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Farnesyl thiosalicylic acid prevents iNOS induction triggered by lipopolysaccharide via suppression of iNOS mRNA transcription in murine macrophages

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ABSTRACT

Inducible nitric oxide synthase (iNOS) is a molecule critical for the development of inflammation-associated disorders. Its induction should be tightly controlled in order to maintain cellular homeostasis. Upon lipopolysaccharide (LPS) stimulation, iNOS, in most settings, is induced by the activation of inhibitor of κB-α (IκB-α)-nuclear factor κB (NF-κB) signaling. Farnesyl thiosalicylic acid (FTS), a synthetic small molecule that is considered to detach Ras from the inner cell membrane, has been shown to exhibit numerous anti-inflammatory functions. However, it remains unclear whether and how it affects iNOS induction in macrophages. The present study addressed this issue in cultured macrophages and endotoxemic mice. Results showed that FTS pretreatment significantly prevented LPS-induced increases in iNOS protein and mRNA expression levels in murine cultured macrophages, which were confirmed in organs in vivo from endotoxemic mice, such as the liver and lung. Mechanistic studies revealed that FTS pretreatment did not affect IκB-α degradation and NF-κB activation in LPS-treated macrophages. The nuclear transport of the active NF-κB was also not affected by FTS. But FTS pretreatment reduced the binding of NF-κB to its DNA elements, and reduced NF-κB bindings to iNOS promoter inside LPS-treated macrophages. Finally, our results showed that FST pretreatment increased mouse survival rate compared to LPS alone treatment. Taken together, these results indicate that FTS attenuates iNOS induction in macrophages likely through inhibition of iNOS mRNA transcription, providing further insight into the molecular mechanism of action of FTS in inflammatory disorder therapy.

1. Introduction

Inducible nitric oxide synthase (iNOS), one of the members of the family of nitric oxide synthase (NOS) [1], has been shown to exert multiple biological effects via promotion of nitric oxide (NO) production [2]. At physiological conditions, the iNOS and NO is beneficial for normal healing in the skin and intestinal mucosa through killing of bacteria [3], and is tightly involved in regulation of T cell proliferation and differentiation [4,5] as well as leucocyte recruitment in the innate immune system [6]. However, at pathological conditions their beneficial effects can be counteracted by over-accumulated iNOS and NO. For instance, the constant production of iNOS has been shown to damage mitochondrial function and induce cellular apoptosis [7,8]. The over-accumulated iNOS can render the vasculature refractory to typical therapies for septic shock, such as epinephrine administration and volume supplementation [9]. Therefore, iNOS induction should be tightly controlled in order to keep the balance of host defense.

Unlike endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), two constitutively isoforms of NOS, little or no iNOS is present in resting cells [10]. The iNOS expression can be induced by various stimuli including lipopolysaccharide (LPS) [11]. In many settings, LPS stimulates the expression of iNOS via the activation of the classical inhibitor of κB kinase (IKK)-inhibitor of κB α (IκB-α)-nuclear factor κB (NF-κB) signaling in immune cells [12]. Through binding with Toll-like receptors, LPS can induce IκB-α degradation using the ubiquitin-proteasome system [13]. The removal of IκB-α from

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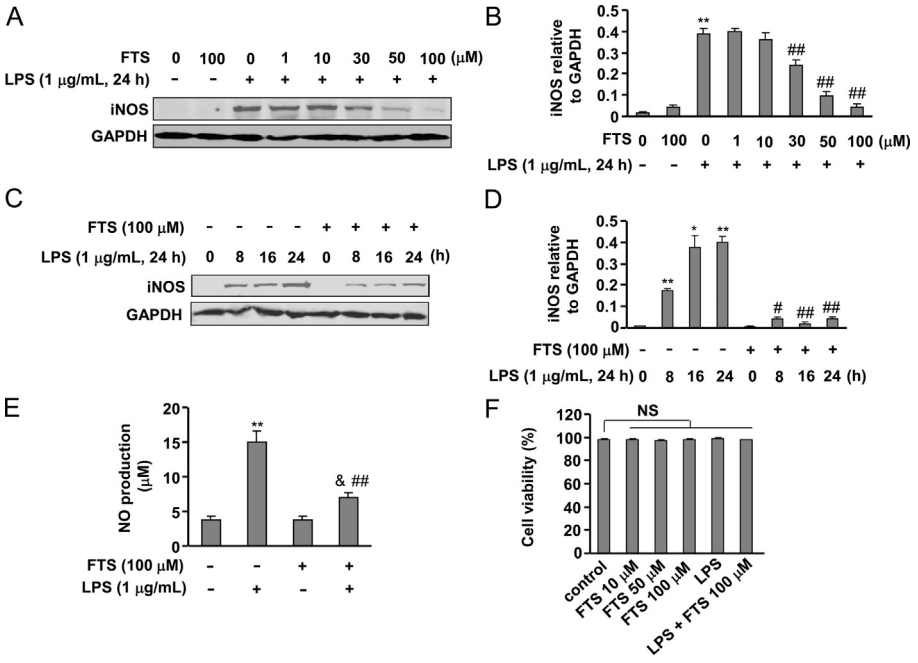

