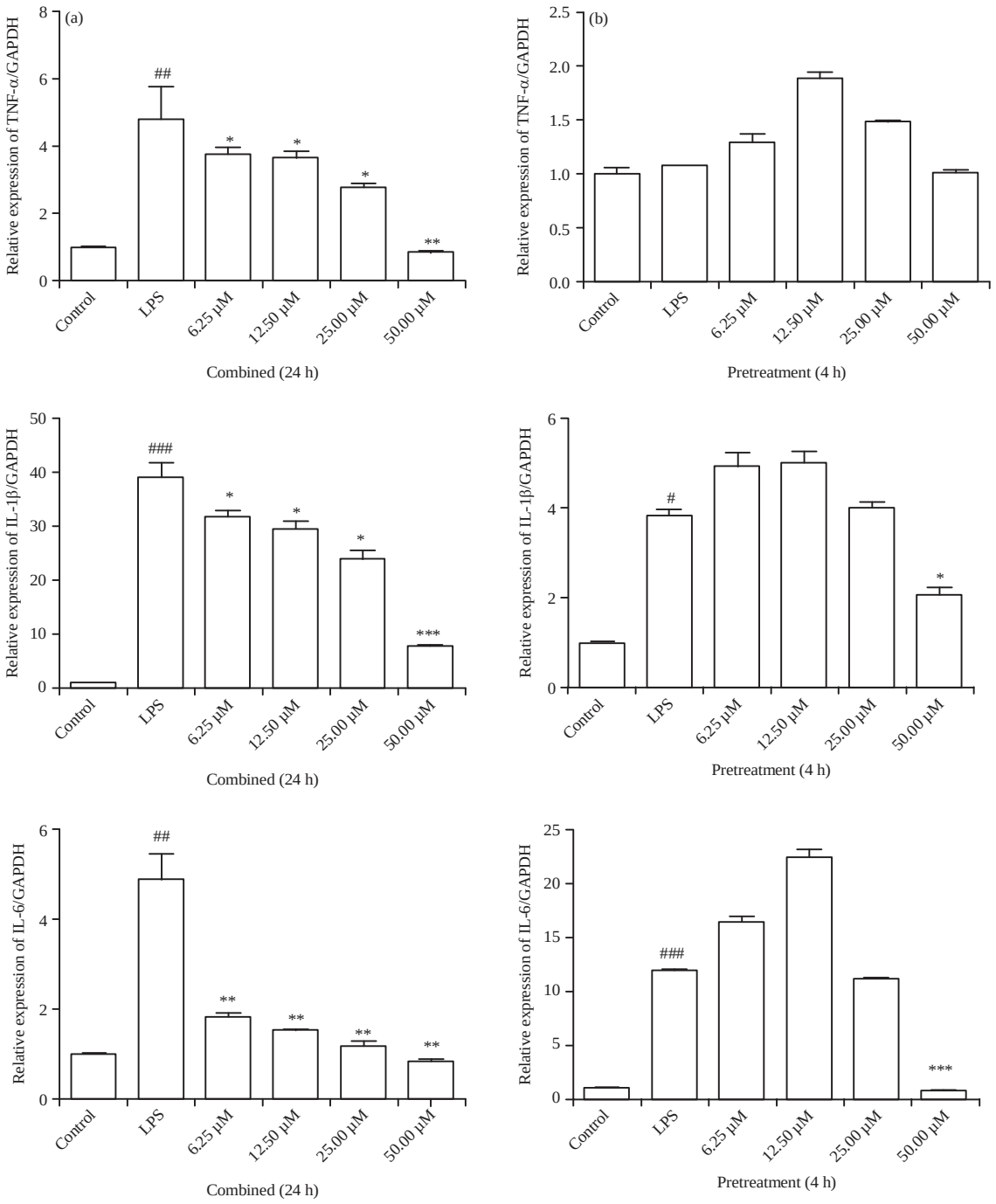

Fig. 2(a-b): Effects of TF-2a on LPS-induced mRNA expressions of TNF-α, IL-1β and IL-6 in PMA-primed U937 cells, (a) PMA-primed U937 cells were treated with LPS only or in combination with different concentrations of TF-2a for 24 h, (b) Before treatment with LPS for 2 h, PMA-primed U937 cells were pretreated with different concentrations of TF-2a for 4 h

Gene expression was measured using qRT-PCR using GAPDH as reference. Data are presented as the Mean±SEM of three independent experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared with the control group; *p<0.05, **p<0.01, ***p<0.001 compared with the LPS-treated group

was conducted to assess the effects of TF-2a on MAPK activation. However, as shown in Fig. 4b, TF-2a didn't inhibit any of the three MAPK pathways phosphorylation in RAW 264.7 cells. Furthermore, only p-JNK and p-Erk1/2 were blocked by 50 μM TF-2a treatment in PMA- primed U937 cells (Fig. 4a). These results suggested that TF-2a didn't through

