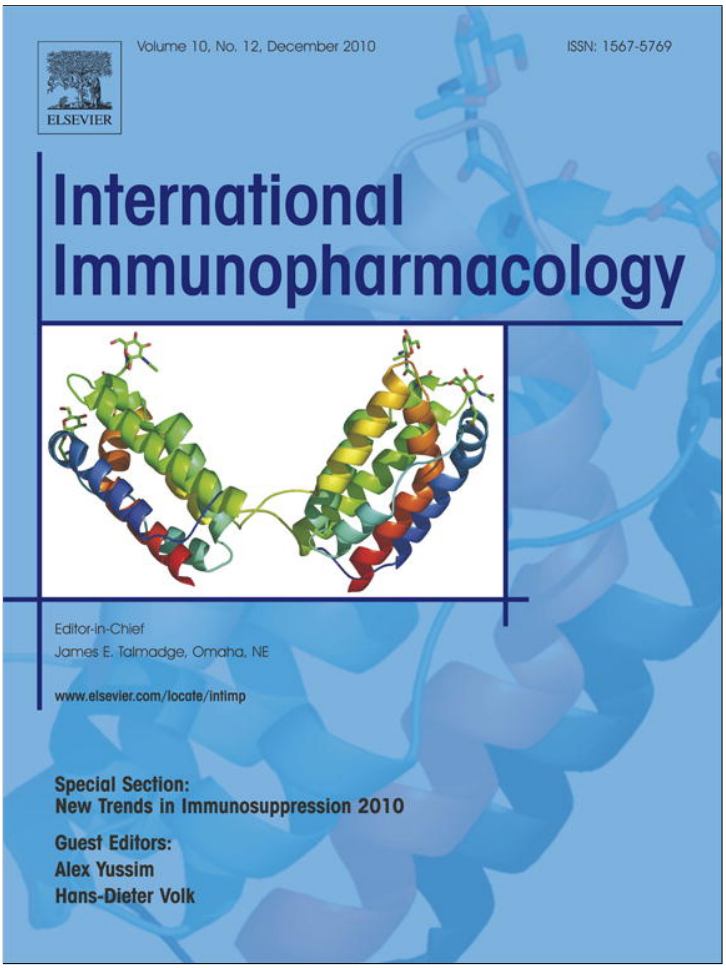

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The fungal metabolite, citrinin, inhibits lipopolysaccharide/interferon-γ-induced nitric oxide production in glomerular mesangial cells

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ABSTRACT

The mycotoxin, citrinin (CTN), is a secondary metabolite of the fermented products of *Monascus*. The mycotoxin can either suppress or stimulate immune responses. In the present study, the immunomodulatory role of CTN in nitric oxide (NO) production, a proinflammatory mediator in the process of inflammation, was investigated. NO is well known as a mediator of immune responses. Overproduction of NO catalyzed by inducible nitric oxide synthase (iNOS) protects host cells against microbial invasion, while aberrant iNOS induction is associated with the pathophysiology of inflammatory events. Herein, we report that CTN significantly suppressed lipopolysaccharide (LPS)/interferon (IFN)-γ-induced NO production in MES-13 cells, a glomerular mesangial cell line. The percentage of NO reduction caused by CTN was far greater than that of the decline in cell viability. CTN decreased iNOS gene and protein expressions in concentration-dependent manners. CTN caused declines in LPS/IFN-γ-induced signal transducer and activator of transcription-1α (STAT-1α) phosphorylation. Furthermore, LPS/IFN-γ's induction of interferon response factor-1 (IRF-1) mRNA expression was inhibited by CTN. Moreover, CTN attenuated IκB-α phosphorylation and reduced NF-κB's translocation to the nuclear fraction. Taken together, our data indicated that CTN significantly suppressed NO and iNOS expressions in MES-13 cells via inhibition of the JAK/STAT-1α and NF-κB signaling pathways.

1. Introduction

Red Koji, a traditional Chinese favoring agent, is the fermented product of a fungal species of the genus *Monascus*. Several metabolites of *Monascus* exhibit beneficial effects to human health while citrinin (CTN), a mycotoxin, causes cytotoxicity and oxidative damage to cultured kidney cells and laboratory animals [1–3]. Mycotoxins can either stimulate or suppress immune responses [4–8]. The mycotoxin, CTN, and gliotoxin differentially affected cytokine production in lipopolysaccharide (LPS)-stimulated human alveolar epithelial cells and a monocytic cell line [5–7]. Exposure to low doses of CTN and gliotoxin inhibited secretion of interleukin (IL)-10 while their effects on the overproduction of tumor necrosis factor (TNF)-α and IL-6 led to an increase in the inflammatory response [7]. Non-toxic doses of CTN reduced LPS-induced IL-10 production in monocytes but had no effect on LPS-induced TNF-α production [5]. Moreover, mycotoxin CTN, gliotoxin, and patulin affect interferon (IFN)-γ rather than IL-4 production in human blood cells [8]. Those reports suggest that mycotoxins such as CTN may cause an imbalance in the secretion of inflammatory cytokines by immune cells.

Nitric oxide (NO), a simple paracrine gas, exhibits a broad range of functions in immune defense [9]. NO is synthesized by three NO synthase (NOS) forms including inducible (i)NOS, neuronal (n)NOS, and endothelial (e)NOS. The induced expression of iNOS protein generates high concentrations of NO when cells are challenged with endotoxins or cytokines. The large amounts of NO produced by iNOS exhibit beneficial antimicrobial, antiviral, and antitumor activities [10]. On the contrary, aberrant iNOS induction is correlated with the pathogenesis of various diseases [9,10]. Regulation of NO synthesis by

Abbreviations: CTN, citrinin; EMSA, electrophoretic mobility shift assay; GAS, gamma-activated sequence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUS, hemolytic uremic syndrome; IL-1β, interleukin-1β; IFN-γ, interferon-γ; γ-IRE, interferon-γ response element; iNOS, inducible nitric oxide synthase; IκB-α, inhibitory factor-κB-α; IKK, IκB kinase; IRF-1, interferon response factor; JAK, Janus kinase; LPS, lipopolysaccharide; MES-13 cells, mouse glomerular mesangial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor κB; NO, nitric oxide; PAA, phenylacetic acid; PVDF membrane, polyvinylidene difluoride membrane; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; Stx, Shiga-like toxin; STAT-1α, signal transducer and activator of transcription-1α; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing Tween 20.