Fig. 1. FTS prevents iNOS induction and NO production in macrophages exposed to LPS. (A) Representative images showing the dose-dependent (1, 10, 30, 50, 100 μM) effect of FTS on LPS (1 μg/mL, 24 h)-induced iNOS protein expression. (B) Quantitative analysis showing the dose-dependent effect of FTS on iNOS protein expression in LPS-treated macrophages (n = 3; $^{**}P < 0.01$ vs. control; $\#\#P < 0.01$ vs. LPS alone treatment). (C) Representative images showing the time-dependent (8, 16, 24 h; 100 μM) effect of FTS on LPS (1 μg/mL, 24 h)-induced iNOS protein expression. (D) A time-course analysis of iNOS protein expression in LPS-stimulated macrophages upon FTS pretreatment (n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $\#P < 0.05$, $\#\#P < 0.01$ vs. LPS alone treatment at different time points, including 8, 16, and 24 h). (E) Quantitative analysis showing the change in NO content in FTS- and/or LPS-treated macrophages (n = 3; $^{**}P < 0.01$ vs. control; $\&P < 0.05$ vs. FTS alone treatment; $\#\#P < 0.01$ vs. LPS alone treatment). (F) Quantitative analysis showing the cell viability of FTS- and/or LPS-treated macrophages (n = 8). Data are shown as mean ± SME. NS: no significance.

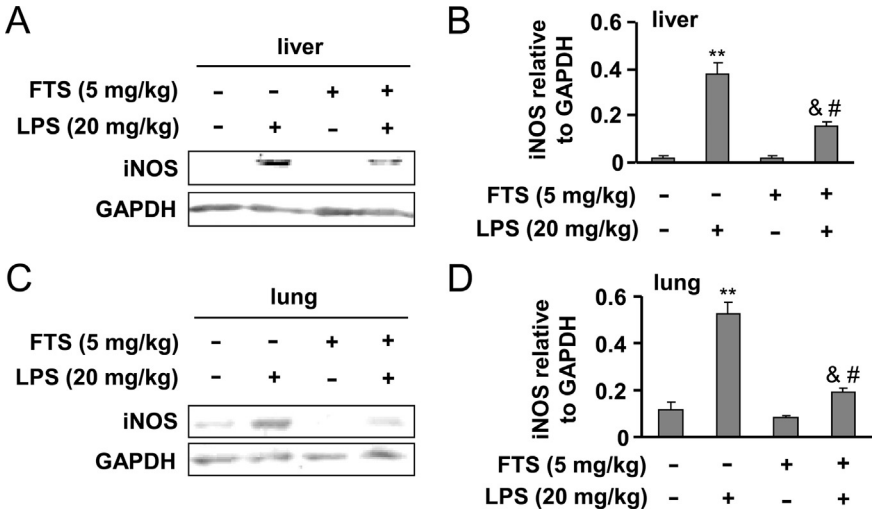

Fig. 2. Effects of FTS on iNOS protein expression in endotoxemia. Mice were divided into sham (saline), endotoxemia (LPS 20 mg/kg), FTS alone treatment (FTS 5 mg/kg), and LPS/FTS co-treatment (LPS 20 mg/kg + FTS 5 mg/kg) group. Mice were sacrificed 20 h after drug injection and iNOS proteins were measured in the liver (A) and lung (C). Results showed that FTS pretreatment markedly suppressed iNOS protein expression in the liver (A) and lung (C) in endotoxemic mice. (B, D) Statistical analysis showing the influence of FTS on LPS-induced expression of iNOS protein in the liver (B) and lung (D) in endotoxemic mice (n = 6; $^{**}P < 0.01$ vs. control; $\&P < 0.05$ vs. FTS alone treatment; $\#P < 0.05$ vs. LPS alone treatment). Data are shown as mean ± SME.

the cytoplasm liberates transcriptional factor NF-κB, whose active form is then translocated into the nucleus for the initiation of iNOS gene transcription. One who wants to develop methods to control iNOS induction should focus on the signaling molecules upstream of iNOS gene transcription.

S-trans, trans-farnesyl thiosalicylic acid (FTS) is a synthetic small compound that resembles the carboxyl-terminal farnesylcysteine of Ras [14]. It has been extensively studied in inhibition of Ras-dependent tumor growth via disruption of the association of Ras with the cell membrane [15–17]. There are also substantial evidences showing that FTS modulates many aspects of immune and inflammatory processes in addition to its well-known anti-proliferative activities. For example, FTS has been reported to prevent pro-inflammatory responses in disease models including experimental autoimmune neuritis [18], MRL/lpr mice [19], allergic encephalomyelitis [20], myocarditis [21], and severe acute pancreatitis [22]. FTS can also inhibit the production of pro-inflammatory mediators in mast cells [23] and T cells [24]. In addition, FTS has been shown to produce anti-arthritic activities in rodent models [25]. Considering the importance of the iNOS/NO-mediated process in host defense and pro-inflammatory responses in both physiological and pathological conditions, we investigated whether the iNOS induction could be regulated by FTS. Our results showed that FTS pretreatment markedly prevents iNOS protein expression and NO production through attenuation of the function of iNOS gene transcription-regulating factors in cultured macrophages and endotoxemic mice. These findings may provide further insight into the pharmacological role of FTS and its possible mechanism in inflammatory disorder therapy.

2. Materials and methods

2.1. Materials

FTS was purchased from Calbiochem (San Diego, CA, USA). LPS and thioglycollate are the products of Sigma (Saint Louis, MO, USA). Antibodies against iNOS, IκB-α, p-NF-κB p65 (Ser536), NF-κB p65, Histone H2A, and glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) are the products of Cell Signaling Technology (Beverly, MA, USA). Protein A/G PLUS-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other related agents were purchased from commercial suppliers. FTS is dissolved in di-H_2O. Prepared FTS stock solutions are protected from light and stored at −20 °C.

