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MOSCATILIN REPRESSED LIPOPOLYSACCHARIDE-INDUCED HIF-1α ACCUMULATION AND NF-κB ACTIVATION IN MURINE RAW264.7 CELLS

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ABSTRACT—In the present study, we investigated the signaling pathways involved in the inhibition of cyclooxygenase 2 (COX-2) and iNOS by moscatilin under LPS challenge in murine macrophage-derived cell line RAW264.7. The results showed that moscatilin (10–100 μM) had a significant inhibition in a concentration-dependent manner on proinflammatory enzymes (COX-2 and iNOS) expression and macrophage activation under LPS (100 ng/mL) treatment. Hypoxia-inducible factor 1 (HIF-1)α was reported to initiate inflammation under cytokine stimulation or hypoxic conditions. In addition, the increase in transcriptional activity and translation process of HIF-1α under LPS stimulation resulted in HIF-1α accumulation. Moscatilin, a purified compound from Chinese herbs, had a dominant repression on HIF-1α expression via down-regulating HIF-1α mRNA without inhibition of cell viability, translation machinery, or proteasome-mediated degradation of HIF-1α. Moreover, the results showed that moscatilin suppressed nuclear translocation of nuclear factor (NF)–κB subunits, p65 and p50, and NF-κB activity by inhibiting phosphorylation of inhibitor of κBα. Taken together, we demonstrated that moscatilin inhibited both COX-2 and iNOS expressions after LPS treatment in RAW264.7. Furthermore, the inhibition of moscatilin seemed to be dependent on the repression of HIF-1α accumulation and NF-κB activation.

KEYWORDS—Moscatilin, macrophage, HIF-1α, NF-κB

INTRODUCTION

Macrophage is one of the myeloid lineages, acting in both innate immunity, cell-mediated immunity of mammals, and removing cellular debris through phagocytosis (1). Because of their role in phagocytosis and cytokine release, macrophages play a critical mediator in many inflammatory diseases of the immune system such as atherosclerosis, sepsis, arthritis, and cancer. So far, LPS, a major component of the outer membrane of Gram-negative bacteria, is one of the best studied immune stimulants in normal animals. LPS can induce strong systemic inflammation through binding to Toll-like receptor and activating downstream signaling cascade, which promote the secretion of proinflammatory cytokines in macrophages (2).

In this cascade, the transcriptional factor nuclear factor-κB (NF-κB) heterodimer plays a key role in binding to the promoter of proinflammatory cytokines and increasing the production of cytokines such as IL-1β, IL-6, and TNF-α and promoting the expression of proinflammatory proteins such as the iNOS and cyclooxygenase 2 (COX-2) (3–5). Under unstimulated conditions, NF-κB locates in the cytosol as a latent, inactive complex with inhibitor of κB (IκB) protein. In response to any inflammatory challenge, activated upstream kinase IκB kinase phosphorylates IκB, which leads to ubiquitination and degradation by the proteasome (6). At the moment, free NF-κB translocates into the nucleus, where it initiates the transcription of inflammatory genes encoding cytokines and proinflammatory proteins (7).

Furthermore, recent studies demonstrated that LPS activates hypoxia-inducible factor 1 (HIF-1) and its downstream genes and proteins under normoxic conditions (8, 9). Hypoxia-inducible factor 1 is a heterodimeric protein composed of an HIF-1α subunit and a constitutively expressed HIF-1β subunit. Unlike HIF-1β, HIF-1α is an oxygen-labile protein and undetectable in normal oxygen, which is rapidly degraded through hydroxylation and proteasomal degradation in a pVHL (the von Hippel-Lindau protein)-dependent pathway (10, 11). However, HIF-1α is also regulated by transcription and translation through receptor-mediated pathway. In mammals, two characterized pathways, mitogen-activated protein kinase (MAPK)/eukaryotic translation initiation factor 4E–binding protein (4E-BP)/eIF4E/p70S6K and phosphoinositide 3-kinase (PI3K)/Akt/4E-BP/eIF4E/p70S6K, precisely control HIF-1α translation (10).