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iNOS is under precise control, predominantly at the transcriptional level [11]. Endotoxin LPS and inflammatory mediators, such as IFN-γ, TNF-α, and IL-1β were shown to induce iNOS gene expression through the activation of several transcription factors. Two major transcription factors, nuclear factor (NF)-κB and the signal transducer and activator of transcription (STAT)-1α were reported to modulate *iNOS* gene expression in cell- and species-specific manners [12].

By binding to the 5′-flanking region of the *iNOS* gene, activated NF-κB and STAT-1α work synergistically to elicit iNOS gene expression. NF-κB is activated by LPS, whereas activation of STAT-1α is triggered by IFN-γ. Through binding to toll-like receptor 4 (TLR4), LPS activates downstream signaling pathways, in a MyD88-dependent way, to trigger activation of inhibitory factor-κB-α (IκB-α) kinase (IKK). IKK then phosphorylates IκB-α proteins leading to their ubiquitination and degradation, followed by the release of its partner, NF-κB. Translocation of NF-κB to the nucleus induces iNOS gene expression through binding to the cis-acting NF-κB-binding element [13]. The activation of STAT-1α is triggered by IFN-γ. Binding of IFN-γ to the IFN-γ receptor initiates activation of Janus kinase (JAK), an IFN-γ receptor-associated protein tyrosine kinase. Activated JAK phosphorylates cytoplasmic STAT-1α at Y701 and then causes dimerization of STAT-1α and its translocation to the nucleus, where it activates transcription of its target genes [14]. One of the target genes of STAT-1α is interferon response factor (IRF)-1, which binds to the IFN-γ-response element (γ-IRE). The γ-IRE is also located in the 5′-flanking region of the murine *iNOS* gene. Moreover, the STAT-1α homodimer recognizes the gamma-activated sequence (GAS) elements on the enhancer region of the *iNOS* region [15]. Thus, the JAK/STAT-1α signal pathway is essential for the transcriptional induction of iNOS by IFN-γ.

Several toxic compounds modulate immune responses through interfering with signaling cascades and suppressing the activation of transcription factors. As reported by Hickey et al. [16], adenylate cyclase toxin of *Bordetella pertussis* inhibits TLR-induced IRF-1 and IRF-8 activation in dendritic cells. Hydroquinone, a toxic metabolite of benzene, interferes with the immune responses by diminishing LPS-mediated NO production and suppressing proinflammatory cytokine production in monocytes/macrophages [17]. Aristolochic acid, a toxic herbal extract from *Artistolochia fangchi*, inhibited NF-κB signaling as shown by a microarray analysis in human kidney HK-2 cells [18]. Most strikingly, gliotoxin, a mycotoxin of fungal metabolites, exhibits anti-inflammatory and immunosuppressive functions through blockade of NF-κB activation [19,20]. Thus, it was speculated that CTN, also a mycotoxin, modulates NO production, a proinflammatory mediator in the process of inflammation.

Herein, we present data showing that the co-incubation of CTN with LPS/IFN-γ significantly suppressed iNOS-induced NO production in MES-13 cells. The percentage of NO reduction caused by CTN was far greater than that of the decline in cell viability. The inhibitory effect of CTN on LPS/IFN-γ-stimulated NO production was not due to a decrease in MES-13 cell viability but was caused by the suppression of iNOS gene and protein expressions. We further demonstrated that CTN inhibited LPS/IFN-γ-stimulated NO production in MES-13 cells via JAK/STAT-1α and NF-κB pathways.

2. Materials and methods

2.1. Materials

Fetal bovine serum was from Highclone (Logan, UT). DMEM medium and Ham's F12 medium and medium supplements were obtained from Gibco BRL (Gaithersburg, MD). LPS and CTN (98.5% purity) were obtained from Sigma Chemical Company (St. Louis, MO). CTN was purified from *Penicillium citrinum* with thin layer chromatography according to the manufacture. IFN-γ was purchased from PeproTech EC Ltd. (London, UK). The specific antibodies for iNOS, inhibitory factor-κB (IκB), and IRF-1 were products from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for p65 (NF-κB) was acquired from BD Biosciences (Franklin Lakes, NJ). Phosphorylated STAT-1α, STAT-1α, phosphorylated IκB-α antibodies were purchased from Cell Signaling Technology Inc.(Beverly, MA). The antibody for β-actin was acquired from Cayman Chemical Company (Ann Arbor, MI). Oligonucleotide primer sequences of iNOS, IRF-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for reverse-transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) were selected by using Primer Select (MD Bio, Inc). The nylon membranes (Hybond N^+) for electrophoretic mobility shift assay (EMSA) were purchased from Amersham Pharmacia Biotech Inc., (Piscataway, NJ).

2.2. Cell culture

The MES-13 cell line (glomerular mesangial cells from an SV40 transgenic mouse) was obtained from American Type Culture Collection (CRL-1927; Manassas, VA, USA) and maintained in culture medium with a 3:1 mixture of DMEM medium and Ham's F-12 medium, supplemented with 14 mM HEPES, 2 mM glutamine, antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) and 5% fetal bovine serum at 37^0 C. The incubation chamber was equilibrated with 5% CO_2–95% air.

2.3. Nitrite assay

Nitrite assay was performed to measure NO production in MES-13 cells after different treatments. Nitric oxide (NO) is rapidly converted into nitrite as the end product. Thus, the nitrite accumulation in culture supernatant was used as indirect measures of the amount of NO produced. The Griess assay was used and the nitrite level was measured in triplicate. Briefly, an aliquot of 100 μl of the culture supernatant of MES-13 cells was mixed with 100 μl of Griess reagent (one part 0.1% N-(1-naphtyl) ethylene-diamine dihydrochloride in water and one part 1% sulfanilamide in 5% H_3PO_4; both purchased from Sigma Chemicals). The mixture was incubated for 10 min at room temperature in the dark. The absorbance at 540 nm was measured and the nitrite concentration was calculated by comparison to standard curves of sodium nitrite in culture medium.