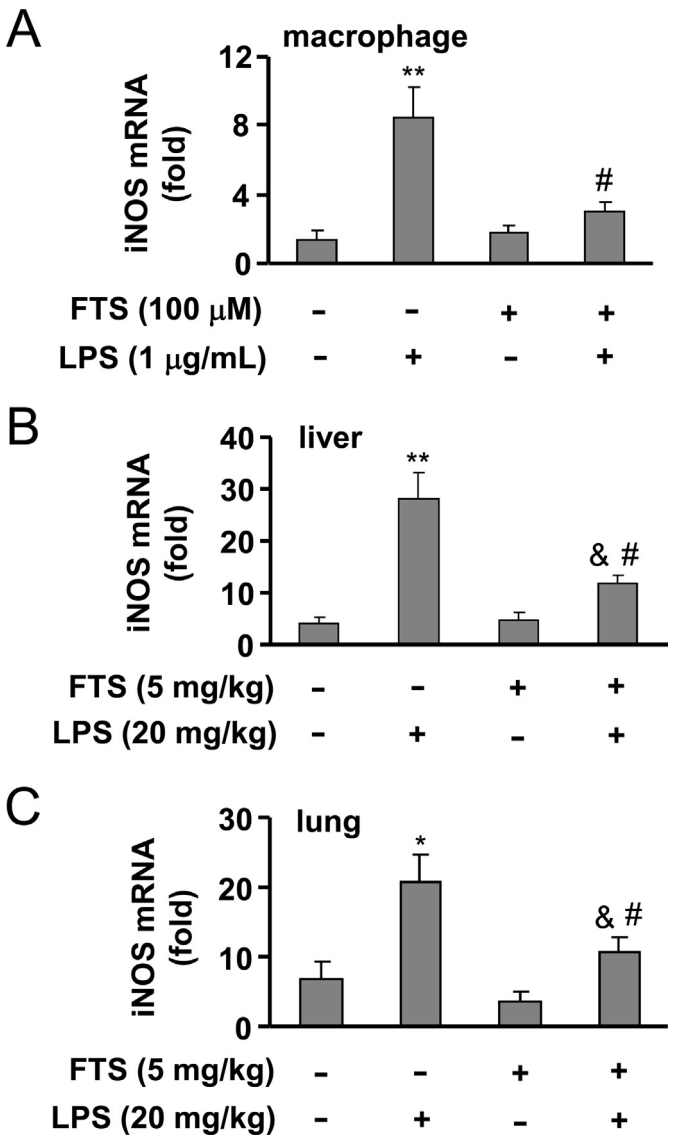

Fig. 3. Effects of FTS on iNOS mRNA expression in LPS-treated macrophages and mice. (A) Quantitative analysis showing the change in iNOS mRNA levels in cultured macrophages upon LPS stimulation (1 μg/mL, 6 h) in the absence or presence of FTS (n = 6; $^{**}P < 0.01$ vs. control; $\#P < 0.05$ vs. LPS alone treatment). (B, C) Mice were divided into sham (saline), endotoxemia (LPS 20 mg/kg), FTS alone treatment (FTS 5 mg/kg), and LPS/FTS treatment (LPS 20 mg/kg + FTS 5 mg/kg) group. Mice were sacrificed 20 h after drug injection and iNOS mRNA levels were measured in the liver and lung of endotoxemic mice. B and C represent the quantitative analysis of the mRNA levels of iNOS in mouse livers (B) and lungs (C), respectively, upon LPS stimulation in the absence or presence of FTS (n = 6; $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. control; & $P < 0.05$ vs. FTS alone treatment; $\#P < 0.05$ vs. LPS alone treatment). Data are shown as mean ± SME.

2.2. Cell culture

Macrophages were separated according to a previous study with some modifications [26]. Briefly, peritoneal macrophages were harvested 4 days after intraperitoneal injection of 3% thioglycollate. Macrophages were washed and plated in 24-well plates at 0.5×10^6 cells per well. After incubation for 2 h at 37 °C, the wells were washed 3 times to remove non-adherent cells. The culture medium was then replaced with RPMI medium containing 10% FBS (Gibco), nonessential amino acid, sodium pyruvate, penicillin G (100 IU/mL), and streptomycin (100 μg/mL). Cells were grown in 37 °C incubator containing 95% air and 5% CO_2. After being treated, cell supernatants were collected and frozen at −80 °C for NO detection.

2.3. Animals and experimental protocol

C57BL6/J mice (6–8 weeks, male) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were randomly divided into four groups. In sham- and LPS-treated groups, mice were injected intraperitoneally with 100 μL of saline + DMSO (vehicle) or 100 μL of LPS (20 mg/kg) + DMSO (vehicle), respectively. In FTS alone-treated groups, mice were administered intraperitoneally with a single dose of FTS (100 μL, 5 mg/kg). In FTS + LPS groups, mice were pretreated with a single dose of FTS (100 μL, 5 mg/kg) 2 h before LPS injection. After that, one set of experiment was designed to investigate whether FTS could affect mouse susceptibility to endotoxic shock via evaluating the survival rate. In a second set of experiment, some parts of the liver and lung in mice in different experimental groups were excised and frozen in liquid nitrogen, and some other parts were stored in RNA stabilization reagent RNAlater (Qiagen GFTSH, Hilden, Germany) for RNA extraction. The dose of LPS was selected according to previous studies [27,28]. The use of C57BL6/J mice was approved by the University Animal Ethics Committee of Nantong University (Permit Number: 2110836).