Moscatilin (4,4′-dihydroxy-3,3′,5-trimethoxybibenzyl) is a bibenzyl compound extracted from orchid *Dendrobium loddigesii* or *Dendrobium nobile*, and both herbs have been used as a Chinese traditional medicine for reducing fever and replenishing body fluid (12, 13). Several studies indicated that moscatilin exhibited antiplatelet aggregation (14) and antimutagenic activities against several cancer cell lines by targeting c-Jun NH2-terminal protein kinase and inducing G_2-M arrest (13). However, no direct evidence has shown how moscatilin prevents bacteria-inducing fever. In the present study, we tried to identify the possible anti-inflammatory action of moscatilin and revealed a novel action of moscatilin involved in the inhibition of HIF-1α expression and NF-κB activation.

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MATERIALS AND METHODS

Materials

Moscatilin with more than 98% purity was extracted, purified, and identified by Chien-Chih Chen. Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), antibiotic, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). LPS, leupeptin, dithiothreitol (DTT), dimethyl sulfoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), cycloheximide, BAY 117082, and nuceolin antibody were ordered from Sigma Chemical (St. Louis, Mo). TRIzol reagent was from Invitrogen (Carlsbad, Calif); random primer and Moloney murine leukemia virus RT were from Promega (Madison, Wis); pro Taq was from Protech (Taipei, Taiwan). Antibodies against iNOS, COX-2, and HIF-1α were purchased from Novus Biologicals (Littleton, Colo). Antibodies against phospho-IκBα was purchased from Cell Signaling Technology (Beverly, Mass). Nuclear factor–κB (p65 and p50 subunit), IκBα, actin, and horseradish-peroxidase–conjugated antimouse, antirat, and antirabbit immunoglobulin G antibodies were ordered from Santa Cruz Biotechnology (Santa Cruz, Calif). An electrophoretic-mobility shift assay (EMSA) kit (NF-κB; AY1030) was purchased from Panomics (Fremont, Calif).

Cell culture condition

RAW264.7 (Murine macrophage cell line) was purchased from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS and antibiotics (penicillin 100 U/mL; streptomycin, 100 μg/mL; and amphotericin B 2.5 μg/mL) at 37°C in a 5%/95% air atmosphere.

Western blot and preparation of nuclear fraction

For analysis of protein expression, cells were seeded in 6-cm dishes overnight, challenged with the indicated agents for different time periods, and then harvested in ice-cold lysis buffer. Nuclear proteins were extracted as the following. Briefly, phosphate-buffered saline–washed cells were resuspended in buffer A (10 mM Hepes [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM PMSF, and 0.5 mM DTT). After incubation on ice for 15 min, cells were centrifuged at 3,000 rpm for 5 min, and then pellets were resuspended in buffer C (20 mM Hepes, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF). After incubation on ice for 20 min, cells were centrifuged at 13,000 rpm for 10 min. The blots were electrophoretically transferred to polyvinylidene fluoride membranes and incubated with antibody in phosphate-buffered saline overnight at 4°C. Signal was detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

Sulforhodamine B Assay

Cells were seeded in 96-well plates with 10% fetal bovine serum medium overnight. After addition of vehicle (0.1% DMSO), LPS, or indicated concentrations of moscatilin for 8 h, cells were fixed with 10% trichloroacetic acid for 10 min and stained with 0.4% sulforhodamine B assay dye for an additional 10 min. Unbound dye was washed with 1% acetic acid, and plate was air-dried. The absorbance was read under 515 nm, and cellular viability was calculated as $[(T_d / T_c) / (T_c / T_c)] \times 100$. T_d represents absorbance of drug-treated group, and T_c is absorbance of vehicle-treated group.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol reagent by a standard protocol (Invitrogen, Carlsbad, Calif). Reverse transcription was performed with 5 μg mRNA and random primer at 65°C for 5 min, then mixed with Moloney murine leukemia virus reverse transcriptase (RT) to react at 37°C for 1 h to obtain cDNA. Gene amplification was followed with RT-polymerase chain reaction (PCR). Primer sequence was as described: *COX-2* sense, 5′-GGAGAGACTATCAAGATAGT-3′; *COX-2* antisense, 5′-ATGGT CAGTAGACTTTTACA-3′; *HIF-1α* sense, 5′-GTCTGGGTTGAAACTCAAG CAACTG-3′; *HIF-1α* antisense, 5′-GGTTTGAGCACAGATTCTGTTTGTT-3′; *GAPDH* sense, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′; and *GAPDH* antisense, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′. Reaction cycles for *COX-2*, *HIF-1α*, and *GAPDH* includes 35 cycles of 94°C for 30 s, 55°C for 30 s (50°C for *HIF-1α*), 72°C for 1 min, and a final incubation at 72°C for 10 min. Polymerase chain reaction products were analyzed on 1.5% agarose gel in the presence of 1 μg/mL of ethidium bromide.