2.4. MTT assay

Colorimetric MTT assay was used to measure the cell viability. In viable cells, the mitochondria are able to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrrazolium bromide (MTT) to formazan. After different drug treatments, the culture medium of MES-13 cells was aspirated and 0.5 mg/ml MTT was incubated with the cells for 3 h at 37 °C followed by solubilization in isopropanol. The formazan product was measured by the absorbance at 570 nm on a VersaMaxTM Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). The absorbance read from cells treated with LPS/IFN-γ alone was used to indicate 100% of cell viability.

2.5. Preparation of whole cell extract and western blot analysis

To detect the protein levels of iNOS after exposure to different stimuli, MES-13 cells were washed with 1× PBS, scraped out, and incubated with lysis buffer. The lysis buffer contained 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail tablet (Roche Applied Science, Mannheim, Germany). Cells suspended in lysis buffer were sonicated. The homogenate was centrifuged at 13,000 rpm for 40 min at 4 °C, and the cell supernatant was collected. The protein concentration was measured using a Bio-Rad protein assay kit. Cell lysate was combined with 5× sample buffer containing 100 mM Tris–HCl (pH 6.8), 20% glycerol, 7% SDS, 5% mercaptoethanol, and 0.1% bromophenol blue. The sample was boiled for 5 min and centrifuged to remove the

debris. Equal amounts of protein samples (60 μg) were subjected to SDS-PAGE using 10% polyacrylamide gels. Following electrophoresis, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% skim-milk in Tris-buffered saline (TBS) containing 10 mM Tris (pH 8.0) and 150 mM NaCl, then incubated with primary antibody at 4 °C overnight. TBS containing 0.02% Tween 20 (TBST) was used to wash out the nonspecific binding material on the PVDF membrane. Finally, the membrane was incubated with secondary antibody for 1 h at room temperature. After washing with TBST, the immunoreactive bands were visualized with a light-emitting kit (ECL, Amersham, UK). The protein amount was quantified by measuring the area of the iNOS band using densitometric analysis with AlphaEaseFC.

2.6. Preparation of cytosolic and nuclear extracts

To determine cytoplasmic IκB-α phosphorylation and nuclear NF-κB protein level, cytoplasmic and nuclear proteins of MES-13 cells were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Company, Rockford, IL) containing proteinase inhibitors cocktail. Phosphorylation of IκB-α in cytoplasmic extracts and the activation of NF-κB in nuclear extracts were analyzed by Western blot. The primary antibodies for phosphorylated IκB-α and NF-κB p65 were purchased from Cell Signaling Technology, Inc. (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. STAT-1α phosphorylation was also determined. The primary antibodies for detection of STAT-1α protein and phosphorylation were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from MES-13 cells using the Tri reagent RNA isolation reagent (Molecular Research Center, Inc, Cincinnati, OH, USA). Total RNA was reverse transcribed to cDNA using Superscript II reverse transcription RT-PCR kit (Life Technologies, Gaithersburg, MD) followed by amplification with PCR. The oligonucleotide primers for the RT-PCR were as followed: 5′-CAGTTCTGCGCCTTTGCTCAT-3’ (forward) and 5′-GGTGGTGCGGCTGGACTTT-3’(reverse) for iNOS; 5′-CGATACAAAGCAGGGGAAAA-3’ (forward) and 5′- TAGCTGCTGTGGTCATCAGG-3′ (reverse) for IRF-1 and 5′- CATCATCTCCGCCCCTTCT-′3 (forward) and 5′-CTCGTGGTTCACACCCATCA-′3 (reverse) for GAPDH. After an initial denaturation at 94 °C for 3 min, 35 cycles of amplification (94 °C for 30 s, 58.3 °C for 30 s, and 72 °C for 1 min) were performed followed by a 7 min extension at 72 °C for iNOS; an initial denaturation at 94 °C for 3 min, 31 cycles of amplification (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min) were performed followed by a 7 min extension at 72 C for IRF-1; an initial denaturation at 94 °C for 3 min, 40 cycles of amplification (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min) were performed followed by a 7 min extension at 72 °C for GAPDH. The amplified PCR products were analyzed on a 1% agarose gel. The PCR product of GAPDH was used as an internal control for quantitation.

2.8. Real-Time RT-PCR

Total cellular RNA was extracted from MES-13 cells using Tri reagent RNA isolation reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). The cDNAs were synthesized from 3.5 μg of RNA of each sample using a SuperScriptTM II reverse transcription system kit according to the manufacturer's protocol. The primers used were as followed: 5′-CCGATTTAGAGTCTTGGTGAAAGTG-3′ (forward) and 5′-TGACCCGTGAAGCCATGA-3′ (reverse) for iNOS; 5′-CCGATACAAAGCAGGAGAAAAAG-3′ (forward) and 5′-TGGCACAACGGAAGTTTGC-3′ (reverse) for IRF-1; 5′-CATCATCTCCGCCCCTTCT-3′ (forward), 5′-CTCGTGGTTCACACCCATCA-3′ (reverse) for GAPDH. The qRT-PCR was carried out in a 25 μl final volume containing: 3 μg cDNA sample, 500 nM primer pairs and 12.5 μl SYBR Green PCR Master Mix, and performed by an initial denaturation at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 60 s in an ABI PRISM 7000 system sequence detector (Applied Biosystems). Each RNA sample was measured in duplication. The specificity of amplified PCR products was evaluated by a comparative Ct method. The threshold cycle value (Ct value), which is inversely proportional to the initial template copy number, is calculated from cycle number at which the PCR product crosses a threshold of detection. The iNOS (or IRF-1) mRNA expression were normalized against GAPDH and gene expression changes induced by various treatments were determined by the $2^{-\triangle\triangle \mathbf{CT}}$ method.