2.4. NO detection

A Griess reagent kit (Invitrogen) was used to measure the total nitrite levels. The reaction mixture consisted of Griess Reagent (20 μL), de-ionized water (130 μL), and serum or cell supernatants (150 μL). After incubation of the above mixture for 30 min at room temperature, nitrite levels were measured at 548 nm using an M2 spectrophotometric microplate reader (Molecular Devices).

2.5. Cell viability assay

Cell viability was measured using MTT Cell Proliferation and Cytotoxicity Assay Kit (Bi Yuntian Biological Technology Institution, Shanghai, China). Briefly, 5 mg/mL of methylthiazolyldiphenyl tetrazolium bromide was dissolved in prepared MTT-dissolved solutions and kept at −20 °C. After washing with PBS, the macrophages in plates were added 20 μL of MTT solutions and kept at 37 °C for 4 h. The blue crystals were dissolved in formazan-dissolved solutions. The absorbance was read at 570 nm.

2.6. Real-time polymerase chain reaction (PCR)

At the end of each treatment, total RNA was isolated from cells or tissues using an RNeasy mini kit according to manufacturer's instructions (Qiagen, GFTSH, Hilden, Germany). The first-strand cDNA was generated by reverse transcription of the total RNA using an RT system (Promega, Madison, WI, USA). Real-time PCR reactions were conducted with mixtures containing 2 μL of diluted cDNA, 2 μM $MgCl_2$, 0.5 μM primers, 1 × FastStart SYBR Green Master mix (Roche Molecular Biochemicals, [29]), and iNOS primers (forward, 5′-CTCACTGGGACA GCACAGAA-3′; reverse, 5′-TGGTCAAAC TCTTGGGGTTC-3′). The PCR products were detected by monitoring the fluorescence increase of double-stranded DNA-binding dye SYBR Green during amplification. The expression levels of target genes were normalized to the housekeeping gene 18S rRNA (forward, 5′-GTAACCCGTTGAACCCCATT-3′; reverse, 5′-CCATCCAATCGGTAG TAGCG-3′). Fold-changes in the target gene expression between experimental groups are expressed as a ratio. Relative gene expression was calculated by the comparative cycle threshold (Ct) method. Melt-curve analysis and agarose gel electrophoresis were used to examine the authenticity of the PCR product.

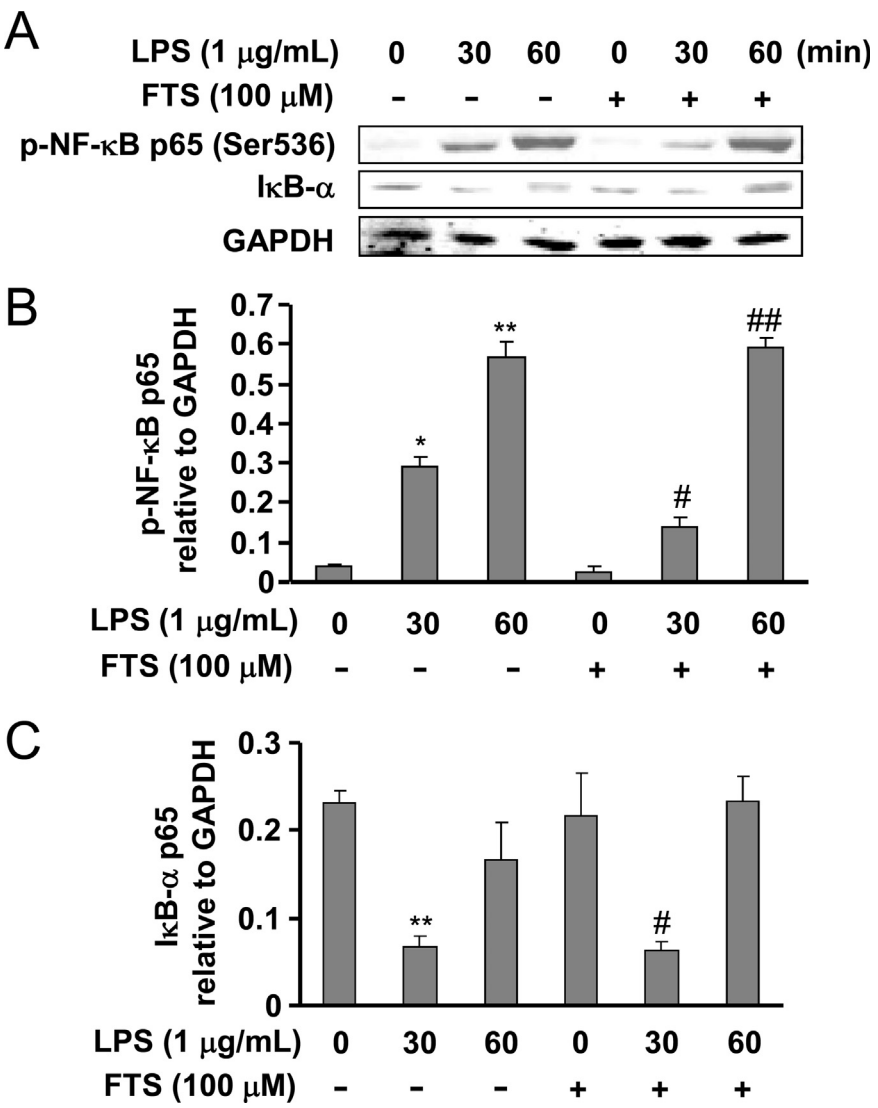

Fig. 4. Effects of FTS on LPS-induced IκB-α degradation and NF-κB phosphorylation in cultured macrophages. (A) As shown, FTS pretreatment (100 μM, 30 min) did not affect LPS (1 μg/mL)-induced increases in IκB-α degradation and NF-κB phosphorylation in cultured macrophages (n = 3). (B, C) Quantitative analysis showing the effect of FTS on IκB-α degradation (B) and NF-κB phosphorylation (C) in LPS-treated macrophages (n = 3; $*P < 0.05$ or $**P < 0.01$ vs. control; $\#P < 0.05$ or $\#\#P < 0.01$ vs. FTS alone treatment). Data are shown as mean ± SME.