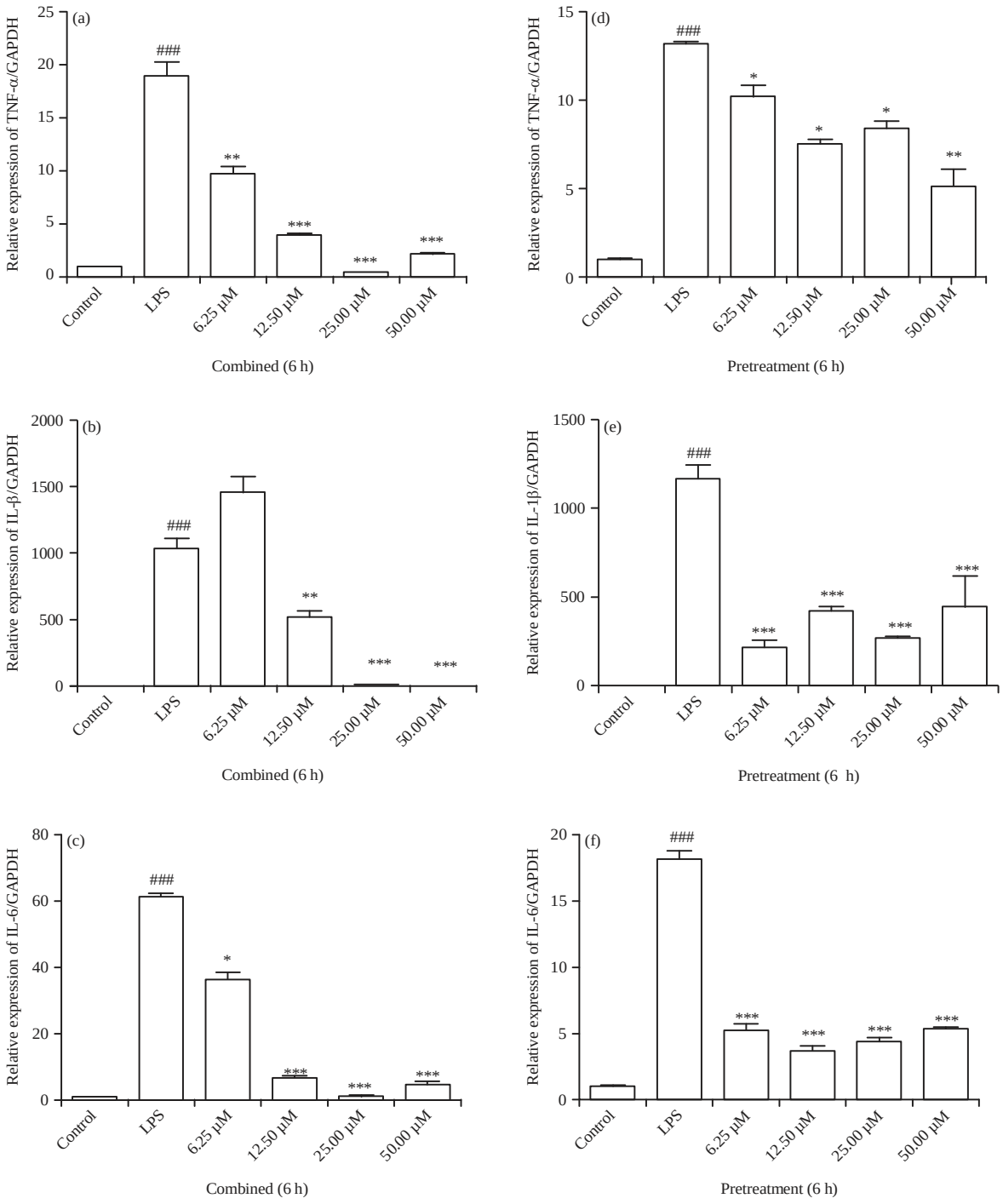

Fig. 3(a-b): Effects of TF-2a on LPS-induced TNF-α, IL-1β and IL-6 mRNA expressions in RAW 264.7 macrophages, (a) RAW 264.7 macrophages were treated with LPS only or in combination with different concentrations of TF-2a for 6 h, (b) Before treatment with LPS for 6 h, RAW 264.7 macrophages were pretreated with different concentrations of TF-2a for 6 h
Gene expression was measured using qRT-PCR using GAPDH as reference. Data are presented as the Mean±SEM of three independent experiments.$^{###}$p<0.001 compared with the control group; *p<0.05, **p<0.01, ***p<0.001 compared with the LPS-treated group

blocking the activation of MAPK signaling pathway to suppress the expression of TNF-α, IL-1β and IL-6 *in vitro*.