Electrophoretic-mobility shift assay

The association of NF-κB with DNA was identified by Panomics' electrophoretic-mobility shift assay kit (Fremont, Calif). Briefly, treated or untreated nuclear extracts were incubated with biotin-labeled probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′), and protein-DNA complexes were separated on polyacrylamide gel. The gel was transferred to nylon membrane and detected using strepatvidin–horseradish peroxidase and a chemiluminescent substrate. The bands were visualized after exposure to film.

Statistical analysis

All data are represented as mean ± SEM. An unpaired Student *t* test was used to compare same data. *P* values less than 0.05 were considered statistically significant for all comparisons.

RESULTS

Effect of moscatilin on macrophage activation and LPS-induced inflammatory response

As described previously, LPS is a well-known potent activator of monocytes and macrophages, which induces lots of cytokines and proinflammatory proteins (5). In accordance with the properties of LPS, we examined the biological effect of moscatilin on LPS-induced inflammatory responses. We showed that treatment of RAW264.7 (murine macrophage cell line) with moscatilin (10–100 μM) reversed LPS-induced macrophage morphology change (Fig. 1A) (15). Next, we explored the effect of moscatilin on LPS-induced proinflammatory enzymes COX-2 and iNOS, which can initiate production of inflammatory proteins. We found that moscatilin inhibited LPS-induced COX-2 and iNOS expressions in a concentration-dependent manner (Fig. 1B). These results demonstrated that moscatilin could reverse macrophage activation and proinflammatory proteins in the presence of LPS.

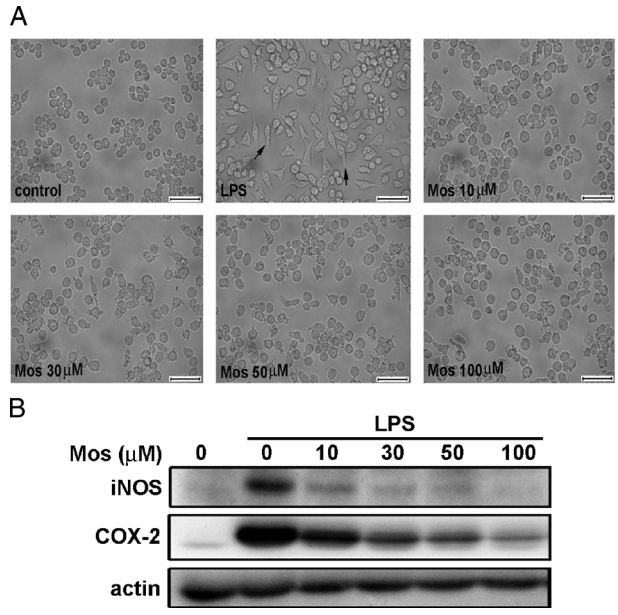

FIG. 1. **Effects of moscatilin on LPS-stimulated activation and proinflammatory protein expression in murine RAW264.7 cells**. A, Murine macrophage-derived cells, RAW264.7, were trypsinized, seeded to 6-well plates, and incubated overnight. Before LPS (100 ng/mL) challenge, cells were pretreated with vehicle (0.1% DMSO) or the indicated concentrations of moscatilin (10–100 μM) for 1 h. After LPS stimulation for 8 h, cells were observed under microscope to obtain the cellular morphology. Upper left, basal; upper middle, LPS alone; upper right, moscatilin (10 μM) + LPS; lower left, moscatilin (30 μM) + LPS; lower middle, moscatilin (50 μM) + LPS; lower right, moscatilin (100 μM) + LPS. B, RAW264.7 cells were seeded on a 6-cm dish until 50% confluence before treatment. Cells were treated with vehicle or different concentrations of moscatilin for 1 h and then exposed to 100 ng/mL LPS for 24 h. Finally, whole cell lysates were harvested for the detection of COX-2, iNOS, and actin expressions by Western blot. Mos indicates moscatilin. Scale bar = 50 μm. The result was representative of three independent determinations.