2.7. NF-κB binding assays

The nuclear compartments in cultured macrophages were obtained by incubating cells with a hypotonic buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1.5 mM $MgCl_2$•$6H_2O$, at 4 °C for 20 min. After complete homogenization, cell homogenates were spun at 3000*g* for 5 min. The supernatants were collected for Western blot. The pellets were recovered, extensively washed, and re-suspended in the nuclear extraction buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate, and protease inhibitors. The NF-κB binding activity of the nuclear extract was measured with a TransFactor NF-κB colorimetric kit (Clontech, Mountain View) according to manufacturer's instructions.

2.8. Western blot

To extract total proteins, cells or tissues were lysed on ice for 30 min in a lyses buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 20 mM NaF, 3 mM Na_3VO_4, 1 mM PMSF, 1% (v/v) NP-40, and protease inhibitor cocktail according to previous studies with some modifications [30–33]. The lysates were then centrifuged at 12,000*g* for 15 min, and the supernatants were recovered. After being denatured, proteins were separated on 10% SDS/PAGE gels and transferred to the nitrocellulose membrane by using a transfer cell system (Bio-Rad, California, USA). After being blocked with 5% nonfat dried milk powder/Tris-buffered saline Tween-20 for 1 h, the membranes were probed with 1:500 primary antibodies against iNOS, IκB-α, p-NF-κB, NF-κB, and Histone H2A or 1:10000 primary antibody against GAPDH overnight at 4 °C. The bindings of the primary antibody were detected with IRDye 680-labled secondary antibodies (1:3000–1:5000). The immunoblots were visualized by Odyssey CLx Western blot detection system. The cytoplasmic and nuclear proteins were separated using a Nuclear Protein Extraction Kit (Bi Yuntian Biological Technology Institution, Shanghai, China), and were normalized to GAPDH and Histone-H2A, respectively. The densities of immunoblots were quantified using Image J software.

2.9. Chromatin immunoprecipitation (ChIP)

This experiment was performed according to previous studies [34]. Cultured peritoneal macrophages were treated with LPS (1 μg/mL) for

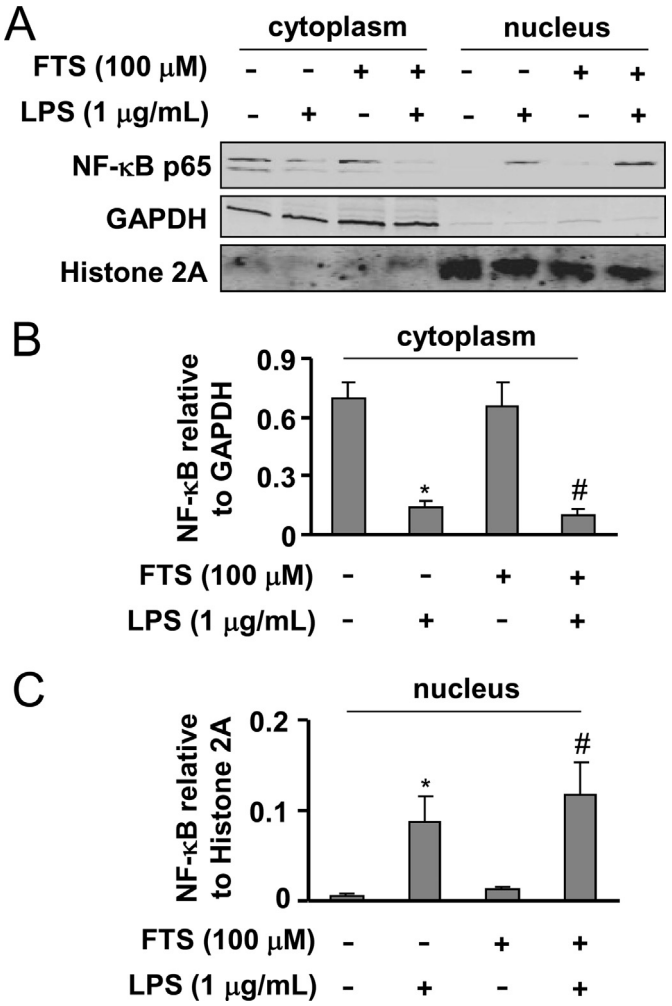

Fig. 5. Effects of FTS on LPS-initiated NF-κB nuclear translocation in cultured macrophages. (A) Representative images showing the effect of FTS on NF-κB nuclear translocation. Cells were stimulated with LPS for 30 min and the levels of NF-κB in the cytoplasm and nucleus were detected. In FTS-interfered groups, macrophages were incubated with FTS (100 μM) for 30 min prior to LPS stimulation (1 μg/mL). (B, C) Quantitative analysis of NF-κB expression in the cytoplasm (B) and nucleus (C) after FTS and/or LPS treatment (n = 3; $*P < 0.05$ vs. control; $\#P < 0.05$ vs. FTS alone treatment). Data are shown as mean ± SME.