TF-2a inhibited LPS induced NF κB activity *in vitro:* Under normal circumstances, NF-κB binds with IκB and is localized to

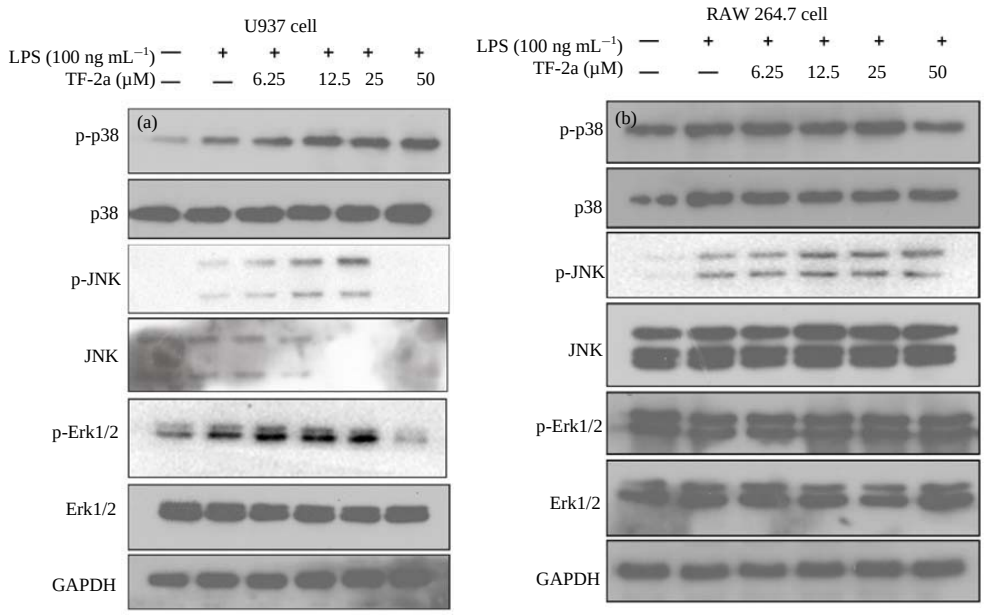

Fig. 4(a-b): Effects of TF-2a on the activation of MAPK in LPS-induced PMA-primed U937 cells and RAW 264.7 macrophages
Protein expression levels of JNK, p38 and Erk1/2 were determined by Western blot assay with GAPDH as internal control

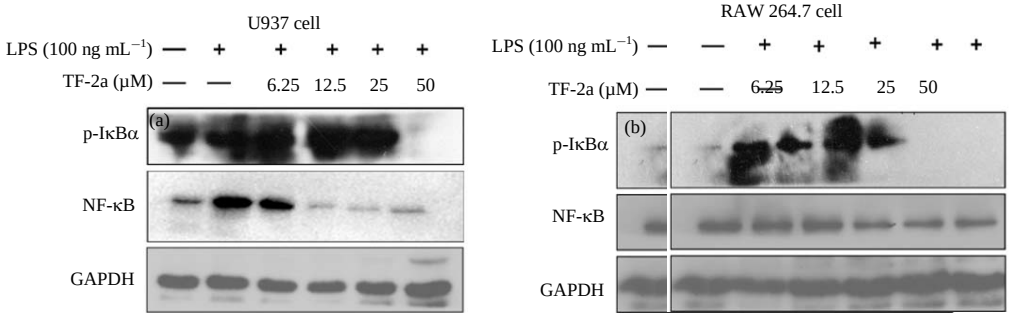

Fig. 5(a-b): Effects of TF-2a on the activation of NF-κB in LPS-induced PMA-primed U937 cells and RAW 264.7 macrophages
NF-κB p65 and phosphorylated IκB-α protein expression were measured using Western blot assay with GAPDH as internal control

the cytoplasm. However, in activated macrophages, IκB is phosphorylated by IκB kinase and degraded rapidly, which causes NF-κB translocate into the nucleus. Furthermore, NF-κB regulates the transcription of TNF-α, IL-1β and IL-6[23,25,26] Thus, to find out whether TF-2a blocks NF-κB (p65) from translocating to the nucleus, western blot assays were conducted to analyze the protein levels of phosphorylated p65 and IκB. The result in Fig. 5 showed that the protein levels of the p-p65 was markedly increased after LPS stimulation, be indicative of LPS activated NF-κB signaling pathway. The NF-κB translocated from the cytoplasm to the nucleus. However, TF-2a reduced the accumulation of NF-κB p65 in the nucleus in LPS-induced PMA-primed U937 cells (Fig. 5a) and RAW 264.7 cells (Fig. 5b) in a dose-dependent manner. In addition, phosphorylated IκB-α was inhibited by TF-2a. These results suggested that TF-2a prevented NF-κB activation *in vitro.*