Effect of moscatilin on LPS-induced HIF-1α expression

A variety of factors are involved in LPS-induced inflammatory responses. Among them, HIF-1 is essential for myeloid cell activation and myeloid cell-mediated inflammation (1, 9, 15). Here, we showed that the induction of HIF-1α by LPS was concentration-dependent, and the induction could be found at the concentration as low as 1 ng/mL, whereas the peak induction was attained approximately 100 ng/mL (Fig. 2A), which was approximately 10-fold potent than what had been observed by Dr. Blouin (8). Next, after different indicated concentrations, moscatilin inhibited the LPS-induced HIF-1α protein in a concentration-dependent manner with a modest inhibition at 30 μM and a significant inhibition at 50 μM (Fig. 2B). With further evaluation, we tested whether inhibition of moscatilin on LPS-induced HIF-1α expression was correlated to cellular viability, and the parallel measurement was done. As shown in Figure 2C, we suggested that the inhibition was independent with cellular viability because there was no significant inhibition of cellular viability at 10 to 100 μM after moscatilin treatment.

Moscatilin inhibited HIF-1α through a proteasome-independent degradation and translation-independent pathway

HIF-1α, a well-known oxygen-labile protein, is rapidly degraded through the proteasome-mediated machinery via hydroxylation of proline residues and ubiquitination. To test the possibility if moscatilin regulated HIF-1α expression by enhancing its degradation, we pretreated RAW264.7 with 0.1 μM MG132 to block proteasome-mediated degradation before the challenge of moscatilin. We observed that combination of MG132 and LPS induced HIF-1α accumulation; however, the inhibition of moscatilin on LPS-induced HIF-1α expression was not reversed by MG132 (Fig. 3A), which indicated that repression of HIF-1α was through a proteasome-independent pathway. To explore more, cycloheximide was used to block new protein synthesis, and the residue of HIF-1α could be the response to the process of degradation. We found that the rates of degradation between the moscatilin-treated group and the vehicle-treated group

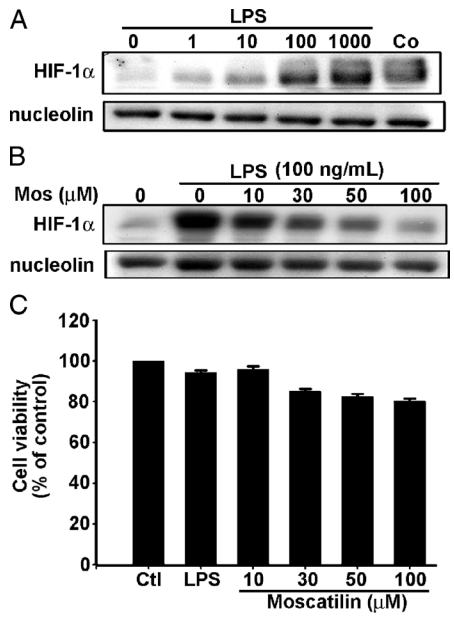

FIG. 2. **Repression of moscatilin on LPS-induced HIF-1α accumulation in murine RAW264.7 cells**. A, RAW264.7 cells were treated different concentrations of LPS (1–1,000 ng/mL) for 8 h. B, RAW264.7 cells were pretreated with moscatilin for 1 h before 100 ng/mL LPS challenge. Nuclear extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and HIF-1α, and nucleolin expressions were determined. Cobalt chloride (100 μM) was used as positive control. C, RAW264.7 cells were seeded into a 96-well plate at the density of 1 × 105 cells/well and incubated overnight. After that, cells were treated with various concentrations of moscatilin (10–100 μM) for 8 h. The cell number was determined by sulforhodamine B assay described in Materials and Methods. The result was representative of at least three independent determinations.