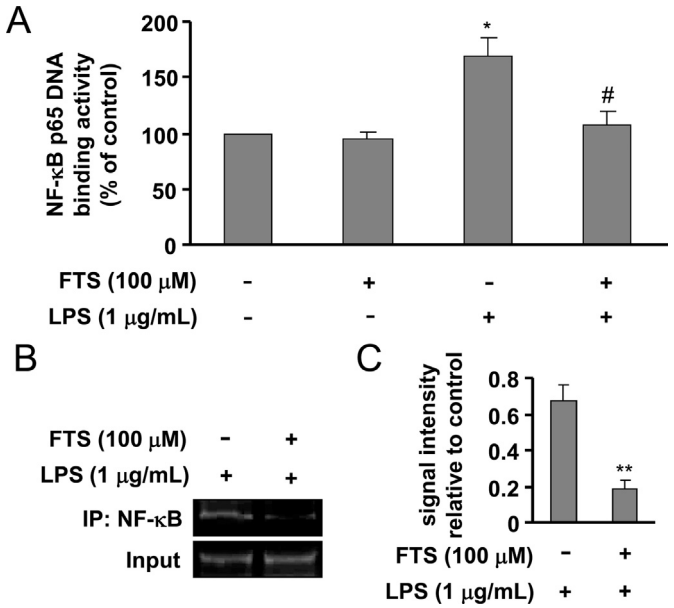

Fig. 6. FTS attenuates the binding of the active NF-κB to their DNA elements in iNOS promoters. (A) Quantitative analysis showing the effect of FTS on NF-κB binding to its DNA element. Macrophages were stimulated with LPS (1 μg/mL) and the binding activity of NF-κB p65 was measured in the absence or presence of FTS (100 μM, n = 5; $*P < 0.05$ vs. control; $\#P < 0.05$ vs. LPS alone treatment). (B) Representative ChIP data show that FTS reduced NF-κB binding to the iNOS promoter in LPS-stimulated macrophages. The chromatin was immunoprecipitated using the anti-p65 antibody. (C) Quantitative analysis showing the effect of FTS on the binding of NF-κB to the iNOS promoter in LPS-stimulated macrophages (n = 5; $**P < 0.01$ vs. LPS alone treatment). Data are shown as mean ± SME.

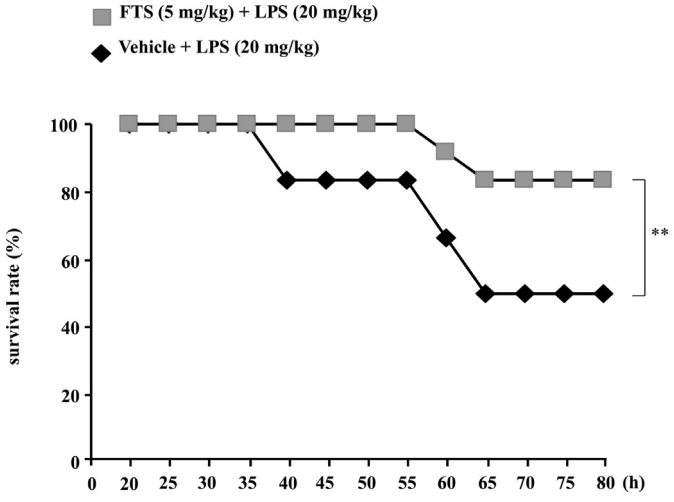

Fig. 7. FTS reduces LPS-induced mouse death. This image shows the change in mouse survival rate after LPS (20 mg/kg) and/or FTS (5 mg/kg) treatment which was recorded at different intervals. Each group contained 12 mice. $**P < 0.01$ vs. vehicle + LPS.

1 h in the absence or presence of FTS. 1% of formaldehyde was then added into the culture medium, and after incubation on the rocker for 10 min at room temperature, cells were rinsed twice in PBS and lysed for 15 min at 4 °C. After sonication, the lysate was used as a DNA input control. The remaining lysates were diluted 10-fold with a ChIP dilution buffer followed by incubation with NF-κB p65 antibody overnight at 4 °C. Immumoprecipitated complexes were collected using protein A/G Plus-agarose beads and extensively washed and incubated in an elution buffer containing 1% SDS and 0.1 M $NaHCO_3$ at room temperature for 15 min. Cross-linking of protein-DNA complex was reversed at 65 °C for 4 h. DNA was extracted with a Qiagen PCR purification kit. PCR primers for ChIP assays addressing NF-κB and STAT1 are as follows: NF-κB, 5′-CAAGCCAGGGTATGTGGTTT-3′ (forward) and 5′-GCAGCAGCCATC AGGTATTT-3′ (reverse). The resulting products were separated by 2% agarose gel electrophoresis.

2.10. Statistical analysis

Data are expressed as means ± SME. The student's *t*-test or two-way analysis of variance (ANOVA) followed by the Bonferroni's post hoc test was used for statistical analysis by employing SPSS 11.0 software. Mouse survival rate was analyzed by Kaplan-Meier survival analysis and log-rank test. Differences are considered significant at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. FTS prevents iNOS induction in LPS-stimulated macrophages

The dose- and time-dependent effects of FTS on iNOS induction in macrophages were first investigated. Cells with or without LPS treatment (1 μg/mL, 24 h) were pretreated with FTS for 30 min at concentrations ranging from 1 to 100 μM. As shown in Fig. 1A, B, FTS pretreatment markedly down-regulated the increase in iNOS protein expression in LPS-stimulated macrophages. Its peak effect was observed at the concentration of 100 μM (Fig. 1A, B). For this reason, 100 μM of FTS was selected to perform the following experiments. A time-dependent response curve showed that pretreatment of macrophages with 100 μM of FTS (30 min) gradually prevented LPS-induced increase in iNOS protein at different time points (8, 16, and 24 h, Fig. 1C, D). In accordance with the effect of FTS on iNOS protein expression, the level of NO released in the medium was also reduced by FTS pretreatment (100 μM, 30 min, Fig. 1E). The cell viability of the cultured peritoneal macrophages was not affected by FTS, LPS or FTS/LPS treatment (Fig. 1F).