2.9. Electrophoretic mobility shift assay (EMSA)

MES-13 cells were pre-treated with CTN (25 μM and 40 μM) for 12 h, followed by stimulation with 1 μg/ml LPS and 10 ng/ml IFN-γ for 40 min. Thereafter, nuclear extracts were prepared by NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Company) and stored at 4 °C until the EMSA was performed. The LightShift Chemiluminescent EMSA Kit was purchased from Pierce Chemical Co. The biotin-labeled and unlabeled oligonucleotides, corresponding to the NF-κB were synthesized. The sequences utilized were as followed: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (for NF-κB). Nuclear extract (10 μg), poly (dI–dC), and biotin-labeled probes were mixed with the binding buffer (to a final volume of 20 μl) and were incubated at room temperate for 30 min. The nuclear protein–DNA complex was separated by a native 6% polyacrylamide gel electrophoresis and then transferred to nylon membranes (Hybond N^+). Next, the membrane was treated with streptavidin–horseradish peroxidase, and the nuclear protein–DNA bands were developed with the use of a SuperSignal West Pico kit (Pierce Chemical Co).

2.10. Statistical analysis

The values were expressed as the mean ± S. E. M. of at least three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett's method for multi-group comparison tests. A value of $p<0.05$ was considered as statistical significantly.

3. Results

3.1. Effects of CTN on cell viability of MES-13 cells

Cell viability of MES-13 cells after exposure to different drugs was assessed by an MTT assay. There was around 20% decline in cell viability of MES-13 cells after treatment with 1 μg/ml LPS and 10 ng/ml IFN-γ. The co-incubation of MES-13 cells with either 3, 15, or 25 μM of CTN and a mixture of LPS (1 μg/ml) and IFN-γ (10 ng/ml) for 24 h did not cause a significant decrease in cell viability; more than 95% of MES-13 cells were viable compared to control cells stimulated with LPS/IFN-γ alone (Fig. 1). On the other hand, exposure of LPS/IFN-γ-stimulated cells to 40 μM CTN caused a further 30% reduction in cell viability.

3.2. CTN suppressed nitrite production in LPS/IFN-γ-stimulated cells

MES-13 cells were co-treated with various concentrations of CTN (3, 15, 25, and 40 μM) for 24 h in the presence of LPS/IFN-γ. The cell medium was collected, and nitrite concentrations within the medium were determined by a Griess assay. Exposure of cells to LPS/IFN-γ resulted in nitrite release to 29.52 ± 3.9 μM. The presence of CTN reduced LPS/IFN-γ-triggered nitrite production in a dose-dependent manner. CTN doses of 3, 15, 25, and 40 μM significantly modulated

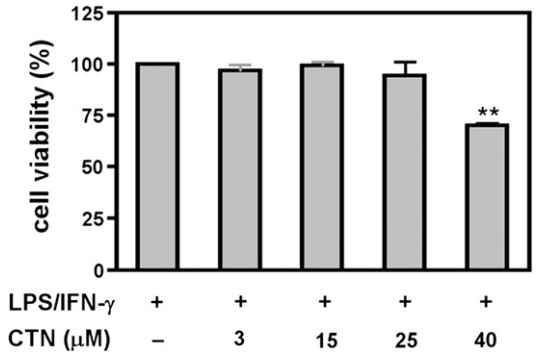

Fig. 1. Effect of citrinin (CTN) on cell viability of MES-13 cells. Cells were treated for 24 h with a mixture of 1 μg/ml lipopolysaccharide (LPS) and 10 ng/ml interferon (IFN)-γ in the absence or presence of various concentrations of CTN. Cell viability was measured by an MTT assay. Data are the mean ± SEM of at least three separate experiments and are expressed as the percentage of the culture treated with LPS/IFN-γ alone. Asterisks indicate a significant difference from treatment with LPS/IFN-γ alone (*$p<0.05$, **$p<0.01$).

levels of nitrite to 30.96 ± 5.3, 24.11 ± 7.2, 17.07 ± 5.7, and 10.06 ± 1.9 μM, respectively. While more than 95% of MES-13 cells were viable compared to control cells stimulated with LPS/IFN-γ, nitrite levels were reduced by over 20% and 40%, upon treatment with CTN at the doses of 15 and 25 μM, respectively. Although the cell viability of LPS/IFN-γ-treated MES-13 cells was further reduced by 30% upon treatment with a CTN dose of 40 μM, nitrite levels were reduced by over 60% (Fig. 2). These results indicate that the inhibitory effect of CTN on NO production in MES-13 cells was not caused by a drop in cell viability.

3.3. CTN downregulated iNOS protein expression in LPS/IFN-γ-mediated culture

The expression of iNOS protein in treated MES-13 cells was determined by Western blotting. As shown in Fig. 3, the iNOS protein level markedly increased in cultures treated with a combination of LPS and IFN-γ. The addition of CTN reduced the expression of stimulated iNOS protein in a dose-dependent way. The value of LPS/IFN-γ-induced iNOS protein as the control level was set to 100%.The signals of iNOS protein significantly decreased to 79%, 61%, 47% and 21% of the control level following treatment with 3, 15, 25, and 40 μM CTN, respectively. On the other hand, LPS, IFN-γ, or CTN alone did not modulate the expression levels of iNOS protein (data not shown). These results suggest that the inhibitory effect of CTN on LPS/IFN-γ-stimulated NO

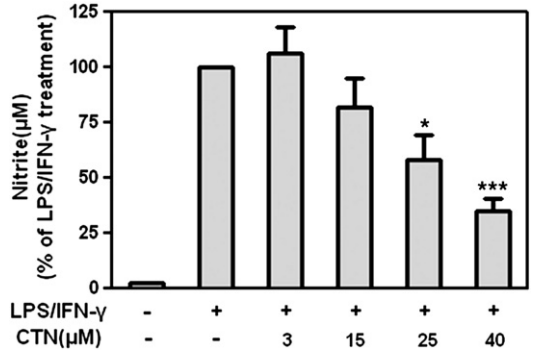

Fig. 2. Effect of citrinin (CTN) on lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated nitrite synthesis in MES-13 cells. Cells were treated for 24 h with a mixture of 1 μg/ml LPS and 10 ng/ml IFN-γ in the absence or presence of various concentrations of CTN. Nitrite levels were measured by the Griess reaction. Data are the mean ± SEM of at least three separate experiments and are expressed as the percentage of the culture treated with LPS/IFN-γ alone. Asterisks indicate a significant difference from treatment with LPS/IFN-γ alone (**$p<0.01$, ***$p<0.005$).