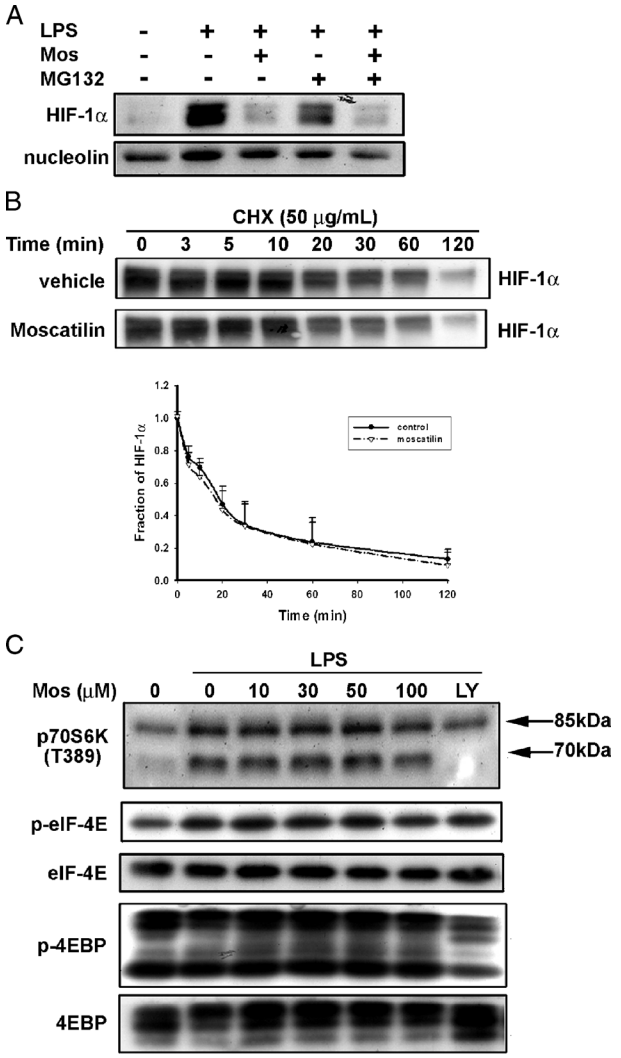

FIG. 3. **Moscatilin has no effect on HIF-1α half-life and translation process**. A, RAW264.7 cells were treated with 100 ng/mL LPS in the presence of MG132, a proteasome inhibitor (0.1 μM), or MG132 and moscatilin (50 μM) before immunoblotting. B, RAW264.7 cells were exposed to 100 μM $CoCl_2$ ovnernight, and then 50 μg/mL CHX was used to block protein biosynthesis after 1-h treatment of moscatilin (50 μM) or vehicle. Nuclear lysates were collected at indicated times after CHX exposure and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis for HIF-1α detection. The lower panel indicates quantification of the HIF-1α levels by densitometry. C, Before immunoblotting, cells were treated with 100 ng/mL LPS for 8 h in the presence or absence of various concentrations of moscatilin or 10 μM LY. Total protein lysates were prepared for detection of phospho-p70S6K, phospho-eIF4E, and phospho-4E-BP. Similar results were obtained in at least three independent determinations. CHX indicates cycloheximide; LY, LY294002.