3.2. FTS suppresses iNOS induction in endotoxemic mice

To explore the functional significance of the inhibitory effect of FTS on iNOS induction in vitro, we investigated the effect of FTS on iNOS induction in vital organs obtained from endotoxemic mice, such as the liver and lung. In accordance with previous studies [10], little or no iNOS was detected in the liver (Fig. 2A, B) and lung (Fig. 2C, D) in sham groups. Injection of LPS (20 mg/kg) into mice induced significant increases in the expression levels of iNOS protein in the liver (Fig. 2A, B) and lung (Fig. 2C, D), and these increases were prevented by FTS pretreatment (5 mg/kg) (Fig. 2A–D). These data indicate that FTS attenuates iNOS induction in endotoxemic mice in vivo.

3.3. FTS does not affect the expression levels of iNOS mRNA in vitro and in vivo

The absence of iNOS protein could be due to the deficiency of either gene transcription or protein translation. To distinguish these possibilities, iNOS mRNA formation was measured in the absence or presence of FTS. As shown in Fig. 3A, FTS pretreatment (100 μM, 30 min) markedly reduced iNOS mRNA formation in LPS (1 μg/mL, 6 h)-treated macrophages. In mice administered with 20 mg/kg of LPS, FTS pretreatment (5 mg/kg) also attenuated the formation of iNOS mRNA in the liver (Fig. 3B) and lung (Fig. 3C). These data demonstrate that FTS prevents LPS-induced iNOS gene transcription in both mouse macrophages and tissues.

3.4. FTS does not alter the IκB-α-NF-κB signaling in LPS-stimulated macrophages

The inhibitory effect of FTS on iNOS gene transcription appears to be mediated by the influence of FTS on IκB-α-NF-κB signaling. Our results showed that LPS stimulation (1 μg/mL) induced a significant degradation of IκB-α in cultured macrophages, which was not affected by FTS pretreatment (100 μM, 30 min; Fig. 4A, B). The effect of FTS on NF-κB phosphorylation in LPS-stimulated macrophages was also investigated. As shown in Fig. 4A and C, FTS pretreatment (100 μM, 30 min) showed no significant effects on LPS-induced increase in NF-κB phosphorylation levels in cultured macrophages. Since the nuclear translocation of NF-κB is necessary for the initiation of iNOS gene transcription, we analyzed the change in NF-κB levels in the compartment of cytoplasm and nucleus in LPS-stimulated macrophages. Results showed that NF-κB was present predominantly in the cytoplasm in unstimulated macrophages (Fig. 5A–C). LPS stimulation (1 μg/mL) induced a significant translocation of the cytoplasmic NF-κB into the nucleus, which was not affected by FTS pretreatment (100 μM, 30 min, Fig. 5A–C).

3.5. FTS attenuates the DNA binding activity of NF-κB in LPS-stimulated macrophages

Since FTS did not affect the IκB-α-NF-κB signaling in cultured macrophages, the possibility that FTS suppresses iNOS gene transcription through interfering with NF-κB binding to its DNA elements in promoters was evaluated. The cultured macrophages were stimulated with LPS to activate NF-κB first. Then, the binding of the active NF-κB with labeled DNA oligos corresponding to their promoters was measured in the absence or presence of FTS. Results showed that LPS stimulation (1 μg/mL) induced a dramatic increase in NF-κB binding activities in nuclei, and this increase was blocked by FTS pretreatment (100 μM, 30 min) (Fig. 6A). These results suggest that FTS may attenuate the binding of both active NF-κB with the DNA elements in its promoters during iNOS gene expression. This hypothesis was further confirmed by measuring the binding of NF-κB to iNOS promoters in LPS-treated macrophages with or without FTS pretreatment using ChIP assays. Results showed that LPS stimulation (1 μg/mL) promoted the binding of NF-κB to the iNOS promoter, and this binding was attenuated by FTS pretreatment (100 μM, 30 min) (Fig. 6B, C). Taken together, these results demonstrate that the FTS inhibits the binding of active NF-κB to iNOS promoters.

3.6. FTS reduces LPS-induced mouse death

Finally, since the LPS-triggered NF-κB signaling in macrophages has been shown to be a critical mechanism for sepsis-related mouse death [35,36], we performed an experiment to assess whether FTS pretreatment could affect mouse susceptibility to endotoxic shock. In this experiment, mice with or without FTS pretreatment (5 mg/kg) were injected intraperitoneal with 20 mg/kg of LPS [27,28], and the mouse survival rate was observed 4 days. As shown in Fig. 7, Along 4 days of observation, the survival rate of FTS/LPS-treated mice was significantly higher than that of vehicle/LPS-treated mice, 94.2 versus 76.9 (%), respectively. Together with the fact that FTS reduces iNOS protein expression in the liver and lung in endotoxemic mice, this result further strengthens the functional significance for the regulation of LPS-triggered NF-κB activation and iNOS expression by FTS.