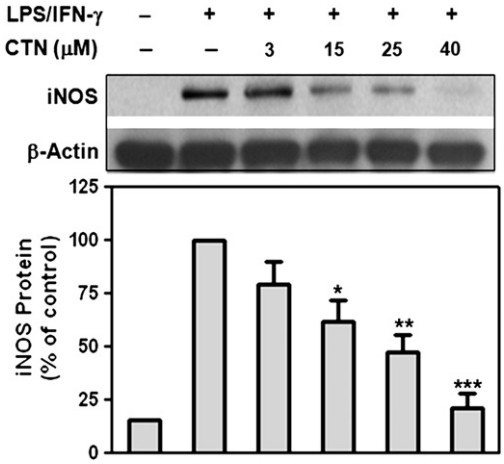

Fig. 3. Effect of citrinin (CTN) on lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated inducible nitric oxide synthase (iNOS) protein expression in MES-13 cells. Cells were treated for 24 h with a mixture of 1 μg/ml LPS and 10 ng/ml IFN-γ in the absence or presence of various concentrations of CTN. iNOS protein expression was determined by Western blot analysis. β-Actin was used as an internal control. Relative protein levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity of the iNOS protein from cultures treated with LPS/IFN-γ alone. Data are the mean ± SEM of iNOS/β-actin from at least three separate experiments, and asterisks indicate a significant difference from treatment with LPS/IFN-γ alone (***$p<0.005$).

production was accompanied by a reduced expression of the iNOS protein.

3.4. CTN decreased iNOS mRNA levels in LPS/IFN-γ-stimulated culture

To examine whether the inhibitory action of CTN on LPS/IFN-γ-stimulated NO production is correlated with the iNOS gene expression at the transcriptional level, a semiquantitative RT-PCR and real-time RT-PCR were performed to determine the iNOS mRNA level in MES-13 cells. MES-13 cells were treated with an LPS and IFN-γ mixture in the absence or presence of various concentrations of CTN (3, 15, 25, and 40 μM). As revealed by a semiquantitative RT-PCR in Fig. 4A, signals of iNOS mRNA gradually diminished with increasing concentrations of CTN. Similar inhibitory effects of CTN were detected by a quantitative real-time RT-PCR. The presence of 15, 25, and 40 μM CTN inhibited levels of LPS/IFN-γ-stimulated iNOS mRNA to 65%, 52% and 16% of the control level, respectively (Fig. 4B). These data indicate that the inhibitory effects of CTN on LPS/IFN-γ-stimulated NO production occurred by transcriptional regulation of the iNOS gene expression.

3.5. Effects of CTN on LPS/IFN-γ-modulated STAT-1α phosphorylation

To investigate whether CTN attenuated transcriptional expression of the iNOS gene through interrupting upstream signaling of the *iNOS* gene, we first elucidated the effect of CTN on activation of the JAK/STAT-1α pathway; tyrosine phosphorylation (Tyr701) of STAT-1α indicated the prime effect of IFN-γ on the iNOS gene expression [21]. MES-13 cells were pre-incubated with or without CTN for 12 h and then treated with LPS/IFN-γ for another 3 h. Cytoplasmic and nuclear fractions of MES-13 cells were separately collected and applied to a Western blot analysis to examine STAT-1α phosphorylation. In Fig. 5, upon treatment of cultures with LPS/IFN-γ, a strikingly high level of phospho-STAT-1α was observed in the nuclear fraction of MES-13 cells. The co-incubation with 25 and 40 μM CTN led to a significant attenuation of STAT-1α phosphorylation induced by LPS/IFN-γ. This result indicated that CTN exerts its inhibitory action on LPS/IFN-γ-triggered iNOS gene expression through the suppression of the STAT-1α signaling pathway. However, neither CTN nor LPS/IFN-γ alone changed the levels of phosphorylated STAT-1α in the cytoplasmic fraction (data not shown).

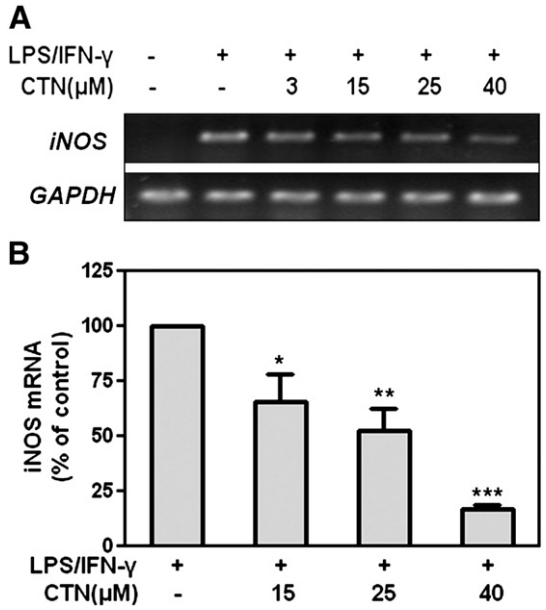

Fig. 4. Effects of citrinin (CTN) on lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated inducible nitric oxide synthase (iNOS) mRNA expression in MES-13 cells assessed by (A) semiquantitative RT-PCR and (B) real-time RT-PCR. Cells were treated for 24 h with a mixture of 1 μg/ml LPS and 10 ng/ml IFN-γ in the absence or presence of various concentrations of CTN. mRNA levels of iNOS were measured by a semiquantitative RT-PCR and real-time RT-PCR. GAPDH was used as an internal control. Relative mRNA levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity of iNOS mRNA from cultures treated with LPS/IFN-γ alone. Data are the mean ± SEM of iNOS/GAPDH of at least three separate experiments, and asterisks indicate a significant difference from the culture treated with LPS/IFN-γ alone (*$p<0.05$, **$p<0.01$).