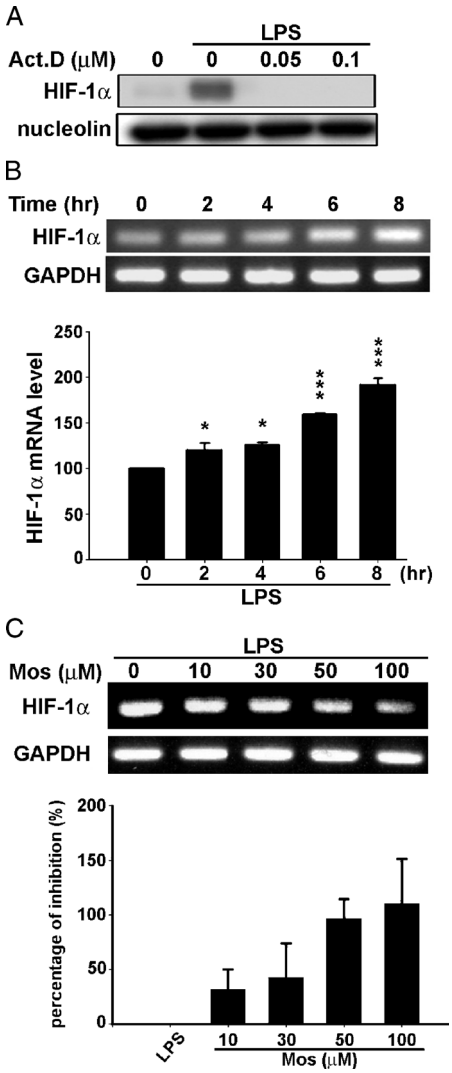

FIG. 4. **Transcription is involved in the inhibition of moscatilin on LPS-induced HIF-1α mRNA expression**. A, RAW264.7 cells were pretreated or not for 30 min with actinomycin D (0.05 and 0.1 μM) and challenged with 100 ng/mL LPS for 8 h. Nuclear lysates were prepared for the detection of HIF-1α by Western blotting. B, RAW264.7 cells were incubated with 100 ng/mL LPS for different periods of time. The lower panel indicates quantification of the HIF-1α mRNA levels by densitometry. C, RAW264.7 cells were treated with different concentrations of moscatilin after LPS (100 ng/mL) exposure for 8 h. The lower panel indicates quantification of inhibition of LPS-induced HIF-1α mRNA levels by densitometry. Total RNA was extracted and HIF-1α mRNA expression was determined by RT-PCR. GAPDH was used as loading control. The result was representative of at least three independent determinations.

were similar with half-life approximately 20 min, which means moscatilin has no significant effect on HIF-1α degradation (Fig. 3B). As previous studies demonstrated, LPS can initiate the translation machinery through activating PI3K/mammalian Target of Rapamycin (mTOR) cascade, and hyperphosphorylating translation regulatory protein p70S6K during macrophage differentiation (7, 15). Consequently, we evaluated the implication of translation machinery in the inhibition of HIF-1α synthesis by moscatilin in RAW264.7 cells. As seen in Figure 3C, LPS phosphorylated eIF-4E and p70S6K but not p-4E-BP or 4E-BP. However, the activation was not affected by moscatilin when compared with LY294002, a potent inhibitor of PI3K/Akt, with significant down-regulation of 4E-BP, eIF4E, and p70S6K. Overall, these findings supported that the inhibition of LPS-induced HIF-1α expression was independent on proteasome-mediated degradation and translation regulatory machinery.

Moscatilin inhibited LPS-induced HIF-1α production

It has been clearly demonstrated that LPS could up-regulate *HIF-1α* mRNA expression at 1 μg/mL and increase HIF-1α expression via reactive oxygen species–dependent pathway (8, 9). Here, we confirmed that 0.05 μM actinomycin D, a transcriptional inhibitor, could completely block 100 ng/mL LPS-induced *HIF-1α* mRNA expression in RAW264.7. This result was similar to the results as the one that has been done in rat alveolar cell line NR8383 (Fig. 4A) (8). Next, we also found that LPS significantly increased *HIF-1α* mRNA expression approximately 20% at 2-h treatment period and increased gradually to 100% in a time-dependent manner up to until 8 h (Fig. 4B). Hence, the increase in *HIF-1α* mRNA predominantly contributed to LPS-induced HIF-1α protein accumulation. Accordingly, the effect of moscatilin on LPS-induced *HIF-1α* mRNA was evaluated by RT-PCR. Before 8-h challenge of LPS, murine RAW264.7 cells were treated with indicated concentrations of moscatilin, and as shown in Figure 4C, moscatilin inhibited *HIF-1α* mRNA expression in a concentration-dependent manner. These results showed that moscatilin repressed HIF-1α mRNA in the presence of LPS.

Effect of moscatilin on NF-κB activation

It has been shown that NF-κB activation is a key regulator on LPS-induced inflammation. As known, NF-κB is an inactive, latent complex with the inhibitor IκBα. If activated, IκBα is phosphorylated, ubiquitinated, and degraded by

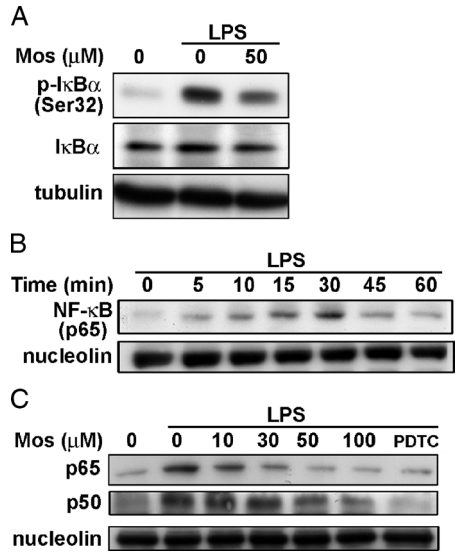

FIG. 5. **Inhibition of NF-κB translocation contributed to repression of LPS-induced HIF-1α and proinflammatory proteins**. A, RAW264.7 cells were exposed to LPS for 5 min in the presence or absence of 50 μM moscatilin for the detection of phospho-IκBα, IκBα, and α-tubulin. B, RAW264.7 cells were incubated with 100 ng/mL LPS for different periods of time. In addition, nuclear protein was extracted for the detection of NF-κB subunits p65 and p50. C, RAW264.7 cells were treated with vehicle or different concentrations of moscatilin (10–100 μM) or 20 μM pyrollidine dithiocarbamate for 1 h and then exposed to 100 ng/mL LPS for 30 min for the detection of nuclear proteins p65 and p50 by Western blot. The result was representative of at least three independent determinations.