4. Discussion

The present study identifies for the first time an inhibitory effect of FTS on iNOS induction in murine macrophages. FTS has been extensively studied in regards to its ability to inhibit Ras-dependent tumor growth [15–17]. Some other studies have revealed that FTS has an ability to modulate immune and inflammatory responses. For example, it has been reported that FTS can inhibit pro-inflammatory responses in various disease models associated with inflammation, such as experimental autoimmune neuritis [18], myocarditis [21], acute pancreatitis [22], allergic encephalomyelitis [20], and arthritis [25]. FTS also reduces the release of pro-inflammatory factors in mast cells and T cells [19,20]. However, whether and how FTS affects iNOS induction remains unknown. Our results showed that FTS prevents iNOS protein expression in murine macrophages stimulated by LPS. Mechanistic studies have indicated that the loss of iNOS protein in FTS-treated cells is due to lack of iNOS mRNA transcription. Prevention of LPS-induced iNOS expression by FTS pretreatment underscores the importance of FTS in iNOS induction regulation. The inhibitory effect of FTS on iNOS induction was also strengthened by the results in vivo, in which FTS was found to inhibit LPS-induced increase in iNOS protein expression in vital organs including the lung and liver. Taken together, these findings establish an inhibitory effect of FTS on iNOS induction in cultured cells and animals in vivo, and exploration of the in-depth mechanism for this

process may be beneficial to understand more therapeutic and side effects of FTS in disease therapy.

Blockade of a gene transcription in stimulated cells is often due to one or multiple interruptions in the signaling pathway from the stimuli to the corresponding transcriptional factors [12,14]. However, FTS pretreatment, while blocking iNOS gene transcription, had no effects on LPS-induced IκB-α degradation and NF-κB phosphorylation and nuclear translocation. These findings suggest that FTS interferes with the signal events downstream of the nuclear translocation of NF-κB. This possibility was further supported by an observation that FTS prevented the binding of the active NF-κB to their DNA elements and iNOS promoters in ChIP assays. These findings show that FTS directly interrupts the binding of NF-κB to the promoters in the onset of iNOS gene transcription, and on the other hand, provide a plausible explanation to why iNOS gene transcription was inhibited, despite that activation of IκB-α-NF-κB signals was not perturbed in FTS-treated cells.

How exactly FTS affects the bindings of NF-κB to their DNA elements in iNOS promoters remains to be determined. FTS has been shown to exert pharmacological effects via targeting a membrane molecule Ras. The functional inhibition of H-Ras, N-Ras, and K-Ras by FTS can inhibit the growth of tumor cells [15–17]. FTS also attenuates atherosclerosis in apolipoprotein E knockout mice [37]. In inflammatory conditions, the activity of Ras is positively associated with iNOS induction, and the expression of a dominant-negative mutant of p21(ras) can prevent iNOS induction in primary cultured astrocytes [38]. Further, inhibition of Ras activity as well as its palmitoylation levels has been shown to abrogate interleukin-1β (IL-1β)-mediated generation of NO in insulin-secreting clonal β (HIT-T15) cells [39,40], and the helicobacter pylori- and ultrasound-induced expression of iNOS is mainly dependent on the presence of membrane Ras [41,42]. In addition, the involvement of Ras in iNOS induction has been shown to be associated with NF-κB activation [38,42,43]. It is therefore reasonable to speculate that the inhibitory effect of FTS on iNOS induction may be due to Ras inhibition. However, whether the attenuation of NF-κB binding to its DNA elements by FTS pretreatment is indeed associated with Ras inhibition remains an open question because Ras has been widely reported to mediate iNOS induction via activation of the classical IKKβ-IκB-α-NF-κB signaling [38,39,43]. Inhibition of Ras by FTS may block IκB-α degradation and NF-κB phosphorylation and nuclear translocation. However, our observations do not support this hypothesis. The off-target effect of FTS still cannot be precluded at least in the present experimental system, which should be investigated in future studies.

It is well known that NF-κB can control the expression of a wide range of pro-inflammatory cytokines [44,45]. Thus, we could ameliorate the pathogenesis of pro-inflammatory disorders via suppression of NF-κB. In this study, the inhibition of NF-κB DNA binding activity by FTS pretreatment strongly indicates that FTS could prevent the production of other pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6, through which it may exert prophylactic and/or therapeutic activities in disorders associated with inflammation. This hypothesis is supported by some previous studies. For example, FTS has been shown to ameliorate adjuvant-induced arthritis via inhibiting the release of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-17, and IL-22 [25,46]. FTS also attenuates dextran sodium sulfate colitis in mice via reducing the production of TNF-α and IL-1β [47]. Our results about the regulation effect of FTS on sepsis-associated mouse survival rate also support this hypothesis because (i) FTS pretreatment can prevent LPS-induced decline in mouse survival rate, and (ii) sepsis-induced mouse death has been confirmed to be triggered by inflammatory storms induced by pro-inflammatory factors, such as TNF-α, IL-6, IL-1β, and NO [35,36,48,49]. In future studies, we will evaluate whether and how FTS exerts prophylactic and/or therapeutic actions in different disease models via affecting the production of pro-inflammatory mediators.

5. Conclusion

Our results present an inhibitory role of FTS in iNOS induction through interrupting NF-κB association with their DNA elements in iNOS promoters in inflammatory settings, which may provide further insight into the pharmacological effect of FTS in disease therapy. The mechanistic study would be beneficial to modify the structure of FTS and optimize its pharmacological effects. In fact, some structure-modified FTS derivatives have been designed in previous studies, and their anti-proliferative activities have also been evaluated [15,50]. Considering the inhibition of iNOS induction needs relative high dose of FTS, we will plan to compare the inhibitory efficiency of different FTS derivatives in iNOS induction in hope of selecting compounds with more excellent anti-inflammatory activities.

Conflict of interest

There is no conflict of interest to declare.

Acknowledgments

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