3.6. CTN reduced mRNA expression of the IRF-1 gene

IRF-1 is one of the target genes of phosphorylated STAT-1α and is able to bind to IREs located in the 5′-flanking region of the murine *iNOS* gene [22,23]. To further confirm that the suppression of STAT-1α phosphorylation by CTN can lead to an ineffective response of its target gene, the effect of CTN on IRF-1 expression was investigated with real-time RT-PCR in MES-13 cells. Levels of the IRF-1 transcript were faintly detected by an RT-PCR in unstimulated MES-13 cells. The stimulation of MES-13 cells with LPS/IFN-γ enhanced IRF-1 mRNA expression while the co-supplementation with 25 and 40 μM CTN reversed this effect as detected with real-time RT-PCR (Fig. 6). After the treatment of cultures with 25 and 40 μM CTN, signals of IRF-1 mRNA respectively decreased to 70% and 45% of that of LPS/IFN-γ-stimulated cells.

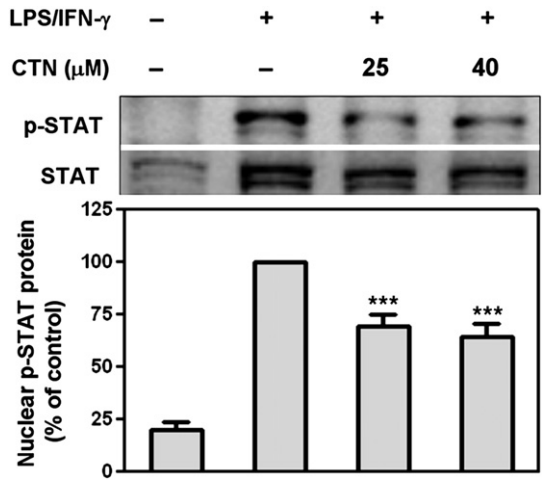

Fig. 5. Effects of citrinin (CTN) on lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated signal transducer and activator of transcription (STAT)-1α activation. Cells were pre-incubated with or without CTN for 12 h and then treated in the absence or presence of 1 μg/ml LPS and 10 ng/ml IFN-γ or 40 min. The nuclear fractions were used to analyze the content of STAT-1α protein expression and phosphorylated STAT-1α level by Western blotting. Relative protein levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity in the culture treated with LPS/IFN-γ alone. Data are the mean ± SEM of phosphorylated STAT-1α/STAT-1α protein from at least three separate experiments, and asterisks indicate significant difference from treatment with LPS/IFN-γ alone (*$p<0.05$).

3.7. The presence of CTN prevented the nuclear translocation of NF-κB and phosphorylation of IκB

Both NF-κB and STAT-1α were reported to modulate the iNOS gene expression in cell- and species-specific manners [12]. To further explore the mechanism underlying the inhibitory effect of CTN on iNOS gene transcription, levels of NF-κB p65 in nuclear extracts were determined by Western blotting. As shown in Fig. 7A, the nuclear translocation of p65 was observed after the addition of LPS/IFN-γ to MES-13 cells. In addition, the presence of CTN (25 and 40 μM) significantly blocked the nuclear translocation of p65 triggered by LPS/IFN-γ.

To confirm the inhibitory effect of CTN on suppressing NF-κB activation, EMSA were performed to assess whether CTN affected the binding of NF-κB to DNA in vitro. In LPS/IFN-γ-activated MES-13 cells, a substantial increase in the DNA-binding activity of NF-κB was observed compared to untreated cells (Fig. 7B). The co-incubation with CTN (25 and 40 μM) led to a lower DNA-binding activity of NF-κB (Fig. 7B). The specificity of binding was assured by a competition with the addition of excessive unlabelled oligonucleotides.

Moreover, phosphorylation of IκB-α, an upstream inhibitor of NF-κB, was examined. As shown in Fig. 8, phosphorylated IκB-α was barely detected in resting MES-13 cells, but upon treatment with LPS/IFN-γ for 20 min, IκB-α phosphorylation was strikingly initiated. Incubation of cultures with CTN (25 and 40 μM) for 12 h moderately suppressed IκB-α phosphorylation in MES-13 cells (Fig. 8). Taken together, these results suggest that CTN inhibited iNOS gene expression through suppression of NF-κB activation by blockade of IκB-α phosphorylation.

4. Discussion

The fermented products of *Monascus* are commonly used in food as flavoring agents as well as in medicine, since one of the bioactive

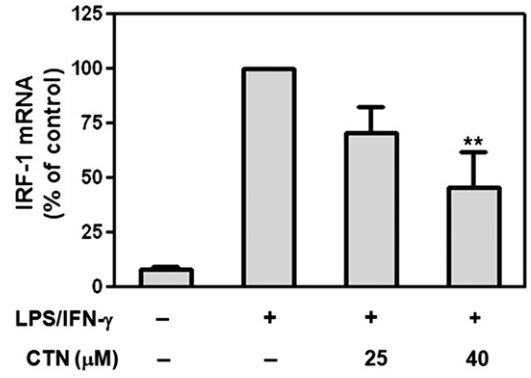

Fig. 6. Effects of citrinin (CTN) on lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated interferon response factor (IRF)-1 mRNA expression in MES-13 cells assessed by real-time RT-PCR. Cells were treated for 24 h with a mixture of 1 μg/ml LPS and 10 ng/ml IFN-γ in the absence or presence of various concentrations of CTN. The mRNA levels of IRF-1 were measured by real-time RT-PCR. GAPDH was used as an internal control. Relative mRNA levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity of IRF-1 mRNA from cultures treated with LPS/IFN-γ alone. Data are the mean ± SEM of iNOS/GAPDH of at least three separate experiments, and asterisks indicate a significant difference from the culture treated with LPS/IFN-γ alone (*$p<0.05$, **$p<0.01$).