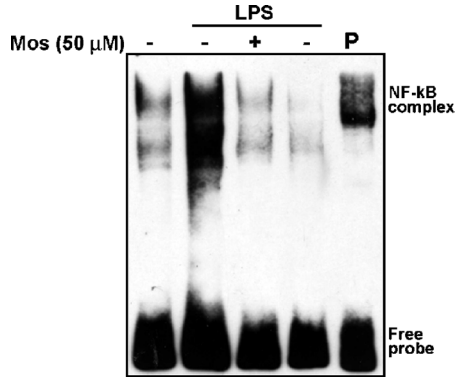

FIG. 6. **Moscatilin inhibited NF-κB–DNA binding**. RAW264.7 cells were exposed to 100 ng/mL LPS for 30 min in the presence of 50 μM moscatilin or vehicle, then nuclear extracts were incubated with transcription factor probe (lanes 1–3, 5) or cold probe (lane 4) and subjected into polyacrylamide gel to detect protein-DNA binding. P indicates positive nuclear extract. The result was representative of three independent determinations.

proteasome. Therefore, we investigated the expression of phospho-IκBα and IκBα. We found that LPS led to phosphorylation after short time treatment as expected, and moscatilin repressed phosphorylation of IκBα (Fig. 5A). Next, we investigated the inhibition of moscatilin on NF-κB activation. As expected, NF-κB subunit p65 translocated into nucleus after short-time exposure to LPS approximately 5 min and reached peak level approximately 30 min (Fig. 5B). In addition, moscatilin modestly inhibited the nuclear translocation of p65 and p50 at 10 to 30 μM and had obvious inhibition at 50 and 100 μM as well as pyrollidine dithiocarbamate after 30-min challenge of LPS (Fig. 5C). To investigate if moscatilin inhibited NF-κB activity, we performed electrophoretic-mobility shift assay to observe the interaction between NF-κB and its specific recognition sequence. As shown in Figure 6, moscatilin repressed LPS-induced NF-κB DNA binding, and the observed signals disappeared in the presence of the cold NF-κB competitor, which indicated these signals were NF-κB specific. Taken together, our data suggested that the inhibition of LPS-induced inflammatory response was through down-regulating two transcriptional factors, HIF-1α and NF-κB.

DISCUSSION

In immune responses, macrophages have been described as a kind of antigen-presenting phagocytes that secrete proinflammatory cytokines and antimicrobial mediators, and its function significantly influences the duration and magnitude of most inflammatory reactions in several diseases after activation (3). In septic shock, Toll-like receptor complex activation triggers the production and release of inflammatory cytokines, in particular, TNF-α, IL-1β, and IL-6, after exposure to LPS. Because of its prominent role in various inflammatory diseases, LPS-activated cascade is a potential drug target model for further investigation (2, 3). In addition, a number of studies have indicated that LPS-stimulated COX-2 and iNOS promoted the release of prostaglandin E2 and a large amount of NO in sepsis and other diseases, which contributed to inflammation and endotoxemia (16, 17). Hence, drug that inhibits iNOS and COX-2 enzymatic activity or gene expression has a lot of therapeutic effects against sepsis, cancer, or other inflammation-related diseases. In the present study, we found that the antagonism of moscatilin against the induction of COX-2 and iNOS in murine RAW264.7 cells could repress inflammation induced by LPS treatment (Fig. 1).