DISCUSSION

The lipopolysaccharide (LPS) endotoxin is a component of cell membrane of the Gram-negative bacterial. The LPS can induce the release of pro-inflammatory cytokines by macrophages and circulating monocytes, resulting in a transient immune activation, which is characterized by elevated levels of TNF-α, IL-1β and IL-6[27,28]. The TF-2a has been obtained to elevate the apoptosis of cancer cells[29]. The TF-2a also suppress tumor growth and metastasis of cancer cells[30]. In this study, the results demonstrated that TF-2a exhibits a

positive protective effect against LPS-induced inflammation *in vitro*. The methods used in this study was just like the previous researchs[28,31]. Before starting to carry out the formal experiments, preliminary experiments were performed, the incubation times and the concentrations of TF-2a were based on the results of preliminary experiments. As is known to all, MTT is a most commonly used method to monitor the cytotoxic effects the drugs and also a lots of articles used this method to verify the cytotoxicity of the small molecule compounds[32,33]. In order to avoid false positives, MTT assays were first carried out to confirm that the positive protective effects of TF-2a were not due to its cytotoxicity. Inflammation is a protective biological response to cell or tissue injury caused by external pathogenic microorganisms, during which large quantities of pro-inflammatory cytokines are produced[23,34]. Out of the many inflammatory factors, TNF-α, IL-1β and IL-6 are always regarded as the most essential cytokines involved in inflammatory reactions and are released in response to bacterial endotoxins. Inhibition of the production of these cytokines is beneficial for the treatment of inflammation. Hence, they are ideal targets for novel anti-inflammatory agents. In the study, the results showed that TF-2a strongly inhibited the LPS-induced increasing mRNA expressions of TNF-α, IL-1β and IL-6 in PMA-primed U937 cells and RAW 264.7 cells. Due to the different methods of treatment, not all of the results showed correlation with increase in the dose of TF-2a. Numerous experiments have proved that co-administration of TF-2a and LPS is beneficial to the action of TF-2a. Thus, this particular mode of treatment was one of the methods adopted in the following study.

It has been reported that MAPK and NF-κB signaling pathways regulate LPS-induced inflammatory reaction[35]. To illustrate the potential mechanisms by which TF-2a exerts positive effect on LPS-induced inflammation, the inhibitory effect of TF-2a on the activation of MAPK and NF-κB signaling pathways in LPS-induced PMA-primed U937 cells and RAW264.7 cells was verified. After exposing to LPS, both NF-κB and MAPKs signaling pathways were activated but co-treatment of TF-2a and LPS only suppressed nuclear translocation of NF-κB (p65) and reduced the phosphorylation of IκB-α in LPS-treated cells. These data indicated that TF-2a exerts its anti-inflammatory activity through reducing the phosphorylation of IκB-α and the nuclear translocation of NF-κB. The underlying mechanism of the inhibitory effect of TF-2a on LPS-induced inflammation may be the same as that of other theaflavins. It was reported that TF-3, another kind of theaflavin, exerts its anti-inflammatory effects via the regulation of NF-κB activation[28]. Similarly, rhein inhibited the production of pro-inflammatory cytokines and attenuated leukocyte infiltration by suppressing the activation of NF-κB[33]. Together, these observations demonstrated that TF-2a inhibits the activation of the transcription factor NF-κB signalling pathway in macrophages, thereby inhibiting the expression of pro-inflammatory cytokines. In turn, the decrease in the release of inflammatory cytokines, for example, TNF-α, would suppress the activation of NF-κB.

CONCLUSION AND FUTURE RECOMMENDATION

It can be concluded that TF-2a have a positive protective effect against LPS-induced inflammation. It could decrease the production of TNF-α, IL-1β and IL-6 by inhibiting the activation of the NF-κB signaling pathway. Moreover, TF-2a had no side effects on the body. Therefore, TF-2a promises to be developed into potential health care products and services for sub-health populations.

However, further investigations, both *in vitro* and *in vivo*, are needed to confirm its anti-inflammatory effects. *In vitro,* further examinations to explore clearer mechanism are indispensable. Besides, it needs to be made certain that the effect on animal models of inflammation as well as investigating different periods of treatment, the optimal concentration of TF-2a and the difference between its activity in human and animals.

SIGNIFICANCE STATEMENTS

This study discovers the possible protective effect of theaflavin-3-digallate on lipopolysaccharide-induced inflammation injury in macrophage cells. This study will help the researcher to know more about the pharmacological effects especially the anti-inflammatory effect of theaflavin-3-digallate. Thus, it may represent a novel therapeutic strategy for the regulation of inflammatory responses.

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