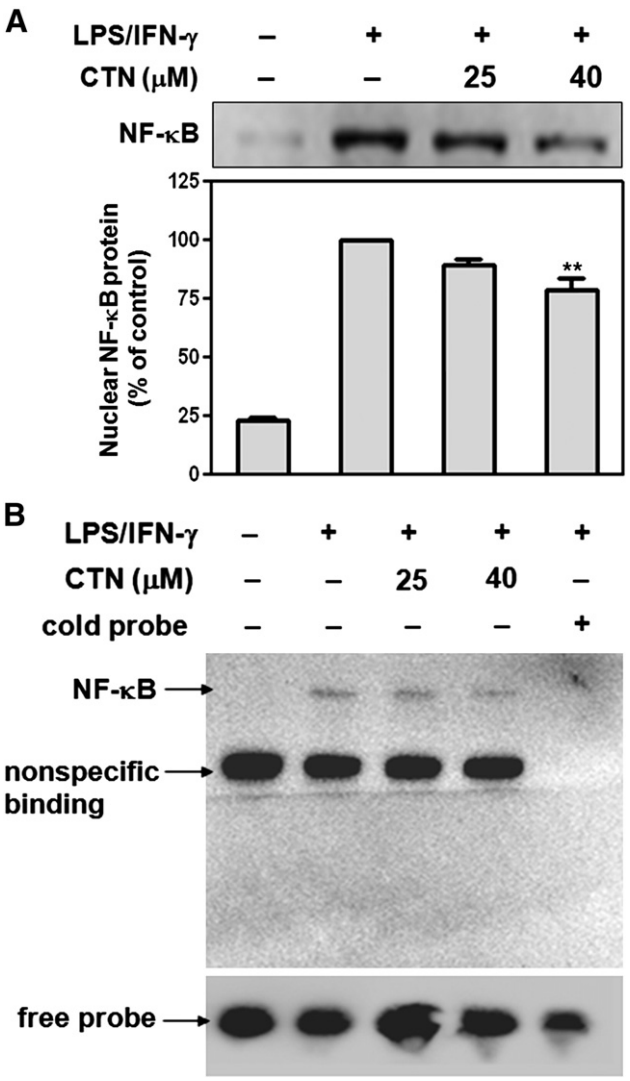

Fig. 7. Effects of citrinin (CTN) on lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated nuclear factor (NF)-κB protein level and DNA-binding activity in MES-13 cells. (A) Cells were pre-incubated with or without CTN for 12 h and then treated in the absence or presence of a mixture of 1 μg/ml LPS and 10 ng/ml IFN-γ for 40 min. The nuclear fractions were used to analyze the protein content of NF-κB protein by Western blotting. Relative protein levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity in the culture treated with LPS/IFN-γ alone. Data are the mean ± SEM of at least three separate experiments, and asterisks indicate a significant difference from treatment with LPS/IFN-γ alone ($^{*}p<0.05$, $^{**}p<0.01$). (B) EMSA experiments of NF-κB DNA-binding activity were carried out using the LightShift Chemiluminescent EMSA Kit from Pierce Chemical. The position of the DNA-protein complex (NF-κB) and the free oligonucleotide are indicated.

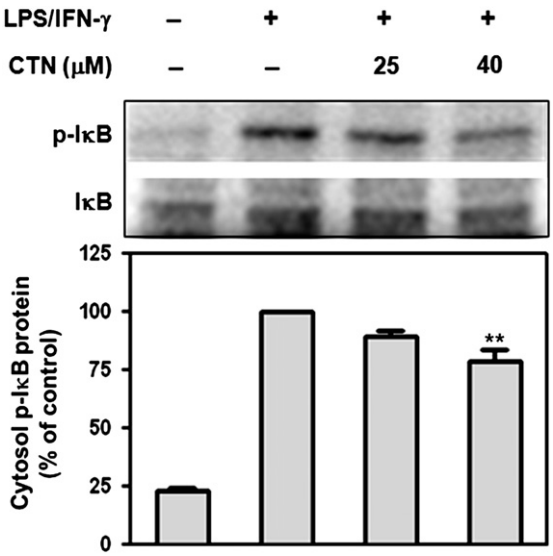

Fig. 8. Effects of citrinin (CTN) on lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated phosphorylated inhibitory factor-κB (IκB)-α protein level and IκB-α protein expression in MES-13 cells. Cells were pre-incubated with or without CTN for 12 h and then treated in the absence or presence of a mixture of 1 μg/ml LPS and 10 ng/ml IFN-γ for 20 min. The cytosolic factions were used to analyze the protein content of IκB-α and phosphorylated IκB-α protein by Western blotting. Relative protein levels were quantified by scanning densitometry and are expressed as a percentage of the maximal band intensity in the culture treated with LPS/IFN-γ alone. Data are the mean ± SEM of at least three separate experiments, and asterisks indicate a significant difference from treatment with LPS/IFN-γ alone ($^{*}p<0.05$, $^{**}p<0.01$).

compounds, named monacolin K, from *Monascus* exhibits antihypertensive and anti-cholesterolemic effects [24]. On the other hand, the presence of CTN, an adverse by-product of *Monascus* fermentation, results in safety concerns with the long-term consumption of *Monascus* products [1–3]. In addition to the by-products of *Monascus*, CTN is also produced by fungal species that belong to the genus *Penicillium*. Yao et al. [25] identified three novel compounds as suppressors of iNOS gene expression from *Penicillium* species. Among them, sporogen exhibited the most potent inhibitory effect [25]. As a fungal secondary metabolite, whether CTN exerts a similar inhibitory effect toward NO production has never been investigated. Based on previous reports from Johannessen et al. [5–7], CTN may cause an imbalance in the secretion of proinflammatory cytokines and anti-inflammatory cytokines in immune cells. In addition, some mycotoxins, such as aflatoxin B1 and patulin, exhibit immunosuppressive effects in different animal models and bovine lymphocytes [4]. Therefore, the immunomodulatory role of CTN in iNOS-derived NO synthesis was investigated in this study.

NO, which acts as a doubled-edged sword, is involved in a range of biological processes. NO generated by iNOS exhibits both protective and toxic effects. Excess bursts of NO as a result of iNOS activation exert antimicrobial activities against various organisms, such as fungi, bacteria, parasites, and viruses [26]. In host defense against infection, interactions of high-output NO with reactive oxygen species (ROS) lead to the formation of multiple antimicrobial metabolites, such as peroxynitrate, 5-nitrosothiols, and nitrogen dioxide, which are able to block microbial DNA replication, oxidize bacterial lipids, and modify protein synthesis [27]. In addition to its protective role against microorganisms, toxic effects of NO toward host cells are observed in parallel. Sustained enhanced production of NO can cause injury to the host and lead to cytotoxicity. Uncontrolled NO synthesis is associated with several human autoimmune and chronic inflammatory diseases [28].