Hypoxia-inducible factor 1 is a master transcription factor controlling multiple functions such as tumorigenesis, cancer metastasis, angiogenesis, and metabolism (11). However, accumulating evidences indicated that HIF-1α played an important role in inflammation and in tumorigenesis (18, 19). Recently, the concept of HIF-1 as an inflammatory mediator is derived from the observation that several proinflammatory cytokines could stabilize HIF-1α and increase HIF-1α synthesis through MAPK- and PI3K-mediated pathways (20, 21). It was demonstrated that HIF-1α protein was stable in rheumatoid synovial macrophages, and Dr. Cramer showed that HIF-1α null cells had less TNF-α release than wild-type cells after LPS treatment, and the activation of HIF-1α was also essential to macrophage differentiation (1, 15, 22). In addition, it has been demonstrated that LPS induced HIF-1α expression through increasing the transcription activity in rat alveolar cell line (NR8383) as we did in rat macrophage–derived cell line (RAW264.7) (Fig. 4), but we still had no idea whether LPS could affect HIF-1α stability (8). Furthermore, we investigated the translational machinery (Fig. 3C) and showed that LPS activated the 4E-BP/eIF4E/p70S6K cascade, which is the downstream proteins of PI3K/Akt. As reported, activation of PI3K/Akt/mTOR or MAPK pathways can stimulate HIF-1α protein synthesis after growth factor treatment in cancer cells (10, 11). Phosphoinositide 3-kinase/mTOR also plays a crucial role in the LPS-stimulated expression of inflammatory cytokine. Hence, it seems that LPS-stimulated phosphorylation of PI3K participated in the translation of HIF-1α, but we did not observe any repression of these translational proteins after moscatilin treatment. This indicated it had no significant effect on translational levels.

Cyclooxygenase 2 and iNOS are responsible for formation of important biological mediators, prostanoids, and a regulatory molecule, NO, which attributed to a variety of pathophysiological functions such as vasodilatation and pain (17, 23, 24). As previously mentioned, free NF-κB translocates into nuclei upon phosphorylation of inhibitor protein, IκBα, and activates COX-2 and iNOS transcription. Therefore, inhibition of IκBα phosphorylation and NF-κB activation by moscatilin would inhibit NF-κB–dependent expression of COX-2 and iNOS, thereby reducing inflammation in LPS-stimulated macrophage (Fig. 5).

Recently, it is getting clear that there is a cross-talk or synergistic effect between the NF-κB pathway and the HIF-1 pathway (25, 26). Stimulated with low concentration of LPS, murine macrophages expressed higher levels of iNOS mRNA when under hypoxic conditions compared with normoxic conditions via hypoxia response element–dependent pathway (27). Now, it was established that HIF-1–induced NF-κB activation via phosphorylating IκB and p65 at residue Ser276 and enhancing p65 nuclear translocation and transcriptional activity (20, 26). Therefore, it was legitimate to suggest that repression of HIF-1α could result in inhibition of NF-κB

activation. In the present study, we observed that moscatilin inhibited LPS-induced HIF-1α accumulation, which may result in inhibition of NF-κB activation (Fig. 2). However, we would need more experiments to demonstrate it in the future.

On the contrary, it has been shown that LPS increased HIF-1α mRNA expression in an NF-κB–dependent pathway by activating upstream p44/42 MAPK (28). Inhibitor of κB kinaseβ deficiency not only resulted in defective induction of HIF-1α target genes but also abrogated HIF-1α accumulation in macrophages while experiencing bacterial infection (25). Under short-term hypoxia or stimulation by cytokines, activated NF-κB binds to a distinct element at −197/188 bp of the HIF-1α promoter and increases production of HIF-1α protein (26, 29, 30). Hence, NF-κB also plays a critical role in the transcriptional regulation of HIF-1α under hypoxic response, linking it to immunity and inflammation. In the present study, we observed that moscatilin inhibited NF-κB activation in the presence of LPS and NF-κB inhibitor, pyrollidine dithiocarbamate, did (Figs. 5 and 6).

Recently, it was demonstrated that YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole) could repress HIF-1α accumulation via inhibiting NF-κB activation in PC-3 (prostate cancer cell line), which links the cross-inhibition between HIF-1α and NF-κB (31, 32). In the present study, we found a similar result that moscatilin inhibited NF-κB activation and HIF-1α accumulation, but we still have to examine which one is the really direct target in the future.

Taken together, our study demonstrated that moscatilin repressed LPS-induced inflammatory response and macrophage activation through inhibition of HIF-1α accumulation and NF-κB activation. This suggests that moscatilin has a great potential as a lead compound for further modification to be a potent anti-inflammatory agent in several diseases.

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