In the current study, MES-13 cells were chosen as a model to study the effects of CTN on iNOS-induced NO production since the kidneys are the pathogenic organ of CTN. MES-13 cells are a glomerular mesangial cell line derived from an SV40 transgenic mouse. They exhibit similar biochemical and morphologic characteristics as primary mesangial cells [29]. The expression of iNOS protein in MES-13 cells was induced by a combination of LPS and IFN-γ. We demonstrated that the incubation of MES-13 cells with CTN inhibited iNOS-induced NO production. As shown in Fig. 1, with the addition of LPS/IFN-γ to MES-13 cells, around 20% reduction in cell viability was observed in several independent studies. The co-incubation of LPS/IFN-γ with CTN caused a further reduction in cell viability (Fig. 1). With ever-increasing dosages of CTN, despite its inhibitory effects on MES-13 cell viability, the percentage of NO reduction caused by CTN was far greater than that of the decline in cell viability (Fig. 2). These results indicated that the inhibitory effect of CTN on LPS/IFN-γ-stimulated NO production was not due to a decrease in MES-13 cell viability.

Coincident with the changes in NO production, CTN dramatically decreased iNOS protein and mRNA expressions as shown in Figs. 3 and 4, respectively. These results suggested that attenuation of NO production by CTN may in part stem from suppression of iNOS gene induction. It is known that LPS induces iNOS gene expression through activation of NF-κB, while IFN-γ works through activation of STAT-1α. By binding to the 5′-flanking regions of the *iNOS* gene, activated NF-κB and STAT-1α work synergistically to elicit iNOS gene expression. We further identified whether CTN reversed IFN-γ-elicited NO generation through preventing phosphorylation of the STAT-1α protein in MES-13 cells. As shown in Fig. 5, the co-incubation with CTN led to a significant attenuation of STAT-1α phosphorylation induced by LPS/IFN-γ. Moreover, as shown in Fig. 6, the presence of CTN further downregulated the expression of the *IRF-1* gene, which is a target gene of the STAT-1α protein [30]. Both STAT-1α and IRF-1 are transcriptional factors which respectively bind to the GAS and IFN-stimulated regulatory elements (ISREs), and three copies of GAS and two copies of ISRE are located within the 1.7-kb fragment of the 5′-flanking region of the murine iNOS gene [22]. Therefore, CTN, by inhibiting STAT-1α activation, may play an important role in modulating various processes of cellular inflammation.

We also identified whether CTN suppressed iNOS-dependent NO generation through the inhibition of IκB phosphorylation and NF-κB DNA-binding activity (Fig. 7A, B). These data suggest an immunosuppressive role of CTN in transcriptional expression of the *iNOS* gene. NF-κB is regarded as a central mediator of the human immune response and regulates the transcription of various cytokine genes encoding IL-1β, IL-6, and TNF-α [31]. Within the promoter region of the murine iNOS gene, two NF-κB-binding sites are located at −961 and −76 base pairs, respectively [22]. Our data indicated that CTN exerts its ability to block iNOS-derived NO synthesis through the inhibition of NF-κB activation.

According to the previous reports, the mycotoxin can either suppress or stimulate immune responses [4–8]. In LPS-stimulated human alveolar epithelial cell line A549, exposure of non-cytotoxic doses of mycotoxins would cause alteration in intracellular oxidant/antioxidant status and imbalance in inflammatory cytokine production, while high doses of mycotoxins are poisonous and eradicate cells directly in various cell types [6]. Moreover, in LPS-stimulated human monocytic cell line, exposure of low doses of CTN led to an increased risk of inflammatory responses with the overproduction of TNF-α and IL-6 while the secretion of the anti-inflammatory IL-10 was strongly inhibited [7]. Imbalance among these immunomodulators may aggravate inflammatory reactions in pathological conditions [7]. From our results in MES-13 cells, the inhibitory effect of CTN on NO, an important proinflammatory mediator in the process of inflammation, may indicate that CTN interfere with the balance of inflammatory responses triggered by LPS/IFN-γ and aggravate inflammatory reactions in pathological conditions, such as glomerulonephritis.

Overproduction of iNOS-induced NO exerts antimicrobial activities and mediates macrophage-dependent killing of bacteria, protozoa, and fungi. Several toxic compounds were shown to impair host defense systems via reducing the macrophage-mediated immune response [17–20,32–35]. Moreover, several reports also indicated the effect of certain toxins on reducing iNOS-stimulated NO synthesis in mesangial cells and macrophages [32–35]. Shiga-like toxin (Stx) produced by *Escherichia coli* strains reduced NO production and cellular iNOS antigen content in mesangial cells during the course of hemolytic uremic syndrome (HUS) development [32]. This unique effect of Stx on reducing iNOS-stimulated NO synthesis by mesangial cells contributes to the aggravation of thrombotic microangiopathy and renal failure observed in HUS [32]. Furthermore, it was reported that urea, a uremic toxin that accumulates in renal failure, impairs macrophage function during renal failure through inhibiting NO production by macrophages [33]. Macrophage dysfunction is an important contributory factor in the increased incidence of infections in uremia [33]. Schmidt et al. [35] identified phenylacetic acid (PAA), as a uremic toxin in patients on regular hemodialysis. The accumulation of PAA in patients with end-stage renal failure inhibits iNOS expression [34,35]. It was suggested that PAA might affect immunoregulatory processes and could play a role in the aggravation of immunodeficiency in patients with end-stage renal disease [34,35]. Taken together, toxic compounds might impair host defense systems via reducing iNOS-induced NO production which exerts antimicrobial activities. Thus, our data may further indicate that the nephrotoxicity of CTN may impair the host immune response by interfering with mesangial cell functions, such as NO release, one of the most important antimicrobial effectors during bacterial infection.

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